Floral Morphogenesis in *Primula*: Inheritance of Mutant Phenotypes, Heteromorphy, and Linkage Analysis.

Margaret Ann Webster

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Faculty of Biological Sciences

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The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract.

Interest in *Primula* flowers from both a horticultural and scientific perspective dates back over 400 years. Floral mutations were first used for ornamental value in the latter part of the 16th Century but had attracted little scientific attention. The phenomenon of floral heteromorphy as a mechanism to promote out-breeding was immortalised by the work of Darwin in the mid 19th Century. Subsequent analysis of this breeding system has attracted much attention, including the genetic definition of the *S* locus as a cluster of tightly linked genes that control pin and thrum flower development and mediate self-incompatibility.

Mutant phenotypes of British *Primula* have been collected by the author for over twenty years. Classical genetic analysis of some of these mutants is included and provides the first detailed analysis of existing and new mutant phenotypes. Genetic analysis of these mutants is presented in the context of the ABC model of flower development. Detailed analysis of the early ontogeny of wild type and mutant flowers by scanning electron microscopy provides new insights into the control of *Primula* flower development. As *Primula* flowers were found to be homomorphic during early ontogeny development of pin and thrum heteromorphic features of *Primula* were investigated to maturity. A new heteromorphic feature was discovered; thrum flowers have a wider corolla tube mouth than pin flowers due to the corolla tube cells above the anthers being wider in thrum flowers than in pin flowers.

Three of the mutant phenotypes *Hose in Hose*, *Staminoid Carpels* and *sepaloid* are predicted to arise through misexpression of a B function gene. The first two are dominant mutant phenotypes and all are linked to the *S* locus. A fourth recently discovered dominant mutant phenotype, *Oakleaf*, affects both leaf and flower development, and is also linked to the *S* locus. As the dominant nature of the *Hose in Hose* mutation precludes complementation tests three point crosses were used both as segregation tests and for mapping genes linked to the *Primula S* locus. Gene order was found to be *Oak Leaf*, *S* locus, *Hose in Hose*, with *sepaloid* either allelic to *Hose in Hose* or very tightly linked. In combination, these analyses have enabled the assembly of the first genetic map of genes around the *S* locus including flanking markers on either side.

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Abbreviations

Genotype	Phenotype
Jig	*Jack in the Green* (leafy sepals)
Hih	*Hose in Hose* (petaloid sepals)
Spr	*Split Perianth* (calyx and/or corolla split)
Stc	*Staminoid Carpels* (anthers in whorl 4)
sep	*sepaloid* (flowers of whorls of sepals and/or carpel)
dbl	*double* (extra whorls of petals)
rdp	*reduced petal* (small reduced petals, loss of colour, broad frilly leaves)
Okl	*Oak Leaf* (lobed leaves/attenuated organs whorls 1 and 2)
gPA	Long homostyle
Gpa	Short homostyle
+	Wild type
Ss	Thrum
ss	Pin
?	Indicates that the genotype is not known

CHAPTER ONE.

Introduction.

1.1. General introduction.

Floral mutations have been recognized as curiosities for over two thousand years. Double flowers were recorded by Theophrastus before 286 B.C. (Meyerowitz *et al.*, 1989). British native floral abnormalities were collected and grown as a means of adding variety to the garden, before introduced species and their modern derivatives became available (Parkinson, 1629), illustrated the range of *Primula* mutations available in the 17th century. The appearance of a floral organ in a whorl normally occupied by a different floral organ was originally termed metamorphosis and became a subject of interest and research. The study of mutant flowers as a means of discovering more about normal floral morphogenesis was known as teratology, and continued throughout the 1800's (Masters, 1869), and into the early 1900's (Worsdell, 1916). As early as 1790, Goethe had proposed that all floral organs are homologous to each other (cited by Coen, 1991).Nearly two centuries later Meyer illustrated that any organ in the flower is capable in some plant or other of developing in the form of any other type of organ in the normal flower (Meyer,1966). He did this by listing all the genera in which the various possible abnormalities were observed. Many of the abnormalities that Meyer listed have been observed in the mutant phenotypes of *Primula* collected by the current author, or recorded in the literature (Table 1.1). There were relatively few studies on the inheritance of plant abnormalities using classical genetics but those undertaken showed that single genes could control floral phenotypes. It was only with the development of methods of molecular analysis that studies of mutant plants began to unravel the process by which floral homeotic genes direct floral morphogenesis.

1.2. Molecular basis of floral morphogenesis.

Floral morphogenesis in flowering plants requires, first, that meristem identity alters from vegetative growth to reproductive growth, and second, that the whorls of floral organs are produced in the correct `order of sepals, petals, stamens, and carpels. Homeotic genes control gene expression during floral morphogenesis so that the appropriate genes for a particular developmental stage are switched on or off as required. Mutations in such homeotic genes result in the development of organs inappropriate to the whorl or to the position that they occupy.

WHORL	1.	2.	3.	4.
Converted to 1. SEPALS		Sepaloid* petals 10	Sepaloid* stamens 4	Sepaloid* carpels 3
2. PETALS	Petaloid* sepals 32		Petaloid* stamens 163	Petaloid* carpels 22
3. STAMENS	Staminoid sepals 4	Staminoid petals 26		Staminoid* carpels 24
4. CARPELS	Carpeloid sepals 1	Carpeloid petals 5	Carpeloid* stamens 65	
GROUND STATE/ LEAVES	Leafy* sepals 57	Leafy* petals 60	Leafy* stamens 22	Leafy* carpels 34

Table 1.1. Floral organ identity mutant phenotypes.

Of the twenty possible mutant phenotypes above, twelve, (marked by asterisks) have been found in *Primula*. One, marked by pink asterisks, was recorded by Masters in 1877 but has not been observed since. The remaining eleven, marked by red asterisks, can still be seen today. In some instances two or more whorls are converted in the one flower, for example many *doubles* have whorls three and four converted to petals, and combinations of mutant phenotypes such as *sepaloid Jack in the Green* have whorls two, three, and sometimes whorl four also, converted to leaves. Numbers in grey indicate the number of genera listed by Meyer in 1966 in which the mutant phenotype had been observed.

The two very distantly related *species* on which initial work was carried out are *Arabidopsis thaliana* and *Antirrhinum majus.* Despite the morphological differences between the two species, molecular and genetic results correlated sufficiently well to suggest an evolutionarily ancient basic process of floral morphogenesis (Bowman, 1997). Many genes found to be involved in floral morphogenesis are members of the MADS-box family. The first MADS-box genes to be identified in plants were *AGAMOUS (AG)* in *Arabidopsis thaliana* (Bowman *et al.* 1989, 1991), and *DEFICIENS (DEF)* in *Antirrhinum majus* (Sommer *et al.* 1990). Both encode proteins with a highly conserved region of 58 amino acids which showed remarkable homology to the known DNA-binding transcriptional regulators of the yeast *MCMI* gene product (Passmore *et al,* 1989), and the Serum response factor encoded by *SRF* of humans and of *Xenopus* (Norman *et al.* 1988). The motif was named the MADS box after MCMI-AGAMOUS-DEFICIENS-SRF. After an intervening region there is a second conserved region, the K box, involved in protein – protein interactions, and MADS–box factors known to control similar processes also have a short stretch of homology at the C terminus.

Both *AGAMOUS* and *DEFICIENS* were found to be organ identity genes, each with a different expression pattern and a different function. The ABC model for floral organ identity (Coen and Meyorowitz, 1991), proposed that three domains of action, each active in two adjacent whorls, control floral organ identity. In the ABC model the A function alone gives sepals, A + B gives petals, B + C gives stamens, and C alone gives carpels. Genes in the A and C domains are mutually antagonistic, while the B domain is established independently (Figure 1.1). The *Antirrhinium* floral organ identity gene *DEFICIENS (DEF)* was identified as a B function gene required for petal and stamen formation (Sommer *et al*, 1990), and the *AGAMOUS (AG)* gene of *Arabidopsis* a C function gene required for stamen and carpel formation (Yanofsky *et al.*,1990), (Figure1.1). As other genes involved in floral morphogenesis were discovered and characterized, both from the two model species *Arabidopsis* and *Antirrhinum*, and from other plants such as petunia, tomato, and tobacco, phenotypes of organ identity gene mutants (Figure1.2) were generally found to be consistent with the ABC model. The model was further tested by controlled crosses between plants with different mutant phenotypes, by manipulation of flower structure in transgenic plants, such as in transgenic tobacco, (Mandel *et al.*, 1992; Davis *et al.*, 1996), and by ectopic gene expression, such as that in *Arabidopsis* (Mizukami and Ma, 1992). *In situ* hybridization also revealed expression patterns of organ identity genes consistent with those proposed

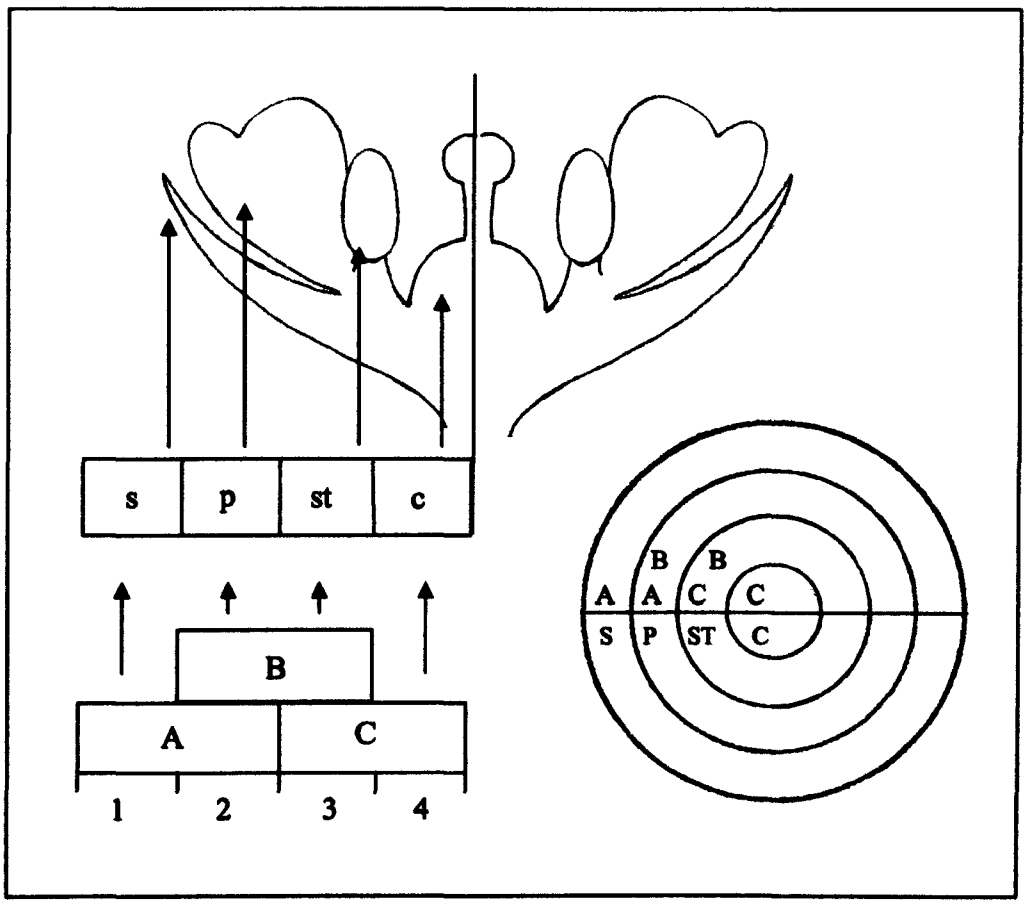


Figure 1.1. Floral organ induction: the ABC model of floral organ identity (after Coen and Meyerowitz).

Schematic representation of the four whorls of floral organs and the combinatorial interactions of the A B and C functions in a normal or wild type flower. s = sepal (whorl 1), p = petal (whorl 2), st = stamen (whorl 3), c = carpel (whorl 4).

in the ABC model (Schwarz-Sommer *et al.*,1990; Sommer *et al.*, 1991; Trobner *et al.*, 1992;Simon *et al.* 1994; Goto and Meyerowitz, 1994; Krizek and Meyerowitz, 1996).

Although genes involved in floral morphogenesis have now been characterized from many plant species, the greatest number has been isolated from *Arabidopsis thaliana.* As new information became available it was necessary to refine the ABC model to include D function for ovules (Colombo *et al.* 1995), and E function for the *SEPALLATA* genes (Pelaz *et.al.* 2000; Honma and Goto 2001; Theisen and Sadler, 2001). A number of the genes involved in floral morphogenesis are shown in Figure 1.3, with *Arabidopsis* genes in red and *Antirrhinum* genes in blue. Many interactions between gene products are required for floral morphogenesis, some promoting or initiating activity of other genes, some repressing activity of other genes, and some establishing boundaries between adjacent domains. Besides MADS-box genes, other types of genes that contribute to the building of a flower have been identified, that encode F box, Zinc finger, AP2 domain and Homeodomain (Lohmann and Weigel, 2002).

A key initiator of the floral meristem is the homeotic gene *FLORICAULA* (Coen *et al.*, 1990) and its orthologue *LEAFY* in *Arabidopsis* (Schultz and Haughin, 1991; Huala and Sussex,1992; Weigel and Nilsson, 1995). In initiating the flower *LEAFY* is expressed throughout the floral meristem and interacts with a number of other genes, including the functionally redundant *FRUITFULL, CAULIFLOWER,* and *APETALA 1* genes (Bowman *et al.*, 1993, Ferrandiz *et al.*, 2000). The *DEFH28* gene from *Antirrhinum* is thought likely to be an ortholog of *FRUITFULL* (Muller *et al.* 2001).

Both *LEAFY* (Weigel *et al.* 1992 and Weigel and Nilsson, 1995) and *APETALA* 1 (Mandel *et al.* 1992; Mandel and Yanofsky, 1995) can cause conversion from vegetative to floral meristem identity when expressed ectopically. The homeotic gene *APETALA 1,* as well as interacting with *LEAFY* to specify meristem identity, is involved along with *APETALA 2* in the determination of petals and sepals (Irish and Sussex, 1990; Mandel *et al.*1992; Bowman *et al.*,1993; Mandel and Yanofsky, 1995). The *Antirrhinum* homologue to *APETALA 1* is *SQUAMOSA*, but while involved in the establishment of the floral meristem *SQUAMOSA* does not play a part in establishing organ identity (Huijser *et al.*, 1992). *LEAFY* and *APETALA 1* together negatively interact with the Arabidopsis gene *TERMINAL FLOWER 1* to maintain the floral meristem (Liljegren *et al.*, 1999). In *Antirrhinum* the homologue of *TERMINAL FLOWER 1* is *CENTRORADIALIS* (Cremer

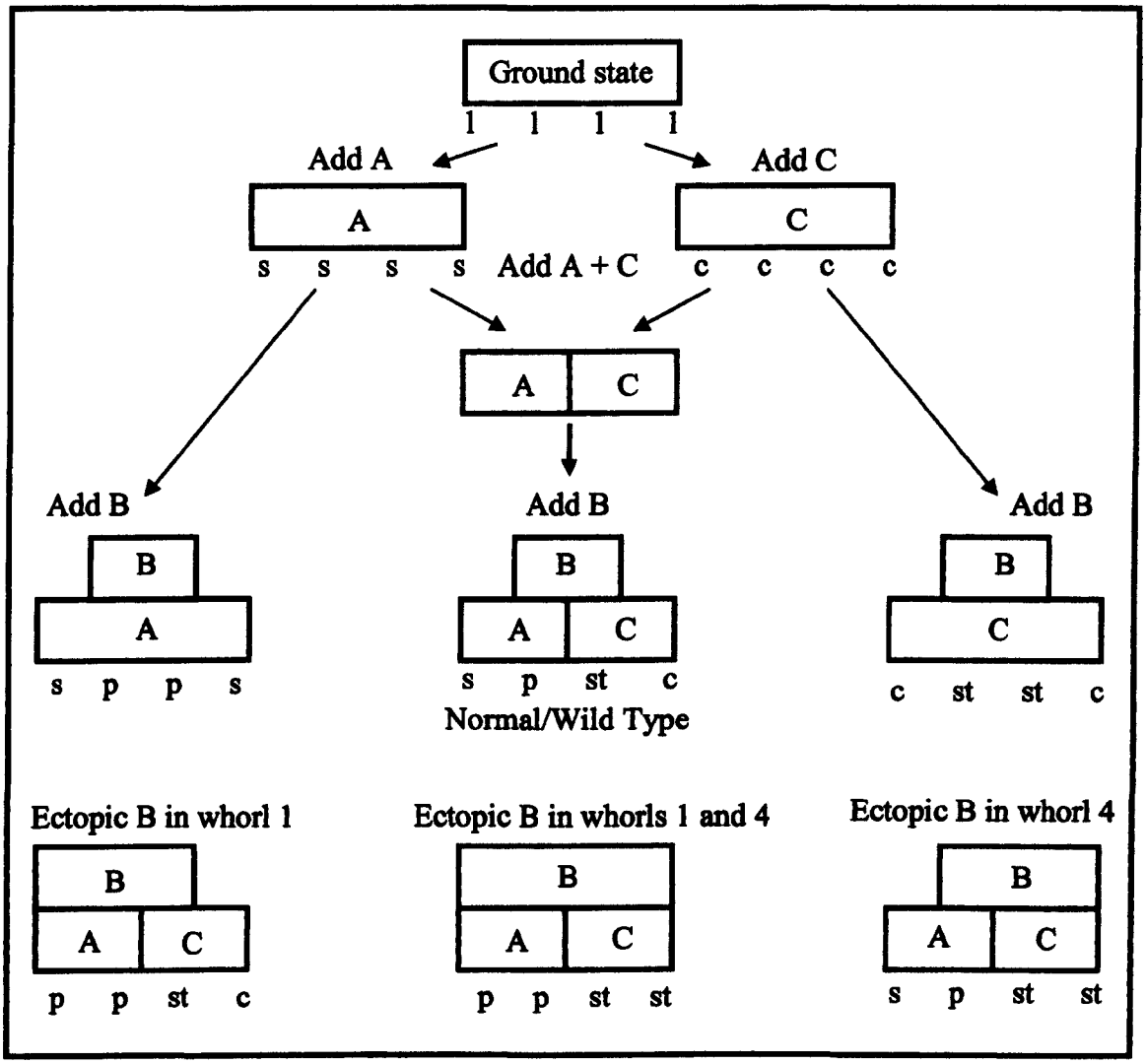


Figure 1.2. A model for floral organ induction (after Coen and Meyerowitz).

The model illustrates the combinatorial interaction of the A, B, and C organ identity gene functions in normal or wild type flowers and in various combinations that result in mutant phenotypes. The triple mutant without A, B, or C function remains as "ground state" or leaves. l = leaf, s = sepal, p = petal, st = stamen, c = carpel.

et al. 2001). Both mutants have a determinate raceme, rather than the normally indeterminate raceme found in these two species (Shannon and Meeks-Wagner, 1991; Bradley *et al.*,1996; Cremer *et al.*, 2001).

In *Arabidopsis* the *APETALA 1* gene product also has a role in establishing the A function, possibly via the formation of protein complexes that act as a transcriptional activator of the B function (Honma and Goto, 2001; Theisen and Sadler, 2001). The other *Arabidopsis* A function gene, *APETALA 2*, is not a MADS-box gene, and besides being involved in establishing organ identity it also negatively regulates C function in the first and second whorls (Drews *et al.*, 1991). A closely related gene *AINTEGUMENTA* shares this function (Elliot *et al.*, 1996; Klucher *et al.*, 1996; Krizek *et al.*, 2000) and may also have a function in determining petal identity (Krizek *et al.*, 2000). Recently two A function genes have been isolated from *Antirrhinum*, *LIP1* and *LIP2*, both of which must be inactivated to give a mutant phenotype. However unlike *APETALA 2*, *LIP* genes are not required for negative regulation of C function in whorls one and two (Keck *et al.* 2003). In Petunia, the gene *PhAp2A* that is considered to be the ortholog of *APETALA 2*, does not fulfill the same function in Petunia as in *Arabidopsis* (Maes *et al.* 2001) illustrating evolutionary functional divergence in A function organ identity genes.

The *Arabidopsis* B function genes that are required for petals in whorl two and stamens in whorl three have been identified as *APETALA 3* and *PISTILLATA* (Jack *et al.*, 1992; Goto and Meyerowitz 1994). It has been shown that these two genes are sufficient to specify B function in *Arabidopsis* (Jack *et al.*1994; Krizek and Meyerowitz, 1996). The *FIMBRIATA* gene in *Antirrhinum* shows extensive homology with *UNUSUAL FLORAL ORGANS* but differences in the functions and genetic interactions were found (Ingram *et al.*, 1995). Both *LEAFY* and *UNUSUAL FLORAL ORGANS* regulate the B function genes, Ectopic expression of *LEAFY* and *UNUSUAL FLORAL ORGANS* was demonstrated to activate *APETALA 3* and *PISTILLATA* outside the flower (Parcy *et al.*, 1998; Honma and Goto, 2000). Since neither *LEAFY* nor *UNUSUAL FLORAL ORGANS* is an absolute requirement for B function (Lohmann and Weigel, 2002), it has been suggested that in addition the MADS-box gene *APETALA 1* directly regulates *APETALA 3* by binding to the *APETALLA 3* promoter (Hill *et al.*, 1998; Tilly *et al.*, 1998; Lohmann and Weigel, 2002). *UNUSUAL FLORAL ORGANS* encodes an F-box-containing protein (Samach *et al.*, 1999) that belongs to a SKP1-cullen F-box (SCF) ubiquitin ligase

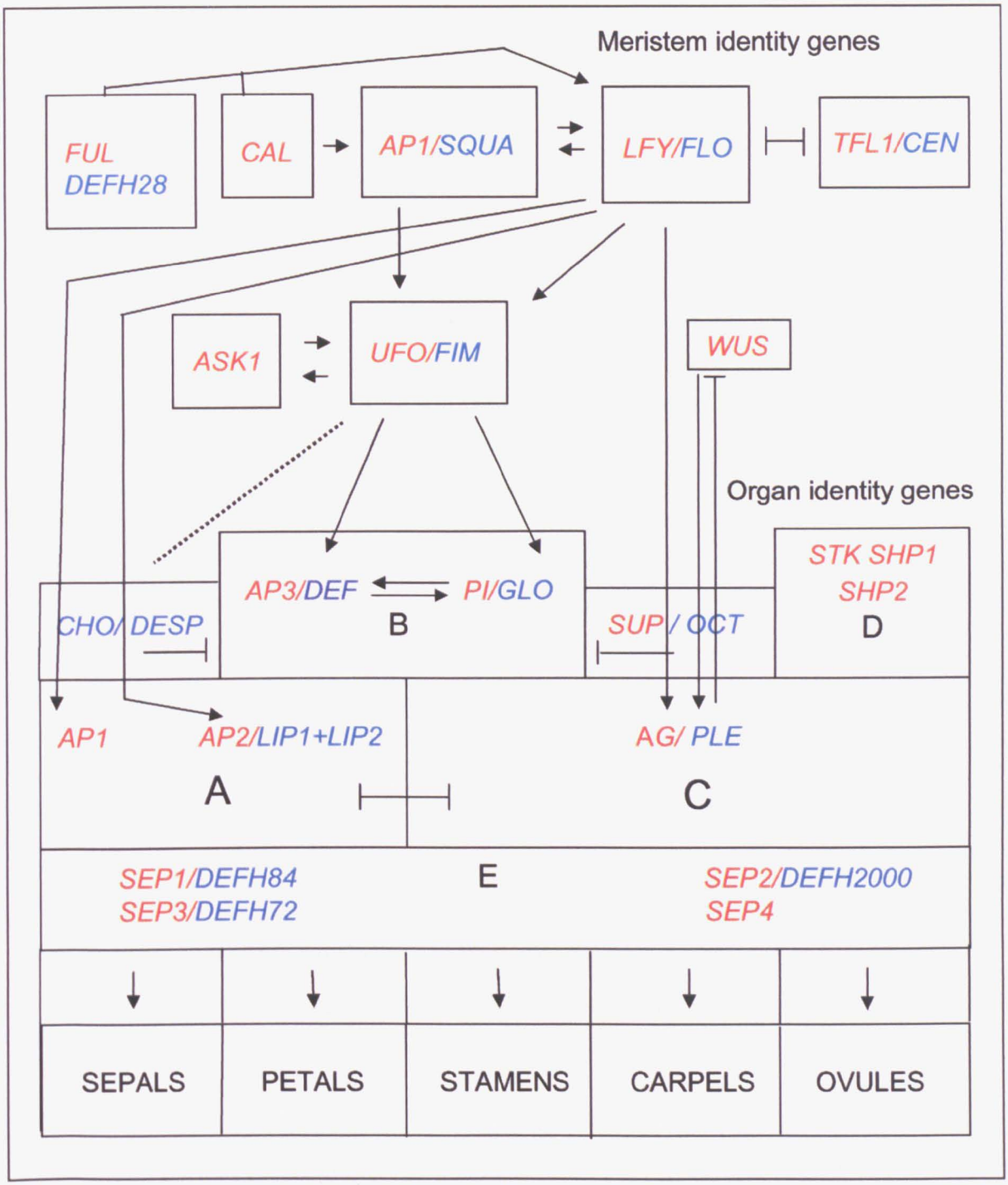


Figure1.3. Genes involved in floral morphogenesis.

Abbreviations. *FUL = FRUITFULL, CAL = CAULIFLOWER, SQUA = SQUAMOSA, LFY = LEAFY, FLO = FLORICAULA, TFL1 = TERMINAL FLOWER 1, CEN = CENTRORADIALIS, UFO = UNIFOLIATA, FIM = FIMBRIATA, WUS = WUSCHEL, CHO = CHORIPETALA, DESP = DESPENTEADO, AP3 = APETALA 3, DEF = DEFICIENS, PI = PISTILLATA, GLO = GLOBOSA, AP1 = APETALA 1, AP2 = APETALA 2, ANT = AINTEGUEMENTA, AG = AGAMOUS, PLE = PLENA, STK = SEEDSTICK, SHP 1 AND SHP2 = SHATTERPROOF 1 AND 2, SEP 1 2 AND 3 = SEPALLATA 1 2 AND 3, SUP = SUPERMAN, AND OCT = OCTANDRA.* (N.B. Not all genes known to be involved in floral morphogenesis could be shown above).

Activation or upregulation of genes is indicated by an arrow. At bottom of the figure, the resulting organ formed is also indicated by an arrow. Repression of genes is indicated by ⊢, and an interaction that is not yet fully understood is indicated by a connecting dotted line. Red = *Arabidopsis* genes; Blue = *Antirrhinum* genes.

complex (Ingram *et al.*, 1995, Lee *et al.*, 1997) and may possibly act in degradation of negative regulators of *APETALA 3* (Ingram *et. al.*, 1997; Zik and Irish, 2003). Two such negative regulators of *DEFICIENS* and *GLOBOSA* have been identified in *Antirrhinum*; these are *CHORIPETALA* and *DESPENTEADO* that may be a target of degradation by the F-box protein *FIMBRIATA* (Wilkinson *et al.*, 2000). In order to regulate B function in *Arabidopsis* UNUSUAL FLORAL ORGANS interacts with ASK 1, another SCF component (Zhao *et al.*, 2001). Recent results suggest that it is a larger SCF complex that besides proteins from *LEAFY* and *UNUSUAL FLORAL ORGANS* includes products from two further genes *AtCUL1* and *AtRbx1* that positively regulates B function (Ni *et al.* 2004). *SUPERMAN* is required for repression of B function in whorl four in *Arabidopsis* (Bowman *et al.*, 1992; Sakai *et al.*, 1995; Yun *et al.*, 2002) and *OCTANDRA* is required in *Antirrhinum*. *SUPERMAN* in turn is under the control of *LEAFY* through *APETALA 3 – PISTILLATA* dependent and independent pathways (Sakai *et al.*, 1995).

The *SEPELLATA* 1, 2, 3, and 4 MADS-box genes, have also been shown to be a requirement for the specification of floral organs (Pelez *et al.*, 2000, Honma and Goto, 2001, Ditta *et al.*, 2004, Castillejo *et al.*, 2005) and have been designated as E function genes (Theisen and Saedler, 2001). The four *SEPELLATA* genes are each functionally redundant but the triple mutant lacking *SEPALLATA* 1, 2, and 3 has flowers composed only of sepals, and the quadruple mutant has indeterminate flowers composed only of leaf like organs (Ditta *et al.*, 2004). Conversion of leaves into petals was achieved by ectopically expressing *SEPELLATA* genes with the A and B functions (Pelaz *et al.*, 2001). The *SEPELLATA* gene products interact with the ABC MADS-box gene products (Davies *et al.*, 1996, Egea-Cortines *et al.*, 1999, Honma and Goto 2001, Causier *et al.*, 2003, Castillejo *et al.*, 2005). The formation of ternary complexes led to the proposal of the "quartet model" of floral organ specification (Theisen and Saedler, 2001).

The C function organ identity gene *AGAMOUS* is expressed early in the apex of the developing flower (Mizukami and Ma, 1997) and specifies determinacy of the floral meristem in addition to organ identity. Expression is regulated by *LEAFY* (Weigel and Meyorwitz, 1993) and a homeodomain containing gene *WUSCHEL* (Zik and Irish, 2003; Jack T., 2004). Another gene, *AtGCN5*, may also be required to regulate floral meristem activity through the *WUSCHEL - AGAMOUS* pathway (Lohmann *et al.*, 2001; Bertrand *et al.*, 2003). Once established, *AGAMOUS* then represses *WUSCHEL* (Lenhard *et al.*, 2001). This interaction, along with *HUA1*, *HUA2*, and *HUA ENHANCER 1*, *HUA ENHANCER 2* and *HUA ENHANCER 4* activity is required to make the floral meristem

determinate (Li *et al.*, 2001; Chen *et al.*, 2002; Jack 2002; Zik and Irish, 2003). C function is required for stamens in the third whorl and carpels in the fourth whorl. However as *LEAFY* is expressed in all whorls, *AGAMOUS* in turn has the potential to be expressed throughout the floral meristem. Restriction of *AGAMOUS* to the third and fourth whorls is dependant on a number of genes including negative regulation by the A function gene *APETALA 2*. Two genes, *AINTEGUEMENTA* (Krizek *et al.*, 2000) and *STERILE APETALA* act redundantly with *APETALA 2* (Elliot *et al.* 1996; Klucher *et al.*, 1996; Krizek *et al.*, 2000). *LEUNIG* (Liu and Meyerowitz, 1995) along with SEUSS (Franks *et al.*, 2002) has also been shown to regulate *AGAMOUS*; and *CURLY LEAF* acts redundantly with *INCURVATA* to repress *AGAMOUS* in both flowers and vegetative tissue (Goodrich *et al.*, 1997; Lohmann and Weigel, 2002).

In *Antirrhinum* there are two C function genes, *PLENA* (Bradley *et al.*, 1993) and *FARINELLI* (Davies *et al.*, 1999) that, unlike *AGAMOUS*, have redundant negative control over the B function genes *DEFICIENS* and *GLOBOSA*. Although *AGAMOUS* and *PLENA* are functional homologues, studies of *AGAMOUS* like genes from fifteen diverse angiosperm species show that *PLENA* and *AGAMOUS* are not orthologs but have come from separate paralogous lineages (Kramer *et al.*, 2004).

The first ovule identity genes *FBP7* and *FBP11* were discovered in Petunia, and these have been designated as D function genes (Angenant *et al.*, 1995b; Colombo *et al.*, 1995). More recently three ovule identity genes have been identified in Arabidopsis, *SEEDSTICK, SHATTERPROOF 1* and *SHATTERPROOF 2* (Pinyopich *et al.*, 2003; Favaro *et al.*, 2003).

Investigation of genes involved in floral morphogenesis from many different plants has provided valuable insight into flower development in general. This has revealed both evolutionary conservation and evolutionary divergence of developmental pathways (Winter *et al.*, 2002; Zhang *et al.*, 2004). Studies of angiosperm flowers with different flower structures, such as the two model species above - *Arabidopsis* with actinomorphic flowers and free petals and *Antirrhinum* with zygomorphic flowers and a gamopetalous corolla - provided opportunity for the discovery of the genes *CYCLOIDEA* and *DICHOTOMA* involved in dorsoventral symmetry (Luo *et al.*, 1996; Luo *et al.*, 1999). Other angiosperm plants exist that differ in the number, architecture and arrangement of floral organs. From studies of *Antirrhinum*, *Arabidopsis*, *Petunia*, and other species similar genes have been found to carry out distinct functions, and in some cases the same

function is determined by different numbers of genes. Further study on plants with different flower structures is essential in order to understand the differences and complete the picture of floral morphogenesis.

The British *Primula* species *Primula vulgaris, Primula veris,* and their derived cultivars differ considerably from both of the two model species above. The gamopetalous corolla of *Primula* is actinomorphic, the stamens are epipetalous, and the flowers are heteromorphic. The *Primula* breeding system has been of interest since the time of Darwin, and mutant flower phenotypes that segregate with either the pin or thrum alleles of the *S locus* have been identified by the author. In addition a number of naturally occurring mutant forms have been collected by the author many of which are consistent with what might be expected from mutations in homeotic genes controlling organ identity. *Primula* is therefore an excellent candidate for further study of floral morphogenesis, both because new information gained from analysis of *Primula* could contribute to understanding of the variations on the ABC model, and because knowledge of floral homeotic genes from other species could enable the identification of genes responsible for the floral homeotic mutations in *Primula.* Such investigation could lead to the identification of genes linked to the *S* locus and enable the first molecular genetic analysis of this locus.

1.3. The *Primula* breeding system.

Primula differs from other species previously studied by molecular means in that many *Primula* species are heteromorphic, with heterostylous flowers, a sporophytic di-allelic incompatibility system and an associated pollen dimorphism. Although heterostyly has been reported from 25 different angiosperm families, *Primula* is the genus that has the largest number of heteromorphic species. Those plants with flowers that have the stigma visible at the mouth of the corolla tube and the anthers approximately half way down are known as "pin" plants. Plants with flowers that have the anthers visible at the mouth of the mouth of the corolla tube and the stigma approximately half way down are known as "thrum" plants (Fig.1.4). The thrum characters of short style, large pollen, and anthers visible at the mouth of the corolla tube are dominant to pin characters of long style, small pollen and anthers half way down the corolla tube. The incompatibility mechanism is stronger in thrums than in pins; thrum x thrum crosses are completely, or almost completely incompatible, while pin x pin crosses have much reduced fertility. Pins are homozygous for the recessive allele, normally written as *ss*, but thrums are heterozygous

for the dominant allele, *S*, written as *Ss*. Thrum homozygotes, *SS*, are extremely rare due to the presence of a lethal gene associated with the dominant S locus (Richards 1986,1993, 1997). All *Primulas* studied in this project are diploid. Awareness of heteromorphy in *Primula* flowers dates at least from the time of Clusius, (Clusius,1583), and has been of scientific interest since the time of Darwin, (Darwin, 1876). Even before the rediscovery of Mendels' work, Darwin (Darwin, 1876) had begun breeding experiments with *Primula vulgaris* and had concluded that the reciprocal placing of the anthers and stigma in pin and thrum plants was conducive to outcrossing between the two morphs. Henslow described a self fertile *P. vulgaris* with anthers and stigma at the mouth of the corolla – the form that is now known as a long homostyle (Henslow, 1878). In 1905 Bateson and Gregory published a paper on the inheritance of heterostyly in *P. sinensis* stating that the long style in that species was a recessive character and the short style a dominant character. Ernst's results (Ernst, 1925, 1936), from working with *P. hortensis* and *P. viscosa* indicated that *S* was not as he had thought a single gene, but a tightly knit suite of at least three separate genes with only rare recombination events between them. These three genes are represented as *G* for the gynoecium, *P* for pollen size, and *A* for the androecium position. Recombination between *G* and *PA* would result in the long homostyle with both anthers and stigma at the mouth of the corolla tube and in a short homostyle with both anthers and stigma half way down the corolla tube. A number of populations of *P. vulgaris* in Somerset contain varying proportions of long homostyle plants have been the subject of much interest and study (Crosby, 1949; 1959, Bodmer, 1958; 1960, Charlesworth, 1979, and Curtis et al. 1985). Ernst originally gave the gene order as *GAP*, but Lewis and Jones (1993) on re-evaluating his data concluded that the order must be *GPA* as the former gene order would require a double cross-over in the thrum parent to generate the most common recombinants. From Ernst's studies the map distance between *G* and *P* was estimated to be 0.19 and between *P* and *A*, 0.37. Lewis and Jones also listed the four anomalous phenotypes obtained by Ernst in some of his crosses. Twenty-seven anomalous plants in all were obtained from crosses involving long or short homostyles, twenty-one of which appeared as mutations from the recessive allele to the dominant (*gp* to *GP*, and *a* to *A*). Ernst's data also showed a significant deficit of short homostyles, and it was concluded that this was due to low viability of the short homostyle morph. In 1956, Dowrick, working on *P. obconica*, expanded the component parts of the *S locus* to *C-G-S-Is-Ip-P-A*, where *C* is the thrum area of conducting tissue, *G* is the style length of thrum, *S* is the papilla length of thrum,

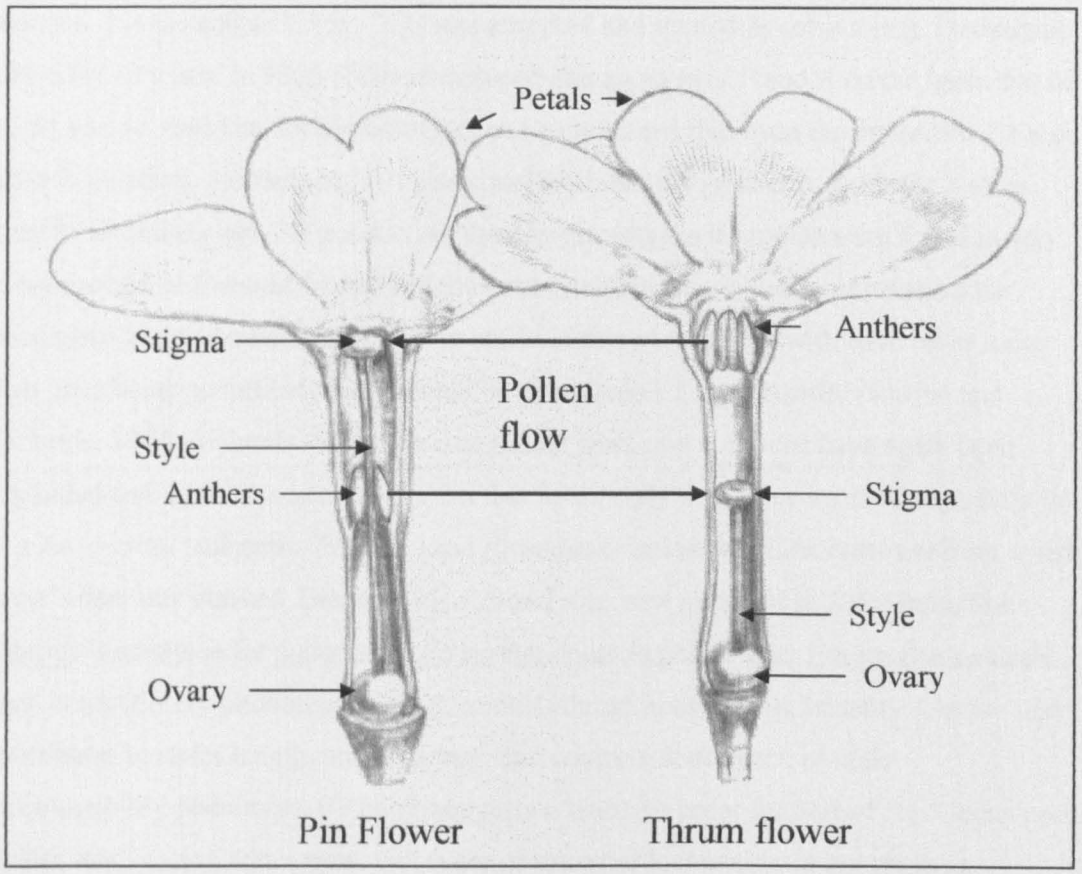


Figure 1.4. Pin and Thrum *Primula* flowers (from Darwin, 1861) annotated to show the parts of the flower and pollen flow between the two morphs.

There are visible differences between the two morphs:

Pin flowers have long styles and the stigma at the mouth of the corolla tube. Thrum flowers have short styles and the stigma approximately half way down the corolla tube. Pin flowers have anthers approximately half way down the corolla tube. Thrum flowers have anthers at the mouth of the corolla tube.

Is is the thrum style incompatibility, *Ip* is the thrum pollen incompatibility, *P* is the pollen size and *A* is the anther height. This was accepted and quoted as correct (e.g. Darlington, 1973) for 30 years. In 1986 Richards reduced this again to *G P* and *A* on the basis that he could find no evidence for the other loci and commented that even the order of *GPA* was open to question. Stebbins in 1971 had concluded that the gene cluster for the *S* locus must be extremely old. He pointed out that the phenotypes it produces are found in very many species of *Primula* throughout the northern hemisphere and he considered the possibility of the characteristics arising and becoming associated with each other more than once being so unlikely that it could be disregarded. More recently (Kurian and Richards, 1997; Richards 1997), the component parts of the *S* locus have again been expanded and it has also been suggested that heterostyly may have evolved separately in *P. x tommasinii* (subgenus *Primula*) and *Primulas* of subgenus *Auriculastrum* from which Ernst's data was derived. Besides *G/g, P/p* and *A/a,* now included is, *Mpm/mpm* that controls dominance for pollen size, *Pp/pp* that controls pollen size, *Pm/pm* that controls male compatibility phenotype, *L/l* that controls thrum homozygote lethality, *Gm/gm* that contributes to stylar length, and *Mpp/mpp* that controls dominance of male incompatibility phenotype. Richards suggests a tentative order for part of the *S* locus gene cluster, as *G/g, A/a, Mpm/mpm* with order of the other loci uncertain but given as possibly *Pp/pp, Pm/pm, Mpp/mpp, l/L,* and *Gm/gm* (Richards 1997). There have been reported differences in the position of the *S* locus in different species of *Primula,* in that in *P. obconica* it is 3% from the centromere and in *P. sinensis* it is completely linked (Lewis and Jones, 1993).

There are features of the *Primula* breeding system that have not yet been indisputably verified, at least not in relation to *P. vulgaris, P. veris,* and their cultivars. The suggested order of loci, *GAP,* for the polyanthus, (Kurian and Richards, 1997) if correct is likely to be the same for *P. vulgaris* and *P. veris* in which the polyanthus had its origin. However, the data was from a smaller sample than that done by Ernst, whose results suggested the order of *GPA* (Ernst,1925,1936). The position of the hypothesized lethal gene, or lethal factor, is also problematic. Richards suggested that the current model for evolution of heterostyly at that time involved *G* becoming linked to a recessive lethal gene (Richards, 1993). Certainly even heterozygous short homostyles *Gap/gap,* are reported to be less viable than heterozygous long homostyles *gAP/gap,* (Ernst, 1957, quoted by Lewis and Jones, 1993), and unlike long homostyles, have not been found in wild populations of *P. vulgaris.* Self-pollinated short homostyles yield one third of the progeny as pins and two thirds as short homostyles (Richards, pers. com.) which would also indicate that the

homozygote might be inviable. Yet results from the work done by Kurian on the Polyanthus suggests that there is a lethal factor associated with *P* (Kurian and Richards, 1997). One problem with mapping the *S* locus by classical genetic techniques has been the absence of suitable outside markers sufficiently closely linked to the *S* locus to obtain indisputable results. It is only molecular analysis that will in time resolve both the question of gene order and the question of whether the *S* locus evolved separately in different *Primula* species or is instead a very old and conserved gene cluster.

1.4. British species of *Primula*.

Although there are over 400 species of *Primula* worldwide, only five species of *Primula* are native to Britain. These are *Primula vulgaris*, the primrose; *P. veris*, the cowslip, *P. elatior*, the oxlip, *P. farinosa*, the bird's eye primrose, and *P. scotica*, the Scottish bird's eye primrose. The two latter species have pink flowers, farinose leaves and chromosome numbers that differ both from each other (2n= 18 and 2n=54 respectively) and from the three other species of native British Primula, so that they can not interbreed (Richards, 1993). The first three all have the same chromosome number (2n=22) and can interbreed to give fertile hybrids. (For details of hybridization between the species see Valentine, D. H. 1947, 1951, and 1955). These three are normally yellow in colour in the wild (Fig.1.5) but old records (Gerard 1597, Parkinson, 1629) also mention white and green forms.

The primrose has the most widespread distribution in the wild, followed by the cowslip, (Richards, 1989), and where they grow together hybrids occur. The *P. veris x vulgaris* hybrid is commonly called "false oxlip" and it is this hybrid that was the forerunner of the garden polyanthus. The true oxlip is restricted in the wild to a small area of East Anglia so has been less widely used for hybridization in the development of commercial cultivars. *P. vulgaris, subspecies sibthorpii* with flowers in pinks reds and purple, was introduced from Eastern Europe in 1640 and commonly used for hybridization in the past. More recently, the discovery of *P. juliae* in the Caucasus in 1900 resulted in the development of the "Wanda" primroses, initially through hybridization of *P. juliae* with our native *P. vulgaris*. Commercial cultivars today are the result of much selective breeding. Not only have they been selected to have a wide range of flower colour, but most have also been rigorously selected for large flower size, high seed yield, large seed size, high and uniform germination, summer germination, uniform growth rate, and uniform flowering time. Some lines will also be expected to have a more uniform genetic background than the *wildtype* species. The wild primrose *P. vulgaris* flowers in spring,

Figure 1.5. Wild British *Primulas*.

There are three wild *Primula* species that can interbreed. A typical wild interspecific hybrid between *P. veris* x *P. vulgaris* is also illustrated..

A. *P. veris*, Cowslip; **B.** *P. elatior*, Oxlip; **C**. *P. vulgaris*, Primrose; **D**. *P. veris x vulgaris*, often called the "False Oxlip". Size bars are approximately 1cm.

with the greatest abundance of wild flowers usually found from late March through to late April. The wild cowslip *P. veris* flowers slightly later, but the flowering time of the two frequently overlaps, and where they grow together natural hybrids between the two species can usually also be found in the wild. Commercial cultivars, although predominantly spring flowering by nature, frequently also produce flowers in the autumn if environmental conditions in the garden are favorable. All will flower for the first time at almost any time of the year if conditions are favourable, depending on when the seed was sown and the maturity of the seedlings. In their second year they become spring flowering, as is the norm.

1.5. Mutant phenotypes of *Primula;* a historical perspective.

All of the mutant phenotypes of *Primula* have occurred naturally. The first *Primulas* grown in gardens were grown for their herbal properties (Gerard, 1596; Culpepper, 1740), but when flowers first began to be grown for ornament many mutant phenotypes of *Primula* were collected, along with other natural wild flower variants, in order to provide variety in the garden. At that time other foreign species had not yet been widely introduced and the possibility of using decorative "bedding plants" was undiscovered. Consequently many descriptions and illustrations of mutant phenotypes of *Primula* can be found in literature dating back to the sixteenth century. Although most of these phenotypes can still be found today, they have always been scarce. Cannell's catalogue of 1880 lists *Hose in Hose* (that has the calyx converted to petals) at five shillings each and *Jack in the Green* (that has the calyx converted to leaves) at three shillings each, which even then was expensive. Today, those few nurseries that still sell the rare *Primula viridis,* the green primrose, generally charge from £5 to £7 per plant. New mutant phenotypes do also appear from time to time in populations of commercially produced plants. Some of the phenotypes found have been previously recorded, but had been lost to cultivation for some time; a few others have never been previously either recorded or illustrated. At least half of the phenotypes used in this project are consistent with what might be expected from homeotic mutations in organ identity genes. One has organs in the correct position but has alteration to the usual form of the organ, and two are pleiotropic mutant phenotypes with abnormal flowers in addition to other observed effects.

1.5.1. *Primula* homeotic mutants.

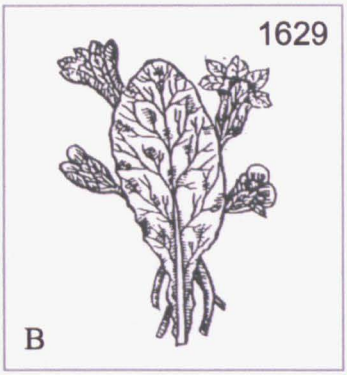
Of the twelve phenotypic mutants available, *Jack in the Green* (sepals converted to leaves) *Hose in Hose* (sepals converted to petals), *Staminoid Carpels* (with partial

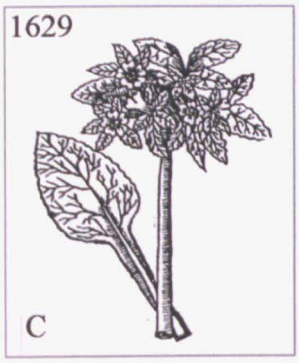
Figure 1.6. *Jack in the Green* (leafy sepals).

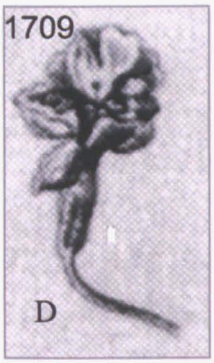
The *Jack in the Green* has leafy sepals and has been illustrated for the past four centuries; the form is still available today.

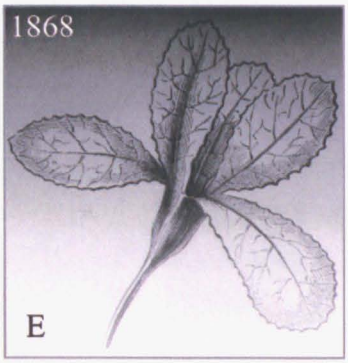
A. *Jack in the Green* plant in flower. **B.** The *Jack in the Green* primrose as illustrated by Parkinson, 1629. **C.** This polyanthus form was also illustrated by Parkinson, 1629, but although it clearly shows leafy sepals it is described in the text as having calyces and bracts that are a mixture of leaf and petal. The name given is "Jackanapes on horseback" – Jackanapes being the name commonly given to plants with calyces of leaf and petal mixture, and "on horseback" referring probably to the fact of the flowers being on a scape with a circle of enlarged leafy bracts below the pedicles. **D.** This is one of the many flowers illustrated in Nuremberger Hesperides, 1709, a *Jack in the Green* flower. **E.** This accurately observed drawing of the *Jack in the Green* calyx is from Masters' Vegetable Teratology", 1868. **F.** This plant from Primroses, Cowslips Polyanthuses and Oxlips, by Philanthos, 1874, is described as "Jackanapes on horseback" and is similar to the plant illustrated by Parkinson in **C** above. The leafy bracts at the top of the scape are clearly illustrated, but while the description comments on the large foliaceous bracts there is no mention of any petal in the calyces or bracts. **G.** The plants in Cannells catalogue of 1880 were all drawn in a very stylized manner. This *Jack in the Green* was advertised for sale at three shillings per plant! Size bar in **A** is approximately 1 cm.


A

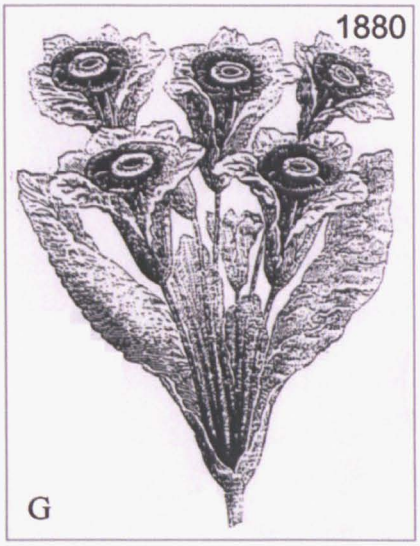

1629
B


1629
C


1709
D


1868
E


1874
F


1880
G

or complete conversion of carpels to stamens), *sepaloid* (two to four whorls of sepals without any petals or stamens) and *double* (with normal sepals and indeterminate whorls of petals), can all be described as organ identity homeotic mutants. In addition the green primrose and the virescent cowslip each have a phenotype consistent with might be expected from mutations in other homeotic genes involved in flower development, rather than in the organ identity genes themselves. References to and illustrations of *Jack in the Green* (Fig.1.6A) can be found from 1629 onwards (Parkinson, 1629). Parkinson's *Jack in the Green* was a primrose, but he also illustrated the "Jackanapes on horseback", which from the description, carries both the mutation that gives *Jack in the Green* and the mutation that gives *Hose in Hose*. This was a scapose plant that may have been a *P. veris x vulgaris* hybrid, or could have been a cowslip *Jack in the Green* (Fig.1.6C), but there has been no specific mention of the latter in the old literature. Gerard too in 1597 described a "Jackanapes on horseback", but his description is different and does not mention a foliaceous calyx (Gerard, 1597). The illustration of a polyanthus in the Leyden catalogue of 1678 is that of a *Jack in the Green* and it bears great similarity to a pressed specimen from Bobart's Oxford herbarium (Duthie, 1988). Both have transformation of the bracts at the base of the pedicles to leaves as well as of the calyces to leaves. I have frequently found this to occur in progeny of *P. veris* x *P. vulgaris Jack in the Green* (see Chapter 4). Other references to the phenotype include Rea, (1665), Masters (1868, 1877), and more recently Genders, (1959), and Fish (1967). An entire page of illustrations of varying degrees of leafiness of *Jack in the Green* calyces can be seen in Tanners book of Woodland Plants (Tanner, 1981). Mention of the phenotype in the wild can also be found in some old floras such as, for instance, White's Bristol Flora that records a *Jack in the Green* found near Easton in 1883 and another found near Shepton Mallet in 1900 (White, 1912). A selection of illustrations of the phenotype throughout history can be seen in Figure 1.6B – G. References to and illustrations of *Hose in Hose* (Fig.1.7.A) can be found from the seventeenth century when Crispin de Passe (1614) illustrated *Hose in Hose* cowslips, (Fig. 1.7B). Parkinson (1629) illustrates *Hose in Hose* Oxelips (i.e. the cowslip, *P. veris* x primrose, *P. vulgaris*, hybrid) (Fig.1.7C). The first reference to a *Hose in Hose* primrose is found in Rea, where a red one is described (Rea, 1665). *Hose in Hose* cowslips are also mentioned by Rea, and again by Bradley (1724), Newton (1752), (Fig.1.7E), The New Botanic Garden, (1812) and Loudon (1822). It is not known whether the flowers illustrated in Nuremberger Hesperides (Hawkes, 1997) were from primrose or polyanthus plants as only the flowers are shown in each case (Fig.1.7.D). The majority of

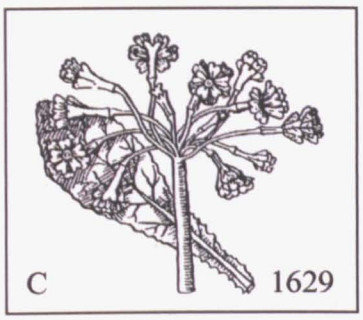
Figure 1.7. *Hose in Hose* (petaloid sepals).

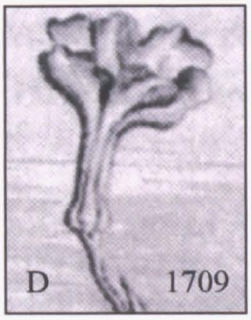
Hose in Hose has part or all of the calyx converted to petal. It has been illustrated for the past four centuries and the form is still available today.

A. Flowers of a *Hose in Hose* polyanthus. **B.** A *Hose in Hose* cowslip from Crispin de Passe, 1614. **C.** *Hose in Hose* "oxelips", from Parkinson 1629. It is generally accepted that these were "false oxlips", hybrids between *P. veris* and *P. vulgaris* rather than the true oxlip, *P. elatior*, that is only found in the wild in an area of East Anglia. **D.** A *Hose in Hose* flower from Nuremberger Hesperides, 1709. **E.** *Hose in Hose* cowslips from Newton's herbal, 1752. Although the plates in this herbal are small, it can be seen that this *Hose in Hose* cowslip does not have the calyx as flower like as in the other old illustrations. **F.** This *Hose in Hose* plant is from Primroses, Cowslips Polyanthuses and Oxlips, by Philanthos, 1874. **G.** *Hose in Hose* polyanthuses from Cannell's catlogue of 1880 drawn in a stylized manner. The plant in **G** was advertised for sale at five shillings per plant! Size bar in **A** is approximately 1 cm.

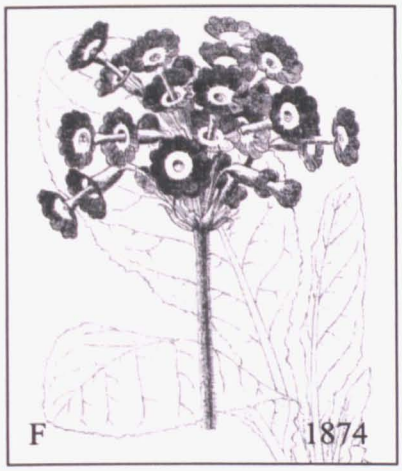

A

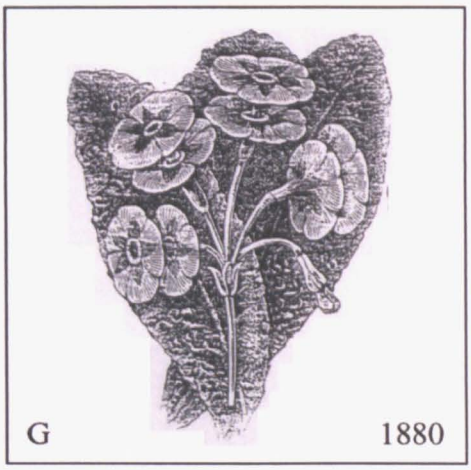

1614
B


C
1629


D
1709


E
1752


F
1874


G
1880

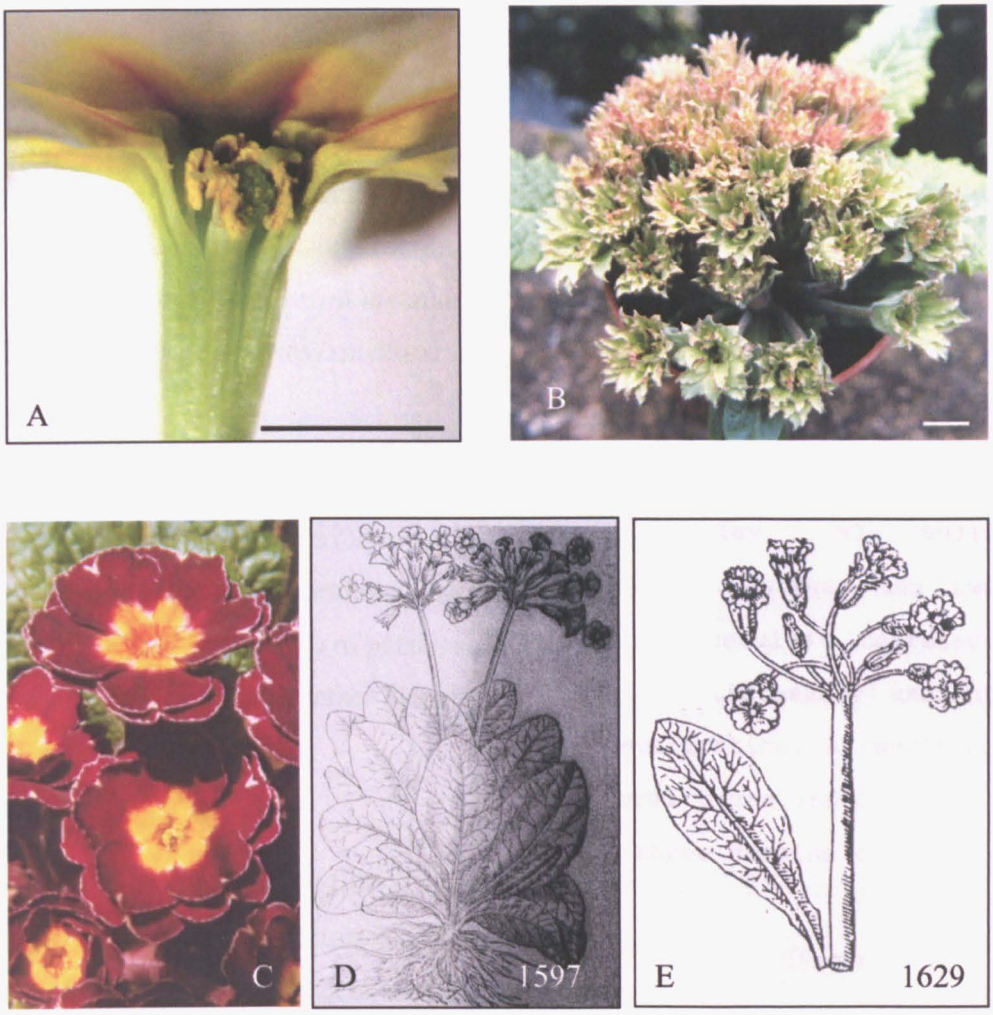


Figure 1.8. *Staminoid Carpels*, *sepaloid*, and *semi-double*.
Not all mutant phenotype have been illustrated in the past, and some of those that have been illustrated have been classified differently in the past to how they would be classified today.

A. *Staminoid Carpels*; there are no references to or illustrations of this phenotype in old literature. **B**. A *sepaloid* plant in flower. There are no references to or illustrations of this phenotype in old literature. **C.** A *semi-double* with organ order of sepal, petal, petal, stamen, stamen (indeterminate). **D** and **E**. Two plants that were both described as "double cowslips hose in hose", by Gerard 1597 and Parkinson 1629 respectively. However in both instances the flowers are illustrated as having calyces as well as two whorls of petals so they are not true *Hose in Hose* with sepals converted to petal. The organ order suggests instead that these are semi-double cowslips. Size bars are approximately 1 cm.

later references and illustrations throughout history are of *Hose in Hose* polyanthuses (e.g. Fig.1.7F and G), but later horticultural works by Genders (1959) and Fish (1967) list both primrose and polyanthus forms. Occasional references to *Hose in Hose* found in the wild can be found in old floras, but even that mentioned in White's Bristol Flora is of one with three flowers on a stalk found near Backwell Hill (White, 1912).There have been no illustrations of either *Staminoid Carpels* (Fig.1.8C.) or *sepaloid* (Fig.1.8D.) mutant phenotypes found in the literature. Meyer (1966) described sepalody as "a relatively uncommon abnormality" and described only two instances of sepaloid petals reported in *Primula*, one in *Primula auricula* and another in *Primula kewensis*. In both of these plants only whorl two was converted to sepals.

There has been some confusion in the past regarding flowers with two whorls of petals. These were sometimes referred to as "Double cowslips two in a hose" (Gerard, 1597). However some of the illustrations (Fig.1.7F and G) clearly show plants with a normal calyx as well as two whorls of petals, so these must have been the form that we call *semi-double* today (Fig. 1.7E), rather than the true *Hose in Hose* that have the calyx converted partly or wholly to petal.

The *double* phenotype (Fig.1.9A) is very well documented in both old and recent literature. Both *double* primroses and *double* cowslips were recorded and illustrated by Gerard (1597), but the woodcut used for the primrose is clearly that of a polyanthus (Fig.1.9B). This apparently was because woodcuts were expensive to produce and if something approximating to the form could be borrowed it helped to keep the cost down. The *double* cowslip was illustrated by Crispin de Passe (1614) (Fig. 1.9D) and again by Parkinson (1629), who also illustrated the *double* primrose (Fig.1.9E). At first *double* primroses were described simply by colour and from Rea (1665) we discover that the *double* red primrose was the rarest. Before there was any knowledge of inheritance or of classical genetics, *Primula* plants had to be propagated either by division or by sowing naturally produced seed. Fertile dominant phenotypes would produce a proportion of the progeny of the same form as the parent from seed. However *doubles* are not normally fertile and so were simply collected and propagated by division whenever they occurred. This is a slower process and they were sufficiently scarce to simply be known by the colour. Some of the 18th century illustrations of *double* primroses can be seen in Fig.1.9 F – H. The New Botanic Garden (1812) lists five different colours of *double* primrose but

Figure 1.9. *doubles.*

There are numerous illustrations of *double Primulas* from the past, both in primrose and in cowslip form..

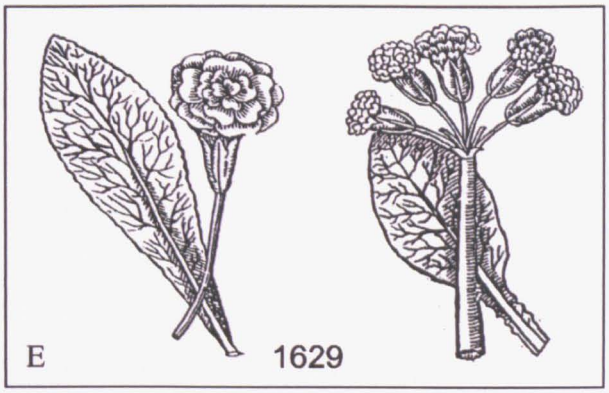
A. A *double* primrose flower. **B.** The *double* white primrose of Gerard 1597.However as can be observed this plant has a scape so is clearly not a primrose! At this time in history many woodcuts, expensive to produce, were frequently re used, even if not exactly correct. **C.** The *double* cowslip of Gerard, 1597. **D.** The double cowslip of Crispin de Passe, 1614. **E.** The *double* primrose (left) and *double* cowslip (right) of Parkinson 1629. **F.** The *double* primrose of Newton, 1752. **G.** The *double* primrose of Hale, 1757. **H.** An early coloured illustration of a *double* primrose *"lilacena plena"*, from Curtis botanical magazine, 1794. This cultivar is reputedly still availablc, but there is no way of being sure of its authenticity although the present day plant does look very similar. Size bar in **A** is approximately 1 cm.


A


6 Primula veris flore pleno.
Double white Primrose.
B
1597


1597
C


1614
D


E
1629


F
1752


G
Double Primrose
1757


H
1794

Louden (1822) was able to list eight different colours, such as for instance the *double* red and the *double* paper white primrose. Towards the end of the century fashion changed to the use of latin names – but still using colour as a criteria. Robinson in1883 lists six double primroses including alba plena, lilacena plena and sulphurea plena. The *double* cowslip was still around at this time and was mentioned by Ellacombe (1884), but it disappeared from cultivation soon afterwards. As travel increased *double* primroses were brought back from the continent, one of the first being Arthur Dumoulins a mauve *double* introduced to Britain around 1880. This was also one of the first individual cultivar names to appear. Another was Marie Crousse, introduced from France, and surprisingly, the latter is still available today. Breeding of *double* primroses only began around the turn of the century. A range of *double* primroses were developed by Murray Thompson but these have now died out. The Cocker brothers developed the "Bon Accord" *doubles* and a few of these are still available today, but are becoming increasingly scarce. More recently the Barnhaven *doubles* were developed in the 1960's and with the advent of micro propagation many *double* primroses are now freely available. The *double* cowslip in contrast disappeared completely for some time. There was a record of an accession by the University of Bristol Botanic Garden in 1982 of *P. veris, double* white, but this has not survived. It was not until the 1990's that Geoff Nicolle, a Primula Society member recreated the *double* cowslip, presumably by hybridization and introgression; this has now been micro propagated and is again available. Occasionally a *double* primrose is still found in the wild, a record in the Flora of Sussex, (1937), records that one with double flowers was found at Bathwick Wood near Hastings.

In the *green primrose* (Fig.1.10A) the transformation of organs is incomplete so that the petals retain the shape and appearance of petals while being green and having some leaf like characteristics. Similarly the lower part of the style also takes on leaf like characteristics. Green primroses and cowslips, both in single and double form, appear frequently in old literature. Gerard (1597) illustrates the green primrose (Fig.1.10B), and describes the flowers as being "somewhat welted about the edges". Dodoen, (1619), mentions green primroses both single and double, and Parkinson (1629), describes both green primroses and green cowslips, single and double. His double green primrose, on the left of Fig. 1.10C. is described as having two "rowes" or whorls, of green leaf like petals. His green cowslip, on the right of Fig. 1.10C. is illustrated as having flowers of normal appearance but described as green in colour. By 1665 Rea tells us that the double green cowslip has become "the rarest and most esteemed of all our English kinds", and

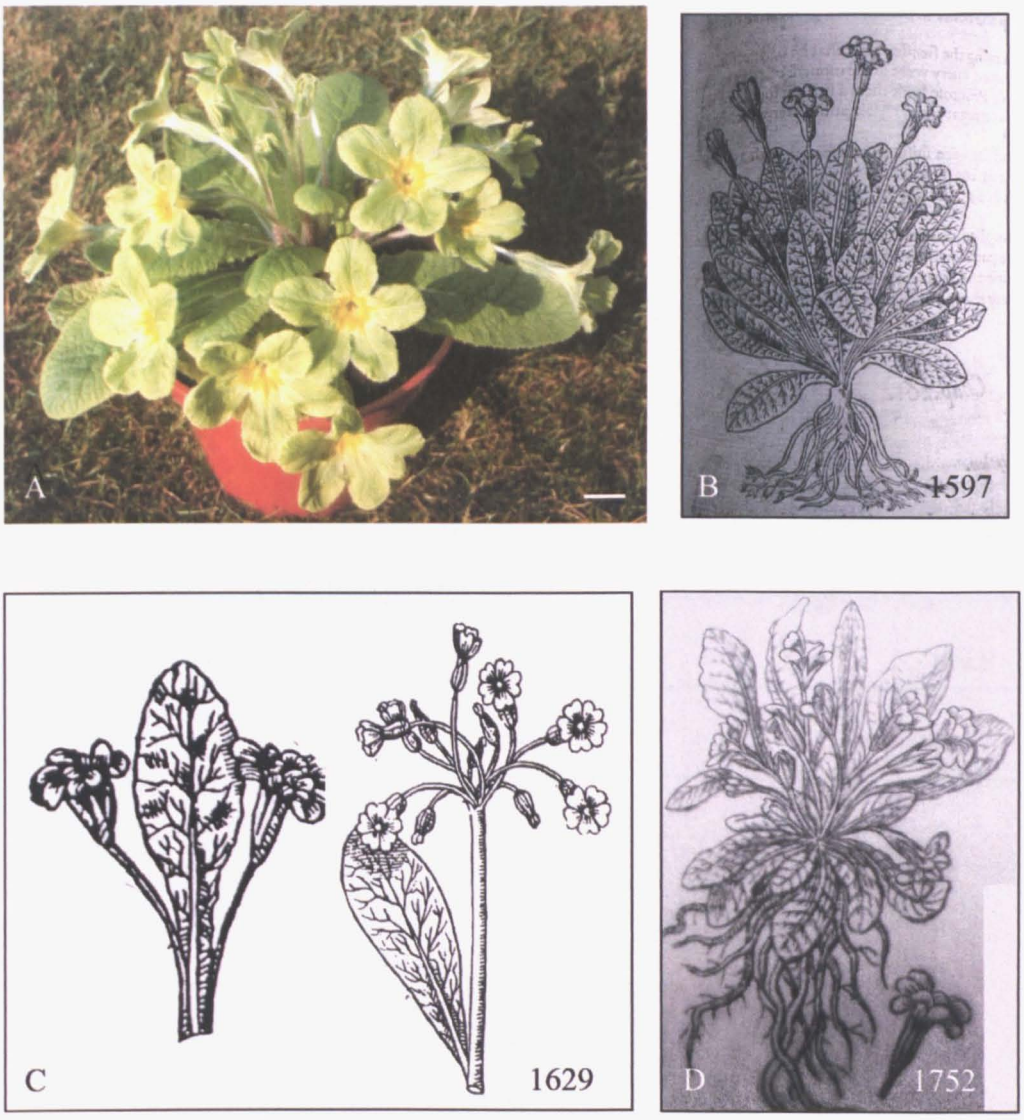


Figure 1.10. Green flowered *Primulas*.

Green *Primulas*, single and double, primrose and cowslip were recorded in the sixteenth and seventeenth centuries. Of these only *Primula viridis*, the green primrose, can be obtained today but it is becoming increasingly rare.

A. *Primula viridis*, the green primrose. Size bar approximately 1cm. **B.** The green primrose of Gerard's Herbal. 1597. **C.** The double green primrose from Parkinson, 1629 (on the left) and his single green cowslip (on the right). **D.** The green primrose from Newton's herbal of 1752.

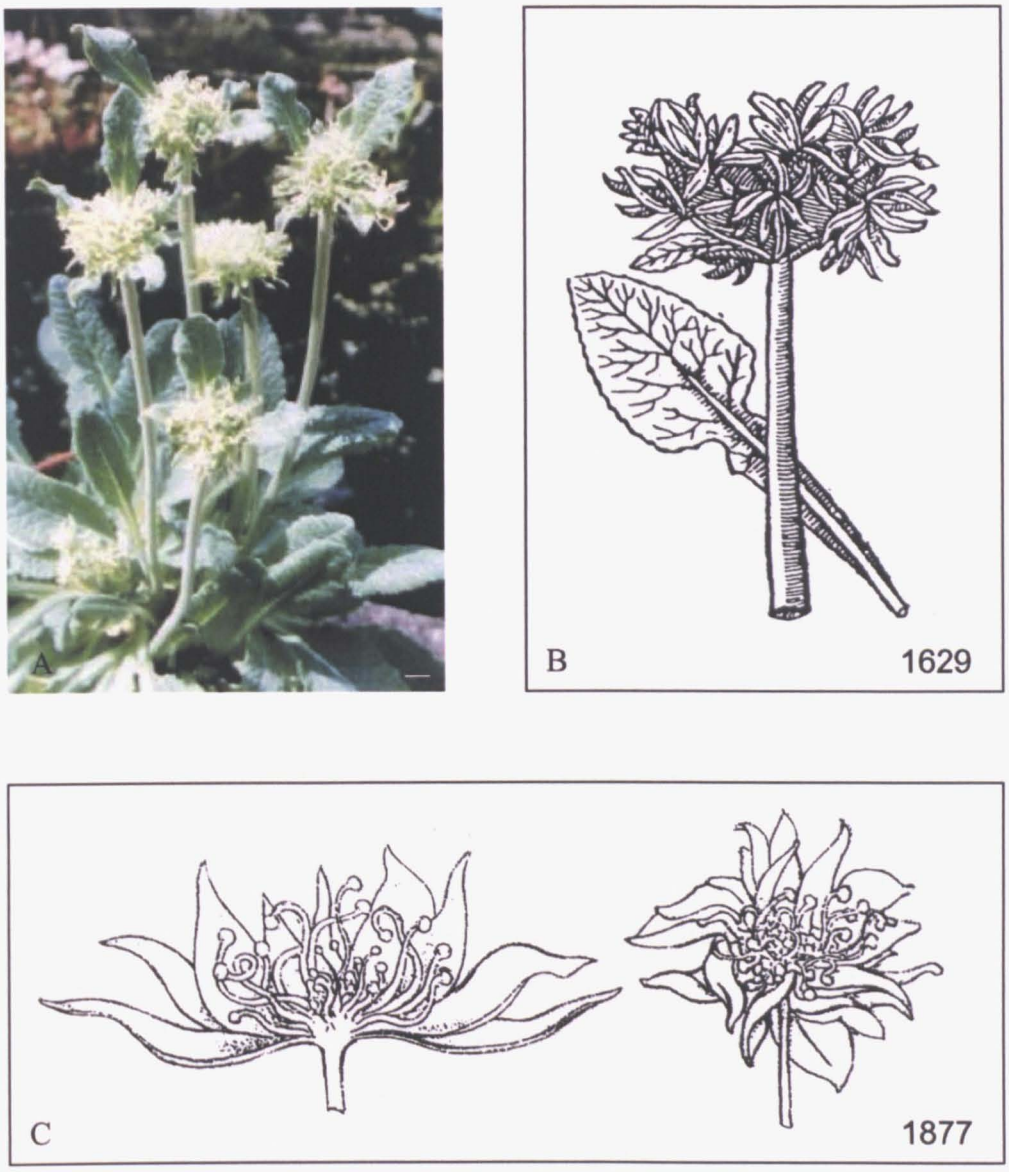


Figure 1.11. The virescent cowslip.

The virescent cowslip, although known since the seventeenth century, is extremely rare and has not been frequently illustrated.

A. Photograph of the virescent cowslip. Size bar is approximately 1cm. **B.** Illustration of the "Greene Rose Cowslip" or "double greene feathered Cowslip" of Parkinson, 1629, which appears to be similar to the flower in A above. **C.** The virescent cowslip as illustrated by Masters in 1877. The similarity to the plant above is more marked in this illustration which shows the mass of stigmatoid organs found in the centre of the flower.

subsequent to this no references to green cowslips, single or double, were found in the literature. Newton, illustrates the green primrose again in his herbal of 1752, and references to the green primrose continue to the present day, for example Taylor (1947) and Genders (1959). In 1984 Nelson lists *Primulas* of Irish origin, including three types of green primrose; "one with very pointed petals, one with rounded petals, and one with a welted calyx". These variations have not been described elsewhere, but a character of the green primrose, that of sometimes producing two whorls of green petals (see also Chapter 4) has led to some horticultural confusion. I have in the past obtained such plants from three different sources; one described as a double green primrose, one as a green *Hose in Hose* and one as a green *Jack in the Green.* Yet all three were of the same form. Green primroses do also occur in the wild from time to time, Gardening Illustrated of May 1909 mentions a green primrose found by W. Eadon of Rugby. The flower is described as pale green with "the character of the petal" as a "primrose leaf in miniature". The Flora of the Isle of Man, 1984, also mentions a number of primroses with green flowers growing in a native population on Shellag Point.

The *virescent cowslip* (Fig.1.11A) exhibits transformation of organs but the nature of the bract like organ type is unclear. There are carpeloid organs that are malformed and infertile surrounded by green organs that may be either bracts or sepals. However in *P. veris* the sepals are normally thin and pale, and these organs are distinctly green in colour, therefore I consider that they are more bract like than sepal like. The form was illustrated by Parkinson (1629) as the "greene rose cowslip" or "double greene feathered cowslip" (Fig.1.11B), and again by Newton (1752). A more detailed drawing of the flower was illustrated by Masters (1877), (Fig.1.11C.), clearly showing the mass of stigmatoid organs in the centre surrounded by bracts or enlarged sepals. His illustrations show more organization in the flower form than was discovered in the specimen described here and again in Chapter 4. This form must also occur in the wild as a reference to a cowslip with a leafy inflorescence was recorded in the Flora of Wiltshire, (1957). The plant that I obtained in the 1980's also occurred naturally among wild cowslips in a Somerset garden.

1.5.2. Mutant Phenotypes with alterations to the usual form of the perianth.

The *Split Perianth* mutant phenotype (Fig.1.12A) has alterations to the usual form of the perianth. Unlike the normal *Primula* flower that has a gamosepalous calyx and a gamopetalous corolla tube below five free petals, the *Split Perianth* mutant phenotype has

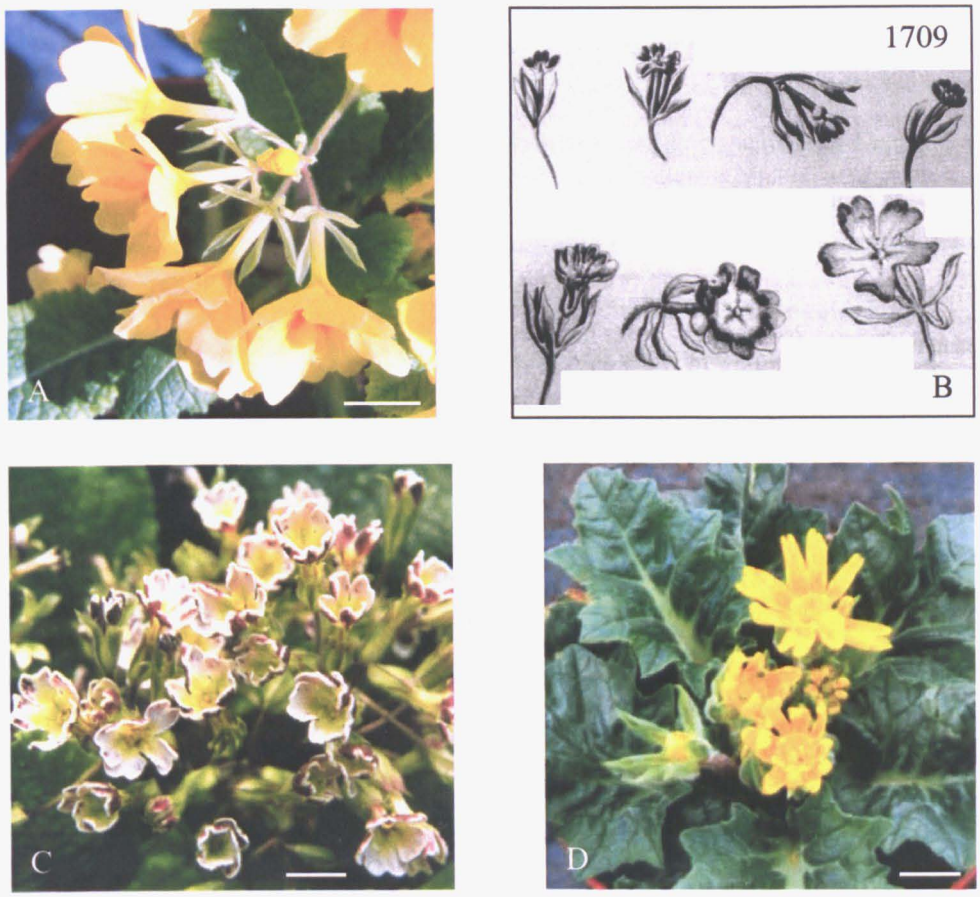


Figure 1.12. Other mutant phenotypes.

Other mutant phenotypes include phenotypes where there are alterations to the usual form of the organ, and pleiotropic mutants where other effects are observed in addition to flower abnormalities.

A. The *Split Perianth* mutant phenotype. This mutant phenotype has the calyx, and in some instances the corolla and the calyx, divided into five separate segments. **B.** Some examples of the form illustrated in "Nuremberger Hesperides" in 1709 are shown. **C.** The *reduced petal* mutant phenotype. This pleiotropic mutant phenotype has the petals thickened and reduced with loss of colour on the adaxial petal surface in addition to effects on leaf shape. No illustrations of the phenotype have been found in old literarure. **E.** The *Oak Leaf* mutant phenotype. Petals are narrow and calyces have streaks of petaloid tissue. Leaves are lobed as are Oak leaves. No illustrations of the phenotype have been found in old literature. Size bars are approximately 1 cm.

the calyx divided into five free sepals and the corolla tube may, or may not be, also split into five parts. The *Split Perianth* phenotype may have been the form that was described, but not illustrated, by Parkinson (1629) as an "Oxelip" that "hath a green huske under the flower but divided into several small long pieces". Gilbert, in 1693 also mentions a primrose with a similarly split calyx that he could find "nowhere described". It was illustrated in the early 1700's (Fig.1.12B) in Nuremberger Hesperides (see Hawkes, 1997), and may have been the form horticulturally referred to in Britain as "Feathers", "Shags", or "Scattered Polyanthus". A form described by Bradley in 1734 also appears to fit the description – "Feathers which seem by nature to be at first designed for *Hose in Hose* have their blossoms so split and curled that they somewhat resemble bunches of feathers. Of these there are many varieties which are multiply'd every year by sowing the seeds". These plants appear to be a combination of two phenotypes, *Split Perianth* and *Hose in Hose*. That they can be propagated by seed fits the fact that these are dominant phenotypes as described later in Chapter 5. The form was described by B. Smith (1986) as a type of polyanthus that was probably extinct "with an elongated calyx that is cut up fine like a fringe around the flower". The phenotype appears to occur naturally from time to time; a dark form of a garden cowslip with the calyx divided to the base is mentioned in The English Flora of 1828, and the current plants are derived from one that appeared in a batch of gold laced polyanthuses in Woodborough Nurseries, Nottingham. In the wild a form of *P. elatior*, termed "calycida", with the calyx "cut to the base" is recorded from Silesia in central Europe by Wright Smith and Fletcher, (1947).

1.5.3. Pleiotropic mutant phenotypes.

There are two pleiotropic mutant phenotypes described in this study . The first is *reduced petal* (Fig.1.12C) that has reduced petals with loss of colour on the adaxial surface, and broader "frilly edged" leaves. The *reduced petal* phenotype bears some resemblance to the *CINCINNATA* mutant phenotype of *Antirrhinum* (Nath *et al*, 2003; Crawford *et al.* 2004).The second is *Oak Leaf* (Fig.1.12D) that has lobed leaves like that of the Oak and flowers with attenuated petals. There is no description of either form in any of the British *Primulas* in older literature, but a form named "oak leaf" was described in *P. sinensis* by Gregory *et al.*, (1923) and by De Winton and Haldane (1933). This form of *P. sinensis* did have lobed leaves but the flower did not have attenuated petals.

1.5.4. Summary.

There are numerous naturally occurring mutant phenotypes of British *Primulas*, a number of which have been documented and illustrated for up to four centuries. Many of these are organ identity homeotic mutant phenotypes. One hypothesis that may partially explain the large number of mutant phenotypes in British *Primulas* is the fact that two species *P. veris* and *P, vulgaris* can, and do interbreed to form fertile hybrids. A third species *P. elatior* can also interbreed with both of the former species but it has a more restricted natural distribution. Meyer in 1966 commented on the disproportionately high number of aberrations to occur in interspecific hybrids, due either to the possibility of the two sets of chromosomes not functioning so well together as those from the same species, or to genes from one species functioning less well in a different cytoplasm (Meyer, 1966). Certainly some of the mutant phenotypes illustrated were hybrids, such as Parkinson's Oxelips *Hose in Hose* and *Jackanapes on Horseback*, and indeed all polyanthus mutant phenotypes are hybrids. Even the *Hose in Hose* cowslip illustrated by Crispin de Passe (1614) appears to also be a hybrid, since the flowers are larger than is usual for a cowslip and the leaves are not narrowed at the base as are cowslip leaves but instead resemble primrose leaves. In addition there is one flower bud on a single pedicle springing from the base of the plant at the right, something that does not occur in a pure cowslip species. The hoverfly pollinator depicted in the same illustration is an accurate observation so presumably other parts of the illustration were also accurately observed. Similarly, more of the recently discovered mutant phenotypes have been found in *Primula* stock that has been developed commercially. However hybridization cannot be the total reason as to why there are so many mutant phenotypes in British *Primulas* since records of mutant phenotypes found from time to time in the wild indicates that mutant phenotypes do occur in the species as well as in hybrids. Molecular analysis of flower development in *Primula* is required in order to answer this question.

1.6. Project Aims.

The *Primula S* locus has been a focus of interest from the time of Darwin (Darwin 1861) and the subject of much subsequent literature (Ernst, 1925,1936; Crosby 1949; Bodmer 1958;Charlesworth 1979 Richards, 1986, 1993, and Kurian and Richards 1997). Despite this intense interest in the *S* locus, little is known about the control of *Primula* flower development and the timing of heteromorphy, and nothing is known about the genes controlling these events. The availability of several mutant phenotypes, including four that are linked to the *S* locus, provided the opportunity to initiate a detailed analysis of the genetic basis of *Primula* flower development and floral heteromorphy in this species.

Specific aims of this project were:

1. To compare of early stages of *wild type* flower development with the development of floral mutant phenotypes in order to define the developmental consequences of these mutations.
2. To undertake a detailed analysis of early and late flower development in *Primula* flowers in order to identify the timing of differential developmental in pin and thrum flowers.
3. To characterize the inheritance of the available floral mutant phenotypes in order to determine dominance relationships, linkage and genetic interactions. While documenting this information is a component of the thesis it is only loci with mutant alleles linked to the *S* locus that are further investigated.
4. Investigation of two of these loci, the predicted gain and loss of B function mutants, *Hose in Hose* and *sepaloid* is of particular importance. They could be allelic mutations or two separate but linked genes.
5. To undertake linkage analysis in order to identify the long sought after flanking markers for the *Primula S* locus, and to establish the first classical genetic map of genes surrounding the *S* locus as a prelude to the molecular genetic analysis of the *Primula S* locus.

CHAPTER TWO

Materials and methods.

2.1. Sources of plants.

Mutant *Primula* collected and bred since 1985 by the author constitute the National Collection of *Primula* "British Floral Variants". The original plants have been obtained from many different sources. The first *Jack in the Green* plants were discovered in a garden hedge in Killay, Swansea in 1980. The first *Hose in Hose* plant was obtained from the late Mary Mottram, North Molton, Devon, in 1985. The first *Split Perianth* plant was a gift from Dr. R. J. Brumpton (Woodborough Nurseries, Nottingham) in 1997. The first *Staminoid Carpels* plant was a gift from Mrs. P. Gossage, Crewkerne, in 1988. The *sepaloid* primroses were a gift from Dr. V. Wooley (Field House Alpines nursery, Nottingham) in 1996. Many *double* primroses are commercially available, and occasionally, if not completely male and female sterile, can be bred from. In addition the following were obtained as gifts. The *semi-double* with organ order of sepal, petal, petal, stamen, stamen, indeterminate, was raised using pollen from a similar *semi-double* obtained from Mr. P. Ward of Saltford in 1993. The very old *double* "Alba plena" was a gift from Mrs. B. Chesney, Charminster in 1995. The *double* Polyanthus "Lin Rogers" was discovered by L. Rogers in a batch of commercial *P. x tommasinii* seedlings from a commercial source and was a gift from L. Rogers, Dovercourt, Essex, in 1997. The *double* cowslip "Katy MacSparron" was a gift from Mr. G. Nicolle, Haverfordwest who bred the cultivar. The green primrose, *Primula viridis,* was purchased from Timpany Nurseries in Northern Ireland. The *virescent cowslip* was a gift from Mrs. P. Gossage, Crewkerne. The *reduced petal* polyanthus and the *Oak Leaf* primrose were gifts from Dr. R. J. Brumpton (Woodborough Nurseries, Nottingham) in 1999 and 2000 respectively.

The following crosses provided mutants in similar genetic backgrounds for scanning electron microscopy. First, a *Jackanapes* Primula (*P. x tommasinii* cultivar) plant was pollinated from a *double P. vulgaris* cultivar "Lilian Harvey". Phenotypes of the progeny were wildtype, *Jack in the Green*, *Hose in Hose* and *Jackanapes*, all were heterozygous for the recessive double allele. Second, a *Jack in the Green* plant from above was pollinated from a sibling *Hose in Hose* plant. Phenotypes of the progeny were wildtype, *Jack in the Green*, *Hose in Hose*, *Jackanapes*, *double*, *double-Jack in the Green*

and *double-Jackanapes*. The first five phenotypes of these progeny were used in the study.

Yellow short homostyle plants were seedlings from self pollinated short homostyles, a gift of Dr. A. J. Richards, University of Newcastle upon Tyne. Long homostyles were derived from crosses using pollen from the Wyke Champflower wild population of long homostyles in Somerset. The blue short homostyle with long stigmatic papillae was identified in a population of plants grown at Leeds University.

Primula vulgaris L. var."Blue Jeans" (F1 hybrid, Thompson and Morgan) were grown at the University of Leeds. *Wild type P. vulgaris* was initially grown from wild flower seed collected locally at Winford, near Bristol, but crosses done from 2002 onwards used plants bought from Round Trees Garden Centre, near Bristol. The red *wild type P. vulgaris* used to provide sibling progeny for measurement of corolla tube mouths of pin and thrum flowers was obtained from Cadburys Garden Center near Bristol, and crossed with *wild type* primrose (*P. vulgaris).*

2.2. Maintenance of plants.

Plants were grown in Levingtons plant protection compost and maintained in pots in an unheated glasshouse under natural daylight. During prolonged periods of sub-zero daytime winter temperatures, a layer of fleece was used to cover plants on the greenhouse benches. Shade during warmer months was provided by applying "Coolglass" to the outside of the glasshouse, and green netting to the inside. After seed collection, plants were placed outside on hard standing throughout the remainder of the summer.

Plants to be used as seed parents were maintained in an isolation unit covered with insect proof mesh, or under a secure net tent, from the time the flowers came into bud until seed set had begun on the hand pollinated flowers.

2.3. Raising of progeny.

Female parents were prepared for crosses by removing the corolla with adnate anthers from those flowers to be pollinated before dehiscence of the anthers occurred. Pollen was obtained from male parents of choice by removing the corolla after dehiscence of the anthers and bending the petals back so that the anthers protruded. By holding the bent half flower by the petal lobe and corolla tube together the pollen was directly transferred to the stigma of choice using the anthers themselves in a brush-like manner. Seed from

controlled crosses was collected, dried, labeled, and stored in sealed packets in the lowest compartment of a domestic fridge until required for sowing. Seed stored in this manner has been known to be viable up to a period of at least five years. Fresh seed will germinate readily on immediate sowing, but if stored in the fridge it should be left for a minimum of three weeks to simulate winter conditions.

Seed was germinated in unheated propagators on a north facing windowsill that gave ample light without overheating from direct sunlight. Seed was sown directly on the surface of the compost as light is required for germination. Germination can generally be observed about three weeks later. However seed from commercial cultivars is likely to germinate faster and more uniformly. Seed from *wild type Primula vulgaris*, or from crosses with wild *P. vulgaris* in the genetic background, can germinate much more erratically. Consequently it is desirable to save the compost, where possible, after pricking out seedlings from any cross that has a wild *P. vulgaris* genetic background, as a second, or even a third wave of germination may take place later in the season or even in subsequent years. Seedlings were pricked out when two to four true leaves had developed. Seedlings were raised either five plants to a 5" pot, and kept on indoor windowsills until growing conditions outside were suitable, or, in seed trays with 24 cell inserts and plastic covers with ventilation holes that were maintained in the cold green houses. The inserts were later transferred outside on to capillary matting on hard standing, where they remained until the plants had flowered and results were recorded. Pots likewise were placed outside on hard standing in a shaded position until plants had flowered and results were recorded. In some cases plants from crosses were transported to Leeds University greenhouses for growing on to flowering stage. The large three point crosses required thousands of progeny from a new mutant form "*Oak Leaf*" (see Chapter four) that has not yet been released to the public. Because of this some 1500 of the seedlings from one cross were transported to the commercial greenhouse belonging to Dr. R.J. Brumpton, where the plants were raised to maturity. For the second large three point cross involving *Oak Leaf*, most of the seed was posted to the above nursery and the progeny raised entirely by them.

2.4. Observation of pollen size.

Pollen was placed on a microscope slide, stained with lacto phenol blue, covered with a coverslip, and examined under a light microscope. Pollen from *wild type* thrum and pin flowers was available for comparison.

2.5. Photography.

Colour photographs of wild type and mutant plants were taken on Activa 400 ASA film using an Olympus OM10 camera with attached close up lenses as required. Photographs of sectioned mutant flowers, and of the development of the wild type flowers were taken as above but using an attached Cosina macro lens. Film was developed by BONUSPRINT Ltd.

Macro photographs of developing *wild type* and homostyle flowers were taken using a Zeiss Tessovar macro photography system (Leeds University) with an Olympus OM2 and dedicated off camera flash. Film was Kodak Royal 100ASA. colour print film.

Some individual flowers scanned directly using a HP scanjet 3670 flatbed scanner. Whole flowers, either entire or cut in two if internal organs were to be observed were placed between two small objects of suitable height (e.g. small wads of folded paper) directly on the scanner. A piece card was placed on top and the lid closed. To prevent light entering from the sides a piece of dark material was draped over the scanner. The flower was then scanned using the zoom and rescan function as needed.

2.6. Scanning electron microscopy.

To analyse flower development, scapes were removed from each plant and floral apical meristems and developing buds dissected out using scalpels and razor blades with a x20 hand lens. For examination of corolla tube growth a range of four developing buds of different sizes along with one mature flower from a pin plant and from a thrum plant of *P. vulgaris* "Blue Jeans" was examined. Corolla tube tissue, upper and lower, was dissected out from the middle portion of each upper and lower corolla tube to be examined using scalpels and razor blades, some cut to a point, and an x20 hand lens. All tissue was prepared for SEM using an Emscope S. P. 2000 Sputter Cryo system. Samples were mounted on a copper stub using a thin layer of 2% aq. Methyl cellulose. Following mounting, the stub was screwed onto the freezing rod and immediately plunged into liquid nitrogen under dry argon gas and subsequently transferred to the microscope cold stage where the temperature was raised to -60 °C until no ice crystals remained. The sample was then transferred to sputter chamber where the sample was sputter coated with gold under vacuum. Specimens were transferred to a Phillips 501B SEM cold stage and maintained at -153 °C to -155 °C during observation. Photographs of the images were taken on FP4 film and processed in Ilford ID11 developer.

2.7. Gene nomenclature.

Gene nomenclature is based on the popular *Primula* names of the phenotypes as the corresponding genes have not yet been isolated and fully characterised. Standard italicised three letter abbreviations have been used with an initial uppercase letter when the phenotype is dominant. Exceptions are long and short homostyles which are designated *gPA* and *Gpa* respectively, as has been used by previous authors (e.g. Richards 1986, 1997) since these are not mutant phenotypes but *GPA/gpa* recombinants. In this instance *G/g*, *P/p* and *A/a* are the three tightly linked loci responsible for the heteromorphic features of *Primula* (see Chapter 1, 1.3).

The *Primula double* mutant is phenotypically similar to the *PLENA* mutant of *Antirrhinum* and the *AGAMOUS* mutant of *Arabidopsis* but popular names of mutant phenotypes of *Primula* (e.g. *Jack in the Green* and *Hose in Hose*) do not always have correlates in *Antirrhinum* and *Arabidopsis*. New *Primula* mutants have been given names that simply describe the phenotype (e.g. *Split Perianth*, *Staminoid Carpels*, *sepaloid*, and *Oak Leaf*). The names used represent a temporary gene nomenclature for *Primula* which will be used only until the relevant genes have been isolated and shown to be orthologs of known genes. During the course of this project the *Primula* genes for *DEFICIENS GLOBOSA* and *PLENA* have been isolated. When the *Primula* genes have been fully classified and characterised and shown to be associated with specific mutant phenotypes, the standard nomenclature for all sequenced plant genes will be adopted following the Gene Nomenclature Guide as published in Trends in Genetics, Genetic Nomenclature Guide, (1995, 1998; http://zfin.org/zf_info/nomen_comm.html). The above system groups all plant genes that encode the same product and have similar coding sequences into the same gene family.

2.8. χ^2 Tests.

All χ^2 tests were done using the interactive calculation tool at http://www.unc.edu/~preacher/chiso/chiso.htm

CHAPTER THREE

Wild type Primulas.

3.1. Introduction.

Wild British *Primula* species and the *Primula* breeding system have been introduced in Chapter 1. This chapter analyses the early ontogeny of the *wild type Primula* flower since no previous study of the temporal development of pin and thrum flowers has been done. Nor has there been any previous study of early *Primula* flower ontogeny using scanning electron microscopy. These detailed scanning electron microscopy studies characterise the early stages of development in normal *Primula* flowers (and later, in Chapter 4, also in the available homeotic mutants). During early ontogeny the individual organs are initiated and become recognizable. Different *Primula* species and cultivars have been observed to ascertain whether any notable differences in development were obvious, but none were found. It was appropriate to include the cultivar *P. vulgaris* "Blue Jeans" in the investigation of late development (Fig. 3.5) as it is the cultivar under molecular investigation in the Gilmartin laboratory.

Primula flowers were found to be homomorphic during early ontogeny therefore pin and thrum flowers were investigated to maturity to reveal the differential timing of divergence of the two flower forms. In addition a new heteromorphic feature was discovered; thrum flowers have a wider corolla tube mouth than pin flowers due to the corolla tube cells above the anthers being wider in thrum flowers than in pin flowers.

3.2. Botanical description of *Primula*.

Primula species and cultivars used in this project are rosette forming hemicryptophytes. Flowers are borne in an umbel and each pedicle has a subtending bract. The flower is actinomorphic, pentamerous, hermaphrodite and heterostylous. The calyx is gamosepalous, five cleft and persistent. The corolla is gamopetalous and the stamens epipetalous. The gyneocium is polycarpellary with a single style and a capitate stigma. The ovary is superior and unilocular with numerous ovules on a free central placenta. The fruit is a capsule, with valvular dehiscence. The seed is endospermous and germinination is epigeal.

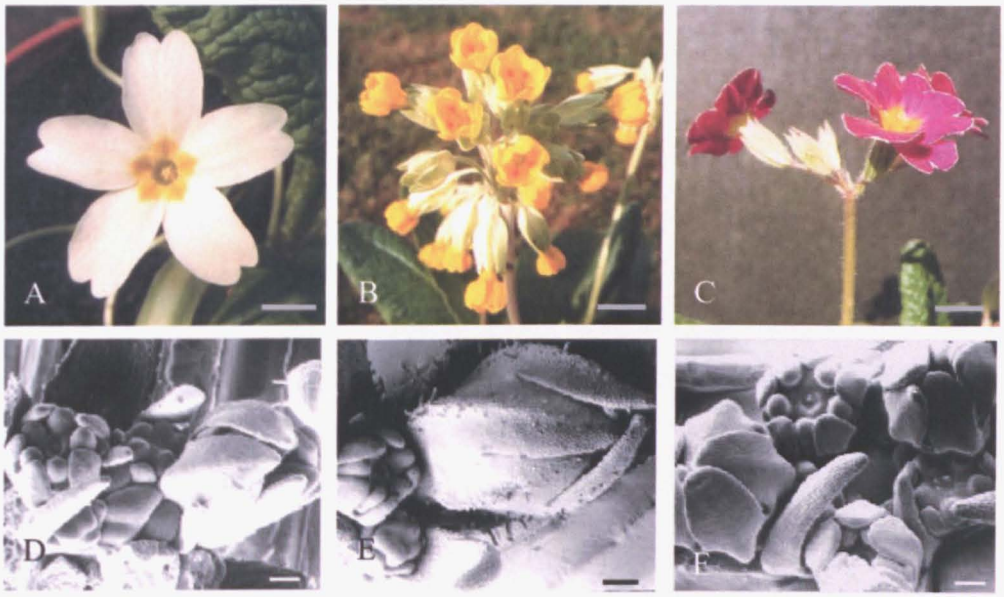


Figure 3.1. A comparison of early ontogeny between *Primula vulgaris* (primrose), *Primula veris* (cowslip), and polyanthus.

Although the size of the flower and the characteristics of the above plants are different the early ontogeny follows the same developmental pattern in each of them.

A. *Primula vulgaris* (primrose). **B.** *Primula veris* (cowslip). **C.** Polyanthus. **D.** A cluster of developing *P. vulgaris* flowers at different stages of development. **E.** A cluster of developing *P. veris* flowers at different stages of development. **F.** A cluster of polyanthus flowers at different stages of development. Size bars **A – C**, approx. 1 cm. Size bars **D – F** 100 μm.

3.3. Early ontogeny of *wild type Primula*.

Early developmental stages of both *Arabidopsis* (Smyth *et al.* 1990) and *Antirrhinum* (Carpenter *et al.* 2001) had already been characterized at the time of this study. The *Antirrhinum* study did not attempt to exactly follow the *Arabidopsis* study (C. Vincent, personal communication). Each stage of *Antirrhinum* was named by the physical appearance, e.g. "Loaf" and "Pentagon" of the developing flower. Stages in the two species do not correspond exactly. For example, in stage 3 in *Arabidopsis* the sepals are clearly visible in the scanning electron microscope image (Smyth *et al,* 1990). In stage 3 of *Antirrhinum*, ("pentagon"), the sepals are not clearly visible in the scanning electron microscope image (Carpenter *et al,* 1995) although it is documented that they do begin to become visible as small bulges towards the end of this stage. Given the differences in flower structure and growth patterns in different species it was considered that attempting to match stages of development of *Primula* to the two different studies above would be inappropriate. Both *Arabidopsis* and *Antirrhinum* bear flowers on racemes, so that development can also be divided into "nodes" (Carpenter *et al.*1995), while *Primula* bears flowers in an umbel. Neither of the two model species above have organs that develop from a common primordium as do the petals and stamens of *Primula*. It was therefore considered preferable to look at specific developmental landmarks of the *Primula* flower and to base each early stage on the development of a new primordium or primordia that would result in a new part or parts of the flower.

Mature flowers of *P. vulgaris* (Fig. 3.1A), *P. veris* (Fig. 3.1B) and polyanthus (Fig. 3.1.C) differ in size and final flower shape, but follow the same early developmental pattern (Fig.3.1. D - F). Developing flowers of the same species can vary slightly in size even when each is at the same point in development, although the pattern is not different. The time taken for flowers to develop from initiation to maturity varies with seasonal and environmental conditions. The stages of development chosen represent convenient static images during continuous development. Although each of the stages represents clearly distinct developmental phenomena there are visible differences within a stage as the individual organs develop, so stages 1 and 2 have been subdivided into early and late, and stages 3 and 4 into early, middle, and late. By stage 4, all four floral organs have been initiated. Beyond this stage there is no further differentiation of new organs, only further development of those that have already been defined. Stage 5 represents the developing flower fully enclosed within the sepals; at this stage the lobes of the anthers become apparent. During stage 6 in whorl 4, the recognisable component parts of stigma style and ovary are initiated; inside the latter, ovules begin to develop on a free central placenta.

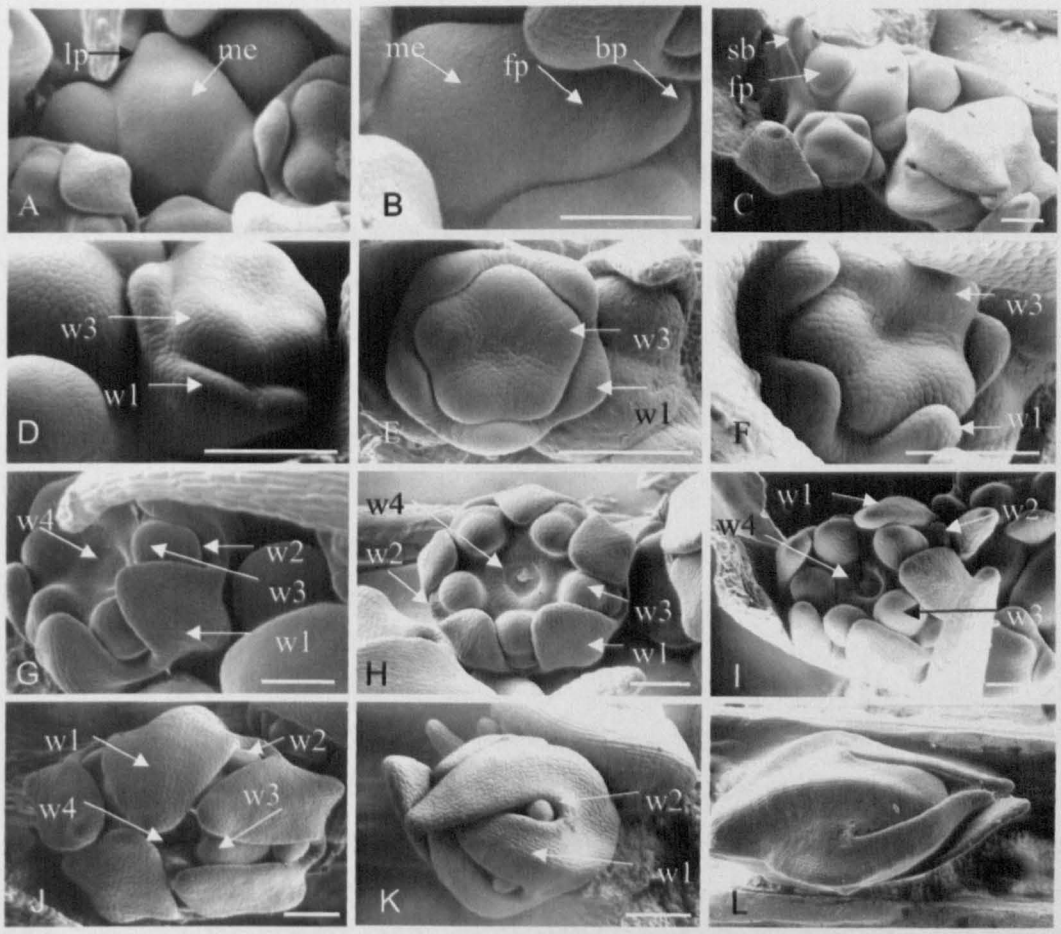


Figure 3.2. Stages of development during early ontogeny in *wild type Primula*.

Stages of development from 1 – 5 have been characterised using developing flowers from the hybrid polyanthus in Figure 3.1.C.

A. Stage 1: Meristematic central primordium (me) forms. Around the central primordium lateral primordia (lp) form. **B.** Early stage 2: the lateral primordia divide into two parts, the bract primordium (bp) and the flower primordium (fp). **C.** Late stage 2: the lateral primordiun has now fully developed into two parts, the flower primordium and subtending bract (sb). **D.** Early stage 3: the flower primordium has initiated whorls 1 (w1) and 3 (w3). **E.** Middle stage 3: **F.** Late stage 3. **G.** Early stage 4: iniation of whorls 2 (w2) and 4 (w4). **H.** Middle stage 4. **I.** Late stage 4. **J.** Early stage 5: growth of the sepals begins to cover the inner whorls of organs. **K.** Middle stage 5. **L.** Late stage 5: inner organs can no longer be seen. Size bars, 100μm .

Stage 1 is defined by an undifferentiated meristematic dome in the centre of the developing inflorescence which around which as it enlarges primordia develop (Fig.3.2A). During stage 2, each primordium that develops around the edges of this dome differentiates into two parts: one will become the flower primordium, the other the primordium of the subtending bract (Fig.3.2B). The flower primordium develops as a rounded mound, while the bract develops as an elongated structure that, when more fully developed, curls protectively over the developing flower (see Fig.3.2C).. Figures 3.2D -F show the early middle and late phases of stage 3. During stage 3, whorls 1 and whorls 3 develop. At this stage, whorl 3 is represented by five developing common stamen-petal primordia (Fig.3.2D). The lower outside edges of the flower primordium grows to form a ring that develops pentagonally into five sepal lobe primordia (Fig.3.2E). The inner region of the developing flower expands into pentagonal symmetry with the points of the inner pentagon adjacent to those on the outer pentagon (Fig.3.2F). The five rounded structures of the inner pentagonal mound will become the common stamen-petal primordia.

Figure 3.2G - I show the early middle and late phases of stage 4. Stage 4 consists of the simultaneous development of whorls 2 and 4. Whorl 2 develops initially as a slight bulge on the abaxial base of each common stamen-petal primordium (Fig.3.2G). At the same time a ring structure forms in the centre of the flower that will develop to form the ovary wall, style and stigma. These changes become more apparent during middle stage 4 (Fig.3.2H). By late stage 4 (Fig.3.2I) all four whorls are clearly visible. During stage 5, the sepals enlarge to conceal the flower within. During early stage 5 (Fig.3.2J), the inner organs are still visible, but by middle stage 5 (Fig.3.2K) only the petal primordia remain visible and by late stage 5 (Fig.3.2L), the flower is fully enclosed. The free central placentation with associated ovules develops subsequently during stage 6 within the carpels (Fig.3.4). More detailed observation was made of the growth of *wild type* whorl 1 at late stage 5 (Fig.3.3.A - D). Although all parts of the organ are growing at this time, the cells at the base of the organ can be seen to be smaller. There is also evidence of more frequent cell division, observed as less deep divisions between many of the cells. Similarly, the middle portion of the sepal can be observed to have a greater number of recently divided cells than the tip of the organ.

Development of whorl 4 into component parts of stigma style and ovary has been designated as stage 6. Because the flower becomes fully enclosed in sepals by late stage 5 (Fig.3.2L), subsequent observation of *wild type* whorls 2, 3 and 4 is only possible by

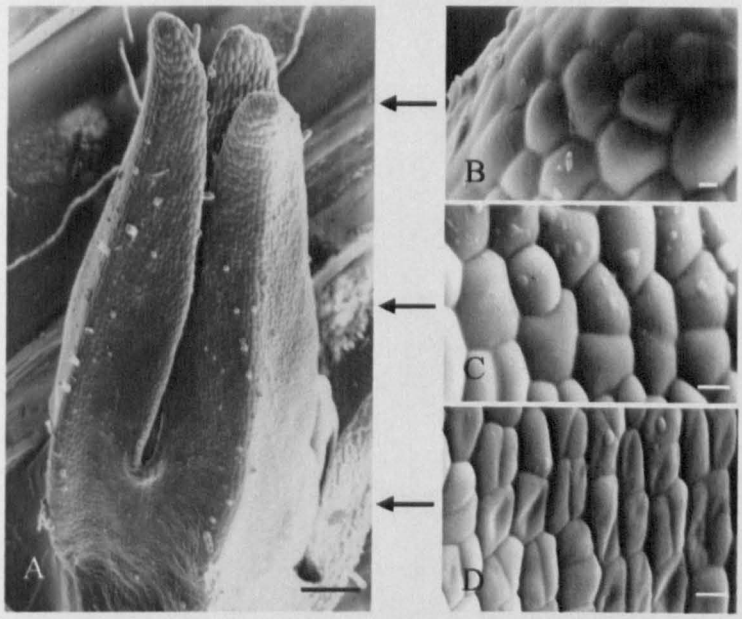


Figure 3.3. Differences in cell shape and size in whorl 1 at stage 6.

One developing flower at stage 6 from the hybrid polyanthus in figure 3.1.C is shown in **A** with the different cell shapes and sizes at the top in **B**, at the middle in **C** and at the bottom of the flower in **D**. Size bars are 100 μm in **A,** and 1 μm in **B – D.**

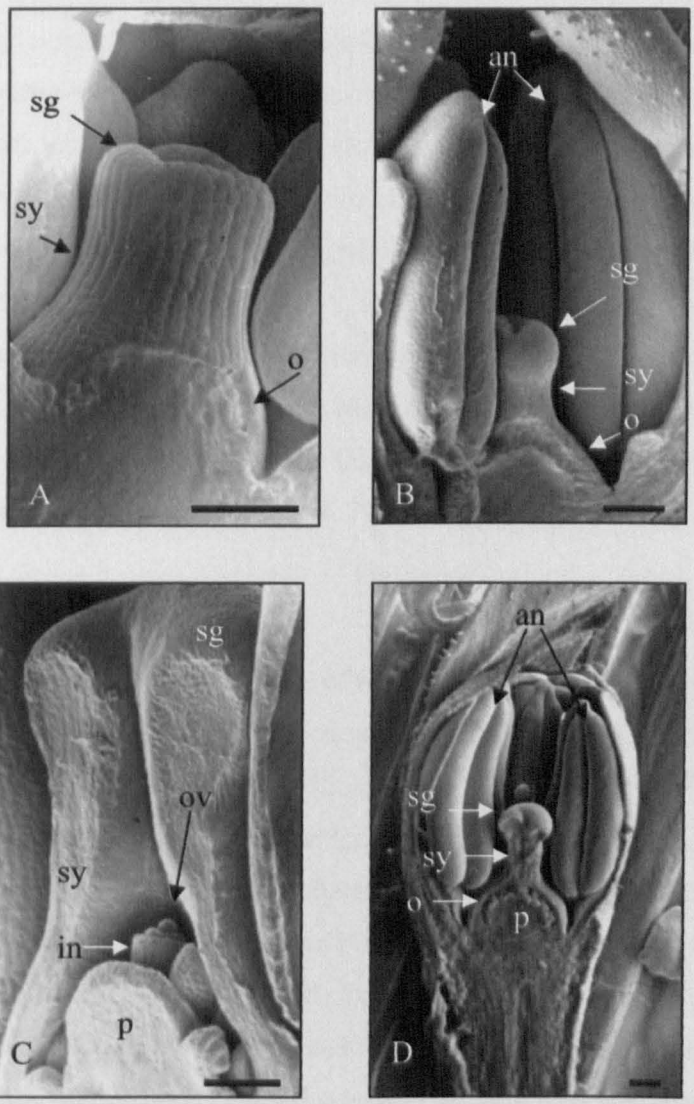


Figure 3.4. Development of whorl 4 into the component parts of stigma style and ovary.

Development of whorl four can not normally be observed without dissecting away part of the outer three whorls.

A. Whorl 4 before the top of the organ has swollen sufficiently to be recognisable as a stigma. A slight enlargement of the top of the organ (sg) indicates early stigm development. **B.** Whorl 4 at early stage 6 is differentiating into the recognisable component parts of stigma (sg) style (sy) and ovary(o). Anthers have elongated and anther lobes are clearly visisble. **C.** In this middle stage 6 flower, the top of whorl 4 has not yet closed although the component parts are differentiating into stigma style and ovary. Inside the ovary ovules (ov) are developing on what will become a free central placenta (p), and the outer and inner integuements of the developing ovules are clearly visible. **D.** By late stage 6 the three component parts of whorl 4 are clearly visible. Ovules can be seen inside on the central placenta that will have become a free central placenta when the flower is mature. Size bars are 100μm .

dissecting developing flowers. The initiation of stage 6, is when the rim of the ovary ring first begins to expand, and is illustrated in Figure 3.4. At first the upper portion of whorl four is only slightly broader than the middle portion (Fig. 3.4A). The differentiation into stigma style and ovary soon becomes clearly visible (Fig.3.4B), although the ovary ring does not always close until the style begins to elongate (Fig.3.4C). There can be variation in the developmental timing for closure of the ovary ring during this stage. Inside the ovary, ovules develop on a free central placenta and at this point the inner and outer integuments are clearly visible (Fig.3.4C). By late stage 6, the component parts of the stigma and style, with the ovary enclosing ovules on a free central placenta, are all easily recognisable (Fig.3.4D). No differences between pin and thrum flowers were observed up to and including stage 6. *Primula* flowers are homomorphic during early stages of development (Webster and Gilmartin, 2003).

3.4. Discussion of early ontogeny.

Floral ontogeny in *Primula* has been a source of interest for many years with an extensive literature dating back nearly 200 years (for example Saint Hilarie 1816; Ducharte 1844; Van Tieghem 1869; Pfeffer 1872; Eichler 1875; Henslow 1876; Masters 1878; Heinricher 1932, 1933; Dickson 1936; Douglas 1936; Sattler 1967; Sundberg 1982). During flower development in *Primula*, stamens and petals develop from a common floral primordium (see Figure 3.2, G, H, and I). The stamen initials develop first, and the petal initials arise later from the abaxial base of the stamen-petal primordium. This observation led Ducharte (1844) to conclude that the stamen and the petal were a single organ, and that *Primula* contained only three whorls of floral organs. This view was later challenged in 1875 by Eichler who suggested that the common stamen-petal primordium probably resulted from congenital fusion of these two organs, and concluded that petals and stamens were indeed two distinct floral whorls, and subsequent authors have supported this view (Eichler 1875).

The normal spatial configuration of floral organs within a flower from the outermost to the innermost whorls is always sepals, petals, stamens, carpels and temporal initiation of the four floral whorls in the two best-studied models of flower development, *Arabidopsis thaliana* and *Antirrhinum majus*, occur in a centripetal manner. Early observations (Ducharte 1844; Pfeffer 1872) revealed that the temporal development of floral organs in *Primula* did not occur in this linear centripetal order. In 1876, Henslow highlighted a number of examples of flower development where it had been observed that the whorls of

floral organs did not develop in an acropetal or centripetal manner, including *Ranunculus acris* and *Veronica chamaedrys* (Henslow 1876). Both of these species show the temporal order of floral organ development as sepals, stamens, carpels, petals. In 1967, Sattler described a number of species with both stamen and petal initials occurring on a common stamen-petal primordium (Sattler 1967). The position of the second initial on the common stamen-petal primordium differs from species to species and ranges from a point on the innermost adaxial surface of the common stamen-petal primordium to a point on the outermost abaxial surface, as described previously for *Primula*. Sattler's observations (Sattler 1967) also revealed that irrespective of which initial developed first on this stamen-petal primordium, the outermost primordium always gave rise to the petal and the innermost primordium to the stamen. This observation reinforced the universality of floral organ sequence and demonstrated that the temporal order of the floral organ initiation does not affect the final spatial order of sepal, petal, stamen, carpel. More recent studies on flower development in *Pisum sativum* (Ferrándiz *et al.* 1999) describe a similar process with common stamen and petal primordia that disrupts the sequential centripetal development of sepals, petals, stamens and carpels.

The images obtained by scanning electron microscopy in this study are in accordance with the images of *wild type P. veris* obtained by Ducharte in 1844. However, the additional detail and resolution offered by the more modern approaches provides greater insight into the early events of floral development in *Primula* such as the differences in cell shape and size in whorl 1 illustrated in figure 3.3. These differences indicate that although growth is occurring in all tissues at this stage, the youngest tissue is at the base of the organ.

The early ontogeny of *Primula* differs from that of *Arabidopsis* and *Antirrhinum* in a number of respects, due, in part, to major differences in the mature flower form in each species. For example a difference visible in early development includes the fact that subtending bracts are found in *Antirrhinum* and *Primula* but not in *Arabidopsis*. It is also important to note that any developmental stage may not be comparable across all three species. Stage 3 for each species is set in different criteria. In *Primula* flower both sepals and stamens begin to develop, in *Arabidopsis* sepals are the only organ to develop, and in *Antirrhinum* sepals only begin to appear at the very end of stage 3. Stage 5 bears some similarity in *Arabidopsis* and *Antirrhinum* in that in both the sepals begin to overarch the dome and petal primordia become visible, but in *Arabidopsis*, and not in *Antirrhinum*, stamen primordia also appear at this stage. Neither *Arabidopsis* nor *Antirrhinum* have a

common primordium from which whorls two and three develop, as is the case in *Primula*. During stage 4 in *Primula* whorl two develops at the same time as whorl four, but in *Arabidopsis* whorl four does not develop until after whorls two and three. These differences illustrate the difficulty of attempting to parallel stages of development in different species by physical appearance. Subsequent to the study on early ontogeny of *Primula* (Webster and Gilmartin, 2003) a more recent study on flower development in *Antirrhinum* (Vincent and Coen, 2004) used the plastochron index to provide a temporal framework for the mapping of developmental events. This system for mapping development could be used as part of a future study on *Primula*.

3.5. Development of the pin and thrum heteromorphic features.

As *Primula* flowers were found to be homomorphic during early ontogeny, development of pin and thrum heteromorphic features of *Primula* were investigated to maturity. This work had been completed before the publication of that done on *Antirhinum* using the plastochron index (Vincent and Coen, 2004) therefore instead of dividing late development of *Primula* into developmental stages the emphasis is on the development of the distinctive thrum and pin architecture (Figure 3.6). Heteromorphic features of mature flowers are shown in figure 3.5.

The developing flowers shown in figure 3.6 are from the cultivar Blue Jeans currently under molecular investigation in the Gilmartin laboratory. That the development illustrated is typical has been verified by analysis of several different cultivars (not shown) and comparing their late development with that of Blue Jeans. The first indication of differences between the two morphs is the elongation of the pin style that begins to raise the stigma above the anthers. This is just beginning in the pin flower in (Figure 3.6A), but is very obvious in the pin flowers in Figure 3.6B, C and D. The thrum stigma normally remains below the top of the anthers throughout development. There is no growth of the corolla tube below stamen attachment in either morph in Figure 3.6A, B or C. When whorl two of the Blue Jeans cultivar has reached approximately 7mm. elongation of the corolla tube below the anthers can be observed in the thrum flower but not in the pin flower (Figure 3.6D). It is not until whorl two has reached 13mm in length that elongation of the corolla tube below the point of stamen attachment can be observed in the pin flower (Figure 3.6E), and at this point the elongation of the corolla below the point of stamen attachment is considerable in the thrum flower. Thus growth of the corolla tube above and below the anthers varies both temporarily and physically in pin and thrum morphs. Both the pin and the thrum flowers in Figure 3.6F are about to open

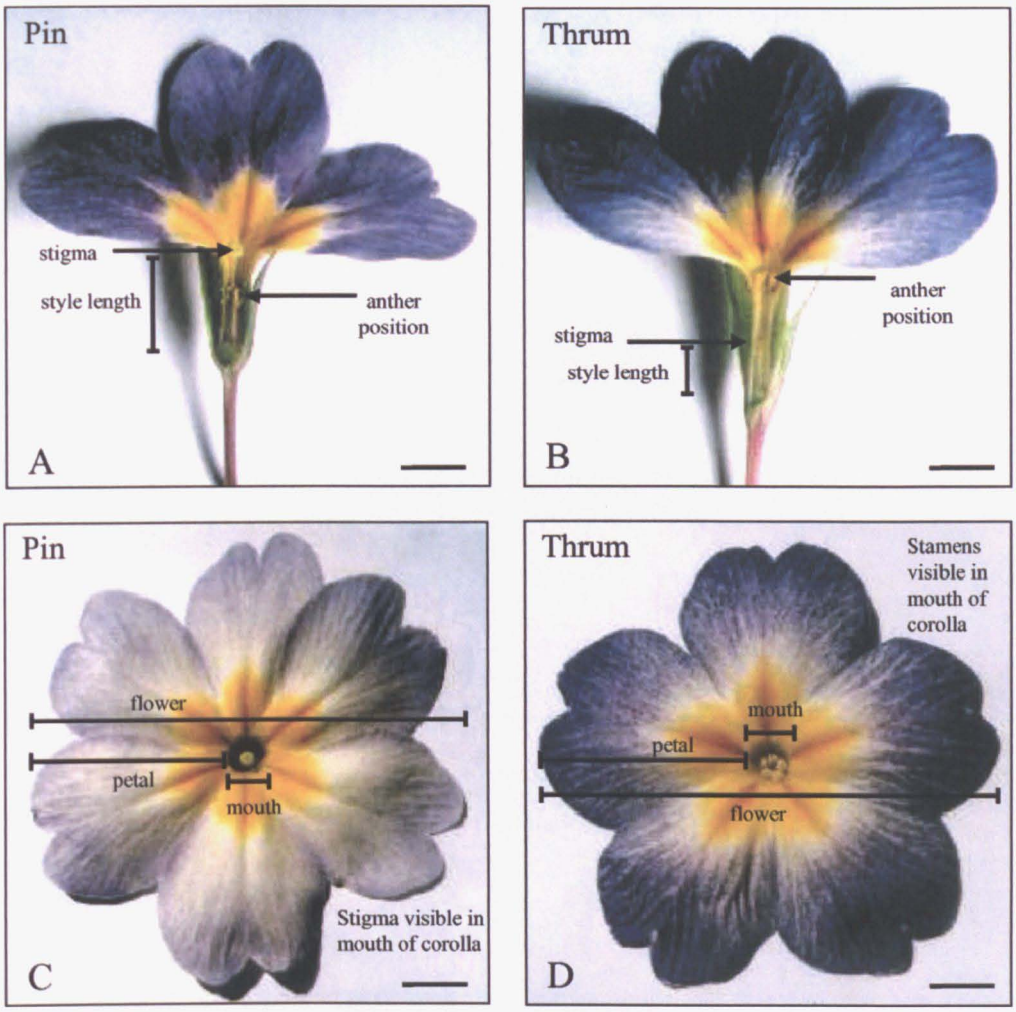


Figure 3.5. Pin and thrum flowers of *Primula vulgaris* var. Blue Jeans

A. Longitudinal section of pin flower. **B.** Longitudinal section of thrum flower. The length of the style and position of the stigma and anthers are shown. The lengths of upper and lower corolla tubes are also indicated. **C.** Top view of pin flower. **D.** Top view of thrum flower. Both C and D show diameter of the flower, the extent of the petal and diameter of the flower mouth. All size bars are 5 mm.

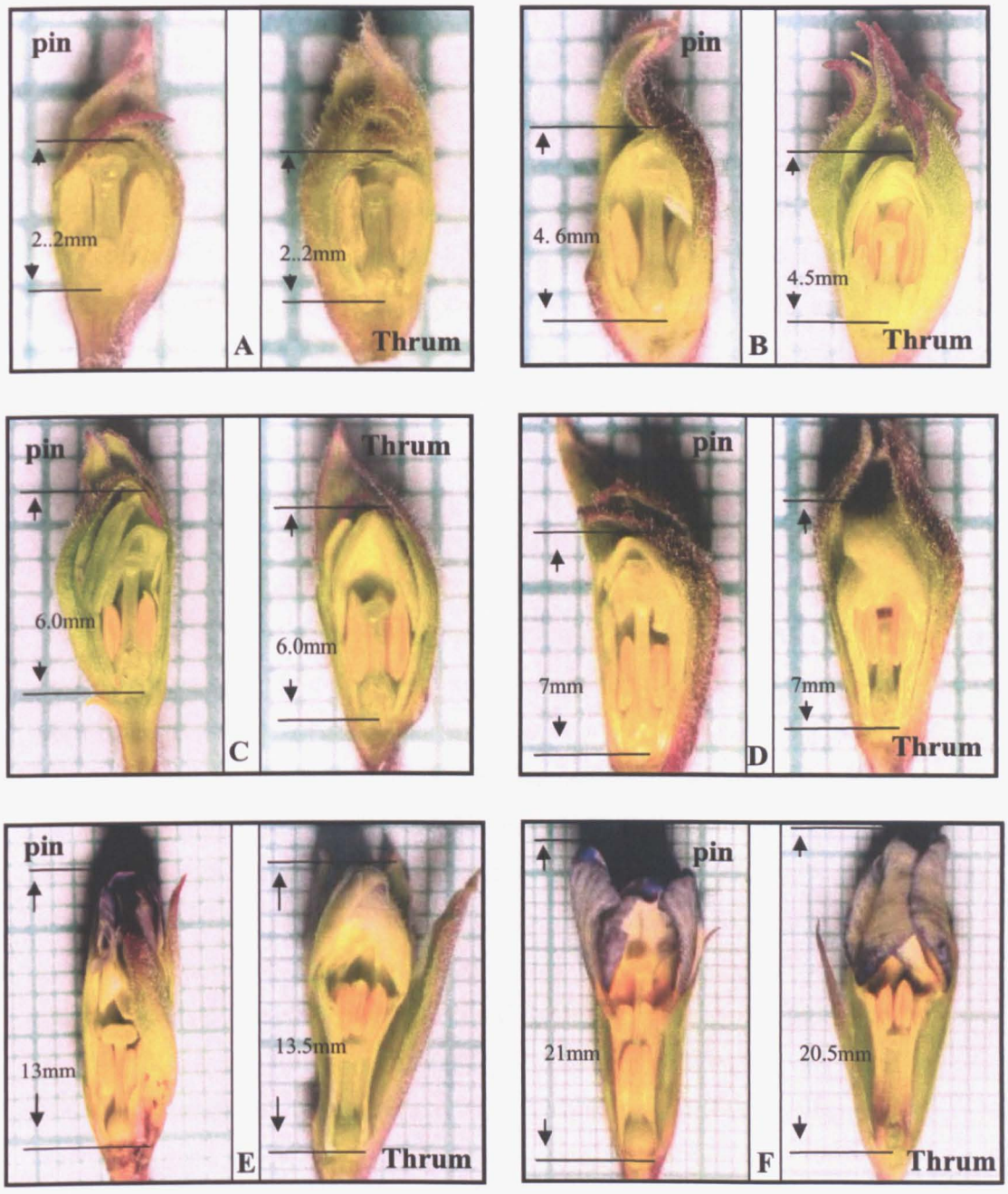


Figure 3.6. Development of pin and thrum sibling flowers.

Differences between pin and thrum flowers are only manifested later in flower development, as shown in **A** (earliest) - **F** (latest) comparison of pin and thrum flower development. The approximate height of whorl 2 has been measured (squares are 1mm.). In the developing flowers shown in **A – C** above the pin style elongates to raise the stigma above the anthers and the thrum stigma remains below the top of the anthers. Petal above anther attachment grows first in both pin and thrum flowers while anthers remain at a similar height, low in the flower. There is no growth of the corolla tube below anther attachment in the developing flowers shown in **A - C**. There is no growth of the corolla tube below the anthers of the pin flowers in **D**, but growth of the corolla tube below the anthers can be observed in all of the thrum flowers in **D – F.** Growth of the pin corolla tube below the anthers has just begun in **E** and continues in the pin flower in **F**. Flowers in **F** will soon open as mature flowers as shown in figure 3.5.

and both the final position of the anthers, at the mouth of the flower in thrum and half way down the corolla tube in pin (as shown in Figure 3.5) and the elongated style of the pin flower and the short style of the thrum flower can be observed.

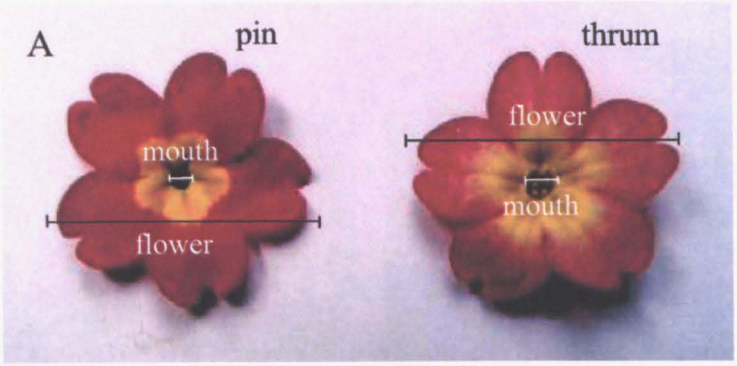
Previous ontogenetic studies on other species revealed that where stamens are attached to the corolla tube, the two parts of the tube above and below the anthers, are formed by two spatially and temporally separate processes. This division of the corolla tube into two parts, the upper corolla tube being the part above anther attachment and the lower corolla tube being the part below anther attachment has been employed by previous authors (e.g. Sporne, 1974); and it has been observed also in *Lactuca sativa* and other members of the Compositae with epipetalous stamens that both parts develop by intercalary growth. The lower part develops later than the upper part, but the timing of its initiation varies between species (Erbar, 1991). As the lower corolla tube develops the stamens are carried up to their final epipetalous position. Erbar further defines the lower corolla tube as the stamen-corolla tube (Erbar, 1991). Work on C function in *Primula* (Cook, 2002) found expression of the C function organ identity gene in the lower corolla tube but not in the upper corolla tube above the anthers. This is in agreement with Erbar's definition, although it was thought that C function was not evenly distributed throughout the tissue but was probably confined to the vascular bundles (Cook, 2002). In *Primula* it has been shown (Figure 3.6) that the upper corolla tube begins to develop first in both pin and thrum flowers. However in flowers of the same or similar size the lower corolla tube of thrum flowers normally begin growth much earlier than the lower corolla tube of pin flowers. Observation of a series of developing flowers from eight thrum plants (not shown) revealed that all had exhibited early growth of the lower corolla tube when whorl two was approximately 10mm in length. In contrast, observation of a series of developing flowers from nineteen pin plants (not shown) revealed only two that exhibited growth of the lower corolla tube when whorl two had reached 10mm. in length. The latter observation indicates some flexibility in the temporal aspect of the growth, but it is the differential physical growth of the corolla tube above and below the attachment point of the stamens that results in different anther positions in the two morphs. The definition "attachment point" is used rather than the definition "insertion point" that has been used by some previous authors (e.g. Mather, 1950) since observations of early ontogeny show that the stamens develop first and that the second whorl subsequently develops attached to the stamens.

3.6. The diameter of the floral mouth is different in pin and thrum flowers.

Study of pin and thrum flowers led to the observation that thrum flowers appeared to have a wider corolla tube mouth than pin flowers of the same size. In order to investigate the possibility of this being a new heteromorphic difference between pin and thrum flowers the diameter of the face of the flower, and the diameter of the corolla tube mouth in 30 pin and 30 thrum flowers from sibling plants, each with the normal complement of five petals, was measured and recorded. Data was collected in parallel from two different lines. The first line was of red cultivars produced from a cross between a red commercial *Primula vulgaris* and a wild primrose (*P. vulgaris*); the second line was the commercial F1 hybrid *Primula vulgaris* var. Blue Jeans. Figure 3.7A shows pin and thrum flowers from the red primrose progeny indicating the measurements taken. The mouth of the corolla is visibly wider in the thrum flower than in the pin flower. Quantification of the data confirmed this initial observation with all measurements made to within 0.5 mm.

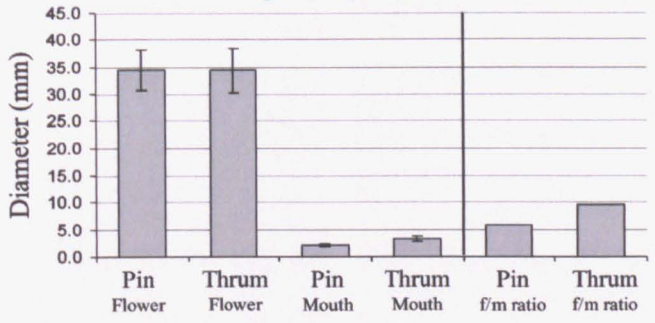
The mean diameter of the flower face in the red cultivar progeny was 34 mm for both pin and thrum flowers (Figure 3.7B). The mean flower diameter in the *Primula vulgaris* var. Blue Jeans flowers was 37 mm (Figure 3.7C). The corolla tube mouth diameters in both sets of samples was greater in the thrum flowers than in the pin with mean values of 2.0 mm and 3.3 mm respectively for pin and thrum flowers from progeny derived from the wild primrose plants and 3 mm and 4 mm from pin and thrum flowers from the larger *P. vulgaris* var. Blue Jeans flowers. In both samples, the ratio of flower diameter to corolla mouth diameter is greater for pin flowers than for thrum flowers of the same size and further emphasises the difference between the two floral morphs (Figure 3.7B and C). This difference in *Primula* heteromorphic floral architecture has not been documented previously.

Examination of corolla tube cells from above the anthers in thrum and in pin flowers using both light microscopy (not shown) and scanning electron microscopy (Figure 3.8) showed the corolla tube cells above the anthers to be broader in thrum flowers than in pin flowers. This observation indicates that the increased width of cells in the thrum upper corolla tube leads to an increased diameter of the flower mouth in thrum flowers as compared to pin flowers.



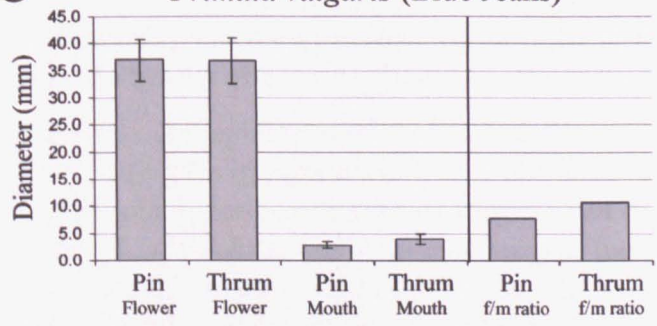
Primula vulgaris (progeny of red cultivar)

B



Size (mm)	Pin flower	Thrum flower	Pin mouth	Thrum mouth	Pin f/m ratio	Thrum f/h ratio
Mean	34.4	34.4	2.0	3.3	5.9	9.6
SD	3.8	4.1	0.2	0.5		

C



Size (mm)	Pin flower	Thrum flower	Pin mouth	Thrum mouth	Pin f/m ratio	Thrum f/m ratio
Mean	36.9	36.8	2.9	4.0	7.7	10.8
SD	3.9	4.2	0.6	0.9		

Figure 3.7. Comparison of flower mouth diameter in pin and thrum flowers

A. Pin and thrum flowers showing flower diameters and mouth diameter. Size bars are 1 cm. **B.** Flower diameter and mouth diameter measurements (mm) for pin and thrum flowers from a wild primrose-commercial cultivar cross. Error bars are based on standard deviations and the flower (f) to mouth (m) ratios are indicated for both pin and thrum flowers. **C.** As in B but data obtained from measurements on *Primula vulgaris* var. blue jeans.

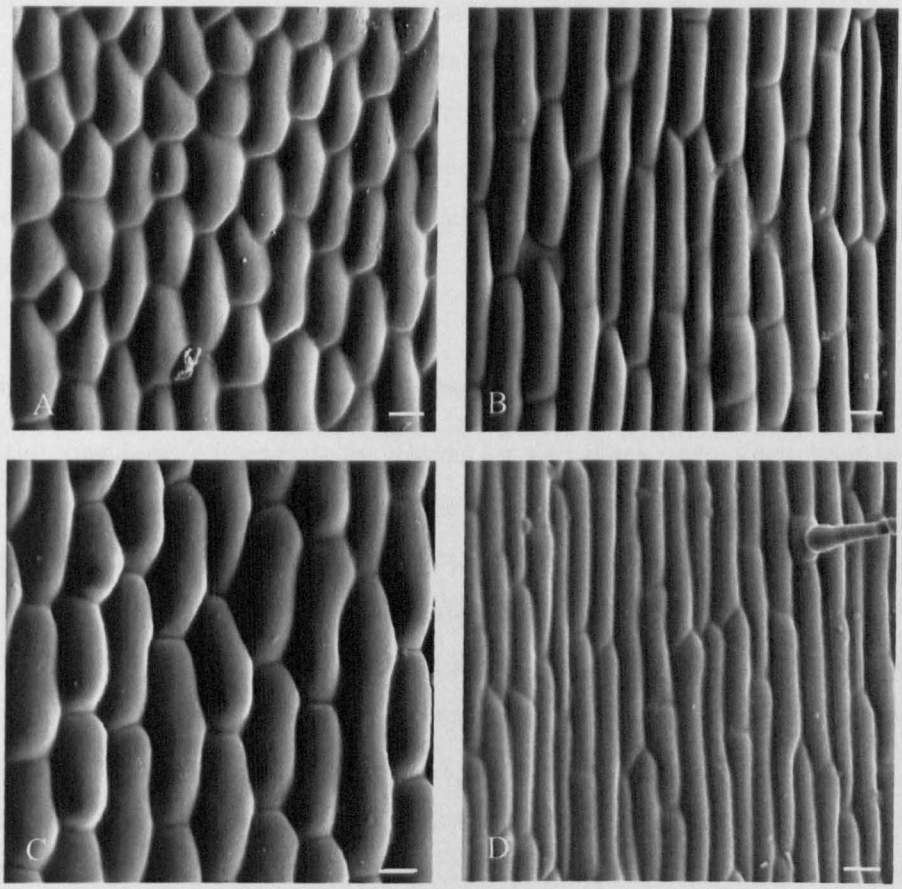


Figure 3.8. Scanning electron microscope analysis of *Primula* pin and thrum corolla tube cells above the anthers.

A. Cells from the outer epidermis of the thrum upper corolla tube of a flower just opening (as in Figure 3.6F). **B.** Cells from the outer epidermis of the pin upper corolla tube of a flower just opening (as in Figure 3.6F). **C.** Cells from the outer epidermis of the thrum upper corolla tube of a mature fully open flower (as in Figure 3.5). **D.** Cells from the outer epidermis of the pin upper corolla tube of a mature fully open flower (as in Figure 3.5).

3.7. Discussion of late development.

The presence of pin and thrum flowers in *Primula* and the phenomenon of heteromorphic flower development has been reputedly known since Clusius, 1583 (van Dijk 1943, quoted in Ornduff 1993), and has been of scientific interest since Darwin, 1876. However there is as yet no insight into the molecular basis of heteromorphy or an understanding of the cellular processes that contribute to the differential development of pin and thrum flowers. This analysis provides new insight into the development of heteromorphic flowers and reveals a previously unreported characteristic of heteromorphic flowers.

3.8. Timing of the development of heteromorphic characteristics.

Despite the considerable interest in the *S* locus for very many years, there have been no previous observations on the timing of events that lead to the final architecture of the mature *Primula* flower and this data provides the first analysis of the developmental timing of heteromorphy. It has been shown that observable differences between pin and thrum flowers are first seen during when the pin stigma shows the first signs of elevation above the top of the anthers and in the corresponding thrum flower the tip of the anthers start to extend above the stigma (Figure 3.6A and 3.6B). The observation that pin style elongation is the first discernable step in heteromorphic development suggests that the effects mediated by the *G* locus may be the first to be implemented, Pin plants are homozygous for the recessive *g* allele, therefore an initial key event in *Primula* heteromorphy may be the action of the dominant *G* allele in inhibiting style elongation in thrum flowers. Pin plants are also homozygous for the recessive *a* allele and do not show significant elevation of anthers from the base of the corolla until later than do thrum flowers. (Figure 3.6, D,E and F) Together these observations suggest that *A* plays a dominant role in promoting anther elevation, while *G* plays a dominant role by suppression of growth. These observations provide the first indication of differential timing of developmental events during heteromorphy, and indicate that temporal control of *S* locus gene expression may be an integral component of pin and thrum flower development.

3.9. Corolla tube mouth diameter, a new heteromorphic character.

This analysis was undertaken using both *wild type* primrose hybrid flowers and a horticultural variety *P. vulgaris* cv. Blue Jeans. In both examples, the observed greater

diameter of the flower mouth in thrum as compared to pin flowers indicated that the presence of this previously undocumented characteristic correlated with floral morph. The selection of equal numbers of pin and thrum flowers of similar sizes and with the normal complement of five petals enabled elimination of differences that could be attributed to different flower sizes or to the presence of extra petals. As examination of corolla tube cells from above the anthers in thrum and in pin flowers using both light microscopy (not shown) and scanning electron microscopy (Figure 3.8) showed the corolla tube cells above the anthers to be broader in thrum flowers than in pin flowers it can be deduced that the increased width of cells in the thrum upper corolla tube leads to an increased diameter of the flower mouth in thrum flowers as compared to pin flowers. It is considered that this may be a direct consequence of the presence of a dominant *A* allele at the *S locus*. This new difference between pin and thrum flowers may have previously gone unnoticed due to the varying sizes of flowers in bloom on a plant at any one time, with the earliest flowers to bloom always being larger than the later flowers.

These studies have illustrated the spatial and temporal functions of the dominant alleles of the *S locus* linked genes *A* and *G*. Analysis of *Primula* floral homeotic mutants will provide evidence for the organ identity specific effects of these *S* locus associated genes *A* and *G*. A new aspect of the *Primula* heteromorphic phenotype has been discovered. The isolation and characterisation of the genes located at the *Primula S* locus, including *G* and *A*, will be required to provide a molecular explanation for these cellular and developmental observations. Work in the laboratory is actively engaged in identifying genes that are linked to *Primula S* locus with the objective of establishing an integrated physical and genetic map to facilitate identification and characterisation of the genes that control heteromorphy in *Primula*.

CHAPTER FOUR

Mutant Phenotypes.

4.1. Introduction.

Ten mutant phenotypes were investigated and are shown in Fig. 4.1. Half of these, *Jack in the Green, Hose in Hose, Staminoid Carpels, sepaloid* and *double*, can be described as organ identity homeotic mutants. The possible basis for *green primrose* and *virescent cowslip* is less straightforward, although both do exhibit transformation of organs. The *Split Perianth* mutant phenotype has organs in the correct position but has alteration to the usual form of the organ, and two, *reduced petal* and *Oak Leaf*, are pleiotropic mutants where both the flower and the leaf form are affected.

4.2. Description of and development of the mutant phenotypes.

4.2.1. The *Jack in the green* mutant phenotype.

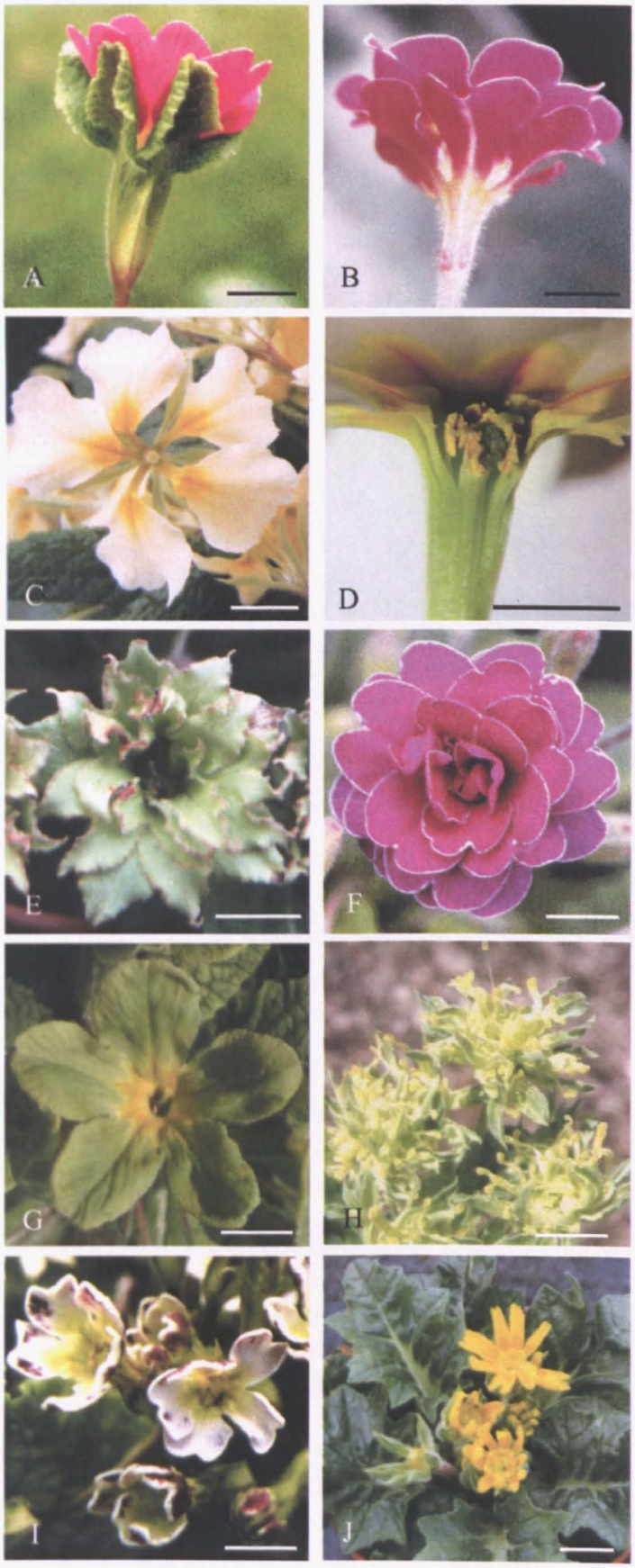
(i) Description of *Jack in the Green.*

The *Jack in the Green* phenotype has the calyx lobes converted wholly or partly to leaves (Fig.4.2A). Expression of the phenotype is variable. In the least extreme phenotypes, only the very tip of each calyx lobe is leafy; in the most extreme, each entire calyx lobe is in the form of a leaf and may be quite large in proportion to the flower (Fig.4.2B). Besides the variation in size of leafy calyx from one plant to another, similar variation can occur on each plant from the beginning of the flowering season to the end. Large leafy calyces are produced early in the flowering season, smaller ones later, and some plants may have normal calyces on the very latest flowers of the season. The leafy calyx is persistent (Fig. 4.2E) and remains intact until after seed has ripened, although it may begin to deteriorate at this time as shown in Fig. 4.2F. When *Jack in the Green* is combined with the genetic background of the cowslip *P. veris,* the bracts at the base of the pedicles, may, in some of the progeny, also show conversion to leaves (compare Fig.4.2C and Fig.4. 2D). The cross is done as *P. veris* x *P. vulgaris Jack in the Green* since crosses done in the opposite direction are not usually successful. (For studies of hybridization between British *Primulas* see Valentine 1947, 1951 and 1955). The first illustration of a polyanthus, drawn from Bobart's herbarium specimen, was of a *Jack in the Green* with large leafy bracts at the base of the pedicles. Conversion of the bracts to leaves is not thought to be a

Fig.4.1. Floral mutant phenotypes of *Primula*.

Ten mutant phenotypes are described. They are arranged in order of the whorls affected, from whorl 1 inwards.

A. *Jack in the Green* – whorl 1 converted partly or wholly to leaves. **B.** *Hose in Hose* – whorl 1 converted partly or wholly to petal. **C.** *Split Perianth* – in which whorl 1, and in some plants whorl 2 also, fail to fuse to form the normal calyx tube and corolla tube structures. **D.** *Staminoid Carpels* – a double mutant in which in addition to having whorl 1 converted to petal, the phenotypes have whorl 4 partly or completely converted to stamens. The form has not been found as a single mutant, and may be an allele of *Hose in Hose* or a separate mutation. **E.** *sepaloid* – in which there are no petals and anthers. There can be four whorls of sepals, or two or three whorls of sepals and a functional or non-functional carpel. **F.** *double* – in which the anthers and carpels are normally replaced by indeterminate whorls of petals. There are a number of forms of doubling with varying organ order (Fig. 4.15). **G.** *Primula viridis*, the green primrose – in which the petals are green, the stamens are degenerate and the carpel has a leafy ovary wall. **H.** *Virescent cowslip* – in which there are no normal organs. The "flower" consists of a mixture of green leafy bracts, ectopic ovules, and stigma like structures with open carpels at the base. **I.** *reduced petal* polyanthus – in which the petals are reduced and there is loss of pigment on the adaxial surface. Leaves are also broader, thinner and more wavy edged than those of wild type siblings. **J.** *Oak Leaf* – in which the leaves are distinctively lobed as are oak leaves and are also thicker than normal leaves. Sepals and petals are generally attenuated. Size bars are approximately 1cm.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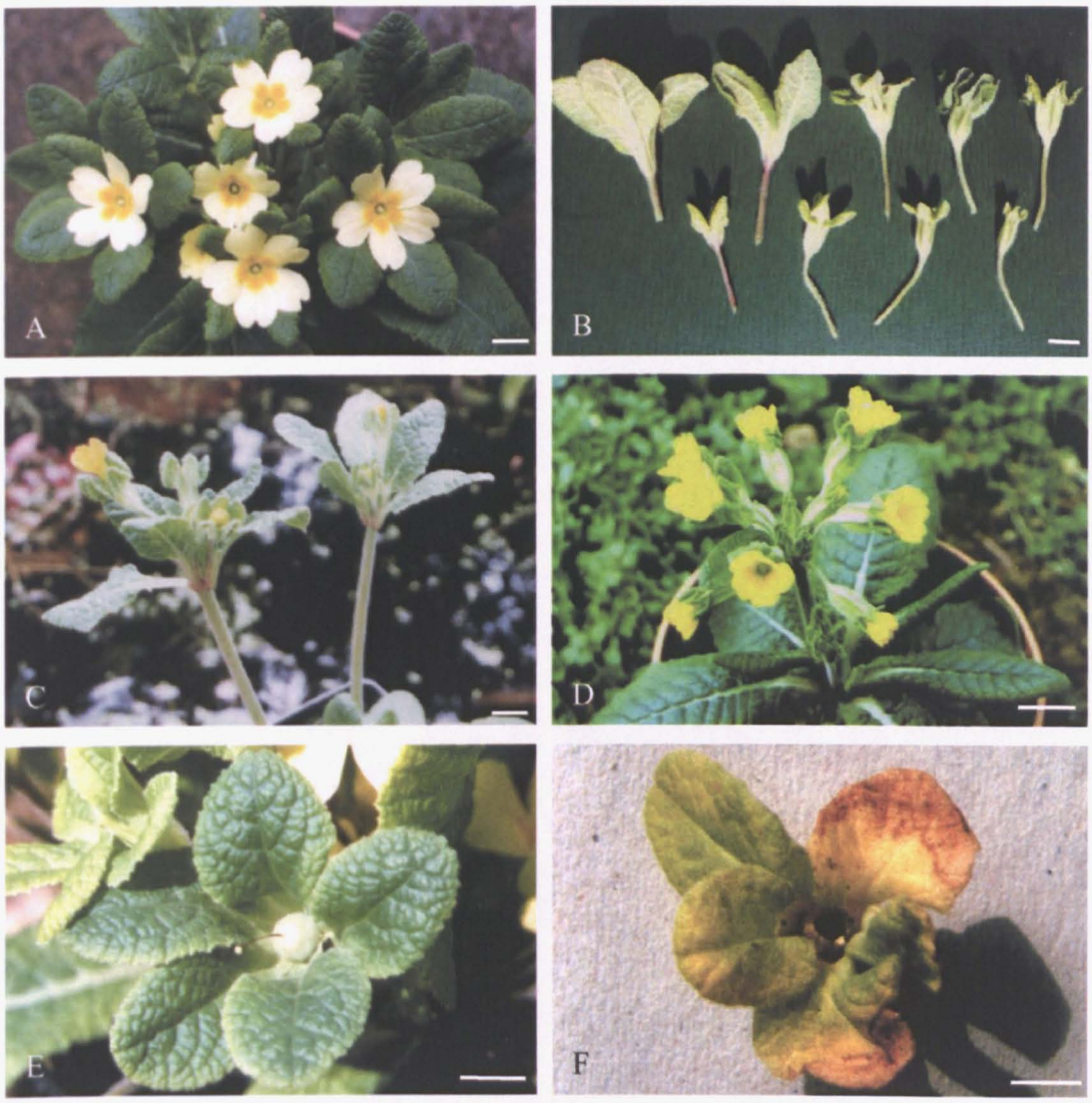


Figure 4.2. The *Jack in the Green* mutant phenotype.

The *Jack in the Green* has part or all of the sepals converted to leaf form.

A. A *Jack in the Green* plant in flower. **B**. Range of expression of *Jack in the Green* leafy calyces. **C.** A *Jack in the Green* cowslip hybrid with the bracts at the base of the pedicles also converted to leaves. **D**. A *Jack in the Green* cowslip hybrid with normal bracts. **E**. A *Jack in the Green* calyx during seed set. **F**. Persistent *Jack in the Green* calyx with open ripe seed capsule. Size bars are approximately 1 cm.

Figure 4.3. Development of the *Jack in the Green* flower.

Early development of the Jack in the Green flower up to and including stage 6 is illustrated. All images are from the same plant as the flower in **Fig. 4.1. A**

A. Cluster of developing *Jack in the Green* flowers.. **B**. Developing *Jack in the Green* flower at middle stage 4. (w = whorl) **C**. Developing *Jack in the Green* flower at late stage 4. **D**. Developing *Jack in the Green* flower at stage 5. **E - H**. *Jack in the Green* calyces in developing flowers of 1.0mm, (**E**), 1.5mm, (**F**), 2.0.mm, (**G**), and 2.5mm. (**H**), in length respectively. All are during stage 6. By the end of stage 6 the calyces are recognizably leaf like and from this point to maturity simply grow larger. **I - L.** *Jack in the Green* whorl 4 development during stage 6, in developing flowers of <1.0mm, (**I**), 1.0mm, (**J**), 1.0mm, (**K**), and >1.0mm (**L**), respectively. Note that although the flowers in **I** and **J** are the same size the degree of development is not the same. Size bars are 100μm .

straightforward Mendelian trait since it does not always occur in all of the *Jack in the Green* progeny, it is more likely to be due to the degree of expression of the phenotype in an individual plant.

(ii) Development of *Jack in the Green* flowers.

Early development of *Jack in the Green* flowers is similar to wild type (Fig.4.3B). During late stage 4 a progressive curling back of the tips of whorl 1 becomes evident (Fig.4.3C). This aspect of the phenotype permits observations of the development of inner organs, such that by late Stage 5 development of the four lobes of each anther are clearly visible (Fig.4.3D). Also visible at this time is a slightly raised ridge present all around the outer edge of whorl 1. Progression of development through some of these stages can be seen in fig. 4.2A. By the time that the developing flowers reach approximately 1.0 mm in length, numerous trichomes are present on whorl 1, on the curled back tips of what will become leafy sepals (Fig.4.3E). This aspect of the phenotype becomes more evident with increased growth of the developing flower (Fig.4.3F - H) and during stage 6 in developing flowers of 2.0 – 2.5mm in size the leaf like tips of whorl 1 are unmistakable. Beyond this stage the leafy whorl 1 organs continue to grow larger and remain leaf like in appearance. Developing *Jack in the Green* flowers during late stage 5 and stage 6 (Fig.4.3I - L) were dissected in order to ascertain whether mutations in whorl 1 had any visible effect on inner whorls. Results show normal development of whorls 3 and 4 in these flowers. The overall sizes of the developing flower in fig. 4.3I and in fig. 4.3J is the same, but variability in rate of development of whorl 4 from one flower to the next is demonstrated by the observation that whorl 4 development remains in stage 5 in Fig.4.3I while whorl 4 development in Fig.4.2 J is at early stage 6.

4.2.2. The *Hose in Hose* mutant phenotype.

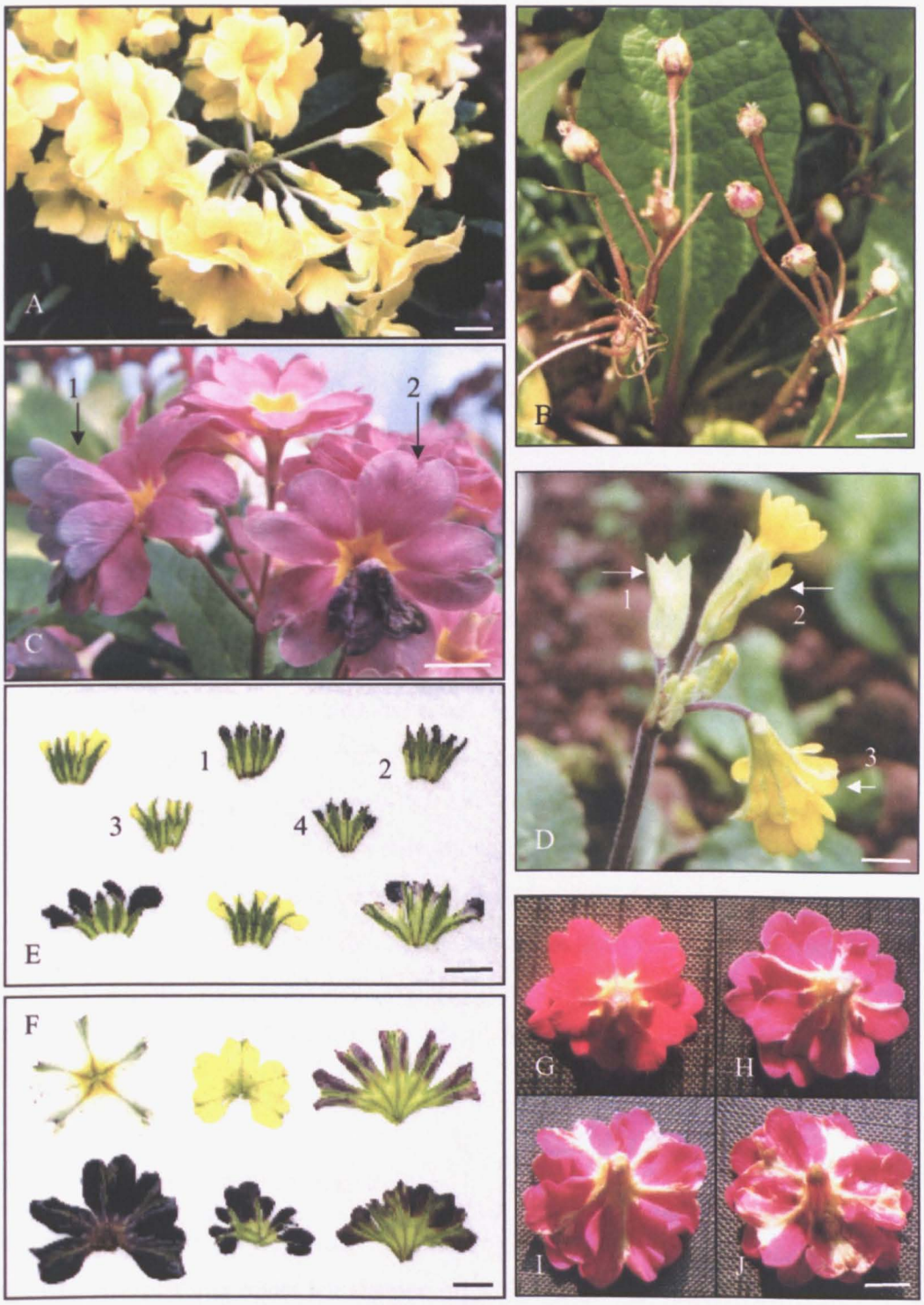
(i) Description of *Hose in Hose*.

The *Hose in Hose* mutant has calyces that are wholly or partly converted to petal. In the most extreme expression of the phenotype one flower appears to grow out of another (Fig. 4.4A). The thrum stigma generally reaches the height of the mouth of the petaloid calyx corolla tube, so that if the true corolla is pulled out what remains looks like a pin flower without a calyx. Environmental conditions can influence corolla tube growth so

Figure 4.4. The *Hose in Hose* mutant phenotype.

The *Hose in Hose* phenotype has the calyces wholly or partly converted to petal. The phenotype exhibits variability in expression both on the same plant and on different plants. The developing seed capsules are unprotected.

A. *Hose in Hose* flowers with fully petaloid calyces, so that one flower appears to grow out of another. **B.** *Hose in Hose* seed capsules, without any protective calyx. **C.** *Hose in Hose* flowers wilting; showing the limited persistence of the petaloid calyx. Flower number 1 is just beginning to wilt and the petaloid calyx is still fresh.. Flower number 2 is completely wilted and the petaloid calyx is beginning to deteriorate slightly at the edges. **D.** A *Hose in Hose* cowslip with different degrees of expression of the phenotype on different flowers on the same scape. Calyx number 1 is as wild type, calyx number 2 is partly petaloid, and calyx number 3 is fully petaloid. **E and F.** Pressed calyces from a number of *Hose in Hose* flowers from different plants showing various degrees of expression of the phenotype. Where expression is very poor, as in numbers 1-4 the phenotype may not be apparent without close examination of the calyces. **G – J.** Four flowers, from four sibling plants, exhibiting different degrees of petalody of the calyx. Size bars are approximately 1 cm.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that under extreme conditions (either of heat or cold) the true corolla does not protrude as fully from the calyx corolla as normal, thus leaving the anthers and the stigma at the same height and giving the appearance of a long homostyle. The petaloid calyx is more persistent than the corolla, (Fig. 4.4C) but begins to deteriorate soon afterwards. Part of the wilted petaloid calyx may cling to the capsule during early seed set but the capsules are generally naked before seed is ripe (Fig. 4.4B). Expression of the phenotype can vary so that in some genetic backgrounds the phenotype is only poorly expressed. There can be considerable variation in the degree of conversion of sepals to petals, and also in the shape of the petaloid calyx (Fig.4.4E and F). Where expression is unstable, expression of the phenotype can not only be variable within a single plant, but even from one calyx lobe to the next. The latter can be observed on some of the calyces in Fig 4.4. E and F. Where poor expression simply results in the calyx being streaked with green the *Hose in Hose* form is still recognizable. When only very tiny amounts of petal are found on some, but not all calyx lobes, (Fig. 4.4E, 1 – 4) the phenotype may not be recognized as *Hose in Hose* without close examination of the calyces. Siblings from the same cross frequently exhibit different degrees of expression of the phenotype (Fig.4.4G – J). Expression of the phenotype can sometimes deteriorate in older plants. Conversely, young plants with flowers showing calyces that have only a thin green midrib to each petaloid calyx lobe may develop flowers with fully petaloid sepals later in the season. In rare instances a single scape may have flowers of the above varying forms (Fig.4.4D). As the plant continues to grow there will be ramets carrying flowers with different degrees of expression so that on one plant there may be ramets with fully petaloid calyces, ramets with partly petaloid calyces, and occasionally ramets in which the calyces have reverted to normal. Other plants exhibit stable expression of the phenotype for very many years. Unlike the *Hose in Hose* plants described by Ernst in 1931, the *Hose in Hose* described here are all at least as floriferous as *wild type* plants, and in many instances more floriferous.

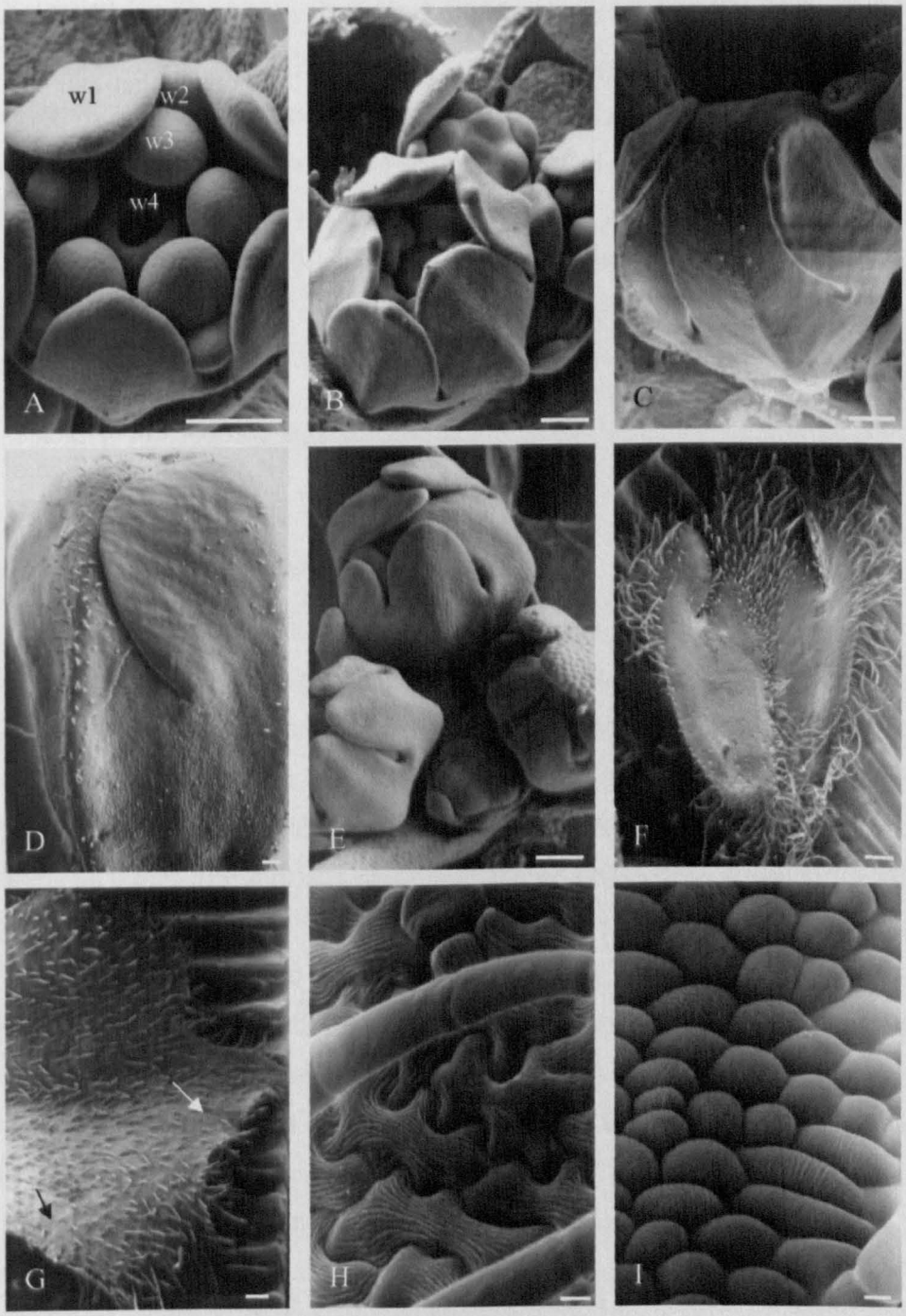
(ii) Development of *Hose in Hose* flowers.

Early development of *Hose in Hose* flowers is similar to *wild type* until Stage 4. By late stage 4 it is evident that whorl 1 is thinner, broader, and shorter than *wild type* (Fig.4.5A). The presence of petals in place of sepals in whorl 1 becomes more evident by early stage 5 (Fig.4.5B) and is unmistakable in late Stage 5 (Fig.4.5C). Beyond stage 5 the developing petaloid whorl 1 continues to grow (Fig.4.5D) without altering radically in appearance. In those plants where the homeotic conversion of sepals to petals is not

Figure 4.5. Development of *Hose in Hose* flowers.

The early development of *Hose in Hose* flowers up to stage 6 is illustrated. Comparison is made of the early development of *Hose in Hose* flowers with incomplete expression of the phenotype with that of flowers that will have fully petaloid calyces.

A. Developing *Hose in Hose* flower at late stage 4. **B.** A developing *Hose in Hose* flower at early stage 5, with a stage 3 developing flower behind. **C.** A developing *Hose in Hose* flower at late stage 5. **D.** A developing Hose in Hose flower at approximately stage 6. (**A – D** were taken from the same plant as were the flowers in **Fig. 4.4.I** and in **Fig. 4.1A**). **E.** A cluster of developing Hose in Hose flowers with incomplete expression of the phenotype (**E – I**) were taken from the same plant as was the flower in Fig. **4.4.L**). **F.** A developing flower of approximately stage 6 from the same plant. The central portion of each calyx lobe is densely covered with long trichomes, and only the smooth edge of the calyx lobe will be fully petaloid. **G.** The adaxial surface of a developing calyx lobe (approx. 3.0mm.in width) from the same plant **H.** Cells from the centre of the calyx lobe in G (shown by a white arrow). **I.** Cells from the edge of the calyx lobe in G (shown by a black arrow). Size bars in **H** and **I** are 10um. Other size bars are 100μm.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complete, Stage 4 and Stage 5 flowers are similar to *wild type* (Fig.4.5E). As the developing flower increases in size (Fig.4.5F, stage 6) it can be observed that the centre of each whorl 1 petal is densely covered in trichomes and only the lower edges are smooth. This contrasts sharply with the fully petaloid stage 6 flower in fig.4.5D. The tip of a larger whorl 1 petal of approximately 3.0mm in width was examined more closely (Fig.4.5G). Cells from the center of the above petal are observed to be not of the normal conical papillate petal cell form (Fig.4.5H). Those closer to the edge of the immature petal are more rounded (Fig.4.5I), and although not conical papillate they do more closely resemble that form than do the cells in the previous figure.

4.2.3. The *Split Perianth* mutant phenotype.

(i) Description of *Split Perianth*.

In this phenotype the calyx, and in many instances the corolla also, is divided into five separate parts without any connective tissue between the sepal lobes or the upper part of the corolla tube (Fig.4.6B). Where the corolla tube is split the division most commonly stops at the point of attachment of the anthers (Fig.4.6C), but occasional plants have been found that have whorl 2 split entirely to the base of the corolla tube making five separate segments (Fig. 4.6D). The split calyx is persistent until seed set and beyond (Fig. 4.6E), but being split and spread open it does not offer much protection to the ripening seed capsules. *Primulas* with divided calyces were illustrated in the past as has been shown in Chapter 1. The form may have disappeared from cultivation for a time; it was no longer commercially available in spring 1997 when a single plant was discovered in a batch of gold laced polyanthus seedlings raised by Dr. R. J. Brumpton (Fig.4.6A). It was from this plant that all subsequent split perianth forms are derived. The splitting of the corolla in the original plant was uneven and erratic, and some progeny also exhibited uneven splitting of the corolla. One plant that was derived from a cross between two split perianth parents, produced flowers that were very much reduced at first flowering (Fig.4.6F-H), the most extreme being reduced to the two reproductive whorls only, (Fig.4.6H). Later in the flowering season the plant produced some flowers that did have all four whorls present.

Figure 4.6. The *Split Perianth* mutant phenotype.

The *Split Perianth* has the calyx and sometimes the corolla also divided into separate parts. The first *Split Perianth* plant discovered is illustrated along with derived *Split Perianth* plants with different degrees of expression of the phenotype.

A. The original *Split Perianth* plant from which all subsequent plants were derived. **B.** A *Split Perianth* plant with normal corollas. **C.** A *Split Perianth* flower with the corolla split to the point of attachment of the anthers. **D.** A *Split Perianth* plant with the corolla totally split to the base of the corolla tube. **E.** A *Split Perianth* ripe seed capsule. **F.** A flower from a *Split Perianth* plant that produced flowers without any perianth whorls on many of the earliest flowers (later flowers did have both perianth whorls). **G.** A flower from the same plant as the flower in F, this one with petal tipped anthers. **H.** Another flower from the same plant as the flowers in F and G, this one without any perianth whorls. Size bars are approximately 1cm.


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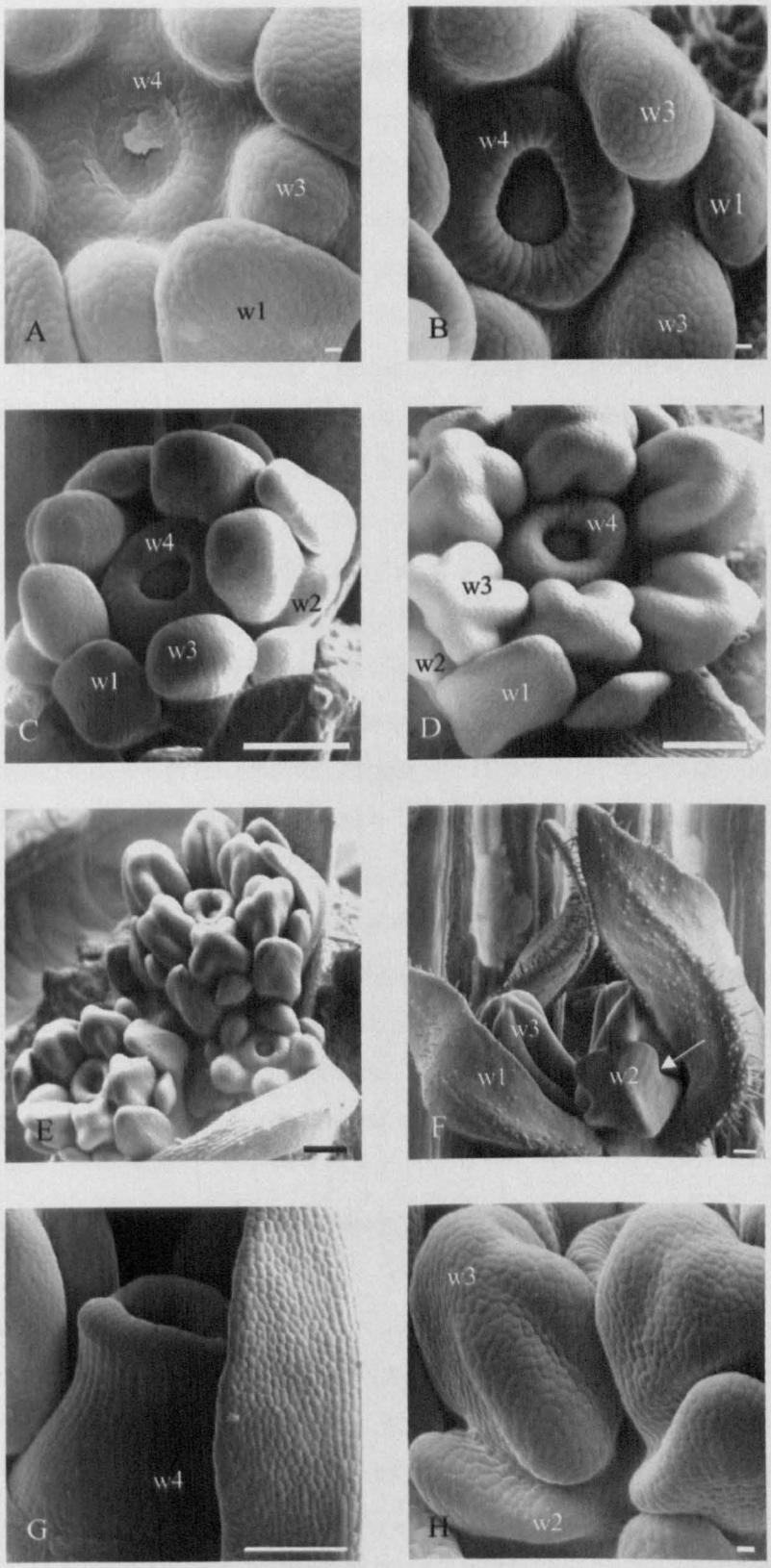
Figure 4.7. Development of the *Split Perianth* flower.

Early development of the *Split Perianth* flower is compared with that of *wild type*, and the development of the *Split Perianth* flower up to stage 6 is illustrated.

A. Developing *wild type* flower at middle stage 4 (for comparison with *Split Perianth*). **B.** A developing *Split Perianth* flower at stage 4. **C.** A developing *Split Perianth* flower at late stage 4. **D.** A developing *Split Perianth* flower at late stage 5. **E.** A cluster of developing *Split Perianth* flowers at different stages of development. **F.** A developing *Split Perianth* flower at approximately stage 6. An arrow indicates the "hour glass" shape of the developing petal. **G.** The tip of whorl 4 enlarging to eventually form a stigma. **H.** Whorl 2 emanating from the base of whorl 3, before the filament of the anther has developed. The size bar in **H** is10μm; all other size bars are 100μm.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(ii) Development of *Split Perianth* flowers.

Early development of *Split Perianth* mutants is generally as *wild type* to stage 3 During stage 4 there are some early differences between *wild type* (Fig. 4.7A) and *Split Perianth* (Fig. 4.7B). In *wild type* whorl 4 is less well developed than in *Split Perianth* at this stage, but whorl 2 is more developed in *wild type* than in *Split Perianth*. Despite the degree of development of whorls 3 and 4 in the *Split Perianth* stage 4 developing flower in Fig. 4.7B, no whorl 2 primordium can be observed behind a whorl 3 organ. There is a similar absence of whorl 2 primordia behind the whorl 3 organs of the small flower in the Fig. 4.7E, that shows a cluster of developing *Split Perianth* flowers at different stages of development. The absence of the whorl 2 primordia makes it difficult to determine whether the flower is at early or middle stage 4. From late stage 4 the separate organs of whorl 1 are evident (Fig.4.7C) and the whorl 2 organs are now apparent. In flowers where the corolla is also divided this becomes evident from late stage 4 (Fig.4.7D). Perianth organs appear less well developed by stage 5 than in other whorl 1 mutant phenotypes, but they do eventually reach the same length as *wild type* perianth organs in mature flowers. When both perianth whorls are split, unique observation of the development of inner whorls is possible. Figure 4.7G shows the very earliest enlargement of the top of whorl 4 that will become the stigma and figure 4.7H shows the point during stage 5 at which the anthers can be observed to be distinct from the corolla, although the anther filament has not yet elongated. The *Split Perianth* is extremely distinctive by stage 6 (Fig.4.7F), and develops a rather untidy appearance as the developing flower grows larger. The *Split Perianth* petal as shown in Fig.4.7F. can be observed to have an "hour glass" shape – the upper part being the normal petal and the lower being the part normally joined to form the upper corolla tube. The images were taken from developing flowers from the same pin *Split Perianth* plant, as was the flower in Fig.4.6C.

4.2.4. The *Staminoid Carpels* mutant phenotype.

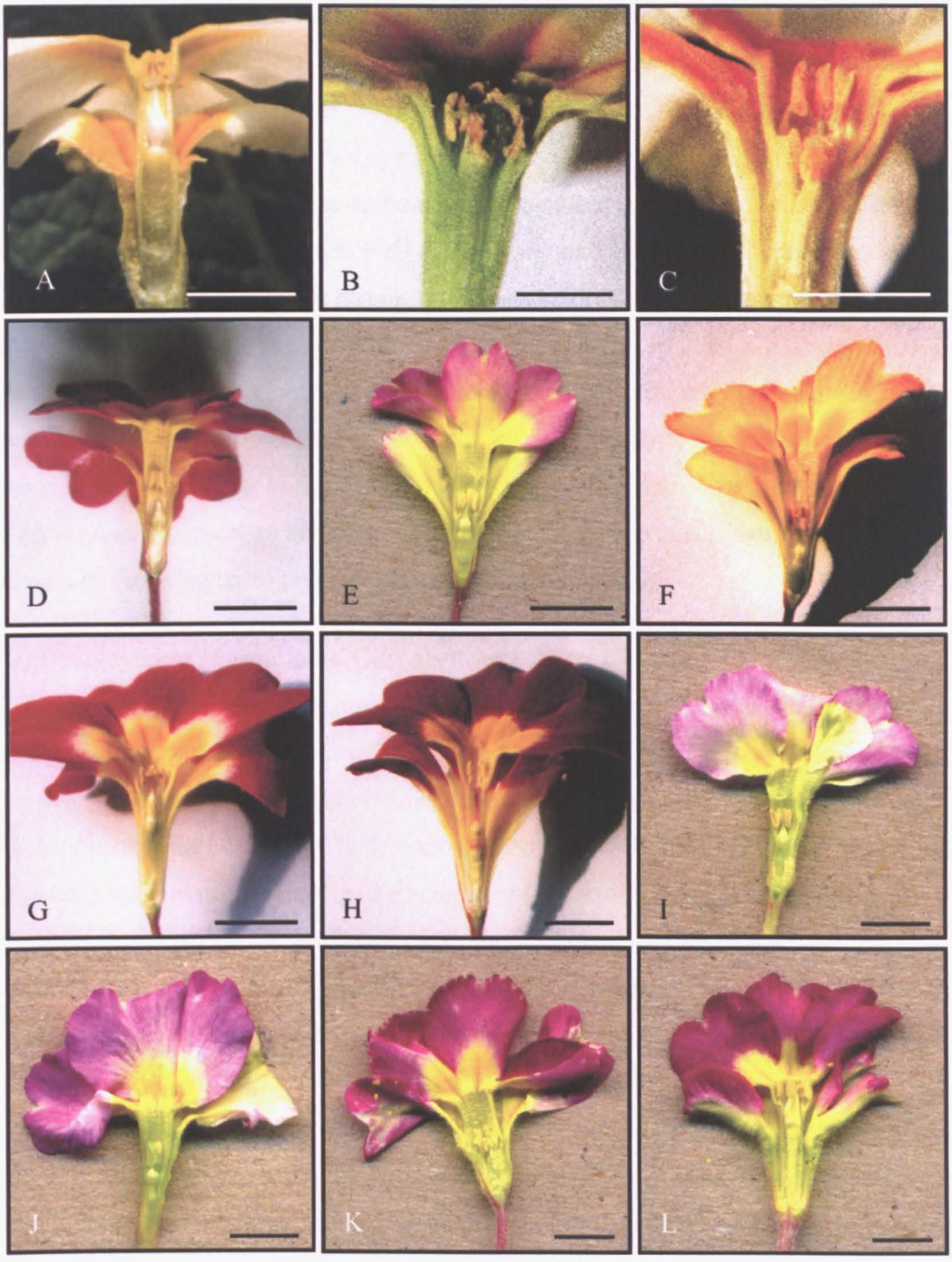
(i) Description of *Staminoid carpels*.

The *Hose in Hose* mutant with homeotic conversion of sepals to petals has been described above. In addition to *Hose is Hose*, a second independent mutant phenotype was obtained in 1988 that shows strong similarity to *Hose in Hose*. This new phenotype, *Staminoid Carpels*, shows aberrant carpel development in addition to the petaloid sepals of *Hose in Hose*. Meyer (1966) describes staminody of the carpel as "quite unusual" and has no reports of the condition in *Primula*. As with other *Primula* floral mutants studied,

Figure 4.8. The *Staminoid Carpels* mutant phenotype

The *Staminoid Carpels* mutant phenotype has so far only been found in conjunction with *Hose in Hose,* and may represent a more extreme allele of that phenotype. Expression is variable in different genetic backgrounds. The least extreme form has an elongated ovary with an occasional anther enclosed inside the upper portion, a more extreme form has the ovary wall converted to anthers enclosing naked ovules, and the most extreme form has both the ovary wall and the ovules converted to anthers.

A. Mature thrum flower with the least extreme form of *Staminoid Carpels.* **B.** Mature thrum flower with *Staminoid Carpels* of the form that has anthers enclosing naked ovules. **C.** Mature thrum flower with the most extreme form of *Staminoid Carpels,* where both the ovary wall and the ovules are converted to anthers. **D.** The original mature pin flower with *Staminoid Carpels* of the least extreme form. **E – H.** Progeny from the cross of thrum *wild type* x *Staminoid Carpels.* Pin flowers, E and F, have elongated ovary walls that are shorter than those shown in thrum flowers, **G** and **H.** Only one thrum *Staminoid Carpels* plant, that shown in **H,** had a shorter elongated ovary wall than is usual for thrum *Staminoid Carpels flowers.* That shown in **G** is typical. **I** and **J.** Mature sibling pin *Staminoid Carpels* flowers homozygous for *Hose in Hose.* **K** and **L.** Sibling *Staminoid Carpels* flowers; that in **K** is heterozygous for *Hose in Hose* and that in **L** is homozygous for *Hose in Hose.* Expression of *Staminoid Carpels* is not generally more extreme in the flowers homozygous for *Hose in Hose.* Thrum *Staminoid Carpels* flowers do elevate the whorl four *Staminoid Carpels* to a higher position in the flower than do pin *Staminoid Carpels.* Size bars are approximately 1 cm.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there is considerable variability in expression of this new mutant phenotype in different genetic backgrounds. The original *Staminoid Carpel* mutant has the least extreme conversion of whorl 4 towards stamens. The whorl 4 organ has a normal stigma, short style, and an elongated ovary wall with an occasional anther inside the upper portion (Fig.4.8A). Subsequent crosses using pollen from this plant onto different genetic backgrounds revealed the more extreme phenotypes of this mutant allele. One of these has the ovary wall converted to anthers enclosing naked ovules (Fig.4.8B), while in the most extreme form the ovules as well as the ovary wall are converted wholly to anthers (Fig.4.8C). All of these plants had thrum flowers as the phenotype segregates with both the *Hose in Hose* allele and the thrum allele of the S locus. In 2001 one pin *Hose in Hose* plant with a *Staminoid Carpel* of the least extreme form occurred naturally as a garden seedling (Fig.4.8D). Its origins are not known. The phenotype has never been found in the absence of the *Hose in Hose* phenotype. It is possible therefore that *Staminoid Carpels* may represent an allele of *Hose in Hose* but the dominant nature of these mutations (see chapter 5) precludes complementation analysis.

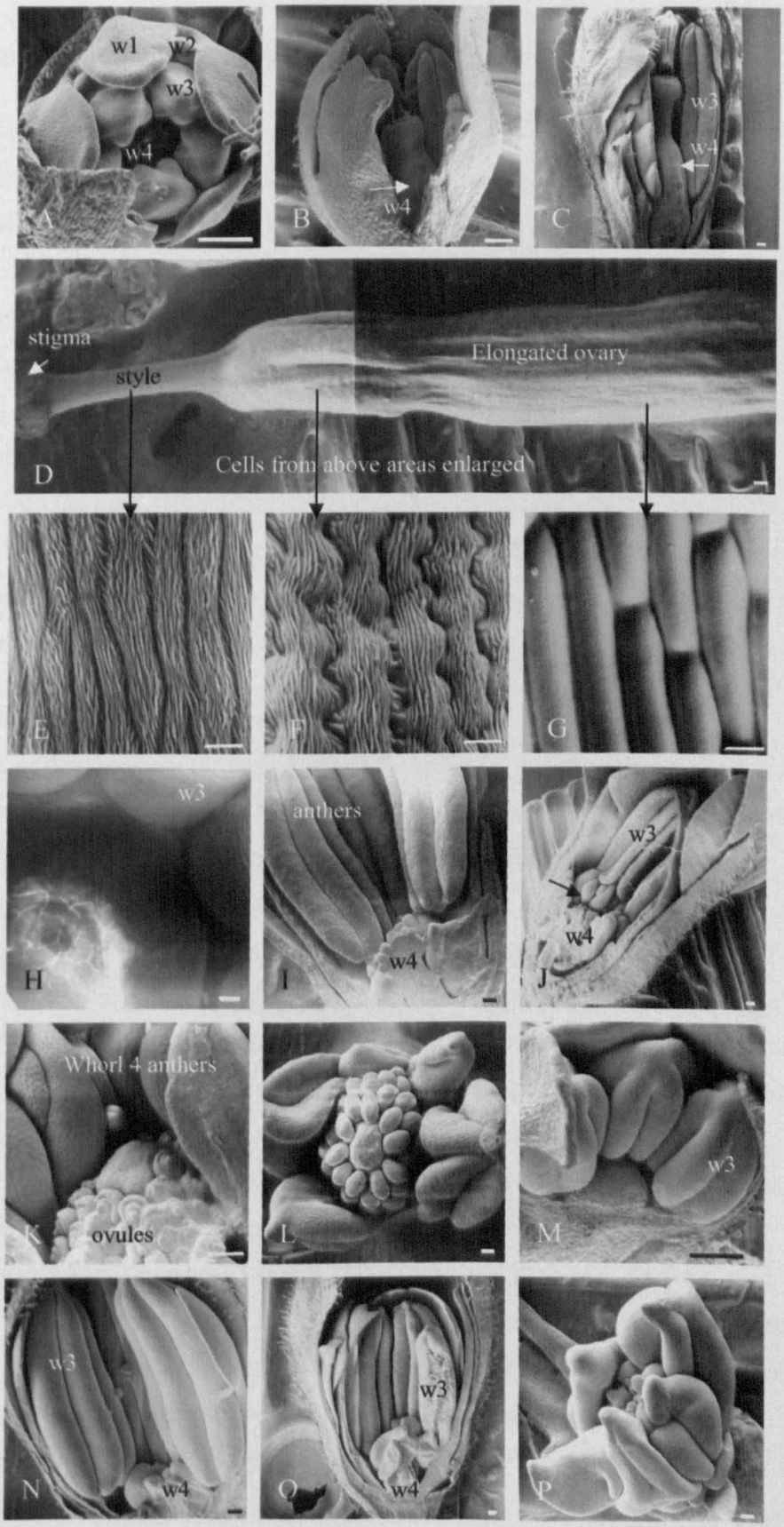
(ii) Development of *Staminoid* Carpels flowers.

Development of the least extreme conversion of whorl 4 to stamens appears normal up to and including stage 5 (Fig.4.9A). From stage 6 and beyond (Fig.4.9B-D) elongation of the ovary becomes increasingly obvious. Examination of the cells from different regions of the mature whorl 4 organ reveals normal stylar cells (4.9E) and elongated lower ovary wall cells with no distinguishing characteristics (Fig.4.9G). Cells from the upper part of the ovary wall are distinctly different, with the shape and surface characteristics of normal anther cells (Fig.4.9F). Development of the two more extreme phenotypes, both of which always have the ovary wall converted to anthers, is dramatically different. At stage 5 no whorl 4 organ has been initiated and the center of the developing flower is empty (Fig. 4.9H and M). Development of whorl 4 organs occurs relatively late in both cases, at what would probably be stage 6 in a normal flower (Fig.4.9I and N). Naked ovules on a central axis surrounded by small developing anthers can be observed in the stage 7 developing flower of approximately 3mm. (Fig.4.9J), and an enlargement of the whorl 4 region shows the outer and inner integuments of the ovules developing at this stage (Fig 4.9K). A mature detached whorl 4 organ of this form can be seen in Fig. 4.9L. Development of the most extreme form of *Staminoid Carpels* follows a similar pattern. By what would probably be stage 7 in a normal flower the whorl 4 anthers are clearly visible on top of the central axis (Fig.4.9O). This axis raises the whorl 4 anthers high inside the corolla tube as seen in Fig.4.9C previously. Detail of the top of a *Staminoid*

Figure 4.9. Development of the *Staminoid Carpels* flower.

There are variations in the development of *Staminoid Carpels* flowers of different extremes of form. Development of three extremes of form is illustrated, along with cells from some of the tissues.

A. A developing *Staminoid Carpels* flower of the least extreme form at stage 5. Whorl 4 appears to be developing normally at this stage. **B.** A developing *Staminoid Carpels* flower of the least extreme form at stage 6. **C.** A developing *Staminoid Carpels* flower of the least extreme form at stage 7. At this point elongation of the ovary can be observed. **D.** A mature whorl 4 organ of the least extreme form of *Staminoid Carpels* flower. **E.** Cells from the style of the above whorl 4 organ. **F.** Cells from the upper part of the elongated ovary of the above whorl 4 organ. **G.** Cells from the lower part of the elongated ovary of the above whorl 4 organ. **H.** Whorl 4 of *Staminoid Carpels* flower at stage 5, of the intermediate form that in the mature flower has anthers enclosing naked ovules. There is no development in the centre of the flower at this stage. **I.** Whorl 4 of *Staminoid Carpels* flower at what would probably be stage 6 in a normal flower (but without a normal whorl 4 the stage cannot be accurately ascertained and must be deduced from development in general). **J.** Whorl 4 of *Staminoid Carpels* flower at what would probably be stage 7 in a normal flower. Whorl 4 anthers are indicated by a black arrow. **K.** Enlarged image of whorl 4 from J above. Whorl 4 anthers surround naked ovules that have the outer and inner integuements developing. **L.** The top of a mature *Staminoid Carpels* whorl 4 organ with anthers surrounding naked ovules. **M.** Whorl 4 of a *Staminoid Carpels* flower of the most extreme form at stage 5. There is no development in the centre of the flower at this stage. **N.** Whorl 4 of a *Staminoid Carpels* flower of the most extreme form at what would probably be stage 6 in a normal flower. Anthers are just beginning to develop in place of the ovary wall and the ovules. **O.** Whorl 4 of a *Staminoid Carpels* flower of the most extreme form at what would probably be stage 7 in a normal flower. The whorl 4 anthers are now clearly visible on top of the central axis. **P.** The top of a mature *Staminoid Carpels* whorl 4 organ of the most extreme form, anthers enclosing more anthers. Size bars in **E**, **F**, and **G** are 1μm; in **H** it is 10μm; other size bars are 100μm.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Carpels whorl 4 organ with the most extreme conversion of whorl 4 to stamens can be seen in Fig.4.9P. Both the ovary wall and the ovules have been converted to stamens.

4.2.5. The *sepaloid* mutant phenotype.

(i) Description of *sepaloid*.

The first *sepaloid* mutants were discovered in 1996 in a batch of "Spectrum" commercial primroses and there is no description of this phenotype in old literature. The majority of the original *sepaloid* plants were sterile, having four whorls of sepals or a whorled flower of sepals. Only three plants that occasionally produced functional carpels were discovered and from these three all subsequent *sepaloid* plants are derived. The most extreme form of *sepaloid* flowers consist of four whorls of sepals (Fig.4.10A). These four whorls are generally concentric but one of the original plants had whorls that spiralled one into the next. Other manifestations of the phenotype may contain either two or three whorls of sepals surrounding naked ovules (Fig.4.10B), may have a chimeric non-functional whorl four organ (Fig. 4.10C), or may have two whorls of sepals and a functional carpel (Fig.4.10D).

(ii) Development of *sepaloid* flowers.

A progression of development through some of the stages of *sepaloid* flower development can be seen in Fig.4.11A. The central meristematic area from which further primordia will develop can also be observed. Development of the *sepaloid* flower is indistinguishable from *Wild Type* up to stage 3. During stage 3 the first observable difference is that at middle stage 3 the depression within the centre of the developing flower is slightly deeper in *sepaloid* flowers than in *Wild Type* (Fig.4.11B). Secondly, there is less differentiation of the organ primordia than in those of *Wild Type* at a similar stage. By late stage 3 *sepaloid* flowers develop a very pronounced hollow in the centre of the flower (Fig.4.11C). At this stage, the bulge on the outer surface that would form petals in *Wild Type* flowers can just be seen. From early stage 4 the developing second whorl is clearly visible, but it is evident that no separation of individual organs has occurred in either whorl 2 or whorl 3 (Fig.4.11D). In *sepaloid* mutants, the inner ring structure that would form the carpels in *Wild Type* flowers is absent. During middle and late stage 4 of *sepaloid* flower development a fourth whorl can be distinguished, but all organs in this particular flower are laterally joined to the inner whorls to form a continuous spiral (Fig.4.11E). By stage 5, the growing whorl 1 sepals of the *sepaloid* mutant gradually obscure the inner whorls (Fig.4.11F and G). Removal of these outer

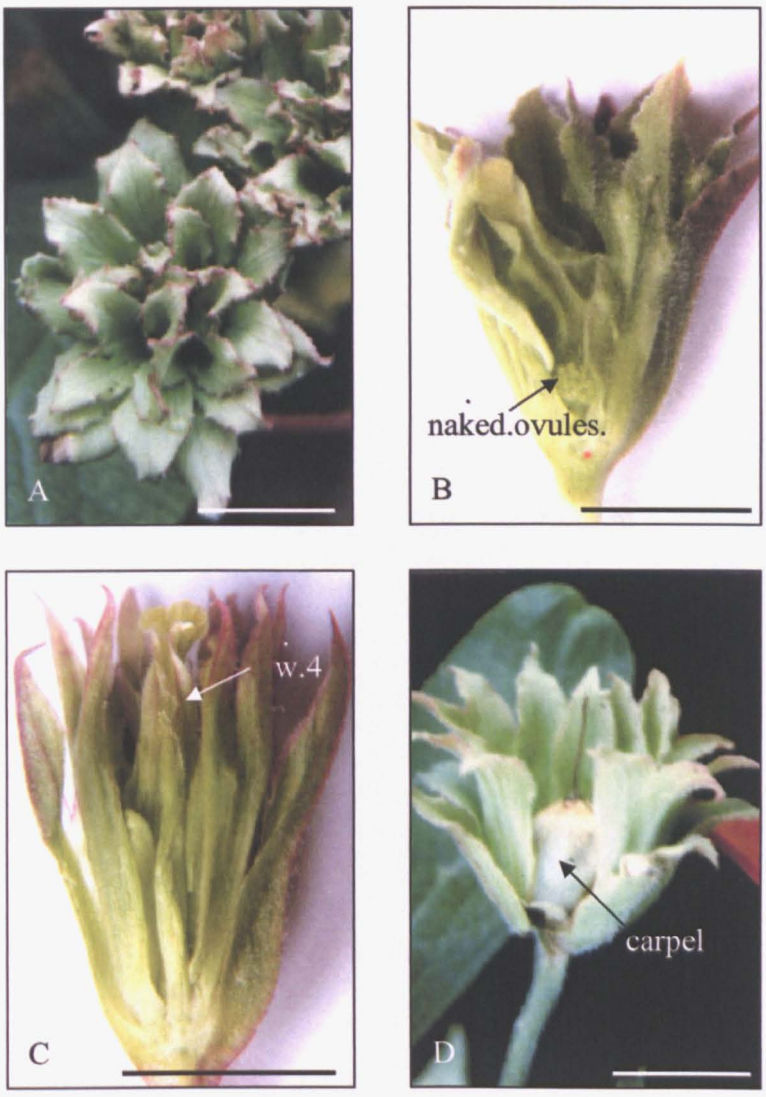


Figure 4.10. The *sepaloid* mutant phenotype.

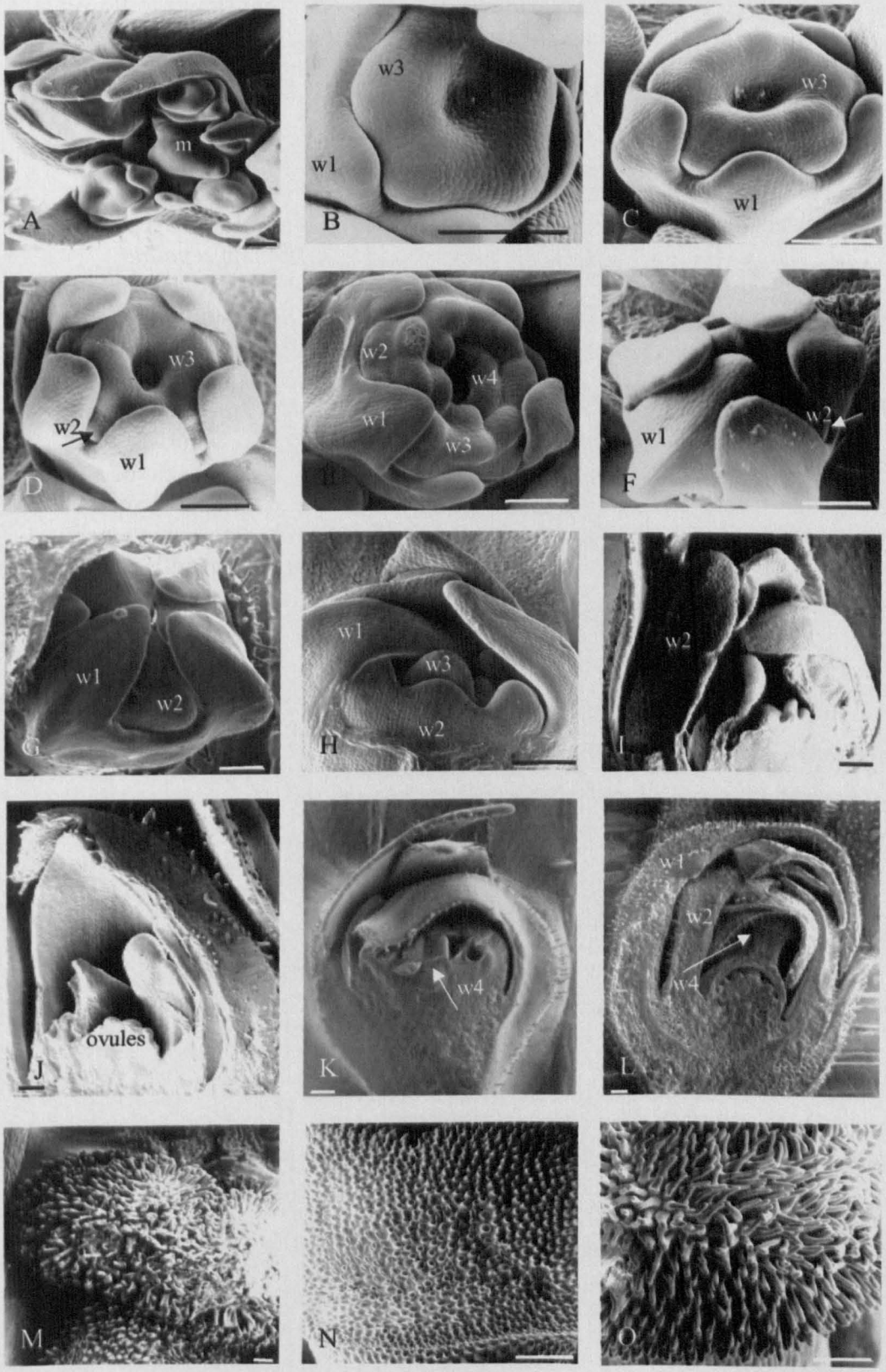
Variations of the form of the *sepaloid* flower range from fully infertile with four whorls of sepals to fertile with two whorls of sepals and a functional carpel.

A. Fully infertile *sepaloid* flower with four whorls of sepals. **B.** Infertile *sepaloid* flower with three whorls of sepals enclosing naked ovules. **C.** Infertile *sepaloid* flower with chimeric fourth whorl organ of mixed sepal and carpel tissue (w.4). **D.** Fertile *sepaloid* flower with two whorls of sepals and a functional carpel. Size bars are approximately 1 cm.

Figure 4.11. Development of the *sepaloid* flower.

Variation in the form of the *sepaloid* flowers occurs from flower to flower on the same plant. Early development of the *sepaloid* flower up to stage 6 is illustrated, along with a comparison of *sepaloid* stigmatic papillae with both thrum and pin *wild type* stigmatic papillae.

A. Cluster of developing *sepaloid* flowers at different stages of development, with a central meristematic area (m) from which new primordia develop. **B.** Developing *sepaloid* flower at middle stage 3. **C.** Developing *sepaloid* flower at late stage 3. **D.** Developing *sepaloid* flower at early stage 4. **E.** Developing *sepaloid* flower at middle stage 4. **F.** Developing *sepaloid* flower at early stage 5. **G.** Developing *sepaloid* flower at late stage 5. **H.** Late stage 5 developing *sepaloid* flower with part of whorl 1 removed. **I.** A *sepaloid* flower at approximately stage 6, showing development of four whorls of sepals. **J.** A *sepaloid* flower at approximately stage 6 that has naked ovules developing in whorl 4. **K.** A *sepaloid* flower at approximately stage 6, showing development of a chimeric carpel and sepal organ in whorl 4. **L.** A *sepaloid* flower at approximately stage 6, showing development of a normal carpel in whorl 4. **M.** Stigmatic papillae of *sepaloid* flower. **N.** Stigmatic papillae of thrum *Wild Type* stigma. **O.** Stigmatic papillae of pin *Wild Type* stigma. Size bars are 100μm.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whorl organs permits observation of the developing flower within and reveals individual sepal lobes beginning to elongate upwards from the inner whorls (Fig.4.11H). The final structure of the mature flower is apparent from what would probably be stage 6 in *Wild Type*. Four dissected developing flowers show inner whorls of sepals (Fig.4.11I), a fourth whorl of naked ovules (Fig.4.11J), a chimeric whorl 4 of carpel and sepal tissue (Fig.4.11K). and a fertile *sepaloid* with a normal fourth whorl containing developing ovules on a free central placenta (Fig.4.11L). Although all organs continue to grow larger there are no significant changes beyond this stage other than the development of stigmatic papillae on fertile whorl 4 organs. All *sepaloid* plants tested so far have been found to be pin, and the *sepaloid* stigmatic papillae in Fig.4.11M more closely resemble the *Wild Type* pin stigmatic papillae in Fig.4.14O, than the thrum *Wild Type* stigmatic papillae in Fig.4.11N. Other *sepaloid* plants occasionally have stigmas with papillae of ambiguous form and it is only by breeding from them that the genotype may be discovered.

4.2.6. The *double* mutant phenotype.

(i) Description of *doubles*.

In horticultural terms, flowers that produce petals in place of other floral organs are normally referred to as doubles. The *plena* mutants of *Antirrhinum majus* (Bradley *et al.* 1993; Davies *et al.* 1999) and *agamous* of *Arabidopsis* thaliana (Bowman Smyth and Meyerowitz1989,1991; Yanofsky *et al.* 1990; Jack *et al.* 1997; Mizukami and Ma 1997), respectively represent well-characterised examples. Descriptions of and illustrations of *double* flowered forms of *Primula* are historically abundant as has been shown in Chapter 1. A number of named cultivars are commercially available; many of which are mass-produced using micropropagation techniques. Some of the older cultivars are conserved by specialist growers and propagated by division.

Variability in the number of floral whorls and of organ identity of the whorls is common. This is reputedly influenced by environmental factors, although no studies have directly addressed this possibility. It is possible to sub divide female infertile *double* flowers into roughly four different categories, in which plants of each form predominantly produce flowers of the morph described. There may sometimes be considerable variation, for example, in the number of whorls of petals or of stamens produced so that there cannot be an absolute criterion for any of the forms of *double* described.

Figure 4.12. The *double* mutant phenotype.

There is considerable variation in the form of the *double* flower, ranging from flowers with indeterminate whorls of petals to flowers with stamens or a functional carpel in the centre of the flower.

A . The fully *double* flower of the *double* cowslip "Katy MacSparron" has never been known to produce any organ other than petal from whorl 2 inwards. **B.** A flower that appears fully *double* from above. **C .** A *semi-double* flower, with two whorls of petals enclosing numerous anthers. **D .** The *double* polyanthus "Lin Rogers" that has a second whorl of sepals in whorl 4. **E.** Flower from A, above in cross section. **F.** The flower from B above when in cross section can be observed to have a mixture of anthers, petals, and petaloid anthers in the centre of the *double* flower. **G.** The flower from C, above in cross section. The centre of the *semi-double* flower contains indeterminate whorls of anthers. **H.** The flower from D above in cross section. The second whorl of sepals can be observed to enclose naked ovules. **I.** Fertile *semi-double* primrose.. **J.** Sectioned fertile *semi-double* primrose. Anthers (a) can be seen on the inner whorl of petals, so that this *semi-double* has five whorls of organs **K.** Fertile *semi-double* polyanthus. **L.** Sectioned fertile *semi-double* polyanthus. This *semi-double* does not have anthers inside the inner whorl of petals, but occasionally an extra petal develops where anthers would normally be attached. Size bars are approximately 1 cm.

The first sub division is that of fully *double* flowers (Fig.4.12A and E). These have the centre of the flower filled with indeterminate whorls of petals. There may be variation in the number of whorls of petals, but they rarely, and in some plants never, produce any stamens. The second is that of *double* flowers with the centre of the flower filled with an indeterminate number of whorls of both petals and stamens, or of chimeric organs composed of both types of tissue (Fig.4.12B and F) such as petal tipped stamens. Great variability has been observed both in the proportion of petals to stamens (or vice versa) and in the number of whorls produced. The third is that of *semi-double* flowers with the centre of the flower filled with indeterminate whorls of stamens (Fig.4.12C and G). There can be variation in the number of whorls of petals surrounding the stamens; it is normally two, but occasionally can be one or three. Variation of this form can occasionally include a determinate flower with naked ovules in the centre. The fourth is that of *doubles* with a whorl of sepals in the fourth whorl. These only extremely rarely produce any stamens. The original, *double* polyanthus "Lin Rogers" (Fig.4.12D and H) rarely varied. However similar progeny (raised as a result of two stamens being produced on one occasion only on the original plant) were less stable. Variation observed included the "flower within a flower" phenotype with occasionally up to three repeats of the organ order, and in a few cases reversion to fully normal and fertile flowers on the same plant as the double flowers.

Recently female fertile *semi-double* primroses and polyanthuses have become available. The primroses, shown in Figure 4.12I and J, are from a commercial line, and have been on sale for the first time during spring 2004. The polyanthus, shown in figure 12K and L, occurred from a horticultural cross of my own plants, and it is not yet known how stable the phenotype will be. Neither of these female fertile phenotypes was available at the time of investigating the development of *double* flowers.

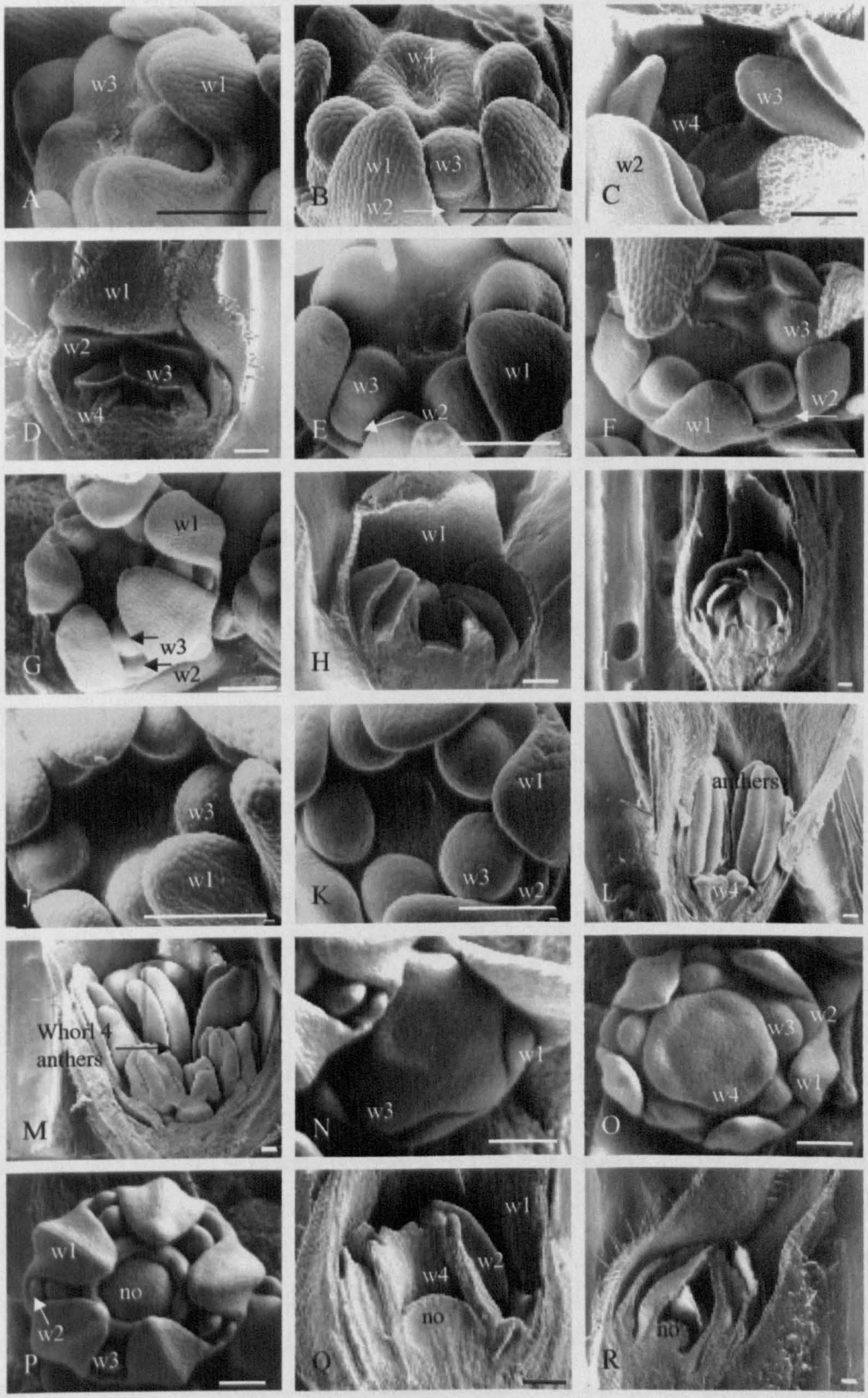
(ii) Development of *double* flowers.

There is no discernible difference from the development of the above *double* flowers and that of wild type flowers up to late stage 3 (Fig.4.13A). Beyond this there are discernible differences in the development of the different forms of *double* flowers. Those *double* flowers that have never been known to produce any stamens have an undulating ring in whorl 4 at middle stage 4 (Fig.4.13B). This will develop into a whorl of petals. Further development results in further concentric undulating rings (Fig.4.13C and D) so that the mature flower becomes as that shown in Fig.4.13A 1 and 2. Both *doubles* that produce a mixture of petals and stamens in the centre of the flower (Fig. 4.13E-I), and *semi-doubles*

Figure 4.13. Development of *double* flowers.

There are differences in the early development of different forms of *double*.

A. The late stage 3 developing *double* flower is indistinguishable from *Wild Type*, (see Chapter 3, Fig. 3.2F,). **B.** The *double* cowslip "Katy Mac Sparron" (organ order, sepal, petal, petal, petal indeterminate) at middle stage 4. **C.** The *double* cowslip "Katy Mac Sparron" at approximately stage 6 showing development of four whorls of petals with a fifth developing whorl in the center of the flower. **D.** The *double* cowslip "Katy Mac Sparron" when slightly further developed. **E.** A. developing *double* flower (from the same plant as was the flower in Fig.4.14.B) at early stage 4. **F.** A developing *double* flower (from the same plant as was the flower in Fig.4.14.B) at middle stage 4. **G.** A developing *double* flower (from the same plant as was the flower in Fig.4.14.B) at late stage 4. Flowers in Fig.4.15.F and G show delayed development of whorl 4 organs at stage 4. **H.** A developing *double* flower (from the same plant as was the flower in Fig.4.14.B) at approximately stage 6. Three whorls of petals are well developed, beyond this development is delayed. **I.** A developing *double* flower (from the same plant as was the flower in Fig.4.14.B) at approximately stage 7, showing concentric whorls of well-developed petals. **J.** A developing *semi-double* flower (from the same plant as was the flower in Fig.4.14.C) at early stage 4. **K.** A developing *semi-double* flower (from the same plant as was the flower in Fig.4.14.C) at middle stage 4. Flowers in Fig.4.15.J and K both show delayed development of whorl 4 organs. **L.** A developing *semi-double* flower (from the same plant as was the flower in Fig.4.14.C) at approximately stage 6. At this stage the whorl 4 stamens are beginning to develop. **M.** A developing *semi-double* flower (from the same plant as was the flower in Fig.4.14.C) at approximately stage 7. The developing stamens in whorl 4 are recognizable at this stage. **N.** The *double* polyanthus "Lin Rogers" at stage 3. **O.** The *double* polyanthus "Lin Rogers" at middle stage 4. **P.** The *double* polyanthus "Lin Rogers" at late stage 4. There is a ring in whorl 4 that will become the inner sepals, enclosing the free central placenta. **Q.** The *double* polyanthus "Lin Rogers" at what would probably be stage 6 in the normal flower, showing development of sepaloid whorl 4. **R.** The *double* polyanthus "Lin Rogers" at a later stage showing further growth of the sepals in whorl 4 that enclose the naked ovules. Size bars are 100μm.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that have an indeterminate number of stamens in the centre of the flower (Fig.4.13J-M) have delayed development of whorl 4 at stage 5. A fourth whorl of petals can be observed in Fig.4.13H when the flower is at what would be approximately stage 6 in a normal flower. In the semi-double flower at approximately stage 6 growth of the central core of stamens has become visible (Fig.13L), and these inner stamens can be observed at a further stage of development in Fig.4.13M. There is no delay in development of whorl 4 in the double polyanthus "Lin Rogers" but the course of development is quite different from either of the above. At middle stage 4 there is a large swelling in the centre of the flower, with a slightly raised undulating ring around the edge that will become the whorl 4 sepals. By late stage 4 the ring that is now enclosing the area that will develop naked ovules is quite distinct. The further development of this ring into an inner whorl of sepals can be observed in Fig.4.13Q, and again in Fig. 4.13R, when the naked ovules have begun to develop.

(iii) Whorl architecture in *double* flowers.

From an aerial view *double* flowers have several whorls of petals (Fig. 4.1F and Fig. 4.12B). Sepals were removed from one flower from each of four commercially available named *double* primroses, "Miss Indigo", Lilacena Plena", "Katy MacSparron" and "Chocolate Soldier", and the inner layers of petals were carefully separated and examined. It was found that the base of each whorl frequently gave rise to several layers of petals attached one upon another as shown in Figure 4.14. Consequently a flower with four whorls at the base plus an indeterminate central area with organs so small that it was impossible to analyse whorl architecture, had up to nine whorls of petals when viewed from above (Fig. 4.14 no. 2). A flower from the cultivar "Miss Indigo" was split open to show the inner layers of petals attached one upon another (Fig. 4.15A) and also the small inner whorl of fused petals that enclosed more petals (Fig. 4.15B). Some of the layers of inner petals appear to develop early in ontogeny as do the anthers and petals from a common primordium in the normal flower. However at least some petals do develop from primordia on inner petals later in development, as can be seen in Fig.4.15C and D. In order to ascertain whether this whorl architecture was a feature of fully *double* flowers only, some sibling *doubles* with a lesser number of whorls of petals and that also produced anthers were examined (Fig. 4.16). All of these had more whorls of petals when viewed from above than they had at the base, and again the extra whorls were achieved by one or more petals growing from various points of another petal. Anthers were present, and appeared to develop randomly in different positions

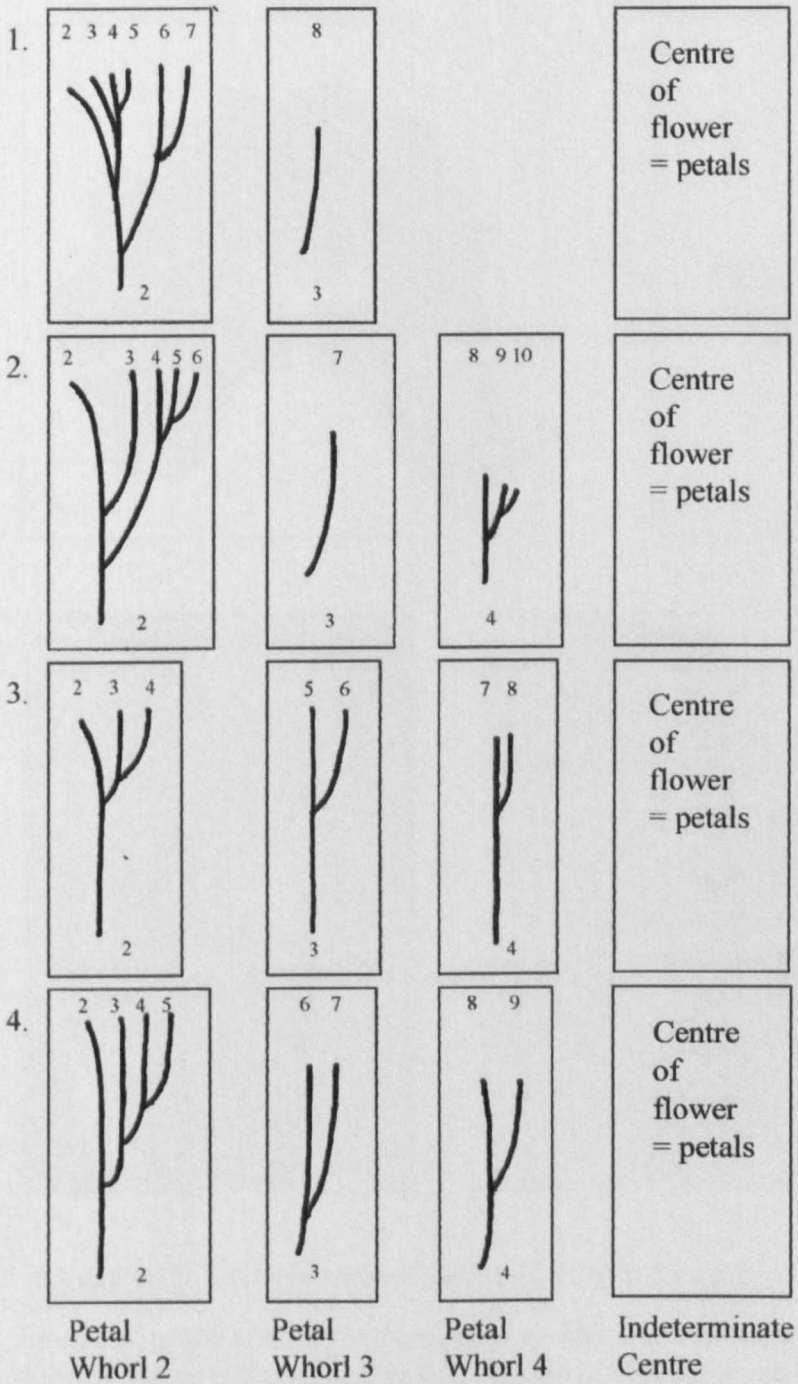


Figure 4.14. Patterns of attachment of extra whorls of petals in four named commercial *double* primroses.

Whorl 1, sepals, is not represented. Whorls of petals are numbered both at the base and at the top of the flower. Although aerially there are many whorls visible, from the base only five are clearly distinguishable. **1.** "Miss Indigo", **2.** "Lilacena plena", **3.** *double* cowslip "Katy MacSparron", this *double* also has the base of all the whorls above fused, and **4.** "Chocolate Soldier".

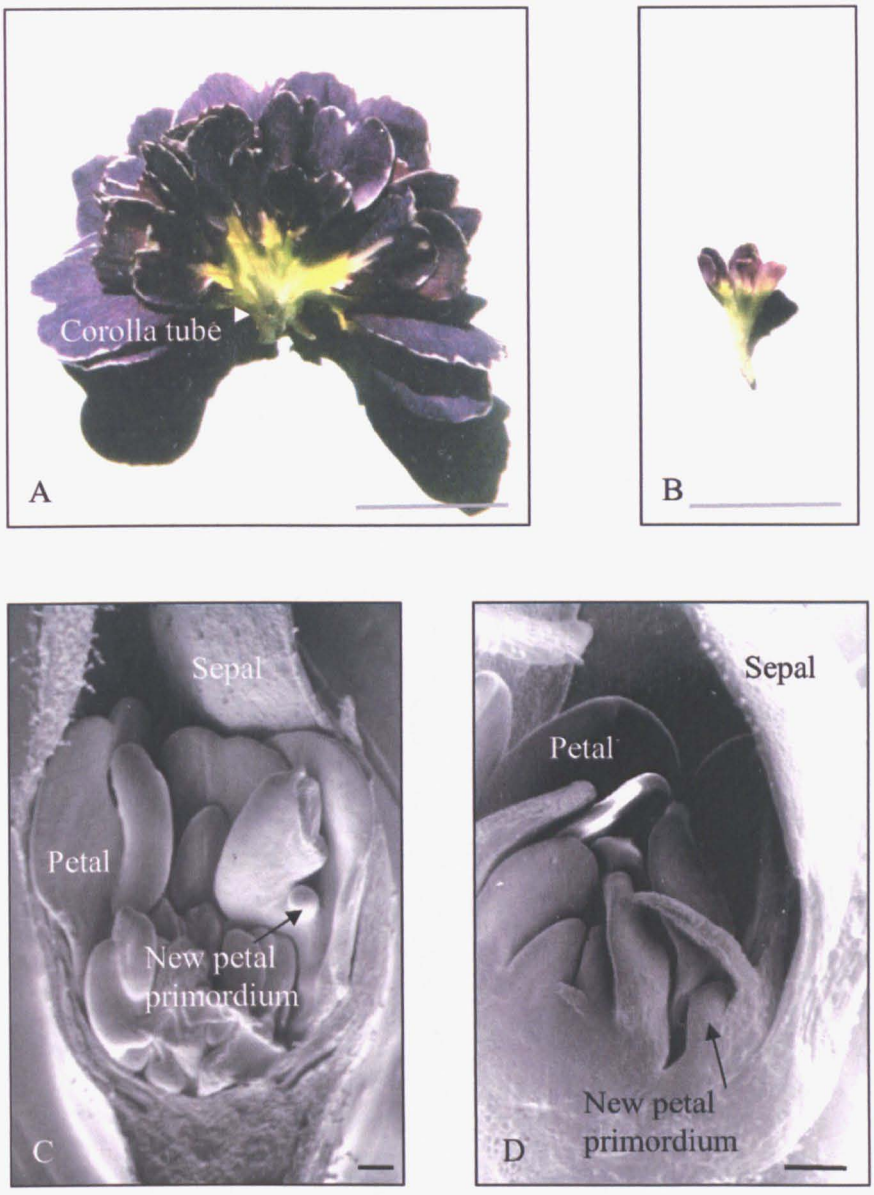


Figure 4.15. Extra whorls of petals in double flowers.

Layers of petals are attached one upon another in the double cultivar "Miss Indigo". A new primordium can be observed to arise from an existing petal in the scanning electron images.

A. Corolla of *double* "Miss Indigo" showing inner layers of petals attached one upon another, and an abnormally short corolla tube. **B.** Central core of small fused petals removed from **A** above. **C and D.** Scanning electron microscope images of developing semi-double **(C)** and double **(D)** flowers that have a new petal primordium arising from an existing petal. Size bars in **A** and **B** are 1 cm., those in **C** and **D** are 100μm .

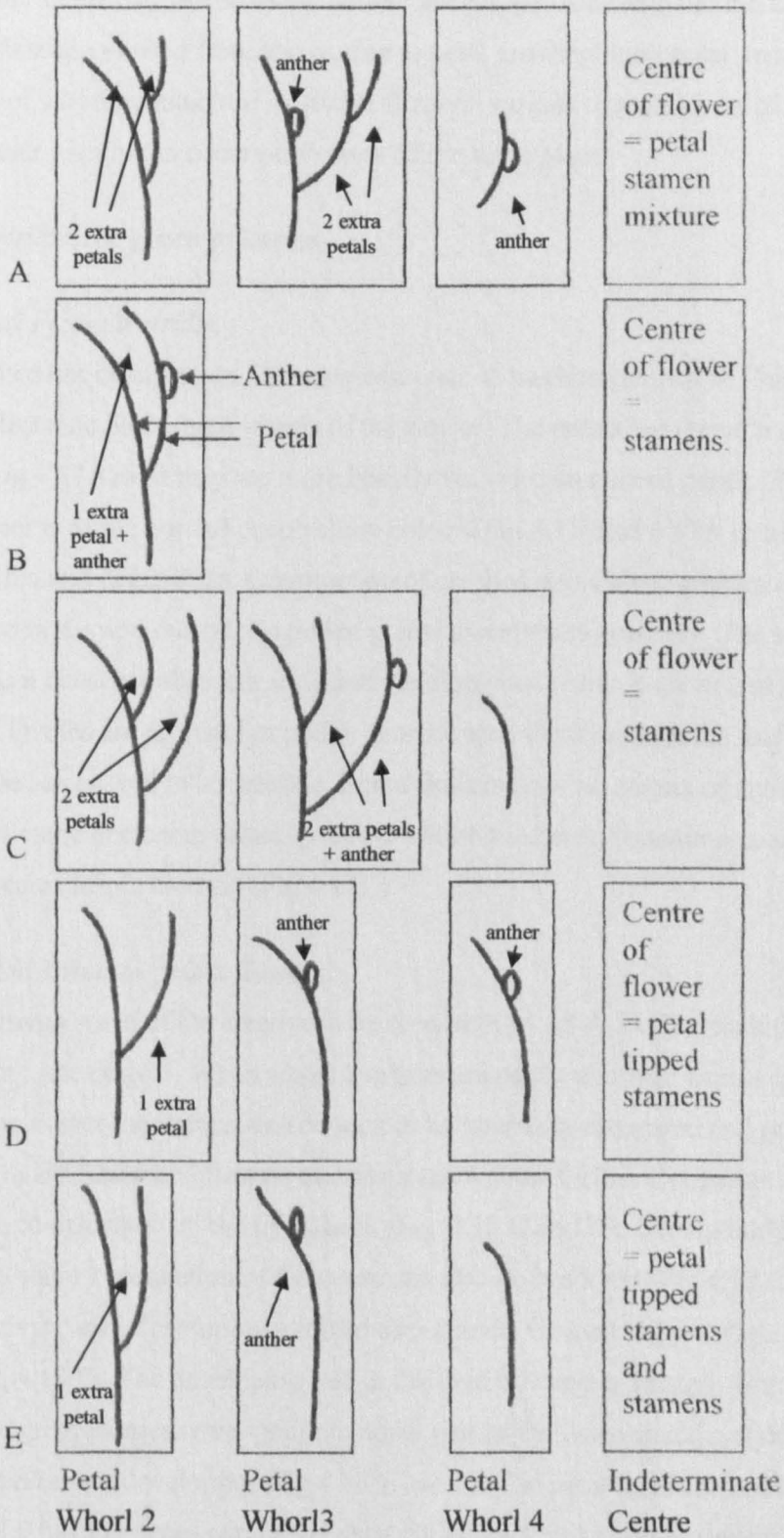


Figure 4.16. Variation in whorl architecture in five sibling *double* flowers, A-E.

The first flower to bloom was examined from each *double*. No two were the same but each had extra petals and occasional anthers.

within the flower. These observations are accurate for the time of observation, but there is much variability in the expression of the *double* phenotype. Although the increased number of whorls when viewed from above, due to petal growing upon petal, remains a constant feature of whorl architecture in *double* flowers, various organisations of attachment of inner whorls can occur on flowers of the same plant.

4.2.7. *Primula viridis*, the green primrose.

(i) Description of *Primula viridis*.

The green primrose has been known for many centuries as has been shown in Chapter 1. The mutation affects the inner three whorls of the flower. The petals are green in colour (Fig.4.1H, and Fig.4.17A) and they are more heavily veined than normal petals. The "eye" of the flower is of the normal deep yellow colour (Fig.4.1H and 4.17A B, and C). The anthers are thin and degenerate. Examination of crushed and stained anthers under the light microscope showed one or two pollen grains that stained normally. The stigma, when present, has a densely pubescent style and the elongated ovary is green and leaf like (Fig.4.17B). Ovules are present but pollen cannot reach them through the leafy tissue and the phenotype has proved to be infertile. Some flowers have no stigma or style but have an open leafy tube enclosing naked ovules in whorl 4 instead. Sometimes a second whorl of petals occurs inside the first (Fig.4.17C).

(i) Development of *Primula viridis* flowers.

A progression through some of the stages can be seen in Fig.4.18 A. Early development is as wild type until late stage 5. When whorl 1 organs are partly removed from a late stage 5 developing flower the anthers can be seen to be unusually elongated and pointed in shape, (Fig. 4.18 B). Sectioned flowers at early stage 6 show further elongation of the anthers and a curved extension on the tip of each (Fig. 4.18 C and D). During middle stage 6 through to stage 7 elongation of the ovary can also be observed (Fig.4.18 E and F) with the leafy ovary wall developing a folded appearance (indicated by a white arrow) from stage 7 (Fig. 4.18 F). The developing bud in this figure is approximately 4mm in size and by this stage trichomes cover both the upper part of the ovary and the style. Inside the ovary ovules are developing (Fig.4.18 E and F). The petal on the left of the flower in Fig.4.18 F has the upper part of the petal curled back in a manner similar to the whorl 1 organs of *Jack in the Green* flowers. Beyond this stage all organs continue to grow larger. A mature anther was detached for examination (Fig.4.18 G), and the lobes can be observed to be thin and shrunken compared to those of normal anthers.

Figure 4.17. *Primula viridis*, the green primrose.

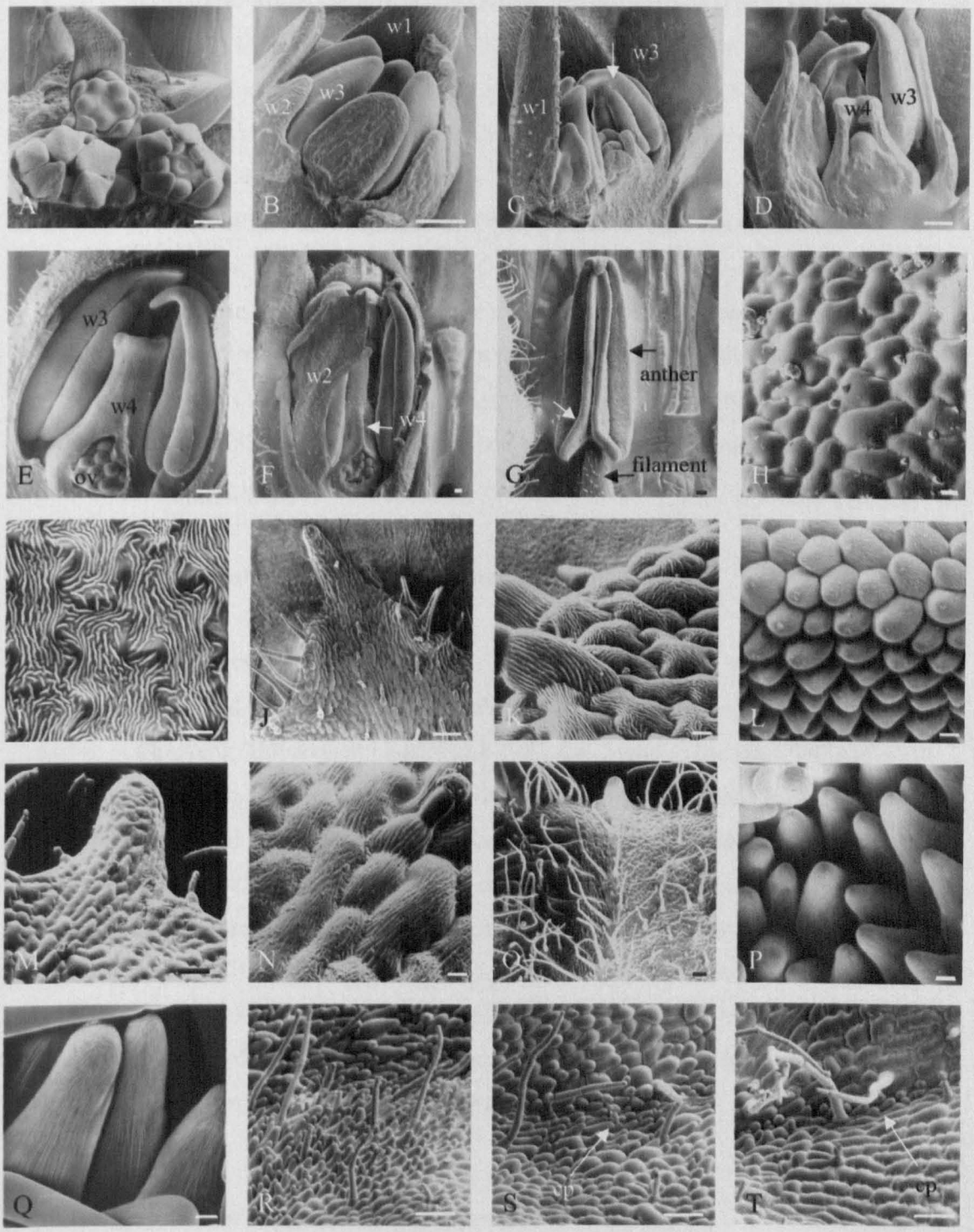
The green primrose *Primula viridis* has the correct organ in each of the four whorls, but the organs of the inner three whorls are abnormal. Petals are green in colour, anthers fail to dehisce and the ovary wall is elongated and leaf-like. Sometimes an additional whorl of petals grows inside the first.

A. *Primula viridis*, the green primrose, in bloom. **B.** Cross section of a typical *P. viridis* flower. **C.** A flower of *P. viridis* with an extra whorl of green petals and an open leafy ovary wall in the centre of the flower. Size bars are approximately 1 cm.

Figure 4.18. Development of *Primula viridis*, the green primrose.

Development of *Primula viridis* differs from that of *Wild Type* from stage 5 when anthers begin to develop elongated tips. With the exception of the cells of the yellow eye of the flower, cells of tissues from the mature flower differ markedly from those of *Wild Type*.

A. A cluster of developing green primrose flowers. Until, and including, late stage 4 they are indistinguishable from *Wild Type*. **B.** Early stage 5 flower with part of whorl 1 removed. The anthers can be observed to be abnormally pointed. **C.** Late stage 5 flower cut in two. The anthers have developed a projecting tip. **D.** Early stage 6 flower cut in two. The stigma is beginning to develop and the anthers now have a long projecting tip. **E.** Middle stage 6 flower. The ovary is slightly elongated, ovules are developing, and the projecting tip of the anther has become hook like. **F.** Stage 7 flower cut in two. The ovary wall has now become folded in appearance and both the ovary wall and the style are covered in trichomes. The anthers have a hooked tip, but not so elongated as previously. A petal visible on the left is covered in trichomes and has curled back edges reminiscent of jack in the green calyces. **G.** An anther from a mature flower. The anther lobes are thin and the filament covered in trichomes. The hooked tip is still visible. **H.** Cells from one of the anther lobes, at the point marked by an arrow. **I.** Normal anther cells from a *Wild Type* primrose. **J.** Tip of a mature green primrose petal. **K.** Cells from the edge of petal tip above. **L.** Normal conical papillate petal cells from a wild type primrose. **M.** Another petal tip from another green primrose flower. **N.** Cells from the center of petal tip above. **O.** Underside of a mature green primrose petal tip. **P.** Cells from the yellow "eye" of a normal *Wild Type* primrose. **Q.** Cells from the yellow "eye" of a green primrose. **R.** Cells from the yellow "eye" of a green primrose with some green cells in the foreground. **S.** Cells further out form the mouth of the corolla tube; some conical papillate yellow "eye" cells can be observed in the center with cells from green tissue on either side. The petal tip would have been some distance to the right of this image. **T.** The last conical papillate yellow "eye"cells, surrounded by green cells. The petal tip was some distance to the right of this image. Size bar in **M** is 1um; size bars in **L, O, P, R, T,** and **U** are 10μm; other size bars are 100μm.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Examination of cells (Fig.4.18 H) at the point marked by a white arrow show them to be unlike normal anther cells (Fig.4.18 I). Green primrose petals, unlike normal petals, have a small projecting point at the end of each main vein that runs through the centre of the petal. Two of these points were examined on the adaxial surface (Fig.4.18J and K, M and N) and one on the abaxial surface (Fig.4.18O). In neither case were the cells at all like the normal conical papillate cells of wild type petals shown in Fig.4.18L. The abaxial surface (Fig.4.18O) is densely covered with trichomes. At the base of each petal and continuing around the mouth of the corolla is the deep yellow area, referred to as the yellow "eye" of the flower. In normal wild type primroses the cells of this yellow "eye" are of the elongated conical papillate type (Fig.4.18.T). Examination of the yellow "eye" of the green primrose reveals the cells here to also be of normal appearance (Fig.4.18Q). Following the central vein of the petal from the mouth of the corolla outwards (Fig.4.18R, S and T) reveals conical papillate cells surrounded by flatter cells. The conical papillate cells decrease in number and become less elongated further up the petal until they stop (Fig.4.18T).

4.2.8. The virescent cowslip mutant phenotype.

(i) Description of virescent cowslip.

The flowers formed by this mutant are similar to those illustrated in the past as has been shown in Chapter1. The present plant (Fig.4.19A) was found in the late 1980's in a naturalized population of cowslips in a garden near Crewkerne. It has leafy bracts at the top of the scape and individual "flowers" composed of one or two whorls of leafy green bract like organs and numerous stigmatoid organs in varying proportions (Fig. 4.19B and C). Naked ovules are present and are randomly attached to the inner organs, especially around the bases of the stigmatoid organs. Sometimes the base of these organs ends in an open carpel with naked ovules visible. No closed carpels or fertile organs have been produced to date and the plant must be propagated by division. Secondary scapes or elongated pedicles may sometimes arise from the main scape.

(ii) Development of virescent cowslip flowers.

Early development shows only two forms of primordia, one form that is densely covered with trichomes, these will become the leafy organs; and one form that is smooth that will become the stigmatoid organs (Fig. 4.20A). When further developed the disorganized

Figure 4.19. The virescent cowslip mutant phenotype

The virescent cowslip has no organised flower structure. Flowers consist of a mass of green bract like structures and pale stigmatic like organs on long styles but without any functional ovary.

A. The virescent cowslip plant in flower. **B.** A scape of virescent cowslip flowers that consist predominantly of stigmatic like organs on long styles interspersed with some green leafy bracts. **C.** A single flower of the virescent cowslip that consists of green leafy bracts, interspersed with fewer stigmatic like organs on long styles. Size bars are approximately 1 cm.

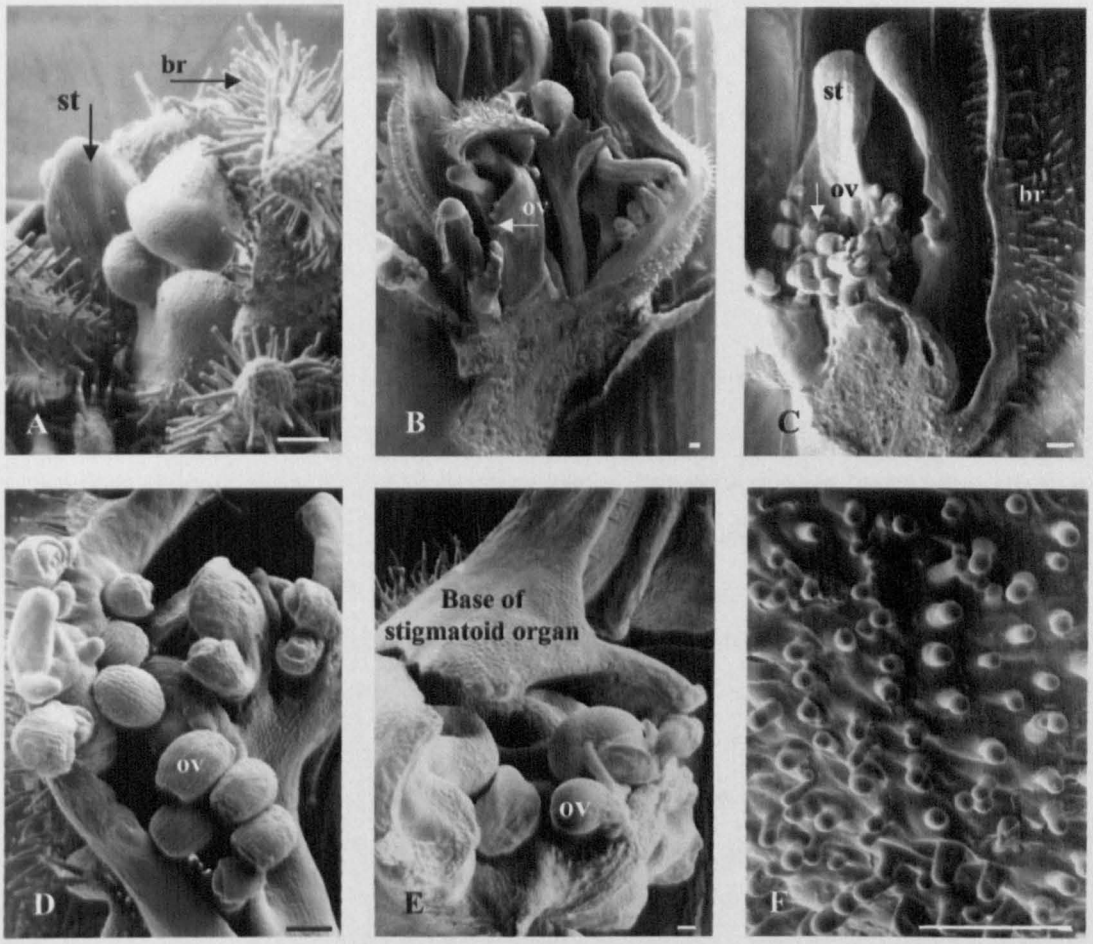

Figure 4.20. Development of virescent cowslip flowers.

Lack of organisation in the developing virescent cowslip flower is evident from the earliest stages. Scanning electron microscopy highlights the profusion of ectopic ovules exhibited by this phenotype.

A. The meristematic area of virescent cowslip flower. Developing bracts (br), and developing stigmatic organs (st), can be observed. **B.** Developing virescent cowslip flower of approximately 3mm. cut in two. Both bracts and stigma like organs can be seen. Numerous developing ectopic ovules (ov), can also be observed. **C.** Part of another flower showing a cluster of naked ovules (ov) developing around the base of a stigma like organ (st). **D.** Ectopic ovules. In some instances the developing inner and outer integuements can be observed. **E.** Ovules developing in another incompletely enclosed ovary at the base of one stigma like organ. **F.** Papillae developing on the tip of a stigma like organ.
The size bar in **F** is 10μm ; other size bars on SEM images are 100μm.

positioning of the ovules on the sides of and at the base of the stigmatoid organs can be seen (Fig. 4.20B and C). Structures resembling open carpels can be seen in Fig.4.20D and E. The tips of the stigmatoid organs were examined and were found to be developing small stigmatic papillae (Fig.4.20F).

2.9. The *reduced petal* mutant phenotype.

(i) Description of *reduced petal.*

A number of plants of the phenotype first appeared in 1999 among both the pin and the thrum progeny (Fig. 4.21C and D) of an inbred line of silver laced polyanthus raised by Dr. R. J. Brumpton, of Woodborough Nurseries, Nottingham. Silver-laced polyanthus and gold-laced polyanthus have the petals outlined around the edges and down the central vein of the petal with either white (silver-laced), or yellow (gold-laced), (Fig.4. 21A). Although laced polyanthuses have been grown and described for many years, there is no previous mention of the mutant *reduced petal* form. The most obvious feature of the original mutant plants was the reduced size of the petals and the loss of pigment from most of, or sometimes from the entire adaxial surface of the petal. The stunted petals also feel slightly thicker than normal. In some instances the perianth may also be split (Fig. 4.21E and F); this is not a constant trait but occurs randomly on plants that do not normally have the perianth split on the majority of the flowers. Stigmas are frequently distorted and female fertility is always much reduced. Leaves are thinner and broader than wild type laced polyanthus leaves, and the edges have an undulating appearance (Fig. 4.21B). Trichomes on leaves of the mutant plants are fewer and shorter than on normal laced polyanthus leaves. Breeding experiments with the *reduced petal* mutants uncovered less extreme forms of the condition. Some plants had flowers with petals of normal form but with reduced pigment on the adaxial petal surface (Fig.4.21G). Others were almost normal, (Fig.4.21H) with only slight loss of pigmentation, so that the lacing remained a feature but without the clean crisp lines normally expected from the form.

(ii) Development of *reduced petal* flowers.

A progression through the development of some of the early stages up to and including late stage 5 can be seen in Fig.4.22A. It can be observed that these flowers would also have had a split perianth, at least in whorl 1. Dissected flowers at early and middle stage 6 can be seen in Fig.4.22B and C. By middle stage 6 the distorted stigma is obvious

Figure 4.21. The *reduced petal* mutant phenotype.

Normal silver-laced polyanthus flower and leaf form is compared with that of the *reduced petal* mutant phenotype. Deterioration in flower form of the most extreme phenotype later in the flowering season is illustrated with examples of two less extreme forms of the *reduced petal* phenotype.

A. Flower of a normal silver-laced polyanthus. **B.** A leaf from a *reduced petal* polyanthus on the left, with a leaf from a normal silver-laced polyanthus on the right. The former is much broader, thinner, and often a lighter green than that of the normal silver-laced polyanthus. **C.** A thrum *reduced petal* polyanthus flower, of the most extreme form. **D.** A pin *reduced petal* polyanthus flower, of the most extreme form. **E.** A *reduced petal* polyanthus flower from C above with a split perianth later in the flowering season. **F.** A *reduced petal* polyanthus flower from D above with a split perianth later in the flowering season. **G.** A less extreme form of the *reduced petal* polyanthus flower. The petals are not so "reduced" but there is loss of colour on the adaxial surface of the petals. **H.** The least extreme form of the *reduced petal* polyanthus flower. The petals are not reduced at all but there is some loss of colour on the adaxial surface of the petals. Size bars are approximately 1 cm.


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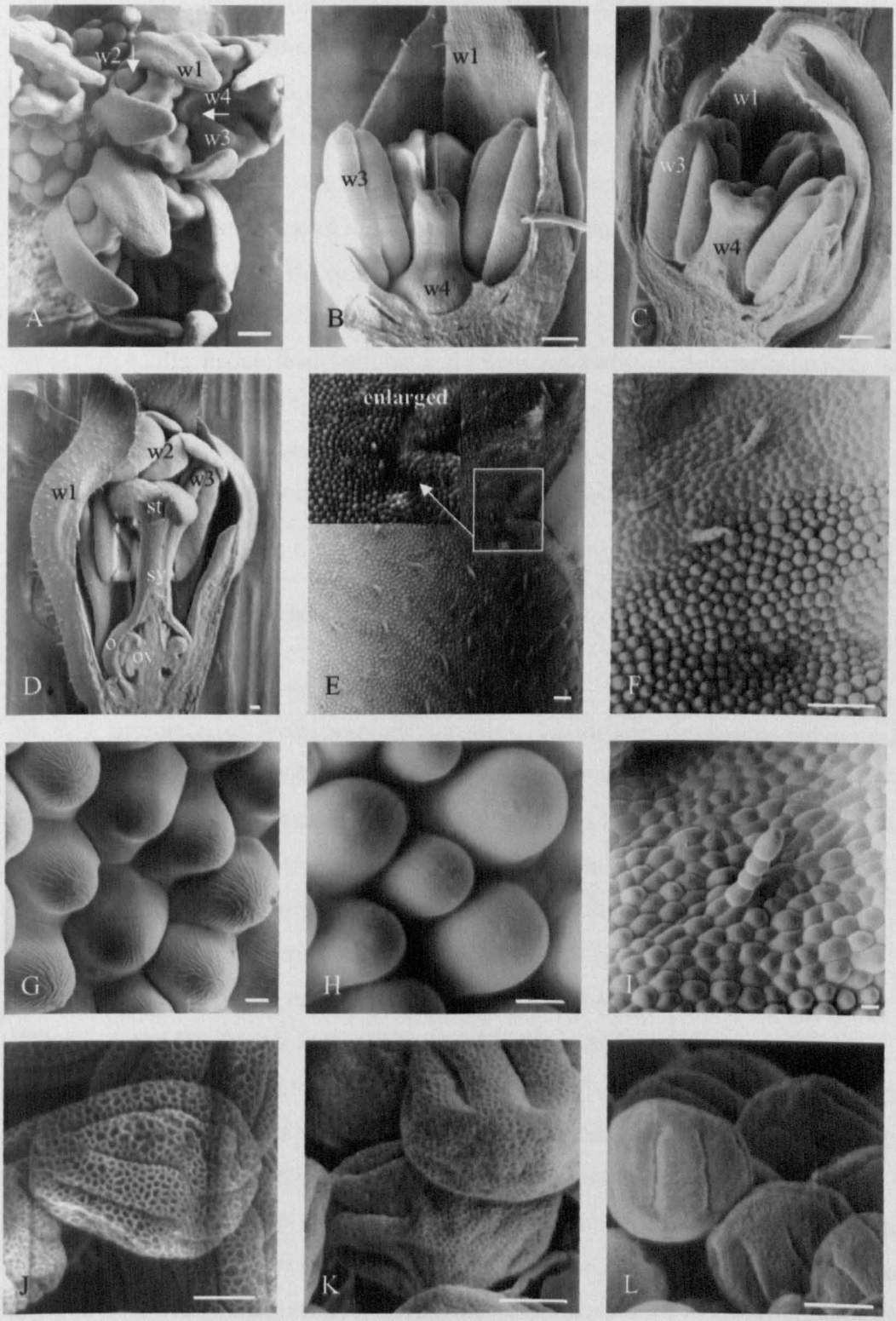
Figure 4.22. Development of the *reduced petal* flower.

There is little difference in the early development of the *reduced petal* flower except when the perianth is split, as observed on the late season flowers in Figure 4.24E and F. Comparison of mature cells from the *reduced petal* mutant phenotype with that of *Wild Type* highlights differences in the form of the mature cells. Pollen appears normal.

A. Cluster of developing *reduced petal* flowers at stages 4 and 5. **B.** A developing *reduced petal* polyanthus flower at early stage 6. **C.** A developing *reduced petal* polyanthus flower at middle stage 6. **D.** A developing *reduced petal* polyanthus flower at stage 7. The stigma can be observed to be irregular. Fewer than normal ovules appear to be developing. **E.** The adaxial surface of the tip of a petal of a normal silver-laced polyanthus. The surface is evenly covered by conical papillate petal cells. An area from the centre of the petal tip has been enlarged in order to demonstrate that the white central stripe on the petal of the silver-laced polyanthus is not achieved by alteration of the cell shape. **F.** Part of a *reduced petal* adaxial petal surface from a flower with small patches of colour as in C and E in previous fig. A patch of conical papillate cells can be observed, but the surrounding cells are flatter. **G.** An enlarged view of cells in the center of the area of conical papillate cells. **H.** An enlarged view of cells at the edge of the area of conical papillate cells. **I.** An enlarged view of the cells that are not conical papillate. **J.** Pollen from a normally fertile thrum *Hose in Hose* polyanthus. **K.** Pollen from a thrum *reduced petal* polyanthus. **L.** Pollen from a pin *reduced petal* polyanthus. Size bars in **H, J, K,** and **L** are 1um; size bars in **G** and **I** are 10μm; other size bars are 100μm.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(Fig.4.22C). A. developing flower at stage 7, of approximately 3mm. in size, also has a large and distorted stigma (Fig.4.22D). It has a split perianth visible in whorl2, and less than the usual number of developing ovules. Mature petal tissue from a normal silver-laced polyanthus was examined to discover whether there was any structural difference between the cells of the laced areas and the pigmented cells (Fig.4.22E). No differences were observed. A piece of mature *reduced petal* polyanthus petal tissue from the plant shown in Fig.4.22C was examined (Fig.4.22F). The latter had some patches of cells that did produce pigment, but most of the adaxial surface was white. Where patches of pigmented cells occurred the cells were of the normal conical papillate form (Fig.4.22G and H), the surrounding cells were flatter and had not developed the conical papillate form (Fig.4.22I). Pollen from both pin and thrum reduced petal polyanthus plants was examined (Fig.4.22K and L). Pollen from a thrum *Hose in Hose* plant known to be a fertile pollen parent was examined for comparison (Fig.4.22J). The *reduced petal* polyanthus pollen appears smoother than the *Hose in Hose* pollen but is of normal stephanocolpate form.

4.2.10. The *Oak Leaf* mutant phenotype.

(i) Description of *Oak Leaf*.

Dr. R. J. Brumpton, of Wood borough Nurseries, Nottingham, discovered the original *Oak Leaf* primrose, shown in Fig. 4.23.A, in a neighboring nursery during spring 1999. From this one plant all subsequent *Oak Leaf* plants have been derived. In spring 2000 I received a division of the original plant and also a number of *Oak Leaf* seedlings raised by Dr. R. J. Brumpton. The leaves of the *Oak Leaf* form are deeply lobed as are oak leaves. They are thicker and firmer than normal leaves and have broader than usual veining. This is especially noticeable in the main vein through the centre of the leaf (Fig.4.23A and D). The abaxial surface of the leaf is extremely pubescent. Flowers frequently have petals that are much attenuated (Fig.4.23A - D). During the first season the original *Oak Leaf* plant appeared to be female infertile, but in the second year seed was set normally from controlled pollination. A few of the progeny from *wild type* x *Oak Leaf* had near normal petals (Fig.4.23.F). A selection of pressed flowers from the first 35 progeny to bloom can be seen in Fig. 4.24A. Only two of these approximate to the normal form, all others have attenuated petals that are sometimes forked or divided. All of the *wild type* progeny from the same cross had normal petals. Flowers of many of the first generation of *Oak Leaf* plants had a tendency to break down in form, developing irregular splitting of the petals, either over the flowering season (Fig.4.23G), or from one season to

Figure 4.23. The *Oak leaf* mutant phenotype.

The flower of the original *Oak Leaf* primrose and those of some representative plants from subsequent progeny exhibit differences in form, but the majority have flowers that are smaller than those of the *wild type P. vulgaris* and have attenuated petals. Other unusual features associated with the *Oak Leaf* mutant phenotype are lobed cotyledons and ectopic shoots.

A. The original *Oak Leaf* primrose at first flowering. **B.** The same plant in the second flowering season showing breakdown of the flower form. **C.** A first generation plant from a cross of *wild type* x *Oak Leaf* showing the most typical flower form. **D.** A first generation plant from a cross of *wild type* x *Oak Leaf* with extremely attenuated petals. **E.** A first generation plant from a cross of *wild type* x *Oak Leaf* with slightly less attenuated petals. **F.** A first generation plant from a cross of *wild type* x *Oak Leaf* with near normal petals. **G.** A first generation plant from a cross of *wild type* x *Oak Leaf* later in the flowering season. Early in the flowering season flowers were similar to those in F above, but flower form has already begun to break down. **H.** An ectopic meristem developing on the main vein of a leaf. **I.** A nother ectopic meristem on the main vein of a leaf of a different plant, at a later stage of development. **J.** An ectopic flower on a leaf tip. **K.** An ectopic seed capsule on the same leaf tip as a consequence of pollinating the ectopic flower. The few seeds produced were not viable. **L.** Seedlings from the cross of *wild type* x *Oak Leaf*. The *Oak Leaf* phenotype is sometimes evident at the cotyledon stage, and is always evident at the first true leaf stage. Size bars are approximately 1 cm.


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Figure 4.24. Pressed flowers and calyces from progeny of *wild type P. vulgaris* x *Oak leaf* primrose.

This figure shows the variability in petal shape, and the varying amounts of petaloid tissue in the *Oak Leaf* calyces. A. One pressed flower from each of the first 35 plants to bloom, adaxial view. **B.** One pressed calyx from each of the first 45 flowers to bloom, adaxial view. All have at least a small amount of petaloid tissue in the calyx. Size bars are 1cm.

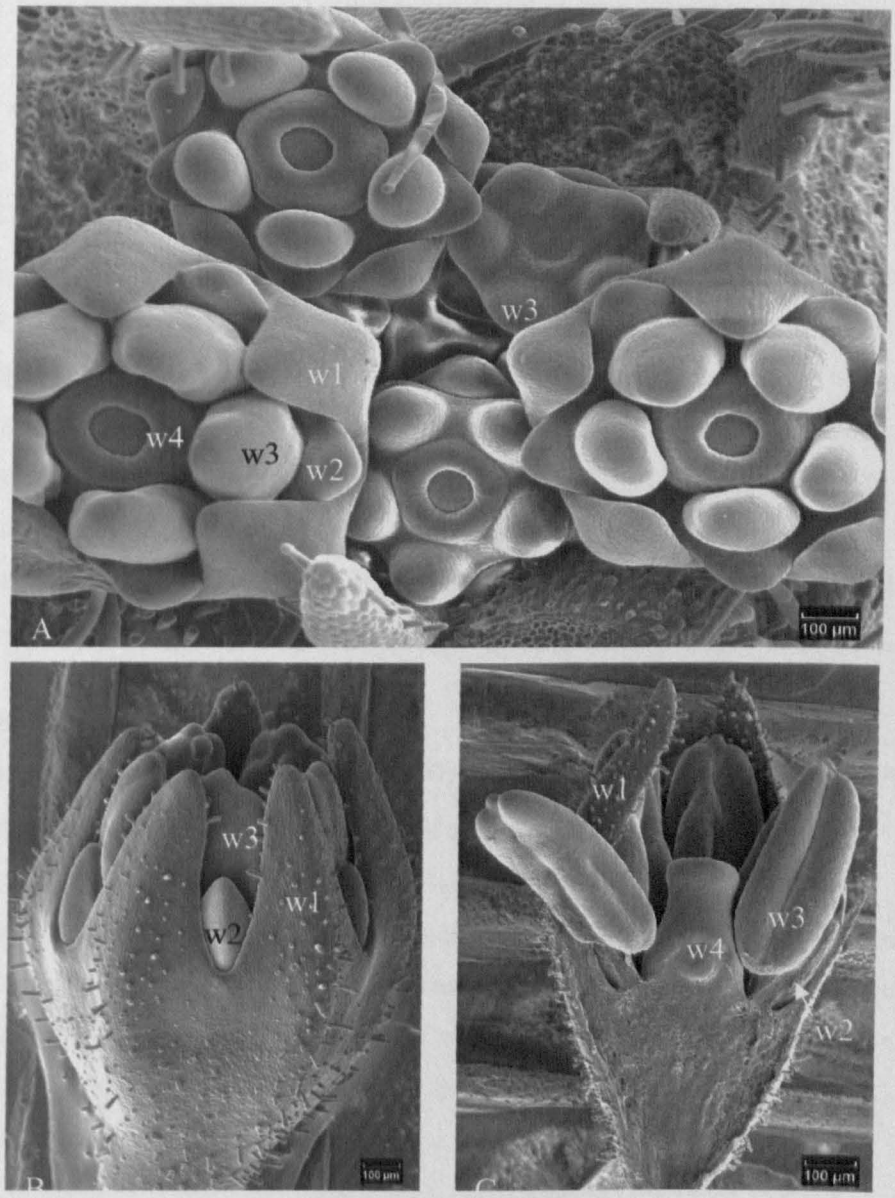


Figure 4.25. Development of *Oak Leaf* flowers.

This figure shows the early development of *Oak Leaf* flowers, up to and including stage 6.
A. A cluster of developing Oak Leaf flowers at stages 3, 4, and 5, showing that they are much more open than wild type flowers at stages 4 and 5. **B.** Side view of stage 6 flower, showing that whorls 1 and 2 already appear attenuated. They are also shorter than wild type whorl 1 and 2 organs at this stage. **C.** Stage 6 flower cut in two. Whorls 3 and 4 appear to be developing normally.

the next. This was first observed in the original plant (Fig.4.23B). The original plant also had an excess number of petals. The most recent generation of *Oak Leaf* plants (from crosses described in Chapter 7) have flowers that do not appear to break down in form. Flowers of *Oak Leaf* are generally smaller than those of *wild type P. vulgaris*, being usually about 2cm. in diameter as opposed to the 3 to 3.5cm. diameter of *wild type P. vulgaris*. Calyces are generally split, but not quite to the base of each calyx lobe (Fig.4.23A). Figure 4.24B shows that some streaks of petal tissue occurred in the calyces of the original plant and in the calyces of the first *Oak Leaf* progeny, although later progeny from crosses with commercial primroses had calyces without any petaloid streaking. Two of the progeny from *wild type* x *Oak Leaf* produced ectopic meristems on the central vein of a leaf (Fig.4.23J and K). One plant from a later cross (see Chapter 7) produced an ectopic flower on the central vein at the tip of a leaf (Fig. 4.23J). At first it appeared to set seed from controlled pollination (Fig.4.23K) but the few seeds produced were not viable. *Oak Leaf* seedlings are sometimes recognisable even at the cotyledon stage, and all are recognisable by the first true leaf stage (Fig.4.23L).

(ii) Development of the *Oak Leaf* flower.

Development of *Oak Leaf* flowers through some early stages up to middle stage 5 (largest flower on left) can be seen in Fig.4.25A. All stages seen are much more open in development than normal flowers of stage 4 and 5. Whorl 4 is broader and more prominent in the stage 4 developing flowers than it is in wild type plants. Petals are more pointed organs than in wild type at stages 4 and 5, and sepals do not grow to cover the inner organs by middle stage 5. A flower of stage 6 (Fig.4.25B) shows the inner whorls still visible. Another stage 6 flower was cut in two and this revealed that whorls 3 and 4 were developing normally (Fig.4.25C).

4.3. Discussion of Chapter 4.

4.3.1. Discussion of the mutant phenotypes.

Jack in the Green plants used in this study had conversion of the upper part of the calyx to leaf (Fig.4.1A), the lower part of the calyx was as normal. The degree of conversion of sepal to leaf in *Jack in the Green* plants varies from small leafy sepal tips to a completely leafy calyx (Fig. 4.2B). This observation could be due to a delay in the expression of the floral organ identity genes at the time of initiation of the cells of the sepal lobes, with the size of the leafy portion in each case correlating with the degree of delay. From the ABC model of floral morphogenesis (Coen and Meyerowitz 1991), therefore, it could be

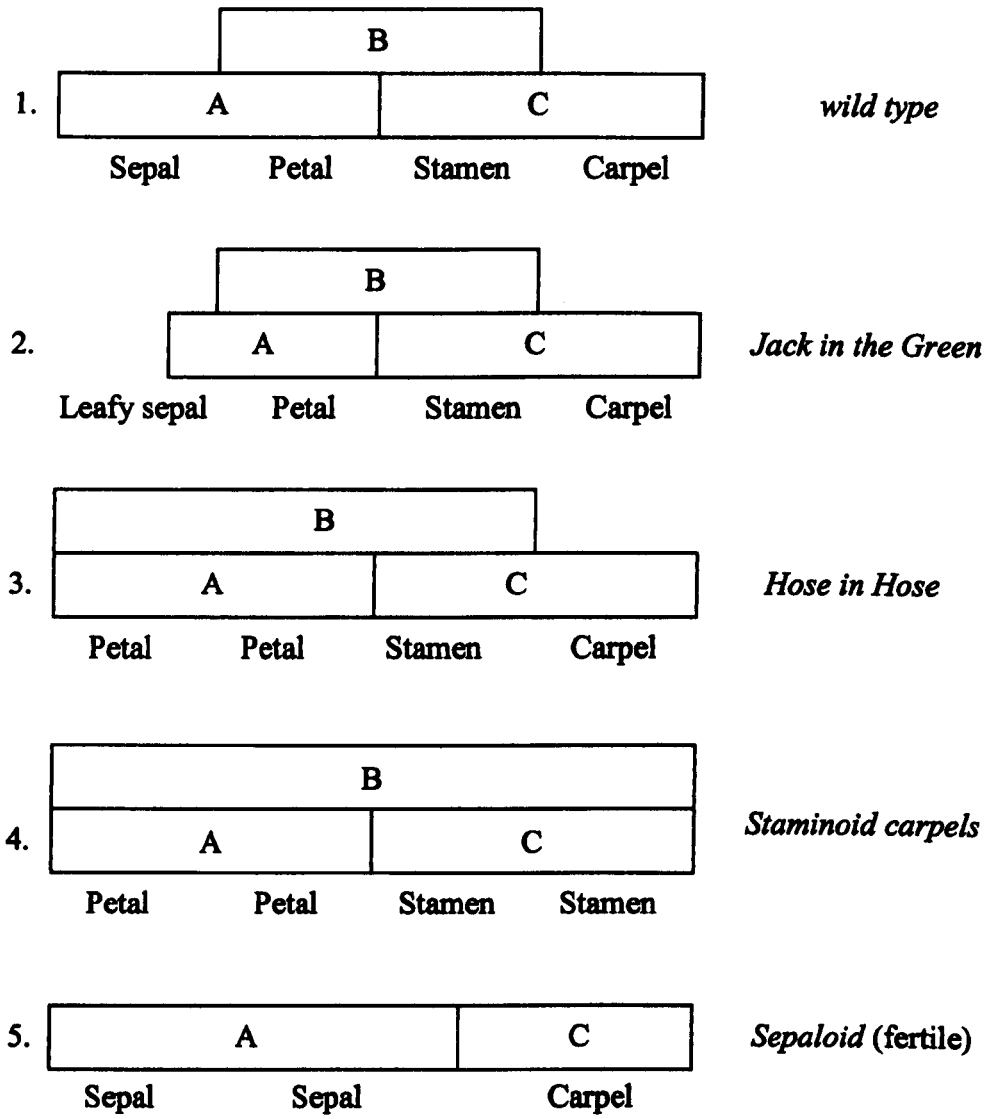


Figure 4.26. Interpretation and prediction of some mutant phenotypes through the A B C model of organ identity.

predicted that this *Jack in the Green* mutant phenotype arises through an absence of floral organ identity gene function in the upper part only of the first whorl (Fig. 4.26 no2), as the top of the organ is the first part to be initiated and it is converted to leaf form. Late expression of the A organ identity function could then result in sepaloid tissue at the calyx base, and in the petals in the second whorl.

It could similarly be predicted that the *Hose in Hose* mutant phenotype is due to ectopic expression of B function genes in the first floral whorl (Fig. 4.26 no 3). The phenotype observed in *Jackanapes* flowers (see Chapter 6), which combines both mutant alleles, further supports these hypotheses. The first whorl organs in these flowers have leaf-like tips and only the basal regions that appeared as a normal calyx in *Jack in the Green* flowers shows homeotic conversion to petals. This observation suggests that the potential for petal identity cannot be established without underlying sepal identity as predicted by the ABC model. A number of studies using Antirrhinum, tobacco, Arabidopsis and Petunia have characterised phenotypes that phenocopy *Hose in Hose*. In Petunia, ectopic expression of the B function MADS box gene *GREEN PETAL* (GP) using 35S-GP results in the homeotic conversion of sepals to petal and the associated up-regulation of other possibly B function MADS box genes (Halfter *et al.* 1994). Similar studies have also been reported using transgenic tobacco over-expressing the Antirrhinum B function MADS box genes *DEFICIENS* and *GLOBOSA*. In these studies, over expression of *GLOBOSA* using *35S-GLO*, produced partial homeotic conversion of sepals to petals. These plants also showed alterations in development of the fourth whorl with some characteristics of both carpels and stamens (Davies *et al.* 1996). Two Antirrhinum mutants, *CHORIPETALA* and *DESPENTEADO* also show petaloid transformation of the first whorl, reduced female fertility and occasionally reduced or unfused carpels (Wilkinson *et al.* 2000). These phenotypes show ectopic B function expression, although interestingly both *CHORIPETALA* and *DESPENTEADO* are recessive mutations. Further studies with ectopic expression of B function genes in Arabidopsis also reveal interesting similarities to *Hose in Hose*. Over-expression of the Arabidopsis B function gene *PISTILLATA* (*PI*) using *35S-PI* produces partial conversion of sepals to petals, whereas over expression of the other B function gene *APETELA-3* (*AP3*) using *35S-AP3* only affects whorl 4 (Krizek and Meyerowitz 1996). However, as with the studies in transgenic tobacco (Davies *et al.*1996), ectopic expression of both B function genes had significant affects on both whorls 1 and 4 (Krizek and Meyerowitz 1996). By analogy to these studies, and the dominant nature of *Hose in Hose*, it is tempting to predict that the *Hose in Hose* phenotype is caused by a mutation that causes either direct or indirect up-

regulation of the B function MADS box genes. The *Staminoid Carpels* mutant phenotype might also be explained as ectopic expression of B function in whorl 4 (Fig. 4.26 no.4). When Arabidopsis B function genes are expressed ectopically using the *CaMV 35S* promoter the *35S::AP3* flowers have staminoid carpels (Jack *et al.*, 1994). It is tempting to predict that *Staminoid Carpels* may represent an allele of *Hose in Hose*, but the dominant nature of these mutations (see Chapter 5) precludes complementation analysis. The cadastral gene *SUPERMAN* in Arabidopsis (Bowman *et al.*, 1992; reviewed by Lohmmann and Weigel, 2002) is required to maintain the inner boundary of expression of the B function gene *AP3* in *Arabidopsis*, and it is possible that a deficiency in a *Primula* orthologue of *SUPERMAN* may be responsible for the *Staminoid Carpels* phenotype.

Although the *sepaloid* mutant phenotype shares some similarities with that predicted by the loss of B function in the ABC model, aspects of the phenotype are different from the *DEFICIENS* or *GLOBOSA* mutants of Antirrhinum (Sommer *et al.* 1990; Tröbner *et al.* 1992) or the *PISTILLATA* or *APETALA-3* mutant of Arabidopsis (Hill and Lord 1989; Jack *et al.* 1992). Also of possible relevance is the phenotype of the Arabidopsis *SEP1/2/3* triple mutant (Pelaz *et al.* 2000, 2001; Honma and Goto 2001). The phenotypes produced by the combination of these different mutant alleles include conversion of the inner whorls to sepals. However, the *Primula sepaloid* mutant differs from the above in remaining determinate, occasionally produces fertile carpels, and is determined by a single locus. Although it is a possibility that the *sepaloid* mutant phenotype arises through either a direct or indirect loss of B function activity (Fig. 4.26 no.6), the possibility cannot be excluded that it arises through the loss of *SEP-like* proteins. However, because *sepaloid* is inherited as a single locus, such a possibility would require *sepaloid* to be a regulator of *SEPELLATA* genes or the *SEP*-function in *Primula* to be determined by a single gene.

The *Primula sepaloid* mutant phenotype also shares some similarity with the most extreme form of the *FIMBRIATA* mutant (Simon *et al.*, 1994) of *Antirrhinum*. The sepals in at least one plant were arranged in a spiral, some of the carpels were incompletely formed or were fused to inner sepals and some of the later generations of *sepaloid* plants had flowers with secondary inflorescences (see chapter 7, Fig. 7.4). The *Arabidopsis* gene *UNUSUAL FLORAL ORGANS* shows extensive homology with *FIMBRIATA* but differences in the functions and genetic interactions were found (Ingram *et al.*, 1995) *UNUSUAL FLORAL ORGANS* is, along with *LEAFY*, an upstream co-regulator of the B

function organ identity gene *APETALA 3* (Parcy *et al.*, 1998; Honma and Goto, 2000). However the *Primula sepaloid* mutant differs from both of the above in that no less extreme forms of the *sepaloid* phenotype have so far been discovered.

The phenotype of the virescent cowslip also appears to be without any B function expression, in that no petals or stamens are ever produced . The form looks similar to the *uniluni* form described in pea in 1997, (Hofer *et al.* 1997). There is a profusion of carpeloid organs in the centre of the flower in both instances, and this is distinctly different from the *sepaloid* mutant phenotype above. Also, unlike *sepaloid*, none of the carpeloid organs are complete and fertile, so that associated disruption of some aspect of the C function can be inferred. The complete infertility of this phenotype made genetical analysis impossible.

No correlates for the developmental mutant *Split Perianth* has been described in the literature. However both the *Arabidopsis* gene *CUC2* (Ishida *et al.* 2000) and the *Antirrhinum* gene *CUPULIFORMIS* (Weir *et al.* 2003), have been reported to be involved in organ separation in both shoot and meristem but there is as yet no evidence that either gene is implicated in *Primula.* Only further molecular analysis of *Primula* will confirm which mutations in which genes are responsible for each different mutant phenotype. Notable is the loss of some or both of the perianth organs in early flowers of two plants (Fig.4.6F-H). Instead of alteration of the whorl to another organ type, these flowers appear to have failed to initiate meristems for whorls 1 and/or 2. This extreme form of the mutation was not stable; later flowers on the same plants did have perianth organs. Also notable is reflexing of the petals in those flowers with the corolla tube divided into five separate pieces (Figure 4.6, C and D). It has been proposed as a possible phenotypic modification necessary for an evolutionary shift from the heterostyly of *Primula* towards the solenoid flowers of *Dodecatheon* (Mast *et al.*, 2004).

The *double* phenotype in a number of other plants has been demonstrated to be due to mutation in the C function gene(s) that are required for normal stamen and carpel development Yanofsky *et al.*, 1990; Coen and Meyerowitz, 1991; Bradley *et al.*, 1993; and Pneuli et al.,1994). Two genes have been isolated from *Primula, PvPLE1* and *PvPLE2* (Cook, 2002) that have expression patterns that appear to correspond to C function (*PvPLE1*) and D function (*PvPLE2*). Some of the variability of expression of the *double* phenotype may be due to mutation that results in varying degrees of expression of

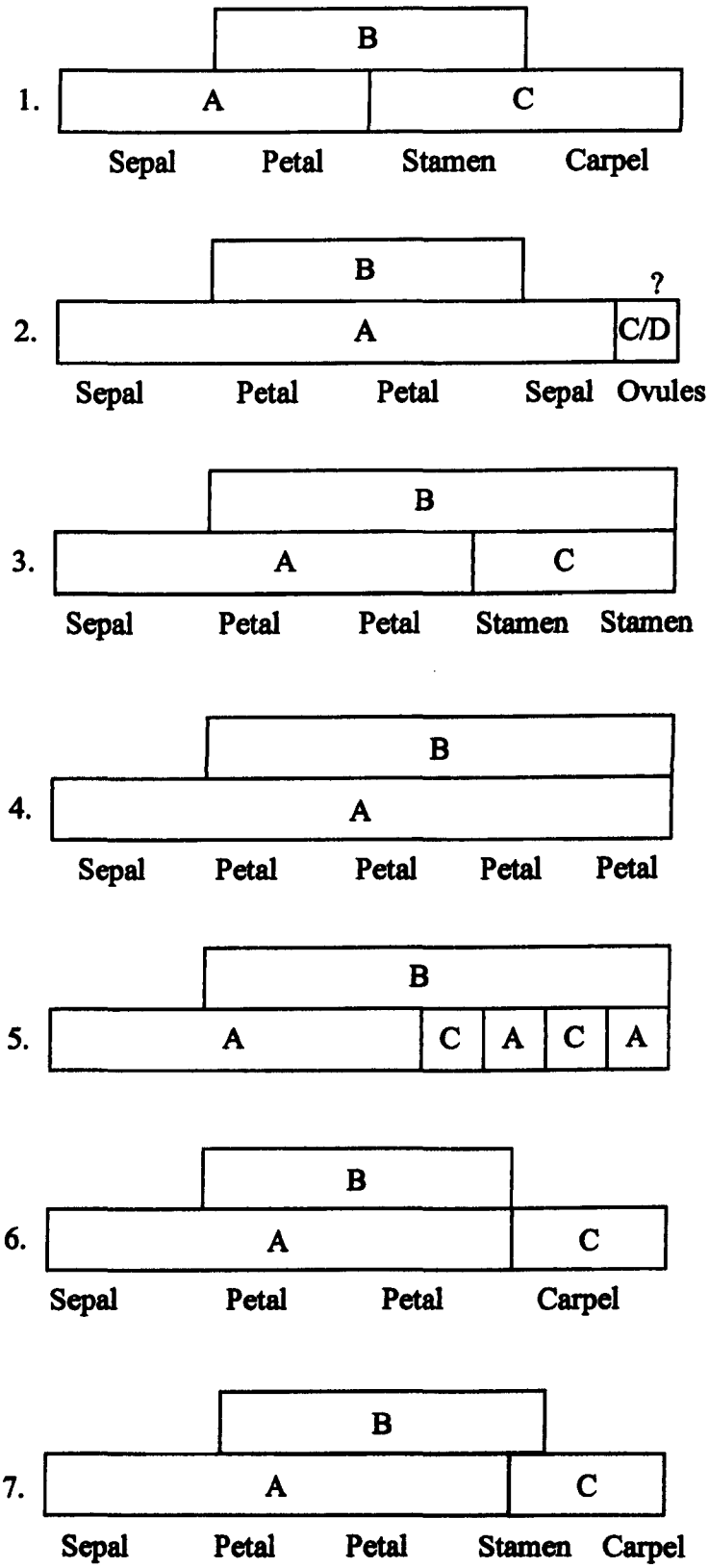


Figure 4.27. Interpretation and predictions of the phenotypes found in doubles through the A B C model of organ identity.

one or both of these genes. Of four *doubles* analysed by RT-PCR (Cook, 2002) "Miss Indigo", "Our Pat", "semi-double" and "Farmen double", only the commercial cultivar "Miss Indigo" was completely deficient in both gene products. This cultivar has not exhibited variation in third or fourth whorl organs; no stamens, carpels, or ovules have ever been discovered and the flower is always indeterminate. The other three *doubles* in which she found weak expression of one or both of the genes were also *doubles* that produced stamens, carpels (infertile) or ovules in whorls three and/or whorl four. From my observations of these three plants, "Our Pat", "semi-double" and "Farmen double", over a number of flowering seasons, the factor common to all of them is that the centre of the flower is frequently determinate. The molecular factor common to all three (Cook, 2002) is the presence of *PvPLE2*, and in the case of the "Farmens *double*" there was also absence of *PvPLE1*. Consequently, it is my opinion that *PvPLE2* is required for determinacy. Absence of *PvPLE2* appears to result in ectopic B function in the centre of the flower, giving indeterminacy as found in the *double* "Miss Indigo". However all *doubles* have variable expression that is predicted to be due to environmental influences, so that if it were possible to do further RT-PCR analysis on flowers of the same plants over a longer time span, they might be expected to show variations in the amounts of *PvPLE1* and *PvPLE2* gene products. Unfortunately the *double* "Lin Rogers" that produces sepals in whorl 4 was not analysed, but the presence of naked ovules in the centre of the flower, from H. Cook's previous results (Cook, 2002), suggests that *PvPLE2* expression must not normally be impaired in this plant. A simplified interpretation of the varying phenotypes found in double flowers through the A B C model of organ identity can be seen in Fig.4.27. It illustrates that only those flowers predicted to lack ectopic B function in whorl 4 are determinate.

The incomplete transformation of the inner three whorls of the green primrose, *P. viridis*, shares some phenotypic characteristics with transgenic tobacco plants expressing antisense RNA for the gene *TM5* (Pnueli *et al*, 1994). Both have normal sepals, but petals are green and do not senesce normally. Both phenotypes also exhibited excessive pubescence of the upper style, and both occasionally produce a more complex form of flower. There are differences as well as similarities, in that in *P. viridis* the complex flowers usually only have one extra whorl of green petals and unlike the transgenic tobacco the stamens are not green in colour. Co-supression of the petunia homeotic gene *fbp2* also gives flowers with modified second third and fourth whorl organs (Angenent *et*

al, 1994), and this gene has been found to be highly homologous to *TM5* of tobacco. The infertility of *P. viridis*, the green primrose, made analysis of the phenotype impossible.

The *reduced petal* mutant phenotype bears some resemblance to the *CINCINNATA* mutant phenotype of *Antirrhinum*. The wider and more undulating "frilled" edges of the leaves of the reduced petal phenotype resemble those described in *Antirrhinum* plants lacking the *CIN* gene (Nath *et al.* 2003; Coen 2003). Similar petal reduction and loss of conical papillate cells has been reported in the most extreme *CIN* allele of Antirrhinum (Crawford *et al.* 2004). Loss of colour on the adaxial petal surface also bears similarities to that described in *Antirrhinum* plants with deficiency in the transcription factor *MIXTA* (Glover *et al,* 1998, Martin *et al,* 2002). We cannot however assume from the phenotypic similarities between *CINCINNATA* and *reduced petal* that the mutation in *Primula* is caused by a defect in the same genes as have been studied in *Antirrhinum*.

The *Oak Leaf* mutant phenotype exhibits some of the characteristics of the phenotype described in *Arabidopsis thaliana* when the *Knotted* gene from maize was expressed ectopically (Chuck *et al*, 1996). Most notable are the lobed leaves in both instances, and the possibility of ectopic meristems. However it is only in the most extreme forms of the *Arabidopsis* transformants that the petals are described as being thin and elongated, while attenuation of the petals in *Primula* is present in the majority of cases. The attenuated *Primula* petals however are of normal colour while the most extreme *Arabidopsis* transformants had greenish petals that abscised early. Another gene, inactivation of which results in lobed leaves in *Arabidopsis thaliana* is *JAGGED* (Dinneny *et al.* 2004), but inactivation of *JAGGED* also results in petals and sepals with serrated edges. *Oak Leaf* flowers do not have serrated edges to either sepals or petals.

It is predicted that *Hose in Hose* (petaloid sepals) and *sepaloid* (no petals or stamens) result from gain and loss of B function. They could be allelic mutations or two separate but linked genes. If the latter, they could be on the same or on opposite sides of the *S* locus. If allelic mutations, they will be the first example of B function complementary phenotypes. If they are two separate genes this will be the first example of a B function gene and a regulator of B being tightly linked. These possibilities will be investigated in Chapter 7.

4.3.2. Discussion of early ontogeny of mutant phenotypes.

Despite the early documentation of several mutant phenotypes of *Primula* (Gerard 1597; Parkinson 1629; Masters 1878) this is the first detailed analyses of early flower development in these plants. Not all of the mutant phenotypes studied exhibited distinctive early ontogeny, the *reduced petal* mutant phenotype only became recognisable later in development, although where the latter also had splitting of the perianth this feature was obvious from stage 4 (Fig.4.22A). *P. viridis,* the green primrose, is as *wild type* up to and including stage four, but by stage 5 the abnormally pointed anthers are distinctive (Fig.4.18C and D), while the virescent cowslip is distinctive in that it lacks organisation of floral organs throughout development. The *Oak Leaf* flower assumes a much more open form than any other mutant phenotype during early ontogeny and this allows observation of inner whorls even at stage 6. Development of whorls three and four appears normal at this stage (Fig.4.25B and C).

The majority of first whorl mutations become clearly visible with expansion of the sepal lobes during stage 4. One exception is *Split Perianth,* in which the divisions between individual sepals are evident at earlier stages of development (Fig.4.7E). However, it is not easy to differentiate between the *Jack in the Green* mutant phenotype, and the *Jackanapes* plants that carry both the *Hose in Hose* and *Jack in the Green* mutant alleles, until stage 5 (the double mutant is shown in Chapter 6, figure 6.3). The developmental progression of whorl 1 is such that the tips are more developed than the bases, and it is not until the lower edges of the sepal lobes have begun to expand later in development that the petaloid nature of this part of the organ becomes evident.

The early architecture of the common stamen-petal primordium for whorls 2 and 3 is common to both the *wild type* (see Chapter 3, Fig.3.2F.) and to the *double Primula* with predicted loss of C function (Fig.4.13A,B,E,F,G,and K). In the *wild type,* anthers develop from the larger initials and petals from the smaller initials on the abaxial bases of these common primordia. In *double* flowers, both of the initials become petals, despite the structure of the initials during stages 4 and 5 being identical to that of the *wild type.* The identity of the second and third whorl organs in this case does not influence the early architecture of the primordium. The persistent presence of a common primordium for the second and third whorls in double flowers suggests that the underlying control of this aspect of floral development occurs independently of genes required for carpel and stamen development. Similarities in *Antirrhinum* between the appearance of whorl 4 in

the *Wild Type* and *plena* mutants at an early developmental stage (stage 6) has also been reported (McSteen *et al.*, 1998). Only when the relevant *Primula* homologues of defined MADS box organ identity genes have been fully characterised and their expression patterns defined in the *wild type* and mutant flowers, will direct comparisons with the well-defined *Antirrhinum* and *Arabidopsis* ABC models be possible.

In striking contrast to the observations of *double* flowers where the initial development of whorls 2 and 3 is identical to the wild type irrespective of the final organ identity in these whorls, development in *sepaloid* flowers that have no petals or stamens shows a dramatically different early ontogeny. Although the second whorl emanates from the base of the third floral whorl, there is no separation of the whorl 2 and 3 organs, and whorl 4 arises from the inner base of whorl 3 as opposed to emerging from the centre of the flower. These observations, together with those of the double flowers that develop sepals within the inner fourth whorl (Fig.4.12H and 4.13O-R), suggest that the programming of sepal identity within a whorl directly affects the development of that whorl. In the absence of programmed sepal identity, the development of whorl 2 and 3 is unaffected by the subsequent identity of the organ that will develop. The phenotype of the *sepaloid* mutant shows no evidence of any B function gene activity, as these flowers never produce petals or stamens. However, the ability of these flowers to produce sepals in the third and, occasionally, fourth whorl would also suggest that C function might also be impaired. These observations also raise the question of whether the B function plays a part in the separation of organs in whorls 2 and 3 during early ontogeny as it is only in sepaloid plants that are unable to produce petals or stamens in which the second and third whorl organs do not separate.

Although the *sepaloid* mutant phenotype shares some similarities with that predicted by the loss of B function in the ABC model, aspects of the phenotype are different from the *DEFICIENS* or *GLOBOSA* mutants of *Antirrhinum* (Sommer *et al.*, 1990; Trobner *et al.*, 1992) or the *PISTILLATA* or *APETALA 3* mutant of *Arabidopsis* (Hill and Lord 1989; Jack *et al.*, 1992). Also of possible relevance is the phenotype of the Arabidopsis sep1/2/3 triple mutant (Pelaz *et al.*, 2000, 2001; Honma and Goto 2001). The phenotypes produced by the combination of these different mutant alleles include conversion of the inner whorls to sepals. However, the *Primula sepaloid* mutant differs from the above in remaining determinate, occasionally produces fertile carpels, and is determined by a single locus. Although it is a possibility that the *sepaloid* mutant phenotype arises through either a direct or indirect loss of B function activity, we cannot exclude the

possibility that it arises through the loss of SEP-like proteins. However, because *sepaloid* is inherited as a single locus, such a possibility would require sepaloid to be a regulator of *SEPELLATA* genes or the SEP-function in *Primula* to be determined by a single gene. An interesting observation arising from analysis of flowers from double flowers that produce stamens in the fourth whorl and *Staminoid Carpels* is that development of stamens in the fourth whorl of *Primula* is associated with the delayed development of the fourth whorl. In contrast, there is no delay in the development of the fourth whorl in *Arabidopsis* flowers when stamens are ectopically produced in this whorl, either by over-expression of *PI* and *AP3* (Krizek and Meyerowitz 1996), or following mutation of *sup-1/flo10* (Schultz *et al.*, 1991; Bowman *et al.*, 1992). In addition, the central area of the developing flower remains undeveloped for an extended period of time in *double* (Fig.4.13F), *semi-double* (Fig.4.13J and K) and in *Staminoid Carpels* (Fig.4.9H and M). This delay is associated with the presence of a mixture of petals and stamens in the centre of the flower in *doubles*, and stamens in *semi-doubles* and *Staminoid Carpels*. Given that both A and B functions are required to produce petals and that both B and C functions are required to produce stamens, these findings reinforce previous observations (Bowman *et al.*, 1992) that B function may be involved in the loss of determinacy of the floral meristem by over-riding the determinacy role of the C function. Previously, loss of determinacy has been associated with loss of C function; however, observations on two different *Primula* mutants, *semi-double* and *Staminoid Carpels*, show that loss of determinacy can occur following expression of B function in the fourth whorl of flowers that also contain C function.

Analyses of mutant *Primula* flowers shows that floral ontogeny in this plant have revealed a number of differences as compared with the two model plants *Antirrhinum majus* (McSteen *et al.*, 1998) and *Arabidopsis thaliana* (Smyth *et al.*, 1990). The ABC model of floral development was formulated primarily from studies on these two species. Observations of the early stages of flower development mutant *Primula* flowers provide a foundation for the possibility to explore the differences in floral MADS box gene expression in this plant as compared with *Arabidopsis* and *Antirrhinum*. Work to identify the relevant *Primula* MADS box genes and to determine the molecular basis of many of the mutant phenotypes described in this work is currently underway in the Gilmartin laboratory.

4.3.3. Discussion of late ontogeny.

Many phenotypes have comparatively uneventful late ontogeny, the leafy calyces of *Jack in the Green* for example simply become larger as growth progresses. Others such as *Primula viridis*, show increased variation from the norm as ontogeny progresses, so that by maturity the only normal cell type in the flower is that of the yellow "eye" of the flower (Fig.4.18Q).

Poorly expressed *Hose in Hose*, in which the central portion of the petaloid calyx is green, appear as wild type during early ontogeny. Comparison of a well expressed *Hose in Hose* flower at stage 6, which in the mature flower has a completely petaloid calyx (Fig.4.5D), with a poorly expressed *Hose in Hose* flower of the same stage (Fig.4.5F) shows that the centre of the abaxial surface of each first whorl organ of the latter is densely covered with trichomes. Examination of the adaxial surface (Fig 4.5G) shows that the cells in the centre are not the expected conical papillate cells of petal tissue (Fig. 4.5H), but that those nearer the edge appear less abnormal (Fig.4.5I). A mature flower from the same plant (Fig.4.4J) clearly shows the first whorl to be petaloid but with green sectors in the centre of each first whorl organ.

Observation of mutant phenotypes, and analysis of development using scanning electron microscopy, provides much useful information. Further information can be obtained by genetical analysis of the mutant phenotypes. As both *Primula viridis* the green primrose and the virescent cowslip were found to be consistently infertile investigation of these two phenotypes cannot be taken further. Future molecular analysis of *Primula* and of *Primula* mutant phenotypes will supply a fully comprehensive picture of floral morphogenesis in *Primula*.

CHAPTER FIVE

Genetic analysis of mutant phenotypes.

5.1. Introduction.

Characterizing the inheritance of the available fertile floral mutant phenotypes by self and test crosses was essential in order to determine dominance relationships and linkage patterns. Genetic analysis was not possible for either the green primrose or the virescent cowslip as neither produced any viable reproductive parts during this project, and both appear to be reproductively inviable. Genetic analysis for some of the phenotypes was already underway before commencing this project, and for two of the phenotypes, was almost completed. Other phenotypes were discovered during the course of this project and require further analysis. Crosses are generally presented in chronological order within each section. Older work is included in order to give a full picture of what is known of each phenotype. Data obtained before beginning this project is included under "Origin and history", and new data under "Subsequent crosses". When beginning analysis of mutant phenotypes, most initial crosses were of small numbers but were sufficient to determine dominance relationships. Larger numbers of progeny were raised in tests for linkage, although in instances where the locus for the mutation was tightly linked to the *Primula S* locus linkage was obvious even with smaller numbers.

5.2. *Jack in the Green.*

The majority of crosses required for genetic analysis of *Jack in the Green* (leafy calyces) were completed before commencing this project. It was already known to be a dominant phenotype with the locus for the mutation unlinked to the *Primula S* locus. It was not certain whether plants homozygous for the dominant *Jack in the Green* mutation were viable, but indications were that they were probably inviable (Webster and Grant, 1990).

5.2.1. Origin and history.

Pin and thrum *Jack in the Green* plants were derived from thrum *Jack in the Green* plants found growing under the garden hedge in Killay, Swansea, (SS 600926) and were subsequently interbred with *wild type* plants. Controlled crosses were carried out (in conjunction with the Department of Botany, University of Bristol) in 1986, and the progeny classified in 1988. Results showed that there was no linkage between Jack in the Green and the Primula S locus. Dominance of *Jack in the Green* was also confirmed with

a better fit for a 2:1 ratio than a 3:1 ratio, indicating possible inviability of the homozygote *Jig/Jig* (Webster and Grant, 1990).

A petal less *Jack in the Green* (designated *Jig*)* that arose in 1985 from naturally set *Jack in the Green* seed was pollinated by a thrum *Jack in the Green* in 1986 and the progeny classified in 1987 (Table 5.1).

pin petal less *Jack in the Green* x Thrum *Jack in the Green*

*Jig** *s* x *Jig* *S*
? *s* ↓ + *s*

Thrum *Jack in the Green*		pin *Jack in the Green*		Thrum *wild type*		pin *wild type*	
Jig	*S*	*Jig*	*s*	+	*S*	+	*s*
?	*s*	*?*	*s*	+	*s*	+	*s*
2		2		1		2	

Table 5.1 . Origin of the two thrum *Jack in the Green* homozygotes (*Jig S/ Jig s*)

The two thrum *Jack in the Green* plants from above set seed from natural pollination. This resulted in 16 progeny that were classified in 1996; all of these were *Jack in the Green*, indicating homozygosity *Jig/Jig*. Further proof of homozygosity was obtained from a further sowing of naturally set seed that yielded 23 progeny from the first plant and 11 progeny from the second plant, all of which were again of phenotype *Jack in the Green.*

5.2.2. Subsequent crosses.

In order to ascertain whether pin homozygotes *Jig/Jig* were also viable, crosses of heterozygous pin *Jack in the Green* (*Jig/jig*) x homozygous thrum *Jack in the Green* (*Jig/Jig*) were carried out under controlled conditions. Progeny were raised and four pin *Jack in the Green* plants subsequently crosses to *wild type* to identify homozygotes. One pin homozygote was identified.

5.2.3. Summary of results.

From the above analysis it can be concluded that:

1. *Jack in the Green*, is a dominant phenotype.
2. No linkage was observed between *Jack in the Green* and the *Primula S locus.*
3. At least some *Jig/Jig* homozygotes are viable, but may be under represented in the population.

5.3. *Hose in Hose.*

As for *Jack in the Green*, the majority of crosses required for the genetic analysis of *Hose in Hose* were completed before commencing this project. Only crosses in section 5.3.8 were done during the period of this project.

5.3.1. Origin and history.

The first *Hose-in-Hose* plant was obtained from the late Mary Mottram, Devon, in 1985. It was a thrum plant and it was subsequently established that Hose in Hose is a dominant phenotype and that tight linkage existed between *Hose in Hose* and the *S* allele of the *Primula S* locus (Webster and Grant, 1990). Incompatibility between thrum x thrum crosses in these *Hose in Hose* plants was successfully broken down at this time so that thrum plants from these progeny could be successfully self pollinated.

From natural seed raised from a *Jack in the Green* plant growing close to the original *Hose in Hose*, plants with a number of phenotypes were raised in 1987/88. These included *Hose in Hose*, *Jack in the Green*, *wild type*, and *Jackanapes* (the latter express both the dominant phenotype of *Hose in Hose* together with the dominant phenotype of *Jack in the Green* and have calyces of both leaf and petal).

One of the *Hose in Hose* plants raised was the first recombinant between *Hih* and *S*, a pin *Hose in Hose*. This was crossed reciprocally to thrum *Hose in Hose* and the progeny classified in 1988. Results reported in 1990 indicated that *Hose in Hose* homozygotes were viable (Webster and Grant, 1990). Subsequently *Hose in Hose* homozygotes, both pin and thrum, were developed.

By hybridization and introgression *Hose in Hose* cowslips were raised. The phenotype can be expressed in a *P. veris* genetic background. This was expected as the first illustrations of *Hose in Hose* were of P. veris *Hose in Hose* (Chapter 1, 1.5.1) and it is

possible that the phenotype originated in *P. veris*. The phenotype can also be expressed in a *P. elatior* background as such cultivars are available commercially from some specialist nurseries.

5.3.2. Isolation of thrum homozygote (SS).

Thrum homozygotes have previously been reputed to be inviable in native British *Primula* (Richards 1986, 1993, 1997), although one was reported from a non British species *Primula sinensis* (Mather and de Winton 1941). The thrum homozygote described here is the first report of a thrum homozygote in a *Primula vulgaris* cultivar.

One of the thrum progeny from a cross of thrum *Hose in Hose* self-pollinated, set seed from natural pollination. The progeny from this seed was raised to test for homozygosity for *Hih* and classified in 1993/4 (Table 5.2).

Thrum *Hose in Hose* #7 x pin *wild type*.

$$\frac{Hih\ S}{?\ ?} \times \frac{?\ s}{?\ s}$$

Thrum *Hose in Hose*	pin *Hose in Hose*	Thrum *Jackanapes*	pin *wild type*
$\frac{Hih\ S}{?\ s}$	$\frac{Hih\ s}{?\ s}$	$\frac{Hih\ S\ +}{+\ s\ Jig}$	$\frac{+\ s}{+\ s}$
63	0	2	0

Table 5.2. Isolation of thrum homozygote (*HS/HS*) Cross no. 1.

Thrum *Jackanapes* (i.e. *Hose in Hose* and *Jack in the Green* are expressed in the same plant giving calyces of leaf and petal – result of natural pollination).

There were no pin progeny, indicating that the parent was a thrum homozygote. The parent thrum homozygote died so some of the progeny above were test crossed to *wild type* pin in order to look for another potential thrum homozygote. Results from one of the progeny, *Hose in Hose* Thrum #2 are classified in Table 5.3.

Thrum *Hose in Hose* #2 x pin *wild type*.

$$\frac{Hih\ \ S}{?\ \ ?} \times \downarrow \frac{+\ \ s}{+\ \ s}$$

Thrum *Hose in Hose*	pin *Hose in Hose*	Thrum *wild type*	pin *wild type*
$\frac{Hih\ \ S}{+\ \ s}$	$\frac{Hih\ \ s}{+\ \ s}$	$\frac{+\ \ s}{+\ \ S}$	$\frac{+\ \ s}{+\ \ S}$
24	0	0	0

Table 5.3. Isolation of thrum homozygote (*HS/HS*) Cross no. 2.

Results confirm that *Hose in Hose* thrum #2 is homozygous for both *Hih* and *S*. Pollen from *Hose in Hose* #2 was stained and examined under a light microscope. Comparison with wild type pin and wild type thrum pollen showed *Hose in Hose* #2 pollen to be of the same size as thrum *wild type* large pollen.

A population of thrum homozygotes was raised in spring 1998 by self pollination of *Hose in Hose* #2 and subsequent raising of progeny. One of the progeny was tested by raising naturally set seed which resulted in 32 thrum *Hose in Hose* progeny, confirming that this plant was homozygous for both *Hose in Hose* and for thrum. The majority of these thrum homozygotes were short lived.

These data provide evidence for the potential of breakdown in the self incompatibility system. Further evidence was observed in *wild type* thrum gold laced polyanthus. As these are "show plants", and only thrum plants are considered attractive, self-pollination has frequently been undertaken by breeders of show plants in order to obtain larger numbers of thrum progeny. Consequently the incompatibility of thrum x thrum crosses appears to have also been broken down in many lines of gold laced polyanthus.

A cross of self-pollination of a gold laced polyanthus was done in 1990. If thrum homozygotes are viable a 3:1 ratio of thrum to pin progeny would be expected.

A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio of 3:1.

observe 46:13; expect 44.25:14.75;

χ^2 for 3:1 (1 dof) = 0.277, P = 0.59.

A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio of 2:1.

observe 46:13; expect 39.32: 19.66;

χ^2 for 2:1 (1 dof) = 3.39, P = 0.066.

Results are closer to a 3:1 ratio, than to a 2:1 ratio indicating that there may be thrum homozygotes in this line of gold laced polyanthus.

This raises the question of whether self-pollination of thrum plants and the breakdown of thrum x thrum incompatibility could be associated with the loss of inviability of thrum homozygotes. It has been suggested in the past that the homozygous thrum *P. sinensis* may have arisen in response to selection for self-fertility in thrum *P. sinensis* in cultivation (Richards, 1993).

5.3.3. Study of expression of *Hose in Hose.*

It has been observed that expression of *Hose in Hose* is not always stable. A *Hose in Hose* plant raised in 1988 developed ramets with different degrees of expression of *Hih*, on the same plant. When sufficiently well grown the ramets were split to give three plants; one had fully petaloid calyces, one had calyces that were a mixture of petal and sepal material and one was as *wild type*. The *wild type* ramet did occasionally produce tiny petaloid extensions to one or two calyx lobes later in the season.

The three ramets were designated R #1, R #2 and R #3 respectively. Reciprocal crosses with *wild type* pin vulgaris were carried out in order to discover whether the loss of petal in the calyx was heritable and to observe and compare the degree of expression of *Hih* in the progeny from the individual ramets. Three sibling *wild type* vulgaris plants were used, one for each ramet. Results were obtained during 1991 and 1992 (Table 5.4).

Cross	Thrum *Hose in Hose*	pin *Hose in Hose*	Thrum *wild type*	Pin *wild type*
	Hih S / + *s*	*Hih s* / + *s*	+ *S* / + *s*	+ *s* / + *s*
R #1 x WT	9	3	0	7
WT x R #1	15	10	0	1
R #2 x WT	1	0	6	11
WT x R #2	8	0	2	9
R # 3 x WT	1	0	2	12
WT x R #3	2	0	8	7

Table 5.4. Reciprocal crosses of three ramets of one *Hose in Hose* plant to *wild type*.

The ramet with the fully petaloid calyces (R#1) produced more *Hose in Hose* plants than *wild type*, while ramets with poorer expression (R#2 and R#3) produced more *wild type* than *Hose in Hose* progeny. Results indicate that the plant from which R #1.2.and 3 were obtained was homozygous for *Hih* but so unstable that *Hih* was not expressed in many of the progeny (Table 5.7.). Since the plant was from a cross between two *Hose in Hose* parents homozygosity was possible. The instability that produced three ramets with different degrees of expression of *Hih*, the fact that expression became poorer with time in a number of the progeny (Table 5.6) and the larger proportion of wild type to Hose in Hose (Table 5.5) supports this view.

If reciprocal results are added together to give the total ratio of to *wild type* plants obtained from each ramet the results are as follows (Table 5.5).

Ramet	*Hose in Hose*	*wild type*
R #1	38	8
R #2	9	30
R #3	3	30

Table 5.5. Combined totals from Table 5.4.

The degree of expression observed on each of the first 40 *Hose in Hose* plants to bloom was evaluated as follows. Fully petaloid calyx = 1. More petal than sepal in calyx = 2. Evenly mixed petal/sepal calyx = 3. More sepal than petal in calyx = 4. Almost wild type calyx, only very tiny amounts of petal in calyx = 5. Results are shown in Table 5.6.

	Hose in Hose Thrum					*Hose in Hose* pin				
degree	1	2	3	4	5	1	2	3	4	5
R #1 x WT	0	2	0	0	2	0	0	1	0	1
WT x R #1	10	1	0	2	0	3	3	0	3	1
R #2 x WT	0	0	0	0	0	0	0	0	0	0
WT x R #2	3	2	2	0	2	0	0	0	0	0
R # 3 x WT	0	0	0	0	1	0	0	0	0	0
WT x R #3	0	0	0	0	1	0	0	0	0	0

Table 5.6. Evaluation of the degree of expression of the *Hose in Hose* phenotype.

Ten of the above plants were observed over several weeks. In most cases the expression of *Hih* became poorer over this period. Five plants placed in expression category 1 now fell in category 4; one placed in expression category 1 now fell in category 2 and one plant placed in expression category 2 now fell in expression category 3. In three plants the expression category improved; from 5 to 3 in one instance, from 3 to 2 in another and from 2 to 1 in the last instance.

Results also show that more plants of phenotype *Hih S* were obtained than of phenotype *Hih s*, and that more of these were obtained from wild type seed parents crossed with

Hose in Hose pollen than from *Hose in Hose* seed parents. The latter suggests that in these plants some genes influencing expression may be carried in the cytoplasm. The former suggest that in this line *Hih* is better expressed in thrum than in pin plants, possibly in part due to *Hih S* on one chromosome (if the unstable plant was a homozygote), having come from a more stable line of *Hose in Hose* plants. It would have been better if three ramets of the same *wild type* plant had been available, as the sibling *wild type* plants used may have had variations in the genetic background that influenced expression of *Hih*. Should such an unstable plant with different degrees of expression of *Hih* on different ramets occur again, the experiment to test the hypothesis that reversion from *Hose in Hose* towards *wild type* is permanent and heritable, could be repeated using individual ramets of one *wild type* plant.

5.3.4. Testing of other lines of *Hose in Hose* plants.

Testing of other lines of *Hose in Hose* plants was done to reveal whether or not different lines might have different characteristics and therefore may be different alleles with different origins, or alternatively whether all *Hose in Hose* may have a common origin. If all are from a single origin testing will reveal linkage of other lines to the *S locus*.

(a) Barnhaven *Hose in Hose*.

Barnhaven is a seed company specializing in primrose and polyanthus seed.

A thrum *Hose in Hose* plant of Barnhaven origin was test crossed to pin *wild type* in 1990 (Table 5.7). No reciprocal cross was done.

Thrum Barnhaven *Hose in Hose* x pin *wild type*.

Hih ? x *+ s*

? ? ↓ *+ s*

Thrum *Hose in Hose*	pin *Hose in Hose*	Thrum *wild type*	pin *wild type*
Hih S / *+ s*	*Hih s* / *+ s*	*+ s* / *+ S*	*+ s* / *+ s*
0	18	18	0

Table 5.7. Test cross of Barnhaven *Hose in Hose*.

Results give a perfect fit of 1: 1 for linkage of *Hih* to the *s* allele of the *S* locus in this plant. It was observed that there was very poor expression of *Hih* in all of the progeny indicating greater instability in this line. Linkage to the *S* locus indicates that the origin for *Hose in Hose* is likely to be the same for this line.

(b) "Wanda", *hose in hose*.

The cultivar "Wanda" was derived from crosses between *P. vulgaris* and *P. juliae*. *P. juliae* was discovered in the Caucasus in 1900 and was introduced to Britain in1911. The genetic background of "Wanda" *hose in hose* might therefore be expected to be different. Wanda *hose in hose* could not be used as a seed parent because of abnormalities in the fourth whorl, which was a chimeric organ of stamen and ovary tissue. It was used as pollen parent to *Wild Type P. vulgaris* and results obtained in 1991 (Table 5.8).

Thrum *Wild Type* x Wanda *hose in hose*

\+ *S* x *hih s*

\+ *s* ↓ *? s*

Thrum *hose in hose*	pin *hose in hose*	Thrum *Wild type*	pin *Wild type*
hih s \+ *S*	*hih s* \+ *s*	\+ *S* \+ *s*	\+ *s* \+ *s*
0	0	11	9

Table 5.8. Inheritance of Wanda *hose in hose* (1).

No *hose in hose* progeny were obtained, indicating that *hose in hose* is not a dominant phenotype in this cultivar, or is not expressed in this new genetic background.

Two of the progeny were crossed together pin x thrum to discover if *hose in hose* was recessive in the "Wanda" cultivars. No *hose in hose* progeny resulted from the cross, but only five progeny were obtained. Time and space did not allow for testing all of the progeny at that time and they were subsequently lost.

In 1994 a cross of Wanda, *Wild Type* x Wanda *hose in hose* was made and results obtained in 1995 (Table 5.9).

Thrum Wanda *Wild Type* x pin Wanda *hose in hose.*

+	*S*	x	*hih*	*s*	
+	*s*	↓	?	s	

Thrum *hose in hose*	pin *hose in hose*	Thrum *Wild type*	pin *Wild type*
hih *s* + *s*	*hih* *s* + *s*	+ *S* + *s*	+ *s* + *s*
0	0	14	10

Table 5.9. Inheritance of Wanda *hose in hose* (2)

Again no *hose in hose* progeny were obtained reinforcing previous results that indicate *hose in hose* is not a dominant phenotype in the cultivar "Wanda" *hose in hose.* If Wanda *hose in hose* was linked to the *s* allele of the *S locus* but was inviable due to the effects of other genes in the genetic background then a deficiency of pin W*ild Type* might occur. A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio of 1:1 for pin : thrum *Wild Type*. The χ^2 for a ratio of 1: 1 for pin : thrum *Wild Type* is 0.67 (1dof) P = 0.4. The results are consistent with 1:1 segregation. There is no deficiency of pin W*ild Type.*

It was not possible to carry out crosses between the 10 pin plants above and 10 of the thrums (in various combinations) to ascertain whether Wanda *hose in hose* was carried as a recessive and expressed in any of the progeny since facilities for raising such a large number of progeny were not available.

Experiments with Wanda *hose in hose* were discontinued until 1999, when whorl four of one plant reverted to normal and it became a fertile seed parent. This was self-pollinated and seed obtained. All but one of these seeds failed to germinate, and this one had *Wild Type* Wanda flowers. The following year the plant was not fertile. It is possible that *hose in hose* in the "Wanda" cultivar may be the result of a different mutation from that in *P. vulgaris*, but further testing is required.

5.3.5. Summary of previous results.

From the above analysis it can be concluded that:

1. *Hose in Hose* is a dominant phenotype in *P. vulgaris*, *P. veris* and their cultivars.
2. *Hose in Hose* is tightly linked to the *S* allele of the *S* locus.
3. Thrum incompatibility was successfully broken down in this line of *Hose in Hose* in 1986 so that thrum x thrum crosses are now possible.
4. *Hose in Hose* homozygotes are viable.
5. Expression can be variable.
6. The occurrence of a thrum homozygote among the progeny of a *Hose in Hose* plant suggests that it is feasible to obtain homozygous (SS) plants.
7. *Hose in Hose* from the Barnhaven range exhibited linkage to the *s* allele of the *Primula S* locus.
8. "Wanda" *hose in hose*, derived from *P. juliae*, is not dominant and does not appear to be heritable but heritability is not fully tested. It is possible that the phenotype may have a different origin in this cultivar.

5.3.6. Subsequent crosses.

Subsequent crosses were carried out to test for presence or absence of an *S* locus linked lethal factor in thrum *Hose in Hose*. The presence of such a factor linked to the *S* allele of the *Primula S* locus has been suggested previously (Richards 1986, 1993, 1997), but occurrence of one thrum homozygote among the progeny of a *Hose in Hose* plant suggests viability of homozygous (SS) plants in this line of *Hose in Hose*.

5.3.7. Testing for presence or absence of an *S* locus linked lethal factor in thrum *Hose in Hose*.

Further investigation of thrum *Hose in Hose* #2, the homozygote *Hih S/Hih S*, (see 5.3.2.above) was undertaken. If the predicted lethal factor (Richards 1997), in coupling with the *S* allele, had recombined so that it was now in coupling with the *s* allele, this would be unlikely to have occurred more than once. Therefore it would seem likely that only one of the two *S* alleles in the homozygous thrum would have lost this lethal factor. To investigate whether any of the progeny from the cross of *wild type* (+ s / + s) x *Hose in Hose* thrum #2 (*Hih S /Hlh* S), that will have genotype of *Hih S/+ s*, (see 5.3.2 above) had lost the lethal factor the following cross was carried out.

Two surviving plants from the cross of *wild type* (+ s / + s) x *Hose in Hose* thrum #2 (*Hih S /Hlh* S),with genotype of *Hih S /+ s*, (see 5.3.2 above) were each self-pollinated and progeny raised from the two duplicate self pollinations (Table 5.10).

Thrum *Hose in Hose* heterozygotes self pollinated.

Hih S / + *s* × ↓ *Hih S* / + *s*

Plant no.	Thrum *Hose in Hose*	pin *Hose in Hose*	pin *wild type*	Thrum *wild type*
	Hih S / ? *s*	*Hih s* / + *s*	+ *s* / + *s*	+ *s* / + *S*
1	31	0	2	0
2	27	2	2	0

Table 5.10. First test for presence or absence of *S* locus linked lethal factor in thrum *Hose in Hose*.

If the *S* allele no longer was in coupling with the lethal factor a 3:1 ratio of thrum *Hose in Hose* to pin *wild type* could be expected. If the S allele remained in coupling with a lethal factor a 2:1 ratio could be expected.

If both parents are *Hih* heterozygotes and if one or both are lacking an *S* locus linked lethal factor expect a 3:1 ratio of *Hih* : *wild type*. A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio of 3:1.

Plant no. 1. χ^2 for 3:1 for *Hose in Hose* thrum to *wild type* pin,

observe 31:2; expect 24.75: 8.25;

χ^2 (1 dof) 6.3, P = 0.012

Results are not consistent with a 3:1 ratio for *Hose in Hose* thrum to *wild type* pin.

Plant no. 2. χ^2 for 3:1 for *Hose in Hose* thrum to *wild type* pin

observe 29: 2; expect 23.25 :7.75

χ^2 (1 dof) = 5.69 P = 0.017

Results are not consistent with a 3:1 ratio for *Hose in Hose* thrum to *wild type* pin.

As results are not consistent with the 3.1 ratio for *Hose in Hose* thrum to *wild type* pin (expected if a lethal factor is associated with the S allele) a χ^2 analysis was done to test

the null hypothesis that there is no significant difference of the data from a predicted 2:1.ratio

Plant no. 1. χ^2 for 2:1 for *Hose in Hose* thrum to *wild type* pin,

observe 31:2; expect 22:11;

χ^2 (1 dof) 11.04, P = 0.0009

Results are extremely deviant from a 2:1 ratio of *Hose in Hose* Thrum to *wild type* pin.

Plant no. 2. χ^2 for 2:1 for *Hose in Hose* thrum to *wild type* pin

observe 29: 2; expect 20.66:10.33;

χ^2 (1 dof) = 10.08 P = 0.0015

Results are extremely deviant from a 2:1 ratio of *Hose in Hose* thrum to *wild type* pin

The results for 3:1 (expected if a lethal factor is no longer associated with the S allele), fits the predicted ratio better than does 2:1 for both plant 1 and plant 2 although both are deviant from the expected ratio.. Both self pollinations resulted in a marked deficit of *wild type* pin plants that cannot currently be explained.

Two plants from the cross pin Hose in Hose (*Hih s/Hih s*) x Hose in Hose #2 (*Hih S/Hih S*) were also self-pollinated and progeny from the duplicate self-pollinations raised (Table 5.11).

Thrum *Hose in Hose* homozygote self pollinated.

Hih S × *Hih S*

Hih s ↓ *Hih s*

Plant no.	Thrum *Hose in Hose*	pin *Hose in Hose*
	Hih S / *Hih ?*	*Hih s* / *Hih s*
1	12	6
2	19	14

Table 5.11. Second test for presence or absence of *S* locus linked lethal factor in thrum *Hose in Hose*.

If both parents are heterozygous for *Hih* and if both lack an *S* locus linked lethal factor expect a 3:1 ratio of *Hose in Hose* thrum to *Hose in Hose* pin. If either of both plants have an *S locus* linked lethal factor a 2:1 ratio of *Hose in Hose* thrum to *Hose in Hose* pin

can be expected. Plant no.1 gave an exact 2:1 ratio for *Hose in Hose* thrum to *Hose in Hose* pin as expected if a lethal factor is in coupling with the *S* allele of the *Primula S* locus. A χ^2 analysis could not be done to test the null hypothesis that there is no significant difference of the data from the predicted ratio of 3:1 for plant no.1 since one of the expected numbers would be less than 5; a χ^2 analysis would not therefore be valid.

A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio of 2:1 for plant no.2.

χ^2 for 2:1 *Hose in Hose* thrum to *Hose in Hose* pin for plant no 2

observe 19:14, expect 22:11;

χ^2 (1 dof) = 1.227, P = 0.267

Results fit the predicted 2:1 ratio.

A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio of 3:1.

χ^2 for 3:1 *Hose in Hose* thrum to *Hose in Hose* pin for plant no 2

observe 19:14, expect 24.75:8.25;

χ^2 (1 dof) =5.34, P = 0.02

Results are slightly deviant from the predicted 3:1 ratio.

It is therefore possible that plant no.1 may lack the lethal factor but plant no.2 is likely to have the *S* locus linked lethal factor. Test crossing of progeny to *wild type* will identify which plants are thrum homozygotes.

5.3.8. Summary of results.

From the above analysis it can be concluded that some *Hose in Hose* plants appear to have lost the *S* locus linked lethal factor and *SS* homozygotes appear to be viable. These can be identified by test crossing to *wild type*.

5.3. Split Perianth.

Both the calyx and the corolla of the original gold laced polyanthus plant discovered by Dr. R. Brumpton in his nursery in Nottingham in 1997 were deeply divided (see Chapter 4, 4.3.3 and Fig. 4.6), the latter to the point of attachment of the anthers. Investigation of the inheritance of the phenotype had begun before this project commenced, but the majority of the work has been done during the project.

5.4.1. Origin and history.

The phenotype had been described and illustrated in old literature but had not been seen for many years. Splitting of the calyx remained stable in each plant, but splitting of the corolla varied considerably even on a single plant. From his own crosses Dr. Brumpton had established by breeding tests that *Split Perianth* (genotype designated *Spr*) is a dominant phenotype that can be expressed with the *Hose in Hose* and *Jack in the Green* phenotypes and also as a triple mutant (*Hih Jig Spr).* A piece of the original *Split Perianth* plant was obtained in spring 1998, and from crosses of this pin plant to *wild type* thrum *Split Perianth* progeny of both pin and thrum phenotypes were raised.

5.4.2. Subsequent crosses.

Subsequent crosses were undertaken first to look for any linkage of *Spr* to the *S* locus and second to investigate variable expression of the phenotype.

5.4.3. Testing for linkage of *Spr* to the *S* locus.

In order to test whether there was any linkage of *Spr* to the *S locus* a cross of thrum *Split Perianth* to pin *wild type* was carried out. This cross will also test for dominance. Results are tabulated in Table 5.12.

Thrum Split Perianth x pin *wild type.*

$+\ s \;/\; +\ s$ x ↓ $Spr\ S \;/\; +\ s$

Thrum *Split Perianth*	pin *Split Perianth*	Thrum *wild type*	pin *wild type*
Spr S / + *s*	*Spr s* / + *s*	+ *S* / + *s*	+ *s* / + *s*
14	16	13	20

Table 5.12. Test for linkage of *Spr* to the *S* locus.

If *Split Perianth* is a dominant phenotype and if there is absence of any linkage of *Spr* to the *S locus* expect equal numbers of all phenotypes. A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio.

χ^2 for 1:1:1:1 for equal numbers of all phenotypes

observe 14 : 16 : 13 : 20, expect 15.75 : 15.75 : 15.75 : 15.75.

χ^2 (3 dof) = 1.825. P = 0.6.

Results fit the predicted 1:1:1:1 ratio. There is no linkage of *Spr* to the *S* locus. Results also confirm dominance of *Split Perianth* to *wild type*.

The *Split Perianth* plant used was one with a split calyx but with a normal corolla. The *Split Perianth* progeny resembled the parent in that in all but a few plants the calyx but not the corolla was split.

5.4.4. Investigation of variable expression of the *Split Perianth* phenotype.

Observation of other *Split Perianth* plants subsequently obtained from Dr. Brumpton showed that both plants with split calyx and corolla completely split to the base of the flower, and plants with the calyx split but with a normal corolla were stable in form. It seemed likely that the splitting of the corolla and the splitting of the calyx were caused by degrees of expression of the same dominant allele. Further investigation was undertaken by self and test crossing the most extreme phenotype that had both the calyx and the corolla split to the base. Results were classified in 2002 and are given in Table 5.13 (self-pollinated), and Table 5.14 (test crossed to *wild type*). Self pollination also tests for viability of *Spr/Spr* homozygotes.

Pin *Split Perianth* self pollinated

Spr s x *Spr s*

\+ s ↓ + s

Split Perianth	*wild type*
Spr s \+ *s*	\+ *s* \+ *s*
15	4

Table 5.13. Self-pollination of the most extreme form of *Split Perianth*.

If homozygous *Split Perianth* plants are viable expect a ratio of 3 : 1 for *Split Perianth* : *wild type*. A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio of 3:1.

χ^2 for 3 : 1 for *Split Perianth* : *wild type*.

observe 15 : 4, expect 14.25 : 4.75.

χ^2 (1 dof) = 0.35. P = 0.55

Results fit the expected ratio of 3:1. indicating viability of *Split Perianth* homozygotes.

Continued observation of above progeny for some weeks showed the majority of the *Split Perianth* progeny to exhibit complete splitting of the corolla. Exceptions were one that had a corolla split only to the point of attachment of the anthers and one that initially had no corolla at all, but had anthers on individual filaments initiating from the base of the ovary. Later flowers had one petal per flower, remaining anthers being on filaments as above. Further observation of *wild type* progeny found that two of the *wild type* exhibited splitting of the corolla only in flowers produced later. These latter were crossed to *wild type* but all progeny were normal. Occasionally splitting of the corolla can be seen in normal plants flowering out of season and it is probably environmental in origin. It is therefore likely that the splitting of the corolla in two of the *wild type* progeny was of environmental origin.

As no linkage was observed between *Spr* and the *S* locus (Table 5.14) expect a ratio of 1:1 *Split Perianth* : *wild type* and equal numbers of all phenotypes. A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio of 1:1.

χ^2 for 1:1 *Split Perianth* : *wild type*

observe 13 : 12, expect 12.5 : 12.5.

χ^2 (1 dof) = 0.04 P = 0.84.

Results fit the predicted ratio for 1:1

A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio of 1:1:1:1 for equal numbers of all phenotypes.

χ^2 for 1:1:1:1 for equal numbers of all phenotypes

observe 7: 6:5:7, expect 6.25: 6.25 : 6.25 : 6.25,

χ^2 (3 dof) = 0.44 P = 0.93

Results fit the predicted ratio of 1:1:1:1.

Split Perianth x *wild type*

Spr s x + S
\+ s ↓ + s

Thrum Split Perianth	pin Split Perianth	Thrum wild type	pin wild type
Spr ? \+ *?*	*Spr s* \+ *s*	\+ *S* \+ *s*	\+ *s* \+ *s*
7	6	5	7

Table 5.14. The most extreme form of *Split Perianth* x *wild type*.

Observation of flowering over a number of weeks showed the *Split Perianth* progeny of the most extreme form of *Split Perianth* x *wild type* to exhibit splitting of the corolla only to the point of attachment of the anthers, with 3 plants also having an occasional flower with a single split to the base of one side of the corolla tube. All *wild type* plants had normal corollas. It is possible that expression of the phenotype may be affected by modifier genes or associations of modifier genes so that when *Split Perianth* is crossed with *wild type* alleles recombination may cause the modifier genes to become disassociated from a position of influence. Conversely, inbreeding the phenotype may enhance the effect of such modifier genes as the alleles of influence will be carried by both parents.

5.4.5. Summary of results.

From the above analysis it can be concluded that:

1. *Split Perianth* is a dominant phenotype.
2. There is no linkage of *Split Perianth* to the *S* locus.
3. Variability of expression was observed. More extreme phenotypes with totally split corollas appeared with inbreeding, and less extreme forms with normal corollas appeared through outbreeding. This may be due to modifier genes in the genetic background.

5.5. *Staminoid Carpels.*

Staminoid Carpels plants, in addition to having petaloid sepals have a fourth whorl that exhibits various degrees of conversion to stamens in different *Staminoid Carpels* plants (see Chapter 4, 4.3.6). Some investigation into the inheritance of the phenotype was undertaken before commencement of this project.

5.5.1. Origin and history.

One plant, reputed to be a very old clone of a thrum *Hose in Hose*, was obtained from Mrs. P. Gossage, Crewkerne, Somerset, in 1989. It was female infertile because of the mutant whorl 4 phenotype; male fertility was normal. The original phenotype had a normal stigma and short style above an elongated ovary with petaloid tissue in the ovary wall and an occasional anther inside the upper portion of the ovary wall. The form has been phenocopied in Tobacco following ectopic expression of the *Antirrhinium* B function genes *Deficiens* and *Globosa* (Davies et al 1996). One indication that it may be an extreme allele of *Hose in Hose* was the appearance of one plant with a whorl 4 mutant phenotype from the reciprocal cross between a pin *Hose in Hose* and a thrum *Hose in Hose*. (See 5.3.1.above) Each of these parent plants was unrelated to the *Staminoid Carpels* plant obtained from Mrs. Gossage. One of the 29 thrum *Hose in Hose* progeny had a *Staminoid Carpels* whorl 4 phenotype, but was lost before being used in any crosses. The original *Staminoid Carpels-Hose in Hose* (genotype designated as Hih*) from Mrs. Gossage was used as a pollen parent to pin *wild type Primula vulgaris* in 1990 and progeny were classified in 1992 (Table 5.15).

pin *wild type* x Thrum *Staminoid Carpels Hose in Hose*.

$\frac{+\quad s}{+\quad s}$ x ↓ $\frac{Hih^*\ S}{?\quad s}$

Thrum *Staminoid Carpels-Hose in Hose*	Pin *Hose in Hose*	Thrum *wild type*	pin *wild type*
$\frac{Hih^*\ S}{+\quad s}$	$\frac{Hih\quad s}{+\quad s}$	$\frac{+\quad S}{+\quad s}$	$\frac{+\quad s}{+\quad s}$
9	0	0	11

Table 5.15. Test cross of thrum *Staminoid Carpels*.

If *Staminoid Carpels-Hose in Hose* is heterozygous for Hih*, with mutant phenotypes in both whorls 1 and 4 segregating with *S* expect a for 1:1 ratio for *Staminoid Carpels-Hose in Hose* to *wild type*. A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio of 1:1.

χ^2 for 1:1 for *Staminoid Carpels-Hose in Hose* to *wild type*

observe 9 : 11, expect 10 : 10.

χ^2 (1 dof) = 0.2, P = 0.65

Results are consistent with the expected 1:1 ratio.

Isolation for the majority of crosses was by use of a net "tent" enclosing the seed parent. Isolation for the following two crosses was by the removal of whorls two and three (petals and anthers) before anther maturity – thus rendering the flower unattractive to insect pollinators. This method is the one used by commercial breeders, but cannot be 100% reliable as an isolation technique unless insects can be effectively excluded from the greenhouse.

5.5.2. More extreme forms of *Staminoid Carpels.*

In 1992 a pin *Hose in Hose* was pollinated by a thrum *Staminoid Carpels-Hose in Hose.* Progeny were reared and classified in 1994/95 (Table 5.16).

pin *Hose in Hose* x Thrum *Staminoid Carpels Hose in Hose.*

Hih s / *? s* x ↓ *Hih* S* / *+ s*

Thrum *Staminoid Carpels-Hose in Hose*	Thrum *Hose in Hose*	pin *Hose in Hose*	pin *wild type*
Hih S* / *+ s*	*Hih S* / *+ s*	*Hih s* / *+ s*	*+ s* / *+ s*
4	3	2	0

Table 5.16. Origin of a more extreme from of *Staminoid Carpels.*

One of the above *Staminoid Carpels-Hose in Hose* was recorded as being a more extreme whorl 4 mutant phenotype in that there was no ovary wall but instead stamens were in whorl 4 on an elongated axis, enclosing naked ovules. This was designated *Staminoid Carpels-Hose in Hose* # 2. Results indicate that the genotype of the female parent must be *Hih s / Hih s.* The 3 thrum *Hose in Hose* raise the question of whether the isolation technique was effective. The more extreme form was used to pollinate a heterozygous pin *Hose in Hose* in 1995 and the progeny classified in 1996/97 (Table 5.17).

pin *Hose in Hose* x Thrum *Staminoid Carpels Hose in Hose* #2

$\frac{Hih\ \ s}{+\ \ s}$ x ↓ $\frac{Hih^*\ S}{?\ \ s}$

Thrum *Staminoid Carpels-Hose in Hose*	Thrum *Hose in Hose*	pin *Hose in Hose*	pin *wild type*
$\frac{Hih^*\ S}{+\ \ s}$	$\frac{Hih\ S}{+\ \ s}$	$\frac{Hih\ \ s}{+\ \ s}$	$\frac{+\ \ s}{+\ \ s}$
2	2	4	0

Table 5.17. Origin of the most extreme form of *Staminoid Carpels*.

One of the *Staminoid Carpels-Hose in Hose* plants from the above progeny had whorl 4 completely converted to anthers (plant # SUPERMAN, because of having no female parts)! Results indicate that the genotype of *Staminoid Carpels-Hose in Hose* # 2 must be *Hih* S / Hih s.* Again the presence of thrum *Hose in Hose* raises a question regarding the efficacy of the isolation technique.

5.5.3. Subsequent crosses.

For subsequent crosses seed parents were maintained in an isolation unit with a hinged lid and covered with insect proof material, until seed set had begun. This ensured complete isolation and tested whether the thrum *Hose in Hose* obtained in previous crosses resulted from failed isolation. Subsequent crosses observed variable expression of the phenotype, investigated the segregation of *Hih** with *S* and *Hih* with *s*, viability of whorl 4 pollen, and investigated the inheritance of a new pin *Staminoid Carpels* plant.

5.5.4. Observations of variable expression of *Staminoid Carpels*.

In 1998 pollen from the original *Staminoid Carpels-Hose in Hose* of the least extreme form was used on a homozygous pin *Hose in Hose*. Progeny were classified in 1999/2000 (Table 5.18). Results both allowed observation of variable expression of the phenotype in the progeny, and tested the efficacy of the new isolation unit.

pin *Hose in Hose* homozygote x original *Staminoid Carpels Hose in Hose.*

$$\frac{Hih\ \ s}{Hih\ \ s} \quad x \downarrow \quad \frac{Hih^{*}\ S}{+\ \ s}$$

Thrum *Staminoid Carpels-Hose in Hose*	pin *Hose in Hose*	Thrum *wild type*	pin *wild type*
$\frac{Hih^{*}\ S}{+\ \ s}$	$\frac{Hih\ \ s}{+\ \ s}$	$\frac{+\ \ S}{+\ \ s}$	$\frac{+\ \ s}{+\ \ s}$
10	5	0	0

Table 5.18. Cross to observe expression of *Staminoid Carpels.*

As the female parent was a homozygous pin *Hose in Hose* a ratio of 1:1 for thrum *Staminoid Carpels-Hose in Hose* : pin *Hose in Hose* would be expected. A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio of 1:1.

χ^2 for 1:1 for thrum *Staminoid Carpels-Hose in Hose* to pin *Hose in Hose*

observe 10 : 5, expect 7.5 : 7.5

χ^2 (1 dof) = 1.667, P = 0.196

Results fit the1:1 ratio of thrum *Staminoid Carpels-Hose in Hose* to pin *Hose in Hose.*

Half of the *Staminoid Carpels-Hose in Hose* plants were as the original form with an elongated ovary and half as *Staminoid Carpels-Hose in Hose* # 2 with the ovary wall converted to anthers enclosing naked ovules. Other genes within the genetic background may modify the degree of expression

5.5.5. Investigation of segregation of *Hih** with *S* and *Hih* with *s.*

In order to observe segregation of *Hih** with *S* and *Hih* with *s* one of the thrum *Staminoid Carpels-Hose in Hose* progeny and one of the pin *Hose in Hose* progeny were crossed to pin *wild type.* Results were classified in 2003 and are shown in Table 5.19 and Table 5.20 respectively.

If *Staminoid Carpels-Hose in Hose* segregates with *S* expect a ratio of 1:1 thrum *Staminoid Carpels-Hose in Hose* : pin *wild type*. A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio of 1:1.

pin *wild type* x Thrum *Staminoid Carpels-Hose in Hose*.

$\frac{+\ \ s}{+\ \ s}$ x $\frac{Hih\ \ s}{?\ \ s}$ ↓

Thrum *Staminoid Carpels-Hose in Hose*	pin *Hose in Hose*	Thrum *wild type*	pin *wild type*
$\frac{Hih^*\ S}{+\ \ s}$	$\frac{Hih\ \ s}{+\ \ s}$	$\frac{+\ \ S}{+\ \ s}$	$\frac{+\ \ s}{+\ \ s}$
18	0	0	27

Table 5.19. Testing of segregation of *Hih with *S*.**

χ^2 for 1:1 thrum *Staminoid Carpels-Hose in Hose* : pin *wild type*

observe 18 : 27, expect 22.5 : 22.5.

χ^2 (1 dof) = 1.8, P = 0.179.

Results fit the 1:1 ratio for thrum *Staminoid Carpels-Hose in Hose* : pin *wild type*.

Results confirm segregation of *Staminoid Carpels-Hose in Hose* with *S*

pin *wild type* x pin *Hose in Hose*.

$\frac{+\ \ s}{+\ \ s}$ x $\frac{Hih\ \ s}{?\ \ s}$ ↓

Thrum *Staminoid Carpels-Hose in Hose*	pin *Hose in Hose*	Thrum *wild type*	pin *wild type*
$\frac{Hih^*\ S}{+\ \ s}$	$\frac{Hih\ \ s}{+\ \ s}$	$\frac{+\ \ S}{+\ \ s}$	$\frac{+\ \ s}{+\ \ s}$
0	13	0	24

Table 5.20. Testing of segregation of *Hih* with *s*.

If *Hih* segregates with *s* expect a ratio of 1:1 pin *Hose in Hose* : pin *wild type*. A. χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio of 1:1.

χ^2 for 1:1 pin *Hose in Hose* : pin *wild type*

observe 13 : 24, expect 18.5 : 18.5.

χ^2 (1 dof) = 3.27, P = 0.07.

Results fit the1:1 ratio of pin *Hose in Hose*: pin *wild type*.

All pin *Hose in Hose* progeny (Table 5.32) had normal carpels.

5.5.6. Investigation of viability of whorl 4 pollen.

In order to evaluate the viability of whorl 4 pollen, pollen from the whorl 4 anthers of *Staminoid Carpels-Hose in Hose* # SUPERMAN was used to pollinate a *wild type* plant. Seed was set and five progeny flowered in 2003. All were *Hose in Hose*, indicating homozygosity (*Hih/Hih*), and three were *Staminoid Carpels-Hose in Hose* confirming viability of whorl 4 pollen.

5.5.7. Investigation of the inheritance of pin *Staminoid Carpels Hose in Hose.*

Until 2001 all *Staminoid Carpels-Hose in Hose* were thrum. At that time one pin *Staminoid Carpels-Hose in Hose* (Fig. 4.10, D) occurred in a flowerbed in the garden. Its origin is unknown. To investigate the inheritance of the new pin *Staminoid Carpels-Hose in Hose* two crosses were carried out. First pin *Staminoid Carpels-Hose in Hose* was crossed to thrum *wild type*. Progeny were classified in 2003 (Table 5.21).

Thrum *wild type* x pin *Staminoid Carpels Hose in Hose*.

$\frac{+\quad S}{+\quad s}$ x ↓ $\frac{Hih^*\ s}{?\quad s}$

Thrum *Staminoid Carpels-Hose in Hose*	pin *Staminoid Carpels-Hose in Hose*	Thrum *wild type*	pin *wild type*
$\frac{Hih^*\ S}{+\quad s}$	$\frac{Hih^*\ s}{+\quad s}$	$\frac{+\quad S}{+\quad s}$	$\frac{+\quad s}{+\quad s}$
6	4	5	8

Table 5.21. Inheritance of pin *Staminoid Carpels Hose in Hose* 1.

Equal numbers of all four phenotypes are predicted with the thrum *Staminoid Carpels-Hose in Hose* progeny having genotype of $\frac{Hih^*\ s}{+\ S}$. A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio.

χ^2 for 1:1:1:1 for all four phenotypes

observe 6:4:5:8, expect 5.75:5.75:5.75:5.75.

χ^2 (3 dof) = 1.52, P = 0.67

Results fit the prediction of 1:1:1:1 for all four phenotypes.

Observation of the progeny above showed that at first flowering all of the thrum *Staminoid Carpels-Hose in Hose* had stigmas at the mouth of the corolla tube, and all pin *Staminoid Carpels-Hose in Hose* had stigmas lower down. Later pin *Staminoid Carpels-Hose in Hose* flowers had longer styles and stigmas higher up the corolla tube. Elongation of the ovary wall however remained longer in the thrum *Staminoid Carpels-Hose in Hose* than in the pin *Staminoid Carpels-Hose in Hose* progeny (see Chapter 4, Figure 4.11, E – H).

Thrum *Staminoid Carpels-Hose in Hose* progeny were crossed to *wild type* pin to confirm segregation of the new *Staminoid Carpels-Hose in Hose* allele with pin. Results are classified in Table 5.22.

pin *wild type* x Thrum *Staminoid Carpels Hose in Hose*.

$\frac{+\ \ s}{+\ \ s}$ x ↓ $\frac{Hih^*\ \ s}{+\ \ S}$

pin *Staminoid Carpels-Hose in Hose*	Thrum *wild type*	Thrum *Staminoid Carpels-Hose in Hose*	pin *wild type*
$\frac{Hih^*\ \ s}{+\ \ s}$	$\frac{+\ \ S}{+\ \ s}$	$\frac{Hih^*\ \ S}{+\ \ s}$	$\frac{+\ \ s}{+\ \ s}$
13	5	1	0

Table 5.22. Inheritance of pin *Staminoid Carpels Hose in Hose* 2.

Results in table 5.22 above are as recorded in May 2004, 32 progeny did not flower. These initial results do show the new *Staminoid Carpels-Hose in Hose* allele segregating with pin. One recombinant, a thrum *Staminoid Carpels-Hose in Hose*, shows that in recombination *Staminoid Carpels* also segregates with *Hose in Hose*. Each of the pin *Staminoid Carpels-Hose in Hose* flowers had an elongated ovary wall that did not reach anther level mid way up the corolla tube.

5.5.8. Testing expression of homozygous *Hose in Hose-Staminoid Carpels.*

In order to test whether homozygosity for *Hose in Hose* affected expression of *Staminoid Carpels*, two further crosses were undertaken. It had already been established that the most extreme form of *Staminoid Carpels* # SUPERMAN was also homozygous for *Hose in Hose* (5.5.6 above, also other results not shown). To do this a pin *Staminoid Carpels* (*Hih* s / hih s*) was crossed to two *Hose in Hose* plants of different genotypes, one that would result in all of the pin *Staminoid Carpels* being homozygous for *Hose in Hose*, and the other that would result in all of the thrum *Staminoid Carpels* being homozygous for *Hose in Hose*. Results indicate that it is not homozygosity for *Hih* that is responsible for the more extreme phenotypes. Some of the progeny from these crosses can be seen in Chapter 4. Both of the pin *Staminoid Carpels-Hose in Hose* homozygous for *Hose in Hose* (Fig. 4.11, I and J) exhibit the same form of expression as the heterozygotes (Fig. 4.11, D-F). The thrum *Staminoid Carpels-Hose in Hose* (Fig. 4.11, L) is not as extreme in expression as the homozygote shown in Fig. 4.11C, but is only slightly more staminoid than the heterozygotes (Fig. 4.11, G and H). Sibling plants of pin and thrum *Staminoid Carpels* continued to show marked differences in elevation of the staminoid carpels in whorl four (Fig.4.11K and L). This demonstrates that the *A* gene of the *S locus* governing anther height is not whorl specific but organ specific.

There were no unexpected phenotypes among any of the progeny when using the new isolation unit. Removal of the perianth organs alone, as commonly used by commercial breeders is demonstrably not a reliable form of isolation unless insects can be effectively excluded from the greenhouse by some other means

5.5.9. Summary of results.

From the above analysis it can be concluded that:

1. *Staminoid Carpels* is a dominant phenotype that originally segregated with both *Hose in Hose* and the *S* allele of the *Primula S* locus.
2. Whorl 4 pollen is viable.
3. One pin *Staminoid Carpels* occurred in 2001, a possible recombinant, it has *Staminoid Carpels* linked to both *Hose in Hose* and the *s* allele of the *Primula S* locus.
4. Thrum progeny from thrum *wild type* x pin *Staminoid Carpels* exhibited a more elongated ovary wall than their pin siblings. This demonstrates that the action of the *A* gene of the *S* locus governing anther height is organ specific rather than whorl specific.

5.6. *sepaloid.*

The *sepaloid* flower never produces any petals or stamens; it may consists of 4 whorls of sepals, or 2 or 3 whorls of sepals and a functional or non functional carpel. (Chapter 4; 4.3.7). Investigation into the inheritance of *sepaloid* had already begun before the commencement of this project.

5.6.1. Origin and history.

The first batch of *sepaloid* primroses arose in the "Spectrum" strain of commercial primroses grown from Nickersons seed in 1996. Dr. V. Wooley of Nottingham obtained a number of these *sepaloid* primroses. All but two of the plants she obtained were infertile, with 4 whorls of sepals; I was given the two plants that had some flowers with carpels and four others. Two of these four did subsequently produce an occasional flower with a functional carpel. All subsequent *sepaloid* plants used in this project were derived by breeding from the fertile siblings above, or from their progeny. Crosses were carried out to investigate inheritance of the phenotype .

5.6.2. Inheritance of *sepaloid.*

The two fertile *sepaloid* plants were pollinated by thrum *Wild Type* pollen in 1996 and results classified in 1997 (Table 5.23).

Two fertile *sepaloid* plants x thrum *Wild Type.*

sep ? x + *s*

? ? ↓ + *S*

Plant	Thrum *Wild Type*	pin *Wild Type*
	+ *S* *sep* +	+ *s* *sep* +
sepaloid No. 1	2	2
sepaloid No. 2	11	5

Table 5.23. First test cross of *sepaloid.*

Results indicate that the phenotype is recessive. Expect equal numbers of pin and thrum plants. The expected 1:1 ratio of thrum *Wild Type:* pin *Wild Type* is perfect in *sepaloid* no 1 results, but numbers are very small. A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio of 1:1 for plant no.2.

χ^2 for *sepaloid* no 2 results 1:1 for Thrum *Wild Type* : pin *Wild Type*

observed 11 : 5, expect 8 : 8.

χ^2 (1 dof) = 2.25, P = 0.13

Results fit the predicted 1:1 ratio for Thrum *Wild Type* : pin *Wild Type.*

In order to investigate further whether the phenotype is recessive the above progeny were crossed together pin x thrum, and the results classified in 1998 (Table 5.24).

pin *Wild Type* (heterozygous for the recessive *sepaloid* allele) x Thrum *Wild Type* (heterozygous for the recessive *sepaloid* allele).

$\frac{sep\ \ s}{+\ \ s}$ x ↓ $\frac{sep\ \ s}{+\ \ S}$

	Sepaloid	Thrum *Wild Type*	pin *Wild Type*
	$\frac{sep\ \ s}{sep\ \ s}$	$\frac{+\ \ S}{sep\ \ s}$	$\frac{sep\ \ s}{+\ \ s}$
sepaloid no.1 progeny results	9	7	4
sepaloid no.2 progeny results	2	13	7
Total numbers	11	20	11

Table 5.24. Second test cross of *sepaloid.*

Results confirm that the phenotype is recessive.

If there is linkage of *sepaloid* to *s* expect a ratio of 1:2:1, for *sepaloid:* thrum *Wild Type* : pin W*ild Type*. A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio.

χ^2 for 1:2:1, for *sepaloid:* thrum *Wild Type* : pin *Wild Type*

observe 11:20:11, expect 10.5:21:10.5.

χ^2 (2 dof) 0.095, P = 0.95.

A ratio of 1:2:1 is consistent with *sep* being in coupling with the *s* allele of the *Primula S* locus.

If there is no linkage of *sepaloid* to *s* expect a ratio of 1:3 for *sepaloid : Wild Type*, with equal numbers of pin and thrum *Wild Type.* We observe 11: 33 *sepaloid: Wild Type* and would expect 11:33 so there is a perfect ratio of 1:3 for *sepaloid : Wild Type.*

If there is no linkage expect a ratio of 1:1 for thrum: pin. A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio of 1:1.

χ^2 for 1:1 for thrum: pin

observe 20 : 11, expect 15.5:15.5

χ^2 (1 dof) 2.6, P = 0.12

Results fit the ratio of 1:1 for thrum: pin.

If there is complete linkage of *sepaloid* to *s* expect 2:1 thrum: pin. A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio of 2:1.

χ^2 for 2:1 for thrum: pin

observe 20 : 11, expect 20.66:10.33;

χ^2 (1 dof) 0.06, P = 0.8

Results fit the ratio of 1:1 for thrum: pin.

The ratio for 2:1 for thrum *Wild Type* : pin *Wild Type* fits the predicted ratio better than 1:1 and indicates complete linkage of *sepaloid* to s. The experiment was continued in order to produce larger numbers and a more conclusive result.

5.6.3. Subsequent crosses.

Two more crosses were carried out to further investigate linkage of *sepaloid* to the *Primula S* locus. These crosses will also confirm that the phenotype is recessive. Total numbers indicated that *sepaloid* was in coupling with the pin allele of the *S* locus. The cross was repeated in subsequent years until a larger number of progeny were classified (Table 5.25).

Wild Type pin (heterozygous for the recessive *sepaloid* allele) x Thrum *Wild Type* (heterozygous for the recessive *sepaloid* allele)

sep s / + s x ↓ *sep s* / + S

Year	Cross (pin x Thrum)	*sepaloid*	Thrum *Wild Type*	pin *Wild Type*
		sep s / *sep s*	+ S / ? s	*sep s* / + s
1998	*sepaloid* 1 progeny	9	7	4
1998	*sepaloid* 2 progeny	2	13	7
1999	*sepaloid* 1 progeny x *sepaloid* 2 progeny	6	6	3
2000	*sepaloid* 1 progeny	10	11	6
2001	*sepaloid* 2 progeny	9	22	5
2002	*sepaloid* 2 progeny	6	15	10
Totals		42	74	35

Table 5.25. First test for linkage between *sepaloid* and *s*.

If linkage exists between *sep* and *s* expect 1:2:1 for *sepaloid:* thrum *Wild Type :* pin *Wild Type.* A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio.

χ^2 for 1:2:1 for *sepaloid:* thrum *Wild Type :* pin *Wild Type.*

observed 42:74:35, expected 39.25:78.5:39.25

χ^2 (2 dof) = 0.709, P = 0.7.

Results are consistent with the 1 : 2 : 1 ratio expected of *sepaloid:* thrum *Wild Type :* pin *Wild Type.*

If there is linkage of *sepaloid* to *s* expect a ratio of 2:1 thrum: pin *wild type.* A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio.

χ^2 for 2:1 thrum: pin *Wild Type*

observed 74:35, expect 72.66: 36.33

χ^2 (1 dof) = 0.07, P = 0.79

Results are consistent with a ratio of 2:1 thrum: pin *Wild Type.*

If there is no linkage of *sepaloid* to *s* expect a ratio of 1:1 for pin *wild type* : thrum *wild type.* A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio.

χ^2 for 1:1 for pin *Wild Type* : thrum W*ild Type*

observed 35: 74, expect 54.5:54.5

χ^2 (1 dof) = 13.96, P = 0.0002

Results are not consistent with a ratio of 1:1 for pin W*ild Type* : thrum *Wild Type.*
Results confirm linkage between *sep* and *s.*

A cross of *wild type* thrum plants carrying the recessive allele for *sepaloid* were back-crossed to a pin *sepaloid* and the progeny classified in subsequent years (Table 5.26). (As the mating type of the *sepaloid* is not always physically obvious, plants that had already been used for other crosses and were known to be genotypically pin *sepaloid* were used).

pin *sepaloid* x Thrum *Wild Type* heterozygous for the recessive *sepaloid* allele.

$$\frac{sep\ \ s}{sep\ \ s} \times \downarrow \frac{+\ \ S}{sep\ \ s}$$

		sepaloid	Thrum *Wild Type*	pin *Wild Type*
		$\frac{sep\ \ s}{sep\ \ s}$	$\frac{+\ \ S}{sep\ \ s}$	$\frac{+\ \ s}{sep\ \ s}$
2000	Thrum *wild type* from *sepaloid* 1	7	18	0
2001	Thrum *wild type* from *sepaloid* 2	3	6	0
2002	Thrum *wild type* from *sepaloid* 2	18	20	0
2003	Thrum *wild type* from *sepaloid* 2	7	1	
Totals		35	45	0

Table 5.26. Second test for linkage between *sepaloid* and *s*.

Ten plants from the 2001 progeny were lost before they could flower.
24 plants from 2003 did not flower.

If there is complete linkage between *sepaloid* and the *s* allele of the *Primula S locus* expect 1:1 *sepaloid* : *Wild Type*. A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio.

χ^2 for 1:1 *sepaloid* : *Wild Type*

observed 35 : 45; expect 40 : 40

χ^2 (1 dof) = 1.25 P = 0.26.

Results fit the predicted 1:1 ratio for *sepaloid* : *Wild Type*.
Results confirm tight linkage between *sep* and *s*.

5.6.4. Summary of results.

From the above analysis it can be concluded that:

1. The *sepaloid* phenotype is recessive.
2. Tight linkage exists between *sepaloid* and the *s* allele of the *Primula S* locus.

5.7. *double.*

Primulas of *double* form usually have inner whorls converted to petals (see Chapter 4, 4.3.8). They have been cultivated from at least the sixteenth century (see Chapter 1, 1.5.1). The inheritance of the phenotype has been known since at least the early part of the twentieth century, and that the allele for the phenotype is recessive has been subsequently commented on in horticultural literature (e.g. Genders 1959). In order to work with the *double* phenotype it is necessary to find viable reproductive organs on what is generally an infertile flower. Some commercial *double Primula* flowers produce an occasional anther on one or more petals, others may have a core of stamens or a petal stamen mix in the centre of the flower. The majority of commercial *double* flowers are fully sterile. Even when not fully sterile, only an occasional flower may produce anthers, so that in order to find pollen very many flowers may need to be pulled apart. The second requirement is that such pollen should be discovered at a time when a suitable female parent is also in flower. Because of the thrum incompatibility system (see Chapter 2) pin *Wild Type* is the ♀ parent of choice for the first cross. Results from this will allow the genotype of the *double* parent to be determined and provide fertile single plants carrying the recessive allele for *double* for future use. It is not known how many alleles for *double* may exist in the population. Before the inheritance of the phenotype was known *double* primroses were propagated vegetatively from plants that occurred naturally. How many of these provided pollen for breeding purposes is unknown and it is possible that the older cultivars provided the *double* alleles found in the more recent cultivars. However new *double Primulas* do still occur naturally from time to time and it is possible that these may have new mutations giving new *double* alleles. Alternatively they could simply be the result of the same older alleles circulating in the population. Some investigation of the *double* phenotype had taken place before the commencement of this project.

5.7.1. Origin and history.

Initially crosses using *double* primroses were undertaken for purely horticultural reasons. The first, in 1993, used pollen from the commercial cultivar "Chocolate Soldier", and pollen from a *double* seedling from Rosetta Jones' seed. Each of these was used to pollinate a pin *Wild Type* gold-laced polyanthus with the intention of breeding some laced doubles. Seed set was low and seed was pooled from the two crosses. The 17 resulting single progeny were all pin flowered with laced edges to the petals. This indicated that both *double* plants used as pollen parents were genotypically pin. The three progeny with

the best lacing were selected and labeled no's 1, 2, and 3 carrying the recessive *double* allele. The *double* no. 1 was self-pollinated in 1995 and of the 12 progeny to flower in 1997 two were laced *double*, both with a central core of stamens. These will also be genotypically pin. Pollen obtained from the *double Jack in the Green* cultivar "Dawn Ansell" in 1995 (and again later in 1997 and 1998), was used on pin *Wild Type* cultivars. Results of these crosses are not shown but all gave only pin progeny, indicating that "Dawn Ansell" too is genotypically pin. One of the progeny carrying the recessive *double* allele from "Dawn Ansell" was self-pollinated and the resulting 11 plants flowered in spring 1999. Five were *double* and 6 were pin *wild type*. If the plant as expected carried the recessive *double* allele from "Dawn Ansell" then expect a ratio of 3:1 for *Wild Type: double*, but numbers above are below the limits for Chi Square testing.

Despite being from a self-pollinated plant that will therefore have had only one *double* allele from the parent "Dawn Ansell", the five *double* plants varied in form at first flowering. One was fully *double* with indeterminate whorls of petals, one had a petal/stamen mix in the centre of the flower, two had a central core of stamens and the last had a central core of stamens surrounding naked ovules. All were raised in the same environmental conditions. This indicates that the background genotype in which the *double* alleles are being expressed must influence expression.

Individual flowers of *double* with pollen were obtained from Peter Ward in 1995. These were used to pollinate pin *Wild Type* flowers. Resultant progeny were of both pin and thrum form indicating that the *double* primroses from Peter Ward were genotypically thrum. In 1996 a thrum laced edge *Jackanapes* (calyx of both leaf and petal) was pollinated by the *double* cultivar "Lilian Harvey" to give progeny of *wild type*, *Hose in Hose* (petaloid calyces), *Jack in the Green* (leafy calyces) and *Jackanapes*, all carrying the recessive allele for double. The *Jack in the Green* was subsequently pollinated by the *Hose in Hose* to give progeny of *wild type*, *Jack in the Green*, *Hose in Hose*, *double*, and the first recorded *Jackanapes-double*. Subsequently *Hose in Hose-double* were also produced. In 1997 pollen from the old *double* "Bon Accord, lilac" (the only instance known of this plant producing any pollen), was used to pollinate another pin *wild type* carrying the recessive allele for *double* from "Dawn Ansell". This resulted in 12 progeny, 6 *double* and 6 *Wild Type* pin. The *Wild Type* carry only the recessive *dbl* allele obtained from "Bon Accord lilac", but the *doubles* have one *dbl* allele from both "Dawn Ansell"

and "Bon Accord lilac". These also showed variability in form at first flowering during spring 1999. Three had fully *double* flowers with indeterminate whorls of petals, one had fully *double* flowers with an occasional stamen on inner petals, and two had a petal/stamen mix in the centre of the flower. None had a core of stamens or naked ovules as observed in the *double* progeny from self-pollination (that flowered the same season), and it is possible that some interaction between the two *dbl* alleles from different parents is also influencing expression.

In 1997, some different forms of *double* flowers were obtained from Peter Ward. There was considerable variation in form, ranging from *semi-double* with organ order of sepal, petal, petal, stamen, stamen, indeterminate, to a central core of mixed petals and stamens, to fully *double* flowers as seen on commercial cultivars. The flowers were from plants that he had bred himself, but not all were from the same genetic background. Also in 1997 a new very recently discovered double polyanthus "Lin Rogers" was obtained. It had flowers with organ order of sepal, petal, petal, sepal, naked ovules, and provided another variation in the form of the *double* flower. The variation in form observed prompted the question of whether or not all forms of *double* were allelic. Peter Ward's flowers, (a *double*, a *semi-double Jack in the Green* and another *semi-double*) were used in various horticultural crosses in order to produce more doubles. The latter *semi-double* was used as pollen parent on my wild type no.3 (carrying the recessive allele for *double* with organ order of sepal, petal, petal, petal, indeterminate), and the progeny classified in 1998 (Table 5.27).

Wild Type pin heterozygous for the recessive *double* allele x Ward *semi-double*.

dbl s x *dbl ?*

\+ *s* ↓ *dbl s*

double	Thrum *Wild Type*	pin *Wild Type*
dbl s *dbl ?*	*dbl S* \+ *s*	*dbl s* \+ *s*
6	4	2

Table 5.27. Determining the genotype of the "Ward" *semi-double*.

Results show that this *semi-double* is genotypically thrum.

Where a plant is homozygous for the recessive *double* allele and is crossed to a plant heterozygous for the recessive *double* allele, expect a ratio of 1:1 *double : Wild Type* Results are a perfect 1:1 ratio.

One of the *double* plants from the above progeny was a *semi-double*, with the same form as the pollen parent from Peter Ward. Pollen from this *semi-double*, of organ order sepal, petal, petal, stamen, stamen indeterminate was used to pollinate pin *Wild Type* no. 2 carrying the recessive *double* allele for a fully double flower. The fully double flower had indeterminate whorls of petals but occasionally produced anthers in the centre of the flower. Twelve progeny resulted, and the first six to flower were classified in 1999 (Table 5.28).

Wild Type pin heterozygous for the recessive *double* allele x *semi-double*.

dbl s x *dbl ?*

\+ *s* ↓ *dbl s*

double	Thrum *Wild Type*	pin *Wild Type*
dbl s *dbl ?*	*dbl S* \+ *s*	*dbl s* \+ *s*
3	2	1

Table 5.28. Test of allelism between fully *double* and *semi-double*.

Results are consistent with the *semi-double* being allelic to fully *double* as *double* plants occurred among the progeny. The three *doubles* classified from the latter cross were of *semi-double* form, with a central core of stamens at first flowering. The presence of *Wild Type* thrum in the progeny indicates that this *semi-double* is genotypically thrum. Those progeny that did not flower in 1999 were lost and could not be classified.

5.7.2. Subsequent crosses.

Continued investigation of *double* flowers followed three lines of inquiry:

1. Continued investigation of whether all forms of *doubling* are allelic.
2. Continued investigation into the variability of expression of *double*.
3. Investigation of whether any linkage exists between *dbl* and the *S* locus.

Results from some crosses give insight into more than one of these.

In order to carry out these investigations it was necessary to obtain pollen from *double* flowers and to raise *Wild Type* progeny carrying the recessive allele for *double* from a number of different *double* plants. This also reveals the genotype of the *double* parent.

5.7.3. Investigation of the new *double* polyanthus "Lin Rogers".

Three cross were undertaken to investigate the new *double* polyanthus "Lin Rogers" of organ order of sepal, petal petal, sepal, naked ovules. The plant produced two anthers on one flower only, late in the flowering season of 1998 (this was the only pollen ever produced by this plant which was later lost in 2003). Pollen was used to pollinate a pin gold-laced *Jack in the Green* polyanthus that was still in bloom when the majority of primroses and polyanthuses were finished. The resulting 11 progeny were classified in 1999 as 5 thrum *wild type*, 2 pin *wild type*, 3 thrum *Jack in the Green*, and 1 pin *Jack in the Green*. Results show the *double* polyanthus "Lin Rogers" to be genotypically thrum. All of the progeny will be heterozygous recessive for the *double* allele from the *double* polyanthus "Lin Rogers" and can be used to test for allelism to other forms of *double* flower.

Wild Type progeny carrying the recessive *double* allele for "Lin Rogers" were crossed together pin x thrum in 1999 and results classified in 2000/2001 (Table 5.29).

pin *Wild Type* (heterozygous for the recessive *double* allele from "Lin Rogers") x thrum *Wild Type* (heterozygous for the recessive *double* allele from "Lin Rogers")

s dbl x *S dbl*
s + ↓ *s* +

double	pin *Wild Type*	Thrum *Wild Type*
s dbl *S dbl*	*s* ? *s* +	*S* + *s* ?
14	25	14

Table 5.29. Investigation of the new *double* polyanthus.

As *double* is a recessive character expect a ratio of 3 *Wild Type* : 1 *double*. A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio.

χ^2 for 3 *Wild Type* : 1 *double*

observed 39 +: 14 *dbl*, expect 39.75 *Wild Type*: 13.25 *dbl.*

χ^2(1 dof), 0.05, P = 0.8.

Results fit the predicted ratio of 3 *Wild Type* : 1 *double.*

If there is no linkage of *double* to the *S* locus expect a ratio of 1:1 pin *Wild Type* : thrum *Wild Type.* A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio.

χ^2 for 1:1 pin *Wild Type* : thrum *Wild Type*

observed 25:14, expected 19.5:19.5

χ^2 (1 dof) = 3.1, P = 0.078

Results fit the predicted ratio of 1:1 pin *Wild Type* : thrum *Wild Type* and indicates that there is no linkage between *double* and the *S* locus.

If there is linkage of *double* to the *S* locus expect a ratio of 1:2 pin *Wild Type* : thrum *Wild Type.* A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio.

χ^2 for 1:2 pin *Wild Type* : thrum *Wild Type*

observed 25:14, expect 13:26

χ^2 (1 dof) = 16.6, P = 0.000046

Results are extremely deviant from the predicted ratio of 1:2 pin *Wild Type* : thrum *Wild Type* and show no linkage of *double* to the *S* locus. However if linkage was very loose very large numbers of progeny would be required to identify the linkage.

Variability of expression of *double* was observed. All *double* progeny had a whorl of inner sepals in the fourth whorl at first flowering. Variations that occurred in later flowers included a reduction in the size of the inner sepals and additional whorls of petals in some plants. Two plants had a flower within a flower phenotype, but not on all flowers; one plant repeating this to give three sets of inner sepals between whorls of petals on occasional flowers.

5.7.4. Tests to discover whether all different forms of *double* are allelic.

The form of fully *double* flowers with indeterminate whorls of petals is notably different from *semi-double* flowers with organ order of sepal, petal, petal, stamen, stamen. Both are notably different from the *double* polyanthus "Lin Rogers" with organ order of organ

order of sepal, petal petal, sepal, naked ovules. Crosses were carried out to investigate whether all of these different forms are allelic.

The first cross to test allelism was a cross between *Wild Type* pin carrying the recessive *double* allele for "Dawn Ansell" (that has indeterminate whorls of petals with sometimes occasional anthers on inner whorls of petals), and a *semi double* (with organ order of sepal, petal, petal, stamen, stamen), carried out in 2001, and classified in 2002 (Table 5.30).

Wild Type pin (heterozygous for the recessive *double* allele from "Dawn Ansell") x *semi-double.*

s dbl x *? dbl*
s + ↓ *s dbl*

Thrum *Wild Type*	pin *wild Type*	*double*
S dbl *s +*	*s +* *s +*	*? dbl* *s dbl*
4	8	15

Table 5.30. Allelism Cross 1.

The two forms are allelic since *double* plants occurred in the progeny.

If no linkage exists between *double* and the S locus expect a ratio of 1:1 for thrum *Wild Type* : pin *Wild Type*. A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio.

χ^2 for 1:1 for thrum *Wild Type* : pin *Wild Type*

observe 4:8, expect 6:6.

χ^2 (1 dof) = 1.33, P = 0.248.

Results fit the predicted ratio of 1:1 for thrum *Wild Type* : pin *Wild Type*.

The *semi-double* is genotypically thrum. The majority of the *doubles* (i.e.11 of 15) from the above cross, had stamens in the centre of the flower at first flowering, the remaining 4 had a petal/stamen mix in the centre at first flowering.

To investigate whether the *double* allele of the *double* polyanthus "Lin Rogers" is allelic to the *double* allele of the commercial *double Jack in the Green*, "Dawn Ansell" the following cross was undertaken. Pin *Jack in the Green*, carrying the recessive allele for *double Jack in the Green* "Dawn Ansell" was pollinated by a thrum *Jack in the Green* carrying the recessive allele for *double* polyanthus "Lin Rogers" in 1999, and progeny classified in 2000/2001 (Table 5.31).

pin *Jack in the Green* (heterozygous for the recessive *double* allele from JIG "Dawn Ansell") x Thrum *Jack in the Green* (heterozygous for the recessive *double* allele from "Lin Rogers")

Jig s dbl / *+ s +* x *Jig s +* / *+ S dbl*

Thrum *Jack in the Green*	pin *Jack in the Green*	*double Jack in the Green*	Thrum *Wild Type*	pin *Wild Type*	*double*
J ? ? / *? ? ?*	*J s ?* / *? s ?*	*J ? dbl* / *? ? dbl*	*+ s ?* / *+ S ?*	*+ s ?* / *+ s ?*	*+ ? dbl* / *+ ? dbl*
8	8	6	2	2	0

Table 5.31. Allelism Cross 2.

Results confirm allelism to the double "Dawn Ansell" since despite the very different organ order observed in flowers of the two plants *double* plants were obtained among the progeny.

No linkage patterns were observed in these crosses, but numbers are low, loose linkage would require larger numbers to identify.

The flowers were examined at first flowering as part of the investigation of variability of expression of *double*. Flowers from all six *doubles* were found to be determinate – all had some naked ovules in the centre. Subsequent flowers sometimes produced occasional inner sepals, or sepal material on inner petals. Some flowers on some plants only, had an occasional inner whorl of inner sepals that were fully developed and leafy as the whorl 1 *Jack in the Green* sepals. One *Jack in the Green-double* produced some flowers that had normal carpels. Fertility of the carpels was investigated (see 5.7.5).

Another smaller cross, *wild type* pin carrying the recessive allele for *double* "Lin Rogers" x thrum *Jack in the Green* carrying the recessive allele for *double* "Lin Rogers" was undertaken at the same time and classified in 2000/2001 (Table 5.32).

Wild Type pin heterozygous for the recessive *double* allele from "Lin Rogers" x thrum *Jack in the Green* heterozygous for the recessive *double* allele from "Lin Rogers"

+ *s dbl* / + *s* +	x ↓	*Jig S dbl* / + *s* +			
Thrum *Wild Type*	pin *Wild Type*	Thrum *Jack in the Green*	pin *Jack in the Green*	*double*	*double Jack in the Green*
+ *S dbl* / + *s* +	+ *s* + / + *s ?*	*Jig S ?* / + *s* +	*Jig s* + / + *s ?*	+ *s dbl* / + *? dbl*	*Jig S dbl* / + *s dbl*
2	5	2	2	7	0

Table 5.32. Allelism Cross 3.

The flowers were examined at first flowering as part of the investigation of variability of expression of *double*. All *double* flowers from the above cross had inner sepals in whorl 4 at first flowering.

5.7.5. Investigation of fertility of the *Jack in the Green-double* with carpels.

The *Jack in the Green-double* with carpels was pollinated in order to ascertain fertility; seed was collected and progeny were subsequently raised from the cross, fertile *semi-double Jig* x *double Hose in Hose* (with a central core of stamens) (Table 5.33). The normal carpels of the *Jack in the Green-double* were found to be fully fertile. As expected all progeny were *double*, 8 were *double- Jack in the Green*, 7 were *double*, 1 was *double-Hose in Hose* and 2 were *double-Jackanapes*. Results show the *Jack in the Green-double* to be allelic to the *Hose in Hose-double*. If recombination is occurring freely between *double* and the other mutant phenotypes *Jack in the Green* and *Hose in Hose* then expect equal numbers of all phenotypes but numbers are too low for statistical analysis. The flowers were examined at first flowering as part of the investigation of variability of expression of *double*. There was considerable variability in the flower structure of the progeny (see Chapter 4, Fig.4.19). The plant did not produce fertile carpels again.

5.7.6. Investigation of linkage between *double* and the *S* locus.

There had been no evidence of linkage between *double* and the *S locus*, but large numbers are required to observe loose linkage patterns. To investigate whether there might be loose linkage patterns between the recessive allele for *double* and the *Primula S locus* a thrum *Wild Type* carrying the recessive allele for *double* flowers was pollinated from a *double* that was known to be genotypically pin. It can be difficult to obtain large numbers due to the fact that little or no pollen is produced by many, if not most, *double* flowers, but in 2002 better pollen production resulted in better seed production and 171 progeny were eventually raised.. Two of these were lost before they flowered. Results were classified in 2003 (Table 5.33).

Thrum *Wild Type* (heterozygous for the recessive *double* allele from *semi-double*) x pin *double*.

S dbl x *s dbl*
s + ↓ *s dbl*

Thrum *Wild Type*	pin *Wild Type*	double
s dbl / *S +*	*s dbl* / *s +*	*s dbl* / *? dbl*
15	21	133

Table 5.33. Investigation of linkage between *double* and the *S* locus.

If there is no linkage of *dbl* to the *S locus* expect equal numbers of pin and thrum progeny and equal numbers of *Wild Type* to *double*. A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio.

χ^2. for 1:1 for pin *Wild Type* : thrum *Wild Type*

observe 21 : 15; expect 18 : 18

χ^2 (1 dof) = 1.0, P = 0.317

Results fit the predicted ratio of pin *Wild Type* : thrum *Wild Type*.

χ^2. for 1:1 for *Wild Type* : *double*

observe 36 : 133; expect 84.5 : 84.5

χ^2 (1 dof) = 55.67, P = 0

Results do not fit the predicted ratio of pin *Wild Type* : thrum *Wild Type*.

The hugely disproportionate number of *double* plants in the progeny is unexpected. It could be due to viability effects from other genes in the genetic background. Alternatively if *double* was not fully penetrant the thrum *Wild Type* parent might have been genotypically *double*, but there is no previous evidence for this hypothesis. As the *wild type* parent died the cross cannot be repeated.

Despite both parent *doubles* having stamens in the centre of the flower all *double* progeny were entirely without any stamens in any position at first flowering.; at later flowering some *doubles* had stamens in the centre of the flower and one plant had a few flowers with normal carpels.

5.7.7. Summary of results.

From the above analysis it can be concluded that:

1. The *double* phenotype is recessive.
2. The phenotype of the *double* flower is extremely variable in form, even in some instances within an individual plant.
3. All *double* plants tested so far have been found to be allelic.
4. No linkage has been observed.

5.8. *reduced petal.*

All investigation of *reduced petal* was done during this project. The phenotype first occurred in the nursery of Dr. R. Brumpton in 1999 in a line of very inbred silver-laced polyanthus. The petals were thicker and much reduced giving them an immature appearance. Colour is fully or partly absent from the adaxial petal surface and the conical papillate cells of the adaxial epidermis are not fully developed (see 4.3.11).Leaf form is broader and more "frilled" at the edges than *Wild Type*. In 1998 plants of this phenotype was obtained in both pin and thrum, but when crossed together seed set was very poor. Seed set was also poor when used as a ♀ parent pollinated by *Wild Type*, but when used as a ♂ parent seed set was normal. A cross of pin silver-laced polyanthus x thrum *reduced petal* polyanthus (genotype designated as *rdp*), produced only three progeny three pin and one thrum *Wild Type*, indicating that the phenotype was likely to be recessive.

5.8.1. Investigation of whether or not there is any linkage between *reduced petal* and the *S* locus.

A cross of thrum silver laced polyanthus heterozygous for the recessive allele for *reduced petal* x pin *reduced petal* was undertaken to test for linkage patterns. The parents were sibling progeny from the cross Pin *Hose in Hose* heterozygous for the recessive allele *reduced petal* x thrum *reduced petal* (shown in Chapter 6, Table 6.5). Results are classified in Table 5.34.

Thrum *Wild Type* heterozygous for the recessive allele for *reduced petal* x pin *reduced petal* polyanthus.

rdp *S* x *rdp* *s*
\+ *s* ↓ *rdp* *s*

Thrum *Wild Type*	pin *Wild Type*	Thrum *reduced petal*	pin *reduced petal*
+ *S* *rdp* *s*	+ *s* *rdp* *s*	*rdp* *s* *rdp* *S*	*rdp* *s* *rdp* *s*
27	34	26	23

Table 5.34. Test to establish whether or not there is any linkage between *reduced petal* and the *S* locus.

If there is no linkage between *reduced petal* and the *S* locus expect a ratio of 1:1 for *reduced petal* : *Wild Type*. A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio.

χ^2. for 1:1 for *reduced petal* : *Wild Type*

observe 49 : 61; expect 55 : 55

χ^2 (1 dof) = 1.3, P = 0.25

Results are consistent with a 1:1 ratio of *reduced petal* : *Wild Type*.

If the ♀ parent is heterozygous Ss expect a ratio of 1:1 for thrum to pin. A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio.

χ^2 for 1:1 for thrum to pin

Observe 53 : 57; expect 55 : 55

χ^2 (1 dof) = 0.15, P = 0.7

Results are consistent with a 1:1 ratio of thrum : pin.

A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio of 1:1:1:1 for all possible phenotypes.

χ^2 for 1:1:1:1 for all possible phenotypes

observe 27 :34 : 26 : 23; expect 27.5 :27.5 : 27.5 : 27.5

χ^2 (3 dof) = 2.36, P = 0.5

Results are consistent with a 1:1:1:1 ratio for all possible phenotypes. Results confirm that there is no linkage between *reduced petal* and the *S* locus.

At the seedling stage the *Wild Type* and *reduced petal* phenotypes could be identified by the different leaf forms. The *reduced petal* leaves were paler in colour, thinner, wider, and more frilled at the edges. Expression of the phenotype was variable in the petals. This was evaluated by dividing the degree of expression into categories as shown in Fig.5.35.

reduced petal Thrum			*reduced petal* pin		
Type1	Type2	Type3	Type1	Type2	Type3
18	4	4	12	5	6

Table 5.35. Evaluation of the degree of expression of the *reduced petal* phenotype in the flowers.

Type 1 = the most extreme phenotype, Type 3 = the least extreme phenotype , and Type 2 = a phenotype intermediate between 1 and 3.

The majority of the progeny were clearly defined but a few of the Type 3 forms were very similar to *Wild Type* and deciding how to categorise the phenotypes became more difficult. This was accentuated by some alteration of the leaf form in a few of the mature progeny that were potted into larger pots for growing on, so that three *Wild Type* plants now had leaves that exhibited some undulation of the edges, and two *reduced petal* plants had leaves that were slightly less undulating. When the phenotype was expressed only in the genetic background of silver laced polyanthus, in which it first occurred, it maintained both flower type and leaf type as being very distinct from *Wild Type*. In the new genetic background, introduced by breeding with *Hose in Hose* (see Chapter 6, 6.3.3) the differences between the leaf phenotypes have become less distinct. The flowers have also become larger and less reduced in appearance, but continue to exhibit loss of colour on the adaxial surface of the petals. Further investigation of variability of expression of the *reduced petal* phenotype is required.

5.8.2. Summary of results.

From the above analysis it can be concluded that:

1. The *reduced petal* phenotype is recessive.
2. Expression of the phenotype is variable.
3. No linkage has been observed.

5.9. *Oak leaf.*

All investigation of the *Oak Leaf* phenotype was done during this project. It was first discovered in 1999 in Ken Foster's nursery Nottingham, by Dr. R. Brumpton. Its parentage and horticultural origin is unknown. The lobed leaves are similar in shape to oak leaves and floral organs in whorls 1 and 2 are attenuated. The original *Oak Leaf* plant was thrum (genotype designated as *Okl*), and crosses carried out by Dr. Brumpton in 1999 established that it was a dominant phenotype (*Okl/okl*) that could be expressed with mutant phenotypes *Hose in Hose, Jack in the Green* and *Split Perianth.* An assorted mixture of young seedlings from the above crosses were given to me in 1999. On flowering they included one thrum *Oak Leaf*, along with a number of pin *Oak Leaf* plants. A double mutant, *Hose in Hose-Oak Leaf* also occurred. I received a ramet of the original plant when it was divided in spring 2000, and it was used along with the above seedlings for genetical analysis of the *Oak Leaf* phenotype.

5.9.1. Genetic analysis of the original thrum *Oak Leaf.*

A test cross of Pin *wild type* x thrum *Oak Leaf* was undertaken, seed sown in Dec. 2000, and initial scoring of seedlings by leaf morphology done in spring/summer 2001 (Table 5.36). As *Oak Leaf* is a dominant phenotype, a ratio of 1:1 for *Oak Leaf*: *wild type* seedlings can be expected.

Pin *wild type* x thrum *Oak Leaf.*

$$\frac{+\quad s}{+\quad s} \times \frac{Okl\quad S}{+\quad s}$$

Oak Leaf	*wild type*
112	54

(An additional 45 seedlings failed to germinate fully in that the cotyledons failed to emerge from the testa).

Table 5.36. Classification of the ratio of *Oak Leaf* seedlings to *wild type* seedlings, 1.

A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio of 1:1 for *Oak Leaf*: *wild type*.

χ^2 for 1:1 for *Oak Leaf*: *wild type*

observe 112:54, expect 83:83

$\chi^2(1 \text{ dof}) = 20.2$. $P = 0.000007$

Results are very significantly deviant from the expected 1:1 ratio for a dominant heterozygote crossed to *wild type*.

The deficit of *wild type* cannot be adequately explained. If it is possible that the 45 failed seedlings may have been all *wild type*, this would give a closer approximation to 1:1, but this cannot be assumed. The original *Oak Leaf* occurred in a commercial line of primroses that would have been rigorously selected for high and uniform germination. The wild *P. vulgaris* in contrast generally has erratic germination. It is possible that the factor(s) responsible for high and uniform generation are segregating with the *Oak Leaf* phenotype.

Progeny were fully classified as they flowered during 2001/2 (Table 5.37).

pin *wild type* x Thrum *Oak Leaf*.

$\frac{+ \quad s}{+ \quad s}$ x ↓ $\frac{Okl \quad ?}{+ \quad ?}$

Thrum *Oak Leaf*	pin *Oak Leaf*	Thrum *wild Type*	pin *wild type*
$\frac{Okl \quad S}{+ \quad s}$	$\frac{Okl \quad s}{+ \quad s}$	$\frac{+ \quad S}{+ \quad s}$	$\frac{+ \quad s}{+ \quad s}$
2	94	25	2

Table 5.37. Test cross of the original Thrum *Oak Leaf*.

In addition 18 *Oak Leaf* and 29 *wild type* were lost before scoring. Results show *Oak Leaf* and the s allele of the *Primula S locus* are in coupling. Four recombinants in a total of 123 progeny scored give a map distance of 3.3%.

As *Oak Leaf* is dominant expect a ratio of 1:1 for *Oak Leaf*: *wild type*. A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio

χ^2 for 1:1 for *Oak Leaf*: *wild type*

Observe 114 : 58; expect 86 : 86

χ^2(1 dof) = 18.2, P = 0.00002.

Results are significantly deviant from the predicted ratio of 1:1 for *Oak Leaf*: *wild type.*

Again it is possible that the factor(s) responsible for high and uniform generation are segregating with the *Oak Leaf* phenotype since the original *Oak Leaf* occurred in a commercial line of primroses that would have been rigorously selected for high and uniform germination. The wild *P. vulgaris* in contrast generally has erratic germination. This hypothesis could be tested by using a commercial *wild type* cultivar rather than a wild *P. vulgaris.*

The original *Oak Leaf* plant did not initially set seed and was thought to be female infertile. However in 2001 the reciprocal cross *Oak Leaf* thrum x *wild type* pin (Tables 5.38 and 5.39) succeeded. Control for this cross was by removal of the perianth organs, thus rendering the flower unattractive to insects. The plant was weak and not suitable for enclosure in an isolation unit. It died after seed harvest.

Original thrum *Oak leaf* x pin *wild type.*

Okl ? x *+ s*

+ ? ↓ *+ s*

Oak Leaf	*wild type*
23	21

Table 5.38. Classification of the ratio of *Oak Leaf* seedlings to *wild type* seedlings, 2.

As *Oak Leaf* is dominant expect a ratio of 1:1 for *Oak Leaf*: *wild type.* A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio.

χ^2 for 1:1 for *Oak Leaf*: *wild type*

observe 23:21, expect 22:22

χ^2 (1 dof) = 0.091, P = 0.76.

Results fit the predicted 1:1 ratio for *Oak Leaf*: *wild type*

Of the 44 seedlings in Table 5.46, 11with *wild type* leaves were lost as seedlings. Thirty three plants were raised to maturity but four *Oak Leaf* and 3 *wild type* plants were lost before flowering. Final classification is given in Table 5.39.

Original Thrum *Oak leaf* x pin *wild type*.

$\frac{Okl\ ?}{+\ ?}$ x $\frac{+\ s}{+\ s}$ ↓

Thrum *Oak Leaf*	Pin *Oak Leaf*	Thrum *wild type*	Pin *wild type*
$\frac{Okl\ S}{+\ s}$	$\frac{Okl\ s}{+\ s}$	$\frac{+\ S}{+\ s}$	$\frac{+\ s}{+\ s}$
3	16	7	0

Table 5.39. Reciprocal test cross of the original Thrum *Oak Leaf*.

Two thirds of the *wild type* progeny (14 plants) were lost before maturity, therefore no accurate map distance can be calculated for this cross as it is possible that the failed *wild type* progeny could have included recombinants.

5.9.2. Testing of one thrum *Oak Leaf* for coupling of *Okl* with the *S* allele of the *Primula S* locus.

One thrum *Oak Leaf* occurred among the assorted seedlings that I was given by Dr. R, Brumpton (see 5.10). This was test crossed reciprocally to *wild type* in 2000 in order to discover whether it was a recombinant that now had *Oak Leaf* in coupling with the *S* allele of the *Primula S locus*. Initial scoring of seedlings by leaf morphology was done in spring/summer 2001 (Table 5.40).

Thrum *Oak Leaf* x pin *wild type*.

$\frac{Okl\ S}{+\ s}$ x $\frac{+\ s}{+\ s}$ ↓

Oak Leaf	*wild type*
14	14
reciprocal	
20	39
Total numbers	
34	53

Table 5.40. Classification of the ratio of *Oak Leaf* seedlings to *wild type* seedlings, 3.

As *Oak Leaf* is a dominant phenotype expect a ratio of 1:1 for *Oak Leaf*: *wild type*. The cross of thrum Oak Leaf x pin wild type had a perfect ratio of 1:1 for *Oak Leaf*: *wild type*. A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio of 1:1 for *Oak Leaf*: *wild type* for the reciprocal cross.

χ^2 for 1:1 for *Oak Leaf*: *wild type* for the reciprocal cross

observe 20 : 39, expect 29.5 : 29.5

χ^2 (1 dof) = 6.12 P = 0.013.

Results do not fit the predicted 1:1 ratio for *Oak Leaf*: *wild type*. There is a deficit of *Oak Leaf*.

A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio of 1:1 for *Oak Leaf*: *wild type* for total numbers.

χ^2 for 1:1 for *Oak Leaf*: *wild type* for total numbers

observe 34: 53, expect 43.5:43.5

χ^2 (1 dof) = 4.1, P = 0.04

Results do not fit the predicted 1:1 ratio for *Oak Leaf*: *wild type*. There is a deficit of *Oak Leaf*.

Final classification was undertaken in 2002 (Table 5.41.).

Thrum *Oak Leaf* x pin *wild type*.

$\frac{Okl\ \ S}{+\ \ s}$ x ↓ $\frac{+\ \ s}{+\ \ s}$

Thrum *Oak Leaf*	pin *Oak Leaf*	Thrum *wild type*	pin *wild type*
$\frac{Okl\ \ S}{+\ \ s}$	$\frac{Okl\ \ s}{+\ \ s}$	$\frac{+\ \ S}{+\ \ s}$	$\frac{+\ \ s}{+\ \ s}$
8	1	0	16
reciprocal			
19	0	0	32
Total numbers			
27	1	0	48

Table 5.41. Testing of thrum *Oak Leaf* for coupling of *Okl* with the *S* allele of the *Primula S* locus

Results show *Oak Leaf* to be in coupling with *S*. One recombinant among 25 progeny gives a map distance of 4%. In this cross three *Oak Leaf* progeny were lost before maturity, so whether they would have been thrum or pin is unknown. There were no recombinants among 51 progeny from the reciprocal cross. If numbers from the reciprocal crosses are combined then a total of one recombinant from a total of 76 progeny gives a map distance of 1.3%.

5.9.3. Investigation of viability of *Okl/Okl* homozygotes.

To investigate viability of *Okl/Okl* homozygotes two crosses were undertaken, Thrum *Oak Leaf* x pin *Oak leaf* (Table 5.42), and thrum *Oak Leaf* x pin *Hose in Hose Oak Leaf* (Table 5.43). Results were classified as seedlings.

Thrum *Oak Leaf* x pin *Oak Leaf*.

$$\frac{Okl\ \ S}{+\ \ s} \times \downarrow \frac{Okl\ \ s}{+\ \ s}$$

Oak Leaf	*wild type*
15	5

Table 5.42. Investigation of viability of *Okl/Okl* homozygotes 1.

The above numbers are exactly consistent with the 3:1 ratio expected if the *Okl/Okl* homozygote is viable.

Thrum *Oak Leaf* x pin *Hose in Hose Oak Leaf*.

$$\frac{Okl\ \ S\ +}{+\ \ s\ +} \times \downarrow \frac{Okl\ \ s\ +}{+\ \ s\ Hih}$$

Oak Leaf	*wild type*
21	5

Table 5.43. Investigation of viability of *Okl/Okl* homozygotes 2.

Two seedlings failed to germinate fully from the above cross.

If *Oak Leaf* homozygotes are viable expect a ratio of 3:1 for *Oak Leaf*: *wild type* A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio of 3:1 for *Oak Leaf*: *wild type.*

χ^2 for 3:1 for *Oak Leaf*: *wild type*

observe 21:5, expect 19.5:6.5

χ^2 (1 dof) = 0.46, P = 0.497.

Results are consistent with 3:1 segregation.

The result indicates viability of *Oak Leaf* homozygotes.

5.9.4. Summary of results.

From the above analysis it can be concluded that:

1. *Oak Leaf* is a dominant phenotype.
2. *Oak Leaf* is in coupling with the *s* allele of the *Primula S* locus. Recombinants had *Oak Leaf* in coupling with the *S* allele of the *Primula S* locus.
3. Map distances obtained were 3.2% and 4.0% respectively from two different crosses (reduced to 1.3% if reciprocal totals are combined). .
4. Numbers indicate that the *Okl/Okl* homozygote is viable.

5.10. Crosses using short homostyles.

The majority of wild primrose plants have genotype *gpa/gpa*, pin; or *GPA/gpa*, thrum (see also Chapter 1; 1.3). Rare recombination events within these main components can result in self fertile short homostyles with genotype *Gpa/gpa*. Two short homostyle primroses were obtained from Prof. A. J. Richards in spring 1998. Self-pollinated seed from these plants resulted in two more short homostyle primroses and one pin *wild type* (seed set was normal but germination was very poor). Self pollination of short homostyles results in progeny that segregate as two short homostyles to one *wild type* pin (J. Richards, 1998; personal communication).

The short homostyle from the line obtained from Prof. Richards, had yellow flowers of the expected form with anthers in the pin position, small pollen and with a short style and short stigmatic papillae. Another was found that was a blue flowered plant with similar characteristics except that the flowers were found to have long stigmatic papillae as expected on long styled pin plants. This was from the Blue Jeans parental line, and was in Leeds University Greenhouses. Flowers of both of these plants can be seen in Figure 5.1.

Figure 5.1. Yellow and blue short homostyle flowers.

Both flowers have anthers and stigma half way down the corolla tube. They are different only in that the yellow flower has short stigmatic papillae while the blue flower has long stigmatic papillae.

A. Yellow short homostyle flower. This has the expected morphological features of low anthers, small pollen, short style and short stigmatic papillae. **B.** Blue short homostyle flower. This also has low anthers, small pollen, and short style, but the stigmatic papillae are long. Stigmatic papillae length and pollen size are not visible in the photographs and were determined by light microscopy. Size bars approximately 1cm.

In order to discover whether either or both of these plants carried the dominant *G* of the *S* locus gene cluster, genotype *Gpa/gpa* the following crosses were undertaken. The yellow short homostyle was crossed to thrum *wild type* (and also to both pin and thrum *Hose in Hose* and pin and thrum *Oak Leaf* - see Chapter 6, 6.4.2). Results of the cross to thrum *wild type* were 10 thrum *wild type* and 6 pin *wild type*. No short homostyle progeny were obtained from the cross. Three of the pin *wild type* had slightly shorter than normal styles and all had long stigmatic papillae. Four progeny were lost before blooming.

5.10.1. Determining the genotype of the blue short homostyle.

The blue flowered short homostyle with long stigmatic papillae was crossed to thrum *wild type*, and the results classified in Table 5.44.

Thrum *wild type* x blue short homostyle

GPA x *?pa*

Gpa ↓ *gpa*

Thrum *wild type*	pin *wild type*	Short homostyle
GPA / *gpa*	*gpa* / *gpa*	*Gpa* / *gpa*
17	11	0

Table 5.44. Determining the genotype of the blue short homostyle.

Ten plants were lost before maturity and twelve had not bloomed at the time of scoring the results. No short homostyles were obtained among the progeny that did bloom. Five of the pin *wild type* had shorter than normal styles though not so short as thrum styles and all had long stigmatic papillae.

5.10.2. Determining the genotype of the yellow short homostyle. Long homostyle x short homostyle.

No short homostyles were obtained from crosses in section 5.12. As one possible reason for this could be low viability of short homostyles (Lewis and Jones, 1993), a cross of long homostyle (*gPA/gpa*) x yellow short homostyle (putative genotype *Gpa/gpa*) was carried out in 2003. If the genotype of the yellow short homostyle is as predicted phenotypes of the progeny should include long homostyle, pin *wild type*, short homostyle, and thrum *wild type* in a 1:1:1:1 ratio. Even if the expected numbers of short homostyles were not obtained, 25% of the progeny should be thrum *wild type* (*gPA/Gpa*). This would

confirm genotype of the short homostyle as *Gpa/gpa*. Plants were classified as they came into flower in 2004 and results are tabulated in Table 5.45.

Long homostyle x short homostyle.

gPA x *?pa*

gpa ↓ *gpa*

Long homostyle	pin *wild type*	Short homostyle	Thrum *wild type*
gPA / *gpa*	*gpa* / *gpa*	*Gpa* / *gpa*	*GPA* / *gpa*
21	19	0	0

Table 5.45. Determining the genotype of the yellow short homostyle.

Results show no short homostyles or thrum *wild type* among the progeny therefore the genotype of the short homostyle can not be *Gpa/gpa*. The characteristics of the flower, low anthers, short style, short stigmatic papillae and self compatibility would therefore have to be achieved by a different mechanism.

5.10.3. Summary of results.

From the above analysis it can be concluded that:

1. No short homostyles were obtained from either cross. This could be due to low viability of short homostyles, but is more likely to that the characteristics of the flower, low anthers, short style, short stigmatic papillae and self compatibility have been achieved by a different mechanism.
2. The long stigmatic papillae of the blue short homostyle may indicate that this plant should be re-classified as a short styled pin plant, with shortening of the style due to factors other than recombination within the *Primula S* locus. Further investigation of short homostyles is required.

5.11. Discussion of the inheritance of the mutant phenotypes.

A surprising find was the discovery of so many dominant mutant phenotypes in *Primula vulgaris* and in *P.veris x vulgaris* cultivars. Of the eight phenotypes investigated only *sepaloid, double,* and *reduced petal* were found to be recessive. If naturally set seed is collected from any dominant heterozygous mutant phenotype 50% of the resultant progeny will exhibit the dominant mutant phenotype. Consequently in the past when

variants of British wild flowers began to be grown for horticultural purposes these phenotypes could easily be increased. In contrast, before understanding of Mendelian genetics, recessive phenotypes could only be propagated vegetatively. For example it was around the turn of the 20th century that breeding of *double* primroses, that have a recessive phenotype, first began. This may be one reason for some of the dominant mutant phenotypes such as *Jack in the Green* (leafy sepals) and *Hose in Hose* (petaloid sepals) surviving as horticultural curiosities. Not all *Primula* species have so many dominant mutant phenotypes; *P. sinensis* was intensively studied early in the twentieth century and only three of the thirty five phenotypes investigated were found to be dominant (De Winton and Haldane, 1933).

A notable discovery was linkage of four loci to the *Primula S* locus. Linkage of *Hose in Hose* (petaloid sepals) was previously known (Ernst, 1931; Webster and Grant 1990), and it is possible that *Staminoid Carpels* that segregates both with *Hose in Hose* and the *S* allele of the *Primula S* locus is an allele of *Hose in Hose*. Investigation into *sepaloid* found *sepaloid* to be in coupling with the s allele of the *S* locus. The newly discovered mutant phenotype *Oak Leaf* was also initially in coupling with the *s* allele of the *Primula S* locus, but recombinant plants were also generated that had *Oak Leaf* in coupling with the S allele of the *Primula S* locus (Tables 5.54 and 5.56). The discovery of these *S* locus linked loci allowed mapping of the linked genes using appropriate three point crosses (see Chapter 7).

Another notable discovery (made before the commencement of this project and investigated further here) was the viability of *SS* homozogotes in a line of *Hose in Hose* plants. All previous reports consider *SS* homozygotes to be inviable and thrum plants to be invariably of genotype *Ss* (Richards 1986), although one such homozygote was reported by Mather and De Winton on one occasion in *P. sinensis* (Mather and De Winton 1941; also quoted by Richards, 1993; Kurian and Richards, 1997).

All mutant phenotypes exhibited variability in expression. The flowers of *doubles* exhibited very great variation in form, yet all *doubles* investigated were found to be allelic. The hugely disproportionate number of *double* plants in the progeny from the second test for linkage of *double* to the *S* locus (Table 5.50) is unexpected and invites further investigation. Should the phenotype fail to be penetrant in some plants the inheritance of *double* would require reassessment. It would, however, explain the result. If the *Wild Type* thrum carrying the recessive allele for the *semi-double* was instead a

double with a non penetrant phenotype then all progeny would be genotypically *double* but in some plants *double* could fail to be penetrant. There is no previous evidence for such a hypothesis, but recent variability in expression on a single *double* polyanthus plant has included the occasional production of single fertile flowers (not shown) on a plant that produces predominantly double flowers. The hypothesis could be tested by investigation of those *wild type* plants obtained from the cross shown in Table 5.50, as these would be also expected to be genotypically *double* but with a non penetrant phenotype. If the *wild type* progeny are both pollinated by *double* pollen and also crossed both together pin x thrum the resultant progeny could be assessed for penetrance of the *double* phenotype.

It has already been observed that the *Hose in Hose* phenotype exhibits variability not only from plant to plant but sometimes also from one ramet to another (see 5.3.3) or from one flower to another on the same scape. It has been observed that the petaloid calyx frequently becomes more petaloid later in the season, and this is likely to be the result of environmental influences as the same effect can be observed on a plant in consecutive years. Variability of expression from one part of the plant to another however could be the result of either somatic recombination or of transposon excision since results of the initial investigation showed the change in expression to be a permanent one (as documented in 5.3.3). Parallel work in the laboratory (Dr. J. Li and Prof. P. Gilmartin, personal communication) has cloned *Primula DEFICIENS* and *GLOBOSA* homologues and analysed linkage to the *S* locus. *GLOBOSA*, but not *DEFICIENS*, was found to be linked to the *S* locus and a restriction fragment length polymorphism associated with *GLOBOSA* was found to co-segregate with *Hose in Hose*. Analysis of genomic clones of *GLOBOSA* from *wild type* and *Hose in Hose* identified a retrotransposon in the promoter of *Hose in Hose* that is predicted to cause upregulation of *GLOBOSA*. Instability of the retrotransposon is suggested by the reversion of *Hose in Hose* to *wild type* both on individual ramets, on individual flowers on the same scape and on individual calyx lobes. This could be due to excision, which is unusual for a retrotransposon, or possibly an epigenetic effect associated with chromatin remodeling or with methylation. However recent results from PCR and sequencing of the revertant allele indicates that transposon excision has occurred (Dr. J. Li, personal communication).

The recessive *reduced petal* phenotype bears some resemblance to the *CINCINNATA* mutant phenotype of *Antirrhinum* (Nath *et al.*, 2003; Crawford *et al.*, 2004). In initial crosses it appeared as a pleiotropic mutant phenotype affecting both flowers and leaves.

Observation of mature plants from later crosses to a different genetic background discovered some plants that appear to uncouple these two effects. This requires further observation over a longer period of time. It may be that different alleles of the *reduced petal* form exist, some alleles being stronger than others. Consequently some further work is required on this mutant phenotype.

The dominant *Staminoid Carpels* phenotype was initially only found in thrum *Hose in Hose* and it was not until 2001 that the first pin *Staminoid Carpels-Hose in Hose* occurred. Results from the cross of pin *Staminoid Carpels-Hose in Hose* to thrum *wild type* (Table 5.34) showed that in a pin linked *Staminoid Carpels-Hose in Hose* the thrum *Staminoid Carpels-Hose in Hose* progeny had more elongated mutant whorl four organs than did pin *Staminoid Carpels-Hose in Hose* (Chapter 4, Fig 4.11). These elongated ovary walls have undergone transformation towards the form of the corolla tube, and an occasional anther can be found inside the upper portion of this organ. Elongation of this organ could therefore be said to elongate the whorl four anthers towards the thrum or the pin position in the flower (see Chapter 3). The *Staminoid Carpels-Hose in Hose* mutant phenotype is predicted to be the result of ectopic *B* function (see Chapter 4; 4.3.1) and anther position is dictated by the *A* component of the *Primula S* locus (see Chapter 3). Differences in the elongation of the pin and thrum *Staminoid Carpels-Hose in Hose* whorl four organ, even though the *Staminoid Carpels-Hose in Hose* alleles are the same, each linked to the pin allele of the *Primula S* locus, indicates that the *A* component is organ related rather than whorl related.

Whether the differences in elongation of the mutant whorl four organ are due to the effects of *A* or *a* is being tested by crosses of pin *Staminoid Carpels-Hose in Hose* to both long homostyle (*gAP/gap*) plants and to a short homostyle *Hose in Hose* with large pollen *S locus* of *GaP/gap* (see Chapter 7 for origin of latter). The former should give 25% of the progeny as *Staminoid Carpels-Hose in Hose* with *S locus* of *gAP/gap* and the latter should give 25% of the progeny as *Staminoid Carpels-Hose in Hose* with S locus of *GaP/gap*. It is predicted that the *Staminoid Carpels-Hose in Hose* carrying the dominant *A* component of the *Primula S* locus will have more elongated mutant whorl four organs than those carrying the recessive *a* component and the dominant *G* component. Results are not expected until 2005/6.

Investigation of short homostyles crossed to other mutant phenotypes is documented in Chapter 6; 6.4.2. Further investigation of short homostyles is required. . Genes outside

the *S* supergene have been found to suppress *S* functions in buckwheat (*Fagopyrum esculentum*) (Matsui *et al.*, 2004). A generation of F2 *Primula* progeny raised to ascertain whether this may be the case in *Primula* has not yet flowered. The importance of stigmatic papillae length as a reliable diagnostic factor of pin or thrum gynoecia also needs to be clarified.

Genetical analysis of mutant phenotypes has yielded much useful information on the inheritance of these forms and provides a sound basis for further analysis at molecular level. Identification of loci linked to the *Primula S* locus enables mapping of the genes around the *S* locus (see Chapter 7).

CHAPTER SIX

Combinations of mutant phenotypes

6.1. Introduction.

Following the detailed characterization of mutant phenotypes affecting flower development (Chapter 4) it was important to discover whether any interactions would occur when mutant phenotypes of *Primula* were combined. Both absence of interactions and discovery of interactions would be informative. The only previous information on combinations of mutant phenotypes in *Primula*, was of the inheritance of *Jackanapes*, a mutant combining the dominant alleles of *Jack in the Green* (leafy calyces) and *Hose in Hose* (petaloid calyces), (Chapter 4 and Webster and Grant 1990). The mature *Jackanapes* flower is fully described and the development of the *Jackanapes* flower has been investigated by scanning electron microscopy (section 6.2.1).

To investigate whether interactions might occur between mutant genes affecting the first whorl, *Jack in the Green, Hose in Hose* and *Jackanapes* (*Jack in the Green-Hose in Hose)* were combined with the developmental mutation that gives *Split Perianth* (section 6.2.2). Mutant phenotypes that affected the whole plant as well as the flower were also combined both with each other and with other phenotypes in order to look for any possible interactions (sections 6.3.1, 6.3.2, and 6.3.3).

Where phenotypes were found to be linked to the *S locus* they were combined with the *S locus* recombinants of long and short homostyle in order to discover whether this would affect expression of either phenotype in any way. (sections 6.4.1 and 6.4.2).

The question of whether the mutant phenotype is associated with the organ or with the whorl in which it occurs was answered by experimental combination of mutant phenotypes (section 6.5). Where linkage between genes was identified it was necessary to combine the relevant phenotypes in order to facilitate undertaking large three point crosses that could determine gene order. These combinations, with results, are dealt with in Chapter 7.

6.2. Combinations of mutant phenotypes that affect the first whorl.

Mutant phenotypes that affect the first whorl include *Jack in the Green* (leafy calyces), *Hose in Hose* (petaloid calyces) and *Split Perianth*. The first two are organ identity homeotic mutants and the last has alteration to the usual form of the Perianth (sections 1.5.1, 1.5.2, 4.2.1, 4.2.2, and 4.2.3). No previous information on combinations involving *Split Perianth* could be found, nor any detailed information on Jackanapes, the *Hose in Hose* and *Jack in the Green* combination.

6.2.1. The combination of leafy calyces (*Jack in the Green*) and petaloid calyces (*Hose in Hose*) that is termed *Jackanapes*.

When plants heterozygous for the dominant leafy calyx mutation (*Jack in the green*) are crossed with plants heterozygous for the dominant petaloid calyx mutation (*Hose in Hose*), then in 25% of the progeny the two dominant mutant alleles will be expected to occur in the same plant (Webster and Grant, 1990). The phenotype has been described under the horticultural name of *Jackanapes* from as early as the 16th century (e.g. Gerard, 1597; Parkinson, 1629; Bradley, 1734), at a time when there was no understanding of genetics or inheritance. In the majority of *Jackanapes* plants the leafy portion is at the top of the calyx lobes and the petaloid portion is at the base (Fig.6.1A, B, and C). In some plants, where the expression of the *Hose in Hose* phenotype is poor, the phenotype will vary from one calyx lobe to the next. Some calyx lobes may be either completely leafy, or only have a tiny segment of petal, while others may exhibit the more usual form (Fig. 6.1D). The junctions between petaloid and leafy tissue do not normally correspond on the adaxial and abaxial epidermal surfaces; there is generally more petaloid tissue on the adaxial surface, often overlying the green leafy tissue underneath (Fig. 6.1A and C). Conversely there is usually more green leafy tissue on the abaxial surface, looking paler on the portions where it overlies a petaloid upper epidermis (Fig. 6.1B). Flowers that are pink or red frequently lose the colour at the junctions between the two cell types (Fig. 6.1C, indicated by black arrows). Where the colour is maintained, but overlies a green leafy portion it appears darker (Fig.6.1C, indicated by white arrows).

Junctions between the two tissue types were examined in more detail using scanning electron microscopy (Fig.6.2A,B,C,F.G.and H). There is a clearly distinguishable difference between the leaf cells (Fig. 6.2D and E) and the conical papillate petal cells.

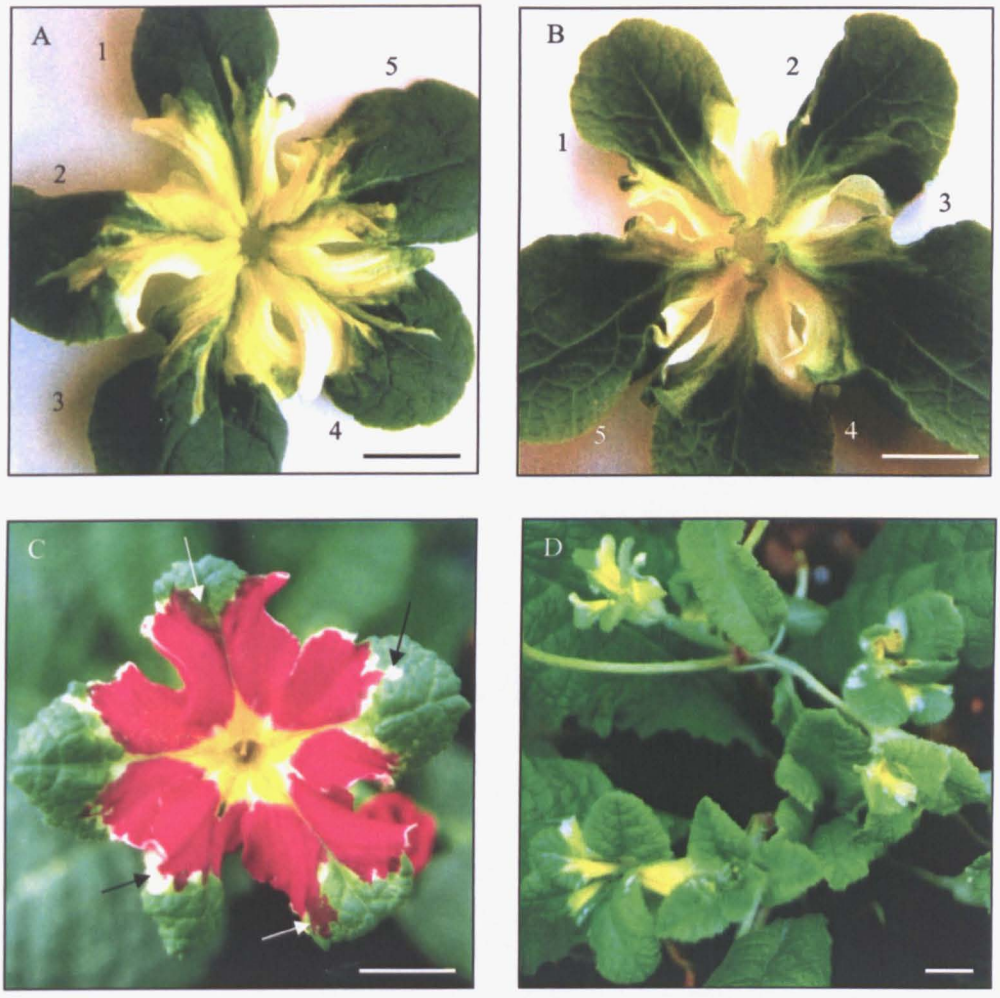


Figure 6.1 Calyces of the double mutant, *Jackanapes*.

The *Jackanapes* calyx combines the dominant phenotype of *Jack in the Green* (leafy calyces) with the dominant phenotype *Hose in Hose* (petaloid calyces).

A. Adaxial view of a yellow *Jackanapes* calyx,. **B.** Abaxial view of the *Jackanapes* calyx shown in A. Calyx lobes are numbered so that the differences in expression of the tissue types on the upper and lower epidermal surfaces can be compared. **C.** Adaxial surface of a red *Jackanapes* calyx, showing loss of red colour at the junctions between petal and leaf tissue, examples are indicated by black arrows. Where the red petal cells overlie green leaf cells the colour appears darker, examples are indicated by white arrows. **D.** *Jackanapes* calyces on a plant with irregular expression of the *Hose in Hose* phenotype. Some calyx lobes are completely devoid of any petal tissue. Size bars1 cm.

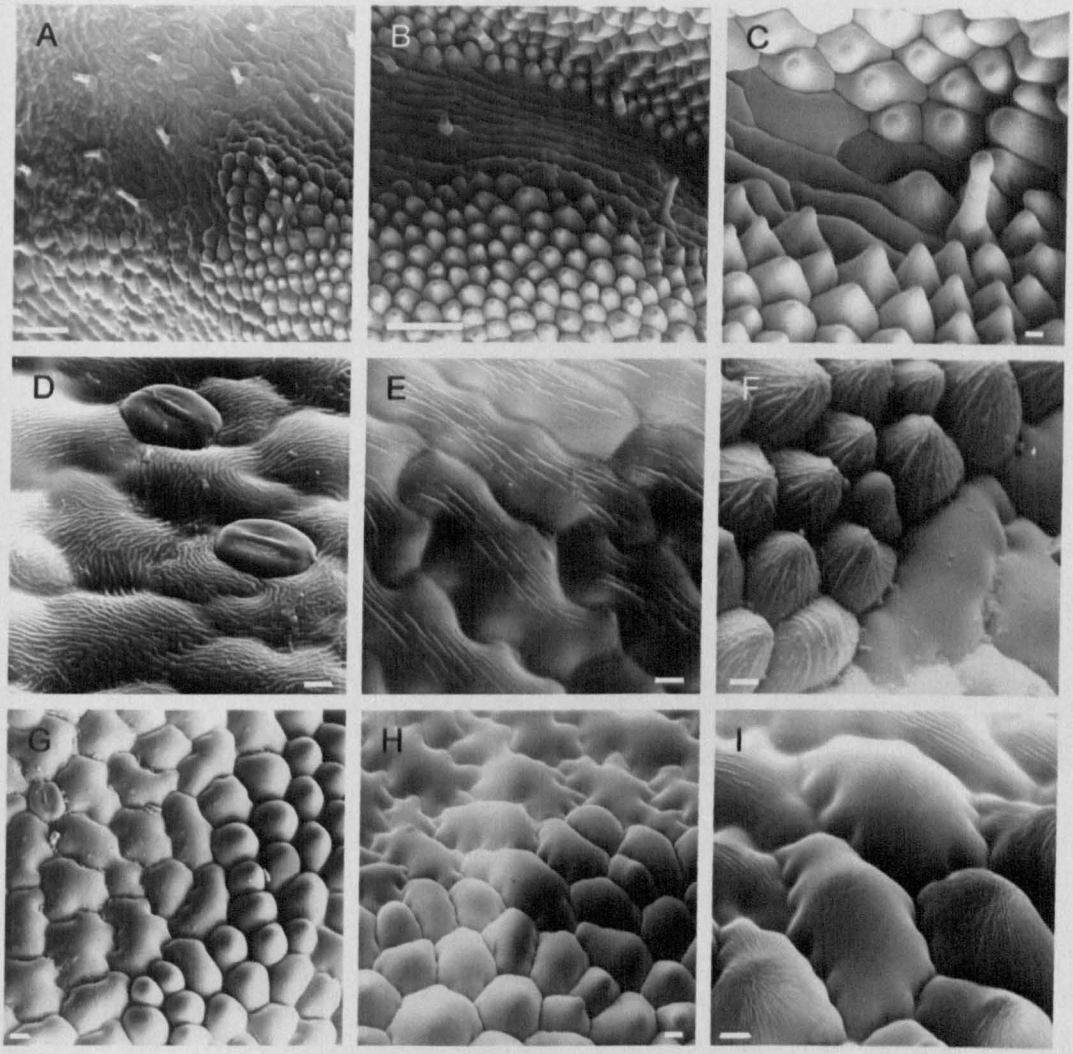


Figure 6.2 The adaxial surface epidermal cells of a *Jackanapes* calyx.

Some junctions between different cell types on the adaxial surface of a mature *Jackanapes* calyx are very distinct, in others cell types merge gradually with chimeric cell forms in between. Normal leaf epidermal cells are also shown for comparison.

A. The upper epidermis at a junction between two cell types, petal and leaf. Conical papillate petal cells can be observed in the lower right hand portion of the image. **B.** A strip of leafy tissue running through petal tissue. **C.** The end of the strip of leafy tissue in **B**. **D.** Normal leaf epidermal cells, including two closed stomata. **E.** *Jackanapes* calyx leaf cells. **F.** A junction between conical papillate petal cells and cells that do not appear as normal leaf cells. **G.** A junction where the changes from one cell type to the other appears less abrupt. **H.** An area of transition from leaf cells (upper left-hand corner) to conical papillate petal cells (lower right hand corner), with chimeric cells between. **I.** Chimeric cells with conical papillate cells at lower right hand side of image. Size bars in A and B are 100um. All other size bars are 10um.

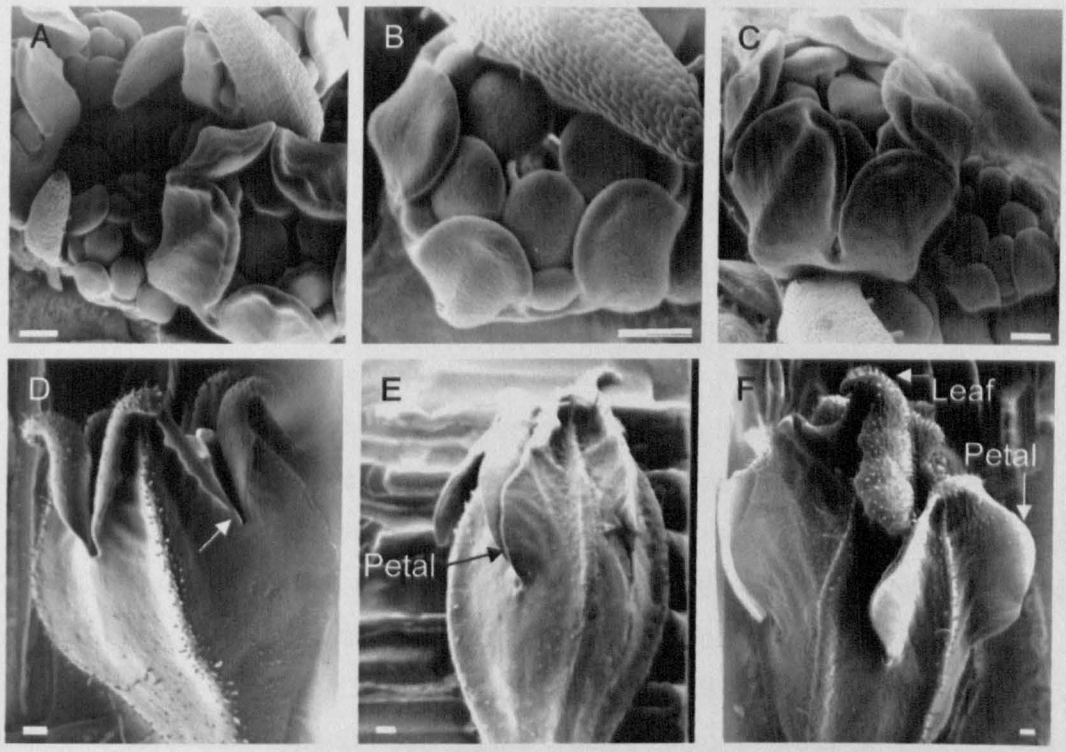


Figure 6.3 Development of the *Jackanapes* calyx.

During early stages of development the *Jackanapes* flower is similar to *Jack in the Green*. This is illustrated by scanning electron micrographs of the development of the *Jackanapes* flower from inception up to and including stage 7 (see Chapter 3 sections 3.3 and 3.5 for stages of development in *wild type*). By stage 7 both the leafy and the petaloid portions of the developing calyx are clearly distinguishable.

A. A group of developing *Jackanapes* flowers at different stages of development. **B.** A stage 4 developing *Jackanapes* flower. The leafy tips of the sepal lobes are just beginning to curl back, and are not easily distinguishable from *Jack in the Green* at this stage. **C.** A stage 5 developing *Jackanapes* flower, again similar to *Jack in the Green* at this stage but the sepal lobes are slightly broader. **D.** A bud of 1.5mm. (at approximately stage 6). The tips of the sepal lobes are clearly leafy but where the base of the lobes join there is a thinner area of tissue that is not curled back and that will become petaloid (marked by a white arrow). **E.** A bud of 2mm. (approximately late stage 6 or early stage 7). The petaloid tissue at the base of each calyx lobe is now beginning to expand (marked by a black arrow). **F.** A bud of approximately 3.5mm (approximately stage 7). The expanding petaloid tissue is very obvious and the leafy tissue at the tips of the sepal lobes has numerous trichomes on the adaxial surface. Size bars are 100um.

In some instances the junction between the cell types, leaf and petal, is quite precise (Fig. 6.2C and F) ; in others there appear to be regions of chimeric cells that are not conical papillate as petal cells, but that are smooth and more raised than leaf cells (Fig.6.2G,H, and I). The colour of the *Jackanapes* flower examined was red (Fig. 6.1C), and it is possible that these are the cell types that show loss of pigment.

Development of the *Jackanapes* flower was studied using scanning electron microscopy (Fig. 6.3). At stage 4 the tips of the leafy calyx lobes are just beginning to curl back and the *Jackanapes* flower is indistinguishable from a *Jack in the Green* flower (Fig. 6.3B). This is further evidence of the tip of the organ developing in advance of the base (see Chapter 3, Fig.3.3). By stage five (Fig. 6.3C) the calyx lobes are slightly broader than those of the *Jack in the Green*, but the difference is not obvious until stage 6 (Fig. 6.3D) when the lower part of each calyx lobe can be seen to be thinner and smoother than that of jack in the green flowers at the same stage. This is the portion of the calyx that will expand and become petaloid, as shown in Figs. 6.3E and F, of flowers of 2mm and 3.5mm in size, and at approximately stage 7. The difference between the smooth petaloid tissue and the leaf tissue with numerous trichomes is particularly evident in the largest flower.

6.2.2. Combining *Split Perianth* with leafy (*Jack in the Green*) and petaloid (*Hose in Hose*) calyces.

During 1998 and 1999, Dr. R. J. Brumpton who discovered the *Split Perianth* phenotype had already carried out combinations between it and the dominant whorl 1 mutant phenotypes *Hose in Hose* and *Jack in the Green* and provided sample plants of *Split Perianth Hose in Hose* (Fig. 6.4A), *Split Perianth Jack in the Green* (Fig. 6.4B) and *Split Perianth Jackanapes* (Fig. 6.4C). The mature petaloid calyx of the *Split Perianth Hose in Hose* reflexes downwards, more so in some plants than in others. This downward reflexing of the *Split Perianth* calyces is also observed in mature *Split Perianth Jackanapes* that has a petaloid portion at the base of each calyx lobe. In contrast the *Split Perianth Jack in the Green* holds each individual calyx lobe upright, or, in some plants, slightly towards the horizontal; in no instance have they been observed to reflex downwards. The mature flower of *Split Perianth Jackanapes* that is shown has only partial splitting of whorl 2, but many flowers produced later on the same plant had the second whorl split down to the point of attachment of the anthers.

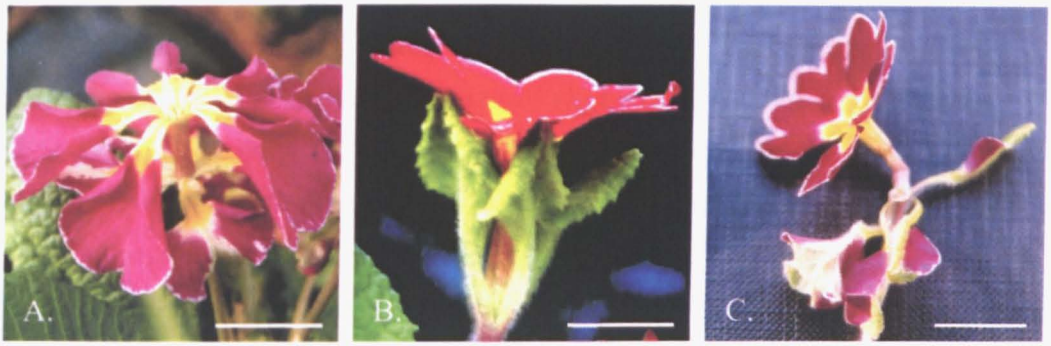


Figure 6.4. *Split Perianth* double and triple mutants.

Split Perianth can be combined with other first whorl mutant phenotypes, *Hose in Hose* (petaloid calyces) and *Jack in the Green* (leafy calyces)

A. *Split Perianth-Hose in Hose*. **B.** *Split Perianth-Jack in the Green.* **C.** *Split Perianth-Jack in the Green-Hose in Hose (Jackanapes).* Size bars are approximately 1cm.

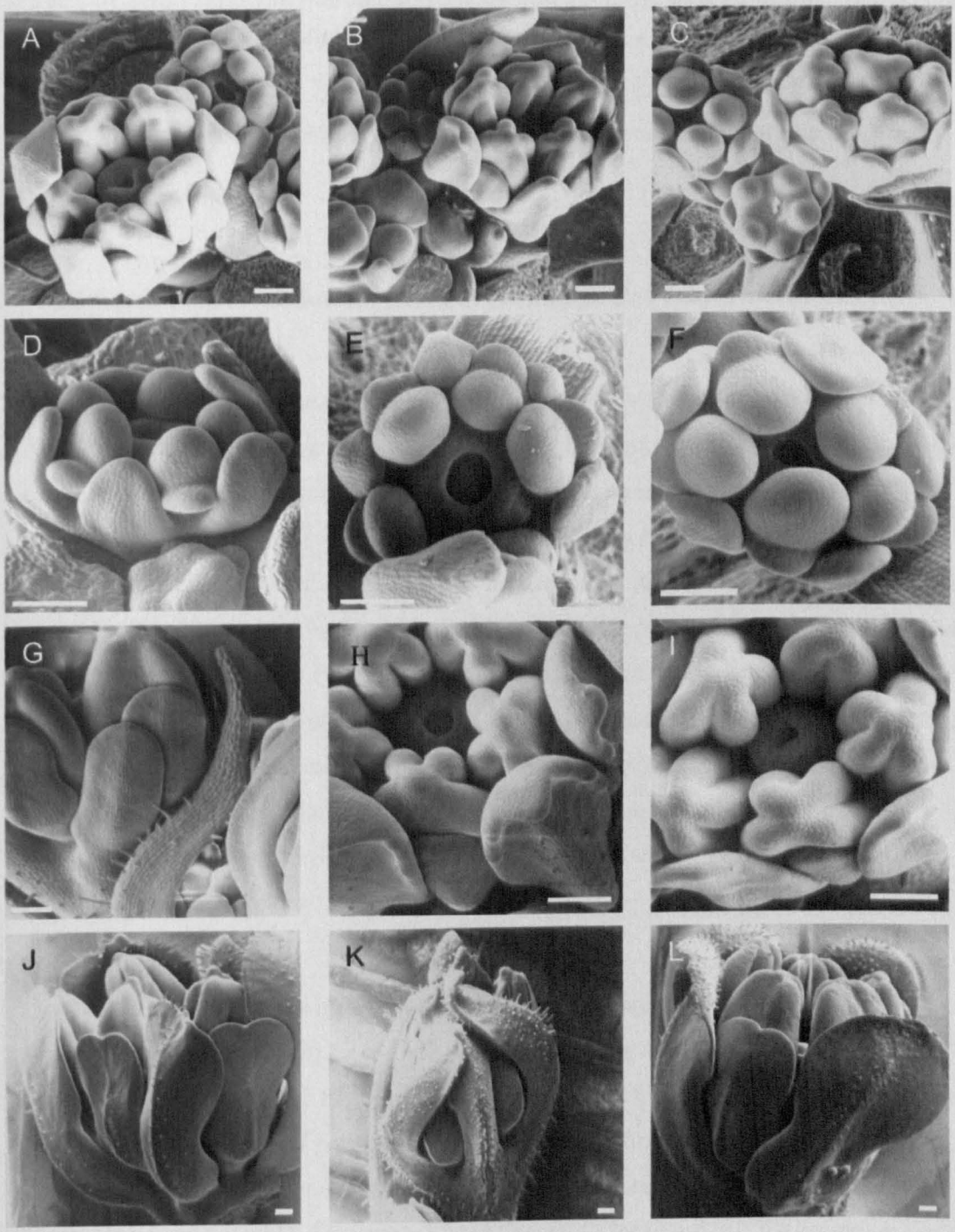
Development of the flowers was studied using scanning electron microscopy. The results (Fig. 6.5) are arranged in three vertical columns so that the developing flowers of each combination, at the same, or at a similar stage of development, are horizontally parallel for comparison. The top of each column shows a cluster of developing flowers on the apical meristem and stages 4, (D, E, and F), stage 5, (G, H, and I), and stage 6 (J, K, and L), follow below. *Split Perianth Hose in Hose* are in the left-hand column (A, D, G and J), *Split Perianth Jack in the Green* in the centre (B, E, H, and K), and *Split Perianth Jackanapes* in the right-hand column (C, F, I, and L).

The fact that the perianth is split is evident in the first whorl from stage 4, and to some extent in the second whorl from stage 5, because of the splitting of the first whorl that allows the second whorl to be visible. By stage 6 splitting of both whorls 1 and 2 is very obvious in both *Split Perianth Hose in Hose* and *Split Perianth Jackanapes* (Fig. 6.5. A4 and D4), but whorl 2 of *Split Perianth Jack in the Green* is sufficiently enclosed by whorl 1 to be partially obscured (Fig. 6.5.K). The differing forms of the three combinations are less evident at stage 4 than if the perianth were not split, but at stage 5 they are clearly differentiated. The petaloid whorl 1 of *Split Perianth Hose in Hose* and the leafy whorl 1 of *Split Perianth Jack in the Green* is narrower than in *Hose in Hose* or *Jack in the Green* without a split perianth. Whorl 1 of *Split Perianth Jackanapes* is slightly broader at this stage than the previous two forms. All forms have a distinctly "keeled" midrib to the first whorl organs by stage 6 (Fig. 6.5, J, K, and L). At this stage *Split Perianth Jack in the Green* has a much narrower first whorl organ than does *Split Perianth Hose in Hose* or *Split Perianth Jackanapes*. Both the first and second whorl organs of the latter two forms have a distinct "waist" to the organ at the point where it would normally have been joined to the adjacent organs in the whorl. This is due to the greater expansion of the petaloid tissue at the upper edges of the first whorl in the region that will become the petal. The petaloid area below that would normally become a petaloid outer corolla tube does not expand to the same extent. The developing flowers of the latter two forms are very untidy in appearance as they are not neatly enclosed by the first whorl as are developing flowers with a normal calyx. The more rigid tissue of the calyx in the *Split Perianth Jack in the Green* fulfills the protective role of the calyx to a greater degree than do the other two combinations.

Figure 6.5 Development of flowers that combine *Split perianth* with other first whorl mutant phenotypes.

Development of *Split Perianth* mutant phenotypes combined with other first whorl mutant phenotypes show distinctive differences between the combinations by stage 6. The scanning electron micrographs are arranged in three vertical columns so that the developing flowers of each combination, at the same, or at a similar stage of development, are horizontally parallel for comparison. The top of each column shows a cluster of developing flowers on the apical meristem and stages 4, 5, and 6 follow below. *Split Perianth Hose in Hose* are in the left-hand column, *Split Perianth Jack in the Green* in the centre and *Split Perianth Jackanapes* in the right-hand column.

A. Cluster of developing *Split Perianth-Hose in Hose* flowers. **B.** Cluster of developing *Split Perianth-Jack in the Green* flowers. **C.** Cluster of developing *Split Perianth-Jackanapes* flowers. **D.** *Split Perianth-Hose in Hose* at stage 4. **E.** *Split Perianth-Jack in the Green* at stage 4. **F.** *Split Perianth-Jackanapes* at stage 4. **G.** *Split Perianth-Hose in Hose* at stage 5. **H.** *Split Perianth-Jack in the Green* at stage 5. **I.** *Split Perianth-Jackanapes* at stage 5. **J.** *Split Perianth-Hose in Hose* at stage 6. **K.** *Split Perianth-Jack in the Green* at stage 6. **L.** *Split Perianth-Jackanapes* at stage 6. Size bars are 100um.


A
B
C
D
E
F
G
H
I
J
K
L

6.3. Combining other mutant phenotypes.

The *Oak Leaf* phenotype was combined both with all of the whorl 1 mutant phenotypes, *Jack in the Green*, *Hose in Hose*, and *Split Perianth* (6.3.1), and with the *reduced petal* mutant phenotype (6.3.2). The *double* mutant phenotype and the *reduced petal* phenotypes were also combined with some of the other mutant phenotypes available (6.3.3).

6.3.1. Combining the pleiotropic mutant phenotype *Oak Leaf* with whorl 1 mutant phenotypes.

The *Oak Leaf* form had been combined with both *Hose in Hose* (Fig. 6.6 C) and with *Split Perianth-Hose in Hose* (Fig. 6.6 F) by Dr. R. Brumpton, who supplied sample plants of these combinations. To make combinations with all available whorl 1 mutant phenotypes, the following two crosses were made. First a thrum *Split Perianth-Hose in Hose Oak Leaf* was crossed with *wild type*, and second a pin *Hose in Hose-Oak Leaf* was crossed with a thrum *Jack in the Green.* Although the crosses were small recombination between the unlinked *Split Perianth* and the *S locus* linked genes provided two *Split Perianth Oak Leaf* mutant phenotypes (Fig. 6.6D). No phenotypes that might be expected from recombination between the genes linked to the pin or thrum alleles of the *S locus* were obtained. Although the calyx of the *Oak Leaf* phenotype is frequently deeply divided it never forms completely separated calyx lobes (see Chapter 4, Fig. 4.26A and Fig. 4.27B) as does the *Split Perianth-Oak Leaf.*

Oak Leaf- Jack in the Greens and *Oak Leaf-Jackanapes* were obtained from the following cross: pin *Hose in Hose-Oak Leaf* x thrum *Jack in the Green* (Table 6.1). Eleven *Oak Leaf* plants and three *wild type* plants did not bloom. No *Hose in Hose, Oak Leaf-Hose in Hose,* or *wild type* progeny, were obtained from the cross, so of the phenotypes should have been present in both pin and thrum form, 6 possible categories are missing from the table. The single thrum *Oak Leaf-Jackanapes* is a recombinant that could have been derived from recombination between either *Oak Leaf* and s, or *Hose in Hose* and s. The pin and thrum *Jack in the Greens* could also have arisen from such a recombination, but other possible phenotypes are absent. *Hose in Hose* and *S* are even more tightly linked (Webster and Grant 1990). One possible explanation could be that some of the *Jack in the Greens* should in fact have been *Jackanapes* but that *Hose in*

pin *Hose in Hose-Oak Leaf* x Thrum *Jack in the Green*

+ *Okl* s +	x	*Jig* + *s* +
+ + s *Hih*	↓	+ + S +

Thrum *Oak Leaf*	pin *Oak Leaf*	Thrum *Jack in the Green- Oak Leaf*	pin *Jack in the Green-Oak Leaf*	Thrum *Jack in the Green-Hose in Hose-Oak Leaf*	pin *Jack in the Green-Hose in Hose-Oak Leaf*	Thrum *Jack In the Green*	pin *Jack in the Green*	Thrum *Jack in the Green-Hose in Hose*	pin *Jack in the Green-Hose in Hose*
+ *Okl* s + + + S +	+ *Okl* s + + + s +	+ *Okl* s + *Jig* + *S* +	+ *Okl* s + *Jig* + *s* +	+ *Okl s Hih* *Jig* + *S* +	+ *Okl s Hih* *Jig* + *s* +	+ + s + *Jig* + *S* +	+ + s + *Jig* + *S* +	+ + s *Hih* *Jig* + *S* +	+ + s *Hih* *Jig* + *s* +
11	13	1	3	1	0	5	7	5	13

Table 6.1. Combining *Oak Leaf* with *Jack in the Green* and *Jackanapes* (calyx of leaf and petal).

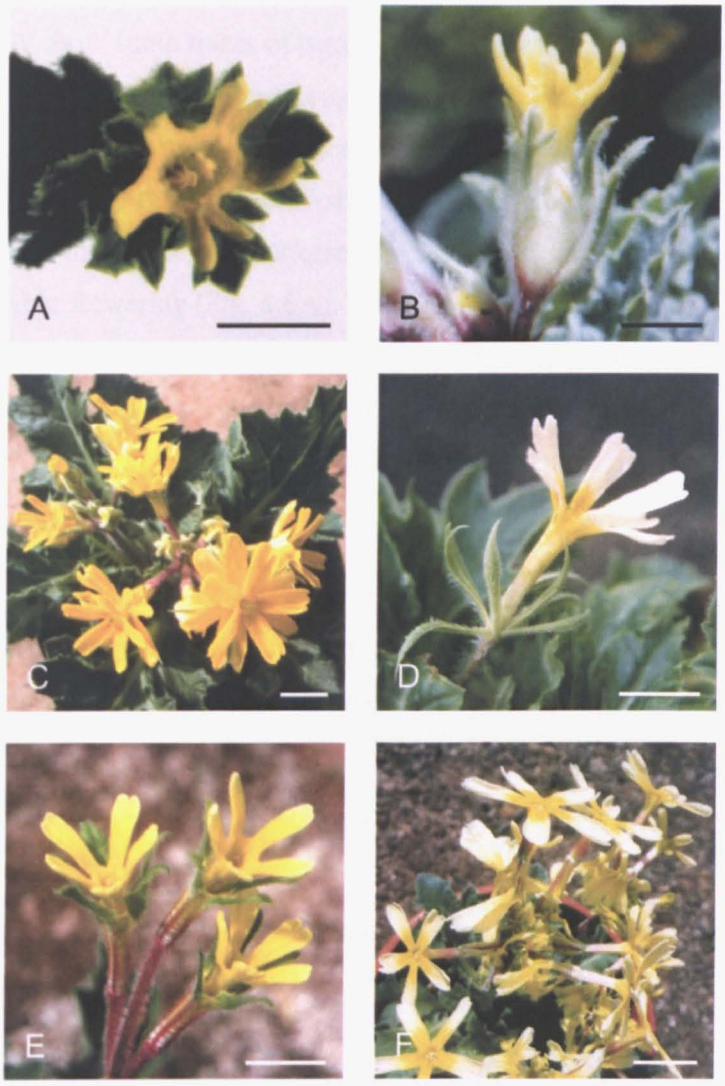


Figure 6.6. *Oak Leaf* combined with whorl 1 mutants phenotypes.

Oak Leaf can be combined with combined with *Jack in the Green, Hose in Hose,* and *Split Perianth* as double or triple mutant phenotypes.

A. *Oak Leaf-Jack in the Green* at first flowering. **B.** A flower from the same plant later in the flowering season. **C.** *Oak Leaf-Hose in Hose*. **D.** *Oak Leaf-Split Perianth*. **E.** A triple mutant combination of *Oak Leaf-Jack in the Green-Hose in Hose (Jackanapes).* **F.** A triple mutant combination of *Oak Leaf-Split Perianth-Hose in Hose.* Size bars are approximately 1cm.

Hose failed to be expressed in that particular genetic background. Observation of the plants over a longer period may have resolved this uncertainty as plants carrying *Hose in Hose* generally show some traces of petal tissue in the calyx at sometime during the flowering season, but the remaining progeny were lost. Thirteen recombinants in a population of 59 plants would give a recombination map distance of 22%. This is much greater than previously recorded for *Oak Leaf* to S (3.2%). *Jack in the Green* is only poorly expressed in an *Oak Leaf* background. Some flowers have a recognizable phenotype at first flowering (Fig. 6.6A), but soon the leafy calyx reduces to thin extensions on the edges of the calyx lobes (Fig. 6.6B) and two of the four *Jack in the Green-Oak Leaf* plants were indistinguishable from *wild type* later in the season. The *Jackanapes* phenotype in *Oak Leaf* also has only small leafy extensions to the calyx lobes (Fig. 6.6E).

6.3.2. Combining the pleiotropic mutant phenotype *Oak Leaf* with another pleiotropic mutant phenotype *reduced petal.*

In 2000 a pin *Oak Leaf,* was pollinated from a thrum *reduced petal.* Seed was sown in Dec. 2000 and progeny obtained (Table 6.2) that flowered in 2001, 2002, and 2003.

pin *Oak Leaf* x thrum *reduced petal*

Okl s x *rdp S*

\+ *s* ↓ *rdp s*

Thrum *Oak Leaf*	pin *Oak Leaf*	thrum *wild type*	pin *wild type*
Okl s / *rdp S*	*Okl s* / + *s*	+ *s* / *rdp S*	+ *s* / *rdp s*
7	7	7	9

Table 6.2. Combining *Oak Leaf* with *reduced petal*

As *reduced petal* is not linked to the *S* locus and is a recessive phenotype, a χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from

the predicted ratio of 1:1:1:1 Thrum *Oak Leaf*, pin *Oak Leaf*, thrum *wild type*, and pin *wild type*.

Chi. squared for 1:1:1:1 for above phenotypes,

observe 7:7:7:9. expect 7.5:7.5:7.5:7.5,

χ^2 (3 dof) = 0.4, P = 0.94

Results fit the predicted 1:1:1:1 ratio for above phenotypes.

All of the above progeny will be heterozygous for the recessive *reduced petal* allele. Pin and thrum *Oak Leaf* progeny (Fig.6.7A and B) were crossed together. Five of the eleven progeny were *Oak Leaf* (Fig. 6.7C-G). It was difficult to be certain which, if any, of these carried both mutant alleles. Given the low numbers obtained only one *Oak Leaf–reduced petal* might possibly be expected. The F1 generation of *Oak Leaf*, (from the cross pin *Oak Leaf* x *reduced petal* shown above) all heterozygous for the recessive for *rdp*, had small flowers with normal broad petals at first flowering (Fig. 6.7A.). The following spring all flowers had the attenuated petals associated with the oak leaf phenotype (Fig. 6.7B). Similarly the early leaves were much broader than the leaves normally produced by *Oak Leaf* at first flowering, but later leaves were attenuated (not shown). The F2 generation (from crossing pin and thrum F1 generation *Oak Leaf* progeny carrying the recessive *reduced petal* allele together) also had some small flowers with broad petals at first flowering (Fig 6.7C, E and G), and two others that were very ragged and split (Fig. 6.7D and F). In the second year, again the petals were again attenuated, both in the entire and in the ragged petal forms (Fig. 6.5N and O). Increased expression of the *Oak Leaf* phenotype with time may also be responsible for the observed breakdown in flower form in progeny from crosses with *wild type* (see Chapter 4, Fig. 4.26G). Results indicate that a combination of these two pleiotropic mutant phenotypes has a very detrimental effect on the flowers.

6.3.3. Other combinations of mutant phenotypes.

Combination of *Hose in Hose* and *sepaloid* was undertaken in order to facilitate using a three point cross both as a segregation test and for gene mapping. This is described in Chapter 7. It was found that *Hose in Hose* and *sepaloid* are not expressed together. The *double* phenotype can be combined with any of the whorl 1 mutant phenotypes (Fig.

Figure 6.7. Combining *Oak Leaf* and *reduced petal*.

Oak Leaf-reduced petal combination has a detrimental effect on flower form. Flowers of the same progeny over two flowering seasons are illustrated.

A Progeny from the cross *Oak Leaf x reduced petal*. An *Oak Leaf* plant heterozygous for the recessive allele for *reduced petal* is on the left and a *wild type* plant from the same cross on the right. **B.** The *Oak Leaf* plant heterozygous for the recessive allele for *reduced petal* (on the left in G) as it flowered during the following spring. **C.** *Oak Leaf* progeny no.1 from the cross between pin and thrum *Oak Leaf* plants heterozygous for the recessive allele for *reduced petal* **D.** *Oak Leaf* progeny no.2 from the cross between pin and thrum *Oak Leaf* plants heterozygous for the recessive allele for *reduced petal*. **E.** *Oak Leaf* progeny no.3 from the cross between pin and thrum *Oak Leaf* plants heterozygous for the recessive allele for *reduced petal*. **F.** *Oak Leaf* progeny no.4 from the cross between pin and thrum *Oak Leaf* plants heterozygous for the recessive allele for *reduced petal*. **G.** *Oak Leaf* progeny no.5 from the cross between pin and thrum *Oak Leaf* plants heterozygous for the recessive allele for *reduced petal*. **H.** *Oak Leaf* progeny no.3 as it flowered the following spring. **I.** *Oak Leaf* progeny no.4 as it flowered the following spring. Size bars are approximately 1cm.

6.8B, and C). The cross *Oak Leaf x double* was undertaken but this combination was not successful due to failure of seed germination . As the reduction in petal in the pleiotropic mutant phenotype *reduced petal* could possibly indicate B function impairment, it was crossed to *Hose in Hose* in order to observe any possible interactions. *Hose in Hose* is predicted to be the result of ectopic B

pin *Hose in Hose* heterozygous for the recessive allele *reduced petal* x thrum *reduced petal*.

Hih s + x + *S rdp*
\+ *s rdp* ↓ + *s rdp*

Thrum *Hose in Hose*	pin *Hose in Hose*	Thrum *wild type*	pin *wild type*	Thrum *reduced petal- Hose in Hose*	pin *reduced petal- Hose in Hose*	Thrum *reduced petal*	pin *reduced petal*
Hih s + / + *S rdp*	*Hih s* + / + *s rdp*	+ *s* + / + *S rdp*	+ *s* + / + *s rdp*	*Hih s rdp* / + *S rdp*	*Hih s rdp* / + *s rdp*	+ *s rdp* / + *S rdp*	+ *s rdp* / + *s rdp*
4	1	2	7	3	1	2	1

Table 6.3. Combining *Hose in Hose* with *reduced petal* polyanthus

function (see Chapter 1 section 1.2. and Chapter 4 section 4.3.1). The first generation (*Hose in Hose* x *reduced petal*) produced *Hose in Hose* plants recessive for *reduced petal*. The following cross was undertaken and the results (Table 6.3) classified during 2000/2001. No interactions were evident, the two phenotypes *Hose in Hose* and *reduced petal* can be expressed together (Fig.6.8A).

6.4. Combinations of phenotypes linked to the *S locus* with both long and short homostyle.

The loci linked to the S locus include those for *Hose in Hose, Staminoid Carpels, sepaloid,* and *Oak Leaf*. Of these only *Hose in Hose* and *Oak Leaf* have both normal male and normal female reproductive organs and so are suitable for combination with long and short homostyle.

Figure 6.8. Other combinations of mutant phenotypes.

Other combinations of mutant phenotypes can be expressed together as is shown above.

A. *Hose in Hose-reduced petal* polyanthus. **B.** *Jack in the Green-double* primrose **C**. *Jack in the Green-Hose in Hose (Jackanapes)-double* primrose. Size bars are approximately 1cm.

6.4.1. Crosses with long homostyles.

Normal plants have genotype *gpa/gpa*, pin; or *GPA/gpa*,thrum, (as described in chapter 2) where, *G/g* style length, stigma papilla length, stylar cell length, female mating type; *P/p* male mating type, pollen size; and *A/a* anther position. Rare recombination events within these main components can result in self fertile long homostyles with genotype *gPA/gpa*. Wild populations of these long homostyles exist in Somerset, one of which is at at Wyke Champflower (ST656339). The site is the west-facing slope of a hedge bank running along a field boundary, with a road on the other side of the hedge and with a convenient public footpath running through the field. In spring of 1999 pollen was collected from the site for use in these crosses. Homozygous pin *Hose in Hose* primroses (genotype *Hih gpa/ Hih gpa*), were pollinated with long homostyle pollen. Seed was sown in Dec. 1999. Results (Table 6.4) were classified in 2000/2001.

Pin *Hose in Hose* x long homostyle

$$\frac{Hih\ \ gpa}{Hih\ \ gpa} \times \frac{+\ \ gPA}{+\ \ gpa}$$

Long Homostyle *Hose in Hose*	pin *Hose in Hose*
$\frac{Hih\ \ gpa}{+\ \ gPA}$	$\frac{Hih\ \ gpa}{+\ \ gpa}$
2	6

Table 6.4. Combining *Hose in Hose* with long homostyle

As *PA* is dominant expect half of the progeny to be long homostyle *Hose in Hose* and half pin *Hose in Hose*. As χ^2 analysis requires expected numbers of progeny to be greater than 5 numbers above are too low for statistical analysis. The results do show that *Hose in Hose* and long homostyle can be expressed together (Fig. 6.7A).

The *Okl* locus is also linked to the *S* locus but not so tightly as *Hlh*. A long homostyle *Hose in Hose* primrose was crossed to a pin *Oak Leaf* primrose and results classified in 2002 and 2003.

Pin *Oak Leaf* x Long homostyle *Hose in Hose*

Okl gpa + x + *gpa Hih*

\+ gpa + ↓ + *gPA* +

Long Homostyle *Oak Leaf*	Pin *Hose in Hose*	Long Homostyle	pin *Hose in Hose-Oak Leaf*
Okl gpa + / + *gPA* +	+ *gpa Hih* / + gpa +	+ gpa + / + *gPA* +	*Okl gpa* + / + *gpa Hih*
7	9	9	2

Table 6.5. Combining *Oak Leaf* with long homostyle

In addition to the progeny recorded in Table 6.5, one pin *Oak Leaf* was recorded, a probable recombinant between *Hih* and *gPA* .

As all the loci are linked expect a ratio of 1:1:1:1 for Long Homostyle *Oak Leaf*: Pin *Hose in Hose*: Long Homostyle : pin *Hose in Hose-Oak Leaf*. A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio.

χ^2 for 1:1:1:1 for the above phenotypes,

observe 7:9:9:2. expect 6.75:6.75:6.75:6.75,

χ^2 (3 dof) = 4.85, P = 0.183

Results fit the predicted 1:1:1:1 ratio.

Results show that *Okl* and *gPA* can be expressed together to give long homostyle *Oak Leaf* plants.

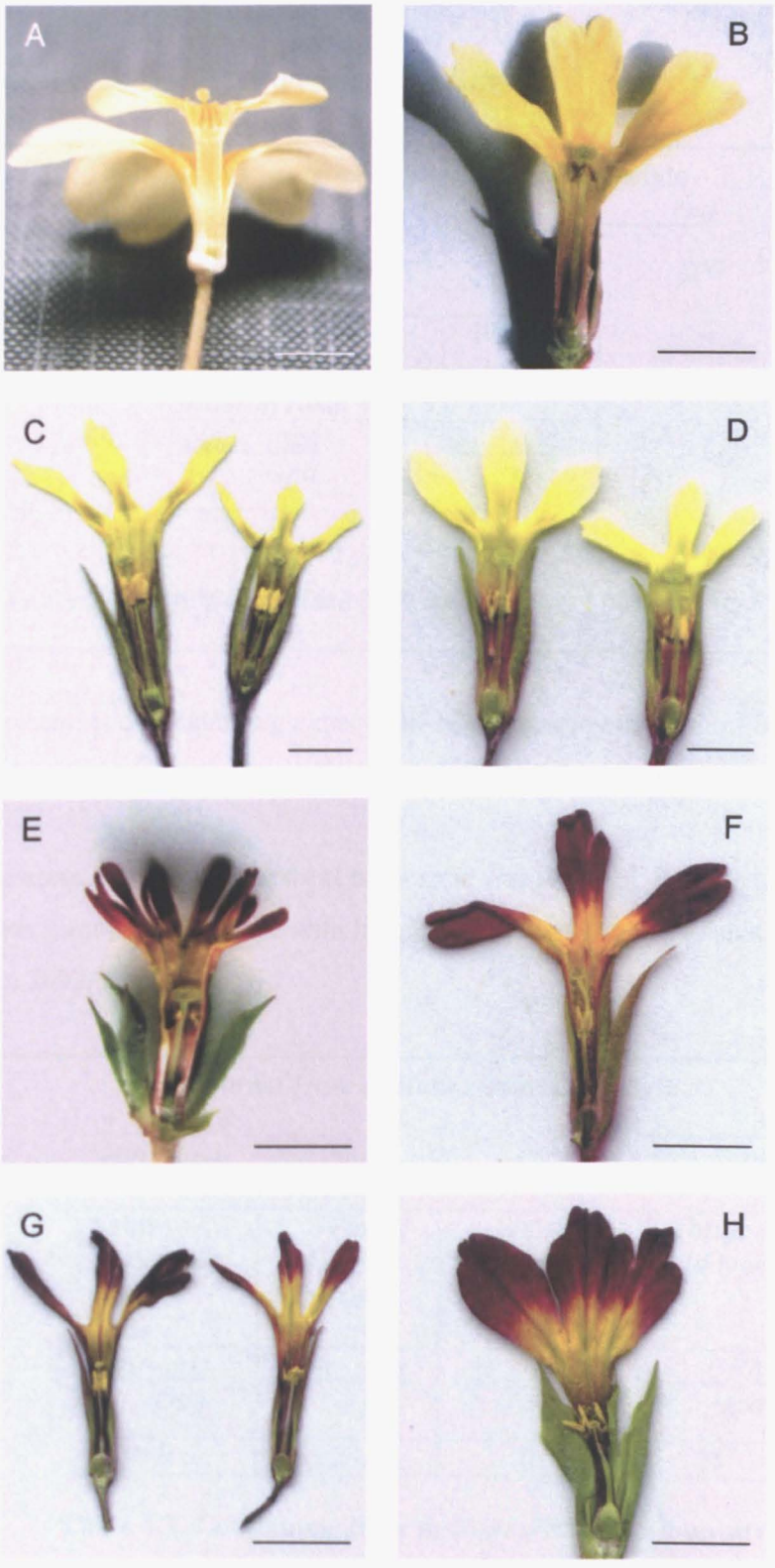
6.4.2. Crosses with short homostyles.

The majority of wild primrose plants have genotype *gpa/gpa*, pin; or *GPA/gpa*, thrum. Rare recombination events within these main components can result in self fertile short homostyles with genotype *Gpa/gpa*. Two short homostyle primroses were obtained from Prof. A. J. Richards in spring 1998. Self-pollinated seed from these plants resulted in two more short homostyle primroses and one pin wild type (seed set was normal but germination was very poor).

Figure 6.9 Combinations of phenotypes with loci linked to the *S* locus with long and short homostyles.

Phenotypes with loci linked to the *S* locus were combined with long and with short homostyles. Each flower is dissected in two to show the position of the anthers and the stigma.

A. Long homostyle *Hose in Hose* flower. **B.** Long homostyle *Oak Leaf* flower. **C.** *Oak Leaf* flowers with shorter than normal pin style in the mature flower (left) from the cross pin *Oak Leaf* x short homostyle. An immature newly opened flower (right) has a style that appears longer, but growth of the corolla tube will raise the anthers further by maturity. **D.** *Oak Leaf* flowers with pin styles of more usual length. **E.** *Oak Leaf* flower no.1 from the cross thrum *Oak Leaf* x short homostyle. Style length is between the expected length for pin and the expected length for thrum. **F.** *Oak Leaf* flower no.2 from the cross thrum *Oak Leaf* x short homostyle. Style length is as pin. **G.** *Oak Leaf* flowers nos.3a and 3b from the cross thrum *Oak Leaf* x short homostyle. Style length is as short homostyle and self pollination appears to be taking place. However examination of the stigmatic papillae under an x20 hand lens showed the papillae to be long. **H.** *Oak Leaf* flower no.4 from the cross thrum *Oak Leaf* x short homostyle. Style length is as short homostyle. Examination of the stigmatic papillae under an x20 hand lens showed the papillae to be short. Size bars are approximately 1cm.


A
B
C
D
E
F
G
H

The cross homozygous pin *Hose in Hose* x yellow short homostyle was carried out in 1999 and progeny classified in 2000. Results are shown in Table 6.6.

The reciprocal cross failed due to infection by *botyritis.*

Homozygous pin *Hose in Hose* x short homostyle

Hih gpa x *+ ?pa*

Hih gpa ↓ *+ gpa*

Short Homostyle *Hose in Hose*	Pin *Hose in Hose*
Hih gpa / *+ Gpa*	*Hih gpa* / *+ gpa*
0	38

Table 6.6. Combining *Hose in Hose* with short homostyle, 1

As *G* , if present, is dominant to *g* expect half of the progeny to be short homostyle *Hose in Hose* and half pin *Hose in Hose*. There is a deficit of short homostyle progeny.

In 2002 the cross *Hose in Hose* x short homostyle was repeated, but this time using a heterozygous thrum *Hose in Hose* with linkage *Hih* to pin. Results (Table 6.7) were classified in 2002/3.

Thrum *Hose in Hose* x short homostyle

+ GPA x *+ ?pa*

Hih gpa ↓ *+ gpa*

Short Homostyle *Hose in Hose*	Pin *Hose in Hose*	Short Homostyle	Thrum *wild type*
Hih gpa / *+ Gpa*	*Hih gpa* / *+ gpa*	*+ Gpa* / *+ gpa*	*+ GPA* / *+ gpa*
0	23	0	28

Table 6.7. Combining *Hose in Hose* with short homostyle 2

If *G* is present in the short homostyle genotype and if *GG* is viable, expect a ratio of 1:1:1:1, of short homostyle *Hose in Hose*, Pin *Hose in Hose,* Short Homostyle, and thrum *wild type*. No short homostyle plants were obtained among 51 progeny therefore the results do not fit the predicted ratio.

In order to investigate whether short homostyle and *Oak Leaf* could be expressed together, both pin and thrum *Oak Leaf* plants were pollinated with short homostyle pollen. The results were classified in 2003 (Tables 6.8. and 6.9).

Pin *Oak Leaf* x short homostyle.

OKL gpa x *+ ?pa*

Plants were + *gpa* ↓ + *gpa*

Short Homostyle *Oak Leaf*	pin *Oak Leaf*	Short Homostyle	pin *wild type*
OKL gpa / + *Gpa*	*OKL gpa* / + *gpa*	*+ Gpa* / + *gpa*	*+ gpa* / + *gpa*
0	16	0	17

Table 6.8. Combining *Oak Leaf* with short homostyle, 1

If no recombination occurs between *Oak leaf* and the *S locus* and as both *Oak leaf* and *G*, if it is present in the short homostyle genotype, are dominant expect a ratio of 1:1:1:1 of Short Homostyle *Oak Leaf,* pin *Oak Leaf,* Short Homostyle, and pin *wild type.* As no short homostyle plants were obtained among 33 progeny the results clearly do not fit the above ratio.

Thrum *Oak Leaf* x short homostyle

OKL gpa x *+ ?pa*

+ GPA ↓ + *gpa*

Short Homostyle *Oak Leaf*	Pin *Oak Leaf*	Thrum *wild type*	pin *wild type*
OKL gpa / + *Gpa*	*OKL* / *gpa* / + *gpa*	*+ GPA* / + *gpa*	*+ gpa* / + *gpa*
1	14	24	1

Table 6.9. Combining *Oak Leaf* with short homostyle 2

As both *Oak Leaf* and *G* if it is present in the short homostyle genotype, are dominant, and if *GG* is viable, then expect a ratio of 1:1:1:1 for Short Homostyle *Oak Leaf*, Pin*Oak Leaf*, Thrum *wild type* and pin *wild type*. With only 1 pin *wild type* and 1 short homostyle *Oak Leaf* results do not fit the above ratio.

All plants from cross 1 above were classified as pin form, but some *Oak Leaf* plants at maturity had flowers with shorter pin styles than normal (Fig. 6.9C), while others had styles of normal or near normal length (Fig. 6.9D). Many of the *Oak Leaf* pin progeny from cross 2 above had shorter than normal styles (Fig. 6.9.E), while others were of normal or near normal length (Fig. 6.9F). At first flowering all were classed as pin, but later in the flowering season a few plants were found to be producing flowers with sufficiently short styles to be classed as short homostyle (Fig. 6.9G and H). These flowers also appeared to self-pollinate. On examination with an x20 hand lens all plants but one (Fig. 6.9H), including that shown in Fig. 6.9G, were found to have long pin form stigmatic papillae. The short homostyle pollen parent had short thrum like stigmatic papillae. No short styled pin *wild type* progeny were found among progeny from *Oak Leaf* crosses. Style length in the short styled pin *Oak Leaf* flowers varied from flower to flower on the same plant, so that some appeared as short homostyle and some as short styled pin (as in e.g. Fig. 6.9E). Style length on the short homostyle pollen parent was not variable in this manner. The stigma was always below the anthers when flowers first opened and subsequent growth of the corolla tube raised the anthers to the point where self-pollination took place. When the stigma is above the anthers as the flower opens, anthers and stigma never meet and self-pollination never takes place. Only the single plant that had short stigmatic papillae as well as a short style was therefore classed as a short homostyle *Oak Leaf* (Fig. 6.9 H), but it requires confirmation by test crossing to *wild type*. Unfortunately the plant was lost before it could be used for experimental breeding. It was already known that style length in *Primula* could be affected by genes that are not part of the *S locus* gene complex (Richards, personal communication 1988) and this may be the explanation for many of the short styled pin *Oak Leaf* progeny.

6.5. Investigation of whether the mutant phenotype is associated with the organ or with the whorl in which it occurs.

The *sepaloid* phenotype provided an ideal medium for the above experiment. The aim was to alter the form of the *sepaloid* organs and observe whether or not the altered form was expressed in the inner whorls of a *sepaloid* flower. Pin *Split Perianth* pollen and pin

Jack in the green heterozygous for the recessive *sep* allele x *Split Perianth* heterozygous for the recessive *sepaloid* allele

Jig + + x + + *Spr*

\+ *sep* + ↓ + *sep* +

Jack in the green	*Jack in the green- Split Perianth*	*Split Perianth*	*wild type*	*sepaloid*	*Jack in the green- sepaloid*	*Split Perianth- sepaloid*	*Jack in the green- Split Perianth- sepaloid*
Jig + + + ? +	*Jig* + + + ? *Spr*	+ + *Spr* + ? +	+ + + + ? +	+ *sep* + + *sep* +	*Jig sep* + + *sep* +	+ *Sep Spr* + *sep* +	*Jig sep* + + *sep Spr*
6	0	5	5	2	3	2	2

Table 6.10. Combining *sepaloid* with *Jack in the Green* and *Split Perianth*

Jack in the Green pollen was used to pollinate *sepaloid* seed parents in 1998. All progeny carried the recessive allele for *sepaloid.* All progeny were also pin, thus confirming the genotype of the parent *sepaloid* plants. A cross between *Jack in the Green* carrying the recessive allele for *sepaloid* and *Split Perianth* also carrying the recessive allele for *sepaloid* was undertaken in 1999 and progeny classified in 2000. Results are classified in Table 6.10. Despite the numbers being small the majority of expected phenotypes were obtained. The absence of *Split Perianth Jack in the Green* is unexpected given that this combination of phenotypes had previously been obtained from other crosses. sep*aloid Jack in the Greens* (Fig. 6.10A), *Split Perianth sepaloids* (Fig. 6.10B) and *Split Perianth sepaloid Jack in the Greens* (Fig. 6.10C) all exhibited the mutant phenotype on the inner whorls of sepals. This demonstrates that the mutant phenotype is associated with the organ rather than the whorl.

Another cross, done and classified at the same time, of *sepaloid x Split Perianth* heterozygous for the recessive allele for *sepaloid* gave results that agreed with the above

sepaloid x Split Perianth heterozygous for the recessive allele for *sepaloid*

sep + x + *Spr*

sep + ↓ *sep* +

Split Perianth	*Split Perianth-sepaloid*	*wild type*	*sepaloid*
+ *Spr* *sep* +	*sep Spr* *sep* +	+ + *sep* +	*sep* + *sep* +
3	3	3	2

Table 6.11. Combining *sepaloid* with *Split Perianth*

in that the *Split Perianth sepaloid* plants from this cross also exhibited the mutant phenotype on inner whorls. Results from the above cross are classified in Table 6.11. As there is no linkage between *sep* and *Spr* expect equal numbers of *Split Perianth, Split Perianth- sepaloid, wild type*, and *sepaloid.* As the number of progeny obtained was small, numbers were too low to do a χ^2 analysis.

Further evidence that the mutation is associated with the organ rather than with the whorl in which it occurs was obtained accidentally, when testing different forms of *double* for allelism. A *Jack in the Green double* was obtained with organ order of leafy calyx, petal, petal, leafy calyx, enclosing naked ovules (Fig. 6.10D). In this plant the sepals in whorl four were also converted to leaf form as were the sepals in the first whorl.

6.6. Discussion of combinations of mutant phenotypes..

It was found that the majority of mutant phenotypes can be expressed together. *Hose in Hose* and *sepaloid* are the only phenotypes that cannot be expressed together (see also Chapter 7), *Hose in Hose* is predicted to be due to ectopic B function and *sepaloid* allele is predicted to be due to lack of B function. If *Hose in Hose* and *sepaloid* are allelic mutations then the *Hose in Hose* allele is dominant to the *sepaloid* allele. If they are two separate genes then *Hose in Hose* is epistatic to *sepaloid* as the presence of *Hose in Hose* masks the presence of *sepaloid.*

Figure 6.10. Combinations of phenotypes that show the mutant phenotype to be associated with the organ rather than the whorl in which it occurs.

Combination of *sepaloid* with two other whorl 1mutant phenotypes show the mutant phenotype to be associated with the organ rather than the whorl in which it occurs.

A. *Jack in the Green-sepaloid.* **B.** *Split Perianth-sepaloid.* **C.** *Split Perianth-Jack in the Green-sepaloid.* **D.** *Jack in the Green-double* with organ order of leafy sepal, petal, petal, leafy sepal, naked ovules. Both the sepals in whorl one and the sepals in whorl four are converted to leaves. Size bars are approximately 1 cm.

6.6.1. Combinations of whorl 1 mutant phenotypes.

Jackanapes calyces that combine *Hose in Hose* and *Jack in the Green* (Webster and Grant 1990) showed interactions between cell types at the junctions between petal and leaf cells. Some junctions were sharply defined (e.g. Fig. 6.2C and F), but the cells adjacent to the petal cells in these instances were not exactly the same in appearance as either normal leaf cells (Fig. 6.2. D) or the green cells of the *Jackanapes* leafy tip (Fig. 6.2E). Other junctions exhibited gradual change in cell shape and many cells appeared chimeric (e.g. Fig. 6.2G, H, and I). In previous studies of *Antirrhinum* (Perbal *et al*, 1996) boundaries between wild type and mutant cells in sectorial chimeras were observed to be sharp. This observation was the basis for their conclusion that there was absence of *DEF*-mediated lateral communication between cells. Images of boundaries that are clearly not sharp in *Primula* may indicate lateral communication in this instance given that *DEF* and *GLO* are required for petal tissue. Transport of *DEF* between cell layers was found to be polar in *Antirrhinum* (Perbal *et al*, 1996; Efremova *et al*, 2001). The epidermal layer of petal cells that can be observed to overlie green tissue on the adaxial surface of *Primula Jackanapes* calyces may indicate similar polar transport (Fig. 6.1 A). Were it otherwise, the product might have been transported to the layer beneath thus converting the tissue to petal. Some differences in the efficacy of B-function-mediated cell communication was found between *Antirrhinum* and *Arabidopsis* (Efremova *et al*, 2001) and so there will be scope for further study on communication between cells in *Primula*, especially investigation of possible *DEF*-mediated lateral communication between cells.

Combining phenotypes first whorl mutant phenotypes with the *Split Perianth* phenotype showed that all of these could be expressed together. The most notable interaction was the reflexing of the calyx in combinations of both *Split Perianth* and *Hose in Hose*, and *Split Perianth* and *Jackanapes* both of which have petal tissue at the base of the calyx. Some were only slightly reflexed as in Fig. 6.4A, but most were extremely reflexed as in Fig. 6.4C.

6.6.2. Other combinations of mutant phenotypes.

Combining *Oak Leaf* with other mutant phenotypes similarly showed that the mutant phenotypes could be expressed together (as shown in Fig.6.4). Notable was the weak expression of *Jack in the Green* in an *Oak Leaf* background, and the deterioration in flower quality in flowers from the cross pin x thrum *Oak Leaf*, recessive for *reduced*

petal. That the petals of the latter flowers became attenuated in the second flowering season is in accord with previous results from crossing *Oak Leaf* and *wild type*. Some flowers from the latter crosses had near normal petals that either became attenuated in the second season or became ragged later in the first season. This phenomenon is therefore unlikely to be the result of combination of phenotypes.

The possibility, suggested by the number of increased number of recombinants in section 6.3.1 (table 6.3) that combining the *Oak Leaf* and *Hose in Hose* mutant phenotypes might increase recombination is interesting. A similar possible increase in recombination was observed in one of the crosses designed to test viability of the *Oak Leaf* homozygote, (Chapter 5, section 5.11.1 and Table 5.59), namely thrum *Oak Leaf* x pin *Hose in Hose Oak Leaf*. Reduction of recombination rate by selection has been reported as long ago as 1921 (Detleson and Roberts, 1921) and increase of recombination rate by selection in 1958 (Parsons, 1958). A similar possible increase in recombination was observed in one of the crosses designed to test viability of the *Oak Leaf* homozygote, (Chapter 5), (namely thrum *Oak Leaf* x pin *Hose in Hose Oak Leaf*). The possibility of *Hose in Hose* failing to be expressed in some genetic backgrounds would also explain the anomaly. If this were the case the *Hose in Hose* progeny would be expected to appear as *wild type*. This could explain the results for the cross in chapter 5, but not the above cross where no *wild type* progeny at all were obtained. If plants are retained for a number of flowering seasons it is usual to discover at least some calyces with traces of petal tissue in any plant that carries the *Hose in Hose* allele, however poorly expressed. It must be noted that 17 seedlings were lost before maturity from the cross shown in table 6.3, and 14 mature plants, of which 11 were oak leaf and 3 *wild type*, had failed to bloom at the time of scoring the results. Results of the reciprocal three point cross of thrum *Hose in Hose Oak Leaf* pollinated by *wild type* (see Chapter 7), may help elucidate the problem of whether combining *Hose in Hose* with *Oak Leaf* does indeed increase recombination rate.

Another combination made was *reduced petal* and *Hose in Hose*. This combination produced flowers with the petaloid calyces of *Hose in Hose* but with the characteristic loss of colour associated with *reduced petal* on the adaxial surface of both the whorl one and the whorl 2 petals. The *reduced petal* phenotype has not yet been combined with *Jack in the Green, Jackanapes, Split Perianth* or *double*. The *double* mutant phenotype has not yet been combined with *Split Perianth, reduced petal* or *Oak Leaf*.

6.6.3. Combination of *Hose in Hose* and *Oak Leaf* with long and short homostyle.

Combinations were made of both *Hose in Hose* and *Oak Leaf* to long homostyle. Each of these was shown to have linkage to the *S locus*, *Hose in Hose* to the *S* allele (Chapter 5, section 5.3.1 and Webster and Grant, 1990) and *Oak Leaf* to the *s* allele (Chapter 5, section 5.11). Consequently it was of interest to discover whether there might be any interactions between these linked genes. None were found both phenotypes were expressed with long homostyle without any interactions (Fig. 6.9A and B).

Combinations of *Hose in Hose* and *Oak Leaf* with short homostyle were problematic. Crosses of both pin *Hose in Hose* with short homostyle, and thrum *Hose in Hose* with short homostyle, failed to produce any short homostyle progeny. Pin *Hose in Hose* progeny had styles of normal length. Previous findings indicate that a deficiency of short homostyle progeny might be expected due to low viability of the morph (Lewis and Jones, 1993), but to obtain none at all from a total of 89 progeny is unexpected.

Crosses of pin *Oak Leaf* with short homostyle, and thrum *Oak Leaf* with short homostyle, resulted in a number of *Oak Leaf* progeny of ambiguous phenotype, particularly among the progeny from the thrum *Oak Leaf*. The *wild type* progeny were unambiguous. The majority of pin *Oak Leaf* progeny from the former cross had normal, or near normal, pin length styles, but some had styles that were slightly shorter than normal (Fig. 6.7C and D). The majority of styles in *Oak Leaf* progeny from the latter cross were of shorter than normal length (Fig. 6.7E), although a few were of normal length (Fig. 6.7F). Later in the flowering season style length in some flowers became sufficiently short as to present as short homostyle (Fig. 6.7G). Although style length is controlled by G/g it is also affected by polygenic modifiers (Ford, 1964; Richards, pers.com. 1989). Examination of the stigmatic papillae found only one plant with short stigmatic papillae, and this one only was originally classified as short homostyle *Oak Leaf* (Fig. 6.7 H). However in spring 2005 the flowers were pin, with long stigmatic papillae and with shorter than normal style. In whorl four stylar cell length and stigmatic papillae length are considered to be developmental correlates of stigma height (Richards, 1993, 1997). Results indicate that the short homostyle phenotype parent does not have the genotype *Gpa/gpa* and that the phenotype must have a different genetic origin. All but one of the short styled pin *Oak Leaf* plants had distinctively long stigmatic papillae. Some of these have been retained for further observation.

6.6.4 Combinations that illustrate the association of the mutant phenotype with the organ rather than with the whorl in which it occurs.

There have been numerous previous reports of homeotic mutants with organs that are normal being produced in the inappropriate whorl (e.g. Bowman *et al.* 1989; Bowman *et al.* 1991; Bradley *et al.* 1993; Jack *et al.* 1997). By combining the *sepaloid* mutant phenotype with both *Split Perianth* (Fig.6.10B) and *Jack in the Green* (Fig. 6.10A) flowers with both abnormal sepals, and sepals in inappropriate whorls were produced. The *Split Perianth/sepaloid* organs, although divided into individual sepals in an abnormal manner, were none the less clearly sepals. Similarly, the *frl 1 ag Arabidopsis* flower (Hase *et al*, 2000) had frilled petals in inappropriate whorls that were clearly petals. In contrast, the *sepaloid/Jack in the Green* (Fig. 6.10A), the *Split Perianth/sepaloid/Jack in the Green* (Fig. 6.10C), each had homeotic conversion of sepals to another organ form that were also produced in inappropriate whorls (see Fig.6.10). In addition the *double/Jack in the Green* with organ order of leafy sepal, petal, petal, leafy sepal, shown in Fig. 6.10D, has both the whorl one and the whorl four sepals converted to leaf. These examples clearly illustrate that each mutant phenotype observed here is associated with the organ, rather than with the whorl in which it occurs.

6.6.5. Summary of results.

From the above analysis it can be concluded that:

1. *Hose in Hose* is either dominant to *sepaloid* (if *Hose in Hose* and *sepaloid* are allelic mutations) or epistatic to *sepaloid* (if they are two different genes) as the two phenotypes are not expressed together, (See also Chapter 7).

2. The mutant phenotype is associated with the organ rather than the whorl.

3. Other interactions include some effects on the phenotype, such as for example, combining *Split Perianth* with *Hose in Hose* results in whorl 1 petals that are extremely reflexed and *Jack in the Green* is observed to be poorly expressed in an *Oak Leaf* background. It was also observed that combining *reduced petal* with *Oak Leaf* has a detrimental effect on flower form.

Having examined dominance relationships and linkage in Chapter 5, and genetic interactions in Chapter 6 above, the majority of the mutant phenotypes are not analysed further. Only three phenotypes, *Hose in Hose*, *Oak Leaf*, and *sepaloid* are used both for

investigation of linkage analysis and investigation of possible allelism of *Hose in Hose* and *sepaloid* (see Chapter 7).

CHAPTER SEVEN

Linkage analysis.

7.1. Introduction.

The discovery of four genes that show linkage to the *S* locus, *Hose in Hose*, *Staminoid Carpels*, *sepaloid* and *Oak Leaf* provided opportunity to develop a linkage map of genes surrounding the *S* locus. It is possible that *Staminoid Carpels* is a second allele of *Hose in Hose*. As this requires further investigation *Staminoid Carpels* was not included in the following linkage analysis.

It could be predicted that *Hose in Hose* and *sepaloid* could be gain and loss of B function. They could be allelic mutations or two separate but linked genes. If the latter, they could be on the same or on opposite sides of the *S* locus. If allelic mutations, they would be the first example of B function complementary phenotypes. If they were two separate genes this would be the first example of a B function gene and a regulator of B being tightly linked. One object of this study was to investigate whether one gene or two is responsible for the phenotypes. A second object, if two genes should be found to be involved, was to discover gene order. As the dominant nature of the *Hose in Hose* mutation precludes complementation tests three point crosses were used both as segregation tests and for mapping genes linked to the *Primula S* locus. Not all *sepaloid* plants are fertile (see Chapter 4, 4.2.7), and those that are frequently have only a limited number of fertile flowers on each plant. Both this, and the very tight linkage of *Hose in Hose* and *sepaloid* to the *S* locus presented impediments to the investigation.

Oak Leaf is less tightly linked to the *Primula S* locus (Chapter 5, 5.11; Tables 5.54 and 5.58) and can be combined with *Hose in Hose*. The genotype (*Okl s* + / + *S Hih*) provided a good basis for undertaking a second three point cross to look for gene order, and was used to pollinate pin *wild type*..

A third opportunity for mapping gene order was provided by pollinating a pin *sepaloid* (+ *sep* s/+ *sep* s) with pollen from a thrum *Oak Leaf* parent (Okl S +/+ + +). The latter was the result of recombination between *Okl* and *s* that brought *Okl* and the *S* allele into coupling. As the *sepaloid* phenotype is recessive, all *Oak Leaf* progeny from this cross will be heterozygous recessive for *sepaloid*. These thrum *Oak Leaf* progeny were used to pollinate pin *sepaloid*. As with all crosses involving *sepaloid*, an initial difficulty was that

of obtaining enough seed from *sepaloid* plants that have limited fertility, in order to produce a sufficiently large number of progeny.

7.2. Investigation to determine whether *Hose in Hose* and *sepaloid* are allelic.

Investigation of the relationships between the tight linkage of *Hose in Hose* and *sepaloid* to the *S* locus was undertaken to determine whether two genes were involved or whether the two mutant phenotypes may be allelic. In order to do this four crosses were carried out, including use of the three point cross for gene mapping, shown in Table 7.1, as an alternative to complementation since *Hose in Hose* is a dominant phenotype. In 1996 a pin *sepaloid* had been pollinated by a *Hose in Hose* plant of genotype *Hih S / Hih S*. Not many progeny resulted from this cross, and of these most failed to survive. Only four thrum *Hose in Hose* plants survived to maturity, each heterozygous recessive for *sepaloid* and providing a suitable genotype (*+ S Hih / sep s +*) for a three point cross. As gene order was unknown, the hypothesis that *Hose in Hose* and *sepaloid* may not be separate genes was tested. In 1998 the following crosses were carried out. Cross no.1: pin *sepaloid* x thrum *Hose in Hose*, heterozygous for the recessive *sepaloid* allele (*sep s + / sep s +* x *+ S Hih / sep s +*). Cross no.2: *Wild Type* pin, heterozygous for the recessive *sepaloid* allele x *Hose in Hose* thrum, heterozygous for the recessive *sepaloid* allele (*+ s + / sep s + x + S Hih / sep s +*). In order to attempt to overcome the difficulty of obtaining enough progeny Cross no. 1 was repeated in subsequent years. Results were classified from 2000 onwards. The same *Hose in Hose* plant (genotype *+ S Hih / sep s +*) was used as parent to plants classified in 2000-2002, but was subsequently lost. A different *Hose in Hose plant* (genotype *+ S Hih / sep s +*), one of the progeny classified in 2002 was used for the final pollinations. Twenty green seedlings from this cross were lost before maturity through fungal infection and could not be classified. If *Hose in Hose* and *sepaloid* are two separate genes then a recombination event could result in an occasional *wild type* plant among the progeny. If linkage is very tight expect 1:1 pin sepaloid : Thrum *Hose in Hose*. A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio.

χ^2 for 1:1 pin *sepaloid* : thrum *Hose in Hose*

Observe 113:82; expect 97.5:97.5

χ^2 (1 dof) = 4.93, P = 0.026

Results show that there is a deficiency of thrum *Hose in Hose*.

Pin *sepaloid* x thrum *Hose in Hose* heterozygous for the recessive allele for *sepaloid* (gene order unknown)

$$\frac{sep\ s\ +}{sep\ s\ +} \times \downarrow \frac{+\ S\ Hih}{sep\ s\ +}$$

	sepaloid	*Thrum Hose in Hose*	*pin Hose in Hose*	Thrum *wild type*	pin *wild type*
	$\frac{sep\ s\ +}{sep\ s\ +}$	$\frac{+\ S\ Hih}{sep\ s\ +}$	$\frac{+\ s\ Hih}{sep\ s\ +}$	$\frac{+\ S\ +}{sep\ s\ +}$	$\frac{+\ s\ +}{sep\ s\ +}$
2000	14	9	0	0	0
2002	48	35	0	0	0
2003/4	51	38	1	0	0
	113	82	1	0	0

Table 7.1. Cross 1. Three point cross to investigate whether *Hose in Hose* and *sepaloid* are allelic.

As the seedlings from 2003 began to germinate it was observed that there were a large number of pale yellow or white seedlings. Some of the pale yellow seedlings survived but all of the white seedlings died. A count was made of numbers of green (110) and of white/pale seedlings (32). If a lethal gene for white seedlings is carried in both parents and is unlinked to either *Hose in Hose* or *sepaloid* then expect a ratio of 3:1 for green to white or pale seedlings. A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio.

χ^2 for 3 : 1 green : white/pale seedlings

Observe 110:32; expect 106.5:35.5

χ^2 (1 dof) = 0.46, P = 0.498.

Results do not quite fit the ratio of 3:1 green : white/pale seedlings.

Twelve of the pale seedlings (Fig. 7.1, A and B) survived to maturity and were classified as seven *Hose in Hose* and five *sepaloid*, confirming that the lethal gene for pale/white seedlings is not linked to either *Hose in Hose* or *sepaloid*. Twelve pale seedlings with leaves that later became green, survived and grew to maturity (Figure 7.1). Seed set from controlled pollination failed, and despite growing strongly at first (Figure 7.1C) the plants began to deteriorate later (Figure 7.1D) and have since been lost.

Figure 7.1. Progress of pale leaf seedlings to maturity.

Pale leaf seedlings appeared able to develop chlorophyll in the leaves, but only slowly. They continued to grow until they flowered, but both failed to set seed and failed to continue to develop chlorophyll later in the flowering season.

A. Pale leaf seedlings. Note the greening of the older leaves. **B.** Pale leaf plants with new leaves pale and older leaves green. **C.** Pale leaf *Hose in Hose* at first flowering. **D.** Pale leaf *Hose in Hose* later in the flowering season. No further greening of the leaves took place and the plants subsequently died. Size bars are approximately 1 cm.

It is possible that there is a problem of viability linked to *Hose in Hose* in this line, but this requires further investigation and the excess number of *sepaloid* progeny obtained from this three point cross cannot currently be explained. One recombinant plant with the phenotype pin *Hose in Hose* was obtained. However a recombination event that gives a *wild type* plant was required in order to establish that there are two genes involved. There could possibly have been other recombinants among the seedlings lost before maturity. Should *Hose in Hose* (predicted ectopic B function) and *sepaloid* (predicted absence of B function) be allelic, with *Hose in Hose* dominant to *sepaloid*, recombination between this locus and the *S* locus would result in a pin *Hose in Hose*. Should the cross be repeated it would be essential to obtain larger numbers of progeny as was possible for linkage analysis of *Oak Leaf, sepaloid* and the *Primula S* locus (see 7.4). The very tight linkage between *sep* and *s* means that a minimum of several hundred progeny is required before a recombinant could actually be expected. Further evidence is required to confirm that *Hose in Hose* and *sepaloid* are two separate genes.

Cross no. 2. Pin *Wild Type* heterozygous for the recessive allele for *sepaloid* was pollinated by thrum *Hose in Hose* heterozygous for the recessive allele for *sepaloid* and the results classified in Table 7.2.

Wild type pin, heterozygous for the recessive allele for *sepaloid* x *Hose in Hose* thrum heterozygous for the recessive allele for *sepaloid*

$$\frac{+\ s\ +}{sep\ s\ +} \times \downarrow \frac{+\ S\ Hih}{sep\ s\ +}$$

sepaloid	Thrum *Hose in Hose*	Pin *Hose in Hose*	Thrum *wild type*	Pin *wild type*
$\frac{sep\ s\ +}{sep\ s\ +}$	$\frac{+\ S\ Hih}{?\ s\ +}$	$\frac{sep\ s\ Hih}{?\ s\ +}$	$\frac{+\ S\ +}{+\ s\ +}$	$\frac{sep\ s\ +}{+\ s\ +}$
6	18	1	1	6

Table 7.2. Results of cross no. 2. Second test to investigate whether *Hose in Hose* and *sepaloid* are allelic

Results from the second cross show two possible recombinants, pin *Hose in Hose* and thrum *wild type*. The latter plant was lost. The tables for crosses 1 and 2 show the genotypes for 2 genes on opposite sides of the S locus but alternative genotypes for the cross should *Hih* and *sep* be allelic, are $\frac{hihsep\ s}{hihsep\ s}$ x $\frac{Hih+\ S}{hihsep\ s}$. In this case recombination could occur between *hihsep* and the *S* locus to result in pin *Hose*

in Hose and thrum *wild type*. The resultant genotype of the pin *Hose in Hose* could be either (A) *hihsep s / Hih+ s* or (B) *Hih+ s / hih+ s*.

If *Hose in Hose* and *sepaloid* are two separate genes it is not known whether the genes are on opposite sides of the *S locus* or on the same side of the *S* locus. If they are on the same side the predicted order would be *hih sep s* since no confirmed recombinants between *sep* and the *S locus* have been obtained so far, thus indicating complete linkage between *sep* and *s*. If *Hose in Hose* and *sepaloid* are on the same side of the *S* locus possible genotypes for a pin *Hose in Hose* resulting from recombination between *sep* and *s* are (C) *hih + s / Hih + s* and (D) *hih sep s / Hih + s*. Possible genotypes for pin *Hose in Hose* from recombination between *hih* and *sep* are (E) *hih + s / Hih sep s* and (F) *hih sep s / Hih sep s*.

If as postulated in the tables (7.1-7.4) there are 2 genes on opposite sides of the *S* locus then recombination between *Hih* and *S* could give possible genotypes for the pin *Hose in Hose* as (G) *sep s Hih / + s hih* and (H) *sep s hih /sep s Hih*.

Cross no. 3. The pin *Hose in Hose* (putative genotype *sep s Hih/+ s +*) was backcrossed to pin *sepaloid* (sep s + /sep s +) in 2000, as shown in Table 7.3, and results were classified in 2001.

Pin *Hose in Hose* backcrossed to pin *sepaloid*

sep s + x *sep s Hih* (putative genotype)

sep s + ↓ *+ s +*

sepaloid	*Hose in Hose*	Pin *wild type*
sep s + / *sep s +*	*sep s Hih* / *sep s +*	*+ s +* / *sep s +*
1	7	17

Table 7.3. Results of cross no. 3. Third test to investigate whether *Hose in Hose* and *sepaloid* are allelic

Only genotypes (from A- H) above that predict results of pin *Hose in Hose* and pin *wild type* can be considered. If *sepaloid* and *Hose in Hose* are allelic then genotype (B) above would give pin *Hose in Hose* and pin *wild type*, but no *sepaloids* could occur. To obtain the *sepaloid* classified in Table 7.3 two genes would be required and the genotype of the pin *Hose in Hose* would be either that as in (E) above if *Hih* and *sep* are on the same side of the *S* locus, with a single recombination occurring between *hih* and *sep* that would give both the pin *Hose in Hose* and the thrum *wild type*, or as that in (G) above, as the putative genotype in Table 7.3, with recombination occurring between *sep* and *s*. However in this instance a second recombination event between *Hih* and *S* would be required to give the thrum *wild type* recombinant. Results from cross no. 3 therefore indicates that there are two genes involved, probably both on the same side of the *S* locus, but another recombinant would give absolute proof, so further tests were undertaken.

Cross no. 4. The reciprocal crosses between thrum *wild type* heterozygous for the recessive allele for *sepaloid* (*sep (?) s Hih* / + s hih) and pin *Hose in Hose* (putative genotype, + *s hih* / *sep s Hih*) was also carried out. Results were classified during 2003.

Thrum *wild type* heterozygous for the recessive allele for *sepaloid* x pin *Hose in Hose* (putative genotype + *s hih* / *sep s Hih*)

sep s + x *sep (?) s Hih*

\+ *S* + ↓ + *s* +

	Thrum *Hose in Hose*	pin *Hose in Hose*	Thrum *wild type*	pin *wild type*	*sepaloid*
	+ S + / *sep s Hih*	*sep s +* / *sep s Hih*	*+ S +* / *+ s +*	*sep s +* / *+ s +*	
2002	20	30	19	26	0
Recip. cross	4	3	14	14	0
2003	6	10	8	10	0
Recip. cross	4	1	15	3	0
Total	34	44	56	53	0

Table 7.4. Results of cross no. 4. Fourth test to investigate whether *Hose in Hose* and *sepaloid* are allelic

Equal numbers of thrum *wild type*, pin *wild type*, thrum *Hose in Hose* and pin *Hose in Hose* can be expected given the results from the cross above. In addition it is possible that recombination could produce an occasional *sepaloid* plant that would confirm the previous results. (*sepaloids* are the only recognisable recombinant phenotypes from the above reciprocal cross). No recombinants were identified. A χ^2 analysis was done to test the null hypothesis that there is no significant difference of the data from the predicted ratio. If no recombination is occurring expect equal numbers of *Hih s* : *Hih S* : + *s* : + *S*

χ^2 for 1:1:1:1 for *Hih s* : *Hih S* : + *s* : + *S*

Observed 44 : 34 : 53 : 56; expect 46.75 : 46.75 : 46.75 : 46.75

χ^2 (3 dof) = 6.31 P = 0.097

Results are consistent with 1:1:1:1 for *Hih s* : *Hih S* : + *s* : + *S*

The *sepaloid* from the cross tabulated in Table 7.3 above indicates that there are two genes involved. Although the very tight linkage between *sepaloid* and *s* means that the chance possibility of a recombinant being obtained from such low numbers is unlikely, it is not impossible. Similarly it is not surprising that no recombinants were obtained from the larger cross shown in Table 7.4 as a minimum of several hundred progeny would be required before a recombinant could actually be expected. The result of this investigation was initially considered inconclusive as there was no way of being sure whether the *sepaloid* could have been the result of experimental error (e.g. a stray seed) or a genuine recombinant. However given the prediction that mutation of *GLOBOSA* is the basis of the *Hose in Hose* phenotype and the possibility of *Hose in Hose* and *sepaloid* being allelic further molecular analysis was undertaken in the Gilmartin laboratory. Sequence of the genomic locus of *GLOBOSA* in *sepaloid* showed no DNA sequences different from the *Wild Type* allele that might be predicted to create a null allele (Dr. J. Li, personal communication). Consequently it now seems likely that there are two genes involved and that the *sepaloid* obtained (see Table 7.3) is a genuine recombinant.

7.3. Investigation of the order of the linked genes *Hose in Hose*, *Oak Leaf*, and the *Primula S* locus.

A homozygous *Hose in Hose* plant that was also a rare homozygote for the dominant *S* allele of the *Primula S* locus, genotype *Hih S /Hih S* (see Chapter 5, 5.3.4), was pollinated using pollen from a pin *Oak Leaf* plant genotype *Okl s* / + *s*. The thrum *Hose in Hose-*

Oak Leaf progeny from the above cross can be expected to have the genotype *Hih S + / + s Okl* (gene order unknown). Three sibling plants of this genotype were used in reciprocal crosses with *wild type* pin plants in an attempt to raise a very large number of progeny. Gene order could be either *Okl Hih S* or *Okl S Hih*, but not *Hih Okl S* as it has already been established that *Okl* is less tightly linked to the *S* locus than *Hih* (see Chapter 5, 5.11).

The *Oak Leaf* plants proved to be less productive seed parents than *wild type*. In contrast the *wild type* plants used as seed parents pollinated with *Oak Leaf* pollen produced abundant seed. A problem both anticipated and encountered with this three point cross was the difficulty of separating true pin *Hose in Hose-Oak Leaf* recombinants from pin *Oak Leaf* plants with streaks of petal in the calyx. Where the calyx is completely petaloid and of the expected *Hose in Hose* form there is no doubt of the classification, but where the streaks of petal are as those found in the calyces of *Oak Leaf* progeny from *wild type* x *Oak Leaf* (see Chapter 4, Fig 4.28) there can be ambiguity. Those pin *Hose in Hose-Oak Leaf* with sufficiently petaloid calyces to be indisputable were recorded as such, and all possible recombinants were maintained for a second flowering season in order to verify results. This worked well with progeny from two of the *Oak Leaf* parents, but pin *Oak Leaf* progeny from a third *Oak Leaf* parent were sufficiently ambiguous for scoring of progeny from this cross to be discontinued and results eliminated from the final classification shown in Table 7.5.

A second difficulty was the possibility of thrum *wild type* actually being *Hose in Hose* with a poorly expressed phenotype. The latter possibility is easier to ascertain, as such plants generally produce at least small amounts of petal in the calyx at some time during the flowering season, so that retaining them for observation throughout the flowering season was sufficient to ensure accurate classification.

Final classification of the three point crosses is tabulated in Table 7.5. Results confirm gene order as *Okl S Hih*.

Results of linkage analysis of *Hose in Hose*, *Oak Leaf*, and the *Primula S* locus.

pin *wild type* x thrum *Hose in Hose - Oak Leaf*

$$\frac{+\ \ s\ \ +}{+\ \ s\ \ +} \times \frac{Okl\ \ s\ \ +}{+\ \ S\ \ Hih}$$

Cross	Parentals		Sco *Okl/s*		Sco *Hih/S*		Dco		Other
	pin *Oak Leaf*	Thrum *Hose in Hose*	pin *wild type*	Thrum *Hose in Hose -Oak Leaf*	pin *Hose in Hose - Oak Leaf*	Thrum *wild type*	pin *Hose in Hose*	Thrum *Oak Leaf*	
***Oak Leaf* 1.**	$\frac{Okl\ \ s\ \ +}{+\ \ s\ \ +}$	$\frac{+\ \ S\ \ Hih}{+\ \ s\ \ +}$	$\frac{+\ \ s\ \ +}{+\ \ s\ \ +}$	$\frac{Okl\ \ S\ \ Hih}{+\ \ s\ \ +}$	$\frac{Okl\ \ s\ \ Hih}{+\ \ s\ \ +}$	$\frac{+\ \ S\ \ +}{+\ \ s\ \ +}$	$\frac{+\ \ s\ \ Hih}{+\ \ s\ \ +}$	$\frac{Okl\ \ S\ \ +}{+\ \ s\ \ +}$	
1A. Pin *wild type* 1 x Thrum *Hose in Hose - Oak Leaf* 1	229	180	0	2	1	0	0	1	0
1B. Pin *wild type* 2 x thrum *Hose in Hose - Oak Leaf* 1	276	152	2	0	0	0	0	0	0
1C. Thrum *Hose in Hose - Oak Leaf* 1 x pin *wild type* 3	215	228	2	0	0	2	0	1	0
***Oak Leaf* 2.**									
2A. Pin *wild type* 4 x thrum *Hose in Hose - Oak Leaf* 2	236	254	2	3	0	4	1	0	0
2B. Thrum *Hose in Hose - Oak Leaf* 2 x pin *wild type* 4	78	77	1	10	0	1	0	0	0
2C. Pin *wild type* 5 x thrum *Hose in Hose - Oak Leaf* 2	65	42	1	2	4	2	0	0	1 short homostyle *Hose in Hose* with large pollen.
Total numbers	1099	932	8	17	5	9	1	2	1
	2032		25		14		3		1
	2075								

Table 7.5. This table depicts results of crosses of pin *wild type* plants x two different thrum *Hose in Hose-Oak Leaf* plants, *Oak Leaf* 1 and *Oak Leaf* 2. Three different *wild type* plants, marked 1, 2, and 3, were used with *Oak Leaf* 1, and two with *Oak Leaf* 2, marked 1 and 2. Crosses 2A and 2B are reciprocal crosses.

Cross	Total numbers	Okl/s recombinants	Hih/S recombinants
Pin *wild type* 1 x Thrum *Hose in Hose - Oak Leaf* 1	413	3	2
Pin *wild type* 2 x thrum *Hose in Hose - Oak Leaf* 1	430	2	0
Thrum *Hose in Hose - Oak Leaf* 1 x pin *wild type* 3	448	3	3
Combined total	1291	8	5
Map distance		Okl/s = 0.6%	Hih/S = 0.4%
Pin *wild type* 4 x thrum *Hose in Hose - Oak Leaf* 2	500	6	5
Thrum *Hose in Hose - Oak Leaf* 2 x pin *wild type* 4	167	11	1
Pin *wild type* 5 x thrum *Hose in Hose - Oak Leaf* 2	116	3	6
Combined total	783	20	12
Map distance		Okl/s = 2.6%	Hih/S = 1.5%

Table 7.6. Map distances of *Hih* and *Okl* from the *S* locus

Map distances vary dramatically between the crosses (Table 7.6) but put *Oak Leaf* further from the *S locus* than *Hose in Hose*.

During the analysis it was noted that as the progeny first began to flower there appeared to be more *Oak Leaf* plants among the earliest plants to bloom. The first *Oak Leaf* plant discovered was in commercial stock as were some of the *wild type* plants used, and many commercial lines have been specifically selected for early flowering. To investigate whether early flowering segregated with *Oak Leaf* a count was made of the plants raised in Woodborough Nursery under the same environmental conditions. Results are shown in Table 7.7.

Progeny of plant 1	flowered	Not flowered	
Oak leaf	349	90	439
wild type leaf	166	286	452
	515	376	891

Progeny of plant 1. χ^2 (1 dof) = 167 P = 0 (significant)

Progeny of plant 2	flowered	Not flowered	
Oak leaf	88	121	209
wild type leaf	63	87	150
	151	208	359

Progeny of plant 2. χ^2 (1 dof) = .008 P = 0.93 (not significant)

Table 7.7. Timing of flowering of *Oak Leaf* and *wild type* leaf progeny.

The results indicate that the *Oak Leaf* progeny from the first thrum *Hose in Hose-Oak Leaf* plant are flowering significantly earlier than the *wild type* progeny. Progeny of the second thrum *Hose in Hose-Oak Leaf* plant show no significant difference in flowering time between the two leaf phenotypes of the progeny. A future experiment could be done specifically to test segregation of early flowering with the *Oak Leaf* phenotype in different plants. However it is also possible that early flowering could have been carried by some of the *wild type* plants and as three different *wild type* plants were used with *Oak Leaf* plant no. 1, (Table 7.5), in a future experiment a single *wild type* parent only should be used.

Progeny of plant 1	parental	Recombinant (Okl / s)	
female	445	3	448
male	838	5	843
total	1283	8	1291

Progeny of plant 1. Yates' χ^2. (1 dof) = 0.042, P = 0.838 (not significant)

Progeny of plant 2	parental	Recombinant (Hih / S)	
female	445	3	448
male	839	4	843
total	1284	7	1291

Progeny of plant 1. Yates'χ^2. (1 dof) = 0.003, P = 0.96 (not significant)

Progeny of plant 1	parental	Recombinant (Okl / s)	
female	156	11	167
male	607	9	616
total	763	20	783

Progeny of plant 2. Yates'Chi sq. (1 dof) =11.885, P = 0.0006 (significant)

Progeny of plant 2	parental	Recombinant (Hih / S)	
female	166	1	167
male	605	11	616
total	771	12	783

Progeny of plant 2. Yates' χ^2. (1 dof) = 0.566, P = 0.45 (very slightly significant)

Table 7.8. Comparison of recombination frequency in pollen and egg of two Oak Leaf plants.

Numbers of recombinants from the two thrum *Hose in Hose-Oak Leaf* plants as male and female parents were also compared. Results are classified in Table 7.8. Recombination in the pollen and seed parents is not significantly different for Thrum *Hose in Hose-Oak Leaf* no. 1 but there is some significant different for Thrum *Hose in Hose-Oak Leaf* no. 2. A future experiment could be undertaken specifically to test recombination in pollen and seed parents of *Primula*.

Of particular interest is the *S* locus recombinant, a short homostyle *Hose in Hose* with large pollen (fig. 7.2.A) obtained from the three point cross (see Figure 7.6). It has the expected characteristics of a short homostyle in that as well as having a short style the stigmatic papillae are also short (Fig. 7.2.B). The majority of flowers that appear as short homostyle (Fig.7.2.C) have long stigmatic papillae (Fig 7.2D). Unfortunately this short homostyle *Hose in Hose* with large pollen does not give us the gene order of the *S* locus as double recombination within all gene orders could give this phenotype.

Examples are set out below (– indicates recombination points).

Okl p – a – g hih to give *okl P a G Hih* and *Okl p A g hih* as gametes of the genotypes
okl P – A – G Hih

Okl g – a – p hih to give *okl G a P Hih* and *Okl g A p hih* as gametes of the genotypes
okl G – A – P Hih

Okl g p – a – hih to give *okl G P a Hih* and *Okl g p A hih* as gametes of the genotypes
okl G P – A – Hih

Okl – a – p g hih to give *okl a P G Hih* and *Okl A p g hih* as gametes of the genotypes
okl – A – P G Hih

No long homostyle *Oak Leaf* plants were discovered among the progeny. All recombinants were checked for pollen size and no further pollen size recombinants were discovered. A random sample of approximately 50 plants of both pin *Oak Leaf* and thrum *Hose in Hose* from each cross were also sampled for pollen size without discovering any pollen size recombinants. Some pin plants (both one pin *Oak Leaf* and one pin *Hose in Hose–Oak Leaf*) were found to exhibit mixed size pollen; but when checked repeatedly over the flowering season the pollen size eventually ceased to be of mixed size and was uniformly small as normal pin pollen. The above plants reverted to producing mixed size pollen again at the end of the 2004 flowering season. They have both been self pollinated and their pollen has also been used on thrum *wild type* flowers in order to test whether

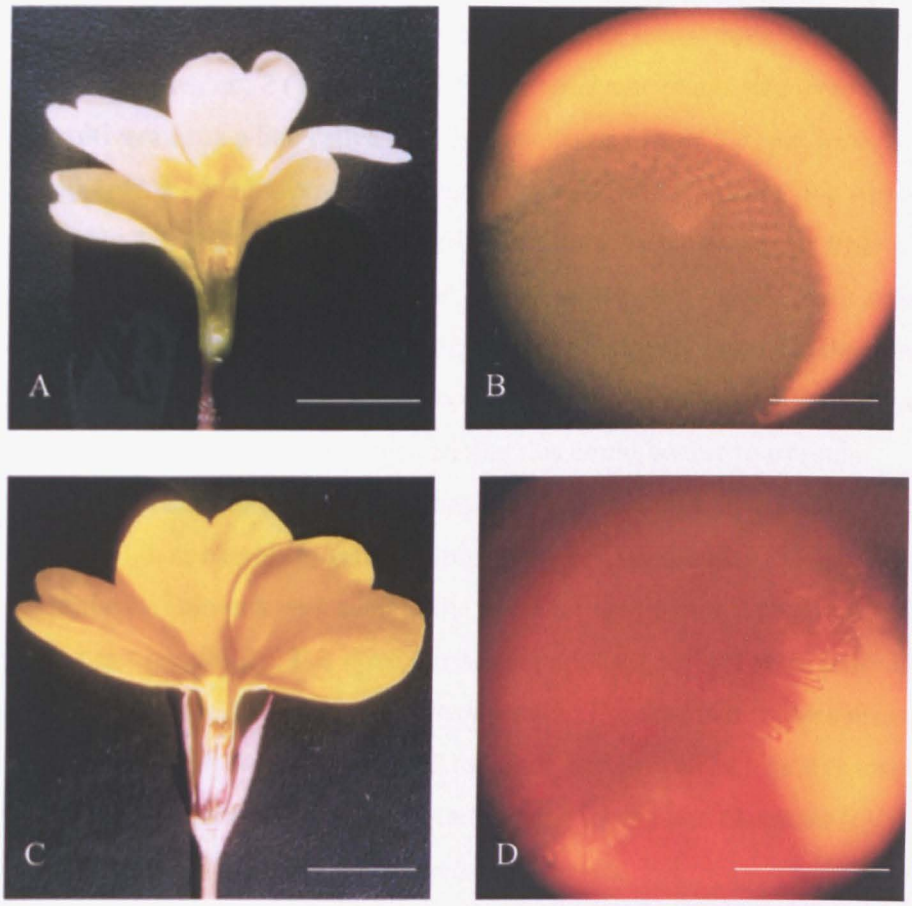


Figure 7.2. Short homostyles and stigmatic papillae length.

The flower and the stigmatic papillae in **C** and **D** are shown for comparison with that of the short homostyle Hose in Hose in **A** and **B**. Short homostyles are normally expected to have short stigmatic papillae, but occasional plants are discovered that present as short homostyles but that have long stigmatic papillae.

A. Short homostyle *Hose in Hose* with short stigmatic papillae and large pollen. **B.** Stigmatic papillae of flower in A as seen under the light microscope. **C.** A short homostyle flower with small pollen and long stigmatic papillae. **D.** Stigmatic papillae of flower in **C** as seen under the light microscope. Size bars are approximately 1cm. in **A** and **C** and approximately 0.5mm. in **B** and **D**.

they might behave as the P* plants described by Kurian and Richards (Kurian and Richards, 1997). There is scope for investigation of pollen size over the flowering season. Five different *wild type* plants were used for the three point cross, three with *Oak Leaf* no. 1 and two with *Oak Leaf* no. 2 (Table 7.5). Of these *wild type* plants two were commercial cultivars from a line called "Emily" primroses (Table 7.5, pin *wild type* numbers 3 and 4- *Oak Leaf* 1 cross C, and *Oak Leaf* 2 Crosses A and B), one was a wild *P. vulgaris* (Table 7.5, pin *wild type* 2 – *Oak Leaf* 1 Cross B) and two were from a cross of *P. vulgaris* x a commercial cultivar (Table 7.5, pin *wild type* 1 – *Oak Leaf* 1 cross A and pin wild type 5 – *Oak Leaf* 2 cross C). It had been previously observed that when crossed to *P. vulgaris* more *Oak Leaf* progeny than progeny with *wild type* leaves were obtained (Chapter 5; 5.10.1). It was suggested that this could be due to irregular germination of *wild plants* in contrast to commercial cultivars that have been rigorously selected for both high and uniform germination. Having crossed both *P. vulgaris* and commercial primrose cultivars with each of the two *Oak Leaf* plants it allowed further investigation of this possibility. The three crosses using *Oak Leaf* no1 were individually tested for a 1:1 ratio of for *Oak Leaf* to *wild type* leaves. The first two crosses using *Oak Leaf* no. 2 were reciprocal, so could be tested together, and the third cross was tested individually. As the *Oak Leaf* plants were heterozygous for the leaf character (*Okl s* + / + *S Hih*) expect a ratio of 1:1 for *Oak Leaf* to *wild type* leaves.

***Oak Leaf* no. 1.**

χ^2 for 1:1 *wild type* no 1 x *Oak Leaf* no. 1. Cross 1A.

observe 233 : 180, expect 206.5 : 206.5

χ^2 (1 dof) = 6.8, P = 0.009

Results do not fit the predicted ratio of 1:1 for *Oak Leaf* to *wild type* leaves.

χ^2 for 1:1 *wild type* no 2 x *Oak Leaf* no. 1. Cross 1B.

observe 276 : 154, expect 215 : 215

χ^2 (1 dof) = 34.6 P = 0

Results do not fit the predicted ratio of 1:1 for *Oak Leaf* to *wild type* leaves.

χ^2 for 1:1 *wild type* no 3 x *Oak Leaf* no. 1. Cross 1C

observe 215 : 232, expect 223.5 : 223.5

χ^2 (1 dof) = 0.6, P = 0.42

Results do not fit the predicted ratio of 1:1 for *Oak Leaf* to *wild type* leaves.

***Oak Leaf* no. 2.**

χ^2 for 1:1 *wild type* no 4 x *Oak Leaf* no. 2 (+ reciprocal). Crosses 2A and 2B

observe 327 : 240, expect 283.5 : 283.5

χ^2 (1 dof) = 13.35, P = 0.0003

Results do not fit the predicted ratio of 1:1 for *Oak Leaf* to *wild type* leaves.

χ^2 for 1:1 *wild type* no 5 x *Oak Leaf* no. 2. Cross 2C.

observe 71 : 45, expect 58 : 58

χ^2 (1 dof) = 5.8, P = 0.016

Results do not fit the predicted ratio of 1:1 for *Oak Leaf* to *wild type* leaves.

It is a cross that involves a commercial cultivar (pin *wild types* number 3) that is least inconsistent with a 1:1 ratio of *Oak Leaf* to *wild type* leaves. The lowest proportion of *wild type* leaf plants is from the cross using wild *P. vulgaris* (pin *wild type* no 2). This indicates that delayed germination of some seed may be associated with wild *P. vulgaris*. The habit appears to be retained in first generation progeny, pin *wild type* numbers 1 and 5 do not fit the predicted ratio of 1:1 for *Oak Leaf* to *wild type* leaves, but the proportion of *wild type* leaf plants to *Oak Leaf* plants in these instances is not so low as that from wild *P. vulgaris*. There is scope for further investigation into germination of wild *P. vulgaris* seed versus selected commercial cultivar seed.

It was essential to confirm the genotype of the short homostyle *Hose in Hose* with large pollen, a recombinant from the three point cross (see Table 7.5 and Figure 7.2) by test crosses, particularly in the light of experiments that found the yellow presumed short homostyle studied in Chapter 5 not to be of the predicted genotype (Chapter 5, 5.10). The short homostyle *Hose in Hose* with large pollen is extremely self fertile and the possibility of it being a short styled pin *Hose in Hose* with large pollen required investigation. Recent results from a small test cross have resulted in two short homostyle *Hose in Hose* plants with large pollen and ten *wild type* pin plants. There are further progeny still to bloom but this result confirms the genotype of the short homostyle *Hose in Hose* with large pollen. The pollen size of the ten *wild type* progeny was examined and one plant was discovered to have mixed size pollen composed of both large and small pollen. This will be further investigated to discover whether it might behave as the P* plants described by Kurian and Richards (Kurian and Richards, 1997).

7.4. Investigation of the order of the linked genes *Oak Leaf*, *sepaloid*, and the *Primula S* locus.

It has been established that *Oak Leaf* and *Hose in Hose* are on opposite sides of the *S* locus (7.3 above). A second investigation to discover whether *sepaloid* is on the same side of the *S* locus as *Hose in Hose* or on the opposite side of the *S* locus to *Hose in Hose* but on the same side as *Oak Leaf*, mapping of *Oak Leaf* and *sepaloid* to the *S* locus was undertaken using a three point cross of *sepaloid* x *Oak Leaf* heterozygous for *sepaloid* (*sep s +/sep s +* x *+ S Okl/ sep s +*). To construct the *Oak Leaf* plants heterozygous for *sepaloid* a thrum *Oak Leaf* (*Okl S / + s*) recombinant from the cross shown in Chapter 5, table 5.54, was used to pollinate a pin *sepaloid* (*sep s / sep s*). The cross failed at the first attempt and so was repeated the following spring when it was successful; seed was collected, sown, and progeny subsequently raised. All five *Oak Leaf* progeny from this cross were both thrum and heterozygous for the recessive *sepaloid* allele. Four thrum *Oak Leaf* plants heterozygous for the recessive *sepaloid* allele (*+ S Okl / sep s +*) flowered in 2003 and were used as pollen parents on *sepaloid* (*sep s / sep s*) plants. In order to obtain as much seed as possible two *sepaloid* seed parents were used for each thrum *Oak Leaf* pollen parent. Not all flowers on *sepaloid* plants are fertile, even on those plants that do produce carpels. A total of 717 progeny were raised, 581 in Woodborough Nurseries and 136 in Winford. Results were scored in mid June 2004 and are classified in Table 7.9.

Numbers of recombinants were smaller than had been hoped for, but indicate that the gene order is likely to be *Oak Leaf* *S* locus *sepaloid*. From the map distances calculated in Table 7.10 it can be observed that there is variation in recombination rate from one plant to another. *Oak Leaf* is further from the *S* locus than *sepaloid*. Map distances vary considerably from plant to plant but generally put *Oak Leaf* further from the *S* locus than *sepaloid*. Like *Hose in Hose*, *sepaloid* is tightly linked to the *S* locus; and from the results shown in Table 7.9 *sepaloid* is on the same side of the S locus as *Hose in Hose*. Since the map distances from the *S* locus are similar, either *Hose in Hose* and *sepaloid* are allelic or they are two separate but very closely linked loci. The 118 plants that had not flowered in 2004 (see Table 7.9) were retained until 2005 and no further recombinants occurred. While all retained plants were as the parental, either Thrum *Oak Leaf* or pin *sepaloid*, due to the loss of a number of labels it was not possible to allocate the correct numbers of Thrum *Oak Leaf* and pin *sepaloid* to the correct parent plants. However for the purpose of

pin *sepaloid* x Thrum *Oak Leaf* heterozygous for the recessive *sepaloid* allele

$$\frac{sep\ s\ +}{sep\ s\ +} \times \frac{+\ S\ Okl}{sep\ s\ +}$$

Cross	Parentals		Sco Okl/S		Sco S/Sep		Dco		
	Thrum *Oak Leaf*	*sepaloid*	Thrum *wild type*	pin *sepaloid Oak Leaf*	pin *wild type*	Thrum *sepaloid Oak Leaf*	pin *Oak Leaf*	Thrum *sepaloid*	Plants not scored
	$\frac{+\ S\ Okl}{sep\ s\ +}$	$\frac{sep\ s\ +}{sep\ ?\ +}$	$\frac{+\ S\ +}{sep\ s\ +}$	$\frac{sep\ s\ Okl}{sep\ s\ +}$	$\frac{+\ s\ +}{sep\ s\ +}$	$\frac{sep\ S\ Okl}{sep\ s\ +}$	$\frac{+\ s\ Okl}{sep\ s\ +}$	$\frac{sep\ S\ +}{sep\ s\ +}$	
pin *sepaloid* x Thrum *Oak Leaf* heterozygous recessive for *sepaloid* **no.1.**	76	69	0	1	0	0	0	0	39
pin *sepaloid* x Thrum *Oak Leaf* heterozygous recessive for *sepaloid* **no.2.**	37	23	0	0	1	0	0	0	20
pin *sepaloid* x Thrum *Oak Leaf* heterozygous recessive for *sepaloid* **no.3.**	157	136	1	3	0	1	1	0	9
pin *sepaloid* x Thrum *Oak Leaf* heterozygous recessive for *sepaloid* **no.4.**	59	35	1	0	0	0	0	0	50
Total numbers	329	263	2	4	1	1	1	0	118
	592		9 recombinants						
	601 plants								

Table 7.9. Results of linkage analysis of *Oak Leaf*, *sepaloid*, and the *Primula S* locus.

Four different *Oak Leaf* plants heterozygous recessive for *sepaloid* and numbered 1-4 were used to pollinate *sepaloid* plants.

Cross	Total numbers	*Okl/S* recombinants	*S/sep* recombinants
pin *sepaloid* x Thrum *Oak Leaf* heterozygous recessive for *sepaloid* **no.1.**	185	1	0
Map distance		0.5%	0
pin *sepaloid* x Thrum *Oak Leaf* heterozygous recessive for *sepaloid* **no.2.**	81	0	1
Map distance		0	1.2%
pin *sepaloid* x Thrum *Oak Leaf* heterozygous recessive for *sepaloid* **no.3.**	308	5	2
Map distance		1.6%	0.6%
pin *sepaloid* x Thrum *Oak Leaf* heterozygous recessive for *sepaloid* **no.4.**	145	1	0
Map distance		0.7%	0

Table 7.10. Map distances of *Okl* and *sep* from the *S* locus.

calculating map distances more accurately these 118 parental phenotypes have been included in the total numbers in Table 7.10.

It is not possible to be sure of whether the *sepaloids* are pin or thrum without breeding or pollination tests, and there is the possibility of infertile recombinant thrum *sepaloid* plants not being identified. Breeding tests were carried out on two *sepaloid-Oak Leaf* and two *sepaloids* that had short styles. One of the thrum *sepaloid - Oak Leaf* plants consistently failed to accept thrum pollen while setting seed from pin pollen. This indicates that this *sepaloid - Oak Leaf* plant is a thrum plant with associated thrum self incompatibility. The number of expected recombinants would be very small. Depending on gene order a thrum *sepaloid* (if order *Okl sep S locus*) or a thrum *sepaloid-Oak Leaf* (if order *Okl S locus sep*) could result from a recombination event between *sepaloid* and the *s* allele of the *S locus*, but linkage between *sepaloid* and the *s* allele of the *S* locus is very tight. Again depending on gene order, a thrum *sepaloid* (if order *Okl S locus sep*) or a thrum *sepaloid-Oak Leaf* (if order *Okl sep S locus*)

could result from a double recombination event. The latter would normally be expected to be a very rare event.

Another difficulty in classifying progeny from the crosses shown in Table 7.9 arises from failure of the first cross attempted (pin *sepaloid* x thrum *Oak Leaf*). Environmental stress affects floral morphogenesis and it is therefore advisable to monitor recombinants and any ambiguous phenotypes for a second flowering season. This could have been achieved if the thrum *Oak Leaf* heterozygous for the recessive *sepaloid* allele had been available a year earlier. To achieve results rapidly it is sometimes necessary to sow seed indoors in autumn or winter, instead of waiting until spring. This results in the progeny beginning to flower in late May or June when the weather is often hot. In hot weather many flowers exhibit restricted corolla tube growth so that thrums appear as long homostyles, especially when grown in small pots. When observed flowering during a cooler period or in a less stressful position, the same plants raise the corolla tube and associated anthers high above the stigma. Examination of stigmatic papillae length under a light microscope can help clarify the phenotype. Potting ambiguous phenotypes into larger pots and observing flowering in a cooler shaded position is the most definitive method of clarifying the phenotype.

Another feature of flowers produced in hot stressful conditions is failure of pollen production. Pollen size tests on plants from the crosses shown in Table 7.6 were carried out during autumn 2003 and spring 2004. Attempts to obtain pollen during June 2004 from progeny of the crosses shown in Table 7.9 consistently failed – all anthers produced in the hot weather of late May and early June 2004 were empty. Consequently were there any pollen size recombinants among the progeny of these crosses they were not identified.

The flower of the pin *Oak Leaf* from the cross classified in Table 7.9 was aberrant in that the petals were split down to the attachment of the anthers and the anthers were at the base of the petal instead of half way up the corolla tube (Fig. 7.3A and B). This may be due to environmental stress: splitting of the corolla has been observed

Figure 7.3. Flowers of some progeny from crosses in section 7.4. that differ from the norm.

A. Aberrant pin *Oak Leaf* with split corolla. **B.** The calyx of the flower in **A** has been completely removed. **C.** A later flower of the aberrant pin *Oak Leaf,* no longer split, but with free anthers low in the corolla tube. **E.** and **F.** Seasonal anomaly of anthers separating from the corolla tube of thrum *Oak Leaf* flowers. Sepals have been removed. Early flowers were of normal form. **G.** Long styled thrum *Oak Leaf flower* no.1. **H.** Long styled thrum *Oak Leaf flower* no 2.. Flowers in **F** and **G** have short stigmatic papillae. Size bars are approximately 1cm.

previously and found not to be either a permanent feature or heritable. One example of this is mentioned in Chapter 5; 5.4.2). Only if splitting of the corolla persisted throughout a second flowering season, or was found to be heritable would this aberration be considered significant. The abnormally low anthers however are a feature not seen previously as an environmental abnormality, although separation of the anthers from the corolla tube on to long individual filaments has been observed on the later flowers of one thrum *Oak Leaf* plant (Figure 7.3D and E). A small amount of pollen was obtained from undehisced anthers of the pin *Oak Leaf* and it was found to be of mixed size both large and small grains. This will require further observation as previous observations found that some plants with mixed size pollen altered to small pollen when flowering during the normal flowering season. The aberrant pin *Oak Leaf* plant was repotted into a larger pot and grown in shade, and by the end of the first week of August 2004 the flowers had ceased to be split (Figure 7.3C). However the anthers remained as before and there was no division of the corolla tube into upper and lower parts as is usual. The free anthers may possibly be the result of a separate mutation. Two thrum *Oak Leaf* plants occurred that had long styles and short stigmatic papillae (Figure 7.3F and G), one from no. 1 on Table 7.9 and one from no. 4 on Table 7.9 but lack of pollen production prevented testing for self compatibility.

One of the four *sepaloid Oak Leaf* recombinants from no.3 on Table 7.9 had some of the flowers with streaks of yellow pigment on the lower part of the inside of the second whorl of sepals (Fig. 7.4C). This is of the same colour as is found on the yellow eye of the normal flower The second whorl of sepals are also much longer than the first whorl. On examination under a light microscope the pigmented cells of the second whorl were found to be of the elongated conical papillate form as those of the yellow eye of the normal flower and of the yellow eye of the green primrose flower. This is the first time that any petal like feature has been observed in a *sepaloid* flower. Although the flowers of this plant bear some similarity to the green flowers of *Primula viridis*, the green primrose, it is different in having fertile carpels on many flowers. Pollination tests from pin and thrum pollen were carried out both in autumn 2004 and spring 2005. Thrum pollen consistently failed, while fertile seed was consistently set from pin pollen. Some of this was sown and has resulted in 12 progeny that have yet to flower. The stigmatic papillae remained consistently short both in 2004 and 2005.

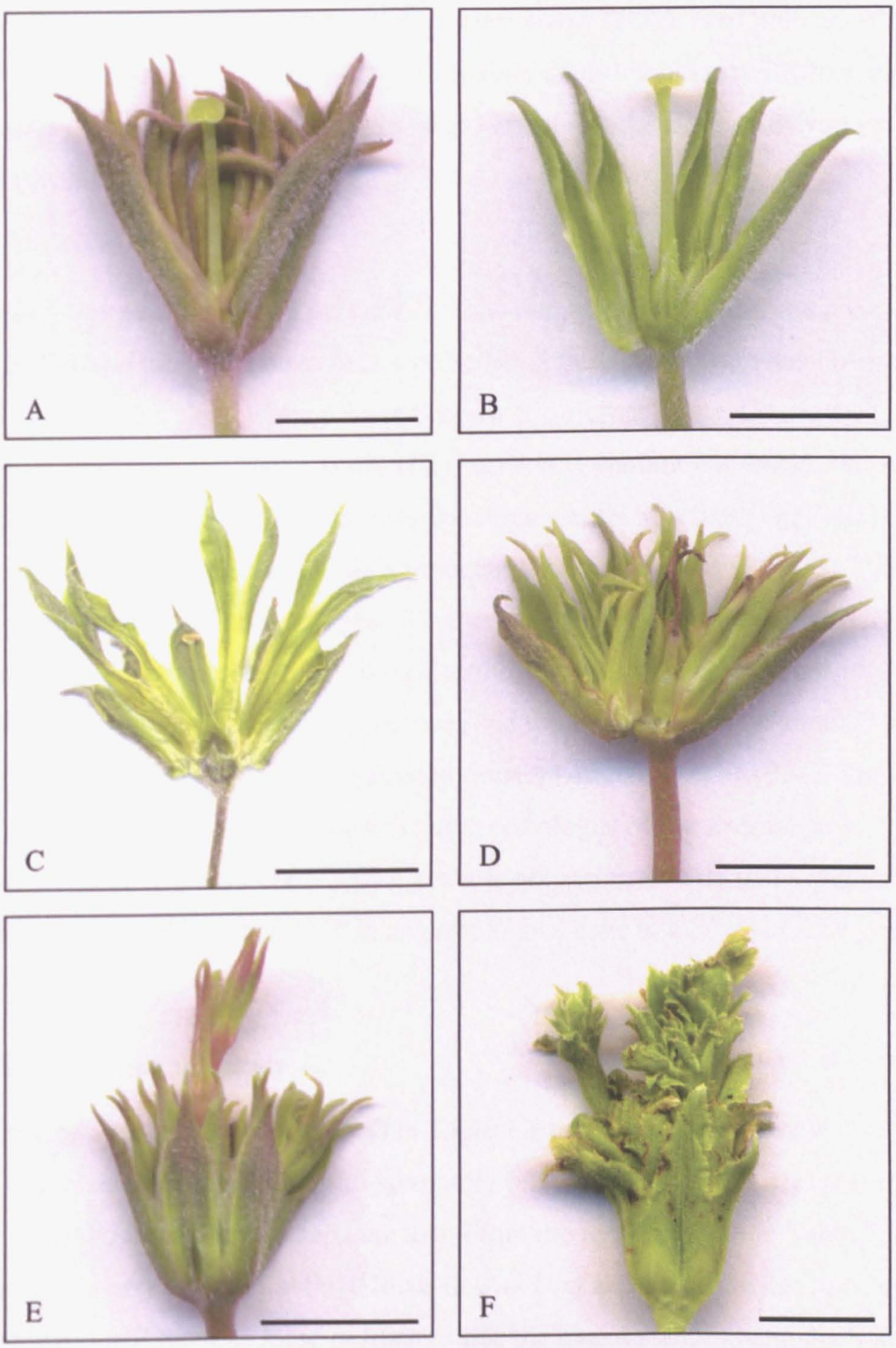


Figure 7.4. Variation in the form of some *sepaloid-Oak Leaf* and *sepaloid* flowers from the cross described in section 7.4.

A. *sepaloid-Oak Leaf* flower with two whorls of sepals and a functional carpel. **B.** *sepaloid-Oak Leaf* flower with whorl 2 sepals reduced in size. **C.** *sepaloid-Oak Leaf* pressed flower with petal cells on the lower part of the whorl 2 sepals. **D.** *sepaloid-Oak Leaf* flower with three whorls of sepals and a normal carpel. **E.** A flower from the same plant that has a secondary inflorescence. **F.** A *sepaloid* flower with two secondary inflorescences. Size bars approximately 1 cm.

Consequently this plant has been classified as a thrum *sepaloid Oak Leaf*. It is possible that the petal material in whorl two is the result of a recombination event that partially restored B function. Results of a cross of the thrum *sepaloid Oak Leaf* pollinated by a pin *wild type* heterozygous recessive for the *sepaloid* allele should reveal which allele of *sepaloid* is dominant, although it might be predicted that the allele with some gain of function will be the dominant one.

The *sepaloid-Oak Leaf* from no.1 on Table 7.9 has two whorls of sepals and a normal carpel (Fig. 7.4A). One of the other four *sepaloid-Oak Leaf* plants from has flowers of this form, but the other three siblings have flowers of different forms. One is the *sepaloid-Oak Leaf* with petal cells in whorl 2 (Fig. 7.4C), another has whorl 2 sepals very much reduced (Fig. 7.4B), and another has three whorls of sepals (Fig.7.4D). The latter also has an occasional flower with a secondary inflorescence (Fig 7.4.E). Two sepaloid plants from Cross no 1 on Table 7.9 also produced one or more secondary inflorescences in some flowers (Fig. 7.4.F). Secondary inflorescences had not previously been observed in *sepaloid* primroses and the form bears some similarity in appearance to the Petunia *FBP2* cosuppression mutant (Angenant et al 1994). The Petunia *FBP2* gene is considered to be a putative orthologue of the *Arabidopsis SEPELLATA* 3 gene (Ferrario *et al.* 2003), and it is not inconceivable that *sepaloid* in *Primula* may be caused by a mutation in an orthologous gene to a *SEPALLATA* gene.

7.5. Discussion of linkage analysis.

The presence of a recombinant (sepaloid) in Table 7.3 in conjunction with the sequence data obtained in the Gilmartin laboratory (see &.2 above) indicates that *Hose in Hose* and *sepaloid* may be two separate loci. From the results shown in Table 7.9 *sepaloid* is on the opposite side of the *S* locus to *Oak Leaf* and therefore has to be on the same side of the *S* locus as *Hose in Hose*. Since the map distances from the S locus for *Hose in Hose* and *sepaloid* are similar (Tables 7.7 and 7.10), either *Hose in Hose* and *sepaloid* are allelic, or, as now seems likely, are two separate but very closely linked loci with *Hose in Hose* epistatic to *sepaloid*. If the cross was repeated, larger numbers of progeny as was possible for linkage analysis of *Oak Leaf, sepaloid* and the *Primula S* locus, would be essential (see 7.4). The very tight linkage between *sep* and *s*

means that a minimum of several hundred progeny would be required before a recombinant could actually be expected.

Results from the cross pin *wild type* x thrum *Hose in Hose-Oak Leaf* (7.3), established that *Hose in Hose* and *Oak Leaf* are on opposite sides of the *S* locus and this result provides the long sought after flanking markers for the *Primula S* locus.

The variation in recombination rates observed from one plant to another is notable. Environmental influences can affect recombination. Ernst (1939) studied chiasma frequency in *Antirrhinum* at different temperatures. He discovered that a change in temperature resulted in a sudden but temporary fall in chiasma frequency (Ernst, 1939; reviewed in Yanney Wilson, 1959). If chiasma frequency is influenced by temperature a contributory factor to the variable recombination rates observed in *Primula* could be that pollination was undertaken over an extended period of time, as flowers became available, and temperature will have varied considerably.

Alternatively variation in recombination rate can have a specific genetic basis. Genes controlling chiasma frequency in *Hordeum* have been documented (Gale and Rees, 1970), and studies on *Petunia hybrida* found that a gene *Rm1* (recombination modulator 1) controlled recombination rate during female gametogenesis (Cornu et. al. 1988). Comparison of recombination frequency in pollen and egg of two *Oak Leaf Primula* plants (Table 7.8) found no significant difference between pollen and egg in plant number 1 and only slight significance in plant number 2. A future experiment could be done specifically to test recombination in pollen and seed parents of *Primula*.

In *Primula* the genetic loci in the *S* locus are very tightly linked and *Hose in Hose* in turn is tightly linked to the *S* locus. Recombination between these loci is therefore expected to be rare. However once chromosomes become paired the enzymes involved in recombination may mediate further recombination throughout a localised region. This could result in variation in recombination rates as not every recombination event could be expected to provoke exactly the same number of additional recombination events. A consequence of recombination occurring in clusters is that the number of double recombinants may be much higher than might be expected. This is termed

"Negative Interference" ("Positive Interference" in contrast being when one recombination event has an inhibitory effect on the possibility of a second recombination event occurring). Negative interference is found when markers are tightly linked, as around the *Primula S* locus, and the extent of interference is expressed as a coefficient of coincidence (i.e. observed double crossover frequency/ expected double crossover frequency). Calculations from the data in tables 7.6 and 7.7 give a coefficient of coincidence of 64.6 for plant no.1 and of 6.5 for plant no. 2. There is high negative interference exhibited in both *Oak Leaf* plants, particularly in plant no.1, in this three point cross. Negative interference is a rarer phenomenon than positive interference but has been reported in other plants, e.g. *Triticum dicoccoides* (Peng *et. al*, 2000), *Hordeum vulgare* (Esch and Weber, 2002), and Maize (Goldman and Doyle, 1995; Auger and Sheridan, 2001),

Pollen size on the pin *Oak Leaf* with low anthers was checked throughout the flowering season, and pollen size was small later in the season. Further observation of the two long styled thrum *Oak Leaf* plants was also undertaken. The latter were found to be self incompatible and later in the season the corolla tubes lengthened to raise the anthers above the stigma, although the styles were longer than is usual for thrum plants. Other genes unlinked to the S locus can alter style length and flowers appear as long or short homostyles (Richards 1988, personal communication), consequently the long styled thrum *Oak Leaf* plants have not been classed as recombinants on Table 7.9 The short homostyle *Hose in Hose* with large pollen (7.3) is self compatible and set seed from controlled self pollination. This would not normally be expected from a plant with large pollen and a short style with short stigmatic papillae. It may be due to the genetic background being from the rare thrum *Hose in Hose* homozygote (*Hih S / Hih S*) that had lost thrum incompatibility (Chapter5; 5.3.4).

Molecular work on the recombinant plants in the Gilmartin laboratory is providing further information on gene order at the *S* locus. Studies of the recombinants from the investigation of the order of the linked genes *Hose in Hose*, *Oak Leaf*, and the *Primula S* locus (7.3) have enabled other *S* locus associated genes to be plotted on the map. The recombinant plants from the cross pin *sepaloid* x Thrum *Oak Leaf* heterozygous for the recessive *sepaloid* allele ($\underline{sep\ s}$ / $\underline{sep\ s}$ x $\underline{Okl\ S}$ / $\underline{+\ s}$) will also be an invaluable aid to further molecular investigation.

CHAPTER EIGHT

General Discussion.

8.1. Introduction.

Two intimately interlinked but distinct aspects to this work were investigation of the mutant phenotypes and mapping of genes around the *Primula S* locus. The availability of a large number of mutant phenotypes allowed opportunity to study flower development in a plant with distinct developmental characteristics. The value of such study had already been demonstrated in *Antirrhinum* where study of the zygomorphic *Antirrhinum* flower led to the discovery of the *CYCLOIDEA* and *DICHOTOMA* genes that are involved in flower symmetry. Subsequent study of the *CYCLOIDEA* and *DICHOTOMA* genes in a related species, *Mohavea confertiflora*, revealed a correlation between changes in gene expression and morphological form (Hileman et al. 2003). Such studies are also of interest in relation to the evolution of flowers and inflorescences (Rudall and Bateman, 2003). Like *Antirrhinum*, the *Primula* flower has a distinguishing morphological feature in that *Primula* is heteromorphic, therefore investigation of *Primula* might be expected to uncover novel data regarding development of heteromorphic flowers. The pin and thrum heteromorphic features of *Primula* have been of interest from the time of Darwin (Darwin 1861) and the subject of much subsequent literature (Ernst, 1925,1936; Crosby 1949; Bodmer 1958;Charlesworth 1979 Richards, 1986, 1993, and Kurian and Richards 1997). Heteromorphy in *Primula* is controlled by a tightly knit suite of genes known as the *S* locus (Ernst, 1925,1936; Dowrick, 1956; Richards, 1986, 1993, 1997, Kurian and Richards 1997). As investigation of the inheritance of the mutant phenotypes of *Primula* revealed that four mutant genes were linked to the *S* locus, study of these four mutant phenotypes is intimately interlinked with linkage analysis of the *Primula S* locus.

8.2. Discussion.

Investigation of the early and late ontogeny of *wild type* and mutant *Primulas* using scanning electron microscopy revealed previously undocumented characteristics of *Primula* development that are different from those observed in other species. Notable is the persistence of the common primordium of whorls 2 and 3 in the *double* flower

even though both whorls will develop as petals (Webster and Gilmartin, 2003). Similarities in *Antirrhinum* between the appearance of the fourth whorl in *Wild Type* and *plena* mutants at stage 6 has also been reported (McSteen *et al.*, 1998). The identity of the second and third whorl organs in *Primula* does not influence the early architecture of the primordium. The persistent presence of a common primordium for the second and third whorls in *double* flowers suggests that the underlying control of this aspect of floral development occurs independently of genes required for carpel and stamen development.

During early ontogeny of *sepaloid*, there is no separation of the whorl 2 and 3 organs, and whorl 4 arises from the inner base of whorl 3 as opposed to emerging from the centre of the flower (Chapter 4; Webster and Gilmartin 2003). The phenotype of the *sepaloid* mutant shows no evidence of any B function gene activity, as flowers never produce petals or stamens. The question is therefore raised as to whether B function plays a part in the separation of organs in whorls 2 and 3 during early ontogeny as it is only in *sepaloid* plants that are unable to produce petals or stamens in which the second and third whorl organs do not separate.

The delay in development of whorl 4 in those mutant phenotypes that produce stamens in the fourth whorl is also novel (Webster and Gilmartin, 2003). There is no delay in the development of the fourth whorl in *Arabidopsis* flowers when stamens are ectopically produced in this whorl, either by over-expression of *PI* and *AP3* (Krizek and Meyerowitz 1996), or following mutation of *sup-1/flo10* (Schultz *et al.*, 1991; Bowman *et al.*, 1992). In addition, the central area of the developing flower remains undeveloped for an extended period of time in *double* (Fig.4.13F), *semi-double* (Fig.4.13J and K) and in *Staminoid Carpels* (Fig.4.9H and M). This delay is associated with the presence of a mixture of petals and stamens in the centre of the flower in *doubles*, and stamens in *semi-doubles* and *Staminoid Carpels*. Given that both A and B functions are required to produce petals and that both B and C functions are required to produce stamens, these findings reinforce previous observations (Bowman *et al.*, 1992) that B function may be involved in the loss of determinacy of the floral meristem by over-riding the determinacy role of the C function. Previously, loss of determinacy has been associated with loss of C function; however, observations on two different *Primula* mutants, *semi-double* and *Staminoid Carpels*, show that loss of

show that loss of determinacy can occur following expression of B function in the fourth whorl of flowers that also contain C function.

The differences in morphology and development from those species already investigated suggest that genes regulating floral morphogenesis in *Primula* may have subtle differences in their functioning. Subsequent molecular study of *Primula* will therefore enlarge understanding of floral morphogenesis generally.

It has been shown that *Primula* flowers are homomorphic during early ontogeny and that the distinguishing features of the pin and thrum morphs develop during late ontogeny (Chapter 3). A new heteromorphic feature was discovered, the mouth of the corolla tube is wider in thrum flowers than in pin flowers. No previous studies report a difference in the size of the corolla tube mouth between pin and thrum flowers, or of the larger cells in the corolla tube above the anthers in the thrum flowers. It is not yet known whether this new heteromorphic feature is under the control of the *A* component of the *Primula S* locus or whether it is under a separate genetic control that will need to be included as another gene in the gene complex. Besides the three components of the *Primula S* locus *G/g* for the gynoecium, *P/p* for pollen size, and *A/a* for the androecium (Ernst, 1925, 1936), other genes suggested as part of the *Primula S* locus gene complex have been described by Richards and by Kurian and Richards (Richards 1997, Kurian and Richards 1997). Besides *G/g, P/p* and *A/a,* now included is, *Mpm/mpm* that controls dominance for pollen size, *Pp/pp* that controls pollen size, *Pm/pm* that controls male compatibility phenotype, *L/l* that controls thrum homozygote lethality, *Gm/gm* that contributes to stylar length, and *Mpp/mpp* that controls dominance of male incompatibility phenotype. Should recombination result in the feature of larger cells and wider corolla tube mouth cells transferring to a pin plant it would show the feature to be under separate genetic control. Such investigation could provide the basis for future study.

The absence of short homostyle progeny from crosses of short homostyle to *wild type* (Chapter 5), and to *Hose in Hose*, is notable (Chapter 6). As none of the progeny were either short homostyle or thrum *wild type* then it must be concluded that the short homostyle phenotype of the parent has to be caused by something other than a genotype of *Gpa/gpa.* Genes outside the *S* locus have been found to suppress *S*

functions in buckwheat, *Fagopyrum esculentum* (Matsui *et al.*, 2004). A generation of F2 *Primula* progeny raised to ascertain whether this may be the case in *Primula* has not yet flowered. The observation that some plants appear as short homostyle but have long stigmatic papillae and the presence of two long styled thrum plants with short stigmatic papillae among the progeny from the cross to investigate linkage analysis of *sepaloid Oak Leaf* and the *S* locus leads the author to consider that stigmatic papillae length may not always be a correlate of stylar cell length as has been previously suggested (Kurian and Richards, 1997), unless the shorter style of the short homostyle with long stigmatic papillae also has long stylar cells and the long style of the long styled thrum with short stigmatic papillae also has shorter stylar cells. This requires further investigation.

The thrum *Hose in Hose* homozygote (*Hih S/Hih S*) discovered before the commencement of this project is remarkable in that thrum homozygotes were previously not thought to be viable (Richards 1986, 1997). Although the original plant was lost in 2003, one seedling from controlled self pollination of the above plant survives. This plant will be useful for molecular investigation of the *Primula S* locus.

Characterization of the inheritance of the available floral mutants revealed an unusual number of dominant phenotypes. The inheritance of two of the dominant phenotypes, *Jack in the Green* and *Hose in Hose*, had already been published (Webster and Grant, 1990) and investigation of the inheritance of *Staminoid Carpels* and *Split Perianth* had been begun before the commencement of this project (see Chapter 5, 5.4 and 5.5). The fifth phenotype, *Oak Leaf*, was discovered in 1999 and obtained for this project in 2000. Three of the dominant mutations were found to be linked to the *S* locus, *Oak Leaf*, *Hose in Hose* and *Staminoid Carpels*. The latter two are predicted to be the result of ectopic expression of B function, in the first whorl, and in both the first and the fourth whorls, and may be allelic mutations. *Jack in the Green* in contrast is predicted to be the result of absence of early floral organ identity gene expression in the first whorl. *Split Perianth* has alteration to the usual gamosepalous and gamopetalous form of the perianth. Flowers with fusion of perianth organs are generally regarded as being at a higher level of evolution than those in which they are free from each other (Sporne, 1974). Consequently it could be postulated that loss of such fusion in the *Split Perianth* mutant phenotype may be considered as a return to a more ancestral

state. The *Split Perianth* mutant phenotype may therefore be useful in evolutionary studies of flower form. Reflexing of the petals in those flowers with the corolla tube divided into five separate pieces (Figure 4.6, C and D) has been proposed as a possible phenotypic modification necessary for an evolutionary shift from the heterostyly of *Primula* towards the solenoid flowers of *Dodecatheon* (Mast *et al.*, 2004). *Oak Leaf* is a pleiotropic mutant phenotype, with both leaf and flower morphology affected, and exhibits some of the characteristics of the phenotype described in *Arabidopsis thaliana* when the *Knotted* gene from maize was expressed ectopically (Chuck *et al*, 1996).

Another species of *Primula, P. sinensis*, was extensively analysed in the past (Gregory, 1911; Gregory, de Winton, and Bateson, 1923; and de Winton and Haldane, 1933), but only three of the thirty five phenotypes of *P. sinensis* investigated were found to be dominant (De Winton and Haldane, 1933). Since then, all of the variant forms of *P. sinensis* appear to have been lost. If they were still available they could have been used for molecular study today. However in *P. sinensis* no recombination within the *S* locus has been recorded (Lewis and Jones, 1993) so it would not therefore be a useful model for study of heteromorphy as are *P. vulgaris* and *P. vulgaris* cultivars in which recombination within the *S* locus does take place. In addition the three dominant mutations, and the one recessive mutation found to be linked to the *Primula S* locus provided opportunity for mapping of the genes around the *S* locus.

Two of the mutations linked to the *Primula S* locus result in the predicted gain of and loss of B function phenotypes *Hose in Hose* and *sepaloid*. No complementary gain and loss of B function phenotypes have been reported for other species, but complementary gain and loss of C function in *Antirrhinum* was found in the *OVULATA* and *PLENA* mutations (Bradley *et al.*, 1993). As the dominant nature of the *Hose in Hose* mutation precludes complementation tests a three point cross (see Table 7.4) was used as a segregation test with the additional aim of mapping genes linked to the *Primula S* locus. Numbers of progeny obtained were insufficient for a conclusive segregation result. However even with larger numbers it would not be possible to prove a negative result as absence of recombinants could be due either to lack of recombination between two tightly linked genes, or to recombination not being possible due to the two mutations being allelic. Mapping of genes to the same or to a different locus would work more efficiently if negative interference was not an

additional factor. In this instance the variable recombination rates resulting from negative interference make precise mapping impossible. If, as is indicated by the single putative recombinant in Chapter 7, Table 7.3, and the sequence data obtained in the Gilmartin laboratory, two genes are involved then larger numbers would at least present greater opportunity for obtaining recombinants that would furnish proof that two genes are involved.

Hose in Hose and *Oak Leaf* are on opposite sides of the *S* locus (see Table 7.5). From the results shown in Table 7.9 *sepaloid* is on the opposite side of the *S* locus to *Oak Leaf* and therefore has to be on the same side of the *S* locus as *Hose in Hose*. The similar distances of each from the *S* locus (Tables 7.7.and 7.10) indicates that they have to be either allelic mutations or two separate but very tightly linked loci.. No tightly linked genes involved in flower development have been reported from other species studied, although loose linkage of the *Petunia* genes *PhAp2A* and *Bl* on chromosome 4 has been reported (*Maes et al.*, 2001). Both the *sep1* and *PISTILLATA* genes of *Arabidopsis* are on chromosome 5 but are not tightly linked, while the *sep2* and *APETALA 3* genes are on opposite arms of chromosome 3 (Arabidopsis TAIR website (http://www.arabidopsis.org/ <http://www.arabidopsis.org/>). If as predicted *sepaloid* and *Hose in Hose* are two separate genes then the *Hose in Hose* phenotype must be epistatic to *sepaloid* (see 7.2, Table 7.3) as the presence of *Hose in Hose* masks the presence of *sepaloid*. If they were allelic it would be predicted that *Hose in Hose* with predicted ectopic B function would be dominant to *sepaloid* that has no evidence of any B function.

Hose in Hose is predicted to result from ectopic B function in the first whorl. Parallel work in the laboratory (Dr. J. Li and Prof. P. Gilmartin, personal communication) has cloned *Primula DEFICIENS* and *GLOBOSA* homologues and analysed linkage to the *S* locus. *Primula GLOBOSA,* but not *DEFICIENS,* was found to be linked to the *S* locus and *GLOBOSA* polymorphisms were found to segregate with *Hose in Hose.* Analysis of genomic clones of *GLOBOSA* from *wild type* and *Hose in Hose* identified a retrotransposon in the promoter of *Hose in Hose* that is predicted to cause upregulation of *GLOBOSA*. Instability of the retrotransposon is suggested by the reversion of *Hose in Hose* to wild type both on individual ramets, on individual flowers on the same scape and on individual calyx lobes. This could be due to

excision, which is unusual for a retrotransposon, or possibly an epigenetic effect associated with chromatin remodeling or with methylation. Further experiments are underway to determine the molecular basis of the instability. Analysis of *GLOBOSA* in *sepaloid* has found absence of *GLOBOSA* expression, but no mutations were discovered within the transcription unit that could cause loss of expression. This suggests that *sepaloid* may be the result of a mutation in a regulator of *GLOBOSA*. However there is a 5bp insertion within the *sepaloid GLOBOSA* promoter that coincides precisely with the insertion point of the retrotransposon in *Hose in Hose*. If *sepaloid* was due to excision of the retrotransposon from *Hose in Hose* conversion of the dominant *Hose in Hose* to the recessive *sepaloid* would be masked by the now dominant *Wild Type* allele, but could appear in a subsequent generation. However there is no evidence for *Hose in Hose* in any *sepaloid* background. The author has now obtained *sepaloid* plants on three separate occasions from commercial primrose stock and no *Hose in Hose* primroses are, or have been, commercially available to date.

The phenotype of *sepaloid* bears some resemblance to the *SEPALLATA 1/2/3* triple mutant (Pelez *et al.* 2000.2001; Honma and Goto 2001). Of interest is the gain of function *sepaloid Oak Leaf* that has some petal cells in whorl two. These cells in turn resemble those of the yellow eye cells of *Primula viridis* the green primrose (Ch 4, Figure 4.21Q), that shares some phenotypic characteristics with transgenic tobacco plants expressing antisense RNA for the gene *TM5* (Pnueli *et al*, 1994). Co-supression of the Petunia homeotic gene *fbp2* also gives flowers with modified second third and fourth whorl organs (Angenant *et al*, 1994), and this gene has been found to be highly homologous to *TM5* of tobacco. Secondary inflorescences have recently been observed in *sepaloid* primrose flowers and these too bear some resemblance to the Petunia *FBP2* cosuppression mutant (Angenant *et al.*, 1994). The Petunia *FBP2* gene is considered to be a putative orthologue of the *Arabidopsis SEPELLATA* 3 gene (Ferrario *et al.* 2003). It is tempting to predict that *sepaloid* will be found to be homologous to *SEPALLATA* 3 and that the green primrose phenotype will be caused by an allelic mutation to *sepaloid*.

The *Primula sepaloid* mutant phenotype also shares similarity with the most extreme form of the *FIMBRIATA* mutant of *Antirrhinum* (Simon *et al.*, 1994). The *Arabidopsis* gene *UNUSUAL FLORAL ORGANS* shows extensive homology with *FIMBRIATA*

but differences in the functions and genetic interactions were found (Ingram *et al.*, 1995) *UNUSUAL FLORAL ORGANS* is, along with *LEAFY*, an upstream co-regulator of the B function organ identity gene *APETALA 3* (Parcy *et al.*, 1998; Honma and Goto, 2000). The *sepaloid* mutant of *Primula* differs from both of the above in that no less extreme forms of the *sepaloid* phenotype have so far been discovered. The possibility that the *sepaloid* phenotype could be the result of an as yet uncharacterized gene cannot be ruled out.

The *Primula S* locus has been a focus of interest from the time of Darwin (Darwin 1861) and the subject of much subsequent literature (Ernst, 1925,1936; Crosby 1949; Bodmer 1958;Charlesworth 1979 Richards, 1986, 1993, Lewis and Jones 1993 and Kurian and Richards 1997). There have been previous attempts to map the main components of the *S* locus (Ernst, 1925, 1936; Dowrick, 1956, Richards 1986, 1997; Lewis and Jones (from Ernst's data) 1993; and Kurian amd Richards 1997). However a major difficulty has been the lack of flanking markers for the locus (Lewis and Jones 1993). This work has revealed that *Hose in Hose* is on one side of the *S* locus and *Oak Leaf* is on the other, and so identifies the long sought after outside markers for the *Primula S* locus. A second marker, *sepaloid,* is also on the opposite side of the *S* locus to *Oak Leaf.* Flanking markers are required to allow classical genetic mapping of the *Primula S* locus. Plants that have been generated that had recombination between these outside loci and the *S locus*, and/or between these loci and within the *S* locus, have provided further valuable tools for molecular analysis of the *Primula S* locus.

The three plants that exhibited mixed size pollen require further investigation to discover if they behave as the *P** plants described by Kurian and Richards (Kurian and Richards, 1997). If they are found to be *S* locus recombinants the gene order of the *S* locus will be *Okl/okl G/g A/a P/p Hih/hih.* To have half of the pollen large a dominant *P* must be present (although if not all pollen is large some of the dominance must have been lost as was reported for the *P** plants of Kurian and Richards (Kurian and Richards, 1997)). Recombination events that could give a pin *Oak Leaf* with some large pollen and a pin *Hose in Hose Oak Leaf* with some large pollen are either,

Okl g a - p - hih to give *Okl g a P hih* and *okl G A p Hih* as gametes of
okl G A – P - Hih the genotypes

Or,

Okl g a - p hih to give O*kl g a P Hih* and *okl G A p hih* as gametes of
okl G A - P Hih the genotypes

If gene order was either *Okl g p a hih* or *Okl a p g hih* or *Okl p a g hih*
okl G P A Hih *okl A P G Hih* *okl P A G Hih*

a triple crossover would be required to give the phenotype of pin *Hose in Hose-Oak Leaf* with some large pollen.

The pin *wild type* with some large pollen (found in the progeny from the test of *wild type* x short homostyle *Hose in Hose* with large pollen) is also possible from the above gene order with a double recombination event.

okl g a - p - hih to give o*kl g a P hih* and *okl G a p Hih* as gametes of
okl G a - P - Hih the genotype

It would appear from these results that *P* is not fully dominant in the absence of *G*. In both generations of short homostyle *Hose in Hose* with large pollen, it could be predicted from the phenotype that there was also a dominant *G* present but no dominant *A* and there was no variability in pollen size. In the three plants generated that had mixed size pollen it could be predicted from the phenotype that there was neither a dominant *G* nor a dominant *A* present. The position for the suggested gene for dominance, *Mpm*, (Richards, 1997), was given as *G A Mpm P*, but from the results obtained above such a gene would have to be either proximal or distal to *G* (*G Mpm A P*, or *Mpm G A P*). However long homostyles, with no dominant *G*, but with a dominant *A* present, also have a dominant *P*. This would both suggest that *P* is fully dominant in the presence of either *G* or *A*, and that *Mpm* is between *G* and *A*. Since this is not consistent with the results obtained by Kurian and Richards (Kurian and Richards, 1997) it raises the question of whether dominance of *P* is indeed the result of a single gene at a given locus or whether instead other factors could be responsible. A possible alternative hypothesis is that *P* is actually a null allele and that large pollen would be the norm without the suppressant factor of *p*. If one allele of *p* was insufficient to suppress pollen size, with the homozygote *pp* being epistatic to *P*,

then one slightly deficient *p* allele may result in *P* not being completely suppressed. This hypothesis requires further investigation.

8.3. Further studies.

It would be possible to repeat a three point cross to confirm that *Hose in hose* and *sepaloid* are two separate genes. A repeat experiment would use a number of plants of *Hose in Hose* heterozygous recessive for *sepaloid* (*Hih + S/ + sep s*) crossed to pin *sepaloid* (*+ sep s/+ sep s*). Large numbers of progeny would be required before recombinants could be expected. Alternatively, as the *SEPALLATA* and *FIMBRIATA* genes are possible candidates for *sepaloid* homologues a molecular approach is also possible. The *Primula SEPALLATA* and *FIMBRIATA* equivalents could be isolated and tested for linkage to the *Primula S* locus. Any gene not linked to the *S* locus could be eliminated from the investigation. If linkage was discovered it would then be possible to look for polymorphisms that co-segregated with the mutant phenotype.

A number of other questions were raised from investigations carried out during this project. Investigation of the inheritance of the new heteromorphic feature of wider cells and correspondently wider corolla tube mouth of thrum plants could be undertaken by a large cross of pin x thrum *wild type*, in order to discover whether the locus responsible can recombine on to the s allele to enable its presentation in pin plants. In addition further investigation could be carried out to discover whether *Oak Leaf* plants are earlier flowering than plants with *wild type* leaves, or whether there is more recombination in the egg than in the pollen of *Primula* cultivars.

Of particular interest is the anomaly in the size of pollen. Variation in pollen size in a single plant over a flowering season has not previously been documented and further observation and investigation of both the pin *Oak Leaf* and the pin *Hose in Hose-Oak Leaf* is required. The mixed size pollen discovered on *P. Tommassinii* (Kurian and Richards, 1997) was considered to be the result of recombination within the *S* locus. The pollen of the plants with mixed size pollen discovered during this project varied between being of mixed size early and late in the flowering season and being small pollen in the middle of the flowering season. By self pollinating the plants and by test crossing to both pin and thrum *wild type*, whether the plants behave as do those *P** plants described by Kurian and Richards (Kurian and Richards, 1997) can be

determined. If these plants are *S* locus recombinants this will give gene order of the main components of the *Primula S* locus.

Alternatively, should study of the plants with mixed large and small pollen fail to confirm them as *S* locus recombinants, the newly discovered flanking markers for the *Primula S* locus will allow the author to undertake classical genetic analysis of the main components of the *Primula S* locus. Order of the main components of the Primula *S* locus has never been verified. Ernst originally gave the gene order as *GAP*, but Lewis and Jones (1993) on re-evaluating his data concluded that the order must be *GPA* as the former gene order would require a double cross-over in the thrum parent to generate the most common recombinants. More recently *GAP* has been suggested as the order of the main component parts of the *S* locus and it has also been suggested that heterostyly may have evolved separately in *P. x tommasinii* (subgenus *Primula*) and *Primulas* of subgenus *Auriculastrum* from which Ernst's data was derived (Kurian and Richards, 1997; Richards 1997), Consequently pollinations have been done by the author to generate seed that will give plants with the genotypes,

1. *Okl g a p Hih / + g A P +*
2. *Okl g a p + / + G a PH* (actual gene order is unconfirmed).

The second genotype is generated using the short homostyle *Hose in Hose* with large pollen, a recombinant plant from the three point cross in Table 7.5. Parallel three point crosses to *wild type* that will give large numbers of progeny should result in sufficient numbers of recombinants to reveal the gene order of *G/g A/a* and *P/p*. Work sheets have been drawn up elucidating possible recombinant phenotypes and showing which parallel recombinant phenotypes will reveal gene order (see Appendix).

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APPENDIX

Work sheets for classical genetical analysis of the *Primula S* locus.

For simplicity, genes have been given only single letter designations in the work sheets.

Flanking markers.
O = Oak Leaf
H = Hose in Hose

Genes within the *S* locus.
G = gyneocium, controls style length and stigmatic papillae length
A = andreocium, controls anther position
P = Pollen, controls pollen size

Two three point crosses are used, one of long homostyle *Hose in Hose-Oak Leaf* crossed to *wild type* and one of short homostyle *Hose in Hose-Oak Leaf* with large pollen crossed to *wild type*. This increases the chance of discovering recombinants that will give the gene order, as besides those few recombinants that would in themselves define gene order, the presence of a particular recombinant from one cross along with a particular recombinant from the second cross would only be possible with one particular gene order.

Work sheets for three point crosses of plants of putative genotypes (S locus gene order unknown) to pin wild type.

1. If G is next to O.

Putative genotype of non wild type plant	Crossed to wild type genotype	No.1.		Putative genotype of non wild type plant	Crossed to wild type genotype	No.2.	
OgapH	ogaph			Ogaph	ogaph		
ogAPh	ogaph	Phenotypes		oGaPH	ogaph	Phenotypes	
	Sco A/P	OP	AH		Sco G/a	OPH	G
	Sco g/A	OAP	H		Sco a/P	OPH	G
	Dco A/P + P/h	OPH	A		Dco G/a + P/H	OP	GH
	Dco O/g + A/P	OAH	P		Dco O/g + G/a or a/P	OG	PH
	Dco in S locus	OAH	P		Dco in S locus	O (as parentals)	GPH
		No. 3.				No. 4.	
OgpaH	ogpah			Ogpah	ogpah		
ogPAh	ogpah	Phenotypes		oGPaH	ogpah	Phenotypes	
	Sco P/A	OA	PH		Sco G/P	OPH	G
	Sco g/P	OPA	H		Sco P/a	OH	GP
	Dco P/A + A/h	OAH	P		Dco G/P + a/H	OP	GH
	Dco O/g + P/A	OPH	A		Dco O/g + G/P	OG	PH
	Dco in S locus	OPH	A		Dco in S locus	OP	GH

Phenotypes that will ONLY be possible if G is next to O are, OAP + H (sco g/A), OPH + G, (sco G/a or a/P or G/P) and OH + GP (sco P/a), the latter two phenotypes are definitive for gene order ogpah.

Combinations of phenotypes (one from each cross) that give gene order are,
OP + AH (sco A/P) with OG + PH (sco P/a)
OP + AH (sco A/P) with OPH + G, (sco G/a or a/P or G/P). These combinations can only occur if gene order is ogaph.

OA + PH (sco P/A) with OPH + G, (sco G/a or a/P or G/P).
OA + PH (sco P/A) with OP + GH (dco G/P + a/H, or dco in S locus)
OA +PH (sco P/A) with OH + GP (sco P/a).
OPA + H (sco g/P) with OH + GP (sco P/a). These combinations can only occur if gene order is ogpah.

2. If G is next to H.

Putative genotype of non wild type plant	Crossed to wild type genotype	No. 5.		Putative genotype of non wild type plant	Crossed to wild type genotype	No. 6.	
OpagH	opagh			Opagh	opagh		
oPAgh	opagh	Phenotypes		oPaGH	opagh	Phenotypes	
	Sco P/A	OA	PH		Sco P/a	OGH	P
	Sco A/g	O	PAH		Sco a/G	OGH	P
	Dco P/A + g/H	OAH	P		Dco P/a + G/H	OG	PH
	Dco O/p + P/A	OPH	A		Dco O/p + a/G	OG	PH
	Dco in S locus	OAH	P		Dco in S locus	O As parentals)	PGH
		No. 7.				No. 8.	
OapgH	oapgh			Oapgh	oapgh		
oAPgh	oapgh	Phenotypes		oaPGH	oapgh	Phenotypes	
	Sco A/P	OP	AH		Sco P/G	OGH	P
	Sco P/g	O	APH		Sco a/P	OPGH	(wild type)
	Dco A/P + g/H	OPH	A		Dco a/P + G/H	OPG	H
	Dco O/a and A/P	OAH	P		Dco O/a + P/G	OP	GH
	Dco in S locus	OPH	A		Dco in S locus	OP	GH

Phenotypes that will ONLY be possible if G is next to H are, O + APH (sco A/g or P/g), OGH + P (sco P/a or a/G), OPG + H (dco a/P and G/H) and OPGH + wild type (sco a/P); the latter phenotypes are definitive for gene order oapgh.

Combinations of phenotypes (one from each cross) that give gene order are,
OA + PH (sco P/A) with OGH + P (sco P/a or a/G).
O + PAH (sco P/g) with OG + PH (dco P/a + G/H or dco O/p + a/G). These give gene order as opagh.

OP + AH (sco A/P) with OGH + P ((sco P/a or a/G),
OP + AH (sco A/P) with OPG + H (dco a/P and G/H),
O + APH (sco P/g) with OPG + H (dco a/P and G/H),
O + APH (sco P/g) with OP + GH (dco in S locus). These give gene order as oapgh.