Investigation of a Glucosyltransferase that Recognises Abscisic Acid

by

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ABSTRACT

Family 1 of glycosyltransferases (GTs) catalyse the transfer of sugars to small acceptor molecules such as secondary metabolites, phytohormones or xenobiotics. Glycosylation changes the solubility, bioactivity or transport properties of the acceptors. In the Arabidopsis genome, 107 GT-encoding genes from family 1 have been identified by sequence analysis. Through an *in vitro* screen, it was found that eight of them could glucosylate the plant hormone abscisic acid (ABA), but only one, UGT71B6, displayed selectivity for the natural (+)-ABA enantiomer.

In plants, ABA is involved in many processes such as seed germination and dormancy, root and shoot growth, maintenance of water relations and stress tolerance. Glucosylation leads to deactivation of the hormone and may play a role its homeostasis. This work has characterised the glucosylation of ABA by UGT71B6 *in vitro,* and investigated the impact of changing the expression of the gene *in planta.*

Detailed biochemical characterisation of the enzyme activity was carried out in which optimum assay conditions and kinetic properties were determined. A range of structural analogues of ABA were used to explore the features of the molecule required for glucosylation by the enzyme. Interestingly, the GT could accommodate extra bulk around the double bond of the ABA ring, but not alterations to the 8′ and 9′ methyl groups.

Transgenic plants constitutively overexpressing (OE) the *UGT71B6* gene were studied with respect to phenotypic characteristics known to relate to ABA. The seedlings of OE plants were found to be more resistant to glucose stress and inhibition of post-germinative growth by exogenous ABA. Additionally, water loss from detached leaves of OE plants was accelerated compared to wild type plants. These observations were consistent with the deactivation of ABA by the transgenic UGT71B6. Profiling of ABA and related metabolites *in planta* revealed that overexpression of *UGT71B6* led to increased accumulation of the ABA-glucose ester. Interestingly, the amount of free ABA in OE plants was only changed in conditions of water stress.

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OPENING QUOTE

Poetry and Hums aren't things which you get, they're things which get you. And all you can do is to go where they can find you.

(Milne, 1928)

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Over the last three years my time, emotional energy and intellectual head-space have been almost fully engaged with the investigation of a glucosyltransferase that recognizes abscisic acid. The physical evidence of this and the advancement in human knowledge attained are summarized in this thesis in addition to a (couple of?) publication(s). However, the achievement over these years would be vastly undersold if it was limited to the physical evidence. Much more important are the transformations that have been occurring within my own self. The process is often more valuable than the end-product: the journey as important as the destination. When I came to the Bowles group, I felt the child-like wonder of the world of plant science and had so many questions about how everything worked. I was not ready to stop asking; and I wanted to learn how to answer the questions myself. I have enjoyed learning how to listen, read, speak, write and think critically and rigorously. I have also had to learn how to organize, prioritise and when things get too much, sit down and make a list. I believe that when the book lies dusty on the shelf, I will still be carrying and using these skills with me wherever I go.

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CHAPTER 1: INTRODUCTION

1 INTRODUCTION

1.1 Glycosyltransferases

Glycosylation is an important process in living cells, involving the transfer of an activated sugar molecule to an acceptor molecule, and is catalysed by the glycosyltransferases (GTs). The activated donor sugar molecules are typically nucleotide diphospo(NDP)-sugars, but may also include dolichol-phopho-sugars, sugar-1-phosphates, nucleoside monophospho-sugars and lipid diphospho-sugars (Coutinho *et al.*, 2003). Acceptor molecules range from small secondary metabolites to large proteins, lipids or growing polysaccharide chains. The GTs have therefore been described to be, quantitatively, the most important enzymes on earth since they are responsible for the bulk of biomass transfer (Campbell *et al.*, 1997).

All the available sequences for NDP-GTs were obtained from the SwissProt and EMBL / GeneBank databases in 1997 and analysed systematically by hydrophobic cluster analysis. The 555 gene sequences were divided into 26 families, of which families 1, 2 and 4 were large (Campbell *et al.*, 1997). As more sequences become available they are classified into new or existing families and can be viewed on the CAZy (Carbohydrate-Active enZYmes) website (http://afmb.cnrs-mrs.fr/CAZY/GT.html). To date, there are 78 GT families.

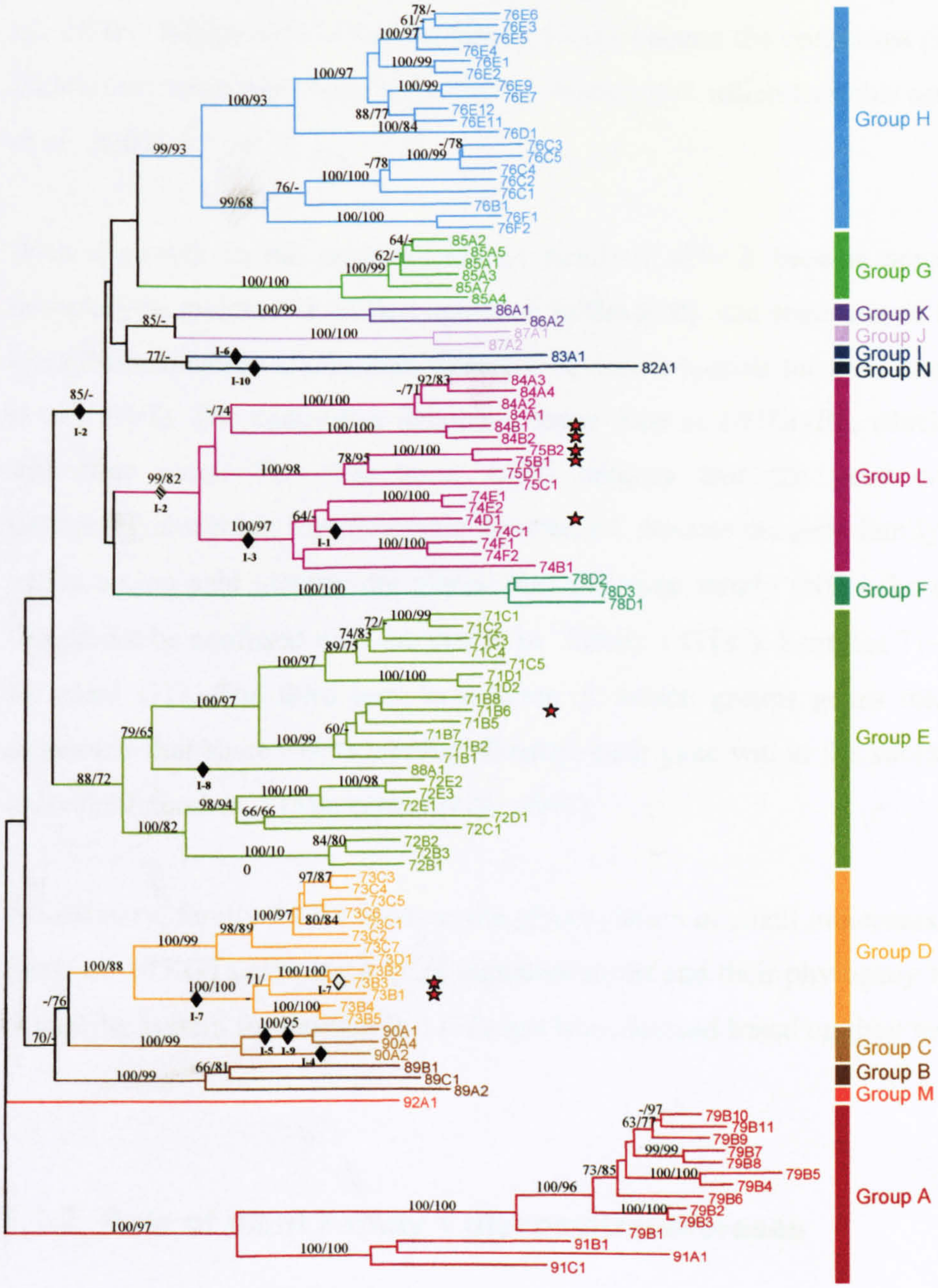
1.1.1 Family 1 glycosyltransferases

Family 1 GTs include those enzymes that catalyse the glycosylation of small molecular weight lipophilic molecules (reviewed in Lim and Bowles, 2004). A subset of family 1 GTs contain a 44 amino acid consensus sequence towards the C terminal region of the polypeptide chain. This consensus sequence has been described as the UDP-glucosyltransferase (UGT) signature motif and was proposed to form the UDP binding

site (Hundle *et al.*, 1992). Photoaffinity labelling studies have since shown that residues in the signature motif of UGT2B4 do interact with UDP (Pillot *et al.*, 1993). Hughes and Hughes (1994) later refined the sequence for plant specific sequences as a Plant Secondary Product Glycosyltransferase (PSPG) motif, believing that UGTs were only involved in secondary metabolism. Interestingly, UGTs containing this PSPG motif have since been found to glycosylate phytohormones (Szerszen *et al.*, 1994; Jackson *et al.*, 2001; Lim *et al.*, 2002) and components of primary metabolism, such as lignin monomers (Lim *et al.*, 2001; Lim *et al.*, 2005), as well as non-natural xenobiotics (Loutre *et al.*, 2003; Poppenberger *et al.*, 2003).

In plants, UDP-glucose was a typical sugar donor for family 1 GTs whereas UDP-glucuronic acid was a typical donor in mammals. In consequence, the family 1 GTs have frequently been referred to as UGTs. It has since been shown that GTs containing the consensus will also utilise UDP-rhamnose, UDP-galactose, UDP-xylose (reviewed in Bowles *et al.*, 2005) and also a dTDP activated sugar, dTDP-glucose (Lim EK, unpublished data). Therefore, it is more accurate to refer to these enzymes as family 1 GTs rather than UGTs. Nevertheless, certain terminology remains including the systematic names given to genes.

The completion of the genome sequencing project for *Arabidopsis thaliana* (Arabidopsis) in 2001 provided a major opportunity for the rapid identification of genes within specific families in the model plant. The Bowles group utilised the UGT signature motif to screen the Arabidopsis genome for all genes containing the 44 amino acid consensus sequence. The results revealed 107 genes, in addition to 10 pseudogenes with interruptions to their open reading frames (ORFs) (Li *et al.*, 2001; Ross *et al.*, 2001). Nine conserved motifs within the genes were used to analyse their phylogeny and 14 main groups were identified (Figure 1.1)(Li *et al.*, 2001; Ross *et al.*, 2001). Consistent with the previous understanding that family 1 GTs in plants are located in the cytosol, none of the Arabidopsis genes contained obvious signal sequences or targeting signals for membranes (Li *et al.*, 2001). Also consistent with previous findings, the N terminal regions were more variable than the C terminal regions, supporting the suggestion



Modified from Ross *et al.*(2001)

Figure 1.1 Phylogenetic analysis of 107 Arabidopsis family 1 GTs

The numbers above the nodes are bootstrap values derived by two independent methods. Hypothetical intron gains ♦ and losses ◊ are indicated by diamonds followed by intron number. GTs with activity towards ABA are indicated with a red star.

that the C terminal part of the polypeptide chain is involved in binding of the NDP-sugar, and the N terminal region is involved in recognising the diverse acceptor molecules (Li *et al.*, 2001). Whilst 48% of known family 1 GTs contain the consensus (Lim and Bowles, 2004), only three have been identified in Arabidopsis which lack this sequence (Paquette *et al.*, 2003).

With a growth in the identification of family 1 GTs it became apparent that it was necessary to maintain a unified approach in the study and reporting of these genes. The UGT Nomenclature Committee therefore devised a system for their naming (Mackenzie *et al.*, 1997). The committee denotes a name such as *UGT84B1*, which can be divided into four parts. The first term, *UGT*, denotes that the gene encodes a UDP-GlycosylTransferase. The following number, *84*, denotes the gene family: sequences with >45% amino acid identity are placed into the same family (NB - the term family here should not be confused with the family in "family 1 GTs"). Families 71-100 are reserved for plant GTs. The third term is a letter, *B*, which groups genes into subfamilies of sequences that share >60% identity. Finally, each gene within the subfamily is given an individual number, *1* (Mackenzie *et al.*, 1997).

In summary, family 1 GTs catalyse the glycosylation of small molecules. In Arabidopsis, there are 107 GTs carrying a UGT signature motif and their phylogeny has been studied. A naming system for the family 1 GTs has been devised based on their sequence identity.

1.1.2 Role of plant Family 1 glycosyltransferases

The roles of family 1 GTs have been reviewed in detail (Jones and Vogt, 2001; Lim and Bowles, 2004; Bowles *et al.*, 2005). These enzymes catalyse the transfer of activated sugar moeities onto small acceptor molecules at –OH, -COOH, -NH_2, -SH or C-C groups. In plants, acceptor molecules include phytohormones, secondary metabolites and xenobiotics. Glycosylation of these small molecules increases their size and polarity,

which can serve to alter their activity, toxicity, solubility and transport properties, both within the cell and throughout the plant.

The activity or toxicity of a small molecule could be changed through multiple mechanisms (Jones and Vogt, 2001). First, the increased size or altered polarity could prevent the molecule from binding to its receptor. Second, if the functional group to which the sugar binds is important for receptor ligand interactions it will be blocked. Third, the reactivity and toxicity of many acceptors relies on the nucleophilic properties of the molecule; the attachment of glucose to an aglycone can reduce the possibility of electron transfer and thus lower the reactivity. Fourth, the glucosylation of the small lipophilic molecules can prevent passive diffusion across membranes or allow access to transporters that will cause them to be exported from the cell or sequestered to the vacuole.

Several members of family 1 GTs have been shown to be capable of glycosylating multiple substrates *in vitro*. Additionally, many GTs may be capable of glycosylating the same substrate. They can glycosylate both endogenous compounds and also molecules which are foreign to the plant, including herbicides and toxins. It can therefore be difficult to determine the 'true role' of a specific enzyme. The catalytic activity of an enzyme towards a substrate *in vitro* may not reflect the substrates which the enzyme would encounter *in planta*. Also, when a GT is in its natural environment *in planta*, it may not be capable of acting on specific substrates identified through an *in vitro* screen. Further, the size of the gene family implies that some redundancy may exist and even if a gene is 'knocked out', enzymes encoded by other GT genes may compensate. Therefore, no loss of function would be observed, and no 'role' could be conclusively assigned. In fact, it could be argued that each GT has multiple roles and that one role of the family is to be able to respond to new, potentially toxic, compounds that have not exerted any prior evolutionary pressure. As such, it is perhaps misleading to describe any enzyme that glucosylates a particular substrate *in vitro* and *in planta*, for example ABA, as 'the' ABA glycosyltransferase, but rather refer to it as 'an' ABA glycosyltransferase.

1.1.3 Strategies to identify GT functions

A range of strategies have been used to identify which GTs have particular functions (reviewed in Bowles *et al.*, 2005). One approach involves the biochemical purification of enzyme activities from plant extracts, followed by the cloning of the corresponding gene (Xu *et al.*, 2002; Loutre *et al.*, 2003). Loutre *et al.* (2003) observed a 50 kDa polypeptide in the fraction of an SDS-PAGE gel associated with activity towards the pollutant 3,4-dichloroaniline. The polypeptide was excised from the gel and MALDI-TOF mass spectrometry used to identify the corresponding gene. Degenerate primers have also been used to clone GTs similar to those known in other species. For example, to identify GTs involved in the glucosylation of the saffron component crocetin, Moraga *et al.* (2004) amplified GT sequences from the cDNA from stigmas of *Crocus sativus*. The glucose ester was known to accumulate in this organ, and the recombinant protein of one of the two GTs identified was shown to recognise the substrate *in vitro*.

Since the identification of the 107 GTs in Arabidopsis, the Bowles group has applied a systematic screening mechanism for acceptors *in vitro*. The enzymes were expressed as recombinant proteins in *E.coli* and purified by means of a GST tag before their activities towards particular substrates determined (Jackson *et al.*, 2001; Lim *et al.*, 2001; Lim *et al.*, 2002; Hou *et al.*, 2004; Lim *et al.*, 2005). Whether the enzymes catalyse the same reactions in plants can be tested by analysing plants in which the genes are overexpressed or knocked out (Jackson *et al.*, 2002).

GTs with particular catalytic activities have also been cloned through unbiased screens. Quiel *et al.* (2003) carried out a genetic screen with mutants of an Arabidopsis line that over-accumulated a fluorescent conjugate of anthranilate. The *ugt74f2* loss-of-function mutant showed reduced fluorescence. The recombinant UGT74F2 protein was then shown to catalyse the glucosylation of anthranilate *in vitro*. Poppenberger *et al.* (2003) functionally expressed an Arabidopsis cDNA library in yeast to screen for candidates that

confer resistance to the mycotoxin deoxynivalenol. UGT73C5 was isolated using this approach and shown to inactivate the toxin by glucosylation. Plants overexpressing the GT also showed increased tolerance to the mycotoxin (Poppenberger *et al.*, 2003). Interestingly, the same plants also displayed a brassinosteroid deficient phenotype, accumulating reduced amounts of brassinosteroids. Transgenic lines silenced in *UGT73C5* expression were less able than wild type to glucosylate exogenously supplied brassinolide. This implied that UGT73C5 may function in the glucosylation of brassinosteroids in wild type plants (Poppenberger *et al.*, 2005).

Therefore, a range of strategies have been applied to identify substrates for GTs. It has been suggested that activation tagging may be useful to identify genes involved in phytohormone metabolism (Nambara and Marion-Poll, 2005). It will be interesting to see whether such a strategy leads to assignment of function and capability to more GTs.

1.1.4 Glycosylation in phytohormone homeostasis

The level of phytohormones in plant cells is controlled through homeostatic mechanisms involving biosynthesis, redistribution and inactivation through catabolism or conjugation. These processes combine to result in controlled levels of each phytohormone in key cells and tissues at specific developmental time-points and in response to particular stimuli.

Glucose conjugates are known to exist for a range of phytohormones including auxins, gibberellins, cytokinins, brassinosteroids, salicylic acid, jasmonic acid and abscisic acid (ABA). Glycosylation of these phytohormones is generally thought to result in deactivation. Glycosylation can be reversible or irreversible, dependent on the individual hormone. It should be noted that results from applying hormone glycosides to plant tissues can be difficult to interpret, since there is uncertainty with regard to both uptake and cleavage by extracellular glycosidases (reviewed in Kleczkowski and Schell, 1995).

Kleczkowski and Schell (1995) suggested that in order to learn more about the conjugation of phytohormones, the traditional method of observing metabolic products from applied phytohormones is not sufficient. They highlighted the importance of studying the kinetics of the metabolic enzymes involved. They also suggested that studies involving mutants or transgenic plants with altered expression of key genes would allow scientists to study phytohormone conjugation without complications arising from hormone application including those related to uptake, transport, wounding and tissue culture.

1.2 Abscisic acid

1.2.1 Role of ABA

ABA plays important roles in the control of growth, transpiration, stress tolerance and the development and germination of seeds (Finkelstein and Rock, 2002). Many of these processes seem to be linked to tolerance of water deficit. For example, a plant that is unable to regulate stomatal opening and transpiration will lose turgor: loss of turgor prevents cell expansion and therefore growth. Stresses caused by excess salt, osmolytes, and freezing all reduce the relative amount of water available to the cell. Developing seeds experience low levels of water, particularly during desiccation.

A range of ABA deficient mutants have been identified through genetic screens that are compromised in these physiological processes (Finkelstein and Rock, 2002; Schwartz *et al.*, 2003; Nambara and Marion-Poll, 2005). In Arabidopsis, typical ABA biosynthetic mutants, such as *aba3-2*, accumulate very low levels of the hormone (2-20% compared with wild type) resulting in poor growth, reduced seed dormancy, lack of stomatal control and wiltiness (Rock and Zeevaart, 1991; Leon-Kloosterziel *et al.*, 1996; Seo *et al.*, 2000). The phenotypic severity of ABA deficient mutants tends to correlate with the level of ABA in the plant (Koornneef *et al.*, 1982; Rock and Zeevaart, 1991; Seo *et al.*, 2000;

Gonzalez-Guzman *et al.*, 2004; Seo *et al.*, 2004). For example, the *aao3-1* mutant accumulated ABA at 60% of wild type levels in dry seeds and exhibited wild type dormancy, but displayed a wilty phenotype (Seo *et al.*, 2000; Gonzalez-Guzman *et al.*, 2004). The *aao3-2* and *aao3-3* mutants, with more severe lesions, accumulated ABA at 30-40% of wild type levels in dry seeds and show reduced dormancy (Gonzalez-Guzman *et al.*, 2004).

The capability of ABA in stress tolerance and dormancy has also been demonstrated using transgenic plants with increased levels of ABA. For example, the level of ABA was increased two to three fold in *Lycopersicon esculentum* (tomato), *Nicotiana plumbaginifolia,* and Arabidopsis, by overexpression of biosynthetic genes. The increased ABA in these transgenic lines resulted in increased seed dormancy, decreased water loss and increased drought tolerance (Thompson *et al.*, 2000; Iuchi *et al.*, 2001; Qin and Zeevaart, 2002). Therefore, mutants and transgenic plants with large changes in accumulation of ABA relative to wild type show strong phenotypic characteristics related to stress tolerance, wiltiness, dormancy and growth rate.

When seedlings are subjected to stress, in the post-germinative phase, they are prone to enter developmental arrest or undergo reduced growth (Lopez-Molina *et al.*, 2001). This developmental arrest requires endogenous levels of ABA to increase (Lopez-Molina *et al.*, 2001). Mutants that are impaired in the biosynthesis of ABA are therefore unable to over-accumulate ABA in response to stress and, in consequence, will not arrest (Arenas-Huertero *et al.*, 2000; Lopez-Molina *et al.*, 2001; del Refugio Ramos *et al.*, 2004). It would be easy to interpret such mutants as being stress-tolerant since they grow more effectively than wild type during this early stage. In reality, it is likely that this post-germinative developmental arrest is required in wild type to maintain germinated seedlings in a quiescent state; thereby protecting them by preventing growth whilst environmental conditions remain unfavourable (Lopez-Molina *et al.*, 2001). An ABA deficient mutant would therefore be unable to adapt its growth and development in a manner appropriate to its surroundings and would be unlikely to survive until maturity.

Interestingly, early developmental growth displays greater sensitivity to small changes in ABA levels than germination, such that ABA is more efficient as an early growth inhibitor than as an inhibitor of germination (Gusta *et al.*, 1992; Lopez-Molina *et al.*, 2001). In consequence, a variety of studies related to ABA function have scored greening or emergence of cotyledons rather than germination (emergence of radical) (Lei *et al.*, 1994)((Arenas-Huertero *et al.*, 2000; Quesada *et al.*, 2000; Lopez-Molina *et al.*, 2001; Duque and Chua, 2003). Duque and Chua (Duque and Chua, 2003) showed that the *imb1* mutant was unaffected by exogenous ABA during germination but was more inhibited than wild type during cotyledon emergence and greening. A range of mutants identified through their ability to emerge cotyledons and green on high concentrations of salt were shown to be ABA deficient mutants (Quesada *et al.*, 2000; Gonzalez-Guzman *et al.*, 2004). Seedlings stressed by growth on media containing high concentrations of glucose, which increases ABA accumulation, will arrest and not green (Arenas-Huertero *et al.*, 2000). Glucose insensitive (*gin*) mutants have been identified that are resistant to the glucose stress. Interestingly, many of the *gin* mutants were shown to be allelic to ABA deficient or ABA insensitive mutants (Arenas-Huertero *et al.*, 2000; Rook *et al.*, 2001; Brocard-Gifford *et al.*, 2004). For example, *gin5* is allelic to *aba1* and accumulated 40% of normal levels of ABA (Arenas-Huertero *et al.*, 2000). Further, the *gin5* phenotype was rescued by addition of exogenous ABA, demonstrating that ABA is involved in the developmental arrest of seedlings induced by high glucose stress (Arenas-Huertero *et al.*, 2000).

In summary, ABA plays important roles in the control of growth, transpiration, stress tolerance, the development and germination of seeds, and the induction of post-germinative developmental arrest. Altering the level of ABA has phenotypic consequences for plants as shown by studies involving mutants and transgenic plants.

1.2.2 Homeostasis of ABA

ABA function is controlled by levels of the phytohormone and sensitivity to the phytohormone. The level of ABA in plant cells is controlled through a homeostatic mechanism involving biosynthesis, catabolism and redistribution. These processes combine to result in elevated ABA in key cells and tissues at specific developmental time-points and in response to stress. It is difficult to estimate the absolute concentration of ABA in a given cellular location. ABA levels are therefore generally described in terms of amount per gram dry or fresh weight. Whilst such measurements avoid large errors in accuracy, they do not necessarily reflect the amount of ABA experienced by receptors within the cell. However, Harris *et al.* (1988) have estimated concentrations to be approximately 0.3 μM, rising to 13 μM during stress.

1.2.2.1 Homeostasis of ABA during stress

ABA is present in all tissues, but levels can rise dramatically and rapidly during stress by between 10 and 50 fold (reviewed in Walton and Li, 1995). In an early experiment, Wright and Hiron (1969) observed that ABA accumulated in the leaves of wilted wheat seedlings. Hiron and Wright (1973) then recorded ABA levels to rise by 10-20 fold during wilting in Dwarf bean leaves, and noted that the levels of ABA correlated with stomatal closure. However, Cornish and Zeevaart (1985) showed that the onset of stomatal closure in *Xanthium strumarium* can occur before total leaf ABA increases. Interestingly, the concentration of ABA in the xylem exudate that could be forced out of the leaf under pressure was shown to increase following loss of turgor. This was interpreted to mean that ABA had redistributed around the leaf, leading to substantial increases in 'apoplastic' ABA. It was therefore proposed that redistribution, rather than biosynthesis, increased the concentration of ABA experienced by the guard cells to induce their closure in the early stages of wilting. Recent experiments have suggested that guard cells may themselves synthesize ABA. Studies in Arabidopsis showed that the promoters of key biosynthetic genes are activated in the guard cells and that mRNA accumulates during stress (Tan *et al.*, 2003; Koiwai *et al.*, 2004; Nambara and Marion-Poll, 2005).

The control of ABA levels is not limited to biosynthesis. Catabolism of ABA is also highly regulated during stress when turnover is high. Harrison and Walton (1975) measured the accumulation of ABA, and two oxidative catabolites of ABA, phaseic acid (PA) and dihydrophaseic acid (DPA) during wilting in *Phaseolus vulgaris* (see section 1.2.4 for information on catabolism of ABA). ABA increased by more than 10-fold over the first four hours and then remained constant. Interestingly, levels of PA and DPA increased throughout the experiment. Therefore, elevated ABA was maintained by high, but balanced rates of both biosynthesis and catabolism. In fact there is evidence that catabolism of ABA can be induced by both stress and ABA itself. Wang *et al.* (2002) showed that catabolism of ^{3}H-ABA fed to maize kernels was increased three-fold during water stress. Uknes *et al.* (1984) showed that pre-incubation of barley aleurone layers with ABA increased the conversion of ^{14}C-ABA to ^{14}C-PA by four-fold. Cutler *et al.* (1997) showed that ABA induces the ABA catabolic enzyme, 8′-hydroxylase, in cultured maize cells. In Arabidopsis, expression of the four *Cytochrome P450* (*CYP*) genes implicated in the 8′-hydroxylation of ABA was induced by exogenous ABA, dehydration and/or rehydration (Kushiro *et al.*, 2004). Therefore, ABA levels, which increase in plants during stress, are highly regulated through rates of both biosynthesis and catabolism.

During drought treatment, stomatal closure can occur without any loss of turgor in the leaves (Gollan *et al.*, 1986; Gowing *et al.*, 1990; Holbrook *et al.*, 2002). It has therefore been suggested that there is a long distance signal, synthesized in the roots, and transported to the leaves. ABA has been proposed to mediate this signal (Davies and Zhang, 1991). However, in grafting experiments Holbrook *et al.* (2002) showed that ABA biosynthesis in wild type tomato roots was not sufficient to induce proper stomatal closure in ABA deficient shoots. In addition, Christmann *et al.* (2005) used an *in vivo* imaging system involving the promoter of ABA-responsive genes coupled to *Luciferase* or *GUS* reporter genes. The reporters were activated by water stress, salt stress and exogenous ABA in a wild type background. These stresses did not activate the reporters in an ABA deficient background, indicating that ABA is required for promoter activity

(Uno *et al.*, 2000; Christmann *et al.*, 2005). Following water stress applied to the roots of the transgenic Arabidopsis plants, the *in vivo* imaging system detected increased levels of ABA sensing in shoots and not roots. These results were then confirmed with physical measurements of ABA, which showed a 50 fold increase in shoot ABA levels whilst root ABA remained largely unchanged (Christmann *et al.*, 2005). This data does not support a role for ABA in long-distance root to shoot signalling. It is therefore uncertain whether ABA is indeed the long-distance signal which mediates stomatal closure in water stressed plants.

1.2.2.2 Homeostasis of ABA during seed development

ABA homeostasis is also highly regulated during seed development, in which there are two distinct peaks of ABA accumulation. In Arabidopsis: the first peak occurs early in development during the seed maturation phase; the second peak occurs later at the time of the onset of dormancy. Levels of ABA then decrease during desiccation and are quite low in mature seeds (reviewed in Finkelstein and Rock, 2002). Interestingly, reciprocal crosses of wild type with ABA deficient mutants have shown that the first peak in ABA accumulation is produced by maternal tissues, whereas the second peak is synthesized by the embryo (Karssen *et al.*, 1983). Control of catabolism as well as biosynthesis is also important during seed development. Setter *et al.* (2001) proposed that the abortion of *Zea mays* (maize) seeds may be caused by over-accumulation of ABA, after showing that the phytohormone increased more in maize kernels that subsequently failed to set. The same group also demonstrated that placental tissue has a greater capacity to catabolise ABA than the embryo (Wang *et al.*, 2002). It was therefore suggested that the rate of catabolism of ABA in the placental tissue regulates ABA loading and transport into the endosperm during seed development (Wang *et al.*, 2002).

ABA is also involved in the maintenance of dormancy during imbibition (Debeaujon and Koornneef, 2000; Grappin *et al.*, 2000; Ali-Rachedi *et al.*, 2004). The ratio of ABA to

gibberellin in seeds is one of the factors regulating dormancy at this stage. High ABA levels, relative to gibberellin, will maintain dormancy: when the relative level of ABA is reduced, germination will be allowed to occur. Experiments have shown that maintenance of dormancy requires continual synthesis of ABA. This is demonstrated by the observation that dormant seeds imbibed in the presence of fluridone or norflurozan, inhibitors of ABA biosynthesis, will germinate (Debeaujon and Koornneef, 2000; Grappin *et al.*, 2000; Ali-Rachedi *et al.*, 2004). Regulation of the rate of ABA catabolism is also important during dormancy breakage. Germination occurs when ABA levels are reduced by suppression of synthesis and increase in catabolism (Nambara and Marion-Poll, 2005). When the level of ABA in seeds decreases during imbibition and/or stratification, levels of catabolites have been observed to increase in barley (*Hordeum vulgare*), lettuce (*Lactuca sativa*), western white pine (*Pinus monticola*) and Arabidopsis (Jacobsen *et al.*, 2002; Feurtado *et al.*, 2004; Gonai *et al.*, 2004; Kushiro *et al.*, 2004). Furthermore, Arabidopsis mutants with T-DNA insertions in the *CYP707A2* 8′-hydroxylase gene accumulated increased levels of ABA in seeds and exhibited a hyperdormant phenotype (Kushiro *et al.*, 2004). This demonstrates the important role that catabolism contributes to the homeostasis of ABA.

1.2.3 Biosynthesis of ABA

Plants synthesise ABA through the modification and cleavage of carotenoids, followed by oxidation of the C4′ and C1 carbons (reviewed in Zeevaart, 1999; Nambara and Marion-Poll, 2005). The carotenoid precursors of ABA are chiral and in consequence, natural ABA is produced in the chiral *cis*-S-(+)-ABA configuration ((+)-ABA) (figure 1.2). The carotenoid zeaxanthin, which is synthesised in the chloroplast, is converted to violaxanthin by zeaxanthin epoxidase (ZEP). The *ZEP* gene was first cloned from *N. plumbaginifolia* by insertional mutagenesis. The *aba1* mutants of Arabidopsis are mutated in the orthologous gene and have a severe reduction in ABA content (Koornneef *et al.*, 1982). Synthesis of 9′-*cis*-neoxanthin and 9′-*cis*-violaxanthin from violaxanthin is not yet fully elucidated (Nambara and Marion-Poll, 2005).

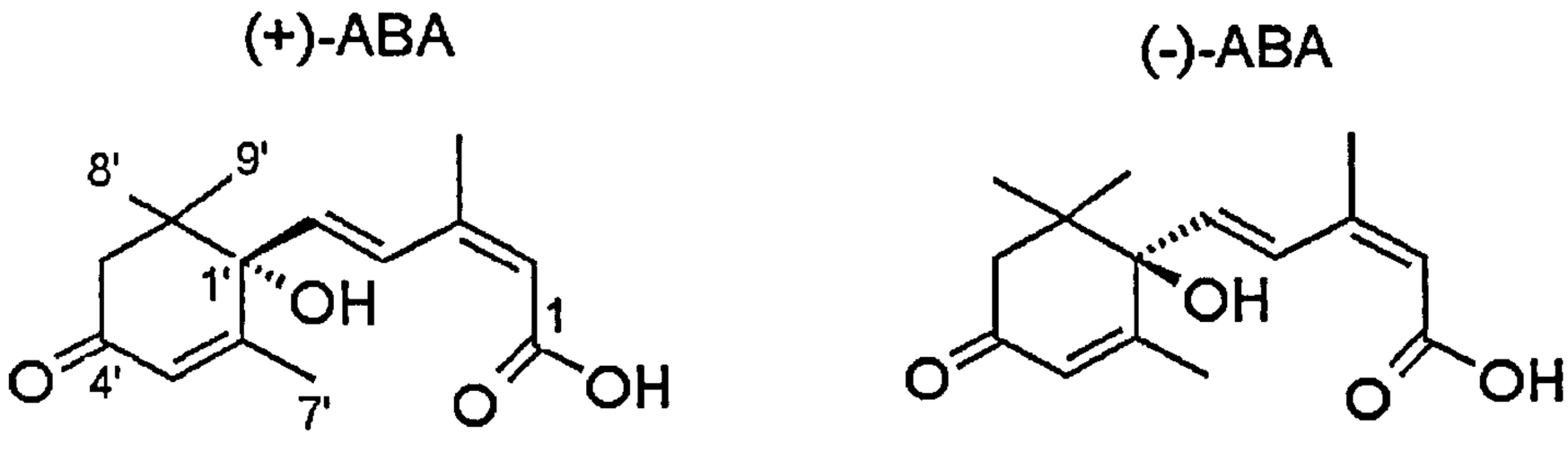


Figure 1.2 (+) and (-) enantiomers of ABA

The cleavage of carotenoids to xanthoxin also occurs in the chloroplast and represents the first committed step in ABA biosynthesis. Whether the parent carotenoid is 9′-*cis*-neoxanthin and/or 9′-*cis*-violaxanthin has been uncertain. Strand *et al.* (Strand *et al.*, 2000) showed that in the leaves of *Spinacea oleracea* (spinach) and *Brassica oleracea* (broccoli) more than 90% of neoxanthin is in the *cis* form whereas more than 90% of the violaxanthin is in the *trans* configuration. This suggests that since 9′-*cis*-neoxanthin is more abundant than 9′-*cis*-violaxanthin, it is more likely to be the precursor of xanthoxin (Schwartz *et al.*, 2003). The cleavage reaction is catalysed by nine-*cis*-epoxycarotenoid dioxygenase (NCED) enzymes. The first *NCED* gene was cloned from maize after isolation of the *vp14* mutant, which displayed a viviparous phenotype (Schwartz *et al.*, 1997; Tan *et al.*, 1997). The NCED enzymes are encoded by a small family of related genes in all species analysed (Nambara and Marion-Poll, 2005). In Arabidopsis, there are nine *NCED* sequences, of which five are probably involved in the biosynthesis of ABA (Iuchi *et al.*, 2000; Schwartz *et al.*, 2003). As such, *nced* mutants tend to show mild deficiency in ABA due to gene redundancy (Tan *et al.*, 1997). The *AtNCED3* gene is induced by stress and control of expression at this stage is thought to represent a rate limiting step in the biosynthesis of ABA (Iuchi *et al.*, 2000; Tan *et al.*, 2003; Nambara and Marion-Poll, 2005). Interestingly, *NCED* genes have been overexpressed in tomato, Arabidopsis and *N. plumbaginifolia* resulting in increased levels of endogenous ABA (Thompson *et al.*, 2000; Iuchi *et al.*, 2001; Qin and Zeevaart, 2002).

Following movement to the cytosol, xanthoxin is oxidised at the C4′ position to produce abscisic aldehyde. This step is catalysed by a short-chain dehydrogenase reductase. In Arabidopsis, there is a single *ABA2* gene encoding this enzyme and, in consequence, *aba2* mutants are severely deficient in ABA (Cheng *et al.*, 2002; Gonzalez-Guzman *et al.*, 2002). The final step in the biosynthesis of ABA involves the oxidation of the aldehyde at C1 to a carboxyl group by an aldehyde oxidase (AO). Whilst there are four *Arabidopsis Aldehyde Oxidase* (*AAO*) genes, only *AAO3* has been demonstrated to encode an Abscisic Aldehyde Oxidase through biochemical studies and phenotypic characterisation of *aao3* mutants (Seo *et al.*, 2000; Seo *et al.*, 2000; Gonzalez-Guzman *et*

al., 2004). In order to function, the AAO3 enzyme requires a molybdenum cofactor (MoCo). Therefore, mutations in genes involved in the synthesis of the MoCo result in ABA deficiency. *ABA3* from Arabidopsis is one such gene, encoding a MoCo sulfurase (Bittner *et al.*, 2001; Xiong *et al.*, 2001).

In summary, the biosynthesis of ABA in plants occurs through the modification and cleavage of carotenoids to xanthoxin, which undergoes two oxidation steps to give ABA. Most of the genes in ABA biosynthesis have been cloned through the use ABA deficient mutants.

1.2.4 Catabolism of ABA

Glucosylation of ABA in Arabidopsis is the subject of this thesis and therefore I will introduce the different metabolic pathways for ABA. Unfortunately, catabolism of ABA has not been well studied in Arabidopsis so I will describe and discuss data generated in other plants.

Catabolism of ABA can occur either through conjugation to glucose or through oxidation of the methyl groups around the ABA ring (Figure 1.3). It is considered that the principal oxidation pathway occurs through hydroxylation of the 8′-methyl to give 8′-hydroxy ABA (8′OH-ABA) which rearranges to form phaseic acid (PA). The 4′-keto group of PA may then be reduced resulting in dihydrophaseic acid (DPA) (Cutler and Krochko, 1999; Zaharia *et al.*, 2004). In addition to the 8′-hydroxylation pathway, the 7′-methyl and 9′-methyl groups can also be oxidized to give 7′-hydroxy ABA (7′OH-ABA) and 9′-hydroxy ABA (9′OH-ABA) (Hampson *et al.*, 1992; Zhou *et al.*, 2004). 9′OH-ABA rearranges to form neo-phaseic acid (neo-PA)(Zhou *et al.*, 2004). The oxidative catabolites of ABA retain the carboxylic acid group at C1 and as such may be referred to as acidic catabolites of ABA. The main conjugation pathway occurs through

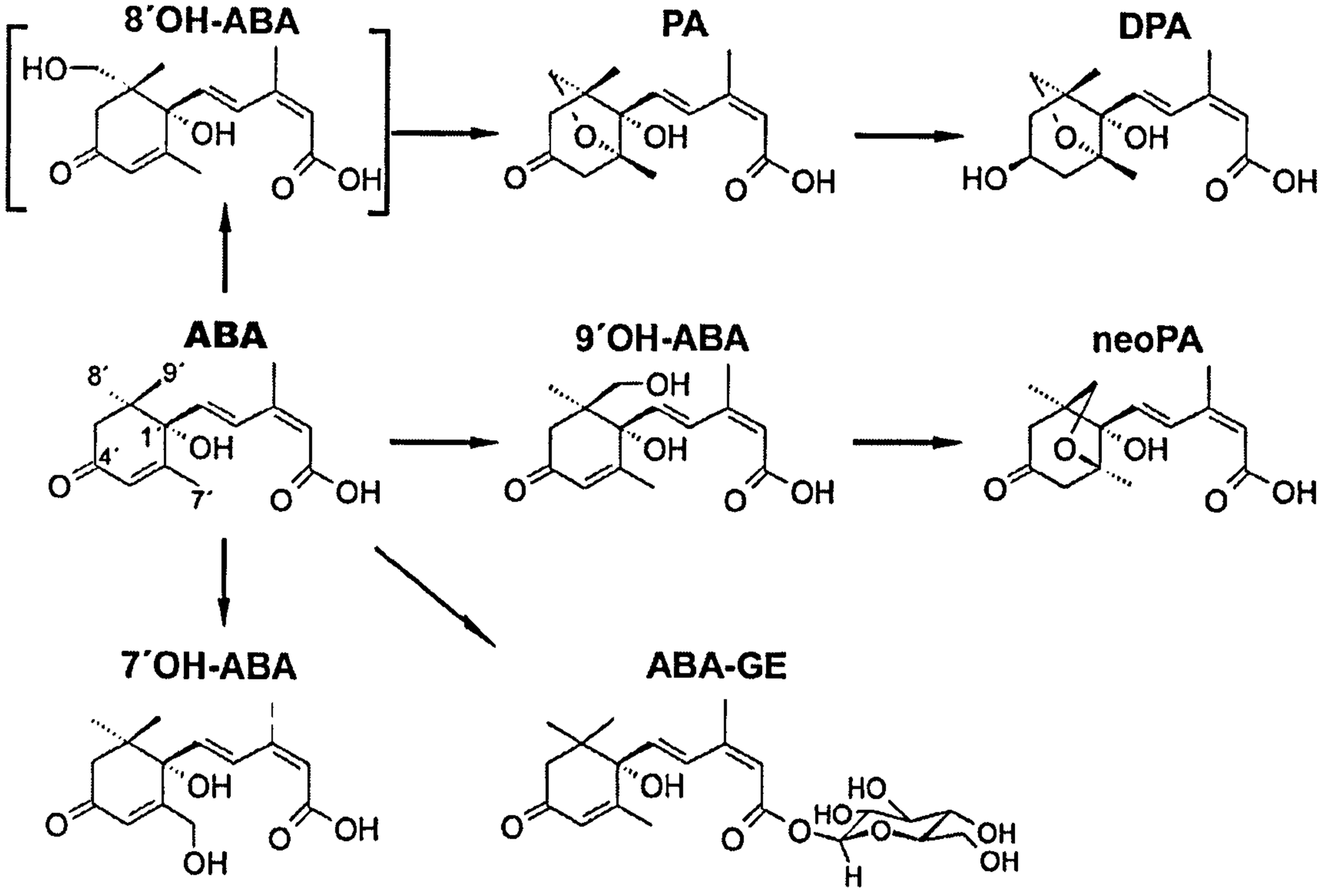


Figure 1.3 Catabolic pathways for ABA

ABA, abscisic acid; 8′OH-ABA, 8′-hydroxyABA; PA, phaseic acid; DPA, dihydrophaseic acid; 9′OH-ABA, 9′-hydroxyABA; neo-PA, neo-phaseic acid; 7′OH-ABA, 7′-hydroxyABA; ABA-GE, abscisic acid-glucose ester.

glucosylation of this carboxyl group to form ABA-glucose ester (ABA-GE, Figure 1.3) (Koshimizu *et al.*, 1968) (Cutler and Krochko, 1999). Other metabolites of ABA have also been described although they have been studied less extensively. These include: ABA 1′4′ diols- with the 4′-keto group reduced to a hydroxyl; epi-DPA- with an alternative chiral configuration at the 4′-hydroxyl of DPA; an ABA 1′-glucoside; PA-glucose ester (PA-GE); DPA-glucose ester (DPA-GE); and DPA-glucoside (DPA-GS) (Zeevaart, 1999; Finkelstein and Rock, 2002). There are therefore many different routes through which ABA can be metabolized, through oxidation and/or conjugation.

Caution must be exercised when drawing general conclusions or comparisons about the importance of the different catabolic and conjugation pathways of ABA in plants. Most of the experimental data is drawn from imperfect experiments in which the techniques are limited. For example, few experiments measure both oxidative and conjugation products and none measure all of the possible catabolites which may be present in the plants. Further, the rates of ABA biosynthesis, catabolism and turnover can be very rapid and a 'snapshot' measurement of ABA and its metabolites at a particular time point may not provide a comprehensive view of these dynamic processes. In addition, a substantial proportion of the experiments did not measure endogenous levels of the compounds, but rather catabolism of exogenously supplied ABA. It should not be assumed that fed ABA will be perceived or metabolized in the same manner as endogenous ABA. Experiments have often been conducted with racemic (±)-ABA, at concentrations that are substantially higher than the plant would experience under 'normal' conditions and/or using cell suspension cultures or excised parts of plants. Whilst many of these experiments did provide valuable and interesting data, it would be incorrect to assume that they necessarily represent the true rates of catabolism of natural ABA at physiological concentrations in intact plants.

1.2.4.1 The 8′-hydroxylation catabolic pathway

Despite the experimental limitations, it is clear that the 8′-hydroxylation pathway is frequently a major catabolic route. PA is the major measured product when exogenous ABA is fed to the kernels or cell suspension cultures of maize (Balsevich *et al.*, 1994; Cutler *et al.*, 1997; Wang *et al.*, 2002). It is also the major product when labelled ABA is fed to the leaves of *P. vulgaris* (Harrison and Walton, 1975; Zeevaart and Milborrow, 1976)) or unlabelled ABA to the seedlings of radish and Arabidopsis (Kushiro *et al.*, 2004; Todoroki *et al.*, 2004). In addition, PA and DPA are major metabolites of endogenous ABA in a range of species including *P. vulgaris* (Harrison and Walton, 1975; Zeevaart and Milborrow, 1976; Zeevaart *et al.*, 1989) *X. strumarium* (Zeevaart, 1980; Boyer and Zeevaart, 1982; Zeevaart, 1983; Creelman *et al.*, 1987) apple (Rock and Zeevaart, 1990) and Arabidopsis (Kushiro *et al.*, 2004). The metabolites of this pathway have also been observed to increase in response to both water stress and rehydration in a range of species including *P. vulgaris* (Harrison and Walton, 1975), *X. strumarium* (Zeevaart, 1980), *N. plumbaginifolia* (Qin and Zeevaart, 2002), and Arabidopsis (Kushiro *et al.*, 2004). Further, when endogenous ABA was elevated in transgenic *N. plumbaginifolia* lines by overexpression of the biosynthetic gene, Pv*NCED1*, from *P. vulgaris*, PA was shown to increase to levels two-fold higher than ABA (Qin and Zeevaart, 2002). The 8′-hydroxylation pathway is therefore an important catabolic pathway for ABA in the vegetative tissues of a range of species.

During imbibition or stratification (moist chilling) of seeds, when levels of ABA decrease, PA was a major metabolite in barley (Jacobsen *et al.*, 2002) and both PA and DPA were major metabolites in lettuce (Gonai *et al.*, 2004). Yellow-cedar embryos metabolized exogenous ^{3}H-ABA to PA, DPA and another major unidentified metabolite (Schmitz *et al.*, 2002). In imbibed seeds of Arabidopsis, Kushiro et *al.* (2004) observed PA and DPA to increase. Feurtado *et al.* (2004) performed a thorough analysis of endogenous metabolites of ABA during dormancy termination in western white pine. They showed that catabolism of ABA occurred through several different routes, depending on the time point and seed tissue. During stratification, PA and DPA increased in the embryo and megagametophyte. ABA-GE and 7′OH-ABA also accumulated in the embryo during stratification and 7′OH-ABA peaked early during germination. The

8′-hydroxylation pathway is therefore also an important catabolic pathway for ABA during dormancy termination and germination in a range a species.

Interestingly, catabolism through the 8′-hydroxylation pathway does not always lead to accumulation of DPA. For example, in *N. plumbaginifolia* levels of PA increased progressively during wilting, but DPA remained very low for at least 24 hours (Qin and Zeevaart, 2002). Further, transgenic *N. plumbaginifolia* lines were generated with high ABA accumulation due to overexpression of the biosynthetic gene *PvNCED1* from *P. vulgaris*. These lines also accumulated high levels of ABA and PA but maintained DPA at low levels. In addition DPA was a minor metabolite in *X. strumarium* and in maize kernels (Zeevaart, 1980; Boyer and Zeevaart, 1982; Zeevaart, 1983; Wang *et al.*, 2002).

Whilst 8′-hydroxylation is frequently a major catabolic pathway for ABA, it should be noted that a range of other experiments have been carried out in which catabolism through other pathways predominates. The relative importance of the different catabolic and conjugation pathways varies between tissues, conditions and species. For example, Rock and Zeevaart (1990) showed that PA was the major catabolite of ABA in the leaves of apple. However, in the fruit of the same species accumulation of PA was low and ABA-GE was the major metabolite to be measured.

1.2.4.2 7′OH-ABA and Neo-PA

Whilst 7′OH-ABA and neo-PA have frequently been overlooked as 'minor' catabolites of ABA degradation (Nambara and Marion-Poll, 2005), they can accumulate to high levels. 7′-OH ABA was originally identified as a metabolite of unnatural (-)-ABA fed to plants. However (+)-7′OH-ABA was later shown to be a natural metabolite of endogenous ABA in the leaves of *Vicia faba* (Lehmann and Schwenen, 1988). Neo-PA was fist identified as a metabolic product of labelled ABA fed to the green siliques of *Brassica napus* (Zhou *et al.*, 2004). Analysis of endogenous metabolites in the developing siliques showed that neo-PA and 7′OH-ABA both accumulated at higher levels than PA. Neo-PA was the

most abundant metabolite to be measured in the seed, while 7′OH-ABA accumulated to higher levels in the pericarp (Zhou *et al.*, 2004). Neo-PA was also shown to occur in a wide range of species including: droughted seedlings from barley and *B. napus*; the fruits of orange (*Citrus sinensis*), tomato, chick pea (*Cicer arietinum*); and the siliques, but not green leaves of Arabidopsis (Zhou *et al.*, 2004). These experiments demonstrate that 7′OH-ABA and neo-PA can be major endogenous metabolites of ABA at specific developmental stages and in specific tissues.

1.2.4.3 ABA-GE

Conjugation of ABA with glucose can also contribute a substantial element to metabolism of the phytohormone. Again, the relative importance of the glycosylation of ABA compared to the hydroxylation pathways varies between species tissues and conditions. ABA-GE, first identified from the fruit of *Lupinus luteus*, is considered to be the most widespread conjugate (Koshimizu *et al.*, 1968; Cutler and Krochko, 1999). In the fruit of apple, ABA-GE was the major catabolic product measured, whereas, PA was the major metabolite shown to accumulate in the leaves of the same species (Rock and Zeevaart, 1990). Neill *et al.* (Neill *et al.*, 1983) measured ABA-GE at levels 25 fold higher than ABA in orange peel, but at levels 10 fold lower than ABA in the fruit of *Persia americana* (avocaodo). In cell-suspension cultures of *V. faba* endogenous ABA-GE is present at levels 50% of ABA (Bray and Zeevaart, 1985), whereas ABA-GE was the major metabolite of ^{14}C-ABA fed to cell-suspension cultures of tomato (Lehmann and Glund, 1986) or intact wheat seedlings (Lehmann and Schutte, 1984). ABA-GE was a major metabolite of ^{14}C-ABA fed to *Beta vulgaris* (silver beet) and tomato shoots but not of endogenous ABA in tomato shoots during wilting (Milborrow, 1978).

Chiwocha *et al.* (2003) measured a range of catabolites during germination of lettuce seeds at 23°C and reported ABA-GE to accumulate at higher levels than PA, DPA or 7′OH-ABA. Interestingly, after induction of thermodormancy, by incubation at 33°C, DPA accumulated to higher levels. ABA-GE was also the major metabolite of ABA in

the seeds of *B. napus* subjected to osmotic stress to induce dormancy (Gulden *et al.*, 2004). In addition, ABA-GE was the major metabolite measured in Arabidopsis seeds during stratification and germination (Chiwocha *et al.*, 2005).

These findings can be contrasted with those of Gonai *et al.* (2004) and Kushiro *et al.* (2004). Gonai reported that PA and DPA accumulated at higher levels in lettuce seeds than conjugates of ABA at temperatures of 18°C, which would not induce theromdormancy. Further, Chiwocha *et al.* (2005) did not observe increases in the levels of PA and DPA during imbibition of Arabidopsis seeds as was seen by Kushiro *et al.* (2004). It is not clear whether these variations in the metabolic routes of ABA are due to different experimental techniques, growing conditions or biological variability.

1.2.4.4 Other metabolites of ABA

It should also be noted that other metabolites of ABA have been reported on occasion (reviewed in Zeevaart, 1999). The 1′4′ diols of ABA were detected as minor metabolites of ABA in the immature seeds of *V. faba* (Dathe and Sembdner, 1982) and apple (Rock and Zeevaart, 1990). Other glucose conjugates have also been described. The ABA 1′-glucoside was isolated as a catabolite of ^{14}C (±)-ABA that had been fed in extremely high concentrations (100 μM) to tomato plants and apple seeds (Milborrow, 1980; Loveys and Milborrow, 1981). To my knowledge the 1′-glucoside has only been isolated on these two occasions from the same laboratory. Whether it constitutes a significant metabolite of endogenous (+)-ABA in plants remains uncertain.

Glucose esters and glucosides of the acidic catabolites have also been identified. The 8′OH-ABA glucoside was isolated from the seed material of Avocado (del Refugio Ramos *et al.*, 2004). The glucoside of DPA was the major metabolite of labeled ABA fed to soybean seeds, sunflower embryos, and tomato (Setter *et al.*, 1981; Milborrow and Vaughan, 1982; Barthe *et al.*, 1993). Whilst the glucoside of epiDPA was a major metabolite of (-)-ABA fed to *P. vulgaris*, the endogenous compound was present at levels

100 fold less than DPA (Zeevaart and Milborrow, 1976). Glucose esters of PA, DPA and epiDPA were also identified in tomato and barley (Carrington *et al.*, 1988; Meyer *et al.*, 1989).

In summary, there are a wide variety of ABA catabolites, of which PA, DPA, 7′OH-ABA, and ABA-GE are the best studied and probably the most widespread. It is difficult to compare the relative roles of each metabolic pathway due to limitations in experimental design and technique. Despite the limitations, it appears that the 8′-hydroxylation of ABA leading to PA and DPA frequently constitutes a major catabolic pathway. However, it is also clear that the importance of each catabolic route is highly variable depending on tissue, species and growth conditions.

1.3 The role and location of ABA-GE

1.3.1 ABA-GE may be a storage form of ABA

Glucose conjugates of many phytohormones are generally thought to be inactive. Whilst exogenously supplied ABA-GE was shown to have an inhibitory effect on the growth of wheat seedlings (Koshimizu *et al.*, 1968) this was thought to be due to hydrolysis of the ester bond to release free ABA (Milborrow, 1970). Direct evidence that glucose can be cleaved from exogenously supplied ABA-GE was shown with the application of the ^{14}C labelled conjugate to cell-suspension cultures of *Lycopersicon peruvianum* (Lehmann, 1983) or to the leaves of *Ricinus communis* (Zeevaart and Boyer, 1984) in which the label became associated with free ABA. It was recognised, however, that exogenously fed ABA-GE belongs to a different metabolic pool from ABA-GE formed *in planta* and therefore it was possible that endogenous ABA-GE may not be hydrolysed (Milborrow, 1970). Consistent with the endogenous ABA-GE being inactive, the feeding of ^{14}C

labelled ABA to wheat seedlings led to retarded growth that was released as the labelled ABA became conjugated (Lehmann and Schutte, 1984).

It has been suggested that some endogenous conjugates of phytohormones can be cleaved to release the free active phytohormone (Kleczkowski and Schell, 1995). This appears to be an economic strategy for a plant to rapidly increase accumulation of a key signal molecule without wasting resources. It was therefore suggested that ABA-GE may be a storage form of ABA (Milborrow, 1970). However, there is very little evidence to support this hypothesis. When contemplating the potential waste of resources, readers should consider the fact that, by their nature, hormones are present at very low levels and therefore that little material is actually wasted. There are also many examples in which plants have been stressed and there is no evidence that endogenous ABA-GE is cleaved.

The feeding of ^{14}C labelled ABA to tomato and silver beet stems led to an accumulation of ABA and conjugates of ABA that could be cleaved by alkaline hydrolysis. It was expected that if the conjugates were a storage form of ABA that they would be hydrolysed during stress to release free ABA; however, wilting led to further increases in these ABA conjugates, rather than decreases, in both species (Milborrow, 1978). It was possible that conjugated ABA was being both synthesised and cleaved simultaneously. If this was the case then the net conjugated ABA could increase while the proportion of radiolabelled ABA conjugates decreased. The proportion of radiolabelled conjugate in the wilted tomato plants did not decrease, but increased, coinciding with a decrease in the proportion of radiolabelled ABA (Milborrow, 1978). Similar results were observed with the feeding of ^{14}C radiolabelled ABA to wheat seedlings, in which most of the radiolabelled ABA became conjugated and did not re-convert to ABA upon stress (Lehmann and Schutte, 1984).

Levels of conjugated endogenous ABA increase upon water stress in a range of species including *X. strumarium* and *P. vulgaris*. The levels of conjugated ABA or ABA-GE in both species remained constant upon re-watering and did not reduce (Zeevaart, 1980; Neill *et al.*, 1983; Zeevaart, 1983). In excised leaf blades of *X. strumarium* the

accumulation of conjugated ABA remained constant on re-watering for at least 24 hours, and then further increased upon a second wilt treatment (Zeevaart, 1980). Successive drought, recovery cycles were performed on intact *X. strumarium* plants up to eight times (Zeevaart, 1983). The accumulation of ABA-GE increased with each cycle and then remained at a constant level for at least 34 days after the final recovery. The stability of accumulated ABA-GE was compared with PA, which returned to pre-stress levels during nine days of recovery (Zeevaart, 1983). Similar experiments were carried out on *P. vulgaris* with similar results (Hiron and Wright, 1973).

Therefore, whilst exogenously supplied ABA-GE can be cleaved to release free ABA (Dietz *et al.*, 2000), this is not evidence that endogenous ABA-GE is readily cleaved after synthesis, accumulation, and storage. The available data has been interpreted by many scientists to conclude that conjugation of ABA is irreversible (Zeevaart, 1980; Lehmann, 1983; Neill *et al.*, 1983; Lehmann and Schutte, 1984).

Recently, however, there have been two reports from the same lab in which levels of ABA-GE rose and then fell during stratification of lettuce and Arabidopsis seeds (Chiwocha *et al.*, 2003; Chiwocha *et al.*, 2005). Whether these findings are significant remains uncertain. It is noteworthy, however, that whilst studying catabolism of ABA in the same species during germination, two other laboratories did not repeat the results (Gonai *et al.*, 2004; Kushiro *et al.*, 2004).

1.3.2 Location of ABA

Early work suggested that ABA conjugates could not leave plant cells and that the bulk of ABA-GE is compartmentalised in the vacuole. For example, most of the radiolabelled ^{14}C ABA fed to intact wheat seedlings became conjugated, and when the wheat seedlings were stressed the conjugates did not redistribute around the plant, which was interpreted to mean that conjugated ABA is not translocated or released from plant cells (Lehmann and Schutte, 1984). Additionally, Zeevaart and Boyer (1984) fed ^{3}H ABA-GE to the

leaves of *R. communis* and found that the labelled conjugate was hydrolysed before entering the phloem and moved as free ABA. Other relevant studies involve measurements of ABA-GE in cell suspension cultures of *X. strumarium* and tomato (Bray and Zeevaart, 1985; Lehmann and Glund, 1986) and in the protoplasts and vacuoles of *V. faba* (Bray and Zeevaart, 1985). Bray and Zeevaart (1985) used a dimethyl sulphoxide (DMSO) compartmentation method to show that ABA-GE was localized to the vacuoles of *X. strumarium* mesophyll cells in suspension cultures. They then showed by direct measurements of vacuoles and protoplasts of *V. faba* cultures that at lest 91% of the ABA-GE was sequestered in the vacuoles. Lehmann and Glund (1986) also showed that ABA conjugates were exclusively found in the vacuoles of tomato cell suspension cultures. The vacuolar location of ABA-GE combined with the lack of evidence that endogenous ABA-GE could be cleaved to release free ABA led many scientists to conclude that ABA-GE is an inactive end-product of ABA metabolism (reviewed in Zeevaart, 1999).

More recent studies from Bano and Hartungs laboratories detected ABA-GE in the vasculature of rice, sunflower, barley, *Anastatica hierochuntica* and maize which contributes to the argument that ABA-GE may be a long-distance transport form of the hormone {Bano, 1994 #349}(Sauter *et al.*, 2002).

1.3.3 ABA-GE may be a transport form of ABA

Regulation of stomatal closure allows plants to respond to water shortages by reducing water loss through transpiration. The phytohormone ABA has been shown to be a key signal molecule that induces stomatal closure (Hiron and Wright, 1973; Davies and Zhang, 1991) and mutants deficient in ABA generally show poor stomatal control (Koornneef *et al.*, 1982; Leon-Kloosterziel *et al.*, 1996). Interestingly, stomata may close when roots sense low water content in the soil, before leaves themselves experience any water deficit (Gollan *et al.*, 1986; Gowing *et al.*, 1990; Holbrook *et al.*, 2002). There is, therefore, a long distance signal from the roots to the leaves. ABA has been implicated in

this process because the phytohormone can cross cell membranes, is known to be transported in the vasculature, and can induce stomatal closure in leaves, when applied to the transpiration stream (Davies and Zhang, 1991).

It has also been argued that ABA-GE may be the long distance transport form of ABA (Sauter *et al.*, 2002). The evidence for this suggestion is lacking and does not consider any of the findings described above (Milborrow, 1978; Zeevaart, 1980; Lehmann, 1983; Neill *et al.*, 1983; Zeevaart, 1983; Lehmann and Schutte, 1984; Zeevaart and Boyer, 1984; Bray and Zeevaart, 1985; Lehmann and Glund, 1986). However, the hypothesis has been sited in many recent publications on ABA metabolism and as such will be discussed.

The argument begins with the observation that a component of the xylem sap of wheat and barley had substantially stronger activity in the induction of stomatal closure than exogenously supplied (±)-ABA (Munns and King, 1988; Munns *et al.*, 1993). The authors speculated that this may be due to a complexed form of ABA or something else. However, it has been shown that (+)-ABA and (-)-ABA display different activities in a range of bioassays (described in section 1.4). It is therefore possible that the activity or transport of (+)-ABA was not effectively mimicked by the racemic mixture. Whilst it was difficult to obtain pure enantiomers in 1988 for experiments, (+)-ABA is now freely available. It is therefore not acceptable to base a controversial proposal on such poor evidence. In addition, the study by Munns *et al.* (1993) showed that the 'something' in the xylem, which was more effective than ABA at inducing stomatal closure, polymerized on freezing. There is no evidence that ABA-GE polymerises on freezing.

The argument continues with the observation that ABA conjugates have been detected in the xylem of many species including rice (Bano *et al.*, 1993), sunflower (Bano *et al.*, 1994) and barley (Dietz *et al.*, 2000). In some species these ABA conjugates were present at higher levels than free ABA and their levels increased upon stress. However, the mechanism by which ABA conjugates pass from the root cytosol into the xylem apoplast is unknown. In addition, the facts that ABA conjugates have been detected in the xylem,

in some species at higher levels than ABA, and that their levels increased on stress does not necessarily implicate ABA-GE as a general transport form of the hormone. Other ABA metabolites have also been detected in the xylem, and they also increase upon stress (Zeevaart and Boyer, 1984; Bano *et al.*, 1994). Further, the level of ABA in the xylem sap of barley is 4–7 times greater than the level of ABA-GE (Dietz *et al.*, 2000). This suggests that even if ABA-GE was cleaved in the leaf to release free ABA, the relative contribution from the conjugate would be small.

Sauter *et al.* (2002) then suggest that ABA would not make an effective long-distance signal molecule, since free ABA readily permeates cell membranes and can be lost from the xylem to surrounding tissues during transport. Conversely, the authors suggest that ABA-GE would make a very effective long distance signal molecule because it is impermeable to cell membranes. Evidence is described which showed that ABA-GE, fed to stem internodes of *Phaseolus coccineus*, was not taken up by stem tissues. However, this evidence is unpublished, and would not be defining for a role. In contrast, the evidence that there is more ABA than ABA-GE in sap collected from the xylem mesocotyl of barley plants (Dietz *et al.*, 2000) shows that ABA was transported to near the top of the xylem. This is consistent with ABA itself being an effective transport molecule, counter to the argument made by Sauter *et al.* (2002).

It is then suggested that, in the leaf, either hypothetical transporters may redistribute ABA-GE to the cell or ABA-GE may be cleaved by apoplastic glucosidases to release free ABA. Evidence is described for the existence of apoplastic glucosidases. This includes the reports in Sauter *et al.* (2002) that, in the xylem sap of excised roots of barley, the ratio of ABA:ABA-GE was between four and seven, whereas in the intracellular washing fluid (IWF) of leaves the ratio was greater (22–33). More importantly, Holden and Rohringer (1985) showed the presence of glycosidases in the IWF. And >30 genes in the AGI have been assigned to be *β-glucosidases* with secretory target signals. Further, Dietz *et al.* (2000) detected extracellular β-glucosidase activity in barley leaves that could release free ABA from its conjugate, and this activity increased seven-fold when plants were salt stressed. This finding is not novel and is consistent with

previous knowledge that extracellular β-glucosidases will cleave a variety of exogenously supplied glucose conjugates, including ABA-GE (Lehmann, 1983; Zeevaart and Boyer, 1984).

In summary, whilst the hypothesis that ABA-GE is the long-distance transport form of ABA is not inconceivable, in the absence of any direct evidence of the action of ABA-GE as a signal molecule, or that endogenous ABA-GE is cleaved to release free ABA it appears that ABA-GE is likely to be an inactive end-product of ABA metabolism.

1.4 (±)-ABA enantiomers

As introduced earlier (section 1.2.3) the ABA molecule has a chiral centre at C1′ and can therefore exist as one of two enantiomers, (+)-ABA and (-)-ABA (Figure 1.2). (+)-ABA is the natural form of ABA which is synthesised by plants. Traditionally, chemical synthesis of the phytohormone produced a racemic mixture of (±)-ABA containing both enantiomers. Until recently it was difficult and expensive to obtain pure enantiomers. As a consequence, a considerable proportion of the studies investigating ABA function and metabolism in plants have utilised (±)-ABA. Whilst (-)-ABA functions in a range of bioassays, there is now considerable evidence that the two enantiomers are not equivalent: neither in relation to their activity nor their metabolism.

1.4.1 Activities of (+) vs (-)-ABA enantiomers

As early as 1971, Sondheimer showed that (-)-ABA was substantially less effective than (+)-ABA at inhibiting the root growth of barley seedlings. The authors also showed a similar, although less pronounced, difference in the ability of the two enantiomers to inhibit the growth of excised bean axes. Since then, differential effects of (+) vs (-) ABA have been observed in experiments investigating a wide range of ABA related responses. For example, a concentration of (-)-ABA ten fold greater than that of (+)-ABA was

required to effect the same growth inhibition in cell-suspension cultures of maize (Balsevich *et al.*, 1994). With regard to seed-specific ABA responses: (+)-ABA was found to be an effective inhibitor of germination in cress seeds and yellow-cedar embryos, whereas the (-) enantiomer had little effect (Gusta *et al.*, 1992; Schmitz *et al.*, 2002). However, both enantiomers were shown to be equally good at inhibiting germination of wheat embryos (Walker-Simmons *et al.*, 1992). In addition, (-)-ABA was not able to mimic the growth stimulatory activity of (+)-ABA during development of white spruce embryos (Dunstan *et al.*, 1992). Accumulation of transcript for the storage protein gene, *napin*, was induced in embryos of *B. napus* to equivalent levels by 1 μM (+)-ABA compared with 30 μM (-)-ABA (Wilen *et al.*, 1993). The two enantiomers have also been shown to display differential effects in stress-related responses. For example, (-)-ABA was less effective than (+)-ABA at inducing stomatal closure in barley leaves, and at inducing freezing tolerance in cultured brome grass cells (Cummins and Sondheimer, 1973) (Robertson *et al.*, 1994). It is therefore clear that (+)-ABA and (-)-ABA show different levels of activity in a range of ABA response assays relating to growth, stomatal function, seed development and germination. These differences have been observed at levels of transcription, cellular response, or whole plants.

1.4.2 Metabolism of (+) vs (-)-ABA enantiomers

The two enantiomers are also metabolised to different products and at different rates when fed exogenously to plants. Zeevaart and Milborrow (1976) fed (±)-ABA to *P. vulgaris* and showed that most of the PA and DPA were derived from the (+) enantiomer, whereas most of the ABA-GE and epi-DPA were derived from (-)-ABA. Since then experiments have been refined with the feeding of pure enantiomers. These experiments have generally shown that both enantiomers can be metabolised to PA, DPA, ABA-GE and 7′OH-ABA. However, PA and DPA were more commonly observed to be major products of (+)-ABA, whereas ABA-GE and/or 7′OH-ABA were produced more rapidly from (-)-ABA (Zeevaart *et al.*, 1986; Balsevich *et al.*, 1994). In addition, the metabolism

of natural (+)-ABA generally occured more rapidly than the metabolism of (-)-ABA (Mertens *et al.*, 1982; Abrams *et al.*, 1989; Dunstan *et al.*, 1992; Balsevich *et al.*, 1994).

In summary, natural (+)-ABA and unnatural (-)-ABA, which are structurally different at the C1′ carbon, are not always perceived and metabolised by plants in an equivalent manner. Therefore, when exogenous ABA is used for ABA related experiments in plants, care must be taken to use the naturally occurring (+)-ABA.

It has been suggested that the differential effects of (+) vs (-)-ABA may be due to the rates at which they are metabolised. Since (-)-ABA is generally metabolised more slowly than (+)-ABA, and therefore persists for longer, it might be expected to have a greater effect. However, while both enantiomers had equal activity in some bioassays, it is clear from the reports described above that (-)-ABA frequently had less activity than (+)-ABA. The fact that (-)-ABA was active in some bioassays, but not in others has led to speculation that there may be more than one ABA receptor in the cell (Walton, 1983). Alternatively, it has been suggested that the metabolites of ABA may themselves be active. The accumulation of different metabolites after feeding (+) or (-)-ABA may therefore be responsible for the apparent activities of the two enantiomers. The possibility that natural catabolites of endogenous ABA may also mediate some or all of the effects of ABA has also been proposed (Zhou *et al.*, 2004).

1.5 Bioactivities of acidic catabolites

A number of studies have investigated the bioactivities of the major catabolites of ABA. Such activities are, however, difficult to interpret because of uncertainty as to the uptake and metabolism of the supplied catabolites. In addition they may be transported to specific cellular compartments, or only be active in specific bioassays (Walton and Li, 1995).

7′OH-ABA has been shown to be inactive at inhibiting cress seed germination (Gusta *et al.*, 1992). However, this metabolite was able to induce the promoter of the *Em* gene and reduce gibberellin stimulated α-amylase activity in protoplasts from the barley aleurone layer (Hill *et al.*, 1995). 8′OH-ABA, has been difficult to study due to its instability. In one study, the catabolite was isolated at acid pH and shown to have equal activity to ABA to induce transcripts involved in seed development in the embryos of *B. napus* (Zou *et al.*, 1995). Cyclization of 8′OH-ABA leads to PA which was inactive for the same bioassay (Zou *et al.*, 1995). PA also had little or no activity in a range of bioassays including germination of cress seeds (Gusta *et al.*, 1992); induction of freezing tolerance in cultured brome grass cells (Robertson *et al.*, 1994) (Zou *et al.*, 1995), induction of the 8′-hydroxylase pathway (Uknes and Ho, 1984) and inhibition of cell elongation (Walton and Li, 1995). PA did, however, show weak activity in the growth inhibition of maize cell suspension cultures (Balsevich *et al.*, 1994) and in the inhibition of germination of embryos from barley (Hill *et al.*, 1992). In contrast to the weak and sporadic activities of 8′OH-ABA and PA, no activity has been detected for DPA in any bioassays (reviewed in Walton and Li, 1995).

The most recently identified metabolites 9′OH-ABA and neo-PA were tested for abilities to induce gene expression in developing embryos of *B. napus*, and to inhibit germination of Arabidopsis seeds. 9′OH-ABA displayed activity comparable with ABA at induction of gene expression, and weak activity in the germination assay. In contrast, neo-PA showed little or no activity in these bioassays (Zhou *et al.*, 2004). Therefore 7′OH-ABA, 8′OH-ABA, 9′OH-ABA and PA have all been shown to display some ABA like activity in specific bioassays. Whereas, neo-PA and DPA appear to be inactive.

1.6 Characterised enzymes in the catabolism of ABA

In order to fully understand how ABA functions in plants, all the genes involved in metabolism of the hormone must be identified. Almost all of the biosynthetic genes have been identified through the use of mutants (Nambara and Marion-Poll, 2005).

Interestingly, mutant screens have not identified any genes involved in the catabolism of ABA. This suggests that there may be considerable redundancy amongst the catabolic genes and/or pathways. Until recently, no catabolic genes had been identified, but now new information is emerging.

1.6.1 Enzymes controlling oxidative metabolism of ABA

In 2004, there were two reports of genes encoding cytochrome P450 (CYP) enzymes that could hydroxylate ABA at the 8′-methyl position (Kushiro *et al.*, 2004; Saito *et al.*, 2004). Like *UGTs*, *CYPs* also form a large multi-gene family, with 272 predicted members in Arabidopsis. The investigators identified a reduced subset of target *CYP* genes through phylogenetic analysis and expression data. Their recombinant proteins were then tested for activity towards ABA *in vitro*. Four genes in the same clade (*CYP707A1-4*) were identified as encoding potential 8′-hydroxylases. All four recombinant enzymes were specific for natural (+)-ABA and no products were detected after incubation with unnatural (-)-ABA. To date, only one of these genes, *CYP707A2*, has been demonstrated to encode an enzyme with 8′-hydroxylase function *in planta*. The *cyp707a2* T-DNA insertion mutant over-accumulated ABA in dry seeds and exhibited a hyperdormant phenotype (Kushiro *et al.*, 2004). Whether these four genes also function in vegetative tissues was not revealed in single knock-out lines, possibly due to overlapping expression patterns.

It is interesting that an enzyme shown to catabolise endogenous (+)-ABA *in planta* would not recognise the unnatural (-) enantiomer *in vitro*. (-)-ABA, supplied to plants, can be converted to PA and DPA (Zeevaart and Milborrow, 1976; Balsevich *et al.*, 1994). This suggests that genes controlling the homeostasis of endogenous (+)-ABA may be distinct from those which respond to the unnatural (-)-enantiomer.

No other genes for enzymes involved in the oxidative catabolism of ABA have been identified. There is, however, some evidence that 7′ and 9′-hydroxylation of ABA are

catalysed by enzymes that are distinct from 8′-hydroxylase enzymes. None of the CYP707A recombinant enzymes produced 7′OH-ABA when incubated with ABA *in vitro* (Kushiro *et al.*, 2004; Saito *et al.*, 2004; Nambara and Marion-Poll, 2005). Further, whilst catabolites of the 8′-hydroxylation pathway generally accumulate to higher levels than 7′OH-ABA or neo-PA, this is not always the case. 7′OH-ABA and neo-PA were present at higher levels in the green siliques of *B. napus* (Zhou *et al.*, 2004). This implies that there may be distinct enzymes for each pathway and that they are expressed in different locations.

1.6.2 Enzymes involved in the glucosylation of ABA

Until recently there had also been little progress in identifying glucosyltransferase enzymes, or genes encoding these enzymes, involved in the glucosylation of ABA. In 1980, an enzyme was partially purified from cell suspension cultures of *Macleaya microcarpa* which exhibited glucosyltransferase activity towards (±)-ABA *in vitro* (Lehmann and Schutte, 1980).

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In 2002, Xu *et al.* purified a glycosyltransferase enzyme from Adzuki bean (*Vigna angularis*) that glucosylated ABA *in vitro*, and cloned the corresponding gene. The recombinant enzyme had greater activity towards 2-*trans*-ABA than towards (+)-ABA or (-)-ABA. (Whilst 2-*trans*-ABA has been identified many times in plant extracts, it is not thought to be an endogenous metabolite of ABA, but rather a product of isomerisation by ultra-violet light during extraction (Abrams, unpublished data)(Nambara and Marion-Poll, 2005). The authors did not demonstrate whether the enzyme glucosylated endogenous ABA *in planta*.

1.6.3 The screening of the Arabidopsis UGTs for activity towards ABA

Following completion of the sequencing of the Arabidopsis genome in 2001, the Bowles group reported the identification and cloning of all the family 1 *GTs* in Arabidopsis containing the *UGT* signature motif (Li *et al.*, 2001; Ross *et al.*, 2001). In order to identify possible ABA glucosyltransferases, 105 of these were expressed as recombinant proteins in *E. coli*, purified as fusion proteins and screened for activity towards ABA *in vitro*. (The remaining two genes (*UGTs 76E9* and *90A1*) did not form soluble proteins and could not be screened). Eight of these enzymes were able to glucosylate the phytohormone and formed a conjugate of ABA that had a retention time on HPLC corresponding with ABA-GE (Lim *et al.*, 2005). (The eight enzymes were UGT71B6, UGT74D1, UGT84B1, UGT84B2, UGT75B1, UGT75B2, UGT73B1, UGT73B3 and their positions in the phylogenetic tree are shown in figure 1.1.) Amongst these enzymes UGT84B1 (hereafter 84B1) displayed the highest catalytic activity towards ABA. This enzyme had previously been studied for its ability to glucosylate indole-3-acetic acid (IAA), for which it had substantially higher activity (Jackson *et al.*, 2001; Jackson *et al.*, 2002). The GT with the second highest activity towards ABA was UGT71B6 (hereafter 71B6). Interestingly, 71B6 was the only GT with preferential activity towards the natural (+) enantiomer of ABA. Lim *et al.* (2005) demonstrated that 71B6 could be used in a whole-cell biocatalysis system to separate (+) and (-)-ABA. It was proposed that this would offer an alternative to chemical synthesis for the production of pure (+)-ABA. Eight UGTs from Arabidopsis have therefore been shown to be capable of glucosylating ABA *in vitro*. However, only 71B6 selectively glucosylated natural (+)-ABA.

1.7 Aim of this thesis

The importance of identifying and studying all the genes and enzymes that may be involved in the metabolism of ABA has been highlighted and the slow progress made, with respect to understanding those responsible for catabolism and conjugation, has been noted. With regard to understanding the metabolism of ABA, the limitations in using exogenously supplied ABA have been discussed.

It was also stated that ABA exists in a chiral configuration, with (+)-ABA being the natural enantiomer. The differences in the activity and metabolism of (+) vs (-)-ABA were described and the importance of using (+)-ABA when studying ABA metabolism in plants was noted. That genes controlling the homeostasis of endogenous (+)-ABA may be distinct from those which respond to the unnatural (-)-enantiomer was proposed in the context of the enantiomeric specificity of the 8′-hydroxylase enzymes identified by Kushiro *et al.* (2004) and Saito *et al.* (2004).

The Bowles group used a new strategy to identify eight family 1 GTs capable of glucosylating ABA *in vitro*. These eight enzymes are therefore candidates for ABA glycosyltransferase enzymes that may function *in planta*. Of the eight, 71B6 was particularly interesting because it displayed selectivity towards the natural (+) enantiomer of ABA and not the unnatural (-) enantiomer. In this thesis, a range of strategies have been used to further investigate the candidate glucosyltransferase for ABA, 71B6. Initially, the activity of the enzyme towards ABA and ABA structural analogues was characterised *in vitro* (chapter 3). The consequences of overexpressing or knocking out the gene were then investigated through analysis of the metabolite profiles and the morphological and developmental phenotypes of transgenic plants (chapters 4 and 5). Finally future work and implications are discussed (chapter 6).

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.2 Charactierisation of recombinant protein in vitro

2.3 Transgenic plants

2.4 Transcript analysis of transgenic plants

2.5 Phenotypic and chemotypic analysis of plants

2.6 Quantification of ABA and ABA metabolites

2.7 Challenge experiments

2.8 Salt challenge from roots

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant tissue

2.1.1.1 Soil grown plants

Plants were grown on Levingtons seed and modular compost in a controlled environment of 16 h/ 8 h light-dark cycle (22°C, 150 µmol m^{-2} sec^{-1}), 65% relative humidity. Plants for experiments were grown in a cabinet and plants for seed production were grown in the glasshouse supplemented with natural sunlight.

2.1.1.2 Sterile grown plants

Seeds were surface sterilized by chlorine gas in a sealed container. Approximately 100–200 µl of seed were placed in a 1.5 ml plastic tube and placed into an air-tight container. Chlorine gas was generated by adding 3 ml concentrated HCl to 100 ml of chlorine (Chloros). The container was sealed for 3–4 h in a fume hood.

Seedlings were grown on 0.8%(w/v) agar plates in a controlled environment of 16 h/ 8 h light-dark cycle (22°C, 80 µmol m^{-2} sec^{-1}). For some experiments the agar plates were supplemented with 1 x Murashige & Skoog medium (MS) (Melford) or ½ x MS.

2.1.2 Bacterial strain

The strain of *Escherichia coli* used in the protein expression work was XL1-Blue. The strain of *E. coli* used in the cloning work was NovaBlue. The strain of *Agrobacterium tumefaciens* used to transform plants was GV3-101.

2.1.3 Protein expression system

The p-GEX-2T vector utilized the Glutathione S-transferase (GST) Gene Fusion System (Pharmacia) to express UGT71B6 as an N-terminal fusion protein tagged with a 26 kDa GST domain from *Schistosoma japonicum*. The GST tag allowed the recombinant protein to be purified using Glutathione Sepharose 4B (Pharmacia) and recovered by exchanging with reduced-form glutathione, following the manufacturers instructions (see below).

2.1.4 Chemicals

The majority of the compounds were purchased from Sigma-Aldrich. (+)-ABA and ABA-glucose ester (ABA-GE) were purchased from OlCHemIm Ltd (Czech Republic). ABA metabolites, and the structural analogues of ABA and deuterated standards were synthesised in the laboratory of Prof. Suzanne R. Abrams at the Plant Biotechnology Institute (PBI), Saskatoon, Canada, as described: phaseic acid (PA), dihydrophaseic acid (DPA) (Zaharia *et al.*, 2005); PBI-49, according to Mayer *et al.* (1976), using 2-trans-3-methyl-penten-4-yn-1-ol (Mayer *et al.*, 1976); PBI-82, PBI-89 (Walker-Simmons *et al.*, 1992); PBI-253 (Lei *et al.*, 1994); PBI-271, PBI-293 (Walker-Simmons *et al.*, 1994; Rose *et al.*, 1996); PBI-372, PBI-493, PBI-524 (Rose *et al.*, 1997); PBI-401, PBI-514 (Cutler *et al.*, 2000); (-)-5, 8′,8′,8′-d_4 ABA (Abrams *et al.*, 2003); (-)-7′,7′,7′-d_3 PA, (-)-5,8′,8′,8′-d_4 7′OH-ABA, (-)-7′,7′,7′-d_3 DPA, (+)-5,4,8′,8′,8′-d_5 ABA-GE (Chiwocha *et*

al., 2003); PBI-233, PBI-287, PBI-410, PBI-413, PBI-703, PBI-705, (-)-8′,8′,8′-d_3 neo-phaseic acid (neo-PA) (Abrams SR, unpublished).

2.2 Charaterisation of recombinant protein in vitro

2.2.1 Expression of GST-71B6 fusion protein in *E. coli*

E. coli transformed with pGEX-2T containing *71B6* was stored in cryovials at -80°C. When required, a sample was streaked onto LB agar plates (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 1.5% (w/v) agar), supplemented with 50 μg/ml ampicillin, and grown at 37°C overnight. In order to purify recombinant 71B6 protein from the bacteria, a culture was prepared. A single colony was transferred to 1 ml of 2 x YT broth (1.6% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl (w/v), 50 μg/ml ampicillin) and grown at 37°C for approximately 8 h (optical density of ~ 0.8). The culture was diluted 1 in 1000 into 75 ml of fresh 2 x YT broth in a 250 ml flask and grown at 20°C for a further 3 days with shaking at ~120 rpm. The culture was then incubated with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 24 h at 20°C to induce expression of the GST-71B6 fusion protein.

2.2.2 Preparation of glutathione-coupled Sepharaose 4B beads

Glutathione-coupled Sepharose 4B was purchased from Pharmacia, supplied as approximately 75% (v/v) slurry. The slurry was transferred to a 15 ml Falcon tube and centrifuged at 1000 x *g* for 1 min to sediment the Sepharose beads. The beads were washed with 15 ml ice-cold phosphate buffered saline (PBS)(140 mM NaCl, 80 mM Na_2HPO_4, 15 mM KH_2PO_4). To obtain a 50% (v/v) slurry, the beads were centrifuged

again and equilibrated with an equal bed volume of PBS. The beads were then stored at 4°C.

2.2.3 Preparation of glutathione elution buffer

Glutathione elution buffer contained 20 mM reduced-form glutathione, 100 mM Tris-HCl pH 8.0, 120 mM NaCl. Protein was eluted from the beads by incubation for 30 min at room temperature in the elution buffer with gentle shaking. The beads were pelleted by centrifugation at 15,000 x *g* for 10 minutes and the fusion protein (in the supernatant) was collected.

2.2.4 Purification of recombinant GST-71B6

2.2.4.1 Disruption of cells by Spheroblast buffer method

The *E. coli* cells, induced to produce GST-71B6 protein, were harvested by centrifugation at 5000 x *g* for 10 min and resuspended in 2 ml of ice-cold Spheroblast buffer (0.5 mM EDTA, 750 mM sucrose, 200 mM Tris-HCL, pH 8.0)(Burger 1993). Lysozyme (1 mg) and 14 ml ice-cold ½ x Spheroblast buffer were added and incubated with gentle shaking at 4°C for 30 min. The cells were harvested again by centrifugation, and osmotically shocked in 5 ml PBS containing 0.2 mM phenylmethylsuplphonylfluoride (PMSF) (a protease inhibitor).

2.2.4.2 Disruption of cells by French Press method

The *E. coli* cells, induced to produce GST-71B6 protein, were harvested by centrifugation at 5000 x *g* for 10 min and resuspended in 10 ml of ice-cold PBS containing 0.2 mM PMSF. The cells were disrupted by single pass through a French Press at 15,000 psi.

2.2.4.3 Purification of GST-71B6 protein

Cell debris was removed by centrifugation at 10,000 x *g* for 15 min and the supernatant was transferred to a 15 ml falcon tube. 100 µl of 50% (v/v) glutathione-coupled sepharose beads (Pharmacia) was added and incubated at room temperature with gentle shaking for 30 min–2 h to allow binding of the GST-71B6 protein to the beads. The sepharose beads were washed three times with 5 ml of PBS and the GST-71B6 protein recovered in the glutathione elution buffer (2.4.2).

2.2.5 Protein concentration assay

Protein assays were carried out with *Bio-Rad* (Hemel Hempstead, Herts., UK) Assay Dye diluted one in five with 10 µl of sample per ml. The absorbance at 595 nm was measured relative to a control without protein and the concentration determined from a standard graph generated using bovine serum albumin (BSA).

2.2.6 UDP-glucosyltransferase assay

The general UGT activity assay was carried out in a final volume of 100 µl containing 1 µg of purified recombinant protein, 5 mM UDP-glucose, 1 mM ABA or other substrate, 50 mM Tris-HCl pH 6.95. When pH was varied, Mes buffers were used for reactions carried out at pH 5.5 to 6.8 and Tris buffers for reactions at pH 6.8 to 8.5. Metal ions and reducing agents were included at concentrations of 5 mM and 10 mM respectively. After optimisation experiments, 10 mM dithiothreitol (DTT) and 5 mM $MgCl_2$ were also

included. ABA analogues and metabolites were assayed under the same conditions as for ABA. Following 1 hour at 30 °C, reactions were stopped by the addition of 10 µl of 240 mg ml^{-1} trichloroacetic acid (TCA). Precipitated protein was pelleted by centrifugation at 15,000 x *g* for 10 min and the reaction mix transferred to a fresh tube before storage at -20°C. All assays were carried out on at least three independent protein extractions.

2.2.7 Alkaline hydrolysis

Alkaline hydrolysis of the glucose ester bond of the reaction product was performed in 1 M NaOH at 30°C for 1 h.

2.2.8 HPLC analysis of UGT assay

Reverse phase HPLC (Waters Alliance 2690 and Waters Tuneable Absorbance Detector 486, Waters Ltd, Herts., UK) analysis involved a 5µm C_{18} column (250 mm x 4.60 mm; Phenomenex). A linear gradient with increasing methanol against 0.1 M acetic acid (pH 3.5 triethylamine) from 10–80% (v/v) over 30 minutes was used and the eluate was monitored at 270 nm.

2.2.9 Quantification of activity towards ABA analogues

In order to compare the activity of UGT71B6 towards ABA and structural analogues of ABA, calibration curves of standard amounts of each analogue were generated. Following the *in vitro* reactions, any glucose ester formed was purified by HPLC and divided into two samples. One sample was cleaved by alkaline hydrolysis and the area of aglycone produced compared to the area of glucose ester in the second sample. This enabled the amount of glucose ester to be related to the amount of aglycone from the calibration curve.

2.2.10 Cleavage of GST fusion domain

Protein was extracted from *E. coli* (as described in section 2.4.3) until the glutathione-coupled Sepharose stage. Once the beads had been washed in PBS, 100 µl of thrombin cleavage buffer was added (Novagen) and the beads were left shaking at room temperature for one and a half hours. To terminate the cleavage, 10 µl PMSF was added and the supernatant, which contained the 71B6 protein, was collected.

2.2.11 Identification of ABA-glucose ester using HPLC – mass spectrometry

HPLC – mass spectrometry was carried out using an Agilent HPLC attached directly to an Applied Biosystems Qstar Pulsar I mass spectrometer with a turbo ion spray source. The HPLC linear gradient was carried out as above except that the solvents were methanol (1% (v/v) acetic acid) against water (1% (v/v) acetic acid). The mass spectrometer was operated in negative ion mode with an ion spray voltage of –2500 V. The nebulisor and turbo gases were both set at 70 units. Parent ions of 425 m/z ratio were fragmented by collision induced dissociation (CID) and product ions analysed over the range 50–660 amu. The low and high energy fragmentation experiments used collision energy settings of -10 units and -30 units respectively. This work was carried out in collaboration with Dr David Ashford, University of York.

2.2.12 Kinetic analysis

The kinetic parameters of 71B6 towards (+)-ABA in the presence of 5 mM UDP-glucose, 50 mM Tris pH 6.95, 10 mM DTT and 5 mM $MgCl_2$ were calculated using Hyperbolic Regression Analysis in the Hyper32 program from homepage.ntworld.com/john.easterby.

2.3 Transgenic plants

The wild type used was Colombia-0.

71B6 was overexpressed in *Arabidopsis thaliana* plants under the control of the CaMV 35S promoter, and homozygous lines generated (71B6-OE), as described for 84B1 (Jackson *et al.*, 2002). Essentially *Arabidopsis thaliana* plants were transformed with *Agrobacterium tumefaciens* GV3-101 by floral dipping (section 2.3.1.13). The *A. tumefaciens* GV3101 were transformed with the binary vector pJR1Ri containing the *71B6* ORF. This was carried out in the Bowles group by Gillian Higgins.

71B6-KO is a transposon insertion line obtained from the JIC SLAT collection (02_26_08). This was carried out in the Bowles Group by Luisa Elias.

84B1-OE is a transgenic line overexpressing *84B1* under the control of the CaMV 35S promoter that had been generated previously within the Bowles group. The line used was the high expressing 13-3 line described (Jackson *et al.*, 2002).

The *aba3-2* mutant is deficient in ABA (Leon-Kloosterziel *et al.*, 1996) and was kindly provided by Steve Penfield, Department of Biology, University of York. This line is in a Landsberg erecta background. Landsberg erecta and Colombia wild type plants responded in a similar manner to glucose stress, detached leaf water loss assays and accumulation of ABA (Leon-Kloosterziel *et al.*, 1996; Jang *et al.*, 1997; Seo *et al.*, 2000). The *aba3-2* mutant responds to the stresses in a similar manner to ABA deficient mutants in a Colombia background (Leon-Kloosterziel *et al.*, 1996; Arenas-Huertero *et al.*, 2000).

The NCED3-OX transgenic line, overexpressing the *NCED3* gene, was kindly provided by Kazuo Shinozaki (Iuchi *et al.*, 2001).

The 71B6-KO lines were complemented by transformation with a genomic fragment containing the *71B6* gene including its own promoter and terminator regions (71B6-RE3 and 12) using the binary vector, pART27 (Gleave, 1992). There are 440 bp of sequence between the start of the *71B6* ORFs and *UGT71B7* — this region was used as the promoter region. For the terminator region, ~1000 bp downstream from the stop codon of the *71B6* ORF was used.

2.3.1 Generation of 71B6-RE lines

2.3.1.1 Genomic DNA extraction

Fresh leaf tissue (100 mg) was ground to a fine powder in liquid nitrogen using a pestle and mortar. Following suspension in 500 µl of filter sterilised DNA extraction buffer (50 mM Tris-HCl pH 7.6, 100 mM NaCl, 50 mM EDTA, 0.5% (w/v) SDS, 10 mM 2-mercaptoethanol), samples were incubated at room temperature for 15 min. An equal volume (500 µl) of phenol/chloroform/isoamyl alcohol (25:24:1) (pH 7.5–8) was added. Samples were mixed by vigorous vortexing, and incubated for 2 min at room temperature. The layers were then separated by centrifugation at 15,000 x *g* for 5 min, and the top layer removed to a fresh tube. The phenol/chloroform/isoamyl alcohol extraction was repeated and the top layer removed to a fresh tube. An equal volume of chloroform was then added to the sample, the sample vortexed vigorously, and incubated for 2 min at room temperature. The layers were then separated by centrifugation at 15,000 x *g* for 5 min, and the top layer removed to a fresh tube. The DNA in the sample was precipitated by addition of 1 volume isopropanol. Following incubation for 5 min at room temperature, the DNA was pelleted in the bottom of the tube by centrifugation at 15,000 x *g* for 10 min. The DNA pellet was washed in 70% (w/v) ethanol, dried and resuspended in 400 µl of sterile TNE (10 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA pH 8.0) containing 50 µg/ml RNase. Following incubation at 37°C for 30 min, RNA was removed by extraction with an equal volume of phenol/ chloroform/ isoamyl

alcohol, and then with chloroform. DNA was precipitated by addition of 2 volumes of ethanol and incubation at -20°C for 10 min. The DNA was pelleted by centrifugation at 15,000 x *g* for 5 min, washed in 70% (v/v) ethanol and the pellet dried under vacuum. Finally the DNA was resuspended in 50 µl of TE buffer (50 mM Tris-Cl, 10 mM EDTA, pH 8.0) and stored at -20°C until use.

2.3.1.2 Amplification of 71B6 sequence from genomic DNA by polymerase chain reaction

The *71B6* sequence, including 440 bp of 5'untranslated region (UTR) and 1000 bp of 3'UTR were amplified from genomic DNA. Primers were designed to introduce *Not1* restriction sites at the termini of the polymerase chain reaction (PCR) product (5Pro71B6 – GAGCTCGCGGCCGCTAGGCCCAAAGTTTAAAATAT and 71B6ter3revN – GCGGCCGCCTCAAATAAGACGGAG). Arabidopsis genomic DNA (100 ng) and 1 µM of each primer were used in a 50 µl PCR reaction containing 1 x Expand High Fidelity buffer, 0.25 mM of each deoxynucleoside triposphate (dNTP) and 2.5 units of Expand High Fidelity enzyme mix (Roche Diagnostics). The PCR was carried out with 2 min at 94°C, followed by 10 cycles of: 30 s at 94°C; 30 s at 55°C; 150 s at 68°C. A further 20 cycles were carried out with an increase of 5 s at 68°C per cycle. Finally the reaction was maintained at 72°C for 7 min.

2.3.1.3 DNA electrophoresis

DNA fragments were separated by gel electrophoresis in a 1% (w/v) agarose gel made in 0.5 x TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA) containing ethidium bromide (0.1 µg/ml). Prior to loading, loading dye (50% (v/v) glycerol, 0.25% (w/v) bromophenol blue) was added at $1/10^{th}$ the final volume. The gel was run at 100 V in a running buffer of 0.5 x TBE. DNA framents were visualised by UV light using a transilluminator.

2.3.1.4 DNA ligation

The *71B6* PCR amplicon was ligated into pGEM-T easy vector DNA (Promega). The ligation mixture consisted of 1 x ligase buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 µg/ml BSA,), 400 units of T4 DNA ligase (NEB), 1 µl vector DNA and the DNA fragments to be inserted. The ligation mixture was incubated at 4°C overnight.

2.3.1.5 Preparation of competent E. coli cells

A single colony of *E. coli* (NovaBlue) cells was inoculated into 5 ml of LB medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl) and grown for 16 h overnight at 37°C with shaking. This starter culture (1 ml) was used to inoculate 200 ml of fresh LB medium, which was then grown at 22°C overnight with vigorous shaking (~300 rpm) till cells had reached an optical density (OD_{600}) of 0.4–0.8. The cells were chilled on ice for approximately 20 minutes before pelleting by centrifugation at ~ 2500 x *g* for 5 min at 4°C. The supernatant was discarded and the cells resuspended in 40 ml of ice cold SEM buffer (10 mM PIPES, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl). Following incubation on ice for 10 min, the cells were pelleted by centrifugation at ~ 2500 x *g* for 5 min at 4°C. The cells were resuspended in 12 ml ice cold SEM buffer and DMSO added to a final concentration of 7% (v/v) (920 µl). The cells were aliquoted into 100 µl samples and frozen in liquid nitrogen before storage at -80°C.

2.3.1.6 Transformation of plasmid DNA into E. coli

An aliquot of competent NovaBlue cells was thawed and the tube kept on ice. The ligation mixture was transferred into the competent cells, mixed gently, and incubated on ice for approximately 30 min. The *E. coli* cells were heat-shocked at 42°C for 45 s and immediately returned to the ice for 5 min. 200 µl of LB was added to the cells. Following

recovery of the cells by incubation at 37°C with shaking for up to 60 min, the cells were plated onto LB agar supplemented with 50 µg/ml carbenicillin, 100 µg/ml IPTG and 100 µg/ml X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) and incubated at 37°C overnight.

2.3.1.7 Isolation of plasmid DNA from E. coli

White bacterial colonies were inoculated into 5 ml LB and grown for ~16 h with vigorous shaking at 37°C. Plasmid DNA was isolated from the culture using a Wizard Plus SV Miniprep DNA purification system (Promega) according to the manufacturers instructions.

2.3.1.8 DNA sequencing

The insert within the plasmid DNA was sequenced by the Technology Facility, Department of Biology, University of York. The *71B6* fragment was sequenced in full using M13 forward and reverse primers and gene specific primers. Sequence analysis and alignments were carried out using Lasergene software packages.

2.3.1.9 Restriction enzyme digestion of plasmid DNA

The pGEM-T-71B6 plasmid was digested with *Not*I to excise the *71B6* fragment from the vector backbone. Since the fragment, and the backbone were both ~3000 bp, the backbone was further digested with *Hae*II. The digestion was carried out with 10 units of each enzyme in a total volume of 20 µl with 1 x NEB buffer 2 (5 mM NaCl, 1 mM Tris-HCl pH 7.9, 1 mM $MgCl_2$, 0.1 mM DTT), and 100 µg/ml BSA at 37°C for 1 h.

The pART27 binary vector was linearised with *Not*I in a total volume of 20 µl with 1 x NEB buffer 3 (10 mM NaCl, 5 mM Tris-HCl pH 7.9, 1 mM $MgCl_2$, 0.1 mM DTT), and 100 µg/ml BSA at 37°C for 1 h.

2.3.1.10 DNA gel extraction

Following separation of DNA fragments by gel electrophoresis (section 2.5.2.3) the *71B6* fragment and the vector backbone were excised from the agarose gel with clean scalpels. The fragments were purified using the QIAEX II agarose gel extraction kit (Qiagen) according to the manufacturers instructions. The method involved the solubilization of agarose and adsorption of nucleic acids to silica gel particles in the presence of high salt. Following a wash, the DNA was eluted in a low salt solution.

The *Not*1 digested *71B6* fragment was ligated to the pART27 vector backbone in a similar manner to that described in section 2.3.1.4. The pART27-71B6 plasmid was transformed into competent *E. coli* (as section 2.3.1.6), plasmid DNA purified (as section 2.3.1.7), and the border regions sequenced using pART27 specific primers.

2.3.1.11 Preparation of competent Agrobacterium cells for electroporation

A fresh colony of *Agrobacterium tumefaciens* GV3-101 was used to incoluate a 10 ml culture of LB supplemented with 0.1% (w/v) glucose and the culture grown for 6 h at 28°C with shaking. 100 µl of this starter culture was used to inoculate a 100 ml culture in the same media, which was then grown overnight to an OD_{660} of 1–1.5. The culture was then cooled on ice for 15 min, and the cells pelleted by centrifugation at ~ 2500 x *g* for 5 min at 4°C. The supernatant was discarded and the cells resuspended in 10 ml of 1 mM HEPES buffer (pH 7.0). The pelleting and resuspending was repeated two more times before a fourth resuspension in 10 ml of 10% (v/v) glycerol. The cells were then pelleted one final time by at ~ 2500 x *g* for 5 min at 4°C and resuspended in 640 µl of 10% (v/v)

glycerol. The cell suspension was then divided into 80 µl aliquots and frozen in liquid nitrogen before storage at -80°C until use.

2.3.1.12 Transformation of plasmid DNA into Agrobacterium

The electroporation apparatus (Bio-rad, Gene Pulser II) was set to 2.5 kV, 25 µF, pulse controller 300 ohms. An aliquot of competent *Agrobacterium* cells was thawed on ice and 1 µg of plasmid DNA added and mixed gently. The cells were then transferred to an ice-cold electroporation cuvette and water removed from the outside of the cuvette by wiping with a tissue. The cuvette was then placed in the electroporator and a pulse of electricity applied. The cuvette was removed from the apparatus and 800 µl of LB added, mixed with the cells, and then removed to a 1.5 ml plastic tube. The cells were then allowed to recover at 28°C with gentle shaking for 2 h, before being plated onto LB agar plates supplemented with spectinomycin (50 µg/ml) and gentamycin (50 µg/ml). The plates were incubated at 28°C for 2 days till colonies had grown.

2.3.1.13 Generation of transgenic Arabidopsis plants by floral dipping

Arabidopsis plants (71B6-KO) were grown in 3 inch pots, covered in muslin, for approximately 4–5 weeks. The first bolt to emerge from the rosette was removed and plants grown for a further week to allow the growth of secondary bolts. Prior to dipping, all siliques and open flowers were removed from the plant.

A single colony of transformed *Agrobacterium* containing the binary vector was inoculated into a 10 ml starter culture of LB (50 µg/ml kanamycin, 50 µg/ml gentamycin). Following incubation overnight at 28°C with shaking, the starter culture was used to inoculate a 400 ml culture. The large culture was incubated overnight at 28°C with shaking. The culture was decanted into 250 ml centrifuge pots and centrifuged at

3000 x *g* for 20 min at 4°C. The supernatant was discarded and the pellet resuspended in 1 litre of infiltration medium (5% (w/v) sucrose, 400 µl triton, water).

Perpared Arabdiopsis plants were dipped in the culture and swirled for 1 min in the infiltration medium at room temperature. The plants were placed on trays and covered with plastic bags for three days, before being returned to the green house and grown to maturity.

2.4 Transcript analysis of transgenic plants

2.4.1 Extraction of RNA

Total RNA was extracted from 4-week-old rosette tissue using TRI reagent™ according to manufacturer's instructions. 200–500 µg of fresh tissue was ground to a fine powder in liquid nitrogen and 1 ml of TRI reagent added. Following vortexing for 1 min, 200 µl of chloroform was added and vortexed vigoursly. Samples were centrifuged at 15,000 x *g* for 10 min and the supernatant collected to a fresh tube. RNA was precipitated by the addition of an equal volume of isopropanol; for the extraction of RNA from root tissue, RNA was precipitated by the addition of ¼ volume 0.8 M NaCitrate: 1.2 M NaCl solution, followed by ¼ volume isopropanol. After resting at room temperature for 5 min the RNA was pelleted by centrifugation at 15,000 x *g* for 15 min. The pellet was washed in 70% (v/v) ethanol and dried, before resuspending in 50 µl sterile water. The concentration was determined using a Biophotometer (Eppendorf) and the RNA stored at -80 °C.

2.4.2 RNA gel electrophoresis

A 1.5% (w/v) agarose gel was made in 1 x MOPS buffer (0.02 M MOPS pH 7.0, 8 mM Na Acetate, 1 mM EDTA pH 8.0) 5% (v/v) formaldehyde and 0.1 μg/ml ethidium bromide. 10 μg of RNA was diluted in 0.5 M MOPS solution, 5% (v/v) formaldehyde, 50% (v/v) formamide and then a 1 in 10 volume of loading buffer added (50% (v/v) glycerol, 1 mM EDTA, 0.25% (w/v) bromophenol blue). Following denaturing by heating for 10 min at 65°C, RNA was separated on the gel by electrophoresis at 100 V for 1 h.

2.4.3 Northern blotting

Following electrophoresis, the RNA was transferred onto Hybond-N nylon membrane using capillary action and 3MM filter paper in 10 x SSC buffer (1.5 M NaCl, 0.15 M NaCitrate) overnight. The RNA was fixed onto the membrane by UV light (120 mJ) in a Stratagene UV Stratalinker 1800.

2.4.4 Probe synthesis and purification

The radio-labelled probe was prepared using the Stratagene Prime-It® II Random Primer Labeling Kit according to the manufacturer's instructions. The probe for RNA figure 4.1 and 5.3b was prepared from the full length open reading frame (ORF) of *71B6*, whereas for figure 5.3a the probe was prepared from the ORF 3′ of the transposon insertion. A volume of 36 μl containing 25 ng of template DNA with 10 μl of random oligonucleotide primers (supplied) was heated at 100°C for 5 min. 10 μl of 5 x dCTP buffer, 3 μl of ^{32}P labelled dCTP (9.25 Mbq in 25 μl) and 1 μl of Exo(-) Klenow (5 units/μl) were added and the reaction incubated at 37°C for 30 min. The reaction was stopped by the addition

of 2 µl of stop mix (0.5M EDTA, pH 8.0). The probe then was purified using an Amersham Microspin™ G-50 Column. The column seal was broken and the column prepared by centrifugation in a collection tube at 600 x *g* for 1 min. The column was transferred to a fresh collection tube and the radiolabelled reaction mix added. The probe was collected, following centrifugation at 600 x *g* for 2 min, and denatured by heating at 95°C for 5 min.

2.4.5 Hybridisation

Prehybridisation of the membrane was carried out in Church buffer (0.25 M Na_2HPO_4 pH 7.2, 7% (w/v) SDS) at 65 °C for at least 30 min. The Church buffer was replaced and the purified radiolabelled probe was then added and samples were incubated overnight at 65°C. The membrane was washed twice in Wash A (20 mM Na_2HPO_4 pH 7.2, 5% (w/v) SDS) for 30 min, then for 15 min.

2.4.6 Signal detection

The membrane was sealed in transparent plastic and bands detected in a phosphorimage cassette, which was analysed using a Molecular Imager FX (Biorad). Alternatively, bands were exposed to X-ray film at -80°C in protective cassettes. The time of exposure depended on the strength of the signal.

2.4.7 Quantitative Real Time PCR

2.4.7.1 DNase treatment

RNA, extracted from plant tissue, was treated with DNase to remove any potential genomic DNA template that might interfere with the quantitative real time PCR (Q-PCR). 10 µg of RNA was incubated in a final volume of 50 µl with 5 µl 10 x DNase buffer (Promega) and 10 units of DNase (Promega) for 30 min at 37°C. The reaction was stopped by addition of 5 µl stop mix (Promega) followed by heat inactivation at 65°C for 10 min.

2.4.7.2 Reverse transcriptase reaction

2 µg (in 10 µl) of DNase treated RNA was mixed with 500 ng of oligo-dT primer and 1 µl of 10 mM dNTPs in a final volume of 12 µl. Following incubation at 65°C for 5 min, samples were chilled on ice and 4 µl of 5 x reverse transcription buffer (Invitrogen), 2 µl of 100 mM DTT and 1 unit of RNase OUT (Promega) added. Samples were incubated at 42°C for 2 min before addition of 1 µl of reverse transcriptase (Invitrogen). The reverse transcription reaction was allowed to occur by incubation at 42°C for a further 50 min, before heat inactivation at 70°C for 15 min. First strand cDNA samples were diluted 1 in 10 to 200 µl and stored at -80°C until use. Additional samples were reverse transcribed in parallel using RNA samples that had not undergone DNase treatment

2.4.7.3 Actin PCR

To confirm that the reverse transcriptase reaction had occurred satisfactorily, before continuing to Taqman Q-PCR, a PCR reaction was carried out on the cDNA with *actin* specific primers. DNase treated RNA was used as a negative control (1 µl + 9 µl water). The reaction was carried out in a final volume of 25 µl and contained 10 µl of cDNA, 2.5 µl of 10 x Thermopol PCR buffer, 0.5 µl of 10 mM dNTPs, 0.25 µl of each actin primer (100 µM stock) and 0.25 µl of *Taq* polymerase. The thermocycling conditions were 2 min at 94°C, followed by 25 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C, and a final 5 min incubation at 72°C. PCR products were separated by gel electrophoresis (as section

2.3.1.3) and a band of ~500 bp was expected in cDNA samples and not in negative controls.

2.4.7.4 Taqman Q-PCR

A 96-well plate was set up to contain a triplicate of each sample including standards and controls. Standards were used to compare transcript levels of unknown samples to a calibration curve made with cDNA prepared from 71B6-OE transgenic lines. DNase treated RNA was used for non-template control samples. Each well consisted of a 25 µl reaction mixture containing 12.5 µl of 2 x Taqman Universal PCR mastermix (Applied Biosystems, Roche), specific forward and reverse gene primers (0.3 µM) and probe (0.05 µM) (Q-71B6-F, CGGTACGAAATAATCTCCGGA; Q-71B6-R, GCTTTAGACTTT GGATGTGGG; Q-71B6-FAM, GAGATCAACAACCAACGGAGC; Actin LH, GATT CAGATGCCCAGAAGTCTT; Actin RH, TCTCGTGGATTCCAGCAGCT; Actin probe VIC, CCAGCCCTCGTTTGTGGGAATGG-tamra) and 10 µl of cDNA for each sample or control. The Q-PCR was carried out in an ABI prism 7000 machine following standard manufacturers protocols.

2.5 Phenotypic and chemotypic analysis of plants

2.5.1 Crude protein extraction from plant tissue and UGT assay

Total soluble protein was extracted from aerial tissue of 4-week-old plant tissue. 1 g fresh weight was ground to a fine powder in liquid nitrogen using pestle and mortar. 2.5 ml of extraction buffer (100 mM Tris pH 6.95, 10% (v/v) glycerol, 20 mM DTT, 1 mM PMSF, 1% (w/v) polyvinylpolypyrrolindone) was added to the powder and the slurry thawed on ice. Subsequently the slurry was mixed vigorously, filtered through 2 layers of miracloth

and centrifuged at 10,500 x *g* at 4°C for 20 min. The protein concentration of the supernatant was determined with Bio-Rad Protein Assay Dye and the crude extract assayed for glucosyltransferase enzyme activity. 50 µl crude extract (20–50 µg total protein) was mixed with 1 mM (±)-ABA, 5 mM UDP-glucose, in a final volume of 100 µl. The reaction was incubated at 30°C for 0 h, 2 h or 6 h and stopped by the addition of 10 µl trichloroacetic acid (240 mg ml^{-1}). The reaction mix was analysed by reverse phase HPLC as in section 2.2.8.

2.5.2 Cotyledon emergence assay

Seeds of the same age were surface sterilized with chlorine gas and plated on 1 x MS medium containing 0.8% (w/v) agar and 0.5 µM (+)-ABA, PBI-514 or PBI-413. Following stratification at 4 °C in the dark for four days, seeds were transferred to a controlled environment of 16 h/ 8 h light-dark cycle (22°C, 80 µmol m^{-2} sec^{-1}). The percentage of seedlings with cotyledons that had emerged from the seed coat was scored. Experiments were performed with 3 replicates of at least 150 seeds.

2.5.3 Glucose sensitivity assay

Seeds of the same age were surface sterilized with chlorine gas and plated on 1 x MS medium containing 0.8% (w/v) agar and 6% (w/v) glucose. Following stratification at 4 °C in the dark for 4 days, seeds were transferred to a controlled environment of 16 h/ 8 h light-dark cycle (22°C, 80 µmol m^{-2} sec^{-1}). The percentage of seedlings with cotyledons to have greened was scored. Experiments were performed with 3 replicates of at least 150 seeds. Photgraphs were taken of representative seedlings from each genotype.

2.5.4 Detached leaf water loss assay

Plants were grown in a climate controlled cabinet for four weeks and for the last two days the humidity was increased to 80%. Five individual leaves were detached from independent plants and placed upside down on a weighing boat. Fresh weight was determined immediately and weight loss measured hourly. Data were based on three independent replicates of 5 leaves (± SE).

2.5.5 Extraction of ABA and ABA metabolites from plants

2.5.5.1 Harvesting of turgid and wilted rosette tissue

Plants were grown for four weeks in a climate controlled cabinet as in section 1.1.1.1. For turgid tissues, rosettes were harvested and frozen immediately in liquid nitrogen. The wilting treatment was carried out according to (Leon-Kloosterziel *et al.*, 1996). Essentially, detached rosettes were dehydrated in dry air at room temperature until 15% of the fresh weight was lost (approximately 10 min). The stressed material was kept in sealed polythene bags at 22°C for 6 h then wrapped in foil and frozen in liquid nitrogen. The frozen material was freeze dried and the dry weight determined. Samples were stored at -80°C in dark, sealed, dry containers with silica gel.

2.5.5.2 Extraction of ABA and ABA metabolites

ABA, ABA-GE, PA, DPA, 7′OH-ABA and neo-PA were extracted for analysis from four-week-old rosette tissue as described: freeze-dried tissue (50–100 mg) was ground to powder in 2-ml screw-cap microcentrifuge tubes (Starstedt) using ¼ in. ceraminc bead (Q-biogene) with a Fast Prep FP 120 machine (Q-biogene)(10 s at 5 ms^{-1}). 20 ng of internal standards ((-)-5, 8′,8′,8′-d_4 ABA; (-)-7′,7′,7′-d_3 PA; (-)-5,8′,8′,8′-d_4 7′OH-ABA; (-)-7′,7′,7′-d_3 DPA; (-)-8′,8′,8′-d_3 neo-PA; (+)-5,4,8′,8′,8′-d_5 ABA-GE) and 1 ml of extraction solvent (80% isopropanol, 19% water, 1% glacial acetic acid (v/v)) were

added. Samples were mixed by vortexing and then incubated, with shaking, overnight. Following centrifugation at 16,000 x *g* for 2 min at room temperature the supernatant was collected and pellets rinsed with a further 0.5 ml extraction solvent. The supernatants were combined in a fresh tube and dried by centrifugation under vacuum. Following resuspension in 1 ml of 99% isopropanol: 1% acetic acid (v/v) by vortexing and sonication, samples were centrifuged at 16,000 x *g* for 2 min and the supernatant transferred to a fresh tube. After being dried by centrifugation under vacuum, samples were dissolved in 50 µl of 99% methanol: 1% acetic acid (v/v) by vortexing and sonication and a further 450 µl of 99% water: 1% acetic acid (v/v) added. Oils in the samples were removed by hexane partitioning. Following the addition of 1 ml of hexane to the samples and vortexing for two minutes the layers were allowed to separate for 30 minutes. Samples were then centrifuged at 16,000 x *g* for 2 min and the remaining aqueous extracts (bottom layer) removed to a fresh tube and dried by centrifugation under vacuum. Extracts were dissolved in 100 µl of 99% methanol: 1% acetic acid (v/v) by vortexing and sonication and topped up to 1 ml with 99% water: 1% acetic acid (v/v). Oasis HLB 1-ml solid phase extraction cartridges (Waters) were conditioned with 1 ml of 99% acetonitrile: 1% acetic acid (v/v) followed by 1 ml of 99% methanol: 1% acetic acid (v/v) and equilibrated with 1 ml of 99% water: 1% acetic acid (v/v). Samples were centrifuged at 16,000 x *g* for 2 min to remove any remaining particulate material prior to loading. Samples were loaded under a vacuum of 0.16-0.19 kPa below atmosphere followed by a wash with 1 ml of 99% water: 1% acetic acid (v/v). Analytes were eluted using 1 ml of 50% acetonitrile, 49% water: 1% acetic acid (v/v) before samples were dried by centrifugation under vacuum and stored at 4°C in the dark.

2.5.6 Quantification of ABA and ABA metabolites

ABA and ABA metabolites were quantified using liquid chromatography, negative electrospray tandem mass spectrometry as described (Feurtado 2004) with the addition of the analyte neo-PA (cone voltage=20V, collision cell=13V; transition 279>205 and d3

neo-PA 282>208). Quantification was performed by Steve Ambrose in the Abrams Group at the Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Canada.

2.5.7 Challenge experiments

Seedlings were grown on sterile ATS media (1% (w/v) sucrose, 5 mM KNO_3, 2.5 mM KPO_4 pH 5.5, 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 0.05 mM Fe-EDTA, 1 mM micronutrients, 0.6% (w/v) agar) for two weeks. They were transferred to 150 ml flask containing 40 ml liquid ATS and shaken at 100 rpm for six h in a climate controlled environment of (22°C, 80 µmol m^{-2} sec^{-1}). To challenge the seedlings with specific stresses, the liquid ATS was supplemented with 300 mM NaCl, 600 mM mannitol, 40 µM cycloheximide, 100 µM ABA.

2.5.8 Salt challenge from roots

Seedlings were grown on sterile ½ x MS media (0.6% agar (w/v)) for one week. They were then transferred to grow on soil for a further two weeks. Whole seedlings were gently transferred to *Star-lab Tip-One* tip boxes with inserts (for 96 graduated pipette tips of 101-1000 µl) filled with ½ x MS liquid media such that their roots would be submerged with their aerial parts resting on the tip rack. Following one week to acclimatise to the liquid media, the P1000 rack was transferred to a fresh box containing ½ x MS supplemented with 150 mM NaCl. After 24 hours of salt challenge seedlings were divided into roots and aerial parts, wrapped in foil, and frozen in liquid nitrogen.

CHAPTER 3: CHARACTERISATION OF 71B6 *IN VITRO*

3.1 Introduction

3.2 Results

3.3 Discussion

3 CHARCTERISATION OF 71B6 *IN VITRO*

3.1 Introduction

Through *in vitro* analysis of 71B6, it was known that the GT had activity towards racemic ABA and that the product was largely derived from the (+) enantiomer (Lim *et al.*, 2005). However, the activity of the enzyme was not characterised in detail. Since this GT is potentially important with regard to the homeostasis of ABA, it was interesting to understand more about its kinetic properties and substrate specificity.

Many analogues of ABA have been synthesised by the Abrams laboratory in the Plant Biotechnology Institute, Saskatoon, Canada. Many of the analogues were designed and synthesized to investigate the features of the ABA molecule required: for bioactivity; for catabolism through the 8′-hydroxylation pathway; or to provide internal standards for mass analyses of ABA metabolites. In addition they can be useful to explore the substrate recognition of enzymes acting on ABA, and also to provide wider insights with regard to the action of the enzymes in the plant.

For example, in the studies of Jackson *et al.* (2001; 2002) the family 1 GT, 84B1, was studied both *in vitro* and *in planta* with regard to its ability to glucosylate IAA. Plants overexpressing *84B1* (84B1-OE) displayed phenotypes characteristic of auxin deficiency, including loss of gravitropism and loss of sensitivity to inhibition of root growth by IAA. From the *in vitro* studies, it was known that 84B1 could glucosylate IAA, but not the auxin analogue, 2,4-dichlorophenoxyacetic acid (2,4-D). The application of 2,4-D to the 84B1-OE lines led to a wild type response in both assays, demonstrating that the phenotypes were a direct consequence of the action of the overexpressed 84B1 enzyme on IAA.

Through a collaboration with the ABA chemist, Suzanne R. Abrams, analogues were provided for this study. The availability of the recombinant 71B6 enzyme and the analogues, therefore, created a major opportunity to study the substrate selectivity of the enzyme, and to identify analogues that might be useful tools in the exploration of phenotypic aberrations in transgenic plants.

3.2 Results

3.2.1 Recombinant 71B6 has activity towards ABA and produces ABA-GE

The Arabidopsis 71B6 enzyme, expressed as a recombinant GST-fusion protein in *E. coli* and purified by affinity chromatography, was incubated with the naturally occurring enantiomer of ABA ((+)-ABA). The reaction mix contained a more hydrophilic compound, eluting earlier than ABA on HPLC, characteristic of a glucose conjugate (peak A, Figure 3.1A), not observed in the control without enzyme. The data shown are for the intact fusion protein; the activity was reduced by up to 60% following cleavage of the GST protein (data not shown). This was considered to be due to decreased stability of the cleaved protein. No activity was detected towards the ABA catabolites, PA and DPA, nor other phytohormones that have been tested, including auxin, cytokinins, gibberellins and salicylic acid (data not shown).

Peak A disappeared on incubation of the reaction mix with 1 M NaOH (Figure 3.1B), consistent with the cleavage of an ester-linked product by alkaline hydrolysis. To confirm the identity of the compound, negative electrospray liquid chromatography- mass spectrometry- mass spectrometry (LC–MS–MS) was used to compare an authentic ABA-GE standard (Figure 3.1C) with peak A (Figure 3.1D). An ion of mass to charge ratio (m/z) 425 was the major component both of peak A and ABA-GE. The value of 425 corresponds to the mass of an intact ABA-GE ion that has undergone negative electrospray ionisation $[M\text{-}H]^-$. Low energy collision-induced dissociation (CID) product ion spectra of the 425 ion in both peak A and the ABA-GE standard show 3 peaks at 425, 305, and 263. Both the 263 and 305 product ions are characteristic of ABA-GE fragmentation. The 263 ion corresponds to loss of the intact glucose moiety ($C_6O_5H_{10}$). Further, the relative intensities of the product ion peaks in both traces are comparable,

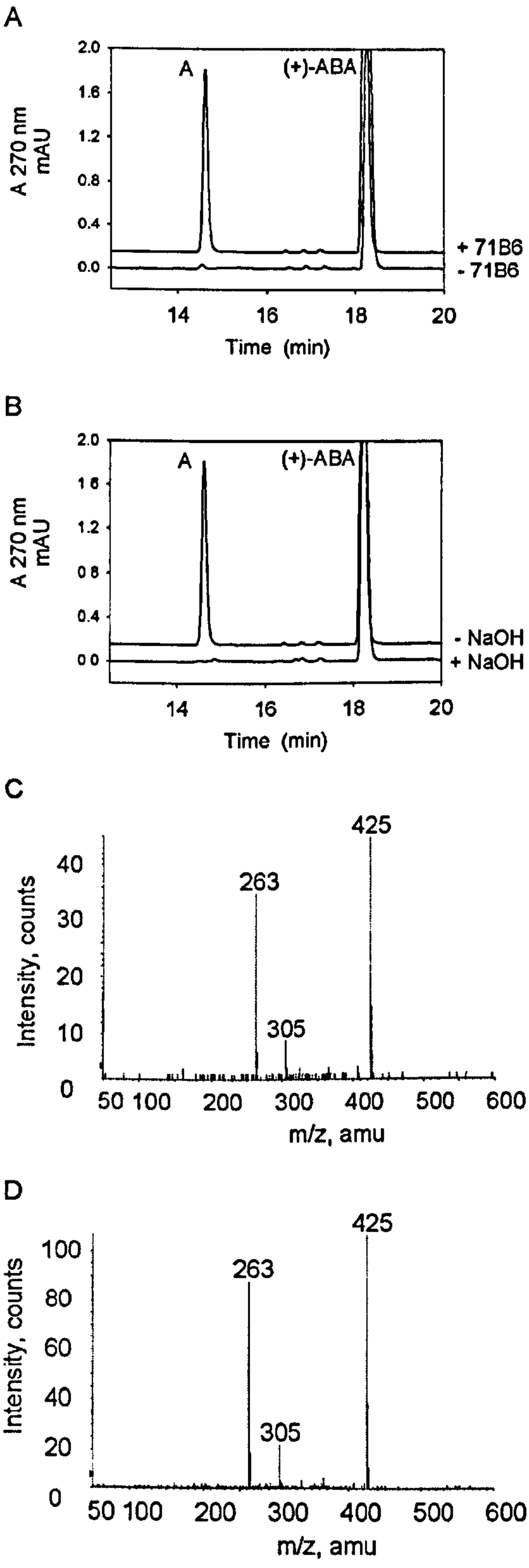


Figure 3.1 Analysis of the reaction mix of 71B6 with ABA and UDP-glucose

Figure 3.1 Analysis of the reaction mix of 71B6 with ABA and UDP-glucose

A reaction mix of 50 mM Tris pH 6.95, 1 mM (+)-ABA, 5 mM UDP-glucose, 5 mM $MgCl_2$ and 10 mM DTT was incubated with or without recombinant 71B6 protein at 30°C for 1 h. The reaction was stopped with trichloroacetic acid (**A**). The reaction was further incubated with and without 1 M NaOH for an additional hour (**B**). The reactions were analysed on a reverse phase HPLC chromatograph. The traces are offset by 0.2 mAU. Negative electrospray LC-MS-MS CID spectra of an authentic ABA-GE standard (**C**) and the major product of recombinant 71B6 protein after incubation with (+)-ABA and UDP-glucose (**D**).

which implies that the glucose is attached at the same position. A high energy CID spectrum of the 425 ion also showed close correlation between the ABA-GE standard and the reaction product of 71B6 (data not shown).

3.2.2 Optimisation of assay conditions

The activity of 71B6 towards ABA was influenced by the assay conditions *in vitro* (Figure 3.2). The enzyme was found to have a pH optimum between 6.5 and 7.0 (Figure 3.2A), and the activity was substantially increased by inclusion of reducing agents (2-mercaptoethanol or DTT) to approximately 150% and 250% of basal levels respectively (Figure 3.2B). The effect of metal ions on activity is shown in Figure 3.2C. A slight increase in activity was observed in the presence of divalent cations, Mg^{++} and Ca^{++}. The activity was decreased by the inclusion of Co^{++}, Ni^{++}, Cu^{++} and Fe^{++}, and completely abolished by Zn^{++}. Combining the addition of DTT with Mg^{++} further increased activity to approximately 400% of levels minus additions (Figure 3.2D).

3.2.3 Kinetic analysis of the activity of 71B6

The kinetic parameters of 71B6 towards (+)-ABA with saturating levels of 5 mM UDP-glucose were investigated with assay conditions of 50 mM Tris pH 6.95, 10 mM DTT, 5 mM $MgCl_2$. The rate of production of ABA-GE was found to be linear from 0–20 minutes for (+)-ABA from 0.05–2.50 mM (Figure 3.3). Initial rates of reaction at each concentration were calculated from four time points and used to determine the kinetic parameters by Hyperbolic Regression Analysis. In the optimum conditions the V_{max} was 1.80 nkatals mg^{-1} (± 0.11) with K_m of 0.28 mM (± 0.04) and the k_{cat}/K_m of 0.5 mM^{-1} s^{-1} (± 0.01) (n=3, ± standard deviation).

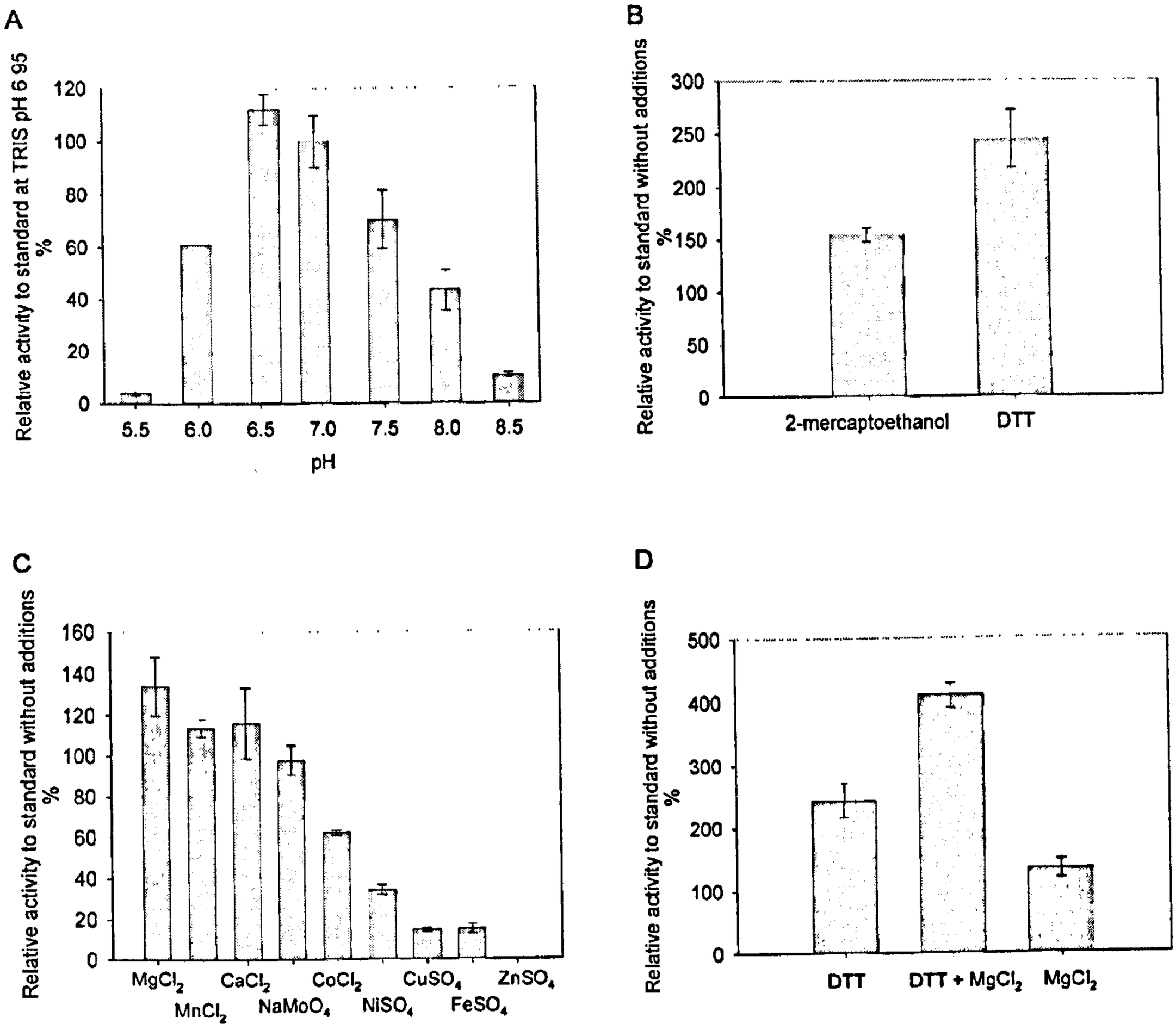


Figure 3.2 Assay parameters for the activity of 71B6 towards ABA

Activity of purified recombinant GST-71B6 was measured towards (+/-)-ABA under conditions of various (**A**) pH, (**B**) reducing agents, (**C**) metal ions, (**D**) combinations of positive treatments. Standard assays were carried out in 50 mM Tris pH 6.95 with 1 mM (+/-)-ABA, 5 mM UDP-glucose and 10 μg/ml protein. Metal ions were added at 5 mM, and reducing agents at 10 mM. Activities were determined using HPLC and calculated relative to standard assays in Tris pH 6.95 and without addition defined as 100%. The data are shown as means (±) standard deviations (n = 3).

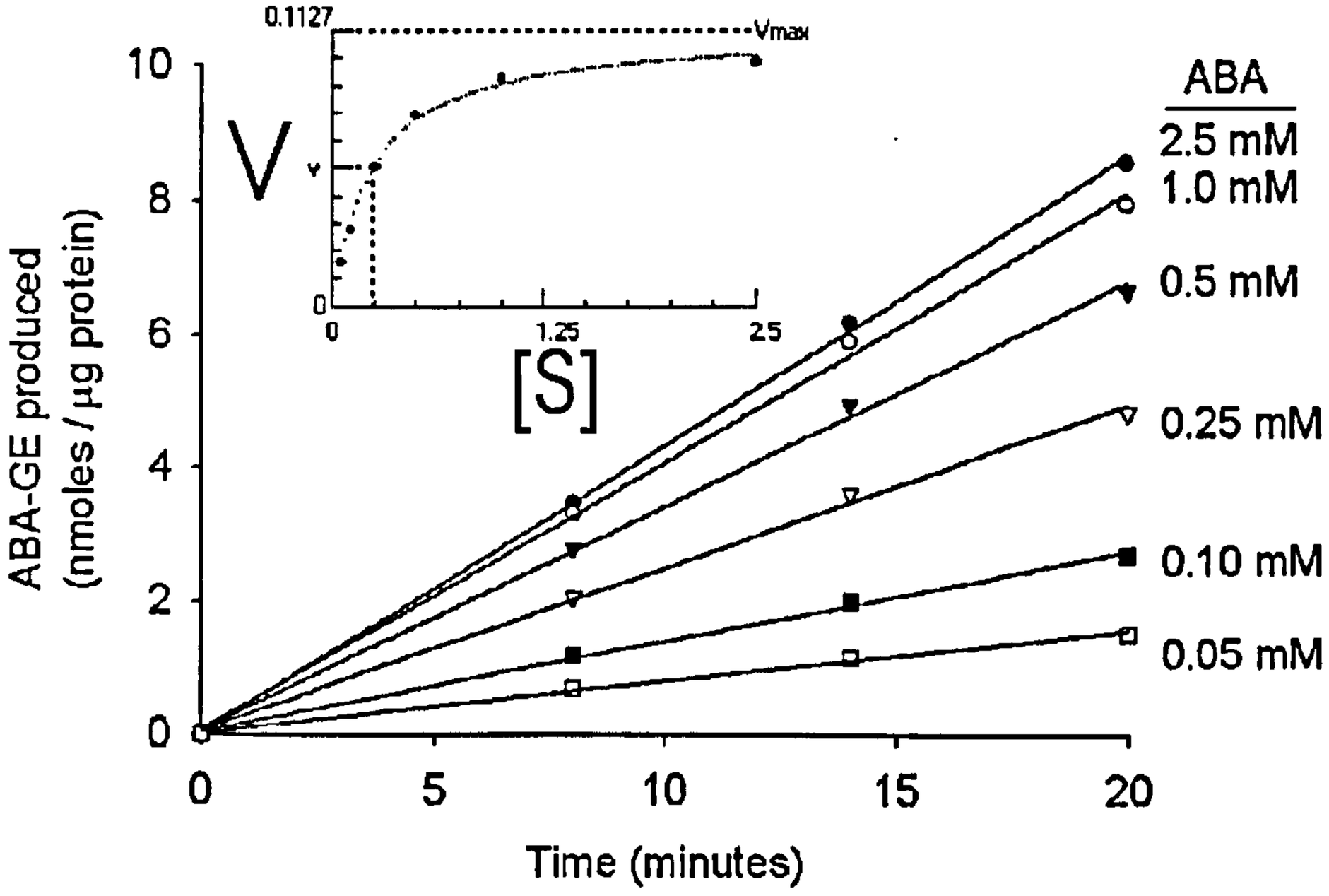


Figure 3.3 Typical time dependency of 71B6 towards (+)-ABA

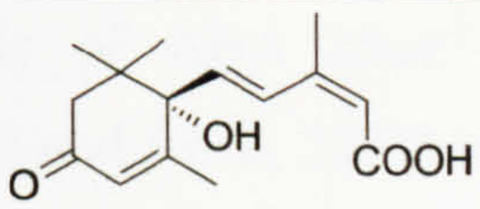
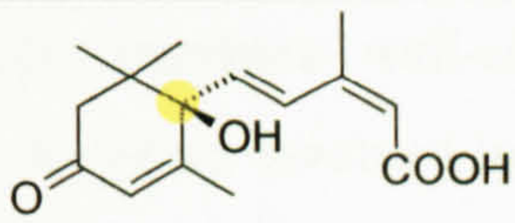
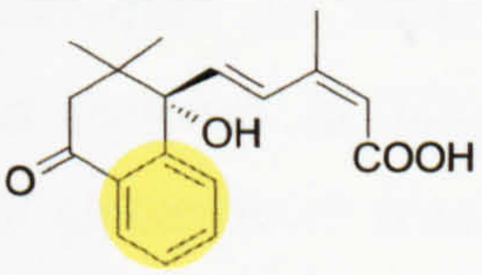
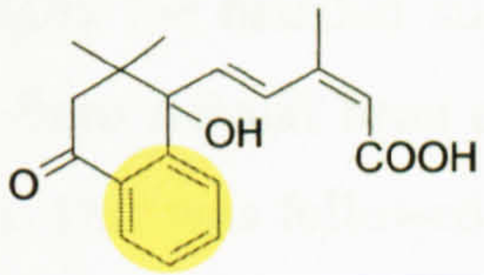
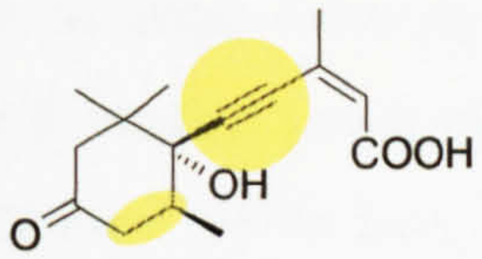
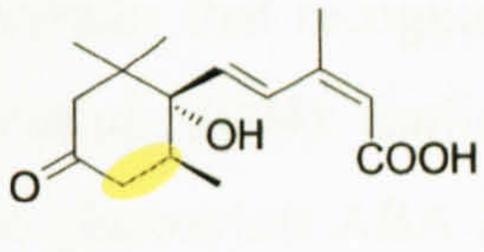
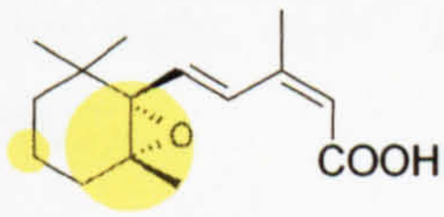
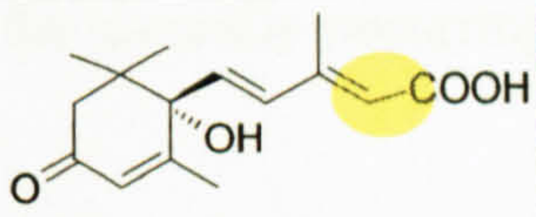
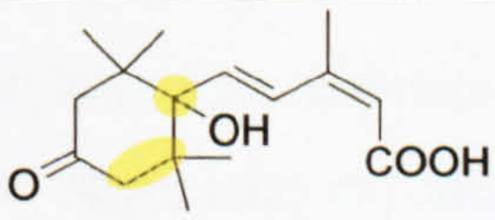
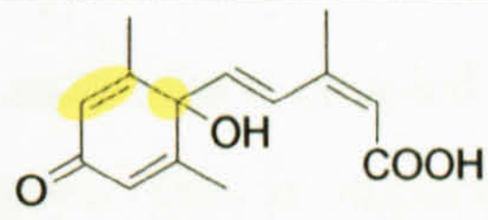
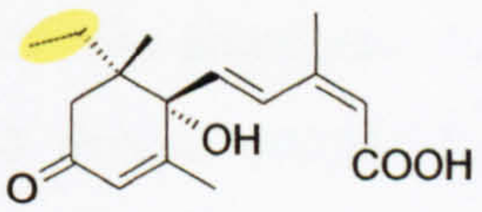
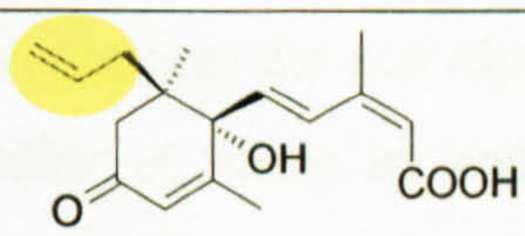
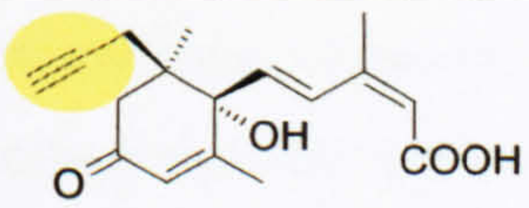
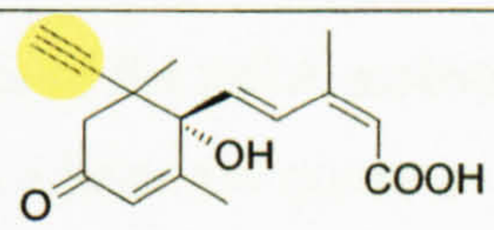
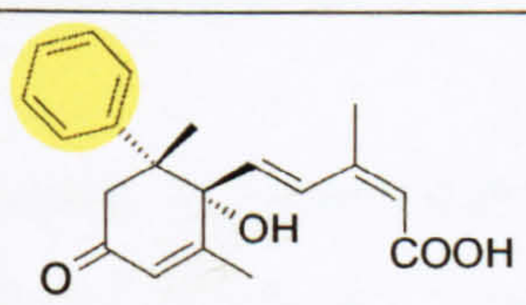
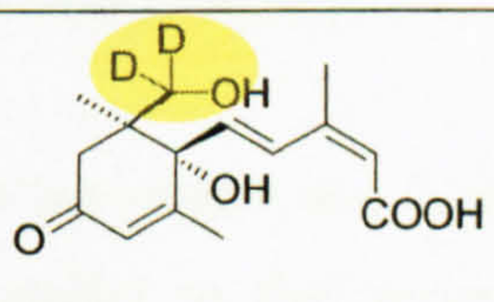
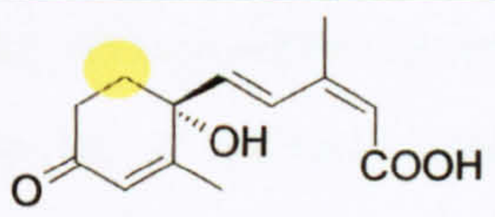
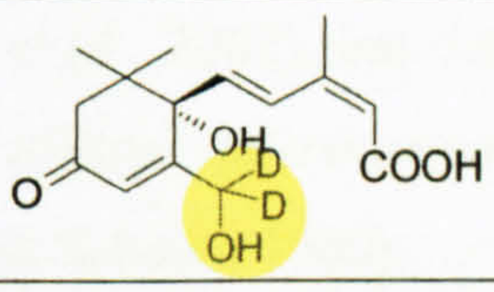
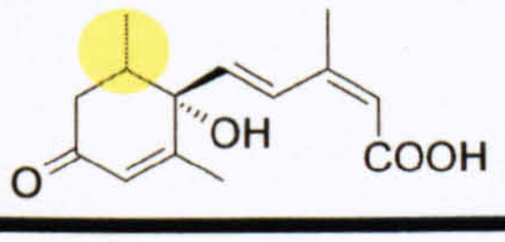
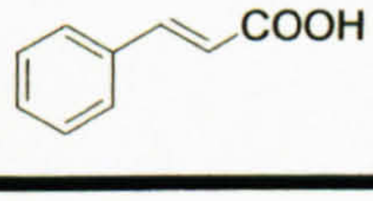
Initial rate of reaction was determined for purified recombinant GST-71B6 at various concentrations of (+)-ABA. Assays were carried out in 50 mM Tris pH 6.95 with 5 mM UDP-glucose, 5 mM $MgCl_2$, 10 mM DTT and 40 µg ml^{-1} protein at 30°C over 0–20 minutes. Activities were determined using HPLC and initial velocities calculated. The corresponding secondary hyperbolic plot is shown in the inset.

3.2.4 Activity towards ABA analogues

The activity of 71B6 towards the individual (+) and (-) enantiomers of ABA, and a diverse range of structural analogues of ABA was analysed (Table 3.1). 71B6 was found to be highly selective towards the natural (+) enantiomer of ABA. The enzyme was able to glucosylate the different structural analogues of ABA to varying degrees. All products were degraded by alkaline hydrolysis implying formation of the respective glucose esters (data not shown). The activity of 71B6 was higher towards PBI-413, PBI-410 and PBI-82, compared to (+)-ABA. The activity of the enzyme towards the analogue PBI-287 was near identical to (+)-ABA and much lower towards the remaining analogues. PBI-49 (racemic 2-*trans*-ABA) was shown to be a poor substrate for 71B6.

Table 3.1 Activity of 71B6 towards enantiomers and structural analogues of ABA

Assays were carried out in 50 mM Tris pH 6.95 with 1 mM ABA enantiomer / analogue, 5 mM UDP-glucose, 5 mM $MgCl_2$, 10 mM DTT and 10 μg/ml recombinant protein at 30°C. Reactions were analysed by HPLC and activities determined relative to the standard substrate (+)-ABA. The results represent means ± SD of at least three independent protein preparations.

Name	Structure	Activity relative to ABA (%)	Name	Structure	Activity relative to ABA (%)
(+)-ABA		100	(-)-ABA		4 ± 2
PBI-413		304 ± 25	PBI-410 (racemic mixture)		234 ± 22
PBI-82		179 ± 27	PBI-89		3 ± 1
PBI-287		102 ± 2	PBI-49 (racemic mixture)		4 ± 2
PBI-233		14 ± 7	PBI-253		3 ± 1
PBI-372		51 ± 9	PBI-401		7 ± 1
PBI-514		5 ± 3	PBI-524		6 ± 2
PBI-493		12 ± 1	PBI-705		0
PBI-271		9 ± 2	PBI-703		6 ± 2
PBI-293		17 ± 8	*Trans* cinnamic acid		12 ± 1

0 100 200 300

0 100 200 300

3.3 Discussion

The homeostatic mechanisms controlling ABA levels in plants have attracted considerable interest over many years. A number of genes that function in the biosynthesis of ABA have been identified and their enzymes well-characterised (Finkelstein and Rock, 2002). In contrast until recently, no genes involved in catabolism had been identified and therefore there was no opportunity for detailed study of their recombinant enzymes. In 2002, Xu *et al.* cloned a gene from Adzuki bean encoding an enzyme that glucosylated ABA *in vitro* (Xu *et al.*, 2002). This was followed in 2004 by two reports on genes encoding cytochrome P450 hydroxylases that recognised ABA *in vitro* at the 8′-methyl group (Kushiro *et al.*, 2004; Saito *et al.*, 2004). Earlier this year, eight recombinant UGTs from Arabidopsis were shown to glucosylate ABA *in vitro*, and only one of the eight, 71B6, displayed preference for the naturally occurring (+)-ABA (Lim *et al.*, 2005).

The availability of recombinant 71B6 has enabled us to undertake a detailed study of its activity and specificity towards a range of ABA analogues. This is important for two reasons. First, the analogues can provide a useful tool to confirm the activity of 71B6 *in planta*, in a similar manner to that shown for conjugation of auxin by another GT of Arabidopsis, 84B1 (Jackson *et al.*, 2002). Second, the availability of such a diverse range of structures allows a detailed investigation of the features of the ABA molecule required for recognition and/or catalysis by the GT. This provides a basis for going on to compare the specificity of this enzyme with others that recognise and/or bind to ABA.

In this chapter, 71B6 was shown to have an optimal pH between 6 and 7, with activity much reduced below pH 6 or above pH 8. This is similar to that reported for the recombinant glucosyltransferase from Adzuki bean (Xu *et al.*, 2002), but different from an activity of a partially purified enzyme from *Macleaya microcarpa* shown to glucosylate ABA with a pH optimum of 5.0 (Lehmann and Schutte, 1980).

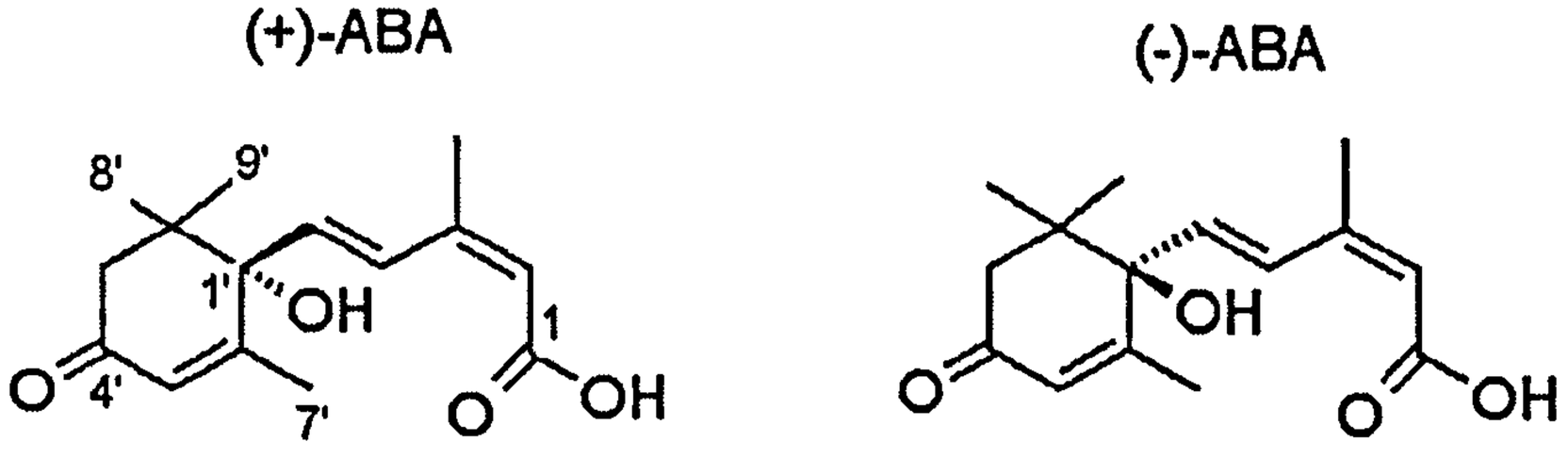
Quantitative TLC analysis of reaction products of the adzuki bean glucosyltransferase had shown that the signal intensity for 2-*trans*-(+)-ABA was 6 fold greater than for (+)-ABA, which was a further 2 fold greater than (-)-ABA (Xu *et al.*, 2002). In contrast, the Arabidopsis 71B6 displayed little activity (4%) towards 2-*trans*-ABA (PBI-49, racemic mixture) compared to (+)-ABA. This is a clear difference in selectivity between the two recombinant enzymes. 2-*trans*-ABA is not bioactive (Sondheimer and Walton, 1970; Kriedemann *et al.*, 1972; Watanabe and Takahashi, 1999; Zeevaart, 1999; Finkelstein and Rock, 2002) and, although found in plant extracts, may be produced as an artefact of (+)-ABA isomerised by light (Wilmar and Doornbos, 1971).

The use of ABA analogues as substrates for 71B6 in this study has enabled a detailed characterisation of the structural features of ABA that are important for binding and/or catalytic activity. The data indicate that 71B6 is specific for ABA rather than its acidic metabolites. No activity was detected towards either PA or DPA. PBI-703 and PBI-705 are deuterated versions of the catabolites 7′OH-ABA and 9′OH-ABA which are used as internal standards for mass analyses of the metabolites (Zaharia *et al.*, 2005). Neither of these analogues were good substrates for 71B6.

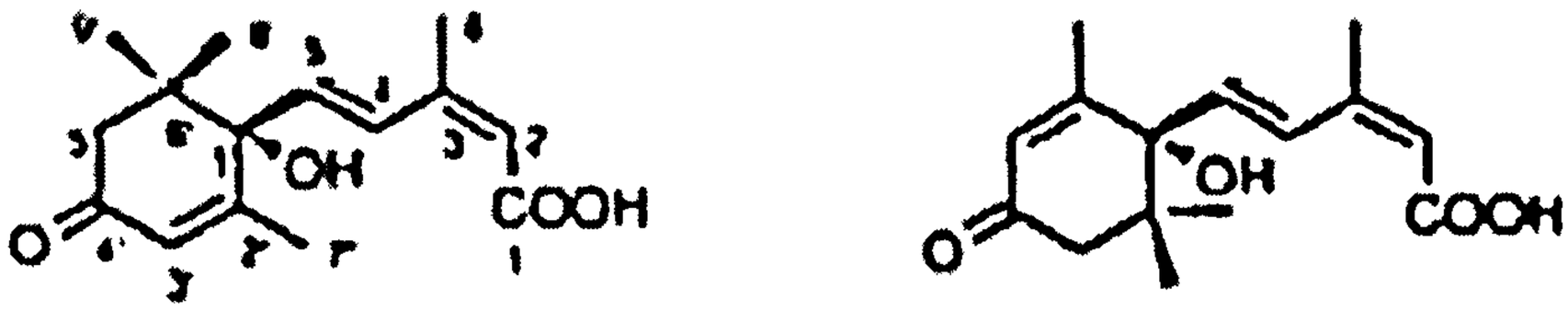
The requirement of 71B6 for the specific chiral configuration of (+)-ABA is striking, and demonstrates that structural changes can have a profound effect on activity (Ueno *et al.*, 2005). The unnatural form (-)-ABA is the mirror image of (+)-ABA. Superimposing the two enantiomers with the hydroxyl groups overlapping reveals that the only change is in the relative position of the methyl groups on the ring (figure 3.4)(Lei *et al.*, 1994). The relative orientations of these methyl groups are also changed in the poor substrates, PBI-233 and PBI-253, which have symmetry around the ring structure and also no chiral centre at the C1′-carbon.

Similarly, the glycosyltransferase also has reduced activity to those analogues with direct modifications to the 8′ and 9′-methyl groups (PBI-372, PBI-401, PBI-514, PBI-524, PBI-493 PBI-705, PBI-271 and PBI-293). This can be contrasted with the analogue PBI-413,

a



b



c

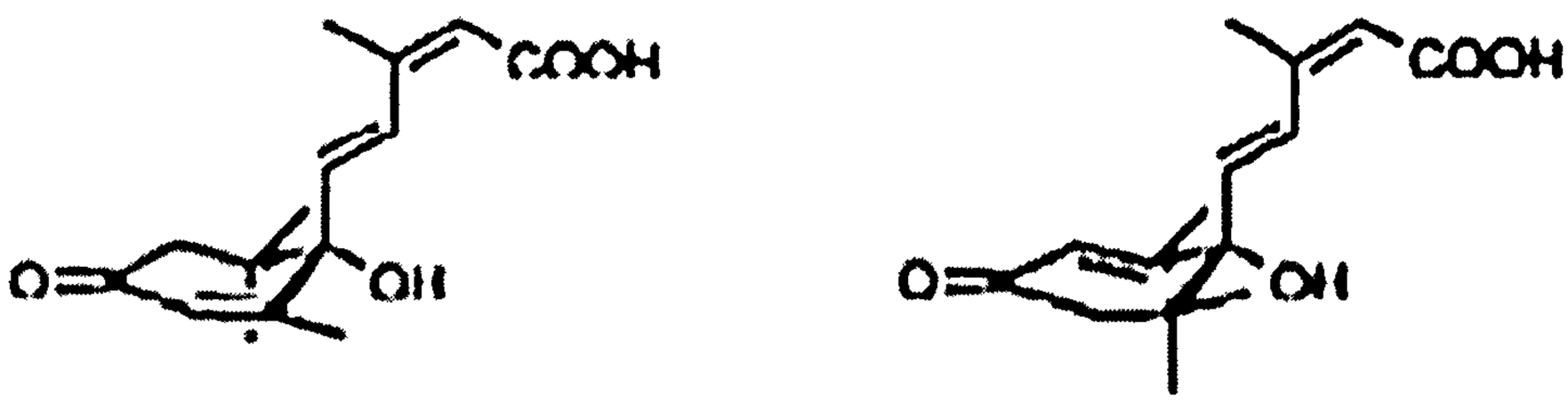


Figure 3.4 Different representations of (+) vs (-) enantiomers of ABA

Part **a** shows the conventional representation. Parts **b** and **c,** taken from Lei *et al.*(1994), show the enantiomers so that the hydroxyl groups overlap when superimposed.

which has an aromatic ring fused to the ABA ring, and is a significantly better substrate than ABA. The lower activity of the racemic form, PBI-410, is consistent with the results for (+) versus (-)-ABA. It appears that whilst the enzyme can readily accommodate extra bulk around the double bond of the ABA ring and maintain activity, even slight alterations to the position or size of the 8′ and 9′-methyl groups result in substantial reductions in activity. Interestingly, a recent study showed that 5′α,8′-cycloabscisic acid, when applied to radish seedlings, was glucosylated, despite modification of the 8′-methyl group (Todoroki *et al.*, 2004). Other UGTs may exist in radish with different activities from that of Arabidopsis 71B6, but it will be interesting to determine whether 71B6 can glucosylate the analogue *in vitro*.

The increased activity towards PBI-413 supports the rationale for the design of a probe for ABA-binding proteins by Nyangulu *et. al.* (2005) in which biotin was linked through the benzene ring of a modified version of PBI-413. The approach contrasted with previous attempts that had linked probes through either the carboxyl group or the ketone (Zhang *et al.*, 2002; Yamazaki *et al.*, 2003). It will be important to investigate whether a modified version of Nyangulu's probe could be used to discover the residues in 71B6 that are involved in ABA binding.

It is interesting that PBI-82 is a better substrate than ABA whereas PBI-89 is not glucosylated by the enzyme. Both PBI-82 and PBI-89 have the ring double bond reduced, however, PBI-82 also has a triple bond in the side chain. As this triple bond changes the disposition of the carboxyl group relative to the ring (Abrams *et al.*, 1996) it can be speculated that it improves the position for binding in the active site. Both of these analogues have shown activity similar to (+)-ABA in inhibition of germination of wheat embryos and both induced expression of ABA-inducible genes in a similar way (Walker-Simmons *et al.*, 1992).

Also of interest, PBI-287, which has no oxygen at C-4′, is as good a substrate for the enzyme as ABA. The analogue resembles xanthoxin with a similar epoxide, but missing

the oxygen at C-4′, and can be converted in plant cells to ABA (Okazaki *et al.*, 2000). This indicates that the ketone of ABA is not required for glucosylation by 71B6.

The analogues PBI-372 and PBI-493 with additional carbon atoms at the 8′-methyl group were designed to be resistant to the major route for ABA deactivation via 8′-hydroxylation (Rose *et al.*, 1997). The analogues PBI-514 and PBI-524 as well as PBI-401 were designed as potential mechanism-based inhibitors of the ABA 8′-hydroxylase (Cutler *et al.*, 2000). It is interesting that these three analogues are also resistant to glucosylation by 71B6. Importantly, the analogues are bioactive when applied to plants (Cutler *et al.*, 2000), yet this study and the earlier study indicates that they will not be susceptible to catabolism by either 8′-hydroxylation or conjugation by 71B6. It is therefore possible the analogues may represent a source of 'long-lasting ABA' agrochemicals for field applications. However, it will first be necessary to confirm that other GTs do not glucosylate them, whether in the model plant, Arabidopsis, or in target crop species. If indeed the compounds prove not to be susceptible to the major pathways of either catabolism or conjugation, they could also be useful in revealing any minor pathways of controlling ABA homeostasis.

The data also establish the foundation for going on to define 71B6 activity in the plant. A number of the analogues that have been assayed as potential substrates for the glucosyltransferase display bioactivity when applied to plants (Walker-Simmons *et al.*, 1992; Lei *et al.*, 1994; Walker-Simmons *et al.*, 1994; Cutler *et al.*, 2000). It is now clear that only a proportion of these analogues can be glucosylated by 71B6. This provides a means for determining whether phenotypes displayed in transgenic plants overexpressing the GT can be reversed by application of an analogue that cannot be glucosylated, in a similar strategy to that of Jackson *et al.* (Jackson *et al.*, 2002).

It will be interesting to discover whether 71B6 plays a role in ABA homeostasis *in planta*, and whether altering the expression of the gene encoding this GT results in changes in flux through ABA the catabolic pathways.

CHAPTER 4: CHARACTERISATION OF 71B6 *IN PLANTA*

4.1 Introduction

4.2 Results

4.3 Discussion

4 OVEREXPRESSION OF *71B6 IN PLANTA*

4.1 Introduction

The previous chapter demonstrated the activity of 71B6 towards (+)-ABA and structural analogues *in vitro*. The data provide the basis for investigating the effects *in planta* if the gene is overexpressed or knocked out. This chapter investigates the consequences of overexpression.

Since ABA-GE is considered to be inactive, it would seem reasonable that overexpression of *71B6* could lead to an increase in the accumulation of ABA-GE and, in consequence, a decrease in the accumulation of free ABA. In these circumstances, ABA deficiency may therefore be observed. A range of ABA deficient mutants have been identified through genetic screens (Finkelstein and Rock, 2002; Schwartz *et al.*, 2003; Nambara and Marion-Poll, 2005). Typical ABA biosynthesis mutants, such as *aba3-2*, accumulate very low levels of the hormone resulting in poor growth, reduced seed dormancy, lack of stomatal control and wiltiness (Rock and Zeevaart, 1991; Leon-Kloosterziel *et al.*, 1996; Seo *et al.*, 2000). However, there are other physiological characteristics, such as early developmental growth, which display greater sensitivity to smaller changes in ABA levels (Lopez-Molina *et al.*, 2001). Seedlings grown on media containing high concentrations of glucose, which increases ABA biosynthesis, will arrest and not green (Jang *et al.*, 1997). Many glucose insensitive mutants are also deficient in ABA and the insensitivity can be rescued by addition of the hormone exogenously (Arenas-Huertero *et al.*, 2000; Rook *et al.*, 2001; Brocard-Gifford *et al.*, 2004).

In this chapter, *71B6* has been constitutively overexpressed in Arabidopsis and the effects assessed on phenotypic characteristics known to relate to ABA. In parallel, the endogenous pools of free ABA, ABA-GE and related catabolites were measured in

stressed and unstressed conditions. The consequences of overexpressing *71B6* on ABA flux are discussed.

4.2 Results

4.2.1 Molecular and biochemical characterisation of plants overexpressing 71B6.

To investigate whether 71B6 can glucosylate ABA in plants, homozygous transgenic lines were generated that overexpressed the gene under the control of the CaMV 35S promoter (71B6-OE). Levels of *71B6* transcript were assessed by Northern blot in the 4-week-old rosette leaves of homozygous transgenic lines, wild type (WT) and empty vector control (Ri) plants. Figure 4.1a shows that wild type, Ri and two 71B6-OE lines (7 and 14) did not accumulate detectable levels of *71B6* transcript, whereas transcript accumulated in six 71B6-OE lines at different levels (10, 11, 12, 13, 17 and 23). To determine whether the accumulation of transcript correlated with increased enzyme activity, crude protein extracts from plants were assayed *in vitro* with the substrates ABA and UDP-glucose, and accumulation of ABA-GE measured by HPLC. Figure 4.1B shows that those lines with the highest accumulation of *71B6* transcript had the strongest enzyme activity towards ABA. Therefore overexpression of *71B6* in plants results in accumulation of active enzyme that can glucosylate ABA *in vitro*.

4.2.2 Phenotypic analysis of 71B6-OE plants

4.2.2.1 Initial observations

We hypothesised that if 71B6 is able to deactivate ABA by glucosylation *in planta*, overexpression would result in increased levels of ABA-GE and, in consequence, decreased levels of free ABA. To determine whether 71B6-OE plants displayed an ABA-

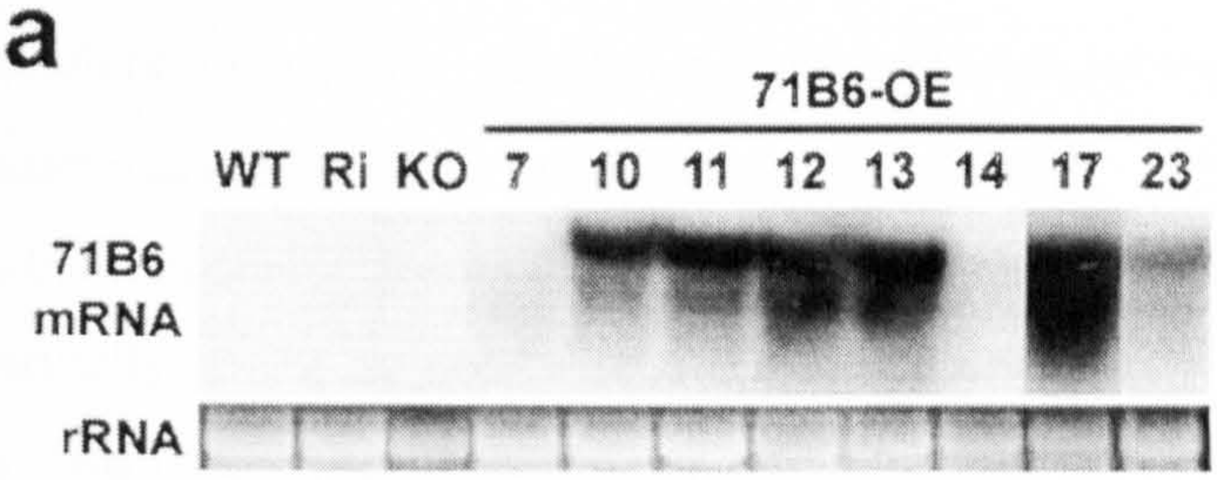


Figure 4.1 Molecular and biochemical characterisation of transgenic lines

a) Northern blot analysis of steady-state levels of *UGT71B6* mRNA in transgenic homozygous lines. RNA was isolated from 4-week-old rosette tissue of wild type (WT), Ri empty vector control (Ri), *71B6* transposon insertion line (KO), and eight independent *CaMV35S-71B6* lines (71B6-OE 7, 10, 11, 12, 13, 14, 17, 23). 10 µg of total RNA was separated by electrophoresis on a formaldehyde gel, transferred to a nylon membrane and hybridised with ^{32}P labelled *71B6*. Signals were detected by autoradioaugraphy.

b) Crude protein extracts were prepared from 4-week-old rosette tissue of the lines described above. The UDP-glucosyltranferase activity towards ABA was determined by incubating the crude extracts with 1 mM (+/-)-ABA and 5 mM UDP-glucose in the presence of 10 mM DTT at 30°C. The accumulation of ABA-GE was measured by HPLC. The data represent the mean of three replicates (± SD).

deficient phenotype, detailed phenotypic characterisation was carried out. Under the parameters analysed and standard growing conditions, 71B6-OE plants were indistinguishable from wild type control plants. For example, the transgenic plants grew normally and were not 'wilty'; transgenic seeds germinated under the same time course as wild type, with or without stratification, and on media supplemented with exogenous ABA, or with the gibberellin biosynthesis inhibitor, paclobutrazol (data not shown).

4.2.2.2 Resistance to exogenous ABA

Whilst germination of seeds is inhibited by high levels of exogenous ABA, the hormone is more efficient as an inhibitor of early post-germinative growth: wild type seedlings are highly sensitive to developmental arrest by low levels of ABA (Lopez-Molina *et al.*, 2001). Therefore, post-germinative growth was measured in terms of cotyledon emergence to test whether the transgenic lines were less sensitive to ABA.

In the absence of ABA both wild type and 71B6-OE displayed 90–100% emergence after 3 days (data not shown). However, Figure 4.2a shows that the cotyledons of 71B6-OE seedlings emerged more rapidly than those of wild type when cultured on 0.5 μM (+)-ABA. To assess whether the accelerated post-germinative growth of the 71B6-OE seedlings was due to enhanced glucosylation of ABA, rather than another unknown substrate, seedlings were grown on media supplemented with the ABA-analogues PBI-514 and PBI-413. Both of these analogues are bioactive (Cutler *et al.*, 2000) (Abrams S.R., unpublished data). However, as described in chapter 3, recombinant 71B6 displayed differential activity towards the two: PBI-514 was a very poor substrate whereas PBI-413 was a better substrate than ABA (Priest *et al.*, 2005). If the accelerated cotyledon emergence of the 71B6-OE seedlings was due to glucosylation of ABA, then the emergence should be tied to the ability of the enzyme to glucosylate and deactivate the compound. Wild type and 71B6-OE seedlings grown on PBI-514 showed no difference in cotyledon emergence (Figure 4.2B). Conversely, the difference in cotyledon

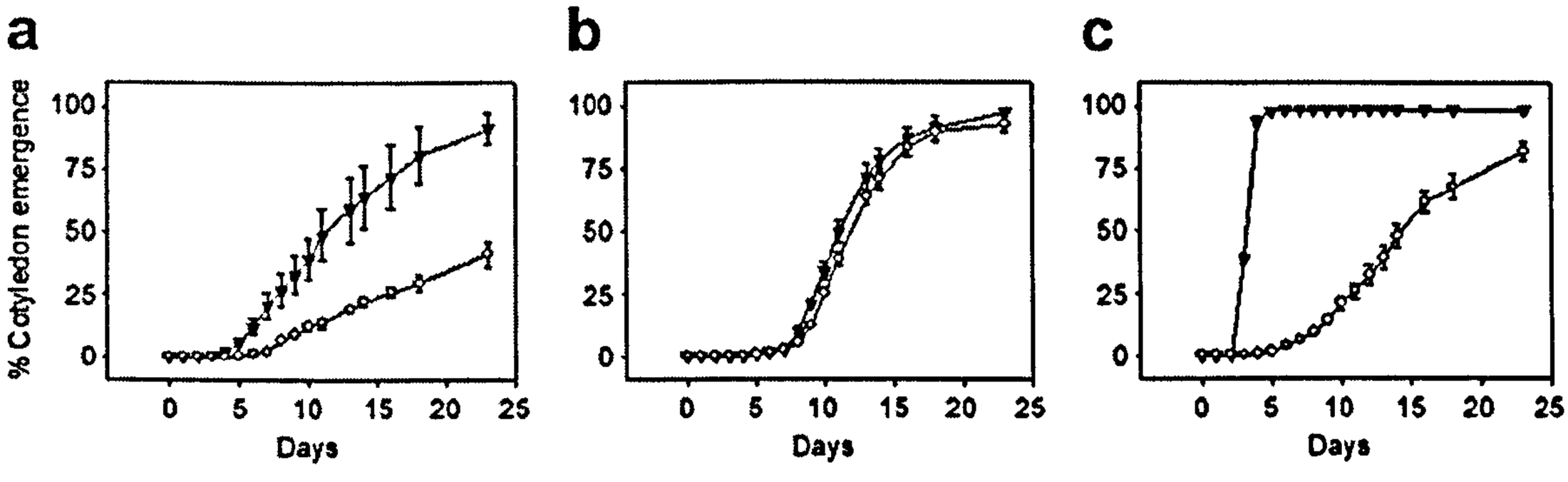


Figure 4.2 Post-germinative growth on ABA and analogues in transgenic lines

Post-germinative growth, as scored by percentage of cotyledons emerged from the seed coat, was determined for 71B6-OE13 (▼) and wild type (o). Surface sterilised seeds were sown on medium supplemented with 0.5 μM a) (+)-ABA b) PBI-514 or c) PBI-413 and stratified for 4 days before transfer to warm light conditions. Experiment also included 71B6-OE 12 and 17 with similar results. Data are based on 3 replicates of at least 150 seeds (± SE).

emergence between wild type and 71B6-OE was enhanced when seedlings were grown on PBI-413 (Figure 4.2c). These experiments demonstrate that 71B6 can glucosylate ABA and an ABA analogue *in planta* and that this activity reduces the susceptibility of seedlings to exogenously supplied ABA / ABA analogue.

4.2.2.3 Resistance to exogenous glucose

It has been shown previously that several glucose insensitive mutants were allelic to ABA-deficient or ABA-insensitive mutants (Arenas-Huertero *et al.*, 2000; Rook *et al.*, 2001; Brocard-Gifford *et al.*, 2004). Therefore, it might be expected that overexpression of *71B6* would lead to glucose insensitivity. To assess this possibility, seeds were germinated on 6% glucose and greening of cotyledons scored. Whilst the wild type seedlings were arrested and none had greened, all of the seedlings from the ABA deficient mutant, *aba3-2*, greened and continued to develop and grow (Figure 4.3). Interestingly, 71B6-OE lines displayed an intermediate phenotype with respect to both greening and growth. These results are therefore consistent with 71B6-OE lines being partially deficient in endogenous ABA when stressed with high concentrations of glucose.

4.2.2.4 Leaf water loss and stomatal function

Assays were carried out to determine whether changing the expression of *71B6* affected water loss from detached leaves and therefore, indirectly, whether there was an affect on stomatal function. Since water contributes a substantial amount towards the weight of a leaf, water loss can be inferred from a reduction in weight over time. Figure 4.4 shows that detached leaves of the ABA-deficient mutant, *aba3-2*, lost weight much more rapidly than wild type. There was also a slight, but consistent increase in water loss from 71B6-OE leaves, suggesting that overexpression of 71B6 marginally reduced the ability of stomata to respond to water deficit. Unexpectedly, the water loss from detached leaves

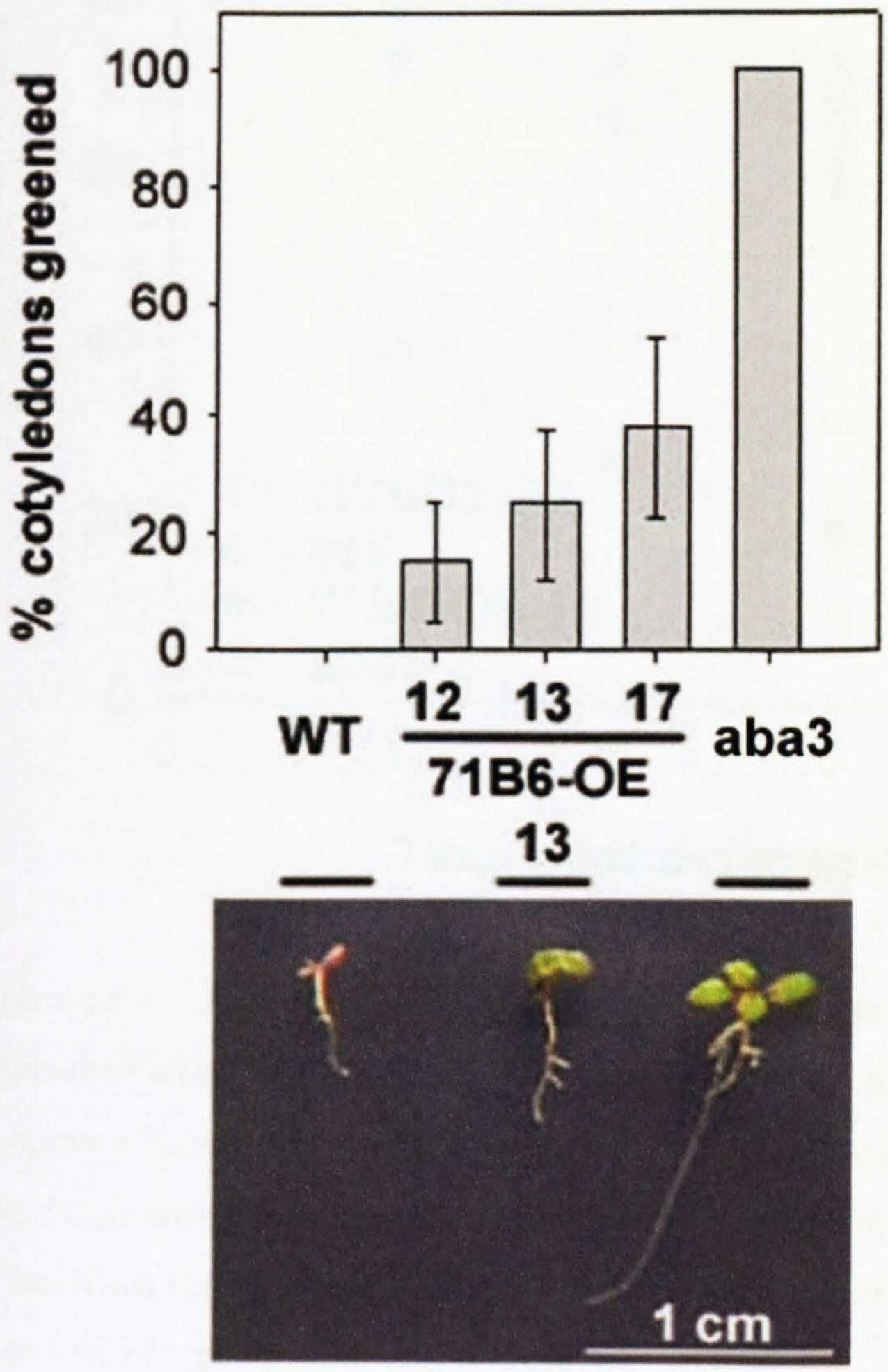


Figure 4.3 The effect of high glucose on seedling development in transgenic lines

Surface sterilised seed were sown on medium supplemented with 6% glucose. Following 4 days stratification they were transferred to warm light conditions. The glucose insensitive phenotype was scored as the percentage of seedlings showing green cotyledons after 10 days. Data are based on 3 replicates of at least 150 seeds (± SE). Wild type (WT); 71B6- OE 12, 13 and 17; ABA deficient mutant *aba3-2* (aba3). Pictures of representative seedlings are shown.

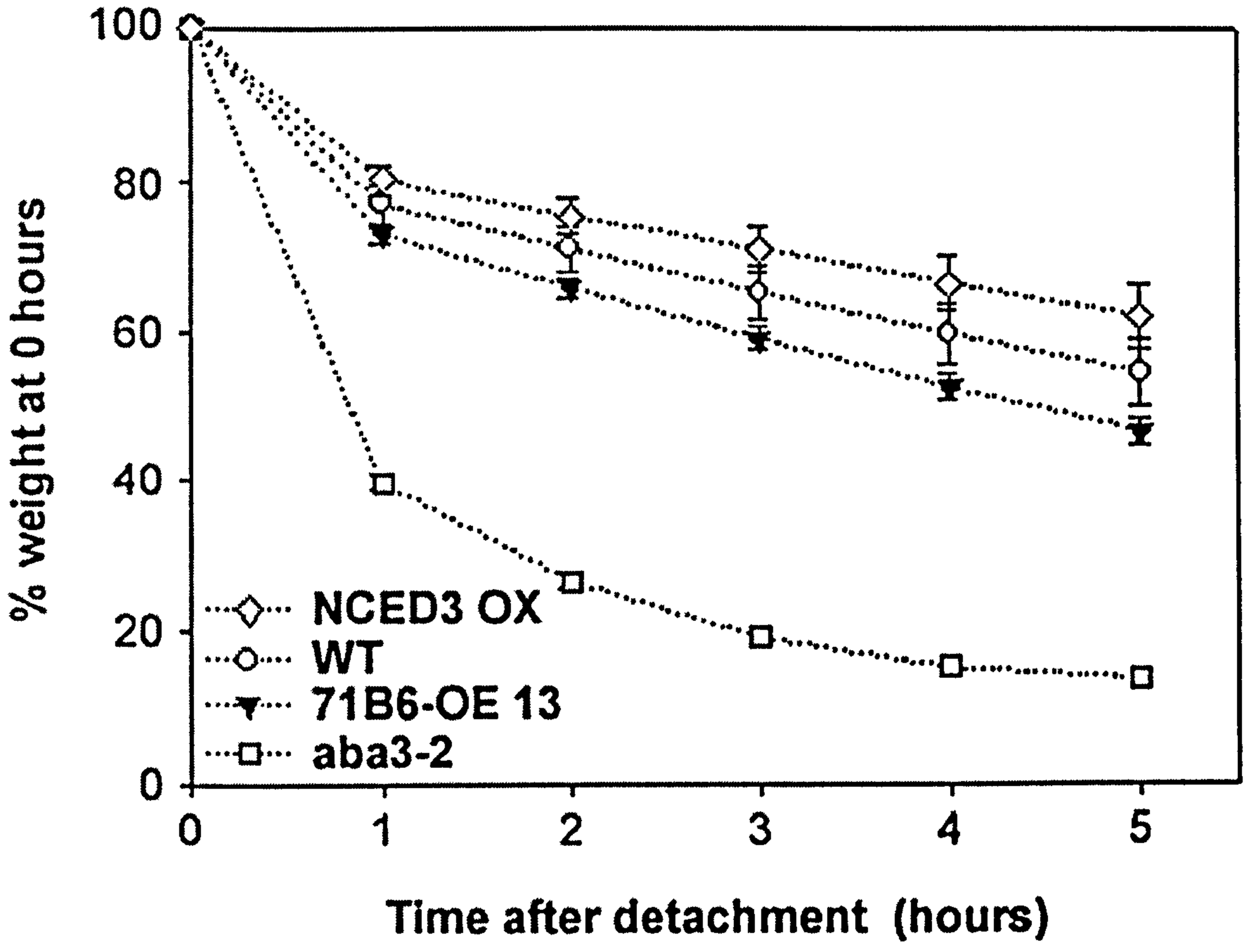


Figure 4.4 Detached leaf water loss assay on the transgenic lines

A detached leaf water loss assay was carried out on the lines described. Five individual leaves were detached from independent plants and placed upside down on a weighing boat. Fresh weight was determined immediately and weight loss measured hourly. Data are based on three independent replicates of 5 leaves (± SE). Experiment also included 71B6-OE 12 and 17 with similar results.

of the NCED3-OX transgenic line, known to accumulate higher levels of ABA in turgid rosettes (Iuchi *et al.*, 2001), was not significantly different from wild type.

4.2.3 Endogenous levels of ABA and ABA metabolites

To determine the extent to which changing the expression of *71B6* affects levels of endogenous ABA and ABA-GE, the hormone and its metabolites were measured *in planta*. A method using liquid chromatography tandem mass spectrometry with multiple reaction monitoring had been developed by the group of Suzanne R. Abrams that allows quantitation of levels of not only ABA and ABA-GE, but also the acidic catabolites PA, DPA, 7′OH-ABA and neo-PA in a single sample (Zhou *et al.*, 2003; Zhou *et al.*, 2004). Given that ABA increases in response to stress (Leon-Kloosterziel *et al.*, 1996) it was possible that the relative flux through the different catabolic pathways would change when levels of the hormone were elevated and therefore the compounds were measured in both unstressed turgid rosette tissue (Figure 4.5a) and stressed wilted tissue (Figure 4.5b).

In wild type turgid rosettes, levels of free ABA were found to be 153 pmoles/gDW with levels of ABA-GE and PA very similar. The catabolite DPA was much higher at 986 pmoles/gDW. Under wilted conditions, as anticipated, levels of free ABA rose substantially to ~4000 pmoles/gDW. Similarly, levels of the two catabolites, PA and DPA, were also high, but ABA-GE remained low. In both turgid and wilted conditions, levels of 7′OH-ABA and neo-PA metabolites were found to be negligible. The *aba3-2* mutant was used as a control for analysing the metabolite profile of an ABA deficient plant. Negligible levels of ABA and its metabolites were observed in this mutant under turgid or wilted conditions, with the exception of DPA, present at 171 pmoles/gDW in turgid and 273 pmoles/gDW in wilted leaves. The NCED3-OX transgenic line was used as a further control, for analysing the metabolite profile of a plant with elevated ABA. In turgid leaves, levels of free ABA in the transgenic line were approximately double those

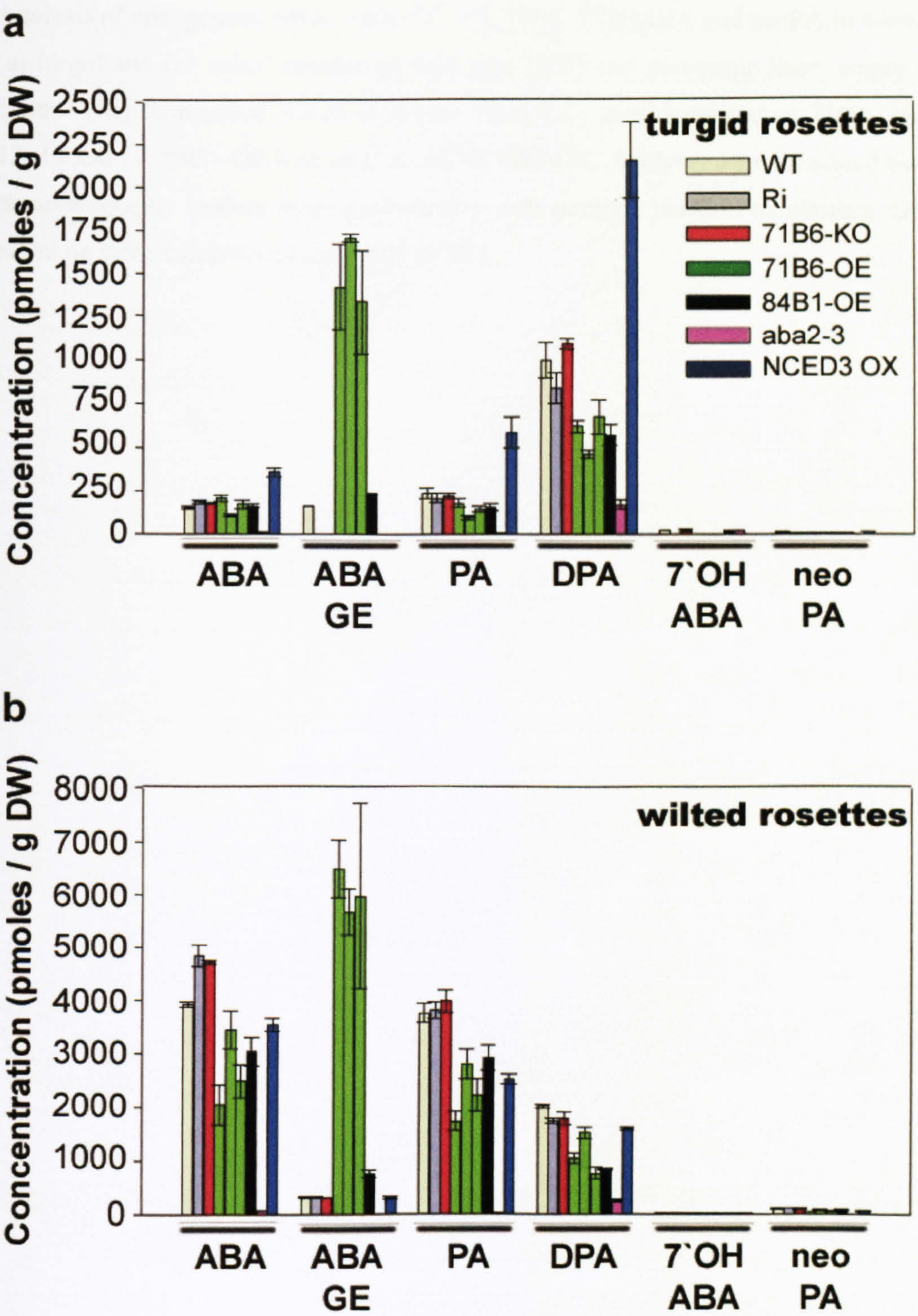


Figure 4.5 **Endogenous levels of ABA and catabolites in transgenic lines**

Figure 4.5 Endogenous levels of ABA and catabolites in transgenic lines

Analysis of endogenous ABA, ABA-GE, PA, DPA, 7′OHABA and neoPA in 4-week-old (a) turgid and (b) wilted rosettes of wild type (WT) and transgenic lines: empty vector control (Ri); transposon insertion mutant 71B6-KO; three independent 71B6-OE lines, 12, 13 and 17; 84B1-OE line; aba3-2 and NCED3-OX. Analytes were measured by liquid chromatography tandem mass spectrometry with multiple reaction monitoring. Data are based on three independent replicates (± SE).

found in wild type. Under wilt stress, levels of free ABA were approximately the same in wild type and the transgenic line. Interestingly, the catabolites PA and DPA were at much higher levels in turgid leaves of the transgenic than in wild type, but this difference was not maintained under wilt stress. In this transgenic line, levels of ABA-GE were similar to those in wild type. Metabolite profiles of transgenic plants overexpressing *71B6* or plants in which *71B6* was knocked out (71B6-KO)(see section 5.2.3) allowed a comparison to be made of the impact of changing the level of expression of the GT compared to the controls of wild type, and high and low ABA backgrounds. The most obvious difference in the profiles was the very substantial increase in levels of ABA-GE in the 71B6-OEs, whether the plants were turgid (nine-fold) or wilted (19-fold). In these transgenic lines, the wilting treatment caused the level of ABA-GE to increase by four-fold compared. Despite the accumulation of ABA-GE, levels of free ABA in turgid conditions were unaffected. When the rosettes were wilted, a significant decrease in free ABA in the 71B6-OEs compared to wild type was observed in two of the three independent transgenic lines. Interestingly, levels of the ABA catabolites PA and DPA were significantly reduced in the 71B6-OEs compared to wild type in both turgid and wilted rosettes. When the 71B6-KO was analysed, very little change was observed in any of the metabolites compared to wild type. The lack of ABA-GE observed in turgid leaves is due to a lack of sensitivity of the analytical technique. For comparison, the metabolite profiles of another transgenic line, 84B1-OE, were analysed. This GT had been found to glucosylate (±)-ABA *in vitro* (Lim 2005). Only a two-fold increase in the level of ABA-GE was observed in the 84B1-OE, and only in wilted conditions. Again, in all of these additional transgenic lines, levels of 7′OH-ABA and neo-PA were negligible.

4.3 Discussion

The homeostasis of ABA requires the coordination of a complex network of processes involving synthesis, transport and inactivation through catabolism or conjugation. Whilst many of the inactivation processes have been defined and enzyme activities described, few enzymes have been purified or their genes cloned. This study represents the first

direct evidence for a specific enzyme, namely 71B6, being able to catalyse the glucosylation of ABA *in planta*.

The activities of 71B6 described in this thesis and in Priest *et al.*(2005) suggested that overexpression of 71B6 *in planta* could lead to an increase in the glycosylation of ABA together with a concomitant decrease in the levels of free ABA. Mutants defective in the biosynthesis of ABA accumulate very low levels of the hormone and have been well characterised (Koornneef *et al.*, 1982; Karssen *et al.*, 1983; Leon-Kloosterziel *et al.*, 1996; Leon-Kloosterziel *et al.*, 1996; Seo *et al.*, 2000; Gonzalez-Guzman *et al.*, 2004; Seo *et al.*, 2004). Whilst high levels of ABA can inhibit growth, a severe reduction in ABA also results in reduced growth, reduced dormancy, poor stomatal control and insensitivity to glucose stress. It was therefore of interest to determine the profiles of ABA metabolites and to investigate the morphological and developmental phenotypes of the 71B6-OE lines.

4.3.1 Metabolite profiles of transgenic lines

Metabolite profiling highlighted the complexity of ABA homeostasis. Abscisic acid, ABA-GE and four additional ABA metabolites were measured in parallel and this provided the opportunity to analyse the impact of changing the expression of a *GT* on the levels of ABA and its catabolites. In addition, the availability of *aba3-2* and NCED3-OX plants allowed the consequences for homeostasis of changing two other metabolic steps to be explored. Given the activity of 84B1 towards ABA *in vitro* it was also of interest to compare the effects of overexpressing this *GT* compared to *71B6*. Interestingly, in wild type (*Colombia*) the levels of ABA, PA and DPA, but not ABA-GE increased substantially during the wilting treatment. This is consistent with a previous study in which water-stressed *Landsberg erecta* showed high rates of biosynthesis of ABA and PA, but not ABA-GE (Rock and Zeevaart, 1991).

4.3.1.1 71B6-OE lines

The profiles showed that 71B6-OE lines accumulated up to 19-fold more ABA-GE than wild type, demonstrating that the catalysis observed *in vitro* (Lim *et al.*, 2005; Priest *et al.*, 2005) reflects the capability of the enzyme *in planta*. The extraordinarily high levels of ABA-GE in the 71B6-OE lines suggest that overexpression of this GT has led to a substantial increase in synthesis of ABA compared to wild type, thereby providing the substrate for glucosylation by the recombinant enzyme. The high levels of ABA-GE were observed in turgid rosettes, but were even higher in wilted rosettes. Free ABA was maintained at wild type levels in turgid rosettes of 71B6-OE lines and only moderately reduced in wilted rosettes.

Interestingly, levels of PA and DPA were reduced in 71B6-OE lines compared to wild type under both turgid and wilted conditions. Three possibilities could be suggested for this decrease. First, PA and DPA may have been glucosylated by 71B6 to form PA-glucose ester and DPA-glucose ester. This is unlikely since PA and DPA were not substrates for the recombinant enzyme *in vitro* (Priest *et al.*, 2005). Second that, as a consequence of the 8′-hydroxylase enzyme(s) and 71B6 acting on the same substrate, there would be less ABA available for conversion to PA and DPA: the 71B6-OE line which accumulated most ABA-GE also accumulated least ABA, PA and DPA in both turgid and wilted tissues. This explanation is possible for the wilted rosettes, since the level of ABA was reduced in the 71B6-OE lines compared to wild type. However, this is unlikely to be the sole reason since, in the turgid rosettes, the level of ABA was not affected by overexpression of 71B6. Third, the plant may have down regulated the 8′-hydroxylase pathway to maintain levels of ABA in response to the forced removal of free ABA from the system by the action of the recombinant enzyme.

Given the metabolite profiles, it would appear that flux through the 8′-hydroxylation pathway was reduced in the 71B6-OE lines. In turgid rosettes, the reduction was not sufficient to account for all the ABA that was conjugated. The rate of ABA biosynthesis must have therefore increased in the turgid rosettes of 71B6-OE lines compared to wild

type. In contrast, in wilted tissue, whilst free ABA was slightly reduced in two 71B6-OE lines compared to wild type, the sum total of ABA, ABA-GE, PA, DPA, 7′-hydroxyABA and neo-PA (total ABA) in the 71B6-OE lines was similar to that in the control lines. It therefore appears that the rate of ABA biosynthesis was near maximum in wilted rosettes and only the reduced flux through the 8′-hydroxylation pathway in the 71B6-OE lines maintained ABA near to wild type levels. It would be interesting to investigate whether overexpression of genes in both of these two deactivation pathways was sufficient to substantially reduce free ABA *in planta*.

4.3.1.2 aba3-2 mutant

As expected from previous reports (Leon-Kloosterziel *et al.*, 1996), the level of ABA in *aba3-2* was substantially reduced (9 pmol/gDW compared with 153 pmol/gDW in wild type). Interestingly however, the data indicated that the mutant accumulated DPA at 171 pmol/gDW. Since DPA is a catabolite of ABA, the mutant must have synthesised more ABA than that present in four-week-old rosettes and converted the hormone to DPA despite an ABA deficit. This therefore suggests that the plant was unable to completely inhibit the action of the 8′-hydroxylase enzyme(s). Alternatively, one could speculate that 8′OH-ABA, PA or DPA are themselves important and play some role in the plant, as has been suggested previously (Zhou *et al.*, 2004).

4.3.1.3 NCED3-OX line

NCED3-OX is a transgenic line in which a gene involved in ABA biosynthesis, *NCED3*, is overexpressed (Iuchi *et al.*, 2001). These authors demonstrated that NCED3-OX lines accumulated twice as much ABA as wild type in turgid rosettes. However, no other metabolites of ABA were measured in that publication. The data described here confirmed the overaccumulation of ABA, and extended the analysis to other metabolites. Interestingly extremely high levels of PA and DPA were observed in the NCED3-OX line, equivalent to an additional 1500 pmol/g DW of total ABA. Therefore, the results

suggest that overexpression of the biosynthetic gene increased flux through the 8′-hydroxylation catabolic pathway of ABA. Since wild type turgid rosettes accumulated 153 pmol/g DW of free ABA, it can be calculated that if the NCED3-OX plants had not increased catabolism, a 10-fold excess of ABA rather than a 2-fold excess would have arisen.

It is interesting to compare this study with that of Qin and Zeevaart (2002), in which another ABA biosynthetic gene, *NCED-1*, from *Phaseolus vulgaris* was overexpressed in *Nicotiana plumbaginifolia* plants. The authors similarly observed that PA increased more than ABA in the transgenic lines. However, in contrast to the data shown here, DPA was not a major metabolite. The 8′-hydroxylation pathway is therefore used in both Arabidopsis and *Nicotiana plumbaginifolia* to respond to and remove forced increases in ABA. Curiously, despite the increased flux through ABA metabolism in the NCED3-OX plants, there was no increase in accumulation of ABA-GE. The Qin and Zeevaart study did not measure ABA-GE.

Whilst overexpression of *NCED3* led to higher levels of free ABA in turgid leaves, surprisingly, the line did not accumulate more free ABA or total ABA than wild type under wilted conditions. This is consistent with the data from the detached leaf water loss assay in which leaves from NCED3-OX and wild type lost water at similar rates (see section 4.2.2.4). Studies on *NCED3* have shown that expression of the gene is massively upregulated during stress, including that imposed by wilting (Iuchi *et al.*, 2000; Tan *et al.*, 2003). It has been suggested that the biosynthetic step catalysed by NCED3 is thought to be rate limiting and upregulation of the gene is required for increased synthesis of ABA (Nambara and Marion-Poll, 2005). Therefore, under wilted conditions it is probable that in wild type expression of *NCED3* is already fully upregulated and is no longer rate limiting, thereby providing the substantial increases observed in ABA. Catabolism of ABA in wilted rosettes of wild type and the NCED3-OX is near identical, suggesting that ABA homeostasis under wilting conditions is controlled in a similar way in the two genotypes.

The data also suggest that the control of ABA biosynthesis is not only regulated by the level of expression of *NCED3*. Constitutive overexpression of *NCED3* led to an accumulation of ABA, but under wilted conditions far greater levels of ABA were synthesised in all of the transgenic lines. Interestingly, the NCED3-OX line has been shown to be more resistant to drought than wild type (Iuchi *et al.*, 2001), yet the data described in this thesis show that under the wilted conditions applied, there were very similar levels of ABA in the wild type and NCED3-OX. One could speculate that this may be due to enhanced stomatal control and water conservation during the early, low-stress, stages of drought.

4.3.1.4 UGT84B1

It is interesting to compare the accumulation of ABA-GE in plants overexpressing *71B6* with those overexpressing *84B1*. Lim *et al.*(2005) identified eight Arabidopsis UGTs that were capable of glucosylating ABA *in vitro*. The recombinant 84B1 enzyme, which had previously been shown to be able to glucosylate IAA both *in vitro* and *in planta* (Jackson *et al.*, 2001; Jackson *et al.*, 2002), was found to have the highest activity towards ABA *in vitro* (Lim *et al.*, 2005). However, the activity of 84B1 towards ABA was less than towards IAA. Whilst the recombinant 84B1 enzyme had a greater specific activity towards ABA than 71B6 *in vitro*, the 84B1-OE plants only accumulated a maximum of two-fold more ABA-GE compared to 19-fold in the 71B6-OE plants. The affinity of 84B1 for ABA is not presently known, but may explain the metabolite profiles observed.

4.3.1.5 Conversion of PA to DPA

From the metabolite profile of plants under turgid conditions, rosette leaves were always observed to have higher levels of DPA than PA. Following six hours of wilt stress, PA accumulated to higher levels than DPA. The 8′-hydroxylation and rearrangement to PA therefore appears to be catalysed more rapidly than the subsequent reduction to DPA under these conditions.

This study therefore shows that plants display remarkable plasticity in their ability to maintain levels of the key phytohormone ABA. This plasticity sheds light on the reasons why no ABA catabolic mutants have been uncovered during genetic screens. The study also contributes to mounting evidence which shows that measurement of a single compound does not give a true picture of the consequences of changing a particular metabolic enzyme. I therefore suggest that in order to understand ABA metabolism, the use of more holistic approaches should become more mainstream.

4.3.2 Morphological and developmental phenotypes

The potential impact of overexpressing *71B6* on a wide range of morphological and developmental characteristics was analysed. Under the conditions used and the parameters analysed, there were few major changes. Typical ABA related phenotypes, such as difference in rates of germination and wiltiness were not observed, suggesting that the impact of *71B6* overexpression did not reduce ABA levels significantly in the plant. This was confirmed by the metabolite profiles.

However, changes were observed when the hormone was supplied exogenously, or when accumulation of endogenous ABA was high due to imposed stress. Thus, 71B6-OE seedlings were more resistant to inhibition of early growth by exogenous ABA than wild type seedlings. This was consistent with the hypothesis that the 71B6-OEs had an enhanced ability to deactivate the hormone by glucosylation. The hypothesis was confirmed through the use of two bioactive analogues of ABA, only one of which could be glucosylated by the GT.

Under conditions of stress imposed on seedlings by high glucose, a second phenotypic change related to ABA was revealed. In assays for glucose sensitivity, 71B6-OE

seedlings displayed intermediate phenotypes between wild type and the ABA deficient mutant, *aba3-2*.

The third phenotypic change related to ABA was also observed during imposition of stress, but in relation to water loss from detached leaves. Again, the 71B6-OE lines displayed intermediate phenotypes between wild type and the ABA deficient mutant, *aba3-2*.

All of these phenotypes are suggestive that overexpression of *71B6* can reduce the free ABA in the plant but the level of reduction is not as great as observed in ABA deficient mutants that have been previously characterised (Koornneef *et al.*, 1982; Leon-Kloosterziel *et al.*, 1996; Seo *et al.*, 2000; Seo *et al.*, 2004). All of those mutations were in genes involved in biosynthesis and it will be of interest to determine the consequences of changing the level of expression of genes involved in other catabolic mechanisms of ABA in addition to glucosylation.

4.3.3 ABA-GE is an inactive ABA metabolite

The data presented here contribute to the argument that ABA-GE is inactive and is not cleaved to release free ABA (Milborrow, 1978; Zeevaart, 1980; Neill *et al.*, 1983; Zeevaart, 1983; Lehmann and Schutte, 1984; Zeevaart and Boyer, 1984; Zeevaart, 1999). First, plants overexpressing 71B6 were more resistant to inhibition by the hormone during post-germinative growth. From *in vitro* studies, it is known that 71B6 could glucosylate ABA but not the structural analogue, PBI-514, and had higher activity towards PBI-413 (Priest *et al.*, 2005). This difference in specificity enabled us to confirm that the phenotype was a direct consequence of the glucosylation of ABA by 71B6. Therefore, the resistance of 71B6-OE seedlings to ABA requires ABA-GE to be inactive. Second, all of the phenotypic aberrations from wild type observed in the 71B6-OE lines are consistent with ABA deficiency, which would only be observed if ABA-GE was inactive. Third, unstressed 71B6-OE rosettes accumulated nine-fold more ABA-GE than

wild type and were unaffected in their ability to grow and develop under normal conditions. The high accumulation of ABA-GE did not appear to affect the 71B6-OE plants under the conditions analysed, suggesting that ABA-GE is inactive. Fourth, during stress by wilting, levels of ABA-GE in the 71B6-OE lines did not decrease: they increased a further four fold. This suggests that endogenous ABA-GE is not readily cleaved to release free active ABA. Lack of cleavage has been previously shown for ABA-GE in *X. strumarium* (Zeevaart, 1980, 1983). In summary, the data in this chapter is consistent with the proposal that ABA-GE is an inactive ABA metabolite (Zeevaart, 1999).

CHAPTER 5: ENDOGENOUS *71B6* GENE

5.1 Introduction

5.2 Results

5.3 Discussion

5 ENDOGENOUS *71B6* GENE

5.1 Introduction

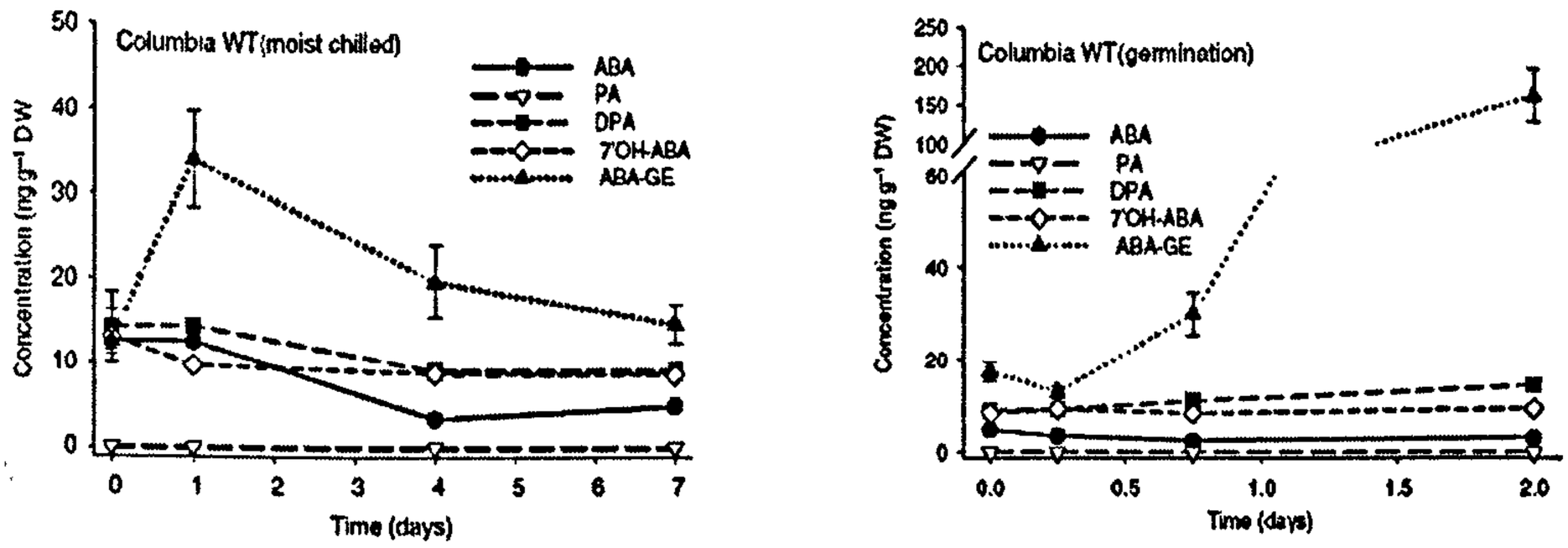
This thesis investigates the ability of 71B6 to glucosylate ABA *in vitro* and *in planta*. Chapter 3 showed that recombinant 71B6, expressed as a recombinant protein in *E. coli*, is capable of glucosylating ABA *in vitro*. Chapter 4 showed that 71B6 is capable of glucosylating ABA *in planta* when the gene was overexpressed with a constitutive promoter. However, whilst 71B6 is able to glucosylate ABA, it does not necessarily mean that the function of the endogenous gene is to encode an ABA glucosyltransferase. The aim of chapter 5 is to investigate whether the endogenous 71B6 is involved in the glucosylation of endogenous ABA in Arabidopsis.

To assess whether *71B6* is involved in the glucosylation of ABA *in planta*, it is important to analyse mutant plants without a functional *71B6* gene; namely a line in which *71B6* is knocked out. If a 71B6-Knock Out line (71B6-KO) accumulated less ABA-GE than wild type, it would strongly suggest that 71B6 is involved in the glucosylation of ABA. To prove the involvement of 71B6 it would then be necessary to show that accumulation of ABA-GE in 71B6-KO could be rescued by transformation with a genomic fragment containing the *71B6* gene under the control of its own promoter and terminator regions. In the case of redundancy, an alternative strategy could involve the use of RNAi to silence *71B6* and other genes which may also be involved in the glucosylation of ABA.

In chapter 4, no difference in the accumulation of ABA-GE was observed between 71B6-KO and wild type. However, the conjugate was only measured in four-week-old rosette tissue that was wilted. It is possible that the gene was not expressed in this tissue under these conditions. The endogenous enzyme will only function in tissues or cell types in which the corresponding gene is actively expressed. It is therefore important to investigate the expression patterns of *71B6*. In a developmental context, a tissue in which

both *71B6* is actively expressed and accumulation of ABA-GE is high would be a good starting point to investigate whether endogenous 71B6 plays a role in the glucosylation of ABA. One study by Chiwocha *et al.* (2005) showed that, in Arabidopsis, ABA-GE accumulated in seeds after one day of stratification and in two-day-old seedlings (Figure 5.1). In both cases, ABA, PA, DPA and 7′OH-ABA were also measured, and ABA-GE was the major metabolite of the five. The two-day old seedlings were particularly striking since levels of the conjugate had increased by more than five-fold over 32 hours (Figure 5.1b). In addition to development, ABA is known to increase massively on stress. However, as shown in chapter 4, ABA-GE did not accumulate at high levels in wilted rosettes of wild type plants. Given the extensive microarray data available — it is possible to search the data for conditions in which the expression of *71B6* is upregulated and then perform analyses in these conditions.

Therefore, chapter 5 approaches the question of whether the endogenous 71B6 is involved in the glucosylation of ABA using two strategies. First, to investigate when *71B6* is expressed, and to measure the accumulation of ABA-GE at those states in both wild type and 71B6-KO plants. Second, to investigate when ABA-GE accumulates in wild type and then measure accumulation of ABA-GE in 71B6-KO at those stages.



Chiwocha *et al.* (2005) *Plant J.* **42**, 35-38

Figure 5.1 Accumulation of ABA and ABA catabolites in seeds and seedlings during stratification and germination

5.2 Results

5.2.1 Developmental regulation of *71B6* gene expression

It was of interest to determine whether the endogenous *71B6* gene is preferentially expressed in particular organs or at particular developmental stages. RNA was therefore extracted from wild type plants across a range of conditions, namely: four-day-old seedlings; eleven-day-old seedlings; eleven-day-old aerial tissue; eleven-day-old roots; four-week-old rosettes; stem; flower; and green siliques. Since no signal was detectable by Northern blot at any of these stages (data not shown), levels of *71B6* transcript were assessed using the more sensitive technique of Quantitative Real-Time PCR (Q-PCR) and compared to levels of *actin* transcript. Figure 5.2a shows that whilst expression was low in all of the stages measured ($\leq$ 3% actin), the level of transcript varied between tissue. Transcript accumulation was greatest in the aerial tissue of eleven-day-old seedlings, and least in the roots of eleven-day-old seedlings. This suggests that *71B6* may function in photosynthetically active tissues.

Chiwocha *et al.* (2005) showed that ABA-GE accumulated in two-day-old seedlings of Arabidopsis (Figure 5.1). To investigate whether *71B6* expression was high around this developmental stage, levels of transcript were assessed in two-day, three-day and five-day-old seedlings. Figure 5.2b shows that there was least transcript at two days with increasing levels at three days and five days. Emergence of green cotyledons occurred between the second and third days. Therefore, figures 5.2a and 5.2b show that accumulation of *71B6* transcript was highest in young aerial tissue of seedlings, and lowest in stages which were not photosynthetically competent. Interestingly, two-day-old seedlings grown by Chiwocha *et al.* (2005), which accumulated ABA-GE, had already emerged cotyledons (Chiwocha SDS, personal communication). Therefore, these data are consistent with the possibility that 71B6 may be responsible for the accumulation of ABA-GE in aerial tissue during early post-germinative growth.

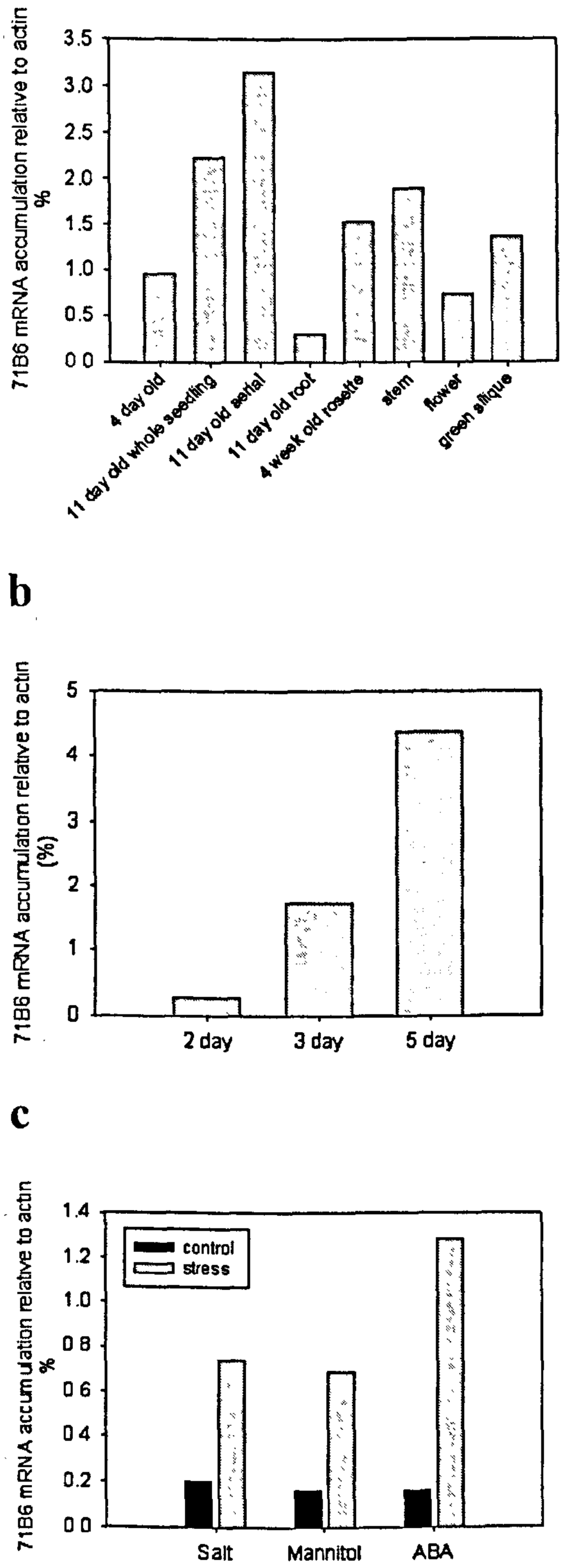


Figure 5.2 Analysis of *71B6* transcript levels by Quantitative Real-Time PCR

Transcript levels were measured by Q-PCR in wild type plants at different developmental stages (**a** and **b**) and in wild type seedlings in response to stress (**c**). For **c**, ten-day-old seedlings were transferred to flasks containing liquid ATS media supplemented with 300 mM NaCl, 600 mM mannitol or 100 μM ABA or without addition and incubated for 6 h.

5.2.2 Expression in response to stress

5.2.2.1 Q-PCR

ABA is known to accumulate in plants in response to stress (reviewed in Walton and Li, 1995). The rate of catabolism of ABA also increases during stress and in response to exogenous ABA (Uknes and Ho, 1984; Cutler *et al.*, 1997; Wang *et al.*, 2002). To discover whether *71B6* expression is induced by stress, transcript levels were measured in seedlings that had been challenged with NaCl, mannitol, or ABA in liquid media for six hours. Control seedlings were treated in liquid media without addition. Q-PCR was used as a sensitive method to measure the accumulation of transcript. Figure 5.2c shows that accumulation of *71B6* transcript increased in each treatment compared to the control, with maximal induction by ABA. It is noteworthy that accumulation of transcript for *71B6* was also very low compared with *actin*, even in conditions of induction. In summary, these data show that expression of *71B6* is induced by abiotic stress and by exogenous ABA.

5.2.2.2 Dot blots

Members of the Bowles group were interested to learn which conditions would induce expression of the 107 Arabidopsis GTs containing the glycosyltransferase signature motif. To that end as a rapid, but less sensitive method, Luisa Elias extracted RNA from wild type seedlings that had been challenged with one of a range of conditions. RNA was radioactively labelled in a reverse transcription reaction and the labelled cDNA was used to probe dot blots containing DNA from each of the 107 GT genes. The dot blots were compared with other dot blots hybridised to cDNA from control seedlings that had not been challenged. In total, thirteen treatments were carried out, namely wounding, cold,

salicylic acid, ABA, indole-3-acetic acid, jasmonic acid, 1-amino-cyclopropane 1-carboxylic acid (ACC), gibberellic acid (GA_3), *trans*-zeatin, hydrogen peroxide, mannitol, salt and cycloheximide. From the thirteen treatments, only cycloheximide (CHX) resulted in substantially increased accumulation of *71B6* transcript: in all other treatments the level was below a threshold considered to be meaningful. Interestingly, the induction of *71B6* by CHX gave one of the highest signals of any of the 107 GTs by any of the treatments, at a level greater than actin. Therefore, the expression of *71B6* in response to all conditions tested by dot blot was negligible with the exception of CHX.

5.2.2.3 Publicly available data

To further investigate the expression of the endogenous *71B6* gene, publicly available information from affymetrix chip experiments and expressed sequence tag (EST) databanks was checked. The bioinformatics specialist in the Bowles group, Elizabeth Wilson, did not find any *71B6* ESTs and did not find *71B6* expression to be induced in any of the Nottingham Arabidopsis Stock Centre (NASC) affymetrix microarray experiments. There is also microarray information available from 63 stress experiments on the Genevestigator website (www.genevestigator.ethz.ch) (summarised in Table 5.1). From these studies, ten conditions were shown to result in increased accumulation of *71B6* transcript and one condition resulted in decreased accumulation. Treatment with high CO_2 resulted in reduced transcript level. *71B6* mRNA was increased by treatment with ozone, silver nitrate, CHX, syringolin, nutrient nitrogen, the biotic pathogens *Botrytis cinerea* and *Phytopthora infestans*, and osmotic, salt and UV stresses. However, the confidence in the data from ozone, nitrogen, osmotic and salt stresses was limited, since the mean level of expression was less than 200 units. Interestingly, the induction of expression by CHX was again substantially greater than the induction of expression by any of the other stresses. The induction of expression by salt was interesting because ABA is known to be involved in salt tolerance. Further, the experimental detail showed progressively increasing levels of expression from 6 to 24 hours following application of

Table 5.1 Analysis of induction of *71B6* expression by stress using affymetrix microarray experiments from Genevestigator. https://www.genevestigator.ethz.ch/

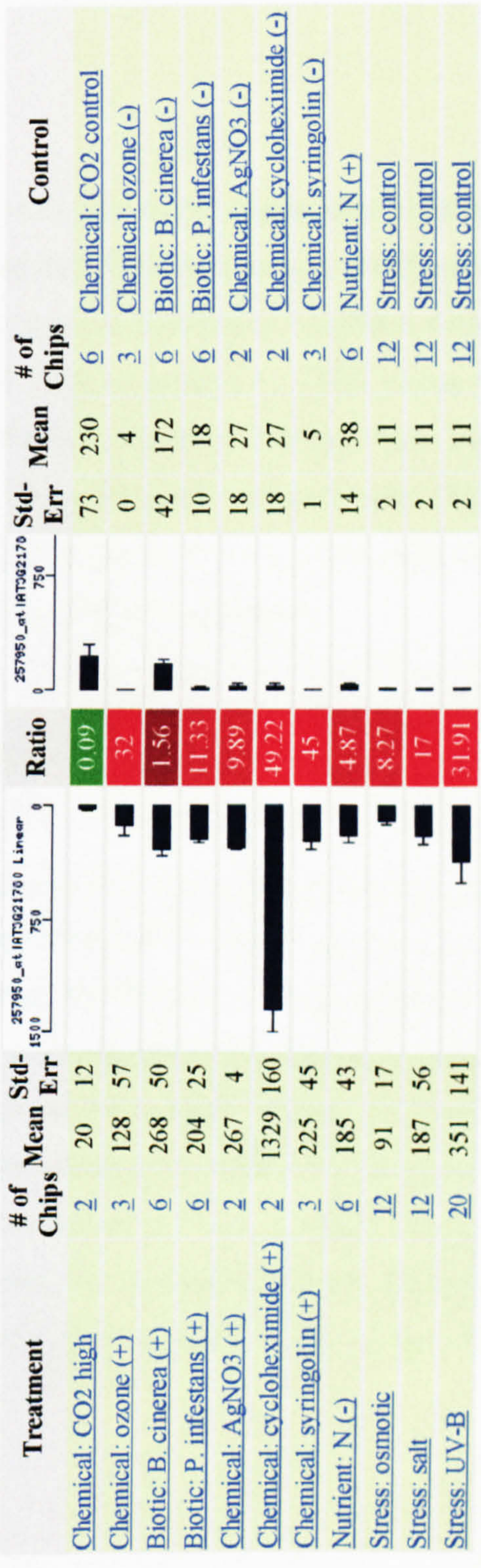
(**a**) A list of stresses to which wild type plants were subjected before microarray analysis.

(**b**) Those stresses which resulted in a significant change in detection of *71B6* transcript.

a

Treatment	
Biotic: A. tumefaciens	Chemical: uniconazole
Biotic: B. cinerea	Chemical: zearalenone
Biotic: E. cichoracearum	Hormone: ABA
Biotic: E. orontii	Hormone: ACC
Biotic: M. persicae	Hormone: BL
Biotic: mycorrhiza	Hormone: ethylene
Biotic: nematode	Hormone: IAA
Biotic: P. infestans	Hormone: MJ
Biotic: P. syringae	Hormone: zeatin
Chemical: 2,4,6 T	Hormone: GA3
Chemical: AgNO3	Light intensity: light
Chemical: AVG	Light quality: blue
Chemical: brz220	Light quality: far red
Chemical: brz91	Light quality: red
Chemical: CO2 high	Light quality: UV-A
Chemical: cycloheximide	Light quality: UV-AB
Chemical: daminozide	Light quality: white
Chemical: ibuprofen	Nutrient: Cs
Chemical: isoxaben	Nutrient: glucose/sucrose
Chemical: MG13	Nutrient: K (-)
Chemical: norflurazon	Nutrient: N (-)
Chemical: NPA	Nutrient: S (-)
Chemical: ozone	PCD: senescence
Chemical: paclobutrazole	Stress: cold
Chemical: PCIB	Stress: drought
Chemical: PNO8	Stress: genotoxic
Chemical: prohexadione	Stress: heat
Chemical: propiconazole	Stress: osmotic
Chemical: salicylic acid	Stress: oxidative
Chemical: syringolin	Stress: salt
Chemical: TIBA	Stress: UV-B
	Stress: wounding

b



Treatment	Mean	Std-Err	# of Chips	Ratio
Control	230	73	6	
Chemical: CO2 control	4	0	3	0.09
Chemical: ozone (-)	172	42	6	32
Biotic: B. cinerea (-)	18	10	6	1.56
Biotic: P. infestans (-)	27	18	2	11.33
Chemical: AgNO3 (-)	27	18	2	9.89
Chemical: cycloheximide (-)	5	1	3	49.22
Chemical: syringolin (-)	38	14	6	45
Nutrient: N (+)	11	2	12	4.87
Stress: control	11	2	12	8.27
Stress: control	11	2	12	17
Stress: control	20	12	2	3191
Chemical: CO2 high	128	57	3	
Chemical: ozone (+)	268	50	6	
Biotic: B. cinerea (+)	204	25	6	
Biotic: P. infestans (+)	267	4	2	
Chemical: AgNO3 (+)	1329	160	2	
Chemical: cycloheximide (+)	225	45	3	
Chemical: syringolin (+)	185	43	6	
Nutrient: N (-)	91	17	12	
Stress: osmotic	187	56	12	
Stress: salt	351	141	20	
Stress: UV-B				

stress to the roots of adult plants. It is curious that not all affymetrix experiments, nor the Bowles group dot blot, revealed that *71B6* was induced by salt stress. This may be due to the different experimental methods used to apply the stress. In summary, some of the publicly available data suggested that expression of *71B6* was induced by salt stress and by challenge with CHX.

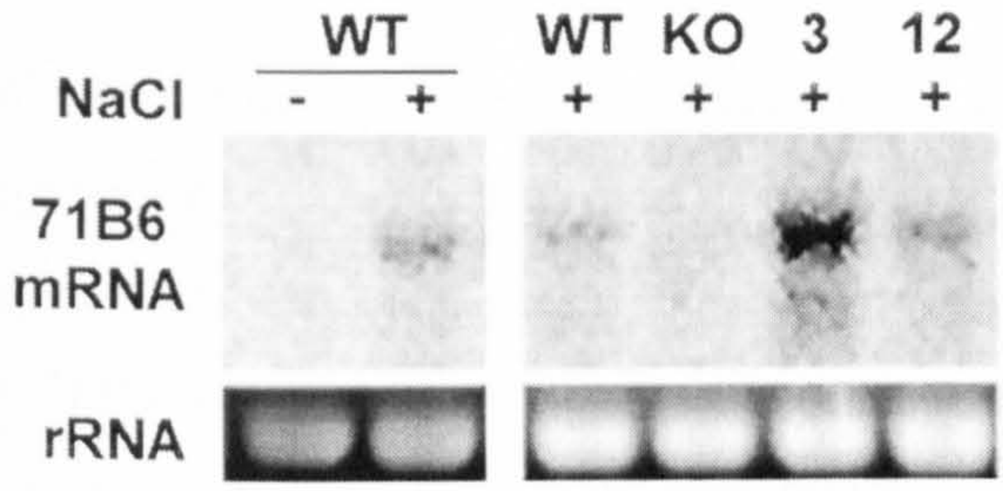
5.2.2.4 Confirmation of affymetrix data

To confirm that the expression of *71B6* is induced by salt stress, plants were challenged from the roots with 150 mM NaCl for 24 hours and their RNA analysed by Northern blot. Figure 5.3a shows that *71B6* mRNA accumulation was increased upon treatment with salt in wild type plants. To confirm that expression of *71B6* is induced by CHX, plants were treated with 40 μM CHX for six hours and their RNA analysed by Northern blot. Figure 5.3b shows that *71B6* mRNA accumulation was increased upon treatment with CHX in wild type plants. Therefore, Northern blots have been used to confirm microarray experiments showing that *71B6* was induced by both CHX and salt stress.

5.2.3 KO and Complemented KO mRNA

To discover whether the endogenous *71B6* gene is involved in the glucosylation of ABA *in planta*, it is important to analyse the accumulation of ABA-GE and phenotypic aberrations in the 71B6-KO plants. Luisa Elias, from the Bowles group, obtained a line from the JIC SLAT collection with a transposon insertion in the open reading frame of *71B6* and confirmed that it was homozygous at this locus (Elias L. personal communication). I then further developed this work during my PhD. This included the complementation of 71B6-KO lines by transformation with a genomic fragment containing the *71B6* gene including its own promoter and terminator regions (71B6-RE3 and 71B6-RE12). Therefore, the tools to investigate whether 71B6 is an ABA glucosyltransferase were available for analysis.

a



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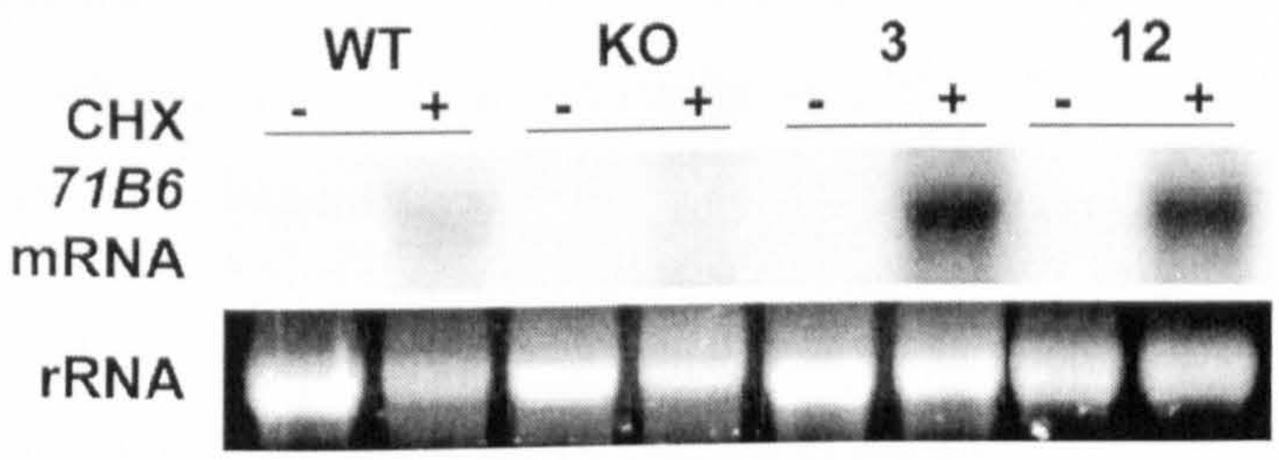

Figure 5.3 Northern blot analysis of the induction of *71B6* transcript by NaCl and cycloheximide

(**a**) Hydroponically grown plants were stressed by submersing the roots in 150 mM NaCl for 24 h. Plants were harvested and *71B6* transcript levels determined by Northen blot with a probe specific to the 3′ end of the open reading frame. (**b**) Ten-day-old seedlings were challenged with 40 μM cycloheximide in liquid media for 6 h. Plants were harvested and *71B6* transcript levels determined by Northern blot with a probe for the complete open reading frame. Wild type (WT), 71B6-KO (KO), 71B6-RE (3 and 12).

5.2.3.1 Analysis of expression of 71B6 in Knock Out lines

To determine whether the transposon insertion in 71B6-KO resulted in reduced expression of *71B6* and whether 71B6-RE3 and 71B6-RE12 had rescued levels of expression, transcript accumulation was investigated by Northern blot. Since expression of *71B6* was low in wild type under standard growth conditions (Figure 4.1), it was necessary to stress the plants with NaCl or CHX before extraction of RNA. As expected, *71B6* mRNA did not accumulate in the 71B6-KO line in response to either treatment (Figure 5.3). The 71B6-RE3 and 71B6-RE12 lines accumulated transcript at levels equivalent to or greater than wild type. Therefore, 71B6-KO plants were confirmed to be 'knocked out' for *71B6* transcript and 71B6-RE3 and 71B6-RE12 lines were confirmed to be complemented.

5.2.3.2 Initial phenotypic characterisation of 71B6-KO

The morphological and developmental characteristics of 71B6-KO and 71B6-RE lines were indistinguishable from wild type under standard growth conditions. The 71B6-KO line also responded as wild type in all of the ABA related experiments carried out on the 71B6-OE lines in chapter 4. There were therefore no obvious phenotypic consequences related to ABA from knocking out expression of the *71B6* gene. It was possible however, that the 71B6-KO plants were compromised in their ability to accumulate ABA-GE. In the analysis of wilted and turgid rosette tissue, accumulation of ABA-GE was low and close to the limit of quantitation (Figure 4.5). There was no quantifiable difference in the accumulation of ABA-GE between the wild type, Ri and 71B6-KO lines. It was therefore important to identify conditions under which accumulation of ABA-GE was high in wild type, so that a difference might be observed.

5.2.4 Analysis of ABA-GE in wild type at different stages

Chiwocha *et al.* (2005) measured the accumulation of ABA-GE in Arabidopsis seedlings during stratification, and over the first two days of germination. The authors showed that ABA-GE accumulated at high levels in Arabidopsis seedlings at two days (Figure 5.1b). This high level of ~150 ng/gDW was equivalent to ~350 pmol/gDW. There was also a smaller peak in accumulation of ABA-GE in seeds after one day of stratification (Figure 5.1a). It was important to check the repeatability of these results and also investigate whether ABA-GE was only high at this time point, or whether there were other stages or tissues in which the conjugate also accumulated. To that end, tissue was harvested from wild type over a time course up to four weeks and also from stem, flowers and siliques of adult plants. The time points analysed were: dry seeds; seeds stratified for one day (1s) and four day days (4s); germinating seedlings after one, two, three, five and seven days in the growth room (1g, 2g, 3g, 5g, 7g); the roots of 14 day-old seedlings (14 root); the aerial tissue of 14 day-old seedlings (14 aerial); and four-week-old rosette tissue. Since the experiment by Chiwocha *et al.* (2005) was carried out on water soaked filter papers, seedlings for the time points up to five days were also grown in this way. It was considered that seedlings would not grow well to fourteen days on water soaked filter paper. In consequence, seedlings for five days, seven days and fourteen days were grown on MS agar. Two time points were included at five days, one on filter paper (5g p) and one on MS (5g M), to provide a control. The adult plant samples of four-week rosettes, stem, flowers and siliques were grown in soil. Chiwocha *et al.* (2005) grew Arabidopsis seedlings at slightly lower light levels and higher temperature than typically used by the Bowles group. To be consistent with other experiments carried out in the Bowles laboratory, including the phenotypic characterisation of the transgenic lines, our standard conditions were used for all of these time points. In addition, one further ABA-GE extraction was carried out on tissue extracted from two-day-old seedlings grown at the conditions of Chiwocha *et al.* Figure 5.4 shows the accumulation of ABA-GE in wild type at the different stages. The other ABA catabolites PA, DPA, 7′OH-ABA and neo-PA were also extracted and their levels determined (Table 5.2).

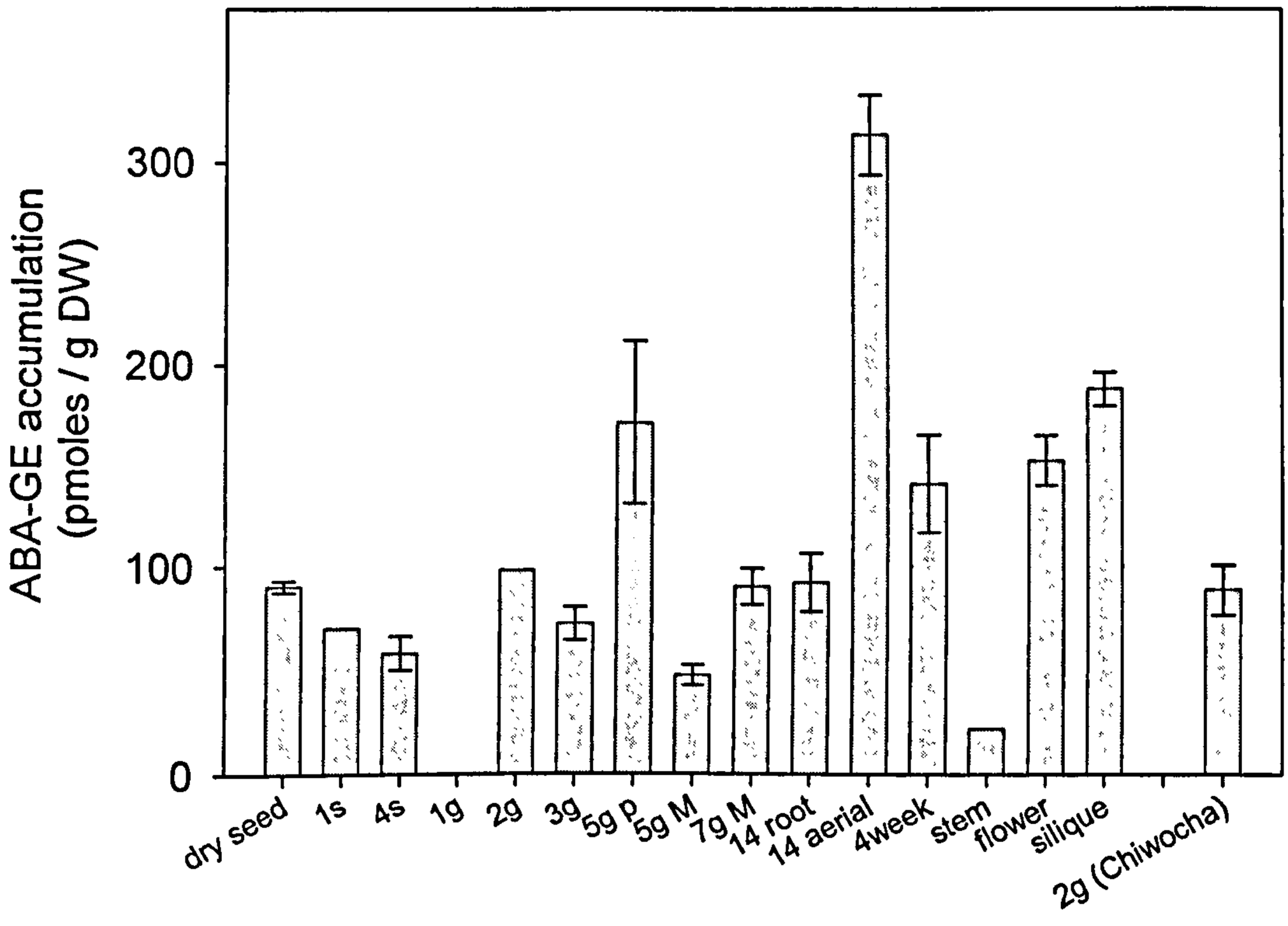

Figure 5.4 Accumulation of ABA-GE at different developmental stages in wild type plants

Levels of ABA-GE were measured in wild type plants by liquid chromatography tandem mass spectrometry with multiple reaction monitoring. For 2 day (Chiwocha), seedlings were grown for two days under the conditions of Chiwocha *et al.* (2005). Values represent the mean of three independent samples ± standard error. Where no value is shown, no measurements were within the limit of quantitation. Where no error is shown, only one or two measurements were within the limit of detection. Seeds stratified for one day (1s) and four days (4s); germinating seedlings after one, two, three, five and seven days (1g, 2g, 3g, 5g, 7g); the roots of 14 day-old seedlings (14 root); the aerial tissue of 14 day-old seedlings (14 aerial); four-week-old rosette tissue (4 week); and 2 day old seedlings grown under the conditions of Chiwocha et al. (2005) (2g Chiwocha). 1s, 4s, 1g, 2g, 3g, 5g P, 2g Chiwocha grown on water soaked filter paper; 5gM, 7gM 14 root, 14 aerial grown on 1xMS, 4 week, stem, flower, silique grown on soil.

Table 5.2 Accumulation of ABA and ABA metabolites at different developmental stages in wild type plants

Levels of free ABA, ABA-GE, PA, DPA, 7′OH-ABA and neo-PA measured in wild type plants are shown. The analytes were measured by liquid chromatography tandem mass spectrometry with multiple reaction monitoring. For 2 day (Chiwocha), seedlings were grown for two days under the conditions of Chiwocha *et al.* (2005). Values represent the mean of three independent samples ± standard error. Where no value is shown, no measurements were within the limit of quantitation. Where no error is shown, only one or two measurements were within the limit of detection.

	Wild Type	ABA pmoles/g DW	ABA-GE pmoles/g DW	PA pmoles/g DW	DPA pmoles/g DW	7'OH-ABA pmoles/g DW	neo-PA pmoles/g DW
stratification (filter paper)	0 day	902 ± 8	91 ± 3	120 ± 29	418 ± 57	29 ± 0	
	1 day	948 ± 330	71	40 ± 3	532 ± 126		
	4 day	141 ± 10	59 ± 7		304 ± 65	24	
germinating (filter paper)	1 day	73 ± 4			443 ± 200		
	2 day	84 ± 3	98		692 ± 127		
	3 day	285 ± 152	73 ± 8		611 ± 86		
	5 day	175 ± 31	172 ± 40		2069 ± 155		
Seedlings (MS)	5 day	105 ± 19	48 ± 5		278 ± 1		
	7 day	154 ± 32	91 ± 9	29	671 ± 183		
	14 day root	497 ± 263	93 ± 14	1214 ± 126	3351 ± 115		
	14 day aerial	334 ± 30	314 ± 19	277 ± 13	1212 ± 52	17	
organ (soil)	4 week rosette	395 ± 15	142 ± 24	820 ± 129	2617 ± 376	14 ± 1	24 ±
	stem	87 ± 15	22	95 ± 11	2713 ± 462		
	flower	1004 ± 101	153 ± 12	891 ± 134	11111 ± 965	22 ± 1	165 ± 6
	silique	860 ± 17	189 ± 8	357 ± 14	11655 ± 1044	56 ± 1	258 ± 20
Other (filter paper)	2 day (Chiwocha)	104 ± 15	89 ± 12		448 ± 146		

Surprisingly, ABA-GE did not accumulate at high levels after one day of stratification, as previously described (Figure 5.1), nor over the first three days of germination. ABA-GE also did not accumulate in the seedlings grown under the conditions of Chiwocha *et al.* There were only five developmental stages in which ABA-GE accumulated at levels greater than 100 pmol/gDW: namely, seedlings grown for five days on filter paper; aerial tissue of fourteen-day-old seedlings; four-week rosettes; flowers and siliques. ABA-GE was highest in fourteen-day-old aerial tissue at 314 pmol/gDW. Accumulation of the conjugate in the other four samples varied from 140-190 pmol/gDW. It was therefore decided that accumulation of ABA-GE would be measured in 71B6-KO from the five stages that showed the highest levels in wild type, namely: five-day-old seedlings grown on filter paper; aerial tissue of fourteen-day-old seedlings; four-week-old rosette tissue; flowers; and siliques.

5.2.5 Analysis of ABA-GE accumulation in 71B6-KO at different stages

To determine whether 71B6 played a role in the synthesis of ABA-GE in wild type plants, it was necessary to ascertain whether less ABA-GE accumulated in 71B6-KO plants. Five stages had been identified in wild type that accumulated high levels of ABA-GE. 71B6-KO plants had been grown in parallel with the wild type for each stage and their tissue freeze-dried and stored at -80°C. ABA and the ABA catabolites were therefore extracted from the 71B6-KO samples and levels analysed. Figure 5.5 shows that ABA-GE accumulated at lower levels in 71B6-KO than wild type plants from four of the five stages, namely: aerial tissue of fourteen-day-old seedlings; four-week-old rosette tissue; flowers; and siliques. (The levels of ABA and the other ABA catabolites PA, DPA, 7′OH-ABA and neo-PA are shown in table 5.3). Only fourteen-day-old aerial tissue of 71B6-KO showed a substantial reduction in ABA-GE: at this stage levels were less than one third of wild type. This experiment is currently being repeated to include the complemented lines 71B6-RE3 and 71B6-RE12.

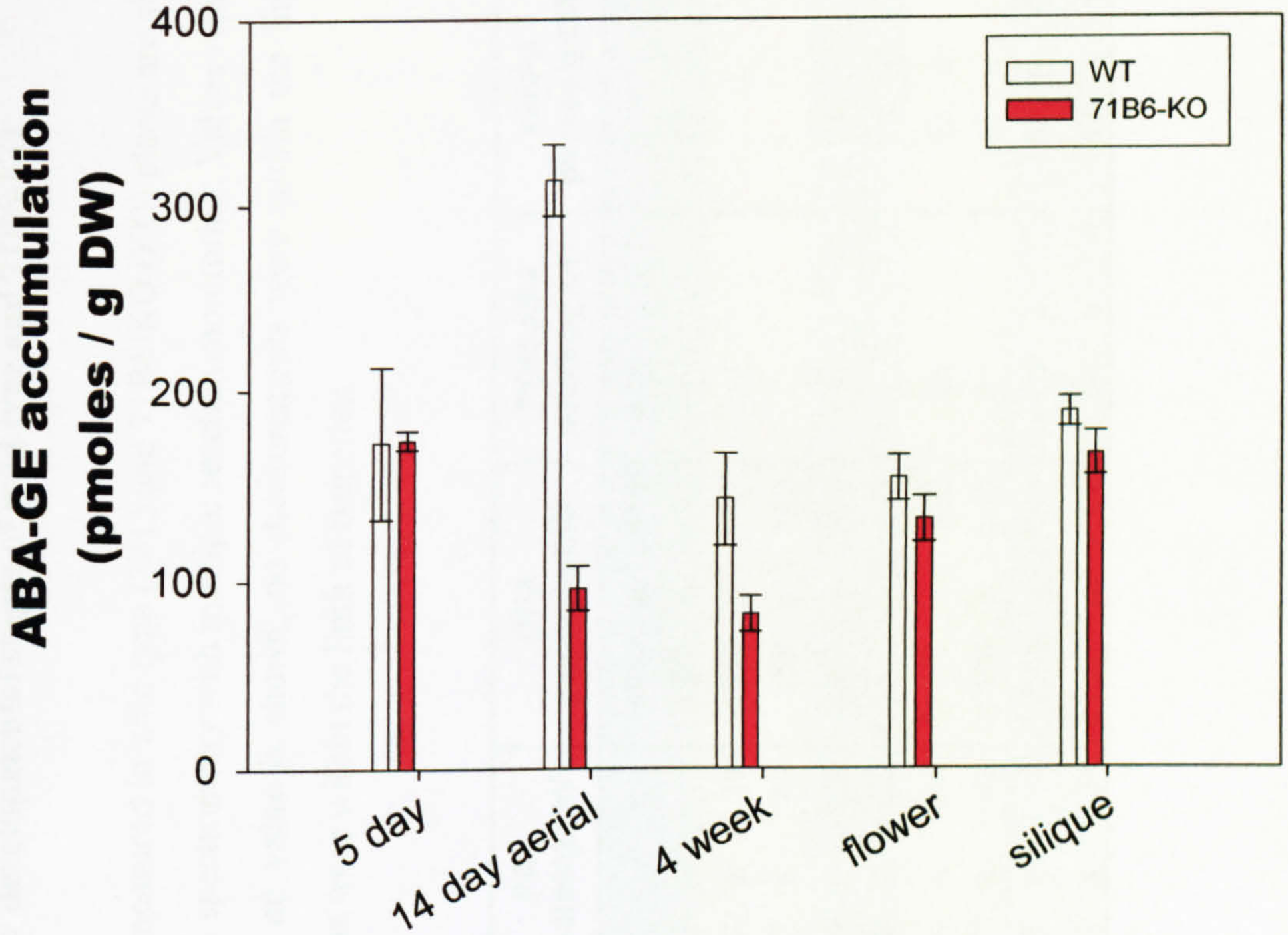

Figure 5.5 Accumulation of ABA-GE in key stages of wild type and 71B6-KO

Levels of ABA-GE measured in wild type (WT) and 71B6-KO (KO) plants are shown. The analytes were measured by liquid chromatography tandem mass spectrometry with multiple reaction monitoring. Values represent the mean of three independent samples ± standard error.

Table 5.3 Accumulation of ABA and ABA metabolites at key developmental stages of wild type and 71B6-KO

Levels of free ABA, ABA-GE, PA, DPA, 7′OH-ABA and neo-PA measured in wild type (WT) and 71B6-KO (KO) plants are shown. The analytes were measured by liquid chromatography tandem mass spectrometry with multiple reaction monitoring. Values represent the mean of three independent samples ± standard error. Where no value is shown, no measurements were within the limit of quantitation. Where no error is shown, only one or two measurements were within the limit of detection.

	Genotype	ABA pmoles/g DW	ABA-GE pmoles/g DW	PA pmoles/g DW	DPA pmoles/g DW	7'OH-ABA pmoles/g DW	neoPA pmoles/g DW
5 day seedling	WT	175 ± 31	172 ± 40		2069 ± 155		
	KO	194 ± 13	173 ± 5	31	1220 ± 91		12
14 day aerial	WT	334 ± 30	314 ± 19	277 ± 13	1212 ± 52	17	
	KO	318 ± 92	96 ± 12	227 ± 43	858 ± 180		
4 week rosette	WT	395 ± 15	142 ± 24	820 ± 129	2617 ± 376	14	24
	KO	202 ± 17	82 ± 9	676 ± 123	3610 ± 349		23 ± 3
flower	WT	1004 ± 101	153 ± 12	891 ± 134	11111 ± 965	22	165 ± 6
	KO	938 ± 107	132 ± 12	767 ± 72	10106 ± 515	25	117 ± 5
silique	WT	860 ± 17	189 ± 8	357 ± 14	11655 ± 1044	56 ± 1	258 ± 20
	KO	958 ± 50	166 ± 12	267 ± 24	11927 ± 139	54 ± 1	343 ± 18

5.2.6 Analysis of ABA-GE accumulation in wild type and 71B6-KO during salt stress

ABA is known to accumulate during salt stress (Zeevaart, 1999; Sauter *et al.*, 2002). In addition, accumulation of transcript for *71B6* was found to be induced during salt stress (Figure 5.3a, Table 5.1). This suggested that, since both transcript and substrate for the enzyme were present at increased levels, 71B6 could play a role in the glucosylation of ABA during salt stress. To test this hypothesis, ABA and the ABA catabolites were extracted and analysed from wild type, 71B6-KO, 71B6-RE3 and 71B6-RE12 plants that had been stressed by salt. Figure 5.6 shows that wild type plants subjected to salt stress accumulated substantial levels of ABA-GE. (The levels of ABA and the other ABA catabolites PA, DPA, 7′OH-ABA and neo-PA are shown in table 5.4). The levels of ABA-GE were significantly higher (2–3 fold) than within unstressed plants at any of the developmental stages. The level of ABA-GE in 71B6-KO was slightly reduced but still very high and near identical levels were observed in the complemented lines. These data show that in the absence of 71B6 (the knock-out), substantial levels of ABA-GE continue to accumulate. Therefore, even if 71B6 is involved in ABA glucosylation, there must be additional or alternative means of synthesising ABA-GE during salt stress. When *71B6* expression was recovered in the complemented knock-out, levels of ABA-GE did not increase to the levels observed in stressed wild type lines. This was unexpected, given that complementation of the knock-out should have resulted in a wild type phenotype, capable therefore of responding in the way of wild type during salt stress.

The principle conclusion that can be drawn from this experiment is that in the absence of 71B6, ABA-GE accumulated during salt stress. Either 71B6 is not involved in this glucosylation reaction under these conditions, or redundancy plays a role and other GTs catalyse the glucosylation of ABA.

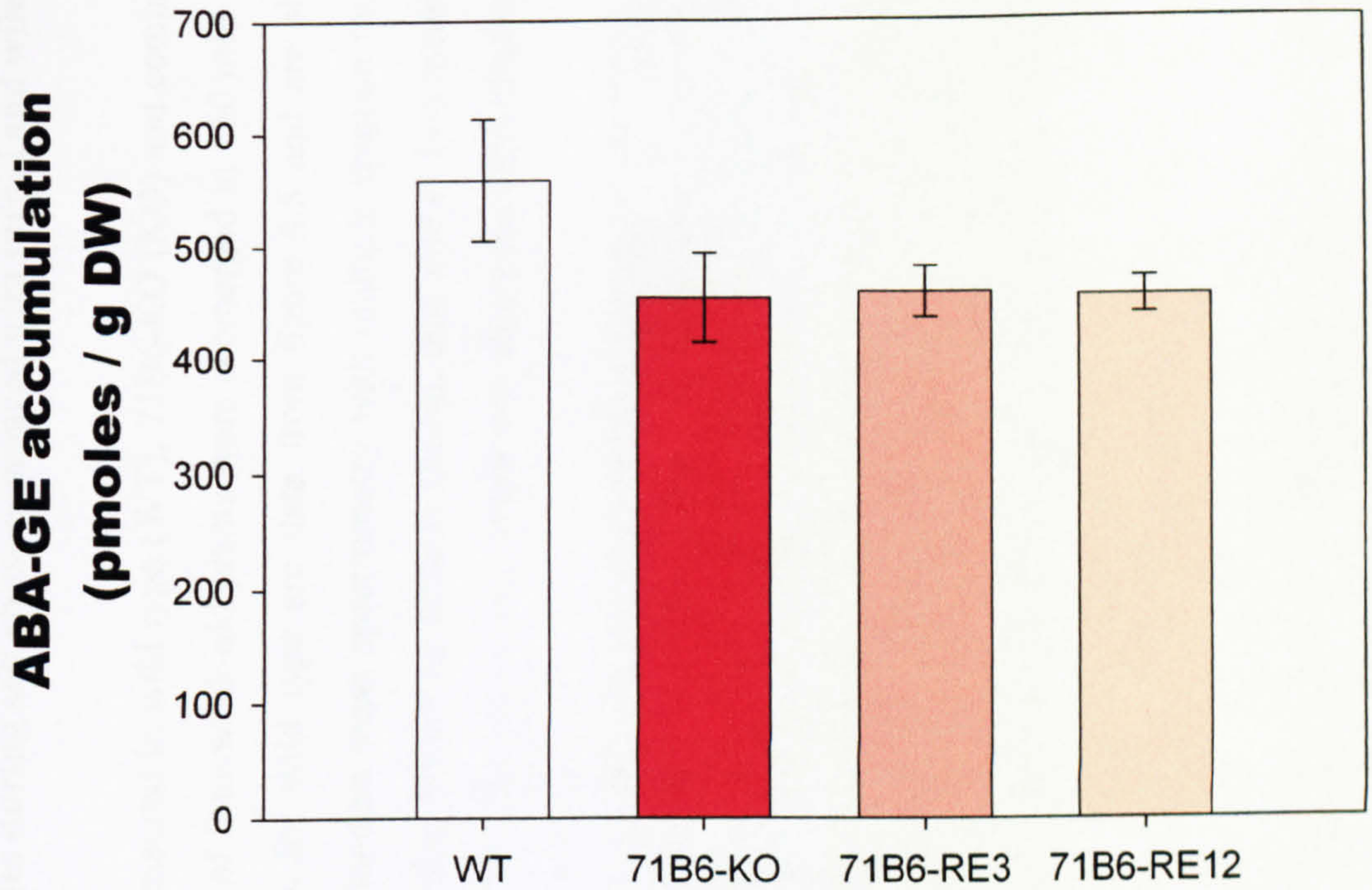

Figure 5.6 Accumulation of ABA-GE in salt-stressed transgenic lines

Levels of ABA-GE measured in wild type (WT), 71B6-KO (KO) and complemented lines (71B6-RE) are shown. The roots of four-week-old plants were submerged in 150 mM NaCl for 24 h before the rosettes were harvested. The analytes were measured by liquid chromatography tandem mass spectrometry with multiple reaction monitoring. Values represent the mean of three independent samples ± standard error.

Table 5.4 Accumulation of ABA and ABA metabolites in rosettes during salt stress compared with turgid and wilted rosettes

Levels of free ABA, ABA-GE, PA, DPA, 7′OH-ABA and neo-PA measured in wild type (WT), 71B6-KO (KO) and complemented lines (71B6-RE) are shown. For salt stressed measurements, the roots of four-week-old plants were submerged in 150 mM NaCl for 24 h before the rosettes were harvested. Turgid and wilted rosettes for wild type are data from figure 4.5 and are shown for comparison. The analytes were measured by liquid chromatography tandem mass spectrometry with multiple reaction monitoring. Values represent the mean of three independent samples ± standard error. Where no error is shown, only one or two measurements were within the limit of detection with the exception of PA in 71B6-RE3**, for which one sample was above the limit of quantitation.

	Genotype	ABA pmoles/g DW	ABA-GE pmoles/g DW	PA pmoles/g DW	DPA pmoles/g DW	7'OH-ABA pmoles/g DW	neoPA pmoles/g DW
rosettes of salt stressed plants	WT	3264 ± 12	559 ± 54	12440 ± 1302	3803 ± 314	36 ± 1	204 ± 23
	KO	3178 ± 509	455 ± 40	10286 ± 816	3257 ± 83	29 ± 2	192 ± 3
	71B6-RE3	3840 ± 345	459 ± 23	15250 ± **	3456 ± 458	33 ± 3	245 ± 21
	71B6-RE12	3043 ± 188	456 ± 16	14714 ± 2114	3150 ± 398	35 ± 2	219 ± 11
turgid rosette	WT	153 ± 10	164	235 ± 30	986 ± 103	22	9
wilted rosette	WT	3927 ± 51	325 ± 10	3769 ± 175	2025 ± 45	32	110 ± 4

5.3 Discussion

5.3.1 Redundancy in the homeostasis of ABA

The homeostasis of ABA requires the coordination of a complex network of processes involving synthesis, transport and inactivation through oxidative catabolism or conjugation. Many of the inactivation processes have been defined and enzyme activities detected, but, until recently, few specific enzymes or their genes had been identified. In contrast to biosynthesis, no genes involved in the inactivation of ABA have been identified through mutant screens. Two possible causes for this lack of progress seem likely. The first being plasticity between the different pathways of ABA catabolism, and the second being redundancy of gene function in a particular catabolic pathway.

5.3.1.1 Many catabolic routes

In addition to conjugation, there are at least three oxidative routes for ABA catabolism involving hydroxylation of the 7′, 8′ or 9′-methyl groups (reviewed in Nambara and Marion-Poll, 2005). The reasons why plants have multiple pathways for the catabolism of ABA are uncertain. One could speculate that, since ABA is an important signal molecule and large fluctuations in accumulation are necessary during the life cycle of the plant, several alternative mechanisms have evolved simply to ensure that inactivation is possible. Each of these routes is capable of removing large amounts of free ABA from the system (Neill *et al.*, 1983; Balsevich *et al.*, 1994; Zhou *et al.*, 2004). The plant can compensate for modification of one pathway by adjusting the flux through the other catabolic pathways and by adjusting biosynthesis. Chapter 4 provides one good example of this. A study by Zeevaart *et al.* (1988) also demonstrated the flexibility of the plant, where inhibition of the 8′-hydroxylase pathway with tetcyclacis led to increased accumulation of ABA-GE. Alternatively, it has been suggested that the catabolites and

conjugates may themselves play roles previously attributed to ABA. (Sauter *et al.*, 2002; Zhou *et al.*, 2004). However, if the catabolites and conjugates did play important roles, then it would be even more surprising that no controlling genes were identified through mutant screens.

5.3.1.2 Many genes per catabolic route

In addition to flexibility between catabolic pathways, there may be multiple genes encoding enzymes that catalyse a single enzymatic step. Members of the cytochrome P450 family (CYPs) catalyse the 8′-hydroxylation of ABA and members of the family 1 GTs catalyse the glycosylation of ABA. Both gene families are large and some redundancy may exist. For example, at least four CYP's from Arabidopsis are capable of catalysing the 8′-hydroxylation of ABA *in vitro*, and the controlling genes display overlapping expression profiles (Kushiro *et al.*, 2004; Saito *et al.*, 2004). Similarly, eight Arabidopsis GTs are capable of glucosylating ABA *in vitro* (Lim 2005). The challenge of showing which of these, if any, are responsible for the glucosylation of ABA *in planta* is considerable.

5.3.2 Strategy to identify 8′-hydroxylase of ABA

To demonstrate that CYP707A2 functions in the 8′-hydroxylation of ABA, Kushiro *et al.* (2004) looked for induction of expression of *CYP* genes during imbibition when 8′-hydroxylation of ABA was known to be active. The authors then narrowed the list of possible genes by determining which of them encoded enzymes that were capable of catalysing the reaction *in vitro*. Finally, the authors then compared accumulation of ABA, PA and DPA in mutants and wild type during imbibition when expression of the candidate *CYP* gene was high and PA and DPA were expected to accumulate. This approach led to the identification of the first 8′-hydroxylase gene.

5.3.3 Is 71B6 an endogenous GT for ABA?

5.3.3.1 At two days?

The previous two chapters showed that 71B6 is capable of glucosylating natural ABA both *in vitro* and *in planta*. The aim of this chapter has been to investigate whether the endogenous *71B6* gene encodes a glucosyltransferase of ABA. The expression patterns of *71B6* and the stages during which ABA-GE accumulated in wild type were investigated. Initially, *71B6* was seen to be expressed in young seedlings around the time of emergence of the cotyledons, which correlated with the developmental stage in which Chiwocha *et al.* (2005) had observed ABA-GE to accumulate. However, seedlings of this stage grown in the Bowles laboratory did not accumulate high amounts of ABA-GE in a similar manner. The experiments measuring catabolites of ABA during germination and imbibition performed for this thesis were not the first to give data that contrasted with that of Chiwocha *et al.* (Chiwocha *et al.*, 2003; Gonai *et al.*, 2004; Kushiro *et al.*, 2004; Chiwocha *et al.*, 2005). Whether the differences observed across the four laboratories of Chiwocha, Gonai, Kushiro and Bowles are a consequence of the different temperature and light regimes is not certain. Although, even when the light and temperature conditions published by Chiwocha *et al.* were used in the Bowles laboratory, the data could not be repeated. In summary, the flux through the different catabolic routes for ABA is highly variable and ABA-GE does not always accumulate in young seedlings of Arabidopsis.

5.3.3.2 At other developmental stages?

Whilst the accumulation of ABA-GE in two-day-old wild type seedlings grown in the Bowles laboratory was low, substantial accumulation of the conjugate was observed at other stages (Figure 5.4). ABA-GE was high in fourteen-day-old aerial tissue, five-day-old seedlings grown on filter paper, rosette tissue, flowers and siliques. It was interesting

that both *71B6* transcript and ABA-GE were high in young aerial tissue. This suggested that 71B6 might be involved in the glucosylation of ABA in these tissues. The hypothesis was supported by the observation that accumulation of ABA-GE in fourteen-day-old aerial tissue was greater in wild type than in 71B6-KO. Further experiments are being carried out to check whether the difference at fourteen days is repeatable and also to determine if the complemented lines 71B6-RE3 and 71B6-RE12 have restored levels of ABA-GE at this stage

5.3.3.3 In response to stress?

It was of interest to determine whether 71B6 functions to glucosylate ABA during stress, since ABA levels are elevated during stress. In chapter 4, it was shown that ABA-GE was present at similar levels in the wilted rosettes of wild type and 71B6-KO. Whilst the level of ABA-GE in wilted rosettes was similar to that in fourteen-day-old aerial tissue, the wilted rosettes also had very high levels of the ABA, PA and DPA. As such the relative level of ABA-GE was low in wilted rosettes.

ABA levels were also elevated during salt stress. Interestingly, transcript levels for *71B6* were shown to be induced by salt stress through Q-PCR, Northern blot and in publicly available microarray data. It was therefore of interest to determine whether the increased expression of *71B6* during salt stress resulted in increased accumulation of ABA-GE. The experiment showed that ABA-GE increased substantially during salt stress, both in the presence and absence of *71B6* transcript. A small reduction in accumulation of ABA-GE was observed in 71B6-KO compared to wild type, however, the complemented 71B6-RE lines did not accumulate wild type levels of ABA-GE despite accumulating *71B6* transcript during the salt stress.

5.3.3.4 71B6-RE lines

It is important to consider why the 71B6-RE lines did not accumulate wild type levels of ABA-GE during salt stress, despite accumulating *71B6* transcript. One possibility is that 71B6 is not involved in the glucosylation of ABA during salt stress. In this scenario, some other insertions in the 71B6-KO line, or expression of the gene for basta resistance, or biological variation may have been responsible for the reduced levels of ABA-GE compared to wild type.

Alternatively, the increased level of *71B6* transcript in the 71B6-RE lines may not have resulted in increased levels of active 71B6 protein. Since there was no tag incorporated within the genomic fragment used to generate the 71B6-RE lines, and antibodies for 71B6 protein were not available, there was no way as yet to test this possibility in the current study.

It is also possible that the genomic fragment used to complement the 71B6-KO line was not sufficient to fully mimic the wild type gene. The endogenous *71B6* is situated directly downstream of *UGT71B7* on the 3rd chromosome with 442 bp of sequence between the two ORFs. Only these 442 bp were included at the 5′ end of the *71B6* ORF in the genomic fragment. It is clear from figure 5.3 that this region is sufficient for induction of *71B6* by salt stress and CHX. However, all six of the transgenic 71B6-RE lines generated displayed a higher level of induction of *71B6* transcript by CHX than wild type (data not shown). It is therefore possible that there were additional controlling sequences within the *71B7* ORF, or even further upstream, which were not part of the fragment used to generate the 71B6-RE lines.

Alternatively, it is possible that in the 71B6-KO background, other glucosyltransferases were upregulated that were capable of glucosylating ABA.

5.3.3.5 Another glycosyltransferase may glucosylate ABA during salt stress

The generally low levels of expression of *71B6* increased the challenge of identifying a role for the gene. Figure 5.2 showed that accumulation of *71B6* transcript can be induced by salt, mannitol and ABA. However, when the signal from *71B6* on the dot blots or during Q-PCR was compared with *actin* or other *GTs* during these stresses, it was clear that the induction of transcript was still very low. Therefore, it was perhaps not surprising to discover that 71B6 was not necessary for ABA-GE to increase during salt stress. The purpose of this thesis was to focus on 71B6 as a potential glucosyltransferase for ABA and, in consequence, the other GTs capable of accepting ABA were not studied. Since it is now clear that ABA-GE accumulated during salt stress, independent of the *71B6* transcript, it would be interesting to investigate which GTs were responsible. Eight of the 107 Arabidopsis GTs are capable of glucosylating the hormone *in vitro* (Lim *et al.*, 2005). The dot blot experiment did not particularly flag any of these eight as being induced by salt. An analysis of the Genevestigator database suggests that *UGT74D1*, *UGT75B1* and *UGT73B1*, are all expressed at higher levels than *71B6* during salt stress (1395, 1381, 552 respectively). Interestingly, the high signals from the three genes do not represent induction by salt stress, since they also gave high signals in controls (2692, 818, 631 respectively). Nevertheless, it is possible that enzyme(s) encoded by one or all of these three genes were responsible for the accumulation of ABA-GE. Providing these data can be confirmed by other means, it would be interesting to measure the accumulation of ABA-GE in the corresponding knock-out mutants or knock-down silenced lines during salt stress. It may also be necessary to generate double or triple mutants to observe any impact upon ABA-GE accumulation. In summary, although *71B6* was induced by salt stress, at low levels, the enzyme was not required for the increased glucosylation of ABA during the stress. One or more other GTs must therefore have catalysed the reaction in the 71B6-KO background. To identify candidate GTs that may control this process, it would be prudent to make use of expression data and *in vitro* substrate specificity data.

In addition, it is possible that 71B6 is involved in deactivating free ABA in plant tissues during recovery from stress. It will be important to investigate whether the transcript is upregulated during recovery. If so, it is possible that the 71B6-KO plants have a

prolonged accumulation of ABA, following stress, compared to wild type plants. This could be challenging to determine since the differences in the levels of ABA and ABA-GE may be small and difficult to observe consistently in the context of the highly variable catabolic pathways for ABA.

5.3.4 Glucosylation of ABA in Arabidopsis

One can only speculate as to the physiological reasons for increased accumulation of ABA-GE in certain tissues in Arabidopsis. The simplest explanation might be that a high level of ABA-GE simply reflects a high concentration of substrate available to the glucosyltransferase enzyme(s). The levels of ABA and ABA-GE were both high in flowers, siliques and salt stressed plants (see tables 5.2 and 5.4). The levels of ABA and ABA-GE were higher in five day old seedlings when grown on paper than when grown on MS media. However, such an explanation would be naïve and not account for all the data. At fourteen days ABA accumulated at comparable levels in roots and aerial tissue, but ABA-GE was only high in aerial tissue. The level of ABA was higher in four-week-old rosettes than in fourteen-day aerial tissue, but the level of ABA-GE was substantially higher in fourteen-day aerial tissue. The level of ABA was very high in dry seeds and in seeds that had been stratified for one day, but the level of ABA-GE was very low at both of these stages.

Whilst ABA-GE did accumulate more in some stages than others, there are no Arabidopsis tissues in which ABA-GE has been consistently shown to be the major metabolite of ABA. Therefore, whilst the evidence suggests that ABA-GE is inactive, the importance of glucosylation as an inactivation mechanism in Arabidopsis is uncertain. The roles of ABA were largely defined through application of exogenous ABA and the study of ABA deficient mutants. ABA-GE cannot be applied exogenously to plants without rapid cleavage to release free ABA (Lehmann, 1983; Zeevaart and Boyer, 1984; Dietz *et al.*, 2000). No mutants compromised in any ABA catabolic routes have been identified through genetic screens. The specific role of ABA-GE is therefore difficult to

determine. A more targeted approach was used to identify an 8′-hydroxylase mutant that over-accumulated ABA in seeds, leading to enhanced dormancy. This demonstrated the importance of 8′-hydroxylation by linking the metabolites involved to a developmental characteristic (Kushiro *et al.*, 2004). The strategy used by the Bowles group may yet reveal mutants that are defective in the glucosylation of ABA. The study of such mutants may highlight the physiological importance of this process to Arabidopsis. Alternatively, ABA-GE may just be an insignificant bi-product of metabolism in this species.

5.3.5 Oxidative metabolites of ABA

There are two other points of interest with regard to the accumulation of metabolites from the data obtained here. First, in chapter 4 it was noted that, in turgid tissue, DPA accumulated at higher levels than its precursor PA. Following the application of water stress by wilting, PA accumulated rapidly to higher levels than DPA. In this chapter, DPA accumulated at higher levels than PA in all the developmental stages (table 5.2). However, following salt stress the level of PA was also higher than DPA. These data suggest that during stress, the synthesis of PA is more rapid than its conversion to DPA. Second, in Arabidopsis, neo-PA has only previously been detected in siliques (Zhou *et al.*, 2004). Chapters 4 and 5 confirm the presence of neo-PA in siliques and also report the catabolites to be present in flowers and rosette tissue (table 5.3, figure 4.5a). Levels of neo-PA increased during stress with salt or by wilting (table 5.4, figure 4.5b).

5.3.6 Cycloheximide

It is interesting to consider why CHX might induce accumulation of *71B6* transcript. CHX causes ribotoxic stress to plant cells and is commonly used in experiments to inhibit expression of proteins. Two possibilities have been suggested for the induction of *71B6* by CHX. First, that under normal circumstances, the expression of *71B6* is being actively repressed by a transcription factor. Upon treatment with CHX the cell no longer synthesises the repressor and transcription of *71B6* is released. Second, that transcription

of *71B6* occurs, but the transcript is being constantly and specifically degraded by enzymes, thereby preventing accumulation of the mRNA. Treatment with CHX would then lead to a loss of enzymes involved in transcript degradation and allow the transcript to accumulate. The likelihood of either mechanism and the physiological significance is difficult to predict. In addition, since wild type Arabidopsis plants would be unlikely to encounter high concentrations of CHX it is unlikely that the role of *71B6* would be to respond to this compound.

CHAPTER 6: GENERAL DISCUSSION

6.1 Use of 71B6 to modify ABA in planta

6.2 Use of 71B6 to modify ABA related responses in planta

6.3 Role of 71B6

6.4 Induction by cycloheximide

6.5 Other applications

6 GENERAL DISCUSSION

This thesis has investigated a candidate glucosyltransferase, 71B6, that may be involved in the conjugation of ABA. The activity of the recombinant enzyme towards ABA *in vitro* was studied in chapter 3. Conditions which affected activity were investigated and kinetic properties determined. The activity was also tested towards a range of structural analogues of ABA and analogues were identified that represented better or worse substrates for the enzyme. If a crystal structure was available for 71B6 or a similar family 1 GT, it would be interesting to model how the analogues might interact with the active site of the enzyme.

Transgenic plants overexpressing *71B6* were studied in chapter 4. Constitutive overexpression of *71B6* led to a substantial increase in the accumulation of ABA-GE, although plants were able to modulate flux through other pathways to prevent major changes in the accumulation of ABA. 71B6-OE seedlings were more resistant than wild type to inhibition of growth by exogenous ABA. Two of the ABA analogues were used to demonstrate that the resistance of 71B6-OE seedlings to the ABA was a direct consequence of glucosylation. The 71B6-OE seedlings were also shown to be less sensitive to post-germinative developmental-arrest induced by glucose stress. This was consistent with overexpressed 71B6 protein being able to glucosylate and deactivate endogenous ABA when it was elevated by the stress, resulting in a mild ABA deficient phenotype.

Chapter 5 went on to examine whether the endogenous *71B6* gene encoded an enzyme involved in the glucosylation of endogenous ABA. In most tissues, the level of expression of *71B6* and the accumulation of ABA-GE was low. This provided a challenge to identify differences in accumulation of ABA-GE between wild type and 71B6-KO plants. The largest difference was observed in the aerial tissue of fourteen-day-old seedlings. Experiments are currently underway to determine whether this difference is rescued in complemented 71B6-KO lines.

6.1 Use of 71B6 to modify ABA *in planta*

In the context of flexibility between the multiple catabolic pathways for ABA, it is perhaps unsurprising that changing the expression of *71B6* did not have a major impact on the plant. The 8′-hydroxylase *cyp707a2* mutant only displayed defects in one developmental phenotype related to ABA (dormancy)(Kushiro *et al.*, 2004). At this stage, mutant seeds accumulated eight fold more ABA than wild type. It could be argued therefore, that it is only necessary to identify the correct stage or tissue in which 71B6 functions. However, the biochemical characteristics of 71B6 and CYP707A2 must be considered. 71B6 had a Km of 280 μM towards (+)-ABA and a Kcat of 0.14 s^{-1} under the experimental conditions used. Whilst the kinetic properties of CYP707A2 have not been determined, the closely related enzyme CYP707A3 had a Km of 1.3 μM and a Kcat of 0.25 s^{-1} (Saito *et al.*, 2004). Bearing in mind that ABA is likely to be present in the low micromolar range, the kinetic properties of the CYP707A enzyme are more suited for the metabolism of ABA at physiological levels (Harris *et al.*, 1988). Assuming the kinetic properties determined *in vitro* reflect the ability of the enzyme to glucosylate ABA in its natural environment in the cell, then 71B6 would not be very effective at rapidly deactivating endogenous ABA. Therefore, in the context of highly redundant and plastic processes involved in its homeostasis and a low affinity for the substrate, it is not surprising that overexpression of this enzyme was unable to impact greatly on the levels of ABA.

There were eight other family 1 GTs that displayed activity towards ABA *in vitro*. Whilst their kinetics are yet to be studied, the specific activity of 84B1 towards ABA was substantially higher than that of 71B6 (Lim *et al.*, 2005). It is unlikely that 84B1 could be used to selectively impact upon the level of ABA *in planta* for three reasons. Firstly, the 84B1-OE line analysed in chapter 4 only accumulated two fold more ABA-GE than wild type (compared with up to 19 fold for 71B6-OE). Secondly, although the specific activity of 84B1 towards ABA is high, a preliminary experiment indicated that the affinity of the enzyme towards ABA was very low, with a Km, 10 fold higher than that of 71B6 (data not shown). Thirdly, 84B1 displayed a substantially greater specific activity towards IAA

than towards ABA, and has been shown to modify the level of IAA *in planta* (Jackson *et al.*, 2001; Jackson *et al.*, 2002). However, a strategy could be employed to bioengineer a new GT from the eight known genes through directed evolution. If a new GT could be developed which selectively catalysed the glucosylation of ABA with a high rate and a tight affinity, then it may be more likely to impact upon levels of ABA when overexpressed *in planta*.

An alternative strategy for using GTs to modify the level of ABA in specific cellular locations *in planta* could involve the use of tissue-specific promoters to drive expression of the *GT* genes. In this context, Phillips *et al.* (1997), found that seed specific expression of a gene encoding single chain Fv antibodies with a high affinity for ABA was necessary to change the ABA levels in seeds: constitutive expression of the gene using the 35S promoter was ineffective.

6.2 Use of 71B6 to modify ABA related responses *in planta*

Despite poor kinetics for ABA, the detailed characterisation of 71B6-OE plants did reveal that the gene is capable of modifying ABA related responses in specific circumstances. These include early growth on exogenous ABA and on glucose. The levels of ABA were only measured in the 71B6-OE plants on a whole rosette basis and it is likely that the overexpressed 71B6 impacted more on the level of ABA in specific tissues, or cell types during these conditions. The low concentrations of ABA make it unlikely that ABA could be measured directly in a single cell or cell type. However, it might be interesting to use an *in vivo* imaging system such as the one of Christmann *et al.* (2005)(see section 1.2.2.1) to determine how overexpression of *71B6* affected relative levels of ABA in particular cells: in unstressed plants; or in seedlings growing on high glucose or on exogenous ABA; or in wilting leaves. Assuming the specificity of the *in vivo* system could be ensured, it could provide a powerful way of following ABA levels in a non-destructive manner. One could imagine using the analogue PBI-413, to which 71B6-OE seedlings are extremely resistant, as a positive control for the *in vivo* imaging system. This system

could be used to answer a number of interesting questions. First, how much does 71B6 change levels of ABA in specific tissues? Second, since the phenotypic consequences of overexpression of 71B6 are subtle, it can be assumed that overexpression only resulted in small changes to the level of ABA. Therefore, it would be interesting if an *in vivo* imaging system could reveal the level of ABA deficiency required for resistance to post-germinative developmental arrest. Third, if the level ABA was seen to be particularly changed in specific tissues in 71B6-OE lines (or 71B6-KO) compared to wild type, then experiments could be designed to look for targeted physiological differences related to these cell types. Fourth, despite much research, it remains uncertain whether the receptor for ABA is located inside or outside the cell. It would be interesting to specifically overexpress an ABA GT in the guard cells of stomata. If an *in vivo* imaging system could be used to show that the level of ABA inside the guard cells was substantially reduced and the plants were not wilty, then it would suggest that the ABA receptor is located outside the guard cell. Alternatively, if the plants were wilty, then the receptor would probably be located within the guard cell.

6.3 *Role of 71B6*

This thesis has not definitively shown that the endogenous 71B6 is involved in the glucosylation of ABA. Even if the experiments did show that 71B6 is involved, the importance of this process for Arabidopsis is difficult to assess. 71B6 was selected as a candidate for an ABA-glucosyltransferase because it displayed activity towards ABA *in vitro* and was selective for the natural (+) enantiomer of ABA. Only enzyme, CYP707A2, has been shown to catabolise ABA *in planta* through the use of 'knock-out' mutants. Interestingly, studies *in vitro* showed that, like 71B6, CYP707A2 was also selective for (+)-ABA. Further work must be carried out to investigate the role of *71B6* in the plant.

Expression studies may provide clues as to the role of the endogenous gene. Whilst the accumulation of *71B6* transcript was induced by salt in wild type, 71B6-KO plants were not substantially compromised in the accumulation of ABA-GE during salt stress. It

would be interesting to further investigate the link between *71B6* and salt stress by determining whether the growth of 71B6-KO plants was altered in saline conditions.

All of the expression studies on *71B6* were carried out on a whole seed, whole seedling, or whole organ basis. It would be interesting to use promoter-reporter lines to investigate promoter activity with greater spatial resolution. If *71B6* was expressed highly in limited cell types then this could explain why previous studies have not revealed the location of *71B6* function.

Alternatively, it is possible that the role of *71B6* is not to inactivate endogenous ABA during abiotic stress, but to remove exogenous ABA produced by fungal pathogens during biotic stress. It has long been known that fungi synthesise ABA (reviewed in Walton and Li, 1995), and the possibility has emerged that ABA forms one of their virulence factors used to weaken the host plants defence mechanisms (reviewed in Nambara and Marion-Poll, 2005). For example, the fungal pathogen *B. cinerea* releases ABA and ABA precursors during infection of tomato plants (Kettner and Dorffling, 1995). The ABA deficient mutant of tomato, *sitiens*, is more resistant to infection by *B. cinerea* than wild type (Audenaert *et al.*, 2002). And application of exogenous ABA can increase susceptibility of both wild type and the ABA deficient mutant to infection (Kettner and Dorffling, 1995; Audenaert *et al.*, 2002). Three pieces of information suggest that 71B6 may function to protect plants, or be capable of protecting plants, from ABA mediated fungal attack. First, concentrations of ABA as low as 1 μM can significantly increase the susceptibility of wild type plants to infection (Audenaert *et al.*, 2002). Second, 71B6-OE lines were more resistant to exogenous ABA applied in this order of magnitude (Figure 4.3 and data not shown), demonstrating that overexpressed 71B6 is capable of detoxifying exogenous ABA at physiologically relevant concentrations. Third, microarray data suggested that accumulation of *71B6* transcript was induced by the pathogens *B. cinerea* and *P. infestans* (Table 5.1). In addition, a role for *71B6* in the inactivation of fungal ABA would be consistent with the general roles of GTs as detoxifying enzymes. It would be interesting to investigate whether overexpression of *71B6* (or the other seven GTs that accept ABA) in tomato or

Arabidopsis conferred enhanced resistance to *B. cinerea* and other pathogens. If resistance was achieved, it would be particularly important since, unlike conventional ABA deficient mutants, 71B6-OE plants are not compromised in normal growth and development. It would also be interesting to determine whether 71B6-KO plants are more susceptible fungal infection than wild type plants.

It is also possible that several of the eight GTs, which accept ABA *in vitro*, are involved in the glycosylation of ABA in overlapping tissues. It may therefore be necessary measure the accumulation of ABA-GE in lines which have more than one of the GTs knocked out or silenced.

Finally, it is possible that endogenous 71B6 is completely uninvolved in ABA related processes and that the ability of the enzyme to glucosylate (+)-ABA is merely an artefact. The enzyme may play an important role in the glycosylation of some other compounds. Further screening for activity of recombinant 71B6 towards other compounds *in vitro* may reveal alternative candidate substrates for the enzyme. However, there are clearly more chemicals present in a cell than can be easily obtained for a screen. It would be interesting to feed radiolabelled UDP-glucose to wild type, 71B6-KO and 71B6-OE lines and look for accumulation of labelled conjugates. It may be possible to identify differences in the 'labellome' or 'glucose-conjugome' between the different genotypes and thereby identify potential new substrates for the endogenous or recombinant 71B6 enzyme.

6.4 Induction by cycloheximide

One of the most striking observations with regard to the expression of *71B6* has been that the transcript accumulates massively upon treatment with CHX. Although this phenomenon is not directly related to the hypothesis that *71B6* may be a glucosyltransferase of ABA it may be interesting to investigate in its own right. Curiously one of the candidate 8′-hydroxylase genes was also induced by CHX (Cutler AJ, personal

communication). If this was due to a *CHX* responsive element in the promoter or a transcriptional repressor, then the two promoters could be compared to identify similar motifs that may be responsible for induction of CHX. Figure 5.3 demonstrates that the key element within the 71B6 sequence must lie within the genomic fragment used to generate the 71B6-RE lines. Since there was only 442 bp of 5'UTR included then it would not be too difficult to narrow down the search.

6.5 Other applications

The process of research for this thesis could lead to a number of potential applications. In addition to those described above, such as disease resistant plants, modification of ABA levels and of ABA related physiological characteristics, three further possibilities are suggested here.

Since PA was the major natural product of ABA fed to cell-suspension cultures of maize, Balsevich *et al.* (1994) used the system to generate large amounts of the catabolite. PA was then easily purified from the cultures. In chapter 4, plants overexpressing *71B6* were shown to accumulate ABA-GE at comparatively high levels. It would be interesting to investigate whether cell-suspension cultures produced from transgenic 71B6-OE lines could be used to synthesise large amounts of ABA-GE in an analogous manner. Currently, ABA-GE is expensive to obtain commercially.

During the analysis of the substrate selectivity of recombinant 71B6 *in vitro* in chapter 3, bioactive analogues were identified that were resistant to 8'-hydroxylation and also to conjugation by 71B6. It would be interesting to investigate whether these analogues are also resistant to glycosylation by the other eight GT's shown to recognize ABA. If analogues can be identified, or designed, that are resistant to multiple inactivation pathways, then they may therefore prove to be useful agrochemicals for field applications.

In chapter 3, the bioactive analogue of ABA, PBI-413, was shown to be glycosylated three times more rapidly than ABA by recombinant 71B6 *in vitro*. In chapter 4, 71B6-OE lines were shown to be very resistant to inhibition of growth by this analogue, since the overexpressed enzyme was able to detoxify it efficiently. Conversely, the analogue effectively inhibited growth in wild type. It would be interesting to investigate whether the analogue could be applied in combination with transgenic crops overexpressing *71B6* as a herbicide / herbicide tolerant crop combination. One could imagine refining the structure of the analogue and directly evolving *71B6* to improve the activity of the enzyme towards the analogue and therefore the power of the herbicide.

LIST OF ABBREVIATIONS

AAO	Arabidopsis aldehyde oxidase
ABA	Abscisic acid
aba3	*aba3-2*
ABA-GE	abscisic acid-glucose ester
ACC	1-amino-cyclopropane 1-carboxylic acid
amu	atomic mass units
AO	Aldehyde oxidase
Arabidopsis	*Arabidopsis thaliana*
avocaodo	*Persia americana*
barley	*Hordeum vulgare*
broccoli	*Brassica oleracea*
bromegrass	*Bromus inermis*
BSA	bovine serum albumin
CaMV-35S	cauliflower mosaic virus 35S promoter
CAZy	Carbohydrate-Active enZYmes
cDNA	Complementary DNA
chick pea	*Cicer arietinum*
CHX	cycloheximide
CID	collison induced dissociation
cis-S-(+)-ABA	(+)-ABA
CYP	Cytochrome P450
DMSO	dimethyl sulphoxide
DPA	Dihydrophaseic acid
DPA-GE	Dihydrophaseic acid-glucose ester
DPA-GS	dihydrophaseic acid-glucoside
DTT	dithiothreitol
DW	dry weight
EDTA	ethylenediamine tetraacetic acid, disodium salt

EST	expressed sequence tag
EST	expressed sequence tag
FW	fresh weight
GA3	gibberellic acid
gin	glucose insensitive
GST	glutathione-S-transferase
GT	glycosyltransferase
GTs	glycosyltransferases
GUS	glucuronidase
HPLC	high performance liquid chromatography
IAA	indole-3-acetic acid
IPTG	isopropyl-β-D-thioglactoside
IWF	intercellular washing fluid
katal	The amount of enzyme which, under specified conditions, catalyses the production of 1 mole of product per second
Kcat	Catalytic constant or turnover number
	number of reaction processes that each active site catalyses per unit time.
Km	Michaelis constant
KO	knock-out
KO	knock-out
LB	lennox broth
LC-MS-MS	High performance liquid chromatography- mass spectrometry-mass spectrometry
lettuce	*Lactuca sativa*
m/z	mass to charge ratio
maize	*Zea mays*
MALDI-TOF	matrix assisted laser desorption/ionization- time of flight
MoCo	Molybdenum cofactor
MOPs	3-[N-morpholino]propane sulphonic acid
mRNA	messenger RNA

MS	Murashige and Skoog plant growth medium
NaCl	Sodium chloride
NASC	Nottingham Arabidopsis Stock Centre
NCED	nine-*cis*-epoxycarotenoid dioxygenase
NDP	nucleotide-diphosphate
neo-PA	neo-phaseic acid
OD	optical density
OE	over-expressor
orange	*Citrus sinensis*
ORFs	Open reading frames
PA	Phaseic acid
PA-GE	phaseic acid-glucose ester
PBI	Plant Biotechnology Institute
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulphonylfluoride
PSPG	Plant secondary product glycosyltransferase
Q-PCR	quantitative real-time polymerase chain reaction
Ri	empty vector control transgenic line
RNAi	RNA interference
[S]	substrate concentration
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	standard error
silver beet	*Beta vulgaris*
SSC	salt, sodium citrate buffer
spinach	*Spinacea oleracea*
TBE	tris-borate buffer
TCA	trichloroacetic acid
TE	Tris, EDTA buffer

TEMED	N, N, N′, N′,-tetramethyl-ethylenediamine
TLC	thin layer chromatography
TNE	Tris, NaCl, EDTA
tomato	*Lycopersicon esculentum*
total ABA	sum of ABA, PA, DPA, 7′OH-ABA, neo-PA and ABA-GE
UGT	UDP-glycosyltransferase
UTR	untranslated region
UV	ultra violet
V	velocity
Vmax	maximum velocity
western white pine	*Pinus monticola*
WT	wild type
x-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
ZEP	Zeaxanthin epoxidase
14 aerial	14-day-old aerial tissue
14 root	14-day-old roots
1g	germinating seedlings after 1 day
1s	seeds stratified for one day
2,4-D	2,4-dichlorophenoxyacetic acid
2g	germinating seedlings after 2 days
2xYT	tryptone and yeast extract broth
35S	cauliflower mosaic virus 35S promoter
3g	germinating seedlings after 3 days
4s	seeds stratified for four days
5g	germinating seedlings after 5 days
5g M	seedlings germinated for 5 days on MS media
5g p	seedlings germinated for 5 days on filter paper
7′OH-ABA	7′-hydroxy ABA
71B6	UGT71B6
71B6-KO	71B6 knock-out line
71B6-OE	plants overexpressing *UGT71B6*

71B6-RE	71B6 knock-out line complemented with the endogenous *71B6* gene
7g	germinating seedlings after 7 days
8′OH-ABA	8′-hydroxy ABA
84B1	UGT84B1
84B1-OE	plants overexpressing *UGT84B1*
9′OH-ABA	9′-hydroxy ABA

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CLOSING QUOTE

"*I don't see much sense in that,*" said Rabbit.

"*No,*" said Pooh humbly, "*there isn't. But there was going to be when I began it. It's just that something happened to it along the way.*"

(Milne, 1928b)

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The use of abscisic acid analogues to analyse the substrate selectivity of UGT71B6, a UDP-glycosyltransferase of *Arabidopsis thaliana*

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Abstract **This study analyses the activity of an *Arabidopsis thaliana* UDP-glycosyltransferase, UGT71B6 (71B6), towards abscisic acid (ABA) and its structural analogues. The enzyme preferentially glucosylated ABA and not its catabolites. The requirement for a specific chiral configuration of (+)-ABA was demonstrated through the use of analogues with the chiral centre changed or removed. The enzyme was able to accommodate extra bulk around the double bond of the ABA ring but not alterations to the 8′- and 9′- methyl groups. Interestingly, the ketone of ABA was not required for glucosylation. Bioactive analogues, resistant to 8′-hydroxylation, were also poor substrates for conjugation by UGT71B6. This suggests the compounds may be resistant to both pathways of ABA inactivation and may, therefore, prove to be useful agrochemicals for field applications.**

Keywords: Glycosyltransferase; Abscisic acid; Glucose ester; Analogues; Enantiomer; *Arabidopsis thaliana*

1. Introduction

Abscisic acid (ABA) is a phytohormone [1] with many functions in plants, including roles in seed development, dormancy and germination, root and shoot growth, maintenance of water relations and stress tolerance [2]. The level of ABA in plant cells is controlled through a homeostatic mechanism involving biosynthesis, catabolism and redistribution [1]. These processes combine to result in elevated levels of ABA in key cells and tissues at specific developmental time-points and in response to stress [1–3]. Biosynthesis of ABA, a monocyclic sesquiterpene, has been studied in detail and occurs through the modification and cleavage of carotenoids [4]. The immediate carotenoid precursors are chiral and in consequence, natural ABA is produced in the chiral *cis*-*S*-(+)-ABA form (Fig. 1A) [4].

Catabolism of ABA can occur through either oxidation and reduction, or through conjugation to glucose (Fig. 1B) and the relative importance of the different pathways is known to vary amongst plant species [5]. It is considered that the principal oxidation pathway occurs through hydroxylation of the 8′-methyl to give 8′-hydroxy ABA, which rearranges to form phaseic acid (PA), that is then further reduced to dihydrophaseic acid (DPA) [4,6]. Other oxidation products of ABA have also been reported, including 7′-hydroxy ABA [7], 9′-hydroxy ABA, and neo-phaseic acid (neo-PA) [8]. The main conjugation pathway occurs through glucosylation of the carboxylic group to form ABA-glucose ester (ABA-GE, Fig. 1) [9]. Other conjugates have been described including an ABA 1′-glucoside and glucose conjugates of the acidic metabolites, PA and DPA [2].

Recently, we have screened a multigene family of UDP-glycosyltransferases (UGTs) of *Arabidopsis thaliana* for activity towards ABA in vitro. Whilst eight enzymes were able to glucosylate the hormone, only one, UGT71B6 (hereafter 71B6), displayed preferential activity towards the naturally occurring (+)-ABA enantiomer [10]. This study uses structural analogues of ABA to explore features of the phytohormone that influence glucosylation by 71B6 and to establish a foundation from which to analyse the role of the enzyme in ABA homeostasis in planta.

2. Materials and methods

2.1. Materials

Chemicals were purchased from Sigma–Aldrich, except ABA-GE (OlCHemIm Ltd., Czech Republic). ABA metabolites and analogues were synthesised as described (PA and DPA [11], PBI-49, according to Mayer et al. [12], using 2-*trans*-3-methylpenten-4-yn-1-ol, PBI-82, PBI-89 [13], PBI-253 [14], PBI-271, PBI-293 [15,16], PBI-372, PBI-493, PBI-524 [17], PBI-401, PBI-514 [18], PBI-233, PBI-287, PBI-410, PBI-413, PBI-703, PBI-705 (Abrams unpublished)). The expression plasmid construction has been described previously [19].

2.2. Purification and assay of recombinant 71B6 protein

Recombinant 71B6 (At3g21780) was expressed as a GST-fusion protein in *Escherichia coli* (XL1Blue) carrying the pGEX-2T expression plasmid. Cells were disrupted through a French Press and protein was purified using GST-Sepharose as described [20,21]. A typical UGT assay involved 1 μg recombinant protein, 1 mM ABA or analogue, metal ions at 5 mM and reducing agents at 10 mM, in a final volume of 100 μl. Following 1 h at 30 °C, reactions were stopped with 10 μl of 240 mg ml^{-1} trichloroacetic acid. Where relevant, alkaline hydrolysis was carried out in 1 M NaOH for an additional hour at 30 °C. The reaction mix was analysed using reverse phase HPLC. All assays were carried out on at least three independent protein extractions. For the kinetic analysis, initial rates of reaction at five concentrations of (+)-ABA were calculated from four time points and used to determine the kinetic parameters by Hyperbolic Regression Analysis.

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Abbreviations: UGT, UDP-glycosyltransferase; ABA, abscisic acid; ABA-GE, abscisic acid glucose ester; PA, phaseic acid; DPA, dihydrophaseic acid; CID, collision-induced dissociation; 71B6, UGT71B6

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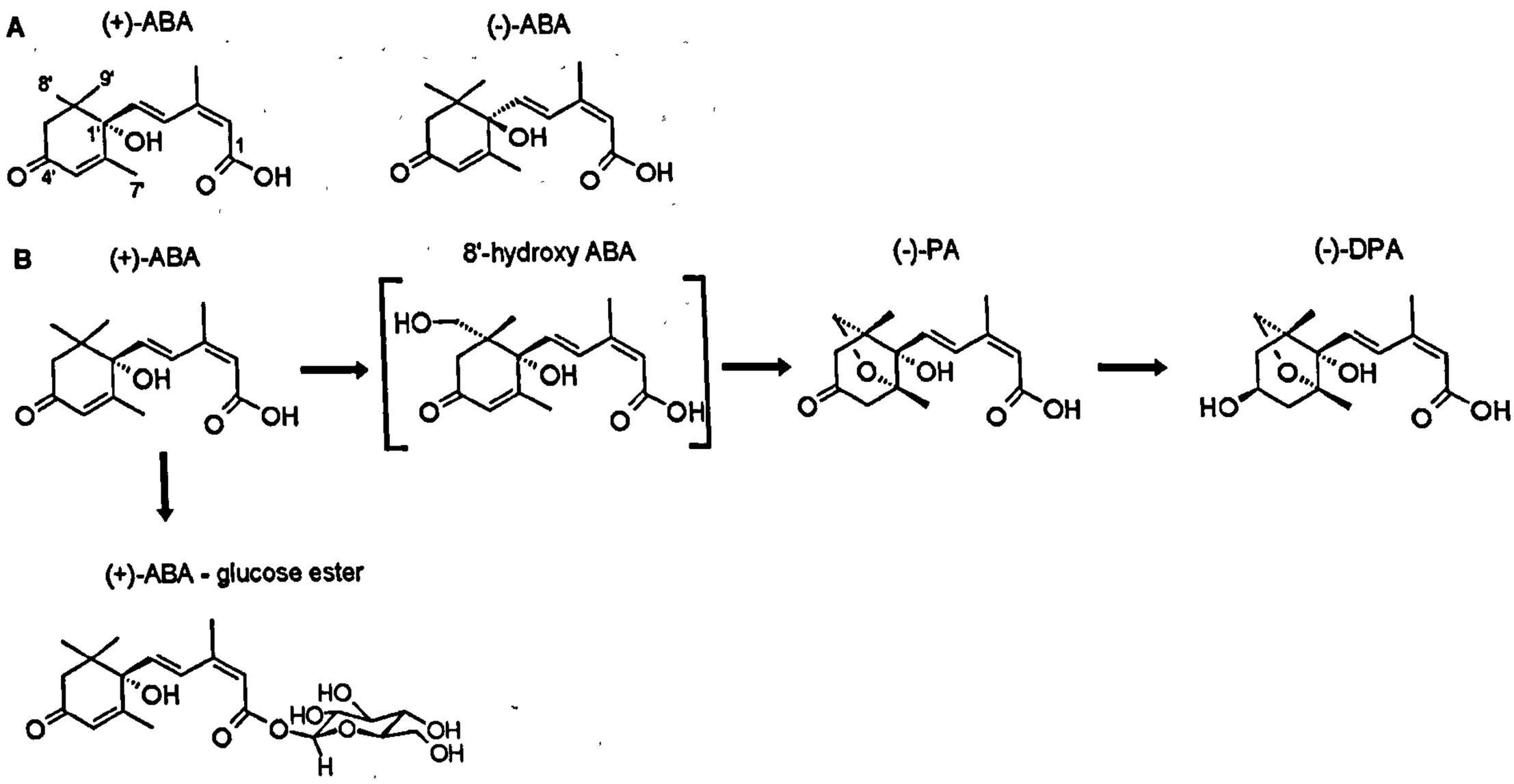


Fig. 1. Molecular structure of ABA and its catabolites. (A) (+)-ABA and (−)-ABA; (B) the deactivation of (+)-ABA to phaseic acid (PA), dihydrophaseic acid (DPA) and the ABA-glucose ester (ABA-GE).

2.3. HPLC analysis of in vitro reaction mixture

Reverse phase HPLC (Waters Alliance 2690 and Waters Tuneable Absorbance Detector 486, Waters Ltd, Herts., UK) analysis involved a 5 μm C_{18} column (250 mm × 4.60 mm; Phenomenex). A linear gradient with increasing methanol against 0.1 M acetic acid (pH 3.5 triethylamine) from 10% to 80% over 30 min was used and the eluate was monitored at 270 nm. To compare the activity of 71B6 towards ABA and its structural analogues, calibration curves of standard amounts of each analogue were generated. Following the in vitro reactions, any glucose ester formed was purified by HPLC and divided into two samples. One sample was cleaved by alkaline hydrolysis and the area of aglycone produced compared to the area of glucose ester in the second sample. This enabled the amount of glucose ester to be related to the amount of aglycone from the calibration curve.

2.4. Identification of ABA-GE using HPLC–mass spectrometry

HPLC–mass spectrometry was carried out using an Agilent HPLC attached directly to an Applied Biosystems Qstar Pulsar I mass spectrometer with a turbo ion spray source. The HPLC linear gradient used methanol (1% acetic acid) against water (1% acetic acid) solvents as above. The mass spectrometer was operated in negative ion mode with an ion spray voltage of −2500 V and the nebulisor and turbo gases set at 70 units. Parent ions of 425 *m/z* ratio were fragmented by collision-induced dissociation (CID) and product ions analysed from 50 to 660 amu. The low and high energy fragmentation experiments used collision energy settings of −10 and −30 U, respectively.

3. Results

3.1. Characteristics of the activity of 71B6 towards ABA

The Arabidopsis 71B6 enzyme, expressed as a recombinant GST-fusion protein in *E. coli* and purified by affinity chromatography, was incubated with the naturally occurring enantiomer of ABA ((+)-ABA). The reaction mix contained a more hydrophilic compound, eluting earlier than ABA on HPLC, characteristic of a glucose conjugate (peak A, Fig. 2A), not observed in the control without enzyme. The data shown are for the intact fusion protein; the activity was not substantially altered following cleavage of the GST protein (data not shown). No activity was detected towards the ABA catabolites, PA and DPA, nor other phytohormones including auxin, cytokinins, gibberellins and salicylic acid (data not shown).

Peak A disappeared on incubation of the reaction mix with 1 M NaOH (Fig. 2B), consistent with the cleavage of an ester-linked product by alkaline hydrolysis. To confirm the identity of the compound, negative electrospray liquid chromatography–mass spectrometry–mass spectrometry (LC–MS–MS) was used to compare an authentic ABA-GE standard (Fig. 2C) with peak A (Fig. 2D). An ion of mass to charge ratio (*m/z*) 425 was the major component both of peak A and ABA-GE. The value of 425 corresponds to the mass of an intact ABA-GE ion that has undergone negative electrospray ionisation $[M - H]^-$. Low energy CID product ion spectra of the 425 ion in both peak A and the ABA-GE standard show 3 peaks at 425, 305, and 263. The 263 ion corresponds to loss of the intact glucose moiety ($C_6O_5H_{10}$).

Further, the relative intensities of the product ion peaks in both traces are comparable, which implies that the glucose is attached at the same position.

The assay conditions of 71B6 towards ABA were optimised as described in Supplementary Figure 1. The enzyme had a pH optimum between 6.5 and 7.0 with activity substantially increased in the presence of DTT and Mg^{2+} (400%). The rate of production of ABA-GE was linear from 0 to 20 min for (+)-ABA from 0.05–2.50 mM (Supplementary Figure 2). In the optimum conditions, the V_{max} was 1.80 nkatals mg^{-1} (±0.11) with K_m of 0.28 mM (±0.04) and the k_{cat}/K_m of 0.5 mM^{-1} s^{-1} (±0.01) ($n = 3$, ±S.D.).

3.2. Activity of 71B6 towards structural analogues of ABA

Table 1 illustrates the relative activity of recombinant 71B6 towards a range of ABA-related structures. The enzyme was found to be highly selective towards the natural (+) enantiomer of ABA and was able to glucosylate many different structural analogues of ABA to varying degrees. All products of these reactions were degraded by alkaline hydrolysis implying formation of the respective glucose es-

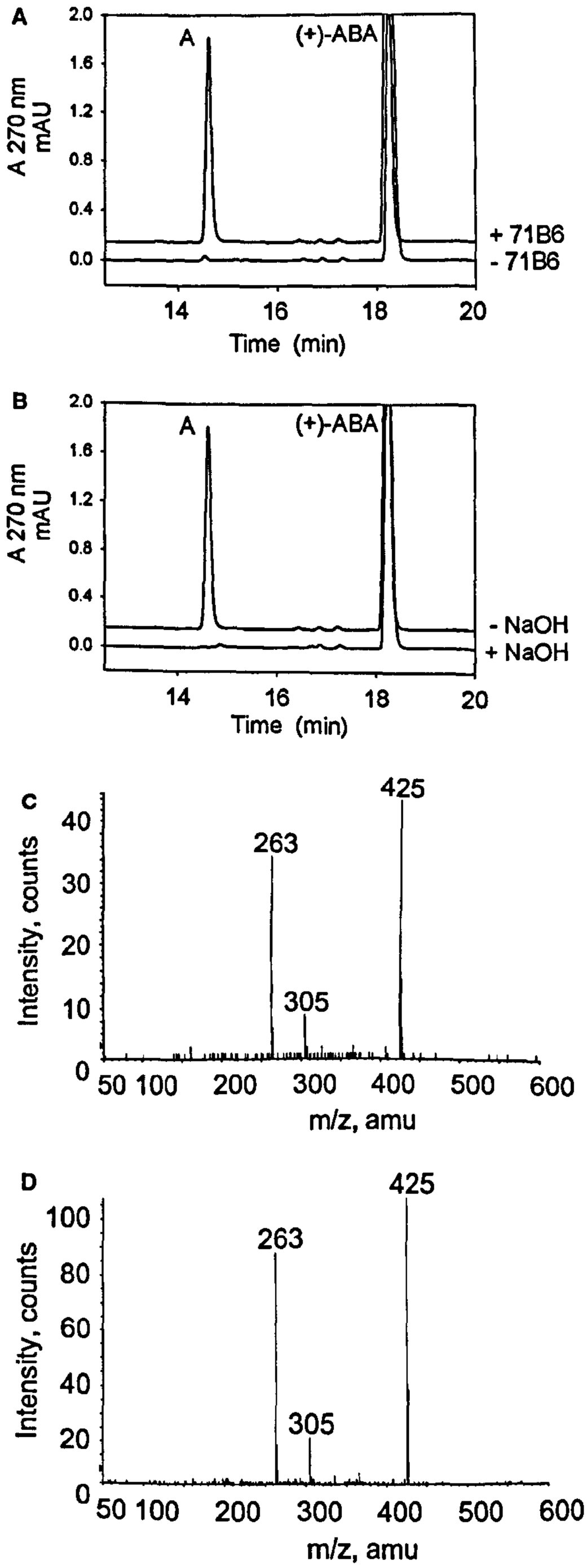


Fig. 2. Analysis of the reaction mix of 71B6 with ABA and UDP-glucose. The reaction mixes were analysed by reverse phase HPLC: with and without recombinant protein (A); following further incubation with and without 1 M NaOH for an additional hour (B). The traces are offset by 0.2 mAU. Negative electrospray LC–MS–MS CID spectra of an authentic ABA-GE standard (C) and the major product of recombinant 71B6 protein after incubation with (+)-ABA and UDP-glucose (D).

ters (data not shown). The activity of 71B6 was higher towards PBI-413, PBI-410 and PBI-82, compared to (+)-ABA. The activity of the enzyme towards the analogue PBI-287 was near-identical to (+)-ABA and much lower towards the remaining analogues.

4. Discussion

The homeostatic mechanisms controlling ABA levels in plants have attracted considerable interest over many years. A number of genes that function in the biosynthesis of ABA have been identified and their enzymes well-characterised [2]. In contrast until recently, no genes involved in catabolism had been identified and therefore there was no opportunity for detailed study of their recombinant enzymes. In 2002, Xu et al. [22] cloned a gene from Adzuki bean encoding an enzyme that glucosylated ABA in vitro. This was followed in 2004 by two reports of genes encoding cytochrome P450 hydroxylases that recognised ABA in vitro at the 8′-methyl group [23,24]. Earlier this year, eight recombinant UGTs from *Arabidopsis* were shown to glucosylate ABA in vitro, and only one of the eight, 71B6, displayed preference for the naturally occurring (+)-ABA [10].

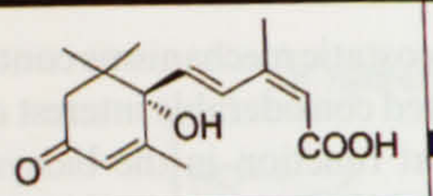
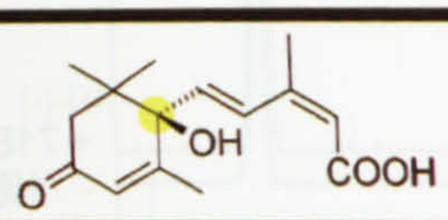
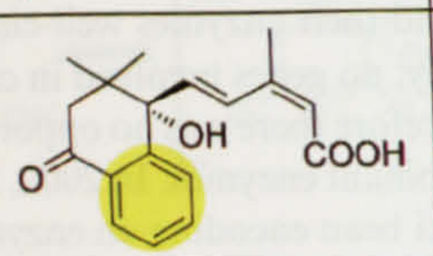
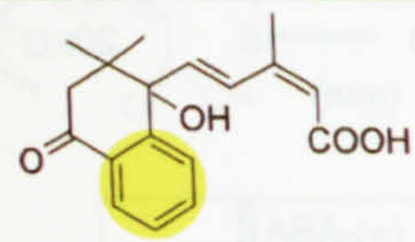
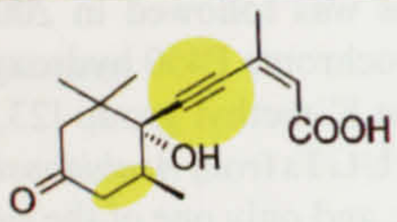
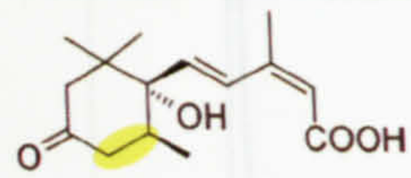
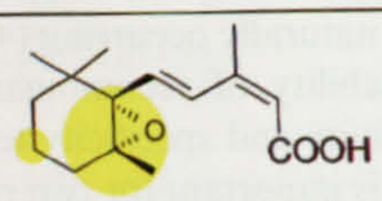
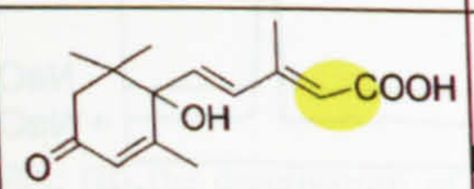
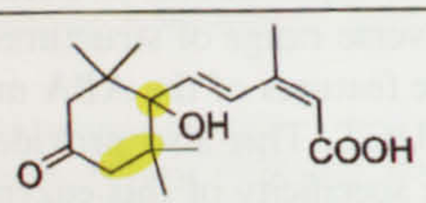
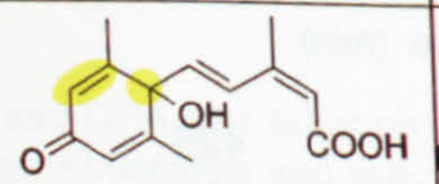
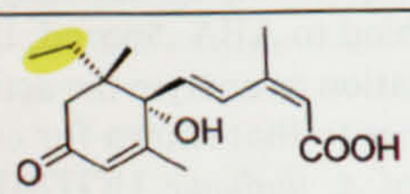
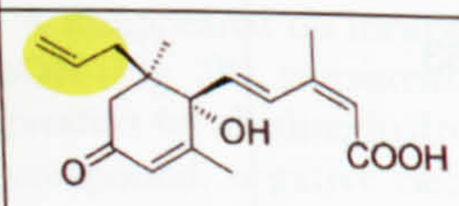
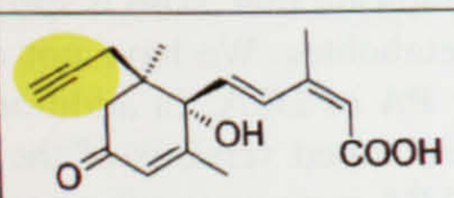
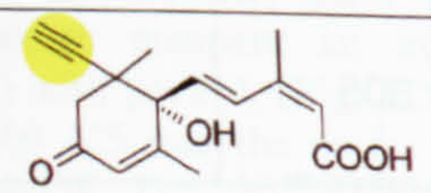
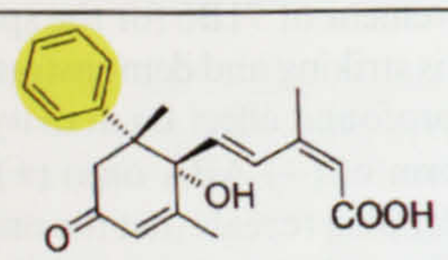
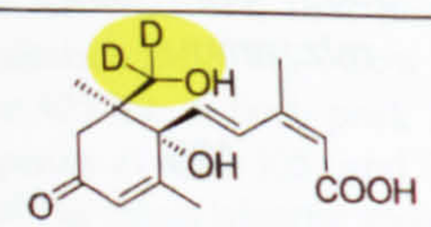
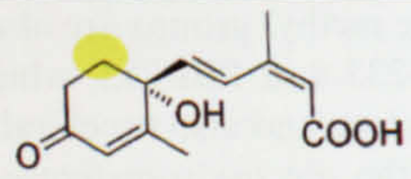
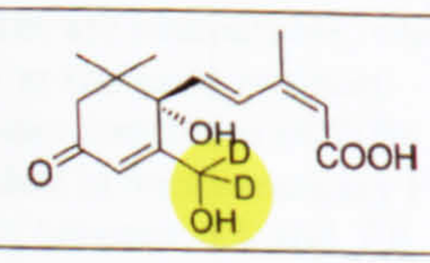
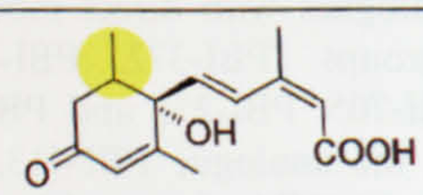
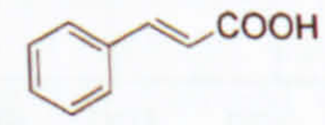
The availability of recombinant 71B6 has enabled us to study its activity and specificity towards a range of ABA analogues. This is important for two reasons. First, the availability of such a diverse range of structures allows a detailed investigation of the features of the ABA molecule required for catalysis by the UGT. This also provides a basis for going on to compare the specificity of this enzyme with others that recognise and/or bind to ABA. Second, the analogues can provide a useful foundation to analyse the activity of 71B6 in planta, in a similar manner to that shown for conjugation of auxin by another UGT of *A. thaliana*, UGT84B1 [25].

The data indicate that 71B6 is specific for ABA rather than its acidic metabolites. We have not detected any activity towards either PA or DPA. In addition, PBI-703 and PBI-705, which are deuterated versions of the catabolites 7′-OH ABA and 9′-OH ABA were poor substrates for 71B6.

The requirement of 71B6 for the specific chiral configuration of (+)-ABA is striking and demonstrates that structural changes can have a profound effect on activity [26]. Superimposing the unnatural form of (−)-ABA onto (+)-ABA with the hydroxyl groups overlapping reveals that the only change is in the relative position of the methyl groups on the ring. The relative orientations of these methyl groups are also changed in the poor substrates, PBI-233 and PBI-253, which have symmetry around the ring structure and also no chiral centre at the C1′-carbon.

Similarly, the glycosyltransferase also has reduced activity to those analogues with direct modifications to the 8′- and 9′-methyl groups (PBI-372, PBI-401, PBI-514, PBI-524, PBI-493, PBI-705, PBI-271 and PBI-293). This can be contrasted with the analogue PBI-413, which has an aromatic ring fused to the ABA ring, and is a significantly better substrate than ABA. It appears that whilst the enzyme can readily accommodate extra bulk around the double bond of the ABA ring and maintain activity, even slight alterations to the position or size of the 8′ and 9′-methyl groups result in substantial reductions in activity. Interestingly, a recent study showed that 5′α, 8′-cycloabscisic acid, when applied to radish seedlings, was glucosylated, despite modification of the 8′-methyl group [27]. Other UGTs may exist in radish with different activities from that of *Arabidopsis* 71B6, but it will be interesting to determine whether 71B6 can glucosylate the analogue in vitro.

Table 1
Activity of 71B6 towards enantiomers and structural analogues of ABA

Name	Structure	Activity relative to ABA (%)	Name	Structure	Activity relative to ABA (%)
(+)-ABA		100	(-)-ABA		4 ± 2
PBI-413		304 ± 25	PBI-410 (racemic mixture)		234 ± 22
PBI-82		179 ± 27	PBI-89		3 ± 1
PBI-287		102 ± 2	PBI-49 (racemic mixture)		4 ± 2
PBI-233		14 ± 7	PBI-253		3 ± 1
PBI-372		51 ± 9	PBI-401		7 ± 1
PBI-514		5 ± 3	PBI-524		6 ± 2
PBI-493		12 ± 1	PBI-705		0
PBI-271		9 ± 2	PBI-703		6 ± 2
PBI-293		17 ± 8	*trans*-cinnamic acid		12 ± 1

The results represent means ± S.D. of at least three independent protein preparations.

It is interesting that PBI-82 is a better substrate than ABA whereas PBI-89 is not glucosylated by the enzyme. Both PBI-82 and PBI-89 have the ring double bond reduced, however, PBI-82 also has a triple bond in the side chain. As this triple bond changes the disposition of the carboxyl group relative to the ring we may speculate that it improves the position for binding in the active site. Also of interest, PBI-287, that has no oxygen at C-4′ is as good a substrate for the enzyme as ABA, indicating that the ketone of ABA is not required for glucosylation by 71B6.

This study has enabled a clear distinction to be made between the activity of 71B6 and that previously described for the UGT from Adzuki bean, which had greater activity towards 2-*trans*-(+)-ABA than towards (+)-ABA or (−)-ABA [22]. In contrast, the *Arabidopsis* 71B6 displayed negligible activity towards 2-*trans*-ABA, (PBI-49, racemic mixture).

Analogues PBI-514, PBI-524 and PBI-401 are bioactive when applied to plants and were specifically designed as potential mechanism-based inhibitors of the 8′-hydroxylase of ABA and, therefore, inhibitors of the catabolic pathway of the hormone involving this enzyme [18]. Importantly, this study now reveals that 71B6 similarly does not recognise these analogues. It is therefore possible the analogues may represent a source of 'long-lasting ABA' agrochemicals for field applications, but first it will be necessary to confirm that other UGTs do not glucosylate them, whether in the model plant, *Arabidopsis*, or in target crop species. If indeed the compounds prove not to be susceptible to the major pathways of either catabolism or conjugation, they could be useful in revealing any minor pathways of controlling ABA homeostasis.

In the future, it will be interesting to determine the phenotypic consequences of changing the level of expression of *71B6* in the plant. These analogues will be helpful to demonstrate a link between phenotype and glucosylation of ABA in these plants in a similar manner to the use of IAA and its analogues in the study of UGT84B1 [25].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2005.06.084.

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