Chapter 6 – Discussion

Drawing meaningful conclusions based on physiological observations in a genetically altered plant is often difficult as there may be a number of direct or indirect effects due to the altered genome which contribute to a given observation. As outlined in the introduction, several members of the EPF secreted peptide family are expressed in the developing leaf and have no other known function besides the control of stomatal development. Although their role as intercellular development signals has been extensively reported, at the start of the current study there was nothing known about their effects on plant physiology. It was important to test a range of possible effects that manipulation of these genes could cause including altered internal leaf structure, leaf size and capacity for carbon fixation. The experiments presented in Chapters 3, 4 and 5 used a range of EPF mutant plants with stomatal densities ranging from approximately 20% to 300% of wild-type values, grown under two different watering regimes and three different atmospheric CO2 concentrations. The most obvious and surprising finding in this study was that plants with significantly reduced stomatal densities produced much larger leaf biomass. However, as evidenced by the A/Ci curves, for a given Ci (carbon dioxide concentration inside the leaf), altering EPF expression levels did not cause significant differences in carbon assimilation rates (Chapter 4). In addition, no structural differences of the mesophyll were observed between plants with altered EPF expression levels (Chapter 3). Cumulatively, these data support the premise that altering EPF expression levels in the leaf cause *isolated* phenotypic changes to stomatal patterning without effecting photosynthetic biochemistry. Thus, the use of this peptide family as a tool to examine mechanical properties of leaf gas exchange in isolation is validated. Given the EPF family is conserved in a wide range of plants (Rychel *et al.*, 2010) including barley (Fig 6.1), it may be interesting to explore similar questions by altering EPF homologue expression in agronomically useful crops.

**6.1 The possibility of engineering desirable traits in economically useful plants by altering stomatal density (*D*)**

Despite the rapid growth in food production leading to a reduction in world hunger over the past half a century (FAOSTAT*,* [*http://faostat.fao.org/default.aspx*](http://faostat.fao.org/default.aspx) *(2011)*), more than one in 7 people are malnourished (Food and Agriculture Organization of the United Nations (FAO), *State of Food Insecurity in the World 2009* (FAO, Rome, 2009)). Although many of the solutions to world hunger are of a political and economic nature increasing yield by adapting farming methods, plant breeding and genetic manipulation of crops is hoped to play a role in increasing food production by a necessary 70 to 100% by 2050, without increasing agricultural land area (Godfray et al. 2010; World bank development report 2008). Agriculture accounts for 80-90% of all fresh water used by humans globally (Morison et al. 2008) but with world population predicted to reach 9 billion by 2050 (Godfray et al. 2010) and fresh water supplies diminishing, one of the major challenges we face is how to make plants yield more with less water (Morison et al. 2008). Selection for increased water use efficiency (WUE) has often led to diminished yields (Blum 2009) as WUE is often driven by traits which reduce gas exchange and thus limit photosynthetic carbon fixation. The experiments reported in this thesis largely confirm this negative correlation between WUE and yield. Although plants engineered with low *D* and *g*wmax (*EPF2*OE) had increased WUE and were able to tolerate drought for longer, they yielded less than a third of the mean seed per plant of the wildtype. Nevertheless, genetically engineering crops with reduced *D* may have a role in drought prone regions of the world particularly in places where subsistence farming and resource-poor cropping systems are employed (Blum 2005). However, severe drought is often prolonged and survival does not necessarily lead to economically viable productivity, meaning that traits increasing drought resistance can be of limited use in commercial farming (Passioura 2002, Passioura 2004, Morison et al., 2008).

Altering expression levels of EPF family peptides to reduce *D* in leaves may be useful for plants for which seed yield is less important. Overexpression of EPF2 in Arabidopsis led to reduced *D*, larger leaves, a larger total leaf area and a higher biomass. In “leafy greens”, such as lettuce where the commodity is the leaf rather than the seed, a larger leaf biomass is clearly desirable. In addition, both larger leaves and reduced stomatal indices have been associated with longer shelf life in lettuce (Zhang et al. 2006). Currently, increasing shelf life of ornamentals can be achieved by application of ABA which closes the stomata thus reducing leaf transpiration and water loss (Pompodakis et al., 2004; Waterland et al., 2010a; Waterland et al., 2010b; Kim and van Iersel, 2011). However, given the wide range of biochemical changes driven by ABA, its use can result in undesirable outcomes. Application of ABA led to early leaf abscission in Silvia (Kim and van Iersel, 2011) and decreased the shelf life of miniature potted roses (Muller et al. 1998). In addition, plants grown in high relative humidity (such as in a greenhouse), develop larger, malfunctioning stomata which are unable to close even with application of ABA and diminish the plant’s shelf life (Nejad and van Meeteren, 2005, 2007). Engineering reduced *D* lowers *g*wmax and minimises transpiration without the need to apply ABA, and in cases where stomatal closure is malfunctioning. *EPF2*OE plants had increased WUE, lower levels of transpiration in all conditions, and survived drought treatment significantly longer than wildtype plants indicating that a lower *D* could indeed increase shelf life. Thus, altering expression levels of the EPF family of peptides to reduce *D* in ornamentals such as Silvia, and leafy greens like lettuce could be used to limit waste, cut costs and in the case of the latter, increase yield.

Plants with increased *D* (*epf1epf2*) did not yield more seed and had a lower total dry biomass (Chapter 5) despite higher leaf conductance and a lower WUE (Chapter 4). This is due to increased epidermal cell division which limited leaf expansion (Chapter 3) and photosynthetic rates, which were not significantly increased with respect to the wild-type rates (Chapter 4). In ambient conditions, leaves with low *D* (*EPF2*OE) were limited more by Ci whereas leaves with high *D* (*epf1epf2*) were limited more by the ability fix carbon, as their higher conductance ensured a high Ci. It seems likely that one major reason *epf1epf2* plants were unable to grow larger, photosynthesise more and increase their seed yield is due to the mutation specifically increasing conductance without altering the leaf’s ability to fix carbon. Nevertheless, yield in cotton and wheat has been associated with cooler leaves due to increased transpiration (Lu *et al.*, 1998). Increasing *D* by altering EPF expression in certain well irrigated crops grown in hot climates could potentially increase yields.

**6.2 Future work**

Despite higher conductance, *epf1epf2* was unable to photosynthesise more than Col-0 indicating that simply increasing *D* alone is not sufficient to increase seed yield. An auxin pathway mutant was found to have higher levels of photosynthesis (Fig 6.2) as well as denser leaves with more chloroplasts per mesophyll cell (personal communication, Dr Brian Jones, University of Sydney). Leaf conductance is not significantly different from wild-type at ambient CO2 but for a given Ci above 250 µmol.mol-1 this mutant has a higher rate of carbon fixation. Attempts to increase *D* by altering EPF expression levels in the mutant are taking place in an effort to boost yields by coupling the augmented photosynthetic capacity with increased conductance.

Steps have also been taken to genetically alter *D* in barley by designing a gene construct for overexpression of two distinct EPF-homologues (Fig 6.2) via *Agrobacterium* mediated transformation of barley. By collaboration with James Hutton Institute in Dundee barley EPF gene homologues were identified in the unpublished genome sequence. Altering *D* in barley would allow similar experiments to be carried out and help determine whether (i) a negative correlation between *S* and *D* occurs in a monocot leaf where cellular division is ranked, (ii) decreasing *D* for a same cultivar increases WUE, and whether this is again accompanied by a reduction in seed yield, (iii) photosynthetic rates are increased alongside *D* or whether like in *Arabidopsis*, carbon assimilation rates do not increase simply by increasing conductance.

Given public resistance to GMOs due in part to studies indicating dangers associated with certain GM crops (Ewen and Pusztai, 1999; Seralini *et al.*, 2012), alternative methods to alter *D* in commercially viable plants, such as targeting induced local lesions in genomes (TILLING)(McCallum *et al.*, 2000) can be persued.

The data set explored in this thesis is far from complete. An adequate examination of root size and structure, psychrometric sampling in the apical meristem, sucrose sampling along the phloem, and xylem mass transfer rates are just a few of the tests which could be carried out to build upon an understanding of how *D* affects development in *Arabidopsis*. In addition, the work could be improved by overexpressing the EPF signalling peptides in the leaf alone, under the direction of more specific gene promoters, to verify that the effect on seed yield is due to the indirect effect of *D* rather than direct ligand-receptor interactions.

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**Fig 6.1: Alignment of *Arabidopsis* epf1, epf2, epfl9 amino acid sequence with 2 barley homologues.** The six cysteine residues conserved in all EPFs and vital to structure and function are also present in the barley homologues. Results of a search of the unpublished *Hordeum vulgare* genome sequence carried out in collaboration with the James Hutton Institute, Dundee.

**Fig 6.2: An auxin pathway mutant has a higher capacity for carbon assimilation.** Methods are identical to methods used for the EPF peptide family A/Ci curves (chapter 4). Error bars represent standard error. (n=4). Unpublished results of an experiement carried out by me in collaboration with Dr B. Jones at the University of Sydney.