The University Of Sheffield.

Molecular genetic studies on the manipulation of Arabidopsis traits for potential crop improvement

By

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Dedication

To my parents who always made me believe I could.

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Summary

Production of food is vital for the sustainability of human kind. Several pressures have been increasing within the last 50 years that have put a stress on food production these include; a large increase in population, soil quality loss, increases in extreme weather conditions and an increase in competition for agricultural land. Ensuring the population is fed currently involves agrichemicals, intensive farming and some non-sustainable farming techniques. With population rising and other pressures increasing there has been a large push, both commercially and politically, to find a more sustainable way to feed future generations. For this we must look to new technologies for example the use of genetically engineered crops.

In this thesis I describe experiments that investigate potential novel herbicides, as well as plants which were engineered to be resistant to the herbicides. The two in co-ordination could be used to increase the sustainability of arable farming. Although some of the chemicals showed some efficacy they were not effective enough to be seen as commercially viable. The genetically engineered plants also showed some resistance to the herbicide, however results were variable and an intrinsic defect caused by the genetic mutations was observed.

The final results chapter of this thesis discusses genetically engineered plants, EPF family mutants with an altered stomatal density that have an increased water use efficiency. I was able to observe a complex relationship between root architecture and transpiration in these mutants. Furthermore I was able to see a relationship between root hair mutants and stomatal traits. These results indicate a potential for both

genetically engineered plants, however they also highlight the necessity to investigate potential knock on effects from the modifications.

Abbreviations

°C	Degree Celsius
µl	microliter
a. thaliana	*Arabidopsis thliana*
ABA	Abscisic Acid
Bicine	N,N'-bis(2-hydroxyethyl)glycine
BLAST	Basic Local Alignment Research Tool
cDNA	complementary DNA
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EPF	Epidermal patterning factor
EPSPS	5-enolpyruvyl-shikimate synthase
HPLC	High Performance Liquid Chromatography
IGPD	imidazoleglycerol-phosphate dehydratase
kb	Kilobase
M	Molar
MAPK	mitogen-activated protein kinase
mRNA	Messenger RNA
n	number in a study or group
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
RNA	ribonuclec acid
RT-PCR	reverse transcription PCR

SDD	Stomatal density and distribution-1
SDS	Sodium dodecyl sulfate
TMM	Too many mouths
Tris	tris(hydroxymethyl)aminomethane
β-gal	β-galactosidase

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1. Chapter 1 – Introduction

1.1 An introduction to food security and sustainable agriculture.

Production of nutritious food is vital for the sustainability of humankind, but several factors are putting an increasing stress on food security. Food security has been defined as "the ability of a country or region to assure, on a long-term basis, that its food system provides the total population access to a timely, reliable and nutritionally adequate supply of food" (Eicher and Staatz, 1986) - thus describing food security as a multi-facetted issue which encompasses technology, environmental conditions, government policies and human population to name just a few. Sustainable agriculture is agriculture that incorporates the protection of the environment, human health and animal welfare while maintaining food security.

There are two types of food insecurity: chronic, long-term malnutrition, and short-term food insecurity, normally caused by crop failure for a season or several seasons (Weber et al., 1988). One of the most prominent examples of short term food insecurity was the great Irish potato famine during the years of 1845-1852. During this time it is not exactly known how many Irish people lost their lives but it is thought Ireland's population fell by around 25% due to around 1 million plus deaths and 1.5 million people emigrating (Woodham-Smith, 1962). The potatoes were infected by *Phytophthora infestans* (Fry and Goodwin, 1997), commonly known as blight, which decreased crop yields, but the severity of the famine was believed to be increased by several different factors. These include: a large increase in population in the early 1800s, poverty leading

to many eating a diet largely dependent on potato and buttermilk and poor economical trading agreements with the rest of Britain (Woodham-Smith, 1962). In this example alone, it's clear just how many facets there are to food insecurity.

One factor that is regularly associated with food insecurity is an increase in population, both within a community and globally. Global population has been increasing exponentially, and in the past 50 years it has trebled from 2 billion to 6 billion and is predicted to continue rising to a peak of around 9 billion in 2050 (U S Census Bureau, 2007)(U S Census Bureau, 2007)(U S Census Bureau, 2007)(U S Census Bureau, 2007)(U S Census Bureau, 2007)(U S Census Bureau, 2007), figure 1.1. The ability to feed so many more people in such a short space of time has widely been accredited to the green revolution.

1.2 The green revolution

The green revolution is usually defined as the period between the 1960's and 2000's where food production increased by 250% to sustain a population that trebled during the same time period (Kendall and Pimental, 1994). Land which had previously been thought of as unsuitable for agriculture was increasingly being farmed with the use of additional fertilisers, pesticides and other new technologies like high-yield crop varieties, see figure 1.2 (Conway and Barbier, 1990, Evenson and Gollin, 2003).

The high yielding cultivars created in the 1960s were mainly in wheat and rice, as research in plant institutes focused predominantly on these crops. The high-yielding breeds were able to compartmentalise their resources differently to increase yield, in the form of grain, and decrease the resources invested into the stem. Varieties were created that were shorter than traditional breeds, leading to the name “dwarf breeds.” Furthermore, the modified breeds responded better to both fertilisers and good irrigation systems, which meant their use rapidly spread globally, particularly in areas like Latin America and Asia where good irrigation and fertilisation were available (Dalrymple, 1986).

Although globally the up-take of the new varieties was fast and widespread this was not true for sub-Saharan Africa, where conditions were not as favourable for the crops – at least until the 1980s when crops were specifically bred for the conditions of that region (R. E. Evenson et al., 2003).

The yield, expressed in Hg/Ha of wheat (figure 1.3A) and rice (figure 1.3B) show an increase from the 1960s in developed countries like the United Kingdom and America; in contrast in South Africa increases in yield did not occur until the 1980s. The United Kingdom is a very good example of just how quickly yield increased during this period. Over the course of 1,000 years, it rose from 0.5 to 2 metric tons per hectare, before shooting from 2 metric tons to 6 in just 40 years (Hazel, 2002).

Figure 1.3 represents yield of both wheat and rice, however, it should be taken into account that different varieties of these crops are grown in the different areas. Particularly the differences in winter and spring wheat

growth in different countries could cause skewing of the data. When looking at percentage difference between yields over the ten year periods, there are no significant differences between the developed countries, USA, UK and China and South Africa. However, South Africa is a relatively developed African country. Unfortunately comparable data is not available for less developed African countries during the same time period.

The rise in plant research globally from the 1960s to the early 1980s, led to an increase in the available types of crops that are high yielding, including sorghum, millet and barley. In developing countries it is estimated that around 20% of the increase in yield was due to the introduction of high-yielding varieties. The other 80% is due to expanding the area of agricultural use and other inputs, like fertiliser, irrigation and agricultural technologies [8].

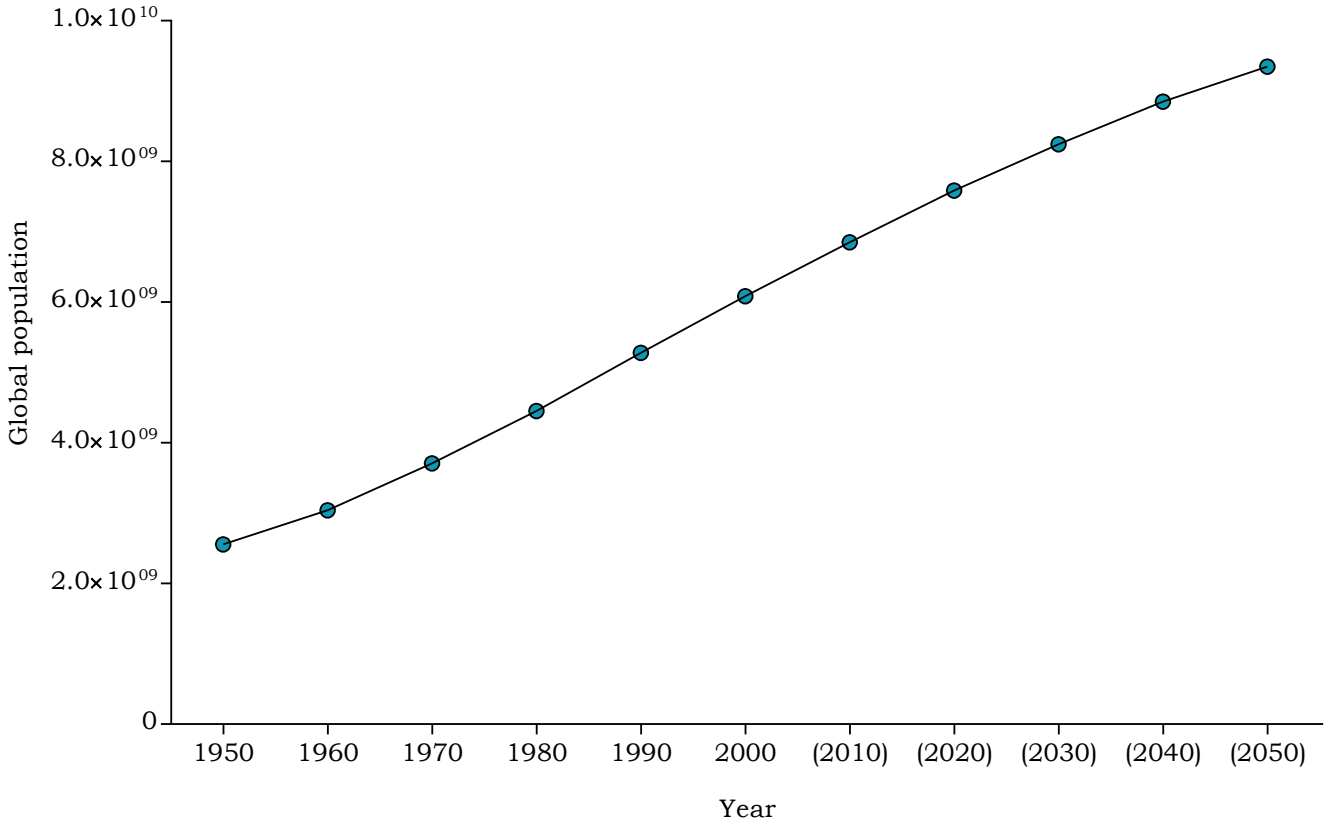


Figure 1-1 global population growth.

Global population increase from the 1950's to 2000 and predicted, indicated with brackets, population from 2010 to 2050. Population has increased from 2 billion to over 6 billion in the last 50 years. It is predicted that this will increase to 9 billion by the middle of the 21st century. Source: U.S. Census Bureau, International Database.

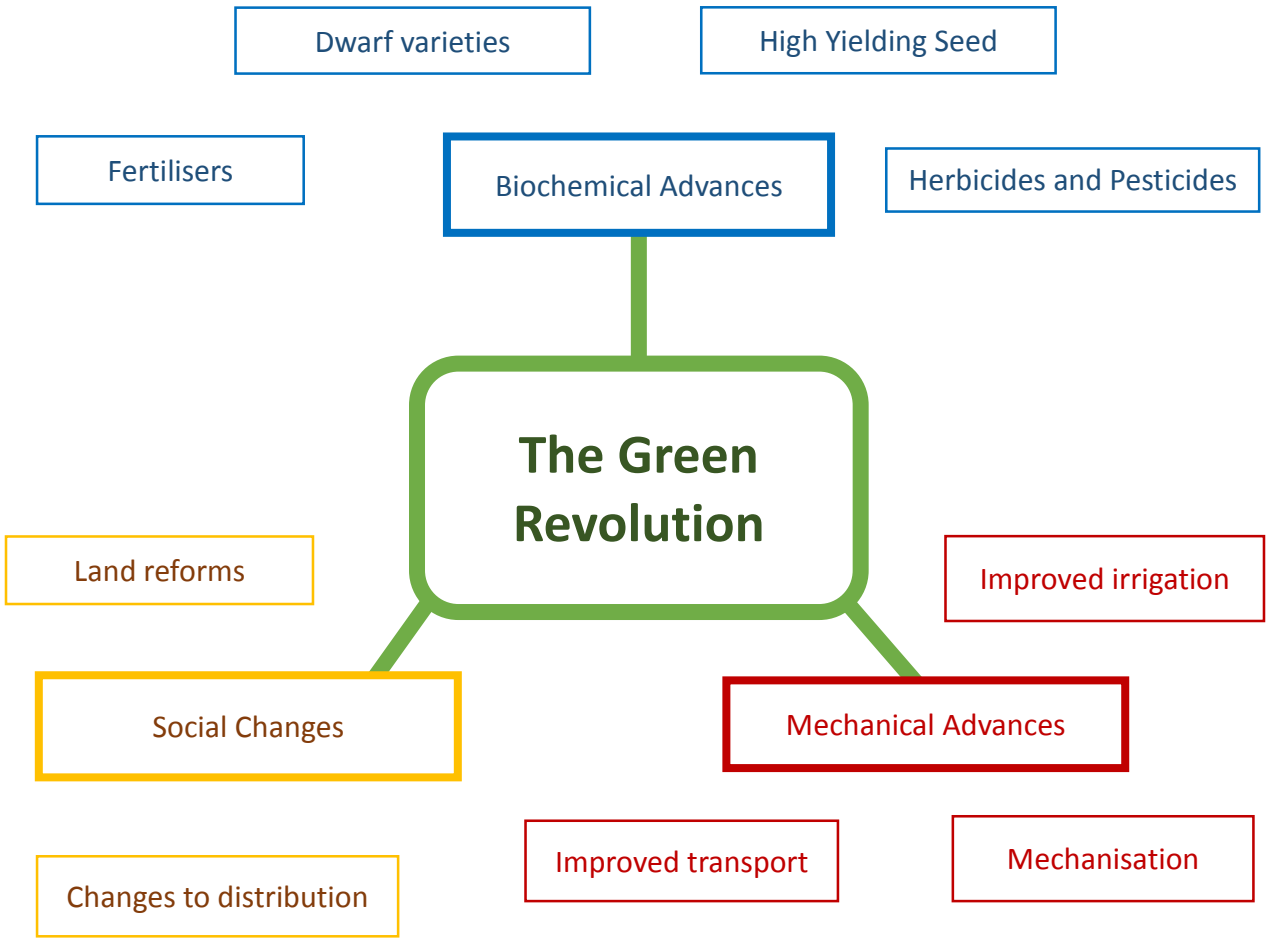
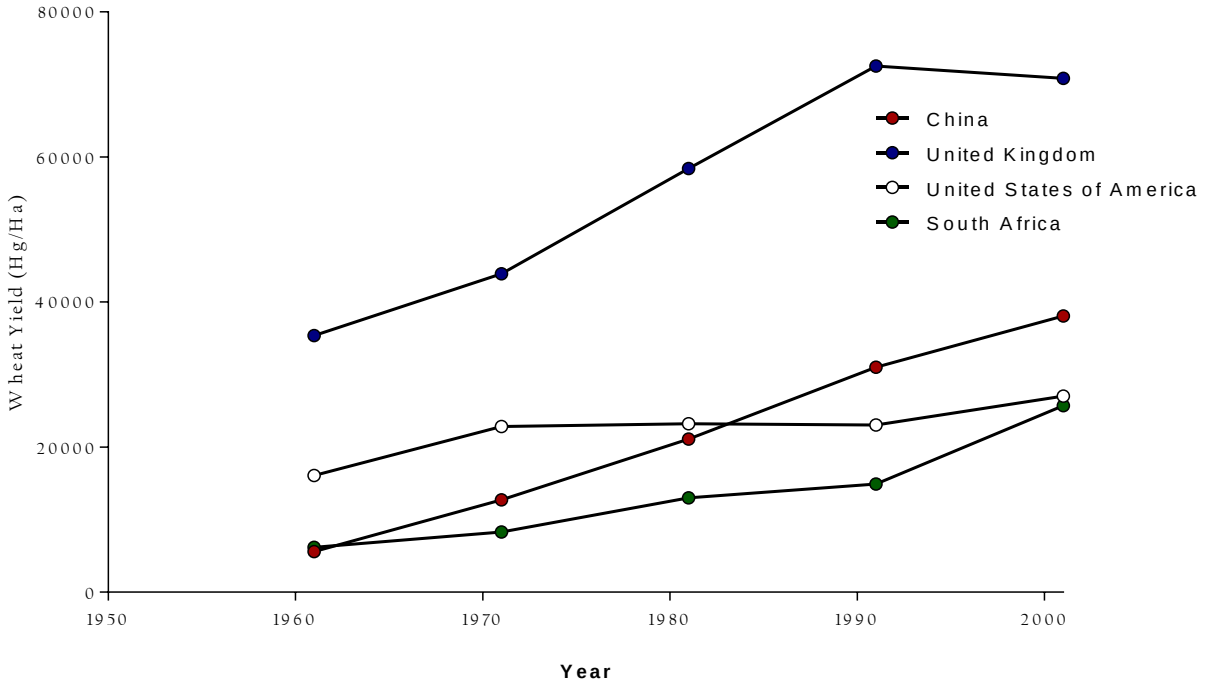


Figure 1-1 the green revolution

Providing enough food for the growing population in the late 20th century is mostly attributed to the green revolution. Figure 1.2 is a diagram showing the three main areas that improved in agriculture to allow for the green revolution. These included biochemical, mechanical and social changes.

A



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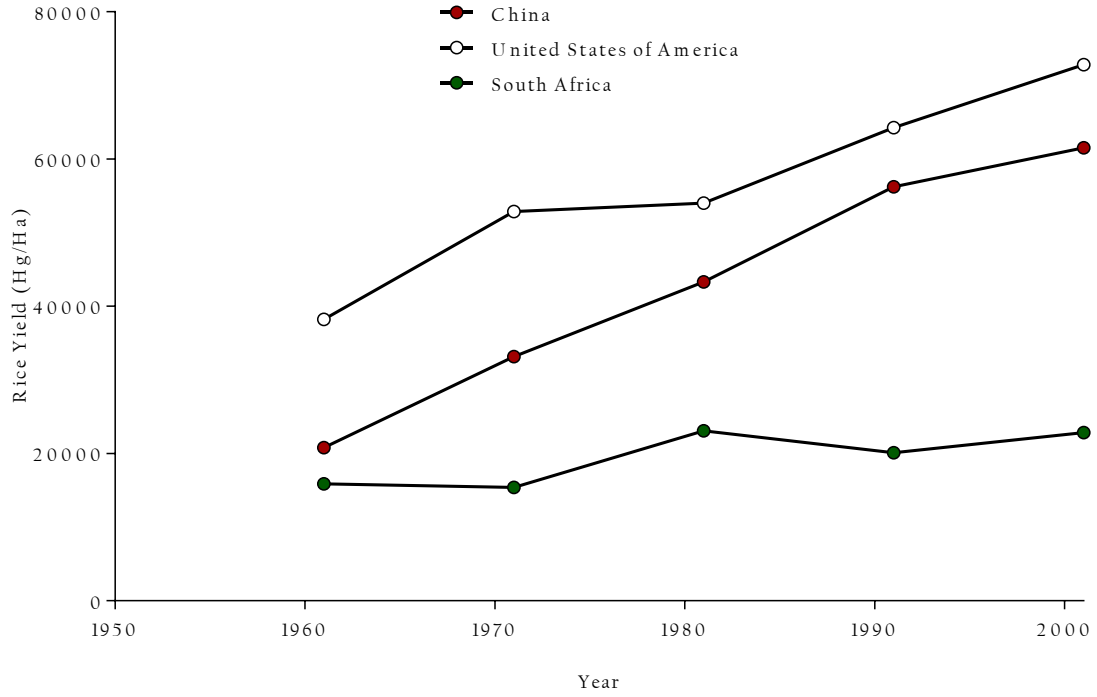


Figure 1-2 Comparing crop yield in different countries over the green revolution

1A, yield increases in wheat in different countries over 40 years. 1B, yield increases in rice yield in different countries during the green revolution. South Africa has a delayed response to the green revolution in comparison to countries like the UK and USA. Data from FAO.

There were clear benefits for both crop growers and consumers. The increase in yield led to an increase in income for the farmers, which then trickled down to the consumer through the decreasing of food prices. This in turn accelerated the amount and diversity of calories consumed and enhanced global nutrition. This often meant that, in places like Asia, where dwarf varieties were particularly popular, livestock, vegetable oils and fruit were consumed more and more (Hazel, 2002).

Although it can be argued that the green revolution saved many lives by providing food to a growing global population, there were some disadvantages. In particular, there were negative socio-economic and environmental impacts. Firstly, as the crops were most productive in well-irrigated land, their adoption dramatically increased irrigation. This had a two-fold effect. Firstly, the sheer amount of water used to irrigate the new crops caused a decrease to the water table. Secondly, it meant only the farms that could afford irrigation were able to grow the higher-yielding crops - often out-competing smaller farms that could only afford to grow the older varieties without irrigation (Lipton and Longhurst, 1989).

There were other concerns, too, about environmental impacts. An increase in the use of fertilisers and other agri-chemicals was associated with pollution of the environment - for example, in lakes and rivers near farms, by nitrification. The leaching of nitrogen into local water streams can be dangerous to humans, if consumed (WHO, 1984); moreover it can cause algal blooms, eutrophication and fish poisoning (Howarth, 1988).

So by intensifying agriculture, farmers also harmed the ecosystems around them and decreased biodiversity.

Many of the same pressures on food production seen in the latter period of the 20th century are cropping up again, however this time climate change brings another dimension to finding a solution.

13. The effects of climate change on food security.

The increasing atmospheric CO_2, mostly associated with the burning of fossil fuels, has led to an increase in global temperatures, exacerbated in recent years. There have been dramatic changes in weather conditions, with an average 0.2°C rise in global temperatures since the 1970s and a 2% increase in global precipitation within the last 100 years (IPCC, 2007a). Modelling results by the IPCC showed it is likely that an increase in atmospheric carbon dioxide and global temperatures by 1-3°C could improve overall yield, yet above those temperatures yields decrease. Experiments in controlled condition chambers have shown an increase in CO_2 and temperature could increase crop yield by around 10-20% in C_3 plants and around 0-10% in C_4 plants (Heller and Zavaleta, 2009). Temperature changes within 1-3°C could nevertheless effect pathogen prevalence, pest outbreaks and crop fires, which are not accounted for in controlled condition experiments (IPCC, 2007b).

Controlled condition chamber experiments have generated a vast amount of data in previous years, particularly looking at responses to changing atmospheric CO_2 levels. Free-air CO_2 enrichment (FACE) experiments have allowed for a more realistic view of what would happen in the field if atmospheric CO_2 levels continue to increase. FACE experiments keep the

majority of field conditions the same, but supplementing the CO_2 levels. FACE experiments have shown that controlled chamber experiments majorly over-estimate the increase in yield that is seen with an increase in CO_2 (Leaky et al., 2009).Moreover, a larger threat to both global food security and sustainable agriculture is predicted due to an increase in extreme weather events. It is expected that the increase in quantity and severity in drought, flooding and heat stress will have a dramatic effect on crop yields, especially in regions where weather conditions are already unfavourable (Parry et al., 2004).

Land use is moving away from a local issue and towards a global issue. With an increase in agriculture to support the growing population, one of the most current estimations of global land use approximates that 30% of land is used for grazing and cropland (Foley et al., 2005). This is associated with negative effects on the global ecosystem, however it will be very difficult to provide food for an estimated 9 billion humans by 2050, without expanding this area.

Other pressures are being placed on the amount of land available to agriculture. With concern growing over fossil fuels adding to climate change, there is a move towards alternative renewable fuels, figure 1.4. These include wind farms, solar farms and biofuels, which are becoming more popular all of which are competing with cropland and pasture.

1.4 Pest control and pathogens

Although very controversial, banned pesticides like DDT used in the 1970s and the more recent worries over neonicotinoids, mean providing sustainable agriculture while decreasing the use of pesticides is a high

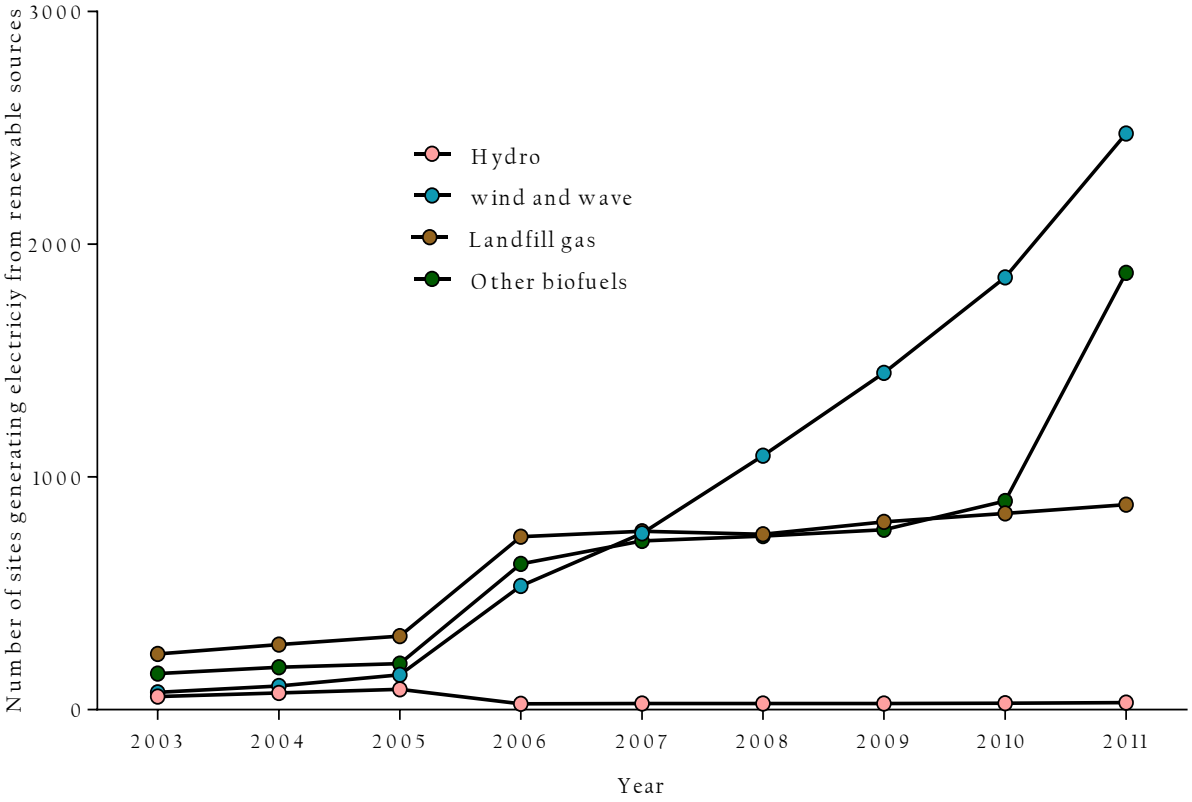


Figure 1-3 Renewable energy sites in the UK

Number of sites in the United Kingdom generating renewable energy from 2003-2011. All renewable energy production has increased with the exception of hydro power. Data from Gov.uk

priority (Lechenet et al., 2014). The majority of agriculture, particularly in temperate regions is intensive and includes the use of pesticides (Tilman et al., 2001). Alternatives to intensive farming have been established - for example organic farming, where no synthetic pesticides or fertilisers are used and integrated farming, which falls in-between organic and intensive farming. However, even though organic farming has increased in both Europe and the US, by 40-50% from 2003- 2010 (European commission, Accessed 19 September 2014) and 270% from 2000- 2008 respectively, the usage of synthetic pesticides has also increased by 3-8% in both Europe and the US from 2000-2007 (FAO Stats, Accessed 19 September 2014, US Environmental Protection Agency, Last accessed Septemeber 2014). Furthermore even the use of organic agrichemicals has its controversies.

Organic agriculture was based on the views of the early founders of the Soil Association in the 1940s and members claim it is a superior way of farming, however a lack of scientific evidence leaves these claims unsupported (Trewavas, 2001). The Soil Association claim that a way of stopping the decline in wildlife within the UK is by supporting organic farming (The Soil Association, Last accessed 26/09/2014). However, by incorporating biodiversity measures into conventional farming, for example field margins, farmers can sustain yields, while having equal benefits to wildlife as organic farming (Communities., 1990). Organic chemistries can have the same negative effects as synthetic ones, like algal blooms. Furthermore the practices that take the place of newer technologies can cause harm to the farm land and the environment, a prime example of this is the increase in ploughing instead of the use of

herbicides, which has negative impacts on the soil and also increases the carbon footprint of the farm (Warne, 1999). The yield deficit in organic farming is also a problem, a conventional farm can match the yields of an organic farm using just 50-70% of the land (Trewavas, 2001). Considering todays pressures on food security organic farming would unlikely be a feasible solution to feeding the increasing population.

60% of crops used for food production are made up of three main cereals - wheat, rice and corn, derived from one weedy species, making global food security highly susceptible to large losses from pathogen infection (Tilman et al., 2002). Fortunately, due to crop rotation and agricultural chemicals, large global losses have been a rarity. One major issue of synthetic agricultural chemistries is the longevity of a chemical before resistance to it arises. In general, some pathogen strains become resistant to antibiotics within 1-3 years, some insects become resistant to insecticide after approximately 10 years (Tilman et al., 2002) and strains of weeds resistant to specific herbicides are observed after around 20 years (Palumbi, 2001).

For successful control of pests, weeds and pathogens, rotation of agrichemicals with different modes of action is vital. Finding new agrichemicals is not an easy matter. For example, since the 1940s, when the first synthetic herbicides were introduced, only 170 compounds have been commercialised. All these compounds share only 17 different modes of action and, of these modes of action, half target just three plant systems: photosystem II, photoporphyrin oxidase and acetolactate synthase (Lein et al., 2004). Solutions need to be found by either

developing more novel agrichemicals, or using other technologies, like using plant biotechnology to modify a plant to be resistant to certain pests.

1.5 Nutrient-use efficiency – Phosphate and nitrogen

The green revolution saw the rise in the use of phosphorous and nitrogenous fertilisers. A large proportion of the phosphorous used in the fertilisers that sparked the green revolution was from the mining of rock phosphate (Cordell et al., 2009). Phosphate is needed for plants to grow and around 90% of the demand for phosphorus is from the agricultural industry. It has been predicted that rock phosphate resources, most commonly used for agricultural fertiliser could be exhausted as early as within the next 50 years, although other estimates predict it could take around 100 years (Steen, 1998, Smil, 2000).

Fertiliser usage increased 7-fold between the years of 1960 and 1995 (Pinstrup-Andersen and Pandya-Lorch, 1996) and is likely to increase 2 to 3-fold by 2050 (Tilman et al., 2001, Cassman and Pingali, 1995). In more developed countries, the over-fertilisation of farming land over the past 40 years has led to the soil having good stocks of nutrients. Therefore, farmers are becoming more aware of over-fertilising, leading to a decrease in use. Yet just as important as the use of fertilisers is their efficiency. Global estimations predict that approximately 45% of phosphate and around 30-50% of nitrogen used in fertiliser is taken up by the crop (Smil, 2000). However, in other countries - for example, countries in sub-Saharan Africa where fertiliser use is not common

practice - soils are starved of nutrients and yield is poor. In places like this, fertiliser use is likely to increase (Cordell et al., 2009).

With increasing pressures and decreasing resources, it is vital for us to increase nutrient use efficiency in agricultural practices. Solutions to this problem include precision agriculture, Figure 1.5 A, and using biotechnology to increase nutrient uptake capacity.

1.6 Water use efficiency

Climate change is predicted to increase drought and water limiting conditions in semi-arid environments (Stocker et al., 2013, Hatfield et al., 2011), which may lead to an increase in land that needs irrigation, decreasing the sustainability of agriculture. Currently, only 18% of farmland is irrigated. Nevertheless, this irrigated farmland is responsible for around 45% of global food production (Döll and Siebert, 2002) making irrigation vital for global food security and likely to become increasingly so. There are large regions, particularly in Asia and Africa, that will soon have an inadequate amount of water to sustain irrigation regimes that will provide, per capita, enough food production (Seckler et al., 1999). Like competition for land use, fresh water competition has increased due to fresh water fisheries, restoration of streams and lakes and urban water use (Tilman et al., 2002). Getting 'more crop per drop' is a rising issue on the global political and scientific agenda, with some people calling for a new, 'blue revolution' to follow the green revolution in the late 20th century (Gagoa et al., 2014).

Water use efficiency is the effectiveness of a crop to fix carbon normalised by the amount of water used. Using different techniques, this can be measured on several different levels from the intrinsic water use efficiency at leaf level to whole canopies and even ecosystems (Gagoa et al., 2014). There have been an increasing number of studies looking at how to improve water use efficiency in different crop models, some using biotechnology to achieve this (Doheny-Adams et al., 2012, Morison et al., 2008, Davies and Sharp, 2000) . Irrigation can be decreased using different methods, including technologies like drip pivot irrigation, figure 1.6 B, and increasing water retention by decreasing ploughing or the addition of manure to the soil (Tilman et al., 2002).

1.7 Soil fertility and degradation

Land is used by humans in many different ways - from urban uses, like housing and infrastructure, to more rural uses such as agriculture. Different uses put different strains on the land (Setälä et al., 2014). Over the past 80 years the global landscape has gone through dramatic changes (Vitousek et al., 1997), with an increase in intensification of agriculture as well as urbanisation (Effland and Pouyat, 1997). Soil resources are finite and prone to degradation, particularly under poor land management systems (lal and Stewart, 2013).

3,500 MHa of land is thought to be prone to degradation globally, due to agricultural practices and climatic conditions (Bai et al., 2008). Agricultural practices have radically increased soil degradation, leaving much agricultural land with a reduced top soil and decreased fertility (lal, 2001).

A

B

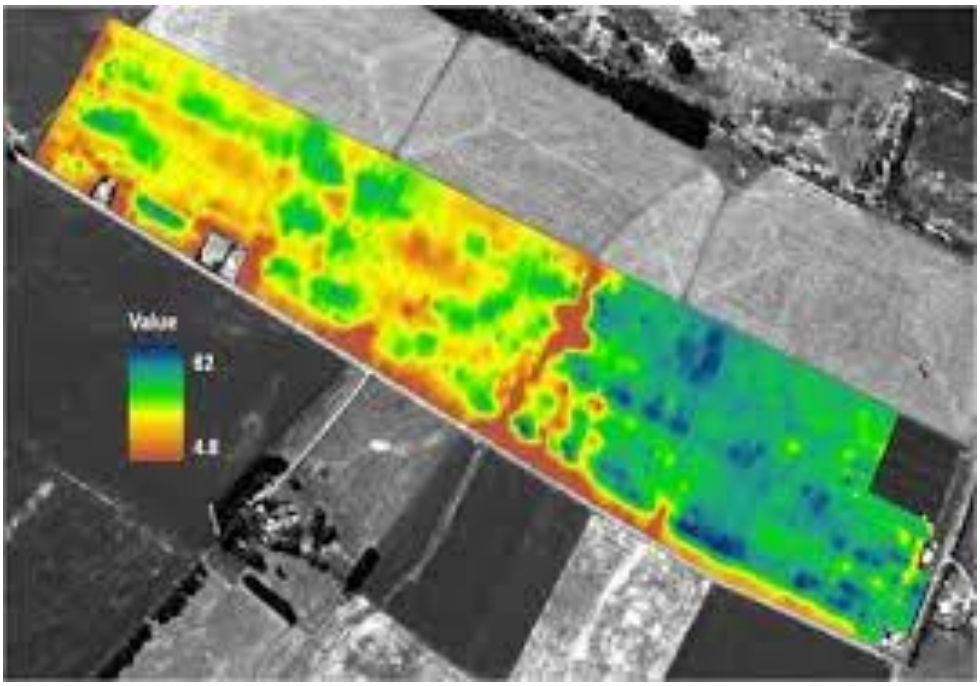


Figure 1-4 Pivot Irrigation and Precision Agriculture

1.5 A, pivot irrigation is thought to use less water and be less labour intensive than other irrigation systems. B precision agriculture uses drones to collect information so application of agrichemicals can be applied where needed and not everywhere. The photo shows a wheat crop field and the density of biomass within the field. Dark green and blue areas show the greatest crop density. (photo from Massey university – Professor Yule)

It is estimated that around 47 million metric tons of good quality soil is lost globally every year. The production of new soil is a slow process taking in the region of 1,000 years to produce 1 cm of top soil and about ten times longer to produce enough top soil to cultivate. Good quality fertile soil is essential for good food production and sustainable agriculture. The maintenance of soil fertility has to be a priority for gaining food security.

1.8 Genetically modified crops

Plant Biotechnology has been recognised as a part of a possible solution for many of the difficulties pertaining to food security outlined above. Genomic data is widely available for many plant species; genome sequences are available from simple algae to a variety of crops that fuel the human population, including grasses and fruit crops. Furthermore molecular techniques are more accessible and inexpensive than ever before. This has led to an increase in interest and research into applying our genetic and molecular knowledge to crop improvement.

In the early 1980's genetically modified crops were beginning to be produced and by 1987 new guidance was published by the National Academy of Sciences followed by guidance from the National Research Council in 1989 to address the general scientific principles, ecological concerns and other key issues, with an emphasis on field trials (Council, 2001).

The FLAVR SAVR was a genetically modified tomato that was able to remain firmer for longer by using an anti-sense gene to down regulate an

enzyme associated with fruit ripening. After a report found that the FLAVR SAVR "did not significantly differ from traditionally bred varieties, and are functionally unchanged except for the intended effects of the FALVR SAVR" (Redenbaugh et al., 1994)- in May 1994 the FLAVR SAVR became the first genetically modified plant on the commercial market (Bruening and Lyons, 2000). In 1996 FLAVR SAVR technology was used to produce a tomato paste, labelled clearly as a genetically modified product, which was introduced into the United Kingdom to be sold in Sainsbury's and Safeway, it was incredibly popular selling approximately 1.8 million cans over 3 years (Bruening and Lyons, 2000).

In 1998 the tomato paste was taken off the shelves. This coincided with, and is often associated with, a scientific report by Arpad Pusztai claiming genetically modified potatoes caused ill-health in rats studies, furthermore he concluded it was the genetic modification that caused the rats to have ill-health and not the specific gene or organism (Ewen and Pusztai, 1999). Further analysis of the data presented in this paper by independent scientists later established the conclusions from the report were incorrect, but this had hit the headlines and the European public still remain concerned about the safety of genetically modified crops.

In other countries GM technology has become widespread and several genetically modified crops are available in countries around the world, see figure 1.6 for more details. There are now many different genetically modified crops available, including BT Cotton expressing a bacterial insecticide gene, Round-up ready Maize with a modified amino acid

biosynthesis gene and others expected to be available soon like golden rice with enhanced pro-vitamin A levels.

1.9 Examples of Genetically Modified crops

During the mid-eighties China invested a large amount of money into biotechnology research (Huang et al., 2002) at a time when they were also having increasing pest control problems with cotton production. *Helicoverpa armigera*, commonly known as cotton bollworm – an insect that ate the cotton plant, was one of the largest problems for cotton growers in Northern China. These were first controlled by using pesticides, but DTT was soon banned and the bollworm became resistant to the other pesticides, pyrethroids and organophosphates (Pray et al., 2002). To endeavour to overcome the issues farmers in China were spraying more pesticides on cotton than any other field crop in the country, with costs of pesticides reaching US$ 101 per hectare of cotton (Huang et al., 2002).

Monsanto in collaboration with The Chinese Academy of Agricultural Sciences produced *Bt* cotton. *Bt* cotton was produced by transforming cotton plants with an altered *Bacillus thuringiensis (Bt)* gene that produces a Cry protein that acts as a pesticide to the bollworm. The majority of literature has found that this has led to a decrease in pesticide usage and an increase in yield for farmers in China (Pray et al., 2001, Pray et al., 2002)- as well as other countries such as the USA (Perlak et al., 2000), South Africa (Ismael et al., 2001), India and Mexico(Traxler et al., 2001). However other countries still remain sceptical questioning the improvement in yield and raising concerns of Bt cotton resistant

bollworms (GRAIN, 2007). Moreover the decrease of sprayed pesticides has led to an increase in other insect pests, like cotton aphid, for which solutions need to be found (Luoa et al., 2014).

Herbicide resistant crops are the most widely grown form of genetically modified crop globally. Monsanto's Roundup ready products which are resistant to the herbicide glyphosate are the most popular selling GM crop trait. Glyphosate resistant crops worldwide (James, 2013). This technology has been most effective in corn, soybean, cotton and rapeseed, reducing farming costs and increasing profitability for farmers (Duke, 2014), increasing income by nearly 5% in rapeseed and soybean between 1996 and 2012 (Brookes and Barfoot, 2014). This technology can also be used for no-till farming, which can help decrease soil degradation and increase sustainability (Bisson, 2012).

To meet a human's metabolic needs around 49 nutrients are needed and under-nourishment is a serious global challenge (Hirschi, 2009, White and Broadley, 2009).The most common dietary deficiencies are iron deficiency causing anaemia and iodine and vitamin A deficiency which cause many health problems. Anaemia for example can cause decreased immunity, mental health problems and increased childbirth mortalities (Puig et al., 2007). Micronutrients can be added to the diet by dietary supplements or the enrichment of food. Although this can often be hard due to the characteristics of the micronutrients and for socio-economic reasons (Bhullar and Gruissem, 2013).

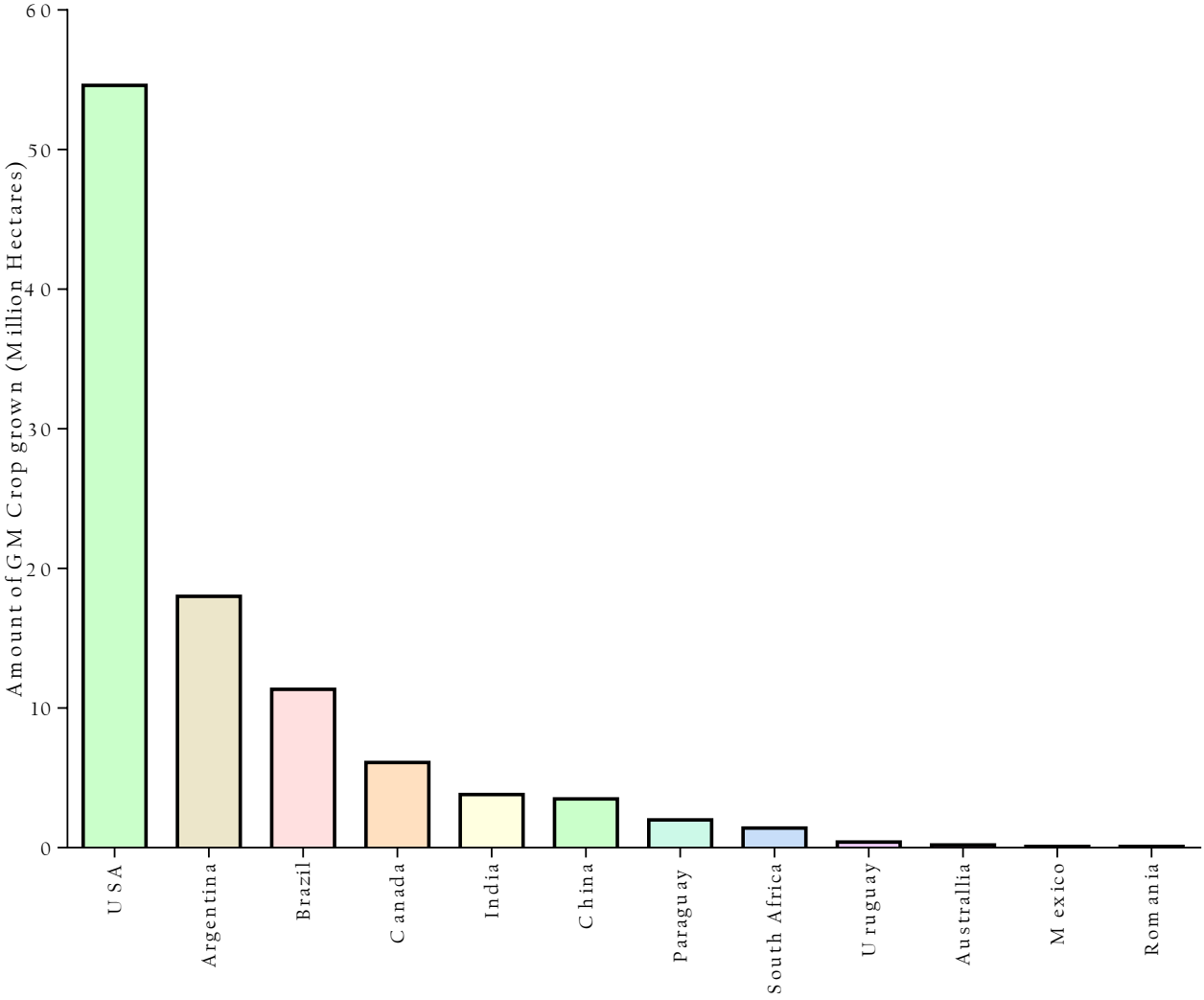


Figure 1-5 Number of GM crops grown worldwide

Genetically modified crops are grown in many different countries worldwide. The USA grows the majority of genetically modified crops in terms of area of GM grown ; growing over 54 million hectares. South American countries also grow a large areas of genetically modified crops, together with several Asian and African countries (Source: ISAAA, Clive James, 2006 (James, 2006))

Rice is a vital food crop for humans providing just under a quarter of per capita energy (Bhullar and Gruissem, 2013). Golden rice, *Orzya sativa, GR*, incorporates a provitamin A synthesis pathway, into the rice endosperm, this is relatively simple as it only involves only two genes (Martin, 2012). The rice is targeted to help people suffering from folate deficiency which is often associated with people in rural areas or poorer populations within cities (Al-Babili and Beyer, 2005).

The above examples are nowhere near an exhaustive list of applications of plant biotechnology. However, these three examples alone show the vast applications of plant biotechnology and the importance of continued research into other technologies that could help the world secure its food supply.

1.10 The role of roots.

Roots provide the main source for many key elements necessary for the plant's life, including water, nutrients and minerals as well as giving anchorage. The proliferation of the roots is majorly dependent on available commodities and their abundance in the micro-environment around the roots, the rhizosphere (Tiaz and Zeiger, 2002). Plants are able to perceive the presence of nutrients, like phosphate, iron, nitrate and sulphate, and alter the proliferation of the roots to enable adaptation to the plants specific environment and needs. Changes in root architecture can therefore have a large effect on the plants ability to uptake water and nutrients from the soil (López-Bucio et al., 2003). Plants can optimise root

growth to grow in soils with low or differential nutrient uptake, frequently leading to asymmetry in the root system (Gruber et al., 2013).

The size and importance of roots became more apparent from the early 20th century. One of the first experiments on roots in 1937 estimated the root system of a rye plant could be as extensive as 200 m^2 of surface area from the primary and lateral roots with an additional 300 m^2 of surface area from the root hairs emphasising that the biomass above ground is just the 'tip of the iceberg' (Dittmer, 1937). Plants can extend their root systems in several ways. Root growth normally begins with the addition and extension of cells to the primary root tip, secondly lateral root growth takes place which increases the foraging ability of a plant, finally root hairs increase the surface area (Dittmer, 1937)

A plant absorbs water when in direct contact with it through its roots, most readily through the meristem of the root. Uptake for water can be manipulated by changing the root architecture, either by increasing the root structure or the root hairs, which are elongations of the root epidermis (Tiaz and Zeiger, 2002). As root hairs can account for 70% of the root surface area, Changes in epidermal cells to form root hairs in root architecture is one of the most predominant root changes, this is very apparent when observing phosphate availability (López-Bucio et al., 2003).

When phosphate is of low availability root hairs become both more dense and longer (López-Bucio et al., 2003). Work on *Arabidopsis thaliana* roots has shown root hairs can have a five-fold increase in density, as well as showing root hair elongation by increasing the differentiation of

epidermal cells into trichoblasts (Ma et al., 2001), this has been shown to be dose dependent (Bates and Lynch, 1996). The importance of root hairs on phosphate uptake has been confirmed using *Arabidopsis* root hair mutants, like *root hair defective2 (rhd2)* that has little to no root hairs, the *rhd2* plants show restricted growth under low phosphate conditions, unlike the wild type *Arabidopsis* control group that can adapt their root hairs accordingly (Bates and Lynch, 2001). Similar responses to low availability of Iron have also be observed (López-Bucio et al., 2003).

1.11 Arabidopsis thaliana as a model organism

Arabidopsis thaliana is a flowering plant that is the member of the *Brassicaceae* (mustard) family which is widely used as an experimental model organism, figure 1.7. Although not an important crop plant, studies of *Arabidopsis* have shown both the importance and convenient role the model plant can play in understanding the biological and molecular aspects of plant biology (Meinke et al., 1998).

Arabidopsis thaliana has some key traits that make it ideal for molecular studies. Physically the plant is around 15 cm tall with a rosette size with an approximate diameter of 12 cm. This small size makes the plant ideal for controlled environment experiments and even where space is expensive and restricted a good number of replicates is still possible. Arabidopsis produces 1000s of seeds with a short life cycle of 6-8 weeks, making the plant ideal for segregation studies as well as speeding up the progress of other genetic studies (Mirza, 2001). As the model plant is used so regularly in scientific studies there are defined growth stages published to ensure some consistency between experiments (Boyes, 2001).

The genome of *Arabidopsis thaliana* qualities also make it an ideal model organism. With only 5 sets of chromosomes it has a relatively small genome size first estimated to be around 70 Mb – 190 Mb (al., 1984, Bennett. and Smith., 1976), the Arabidopsis genome initiative has since shown the DNA excluding telomeric, nuclear and centromeric tandem repeats is around 120 Mb (The Arabidopsis Initiative, 2000), with only 10% being repeated sequences (Dean. and Schmidt., 1995). Because of its relatively small genome size *Arabidopsis thaliana* was the first plant, and one of the first eukaryotic organisms to have its genome fully sequenced (AGI, 2000). Other genomes for plants are now available and crop plants are growing in popularity, like rice (*Oryza sativa)*, which was the second full plant genome sequenced. However, *Arabidopsis* still has a relatively small genome in comparison with other model organisms for example rice which has a genome size of 430Mb. Seeds are available for over 750 accessions from stock centres around the world, furthermore a substantial number of mutant strains with knocked out genes are available from ABRC and NASC.

A large number of experiments and a lot of data is available for *Arabidopsis thaliana*, as well as many laboratory manuals outlining many molecular techniques, including genetic transformations. Transformations using *Agrobacterium tumefaciens* are routine procedures in plant laboratories across the globe. With an easy whole plant 'floral dip' approach, there is no need for tissue culture and many progeny can be produced with T-DNA inserts, inserted into the genome at random (Davis et al., 2009, Gelvin, 2003). These random insertions can be used for both

overexpression of a particular gene, by the addition of an appropriate promoter, or for gene disruption studies.

1.12 GM strategies for gene manipulation

For a crop plant to be transformed there must be three main requirements. The first a gene most be inserted into the crop. The gene must be transformed into cells that can regenerate into a whole plant and finally it must be possible to trace the gene (Shewry and Lazzeri, 1996). This can be done in several ways. The most popular is mentioned in the previous chapter, by *Agrobacterium tumefaciens* transformations.

Some cereals are not amenable to *Agrobacterium tumefaciens* transformations, so other methods are used for example particle bombardment. Particle bombardment involves the firing of DNA coated in gold or tungsten particles into the plant tissue. Once in the plant tissue some of the DNA is taken up into the genome. this has been successful at transforming several different cereals, for example barley(Wan and Lemaux, 1994).

As transformations often lead to random insertions it is highly improbable that you can knock out genes where genomes are polyploidy. This is something that can be overcome by using other techniques like RNA interference. RNA interference is a process of gene-silencing by targeting double stranded RNA to a specific gene sequence. The double stranded RNA often forms a hairpin formation which causes the interference and knock down of the gene expression. It has become a very popular technique as it has several advantages including the ability to target and

supress the expression of multigene families in a regulated manner (Kusaba, 2004).

1.13 Aims and Objectives of the study

The aim of the work described in this thesis was to assess transgenic *Arabidopsis thaliana* plants that had been modified in order to attempt to improve either water use efficiency or to generate herbicide resistance. These two distinct aims are discussed separately below.

For herbicides to remain effective they need to be rotated with herbicides with different modes of action to help prevent resistant weeds arising. The first line of enquiry in this thesis is to investigate potential herbicides with a potentially new mode of action. These herbicidal compounds have been designed using X-ray crystallography studies and synthesized with collaborators within the University of Sheffield. The potential herbicidal compounds are produced to inhibit the enzyme Imidazoleglycerol-phsophate dehydratase (IGPD) in the plant histidine pathway.

Arabidopsis thaliana plants have been modified to over-express IGPD to try and make them out-compete the herbicidal compound and become resistant. This would allow for no tillage forms of agriculture, decreasing soil degradation and improving soil quality.

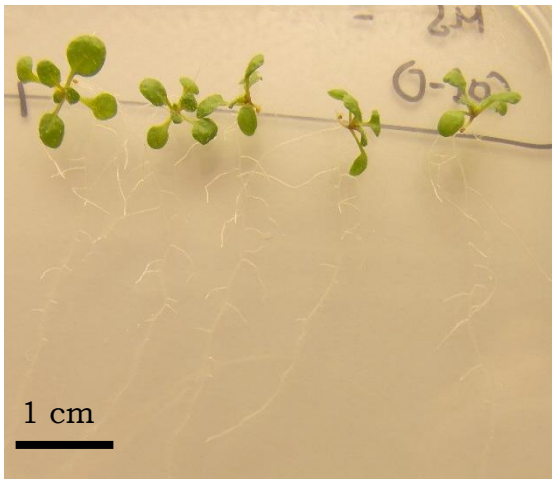
The second distinct line of enquiry involved plants modified to have a changed stomatal density. Stomata are the pores on plants that control gaseous exchange and by modifying stomatal density changes in transpiration rates have been achieved. Previous studies have shown plants with an altered stomatal density have an altered water use

efficiency, moreover plants with a decreased stomatal density have an increased rosette area in both drought and well watered conditions (Doheny-Adams et al., 2012). Experiments were carried out to investigate this altered size, concentrating on links with the below ground architecture.

The objectives of this thesis are:

1. To investigate potential herbicidal compounds that were designed to have a novel mode of action. (Chapter 3)
2. To test the potential herbicides on the plants modified to over-express IGPD. (Chapter 4)
3. To examine if the over-expression of IGPD has an effect on the plant. (Chapter 4)
4. To investigate if altered stomatal density and transpiration has an effect on root properties. (Chapter 5)

A



B

C

Figure 1-6 Arabidopsis thaliana

1A, 10 day old Arabidopsis thaliana plant, grown on agar B, 7 week old fully expanded *Arabidopsis* plants C, A photo of *Arabidopsis thaliana* plants when flower and beginning to set seed.

2. Chapter 2 – Materials and Methods

2.1 General laboratory chemicals

General lab chemicals and reagents were purchased from Sigma, BDF, Fisher or BioRad unless stated otherwise.

2.2 Plant material

All plant genotypes were in *Arabidopsis thaliana* Col-0 background, except *ost1-4* which is in the closely related Col-2 background. Stomatal development mutants utilised in this thesis were first described by Hunt & Gray 2009 (Hunt and Gray, 2009), root hair density mutants in*(Yi et al., 2010)*. *ost1-4* and *abh1* mutants which are ABA insensitive and hypersensitive in stomatal closure respective were described in papers *(Hugouvieux et al., 2001)*. *IGPD OE* plants were described in the thesis of Claudine Bisson (Bisson, 2012).

2.3 Growing conditions

Seeds were sterilised by adding 1ml of 100% Ethanol to an Eppendorf with 10-100 seeds in. Ethanol was replaced with 50% bleach for 10 minutes. Seeds were then washed 3 times with double distilled water. Seeds were stratified (48 hours at 3-5°C in dark) before being transferred to an environmentally controlled growth chamber (Conviron model BDR16) at 22°C/16°C, 9 hours light, and 15 hours dark at 450ppm CO_2.140 µmol m^{-2} s^{-1} light intensity, relative humidity 60% and the lights were fluorescent. Plants were then grown in M3 compost (John Innes)/perlite (3:1) and watered every 3-4 days when needed with water. Experiments carried out on mature plants (stage 3.9 Boyes scale (Boyes, 2001). Plants in all experiments were randomised weekly in

trays. Trays for comparable experiments were kept on the same shelf in the growth chamber. Infrared images of the growth chamber showed a change in temperature across shelves so trays were alternated with each other weekly to dampen any of these effects.

For growth on agar, seeds were sterilised, sown on 0.5 x Murashige and Skoog Basal salt Mixture phytoagar in polystyrene Petri dishes, stratified and then transferred to a controlled environment growth chamber to grow. Murashige and Skoog Basal salts mixture is a salt mixture with macro and micro-nutrients described in 1962 (Murashige and Skoog, 1962).

2.4 Agar herbicide analysis

All seeds were sterilised and sown on 0.5 x MS phytoagar made with potential herbicides (except controls with no potential herbicides). Herbicides were dissolved in distilled water and filter sterilised before being added to “hand hot” liquid agar and left to solidify. Seeds were stratified for 48h at 3^{o}C- 5 ^{o}C in the dark. Plates were then transferred to a controlled environment growth chamber (Conviron model BDR16) to germinate with the same growth chamber conditions as previously mentioned. Plants analysed 7 days after germination. Further experiments were done on mature plants outlined in method 2.5.

2.5 External Novel Herbicide Application on Mature plants

Arabidopsis plants (Col-0 and IGPD OE) were grown on agar plates for 1 week, transferred to soil and measurements taken weekly. 2 weeks after transplanting individual plants were sprayed in a fume cupboard

directly on their shoots once with a plant spray bottle (equivalent to 1mm^2 of liquid) of 0.1% tween (surfactant) with the appropriate concentration of herbicide and returned to the tray. Final measurements were taken 6 weeks after spray treatment.

2.6 Manganese Supplementation

Plants grown and sprayed as described previously (External Potential Herbicide Application on Mature Plants). Plants were supplemented with 0.05% Manganese once a week for three weeks after herbicide spray. Measurements taken 6 weeks after herbicide spray.

2.8 Rhizotron analysis

10 day old seedlings were transferred from agar plates to rhizotrons, consisting of a 150mm diameter polystyrene Petri dish filled with vermiculite, with rock wool plugs placed over two 10mm diameter holes at the top and bottom of the dish. A 150mm in diameter glass microfiber filter (Whatman G/FA grade) was placed between the vermiculite and the wall of the Petri dish. Seedlings roots were placed between the glass fibre and the wall of the dish with the leaf rosette placed through the hole at the top of the dish. The dish was wrapped in aluminium foil so that the roots were in darkness and incubated vertically in the growth chamber. Seedlings were watered biweekly with 0.25x MS solution. Rhizotrons were opened 5 weeks after seedling transfer, and the glass fibre disks with roots attached were scanned and analysed using GIMP and ImageJ software. Roots of 7 plants of each genotype were analysed.

GIMP is photo editing free software and was used to enhance the contrast between the root and the microfiber paper so the root area could be easily defined by free ImageJ software.

2.7 Root hair analysis

All plants were sterilised and sown on agar. Seeds were stratified for 48h at 3°C- 5 °C in the dark. Plates were then transferred to a controlled environment growth chamber (Conviron model BDR16) to germinate. 2 days after germination plates were turned vertically. Root hair measurements were taken on plants 8 days after plates were turned vertically.

2.9 Stomatal Density and Index

All stomatal counts were taken from the abaxial surface of the leaf. Dental resin (Coltene Whaledent, Switzerland) was applied to the surface of a fully expanded leaf and left to dry for a minimum of 5mins. Dry dental resin prints were removed from the leaf and nail vanish applied to the resin where the leaf is at its maximum. Stomatal and epidermal cell counts were determined by light microscopy (Olympus BX51) on the nail vanish peels and photos taken with digital microscope eyepiece (HiROCAM MA88-300A 3.0 Mega Pixels) minimum of 3 areas per leaf, 3 leaves per plant and 3 plants per genotype were used for analysis.(Woodward, 1986)

Stomatal density = stomata /mm^2

$$\text{Stomatal index} = \frac{\text{stomatal density}}{\text{Stomatal density + epidermal cell density}} \times 100$$

2.10 Rosette and Leaf Area Measurements

Both rosette and leaf areas were taken on mature plants (stage 3.9 Boyes scale (Boyes et al. 2001)) unless stated otherwise. Whole rosettes were cut from the roots of the plant and scanned. After scanning the rosette area individual leaves were cut from the rosette spaced on a white background and again scanned. Both rosette and leaf area were calculated using the software GIMP and imagej using the same method as mentioned earlier.

2.11 Root Hair Measurements

Four Arabidopsis *col-0* plants were sown alongside 4 mutant plants and grown vertically on 0.5 MS square agar plates. Root hair length and density (root hairs/mm^2) were determined 1cm above the first root hair from the root tip by light microscopy (Olympus BX51) and photos taken with digital microscope eyepiece (HiROCAM MA88-300A 3.0 Mega Pixels) while still attached to the agar plates.

2.12 Arabidopsis DNA Extraction

700 µl of extraction buffer (Tris-HCL, pH 8.0, 250mM NaCl, 250mM EDTA, pH 8.0 and 0.5% SDS) was added to one young leaf from a mature Arabidopsis or a single seedling ground in an ependorf with a micro pestle. The mixture was vortexed briefly and then centrifuged for 1 min at 16,000g. In a fresh tube 600µl of supernatant was added to 600µl of absolute isopropanol and centrifuged at 16,000g for 5 minutes. Again supernatant removed and 100µl of sterilised distilled H_2O used to re-suspend the DNA pellet. DNA was stored at -20^{o}C

2.13 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction was used to amplify specific DNA fragments for genotype diagnosis using Taq 2 X Master mix (Sigma). 50-100ng

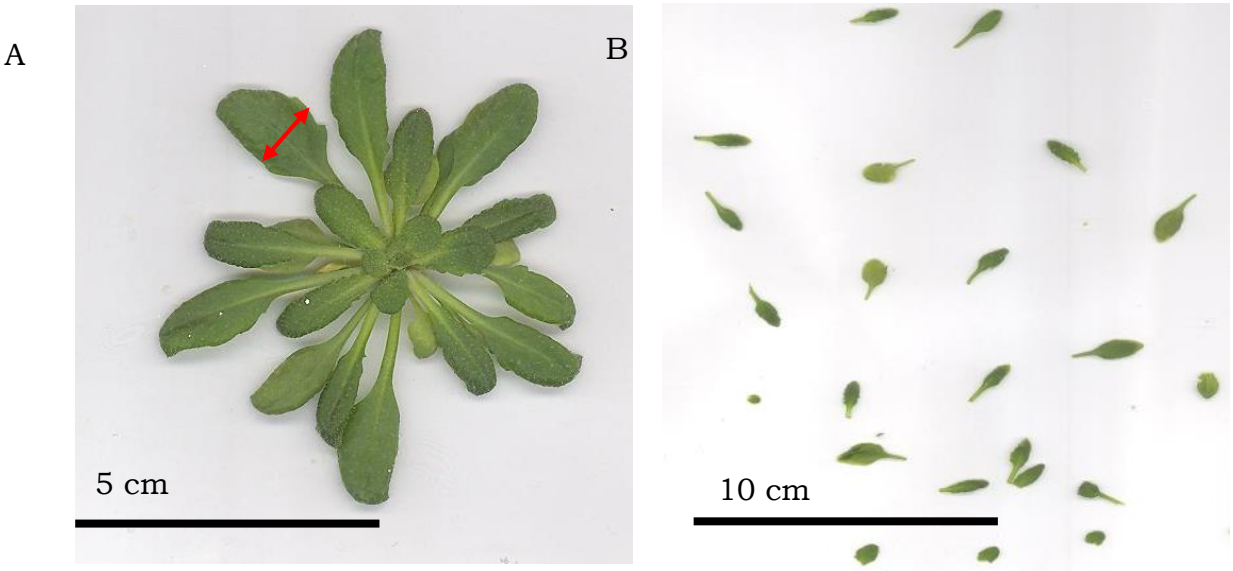


Figure 2 -1 Plant area measurements

Plant rosette and leaf area were measure by scanning A, the whole rosette of a mature *Arabidopsis thaliana* plant. The red arrow shows the widest part of the individual leaf which was used for stomatal density and index measurements. B, by separating individual leaves from a mature *Arabidopsis thaliana* plant and scanning them. Software GIMP and imageJ was used to determine area.

Template DNA, 25pmol of each primer was added to 10µl 2 X taq master mix and made up to 20µl with sterile H_2O.

PCR denaturation at 95 °C for 15 seconds, annealing at approximately 5°C below the calculated Tm of specific primers for 15 seconds and elongation at 72°C for 0.5- 1 minute depending on expected product side (allowing 30 seconds per 1000bp). 35 -40 cycles were performed depending on application of PCR.

2.14 Agarose gel

Analysis of PCR products and RNA integrity were done using DNA separation on an agarose gel by electrophoresis in a Bio Rad mini subcell. 1% Agarose mixed with 1 X TAE (made from 50 X stock 2MTris bas, 57.1 mL glacial acetic acid, 0.05M EDTA pH 8.0) made up with H_2O and dissolved in the microwave (700 watts) for 1 minute 15 seconds. Prior to gel setting 3µl ethidium bromide (5ng/µl), an intercalating agent, was added. The gel poured into a UV transparent gel tray and a comb added to form wells.

Once solidified the gel placed into the gel tank and submerged with 1 X TAE. 10µl of each PCR sample added to individual wells and the gel run at 100 V for approximately 35 minutes using power supply (Bio Rad). DNA visualised using UVitech transillimnator and photos taken with an attached digital camera.

2.15 RNA Extraction

Spectrum Total Plant RNA extraction kit (sigma) was used for all RNA extractions. 100mg of young plant tissue was harvested and grounded to a powder in liquid nitrogen. 500µl of prepared Lysis Buffer (reagent kit) was added to each sample, vortexed for approximately 1 minute, before incubation at 56°C for 5 minutes. All samples spun at 16,000g for 3 minutes. The supernatant transferred to the filtration column seated in a 2 mL Eppendorf tube and centrifuged at 16,000g for 1 minute. 750µl of binding solution (kit reagent) added to the filtered supernatant and mixed well. 700µl of the solution added to the binding column seated in a 2mL Eppendorf and centrifuged at 16,000g for 1 minute, flow through discarded and step repeated until all of the solution has been through. 500µl of Wash Solution 1 (kit reagent) was then loaded onto the binding column and the column centrifuged for 1 minute. Following this 500µl of prepared Wash Solution 2 loaded and centrifuged for 30 seconds, this step was repeated once more. The empty Binding Column was then centrifuged for 1 minute at 16,000g to dry the column. The dry column placed into a new clean Eppendorf and 50µl of Elution Buffer (Kit reagent) loaded directly onto the centre of the column and left to stand for 1 minute. The column centrifuged for 1 minute at 16,000g for final elution. RNA samples were quantified by measuring absorption at A_{260} and quality assessed by running 5µl on a gel.

RNA samples stored at - 20 °C for short term use and – 80 °C for long term storage.

2.16 cDNA synthesis

RT-PCR was used to show over-expression of IGPD in IGPD over expressors. Extracted RNA was used as a template for synthesising complementary DNA (cDNA) by Thermo Scientific Maxima H Minus Reverse Transcriptase.

The following components were added to a sterile, nuclease-free tube on ice in the order listed:

Template RNA	1 µg
Oligo (dT) Primer	100pmol
dNTP Mis (10mM)	1 µL
nuclease free Water	up to 14.5 µL
5X RT Buffer	4 µL
Maxima H Minus Reverse Transcriptase	1.5 µL

The solution mixed gently and centrifuged briefly, before incubating for 15minutes at 50 °C. The reaction terminated by incubating 85 °C for 5 minutes. cDNA stored at -20 °C

Below is a table of primers that were used for PCR and RT PCR after the extraction of DNA or cDNA conversion.

Primer name	Primer sequence	Tm
IGPD F	GCAACTTGCTTCGCATGGCTTGT	73 °C
IGPD R	TCCCTTTACGTCACCAAGAGCC	71 °C
EPF2 F	CAATGGCGGAGAAAAGAAAA	63 °C
EPF2 R	TACATGATTCGGCGACTGAG	63 °C
ACTIN F	GGTAACATTGTGCTCAGTGGTGG	67°C
ACTIN R	AACGACCTTAATCTTCATGCTGC	65 °C
UBIQ F	AAAAGCGGCCGCAGTAATAAACGGCGTCAAAGT	79 °C

UBIQ R	AAAAAGCGGCCGCTGTTAATCAGAAAAACTCAGATT	76 °C
RHD6 F	AGCCACTTTTCGTCAACAAGA	63 °C
RHD6 R	TGTTGGCTTAGGCTTGGTCT	63 °C

2.17 Histochemical Staining

epf1::GUS and epf2:: GUS promoter constructs obtained from Lee Hunt published in Hunt and gray 2009 (Hunt and Gray, 2009) were grown vertically on agar for ten days, removed and placed in x-Gluc reagent (50mM potassium phosphate, 1mM potassium ferrocyanide, 1mM potassium ferricyanide, 0.2% triton x-100, 2mM 5-bromo-4-chloro-3-indolyl-b-d-glucuronic acid and 10mM EDTA) and vacuum infiltrated for 15 minutes. Samples were then incubated at 37°C in the dark for 3 hours. To visualise the staining plant tissue was cleared with 70% ethanol for at least 24 hours. Images of roots and shoots were taken with an Olympus BX51 microscope connected to a DP51 digital camera.

2.18 Infra-red Gas Analysis (IRGA)

Measurements were taken with a LI-6400 potable photosynthesis system (lincoln, NE) on mature leaves (Boyes scale 3.6 (Boyes, 2001). Relative humidity was kept at 65%-75% using self-indicating desiccant, flow rate was 500µmol s^{-1} and block temperature at 20°C. Light intensity was set to 1000 µmol.m^{-2}.s^{-1}, CO_2 set to 450ppm and a single leaf from the plant was left to equilibrate in the leaf chamber for at least 45 minutes in the lab. Measurements were taken every 30 seconds and the IRGA matched every ten minutes on 3 plants per genotype.

2.19 Amino Acid Quantification Using HPLC

High performance liquid chromatography was used for amino acid quantification on 7day old cotyledons. Cotyledons were crushed using liquid nitrogen and amino acids extracted with 20 mM bicine buffer pH 9.8 20µl of the sample was mixed with 30µl OPA and 200µl of borate buffer. 25µl of the sample was loaded onto the column LUNA C8 (Phenomenex - Macclesfield). Samples were eluted with grad of 25% MeOH, 75% $C_2H_3NaO_2$ to a 90%, at a flow rate of 1.4 mL min^{-1} over 60 minutes. Detection of amino acids by fluorescence using excitation wavelength 340nm and emissions wavelength of 455nm. An amino acid solution standard was run in the same conditions to quantify each amino acid.

2.20 Statistical Analysis

One, two and three way ANNOVAs with a post analysis TUKEY test to compare individual means were performed by GraphPad prism or using spss statistics. Variance was considered statistically significant when $p=<0.05$. Individual un-paired student T-tests or a one-way ANOVA with multiple comparisons were performed using GraphPad Prism (significance considered $p=<0.05$). Details of statistical tests throughout results chapters where appropriate.

Chapter 3 - Exploring the potency of novel herbicides

3.1 Introduction

This chapter starts with an introduction to the necessities of creating new agricultural technologies with a focus on novel herbicidal chemistries; followed by a description of an investigation into novel herbicides designed to inhibit the histidine biosynthesis pathway in plants. The aim of the research described in this chapter was to investigate the herbicidal activity of new chemicals beginning with the herbicidal activity of a published molecule which has been shown to actively inhibit the activity of the enzyme imidazoleglycerol-phosphate dehydratase (IGPD) *in vitro*. The novel chemicals were designed and provided by Dr Claudine Bisson and Professor David Rice (University of Sheffield), working in collaboration with Dr Timothy Hawkes (Syngenta, Jealott's Hill International Research Station, Berks, UK).

3.2 Food Security

Although the increase in global population has driven up the demand for food there are other ongoing pressures on crop production, such as, an increase in cultivated land being used for feed for livestock and biofuels. This has led to the levelling off of global food prices in 2005 (Bank, 2007) which had been steadily decreasing since 1975, and even food price spikes like those witnessed in 2008 (Pingali, 2012). These pressures have led to an increased push in research and devolvement in agricultural technologies in both developed and low income countries, as shown in recent policies such as the UK Agricultural Technologies Strategy (Governement, 2013) and the New Partnerships for Africa's development

initiative (NEPAD, 2006). Improving the number and efficiency of pesticides is currently amongst high priorities for agricultural technology.

Herbicides kill weeds which compete with crops for light, water and nutrients, this is in comparison to pesticides which target pests, bacteria and fungi which can threaten crop yield. Herbicides normally inhibit essential biochemical pathways, not found in animals but vital in plants such as *de novo* synthesis of amino acids (Ruegg, 2007). Herbicides must be cost effective, relatively potent and confer low toxicity to animals to become successful.

Herbicides are normally classified by both their mode of action and target. An herbicide's mode of action can either be systemic or contact. Contact herbicides kill the plant solely at the site of contact and are relatively unpopular in comparison to systemic herbicides. Systemic herbicides are absorbed into the plant, travel through their vascular system and are therefore able to target the whole of the organism (Gressel, 1990).

The specificity of individual herbicides is determined by many factors such as the uptake of the herbicidal compound into the plant, but also includes factors relating to the nature of the target enzyme being inhibited, and the inhibitor itself. For example different species of plants may have slightly differing target enzymes or they may be able to turnover or export the herbicidal compound more efficiently than other species (Caseley, 1991).

3.3 Glyphosate

The most widely used broad-spectrum systemic herbicide used globally is glyphosate. Post World War II, crop farming turned increasingly to chemistry to improve crop yields per hectare (Marjo et al., 2012). Glyphosate (N-(phosphonomethyl) glycine), a broad spectrum herbicide, was first commercialised in the 1970s as the product Roundup® and has since become the best-selling herbicide globally. It's popularity comes from its low toxicity, broad activity and relatively low cost (Duke and Powles, 2008).

Glyphosate is very efficient at inhibiting 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) of the shikimate pathway for the biosynthesis of aromatic ring amino acids (Figure 3.1). The method of how inhibiting EPSPS becomes fatal to plants is still unclear (Duke and Powles, 2008). The majority have assumed that the lack of aromatic ring amino acids causes fatality. However, more recent studies indicate that blockage of the shikimate pathway leads to a decrease in negative feedback causing an increase in shikimic acid (Duke, 1988) which in turn causes a large carbon sink leading to other essential pathways being starved (Siehl, 1997).

Glyphosate is generally considered to have relatively low toxicity to humans with an LD_{50} of 5 g kg^{-1}, only slightly higher than common baking soda (LD_{50} 4.2 g kg^{-1}) (Tomlin, 2006). Several studies have shown it binds tightly to soil particles and degrades within 2 weeks (Duke and Powles, 2008). Although there are many reports on the lack of environmental impact of glyphosate (Franz et al., 1997, Williams et al., 2000, Geisy JP, 2000, Soloman KR, 2007) there have been some studies connecting

glyphosate, and it's surfactant with negative impacts to amphibians (Reyla, 2005) and fungi (Soloman KR, 2007, Franz et al., 1997).

3.4 Herbicide Resistance with a Focus on Glyphosate

The most common reason for the acquisition and spread of herbicidal resistance in weeds is a lack of variation in the management practices of weed control (Powles, 2008). Herbicide management practices come in several forms including; crop species rotation over seasons, application of different herbicides with varied modes of action within a growing season and the usage of non-herbicidal modes for weed killing (Beckie and Reboud, 2009, Diggle AJ, 2003, Neve, 2008).

Glyphosate resistant weeds were first reported in the species *Lolium rigidum* on an Australian farm in 1995, 20 years after the commercialisation of Roundup®. It was reported that this resistance arose after 15 years of repeated treatment with glyphosate at lower levels than recommended (Pratley J, 1996, Pratley J, 1999). One case of glyphosate resistance arising over 20 years of glyphosate usage would indicate that the rate of evolution of weed resistance is relatively low. Since 1995 the usage of glyphosate has increased due to the lapse of the glyphosate patent decreasing its cost (Duke and Powles, 2008, Duke and Powles, 2009) and the introduction of genetically-engineered glyphosate resistant crops (Shaner D, 2000). This has led to repeated use of glyphosate, combined with a lack rotational management leading to the likelihood of weeds evolving glyphosate resistance increasing.

Glyphosate resistance is normally conferred by genetic mutations causing either a change in the active site of 5-enolpyruvylshikimate-3-phosphate (EPSPS) synthase leading to glyphosate no longer binding or a decrease in translocation of glyphosate to its target site (Dill, 2005). Since the first report of glyphosate resistance many user guidelines have been put in place to decrease the evolution of glyphosate resistant crops, including increasing crop rotation and ensuring herbicide usage at recommended concentrations. However, currently there are 24 known different weed species that have been shown to have acquired glyphosate resistance (Heap, 2013). This has led to an increase in commercial interest in finding novel herbicides to replace glyphosate.

3.4 Genetically modifying crops to become herbicide resistant.

Crops can be modified in several different ways to confer resistance which can then be utilised in methods to lower application rates or lower the number of applications. Methods to modify crops to become herbicide resistant include; over-expressing the gene of the targeted enzyme to give a larger pool of enzyme for the herbicide to block. Over-expressing a mutated form of the gene of the targeted enzyme; the mutation is generally in the active site and allows the enzymes' substrate to interact, but not the inhibitor. Finally crops can be genetically modified to incorporate a detoxifying agent which can detoxify the herbicidal inhibitor (Slater et al., 2008).

3.5 IGPD as an Herbicidal Target

Imidazoleglycerol-phosphate dehydratase (IGPD) was first recognised as a potential herbicide target when ICI Agrochemicals, now Syngenta, endeavoured to make an analogue of glyphosate to inhibit EPSPS synthase, but instead they synthesised a chemical that appeared to have a different mode of action. Structural studies indicated the analogue may inhibit IGPD in the histidine biosynthesis pathway, which was later confirmed with histidine reversal assays (Bisson, 2012). The histidine *de novo* biosynthesis pathway (Figure 3.2) which includes the IGPD enzymatic step is present in plants, prokaryotes and yeast but not found in other higher eukaryotes, which acquire

histidine through diet, making it a prime candidate for a potential herbicide.

IGPD has been structurally characterised in *Arabidopsis thaliana* and is a 24 subunit containing (24mer) manganese dependent enzyme targeted to the chloroplast by an N-terminal transit sequence (Glynn et al., 2005). Several chemicals have been synthesised to inhibit IGPD activity including C348 (Figure 3.2) (Ohta et al., 1997, Mori et al., 1995, Cox et al., 1997), however, only *in vitro* chemical assays have been published. No experiments to characterise the toxicity of C348 towards living organisms have previously been reported.

3.6 Results: C348 as a Potential Herbicide

The aim of the experiments described in this section was to test the efficacy of the toxicity of chemical C348 on *A. thaliana* plants. C348 has

previously been shown to potently inhibit IGPD activity in *in vitro* experiments with a Ki of 0.6 nM (Hawkes and Lewis, 1993). It has previously been speculated that the strong potency from the short structure comes from C348 mimicking the structure of the intermediate of IGP and IAP in the histidine pathway (John M. Cox et al., 1996). The structure has a shorter backbone, however shows similarity in its functional group and phosphonate group which is thought to bind to the active site of IGPD (Figure 3.2 B-C). Initial investigations took place using *A. thaliana* grown on agar plates with C348 dissolved into the plant agar. The seeds were stratified (to allow consistent germination) and root and shoot length measured 1 week after germination.

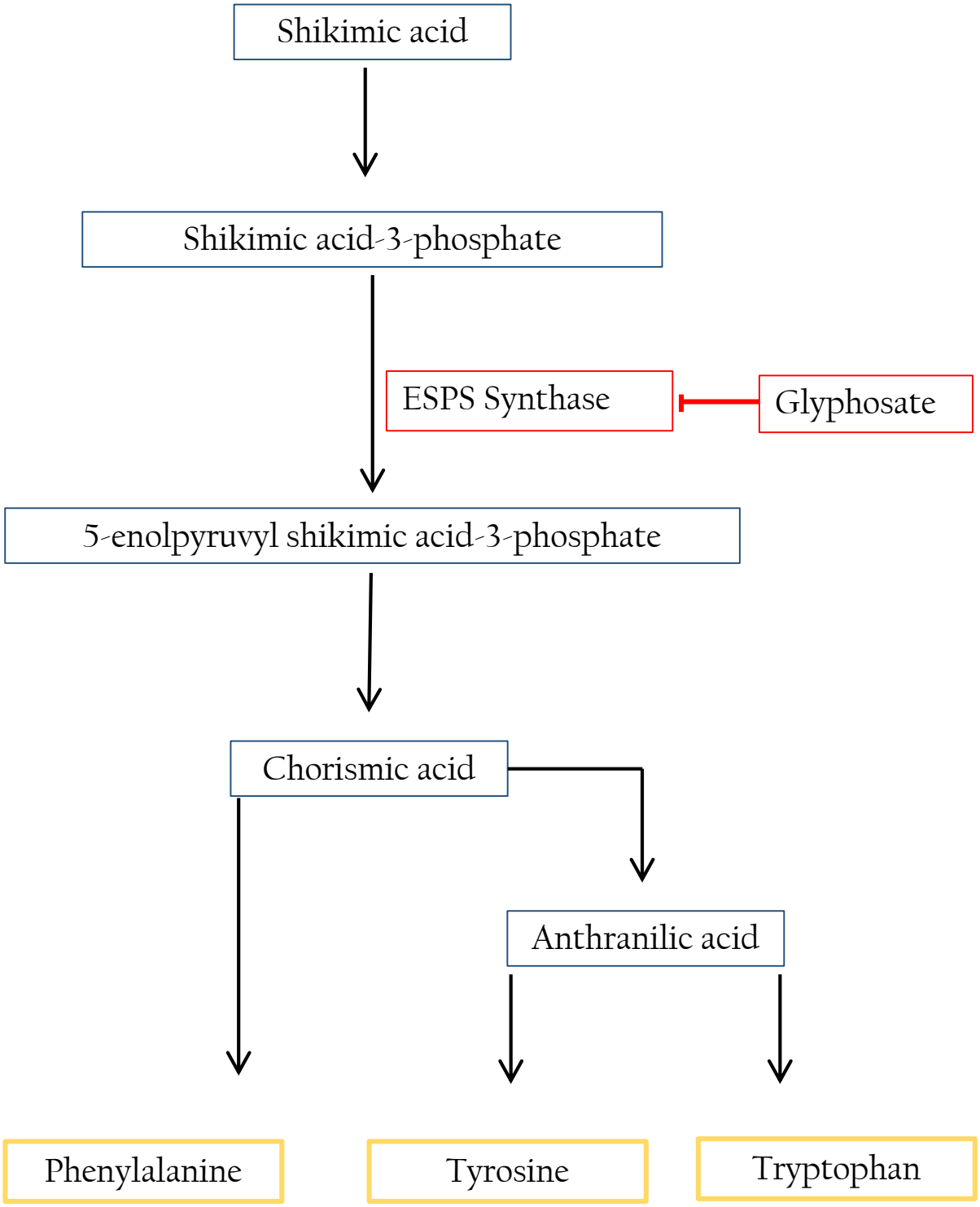
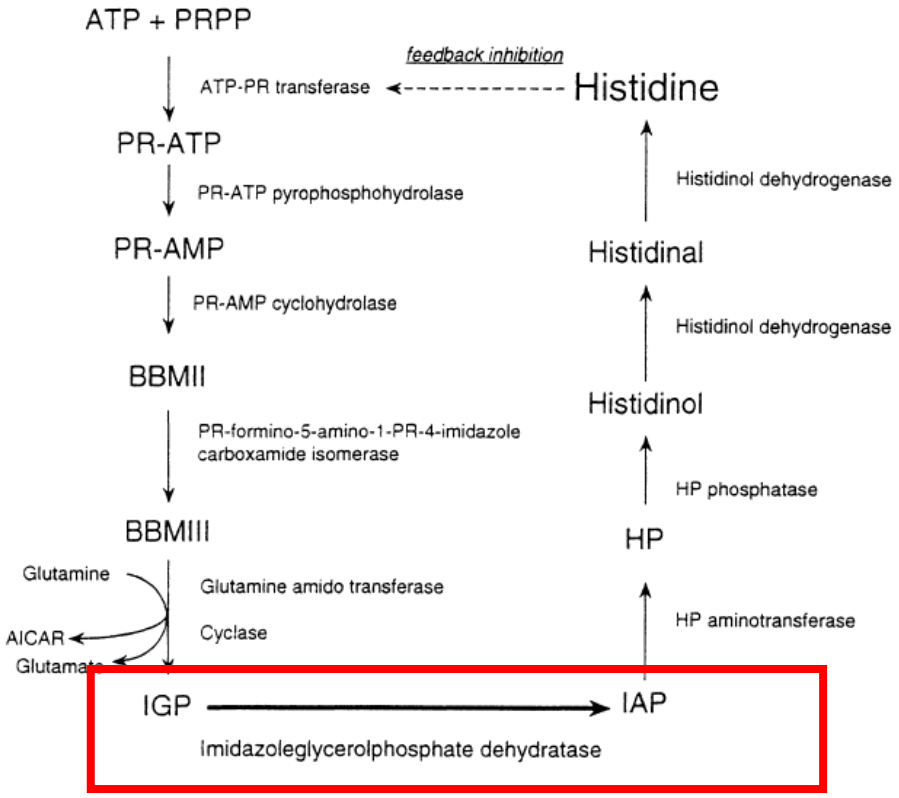


Figure 3.0-1 Shikimate pathway

The Shikimate pathway of plants and microbes is responsible for the production of aromatic amino acids, and is the target for inhibition by the herbicide glyphosate. This diagram of the pathway of aromatic ring biosynthesis indicates the target for glyphosate inhibition with red boxes and products in yellow boxes.

A



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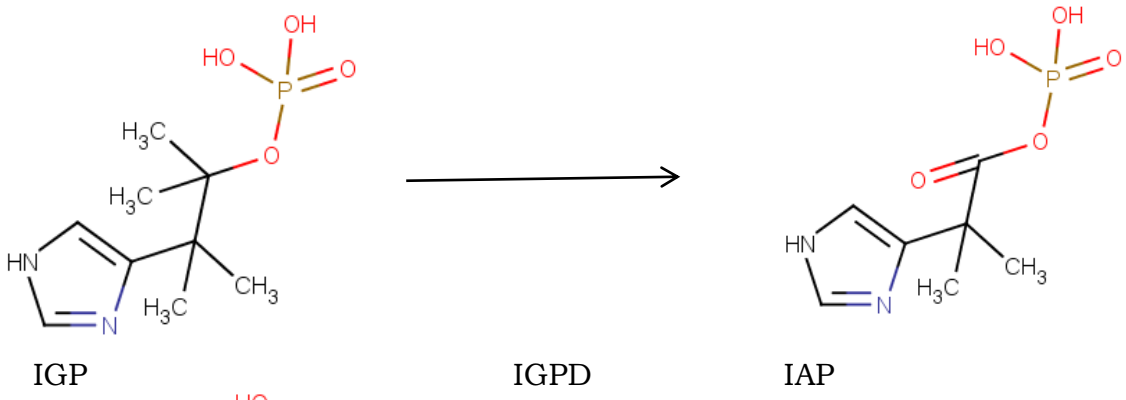

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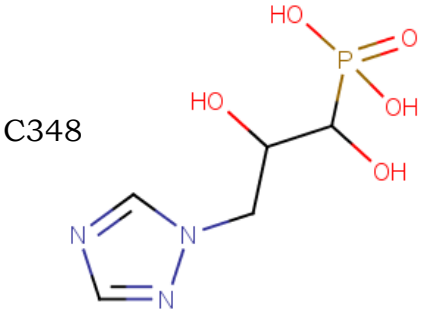


Figure 3-2 Histidine biosynthesis pathway.

A, Schematic of histidine biosynthesis in plants adapted from Ohta et al., 1997 *(Ohta et al., 1997)*. The red box highlights the dehydration step from IGP to IAP catalysed by IGPD the potential herbicide target in this study. B, Schematic of chemical structures of IGP to IAP whose conversion is catalysed by the enzyme IGPD. C, Synthetic chemical 348 (C348) which inhibits IGPD (structure drawn using Chemdoodle.com). Note the structural similarity of C348 with IGP. s

Col-0 *Arabidopsis* seedlings showed sensitivity to C348 across a range of concentrations from 25-200 μM (Figure 3. 3). Roots showed high sensitivity to C348 with severe stunting of root growth from 25 μM. Shoots also showed a significant decrease in rosette diameter from 25 μM, however higher concentrations of C348 were needed for more obvious stunting of rosette growth. Plants also showed discolouration and appeared yellow from 50 μM which become more obvious at higher concentration. At a concentration of 200 μM C348 plants either did not germinate or died soon after germination.

These initial trials showing high levels of toxicity to plants suggested that C348 could be a suitable molecule for a new herbicide. To investigate this further and in an attempt to mimic more agricultural practices experiments were carried out post emergence, with C348 sprayed onto plant leaves.

To gain a clearer understanding of the effects of C348 on plant growth, both plant leaf rosette diameter (Figure 3.4) and leaf number (Figure 3. 5) were measured weekly for two weeks before C348 spraying and for 6 weeks after application. C348 was mixed with a dilute tween solution which was used as a surfactant, to aid chemical access to the plant. Digital images of plants were recorded 8 weeks after plant germination (6 weeks after application of C348 or control tween solution) (Figure 3.5).

As expected Col-0 plants, sprayed with dilute tween solution as a control, showed an increase in rosette diameter from week 2 to week 8 until growth plateaus as the plants reach maturity. The addition of C348

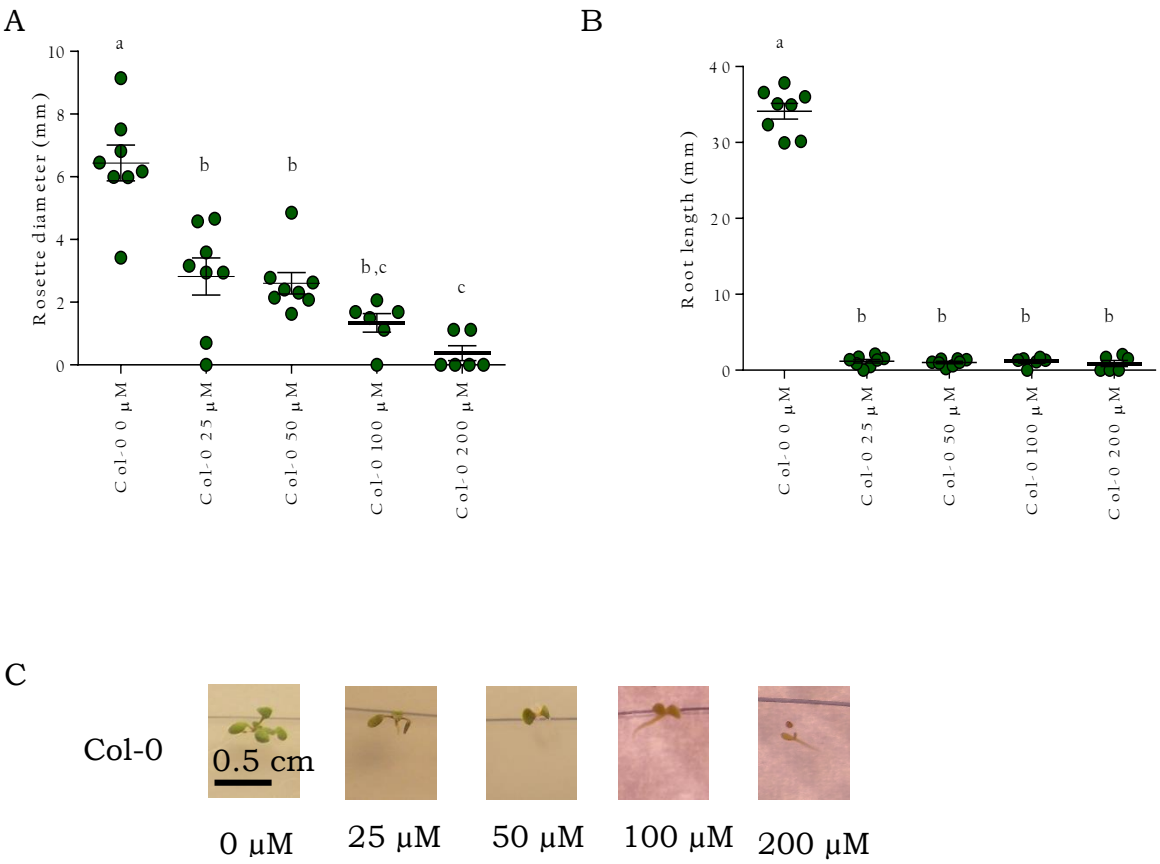


Figure 3-3 C348 is a potent herbicide.

C348 shows herbicidal activity towards germinating Arabidopsis seedlings at low concentrations. Individual green dots represent individual plants, the spread of dots is for visualisation reasons only. A, Significant reductions in shoot growth at 25 -200 µM C348 ($P < 0.0001$). At 200 µM C348 many seed either did not germinate or died soon after germination. B, Roots are extremely sensitive to C348 showing severe stunting of growth at 25-200 µM ($P =< 0.0001$). C, Images of representative plants at each concentration of C348. Scale bar is for all images. Plants showed discolouration (i.e. were less green) at higher C348 concentrations. n=6-8 depending on death rate. Individual concentrations are calculated using data from 2 individual plates. Error bars represent mean and standard error. One way ANNOVA tests followed by individual mean comparisons using a TUKEY test.

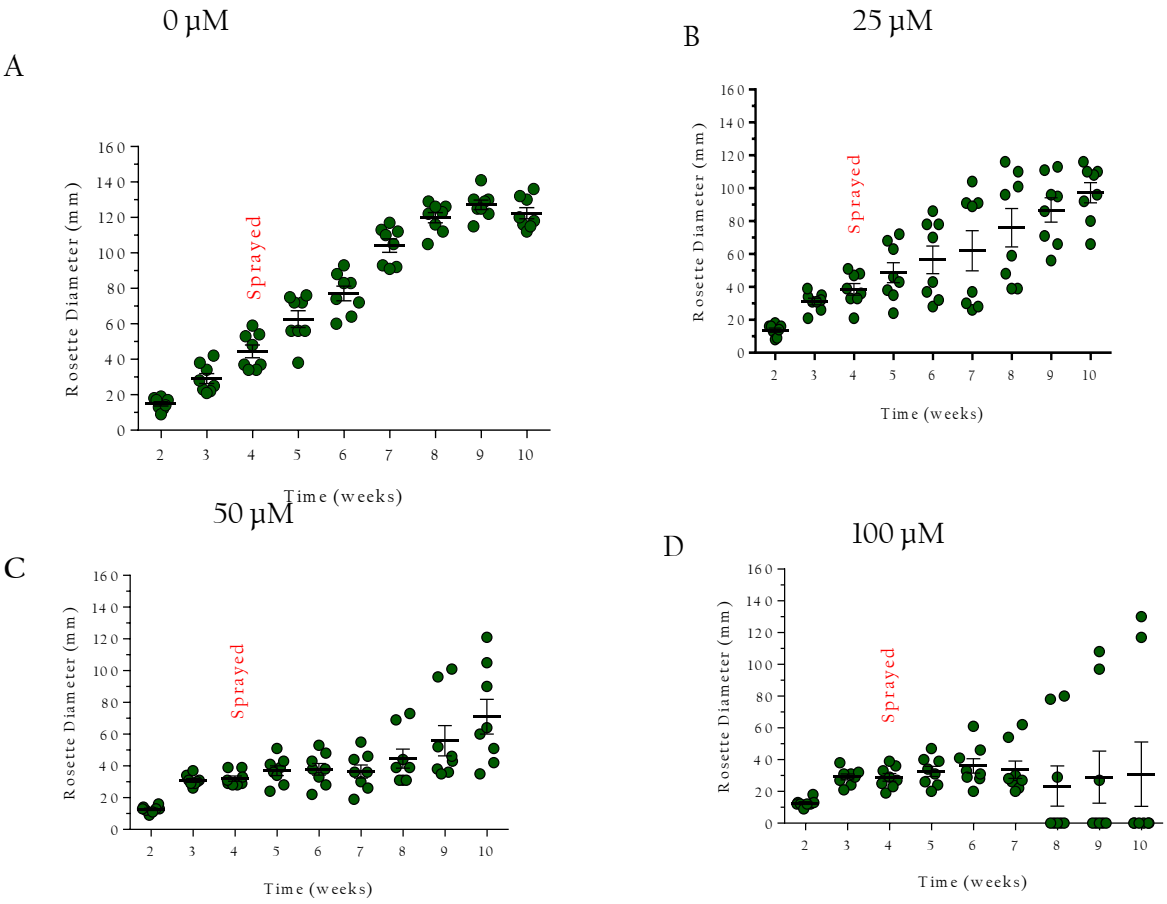


Figure 3-4 the effect of increasing C348 concentrations on size of A. *thaliana* seedlings.

Plant leaf rosette diameter was measured weekly, before and after application of C348. A-D, Plots of individual plant widths (represented by dots) over ten weeks. Plants were sprayed with either A, 0 µM, B, 25 µM, C, 50 µM or D, 100 µM C348 at 4 weeks after germination. The higher concentrations of C348 increased growth inhibition and seedling fatality. p of final time point = >0.0001; n=8. Error bars represent mean and standard error. One way ANNOVA tests were performed followed by individual mean comparisons using a TUKEY test. Individual green dots represent individual plants, the spread of dots is for visualisation reasons only. This graph is not linear.

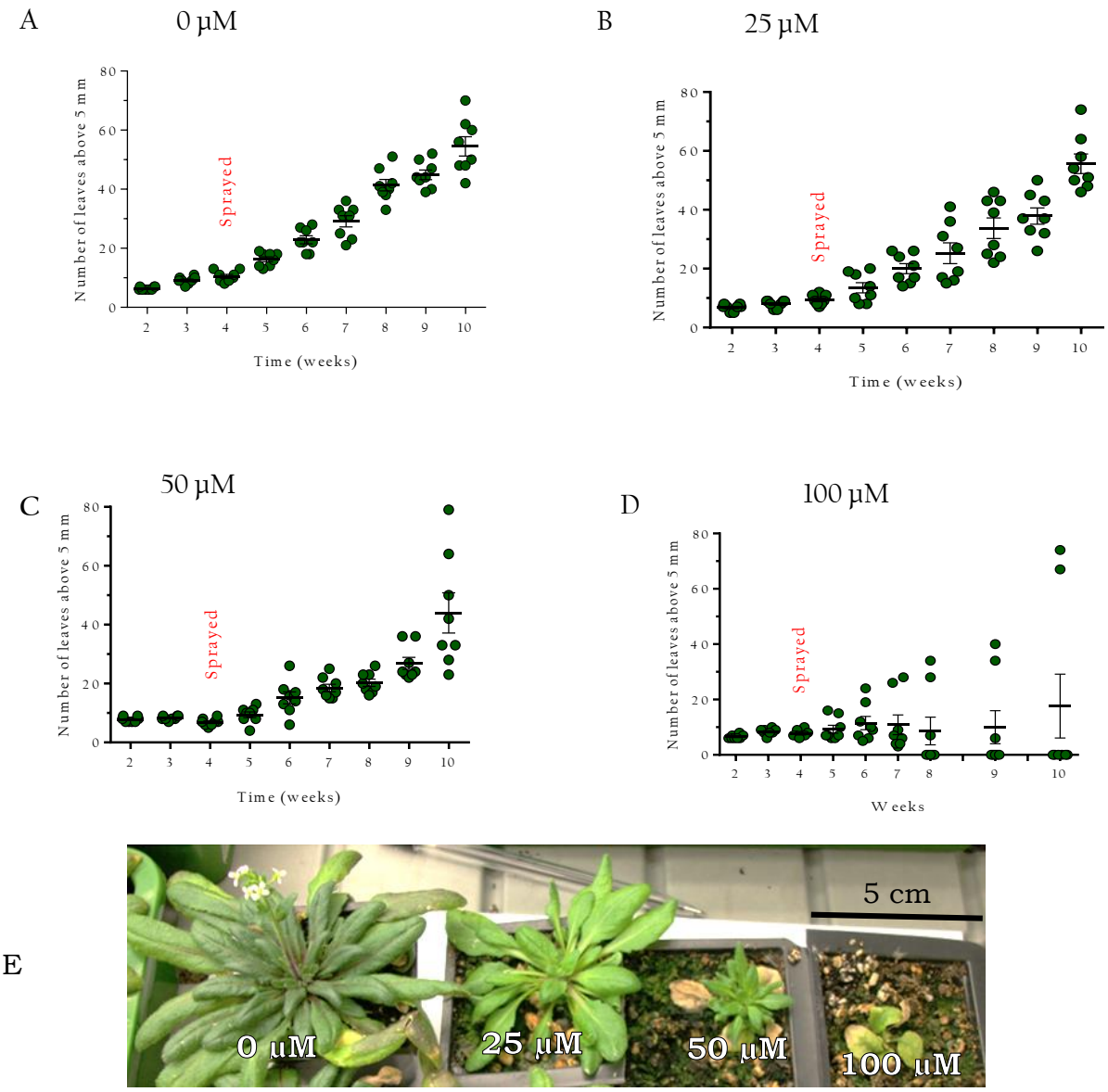


Figure 3-5 the effect of increasing C348 concentrations on leaf number of *A. thaliana* seedlings.

The number of plant leaves, greater than 5mm in length, was counted weekly, before and after application of C348. A-D, Plots of individual plant leaf number (represented by dots) over ten weeks. Plants were sprayed with either A, 0 µM, B, 25 µM, C, 50 µM or D, 100 µM C348 at 4 weeks after germination. The higher concentrations of C348 decreased leaf initiation. E, Representative images of Arabidopsis plants 6 weeks after application of C348. P of the final time point 0.0023; n = 8. Error bars represent mean and standard error. One way ANNOVA tests followed by individual mean comparisons using a TUKEY test were performed. Individual green dots represent individual plants, the spread of dots is for visualisation reasons only. This graph is not linear.

decreased the growth of Col-0 at concentrations of 25 μM and higher. The degree of inhibition of growth increased as the concentration of C348 increased. At 100 μM 75 % of the Col-0 plants died. Interestingly, there was a large variation in individual plant sensitivity with 25% of plants reaching full maturity without an effect on growth in comparison to the majority which died within 4 weeks of spraying.

Variability between Col-0 plants at 100 μM is unusually high this could be due to a poor spraying method. However, this large variability is charactersitic of all of the data collected in reference to C348 spraying. An alternative explanation could be there is a certain freshhold the plant can withstand. The threshold for an indivdual plant could be due to a difference in the ability to incease expression of it's native IGPD.

In addition, leaf number increased in the tween control (0 μM C348) and appeared to plateau after 8 weeks when the plants reached maturity. Leaf number was not affected significantly by application of 25 μM C348, but higher concentrations (50 and 100 μM) induced a significant decrease in leaf production. Leaf number showed a large variation at 100 μM similar to rosette diameter. At 25 μM leaf discolouration was seen and this yellowing increased with an increased concentration in C348. As leaf number appeared less sensitive to C348 than rosette diameter in the following experiments the impact of potential herbicides on plant growth was assessed by measurement of rosette diameter.

Although C348 appears to be relatively potent at the μM concentrations used in the above experiments, for a compound to be commercially valuable it must have a 100% fatality rate. As IGPD is a desirable target

for a future herbicide a group of structural biologists have been designing and synthesising chemicals to inhibit it. All structures discussed in the rest of this chapter were designed and provided by Dr Claudine Bisson and collaborators using knowledge from X-ray crystallography experiments that were designed to investigate the molecular interactions of IGP and C348 with IGPD. Structures were designed to mimic the interactions of either IGP, the intermediates between IGP and IAP or C348. These additional unpublished chemicals were synthesised and then examined for their herbicidal activity in this study. Plants were sprayed with each chemical at four weeks after germination and rosette diameter was measured when the plants were 10 weeks old.

The chemicals which were synthesised as potential herbicides were moderately expensive and in short-supply. Therefore experiments were limited. Concentrations for application to plants were chosen based on previous *in vitro* studies and Inhibitory constants (Ki) values produced by Dr Claudine Bisson University of Sheffield (Personal communications). A Ki value is a measure of the efficiency of an inhibitor, Ki is the amount of inhibitor it takes to decrease the maximum rate of the reaction by half. A part from the concentration the experimental protocol was kept the same for all chemicals. Ki values were calculated by Claudine Bisson using enzyme assays.

Chemical 358 (C358) was shown to have a Ki of approximately 0.5 times the previously tested chemical, C348, and therefore investigation of potency took place at 50 μM. At 50 μM C358 showed no significant effect on rosette diameter (Figure 3. 6). Although the chemical was expected to

be potent from *in vitro* enzyme activity studies, as a spray-on herbicide it appeared to lose its potency. This could be due to the extra aromatic ring that C358 has in comparison to C348. It is possible that the extra aromatic ring restricts the movement of the chemical inside the plant. As no significant herbicidal effect was seen in this experiment, chemical 358 was deemed not to be commercially valuable and therefore no further investigations were carried out.

In the crystal structure analysis of IGP binding to IGPD a nitrate group was observed in the active site and it was thought this was characteristic of the reaction intermediate (personal communication, Claudine Bisson). Therefore Chemical 367 was designed with a nitrate bound to the phosphonate group. C367 was applied to plants in similar experiments to those described above for C348 again using concentrations that were worked out considering Ki values using enzyme bioassays from Claudine Bisson, University of Sheffield (verbal communication),and the amount of chemical available.C367 caused a significant decrease in rosette diameter 6 weeks after spray application (Figure 3. 7). Although some affect was seen at 1 mM, this is a high concentration for a spray herbicide without seeing fatality or a large decrease in size. C367 has a longer chemical backbone meaning it may not bind as well to the active site of IGPD making it less potent than C348. Again due to its lack of potency C367 would not be commercially viable and no further experiments were done to investigate this chemical.

Chemical 368 (C368) was synthesised while attempting to synthesis C367. It was not expected to be as potent as C367, but investigated

nonetheless. C368 caused a significant decrease in rosette diameter growth when measured 6 weeks after spraying with the chemical (Figure 3.8). C368 decreased the growth of the rosette by the largest margin after C348, but again individual plants showed a large variation in their sensitivity to the chemical. C368 lacks the phosphonate group seen in IGP and the other chemicals examined, it also has an aromatic ring, which may cause movement restrictions within the plant. Again C368 was sprayed at moderately high levels and therefore would not be commercially viable.

3.7 Conclusions

Novel herbicides are of commercial interest due to pressures on food security and the rise of herbicide resistant weeds. I have shown C348, a chemical previously shown to inhibit IGPD in *in vitro* experiments has herbicidal effects at μM concentrations. Furthermore, rationally designed chemicals C367, C368 and C358 show limited herbicidal activity and are unlikely to be commercially viable.

Structural design using X-ray crystallography to investigate the target binding site may help to design potential herbicides, but it will be important to team this with plant biological studies and to take into account the transition of the chemical to its target site.

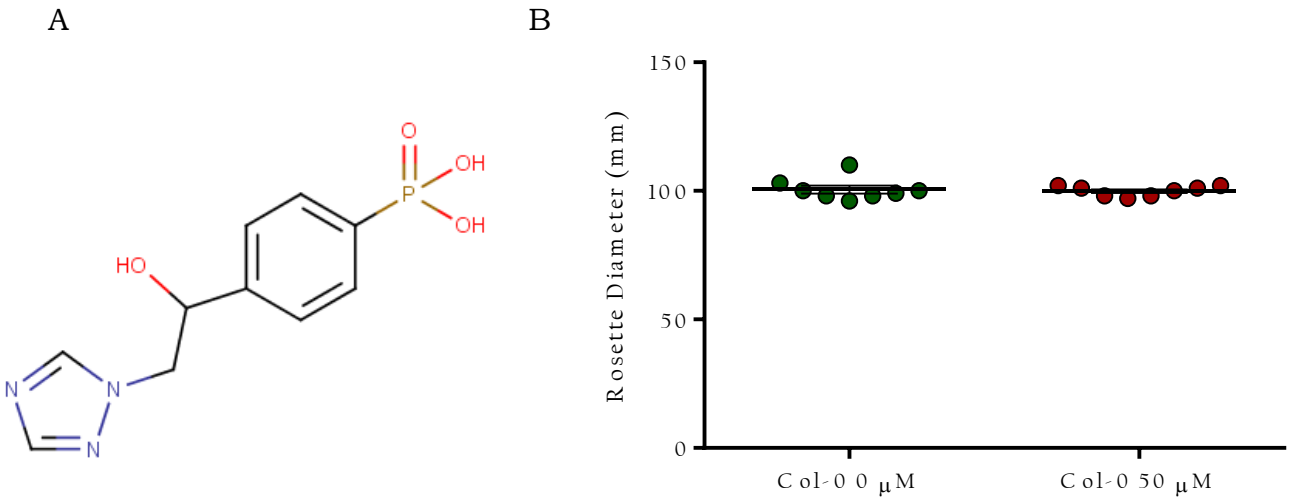


Figure 3-6 investigating the herbicidal efficacy of chemical 358

A, Chemical structure of C358 which was designed from structural studies of IGPD (produced using chemdoodle.com). B, Leaf rosette diameter measurements 6 weeks after application of C358 at 50 µM. C358 showed no significant effects on plant growth ($P > 0.5$; n=8).

A B

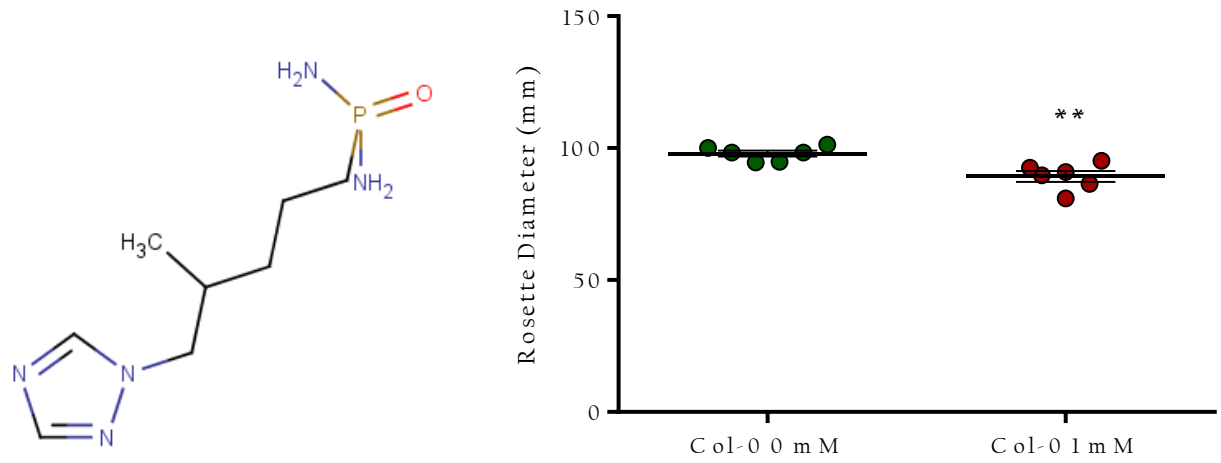


Figure 3-7 investigating the herbicidal efficacy of Chemical 367

A, Chemical structure of C367 (produced using chemdoodle.com). B, Leaf rosette diameter measurements 6 weeks after application of C358 at 1 mM. C367 showed a significant effect on plant growth. p = 0.0041; n=8.

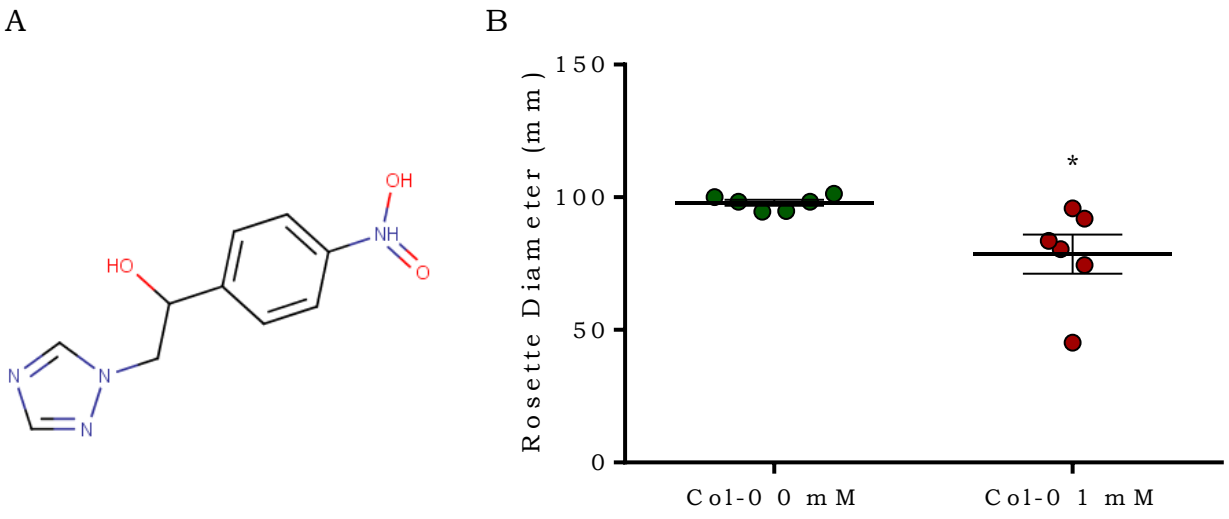


Figure 3-8 investigating the herbicidal efficacy of Chemical 368

A, Chemical structure of C368 (produced using chemdoodle.com). B, Leaf rosette diameter measurements 6 weeks after application of 1 mM C368 ($P = 0.0268$; $n=8$).

Chapter 4 – Investigating the effects of over-expressing IGPD

4.1 Introduction

In this chapter I discuss two forms of agricultural practice, the first conventional farming, with tillage, also referred to as ploughing and the other conservation farming, without tillage. I then describe a set of experiments designed to characterise plants that have been genetically modified with the purpose of increasing their level of tolerance to C348, the novel herbicide discussed in Chapter 3.

It has long been known that tillage, can cause damage to soil structure, soil fertility and increase the soil degradation rate. No-till farming negates this problem by using an herbicide and an herbicide resistant plant system. Currently the most widely used herbicide in this system is glyphosate, trade name Roundup, and glyphosate resistant plants, commercially known as Roundup Ready plants.

4. 2 Conventional agricultural practices and Soil degradation

Agricultural practices are variable and I will not discuss them in detail in this thesis, however I will summarise two main categories; conventional farming and conservational farming. Conventional farming utilises tillage which is a process where farmers invert the soil, usually using a plough, to bury unwanted plant residues; weeds and unwanted crop left from the previous harvest (Carter et al., 2003). Conservation tillage, also known as no-till farming or non-inversion tillage consists of at least 30% of crop residue or a cover crop being left on the field after harvesting (Davies B and B, 2002). Conservation tillage again has many derivatives from

minimal tillage to direct drilling of seeds through the residual or cover crop, See Figure 4.1 for the direct drilling approach.

Farming is thought to have played a role from the very first civilisations, however, the first known “ploughing” tool is thought to have been a wooden plough named an “ard” estimated to be developed between 4000 and 6000 BC (Lal et al., 2007). Ploughs are now much larger and moldboard ploughs are relatively common on the majority of arable farms in developed countries.

The primary use of tillage is weed control, nonetheless, with new herbicidal products it is becoming less necessary and alternative weeding methods are becoming more popular (Cannel and Hawkes, 1985). Conventional farming and intensive tillage has a two-fold effect on soil erosion. It leaves the soil surface exposed as well as disturbing the soil structure, this makes the top soil very susceptible to wind and rain runoff (Lal et al., 2007). Soil erosion is a natural process and the rate of erosion is dependent on several factors including; the amount and type of vegetation on the ground, soil structure, and weather conditions, in particular the amount and intensity of precipitation (Marsh, 1864). Soil is a vital part of agriculture and crop yield production is extremely variable on different soil types.

Tillage has a negative effect on the environment, as well as the growth of crops on a farm. It increases weathering and run-off by disrupting the plant soil interactions which hold the soil together. Runoff has many negative effects it causes removal of nutrients, pesticides and organic matter, which inevitably end up in rivers and lakes leading to

environmental issues (Lal et al., 2007). Studies have also shown that tillage causes a disruption of micro flora in the soil bed, in particular worm ecosystems, which are vital for natural aeration, improvement of soil structure and the recycling of organic matter (Jordan et al., 1997). Furthermore it reduces soil organic carbon (SOC), as SOC is concentrated in the soil surface leading to a lower soil productivity (Lal, 2004). Thus ploughing releases carbon dioxide from the soil which as a greenhouse gas contributes to global warming. Soil degradation is thought to be a serious issue globally and its severity is summarised in Figure 4.3.

With the increase in knowledge about the damage tillage can cause more farmers are turning to no-till farming. The prevalence of no-till farming has been increasing in all countries where records are held over the past ten years, in terms of percentage of hectares farmed (FAO, Website accessed on 24/06/2014 20:18), particularly in the Americas, see Figure 4.4. The UK has relatively low levels of no-till farming with less than 5% of cultivated land being conservational farmed. However, recent studies have suggested soil degradation is a problem in the UK, particularly in the south west where 38% of sites surveyed showed soil structure degradation which was severe enough to cause noticeable changes in surface water runoff (Palmer and Smith, 2013). Concerns have also hit the media with a spokesperson from Farmers Weekly being quoted saying "British soils are reaching crisis point" (Carrington, Website last accessed 24/06/14 19:23). These could be the first signs of Britain leaning further towards more conservational agriculture.

Seeds are drilled into the stubble/ mulch from the cover crop.

Crops grow through mulch and stubble. Often plants are sprayed with an herbicide that they are genetically modified to be

The main crop is harvested.

A cover crop is planted. This is a crop which may or may not be harvested.

The cover crop is harvested or a broad spectrum herbicide is used

No-Till Farming Process

Figure 4-1 No-Till Farming Process

No-till farming, also referred to as conservation tillage, is an alternative agricultural practice to conventional farming using a plough. After the main crop has been harvested at least 30% of the crop is left, or a different cover crop is planted, so the soil is never left bare. This cover crop is killed using an herbicide and herbicide resistant seeds are planted in the mulch. This has several benefits on soil properties as well as acting as a carbon sink.

4.3 Creating an herbicide resistant crop: using glyphosate resistance as a case study.

Several herbicide resistant crops have been produced and commercialised, including both transgenic and non-transgenic. In the majority of countries transgenic plants are heavily regulated and non-transgenic plants are unregulated (Green and Owens, 2011). The first commercialised herbicide resistant crop was non-transgenic and conferred resistance to the herbicide that inhibitis acetyl Coenzyme A carboxylase (ACCase), a vital enzyme in fatty acid biosynthesis in chloroplasts. The resistance was induced and selected for in tissue culture and transferred exploiting traditional breeding (Somers, 1996). Herbicide resistance can be conferred in several different ways including; finding a gene that can break-down the herbicide, inserting an additional copy of the targeted gene with a stronger promoter in an effort to out compete the inhibitor or inserting an analoFigugue of the target gene which is not sensitive to the herbicide, but can still effectively function in the enzymatic pathway the herbicide is targeting.

The United States has adapted its farming practices rapidly and the availability of herbicide resistant plants has allowed farmers to effectively control their weeds while simultaneously conserving soil. In 2012 figures showed that globally 81% of cotton, 35% of corn and 81% of soybean crops were genetically modified crop varieties (Nature, 2013). Although some moderately successful herbicide resistant crops have been produced, the largest success was produced in 1996 and is the glyphosate resistant soybean (Green and Owens, 2011).

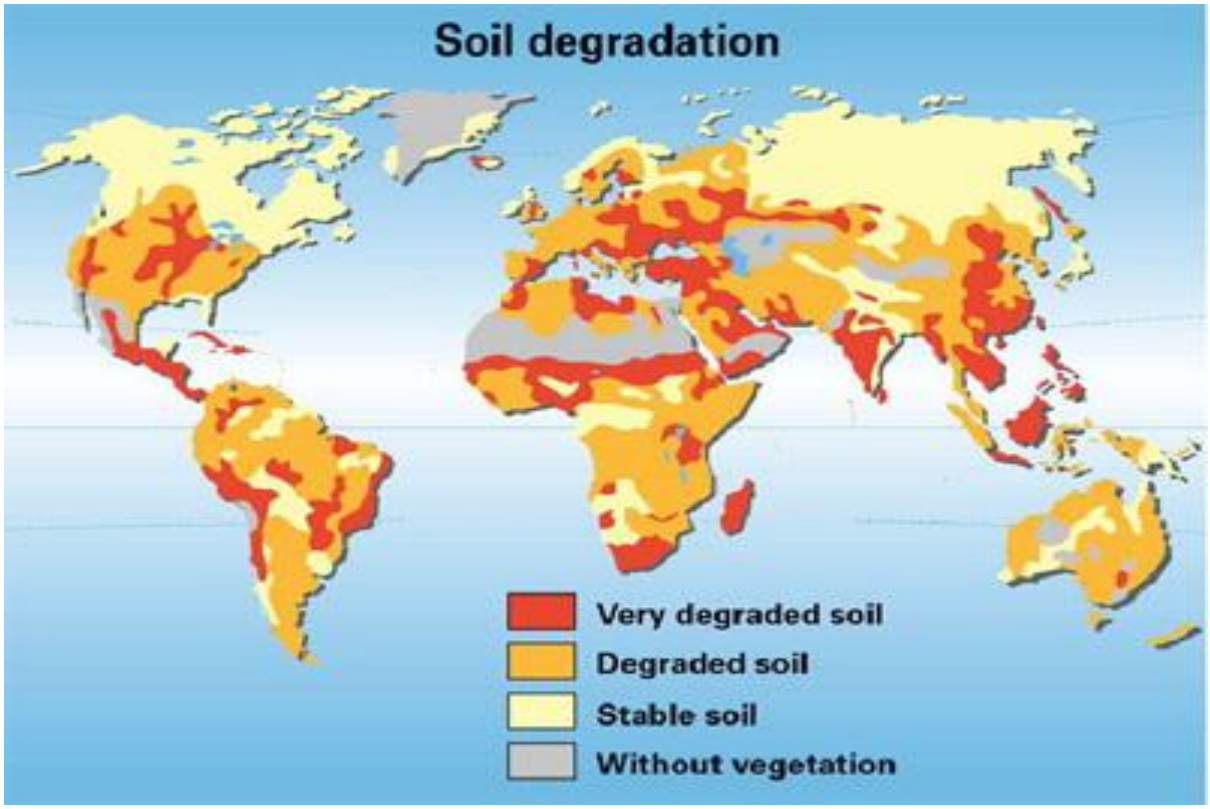


Figure 4-2 Global soil degradation

Soil degradation is a global issue and is summarised in this map. Degradation is particularly severe in countries that have extreme weather. Central America is particularly affected by soil degradation (UNEP, 1997).

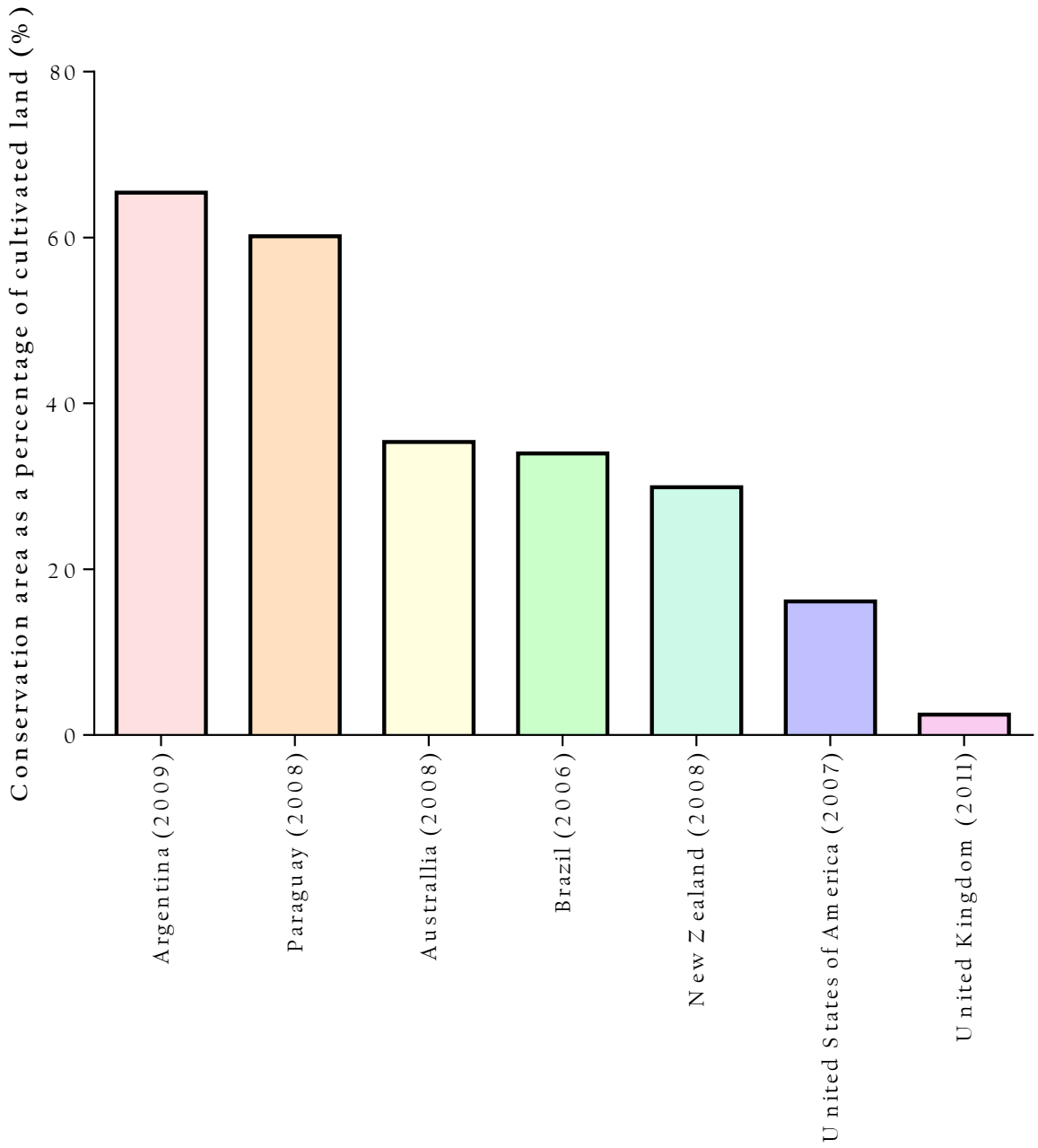


Figure 4-3 prevalence of Conservation Agriculture

Comparison of the use of conservation agriculture between countries as a percentage of arable farmed land. Countries in North and South America show high levels of conservational agriculture. The United Kingdom has relatively low levels with less than 5% of farmland being under conservational farming practices. Data from the FAO (FAO, Website accessed on 24/06/2014 20:18)

In Chapter 3 the herbicide glyphosate, commercially known as Roundup®, was discussed extensively. This is the most widely used herbicide and is often used in no-till farming in conjunction with glyphosate resistant, Roundup Ready®, plants. Glyphosate acts as an herbicide by targeting the enzyme EPSP synthase, glyphosate resistant plants are genetically modified to incorporate an *Agrobacterium sp* EPSP synthase gene on a constitutive 35S promoter that produces an enzyme that is not sensitive to glyphosate. (Funke et al., 2006)

Matthews et al 2006 (Funke et al., 2006) reported several studies in to the mechanism behind the loss of sensitivity to glyphosate in the *Agrobacterium sp* EPSP synthase protein. They concluded that glyphosate still binds to the *Agrobacterium sp* EPSP synthase, however as the *Agrobacterium sp* EPSP has a single-site mutation in the active site (ALA-100-GLY) glyphosate is no longer able to deactivate the enzyme.

The increase in glyphosate resistance in weed species, discussed in Chapter 3, has led to a large commercial push to find a novel herbicide and herbicide resistant system that could perhaps replace the Roundup Ready products. To this end, potential herbicide resistant plants were created by Dr Claudine Bisson, which are described in her PhD Thesis (Bisson, 2012), but only preliminary experiments were completed. The next section of this Chapter describes experiments to characterise the level of herbicide tolerance generated in these plants.

The plants discussed in this chapter, are known as IGPD over-expressors (*IGPD OE).* These are *Arabidopsis thaliana* plants which are expressing an additional Arabidopsis IGPD gene under the control of a constitutive

highly expressing CaMV 35S RNA promoter. In each independently transformed plant the gene construct has been randomly inserted into the nuclear DNA, using *Agrobacterium tumefaciens* transformation. Numbers after *IGPD OE* refer to independent plant lines. These plants are compared with their wild-type background Col-0 which was the ecotype transformed to create the *IGPD OE* lines.

4. 4 Results: IGPD transcript levels are higher in *IGPD OE* than in Col-0 plants

Insertion of the IGPD transgene into the plant genome had previously been confirmed using PCR (Bisson, 2012) and preliminary RT-PCR results had suggested that the gene was transcribed. However, total IGPD expression level (endogenous plus transgene levels) had not previously been investigated nor quantified. RNA was extracted from seedlings of *IGPD OE* – 4.4 and Col-0 controls and use as a template in quantitative RT-PCR reactions. Expression levels of *IGPD* relative to that of the actin gene, were significantly higher in the *IGPD OE* plants; 2.8 fold that of Col-0 levels when quantified using qPCR, Figure 4.5 A. As the level of transcription driven by the CaMv 35S promoter has previously been described as being 'at least 30-times stronger than the nopaline synthase promoter', which is also regarded as a strong plant promoter (Fang et al., 1989), the level of IGPD transcript expression in these plants is relatively low. The lower than expected level of *IGPD* mRNA observed in the over-expressing plants perhaps indicates that Arabidopsis plants are unable to tolerate high levels of IGPD activity, and that potentially highly expressing transformants did not survive the transformation and selection procedure.

4.5 Phenotypic effects of IGPD overexpression.

Seedlings were germinated and grown on agar for 1 week before being transferred to 3:1 soil: perlite. No initial differences were seen in visual comparisons between *IGPD OE* and Col-0 in shoots when mature, see figure 4.6 Chlorophyll fluorescence has become a widely used and a powerful technique to determine the efficiency of photo-chemistry (Maxwell and Johnson, 2000). Experiments investigating light adapted chlorophyll fluorescence, were used to gauge if the over expression of IGPD was having an effect on the photo-chemistry of the plant, which could be an indicator of the plant stress. The experiments showed no significant differences in chlorophyll fluorescence values between the two sets of mature plants, figure 4.6 B.

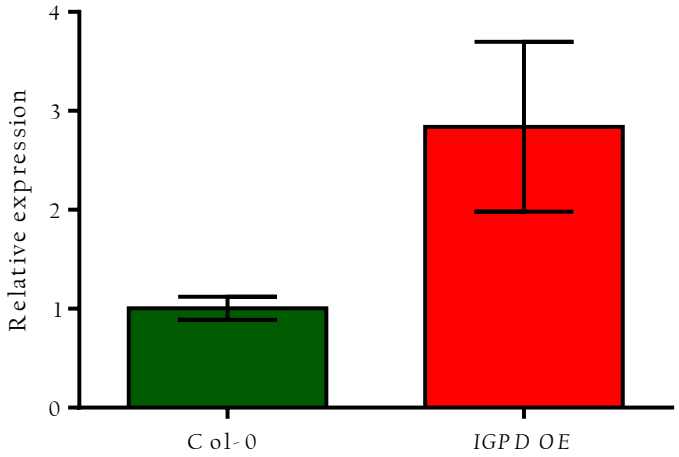
Concentrations of amino acids were measured on 7 day old seedlings germinated on 0 μM C348 plant agar or 50 μM C348 plant agar, figure 4.7. Unexpectedly histidine levels did not significantly decrease, this may be due to the low levels of histidine and the sensitivity of the HPLC. However, glutamine levels, found further up in the histidine synthesis pathway, do significantly increase after the application of C348 indicating some sort of blockage in the pathway. Other changes are observed which show differences between the treatment and the genotypes indicating both the over expression of IGPD and the C348 treatment are having a biochemical impact on the plant. Protein activity does not normally increases linearly with the increase in gene number (Peretti et al., 1989)The levels of most specific amino acids are hard to explain, however it is not rare for metabolic levels to change without an obvious explicable

reason (Sweetlove et al., 2008, Zhu and Galili, 2003). Furthermore, flux through a pathway is not always demonstrated well using metabolite levels as many things influence metabolic flux. A future enzyme assay may give more insight into the pathway flux (Byrne et al., 2014).When agar grown seedlings were examined again there were no obvious visible differences between shoots of IGPD over expressers and controls, further measurements are described later in the chapter. However, there were distinct differences in root length and root width. Primary root length in 7 day old seedlings was significantly shorter in *IGPD OE* plants in comparison with Col-0. The roots of the transgenic seedlings were approximately half the length of controls, Figure 4.8 A. The *IGPD OE* seedling roots also showed an obvious difference in morphology, appearing thinner, Figure 4.8 B. It was evident that the over expression of IGPD was having an adverse effect on root growth, however the growth of shoots appeared to be unaffected.

4.6 Growth of *IGPD OE* plants is less restricted than Col-0 on agar containing C348

Plants were grown on agar containing a several of concentrations of C348, ranging from 0 µM to 200 µM. Both rosette diameter and root length were measured 7 days after germination. At levels of 0 µM of C348, *IGPD OE* seedlings showed no significant difference in shoot diameter, but a significant decrease in primary root length in comparison to Col-0, Figure 4.9. At C348 concentration of 25 µM Col-0 showed a significant decrease in both rosette diameter and root length in comparison to *IGPD OE.* Primary root length was more significantly affected than shoot diameter at all concentrations. At higher concentrations of C348 the difference

A



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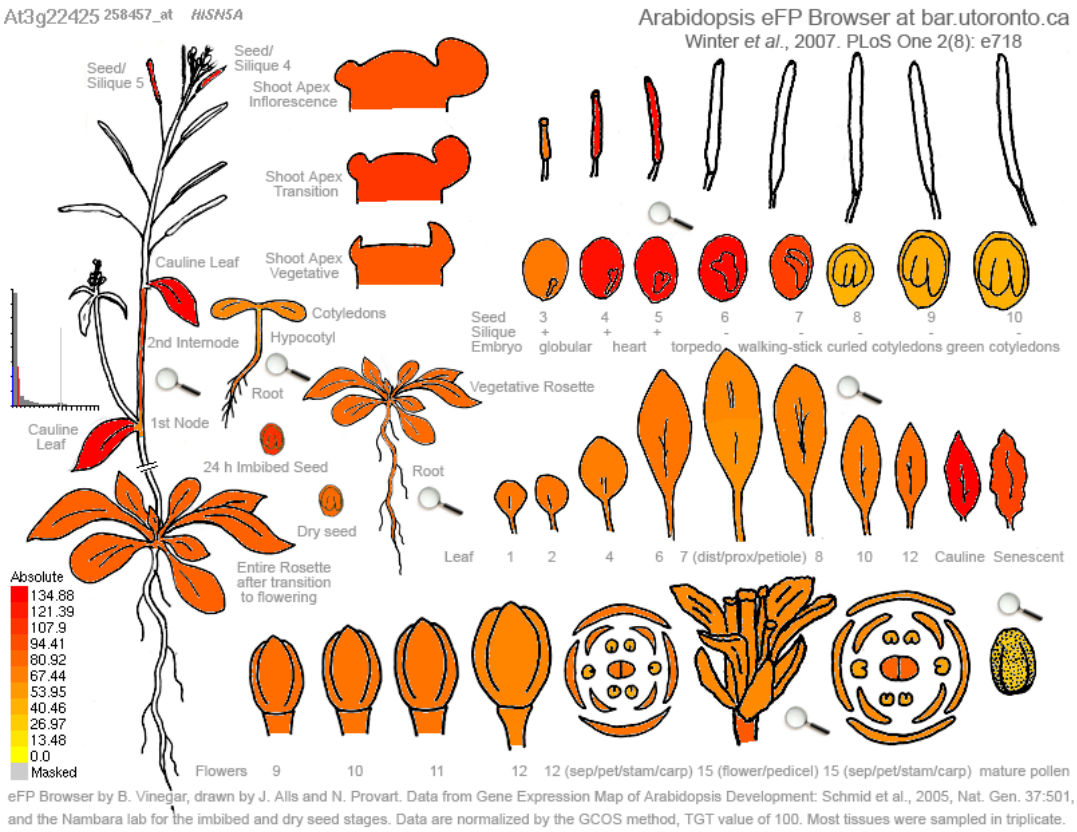


Figure 4-4 Expression levels of IGPD

A, relative expression of *IGPD* in *IGPD OE* and Col-0. IGPD expression measured using qPCR with an actin standard. IGPD expression levels are 2.8 fold increased in *IGPD OE* plants in comparison to Col-0 plants. n=3 p = 0.0213. B, Expression levels of IGPD in Col-0, Affymetrix microarray experiment data from Arabidopsis eFP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). IGPD expressed at moderate levels throughout most of the plant with the exception of the older tissues of the reproductive shoot.

A

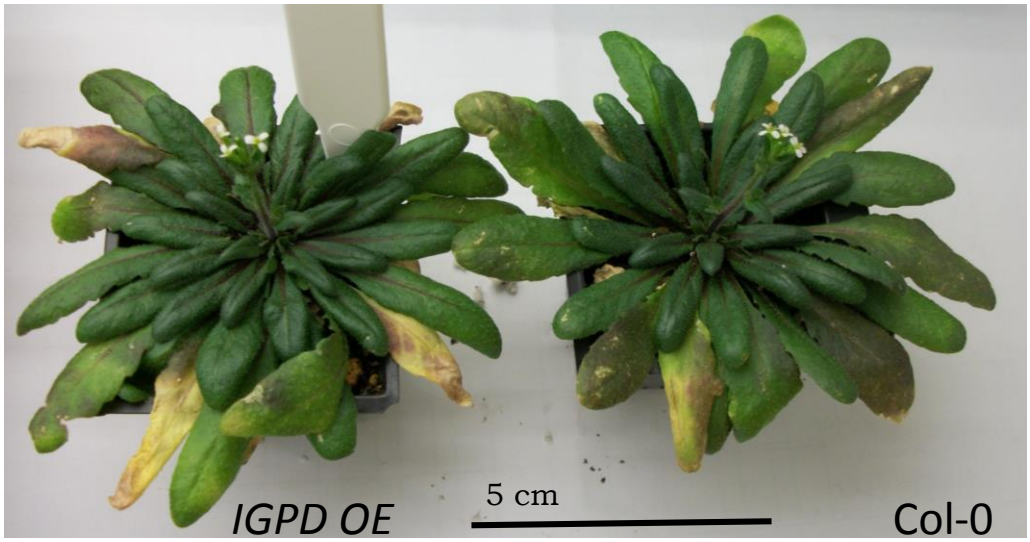


Figure 4-5 no obvious differences are observed in mature plants between IGPD OE and Col-0

A, a photograph of an *IGPD OE* plant and a Col-0 plant flowering. There are no obvious visual differences.

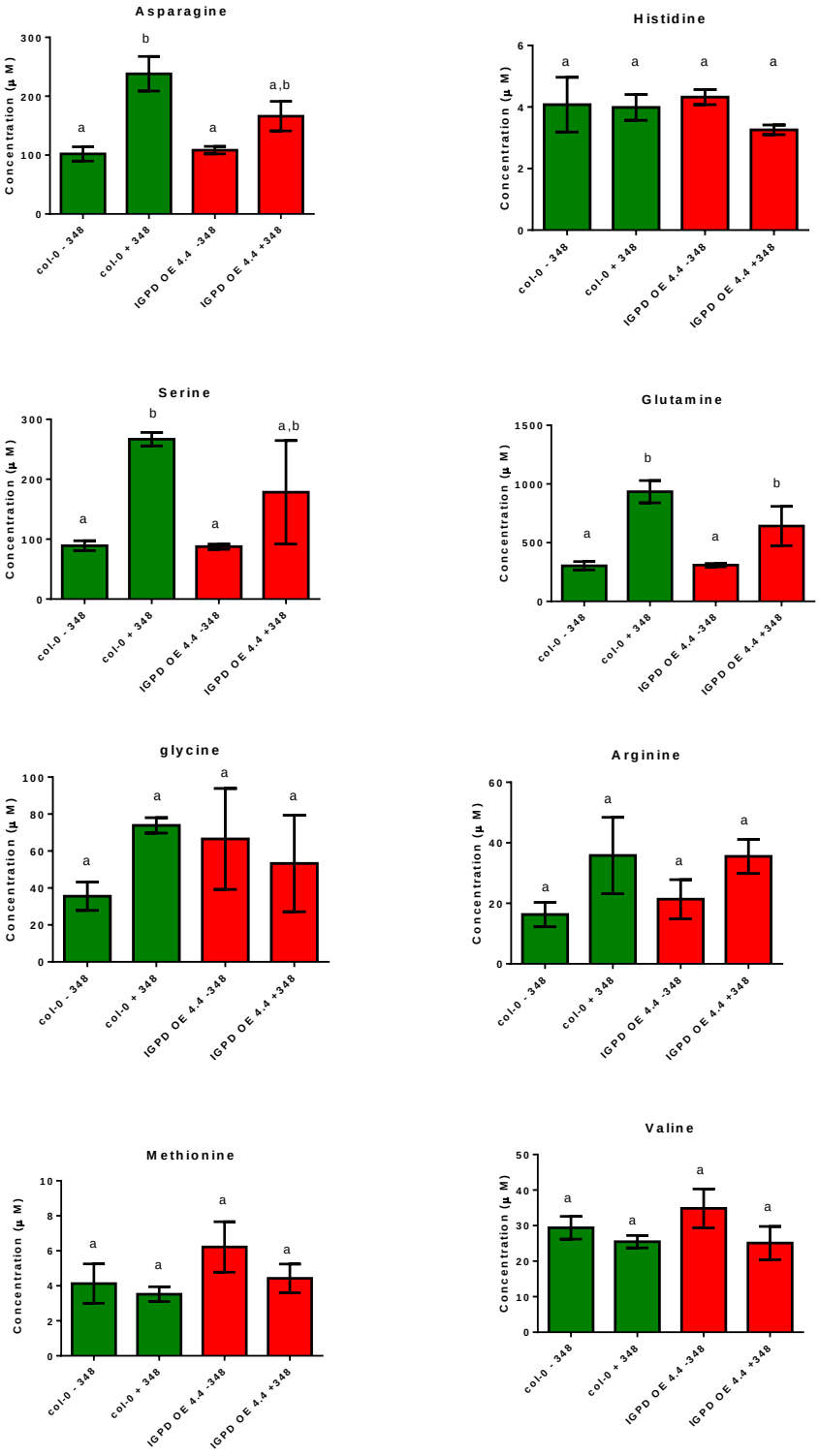


Figure 4-6 Changes in amino acids are observed between genotypes and conditions

Some changes in amino acid levels are seen between genotypes and between the control and C348 treatment group. These are significant in glutamine which is in the histidine biosynthesis pathway, however significant changes in serine and asparagine are hard to explain.

between Col-0 and *IGPD OE* growth was less pronounced as C348 had affected growth of both Col-0 and *IGPD OE* seedlings. At 200 μM Col-0 seeds either did not germinate or sprouted and died soon after. Germination of *IGPD OE* seedling showed more variation with some dying, at 0 μM, and others reaching the size of Col-0 plants not grown on herbicidal media. This wide variation in growth between individual over expressing plants made statistical analyses and data interpretation more difficult.

To compare the effect of C348 on mature plants, seedlings were transferred into pots containing 3:1 perlite to soil and measured weekly for five weeks. Three independently transformed lines of *IGPD OE* were studied. When plants were 4 weeks old leaf rosettes were sprayed with 1 ml of dilute tween containing one of several concentrations of C348. At the end of the experiment several plants of all three *IGPD OE* had grown larger rosettes at all concentrations except 0 μM. However, because of the more variable growth of the over expressing plants their mean size was not significantly different to controls, figures 4.10-4.12.

The three *IGPD OE* lines showed large variation in sensitivity to C348, as did individual plants within lines. The variation in *IGPD OE* plant size was greater than the variation in Col-0 size. A subset of IGPD OE plants exhibited very poor growth and there was an increased level of fatality in the over-expressing lines, even at 0 μM in the absence of C348. *IGPD OE* fatality had also been observed on the seedling growth agar experiments described above. This subset of unviable *IGPD OE* plants died more quickly than the Col-0 plants treated with C348, and were visually

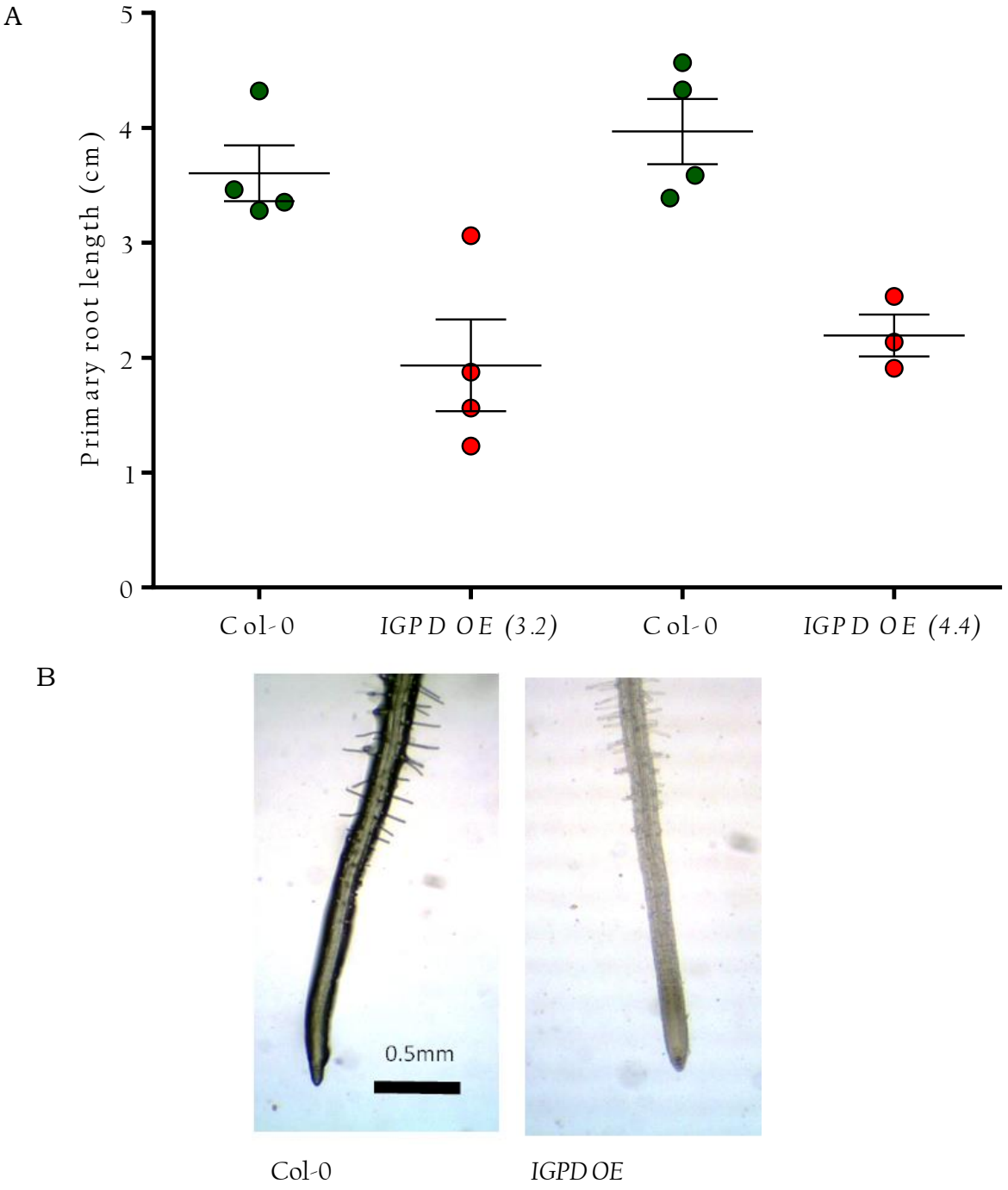
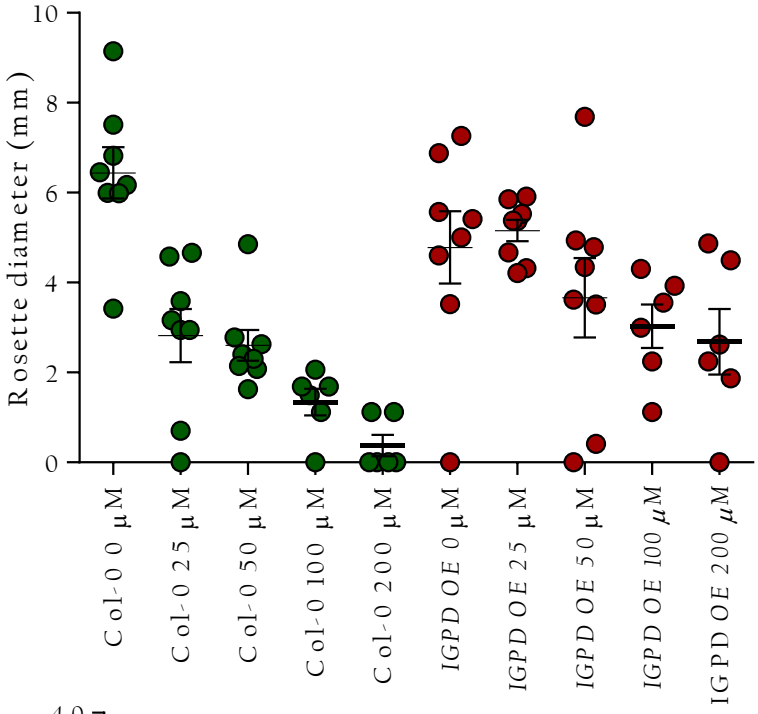


Figure 4-7 there are significant differences in root length in IGPD OE plants

A, individual dots represent individual plants. Green dots represent Col-0 plants and red dots *IGPD OE* plants. Primary root length is shorter in *IGPD OE* plants. n = 4 *IGPD OE* 3.2 p = 0.0116 *IGPD OE* 4.4 p = 0.0048. B, A micrograph of root types showing thinning of roots in *IGPD OE*

A



B

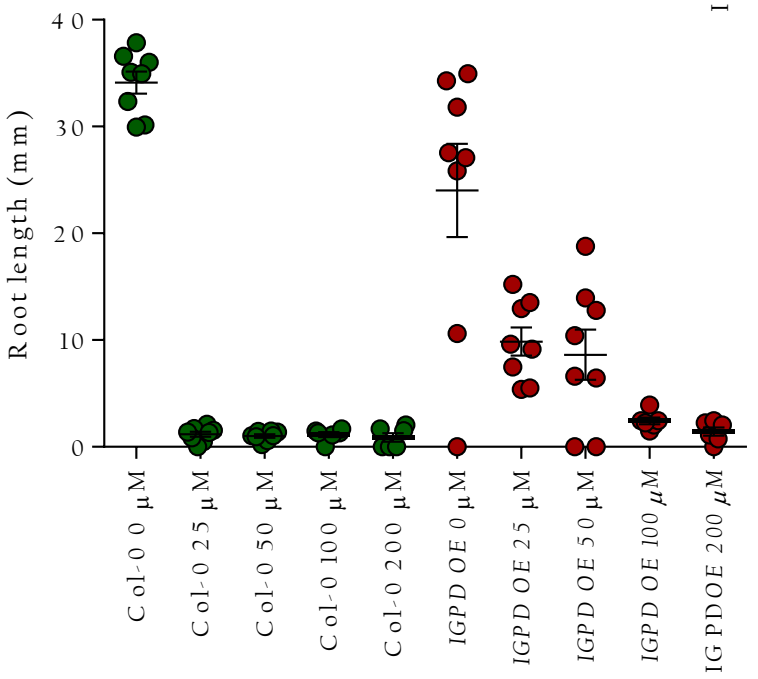


Figure 4-8 IGPD OE plants grow larger than Col-0 on agar containing C348

A, seedlings were grown on herbicidal agar at varying concentrations and rosette diameter was measured 7 days after germination. Individual dots represent individual plants. Green dots represent Col-0 plants and red dots IGPD OE plants. Col-0 are effected more by C348 even at low levels of 25 µM. n = 8 unpaired T test 0 µM P= 0.3879 25 µM p= 0.0026 50 µM p= 0.0240 100 µM p= 0.0138 200 µM p=0.0134 B, root length was measured 7 days after germination. Root length shows the most severe differences, with complete stunting of root growth in Col-0 from 25 µM and significant root growth inhibition from 100 µM in *IGPD OE.* n = 8 0

µM p= 0.043 25 µM p= <0.001 50 µM p=0.0061 100 µM p= 0.0111 200 µM p= 0.3413

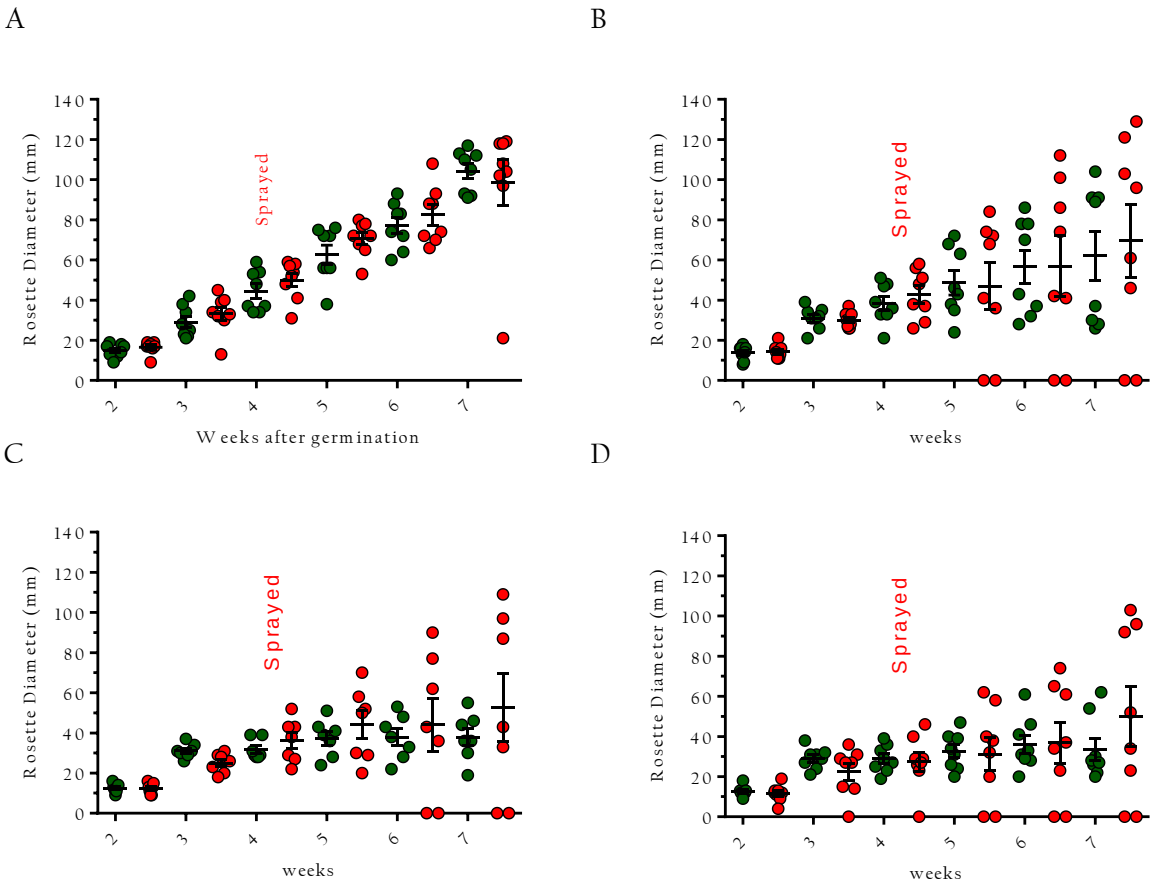


Figure 4-9 IGPD OE plants show large variation in their sensitivity to C348.

Line 4.4. Individual dots represent individual plants. Green dots represent Col-0 plants and red dots *IGPD OE* plants. Plants were sprayed with different concentrations of C348 and measured weekly. A, plants were sprayed with a dilute Tween control. Col-0 and *IGPD OE* show no significant difference. Unpaired T tests were performed on final measurements. P=0.6401 B, 25 µM large variation between individual plants. p = 0.7358 C, 50 µM IGPD OE shows larger variation than Col-0 p = 0.4222. D, 100 µM p = 0.3239

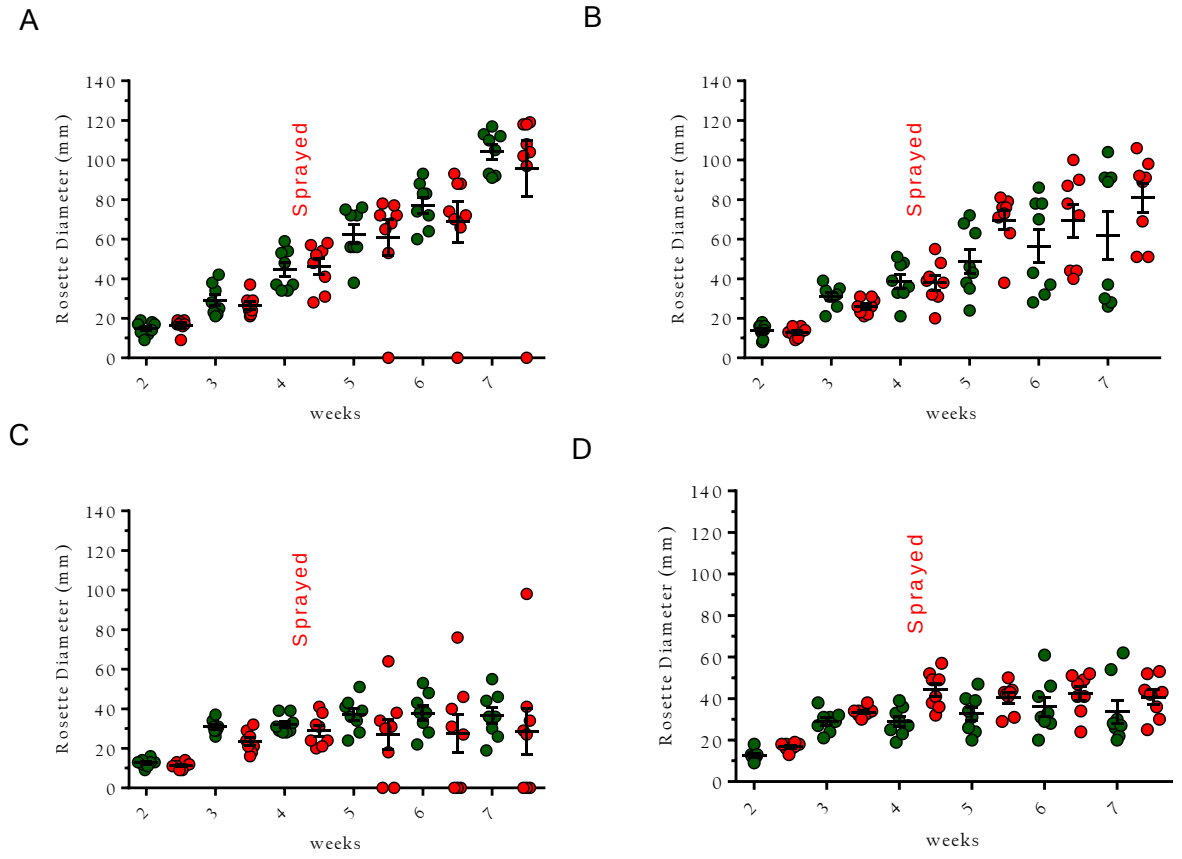


Figure 4-10 IGPD OE plants show large variation in their sensitivity to C348.

Line 3.2. Individual dots represent individual plants. Green dots represent Col-0 plants and red dots *IGPD OE* plants. Plants were sprayed with different concentrations of C348 and measured weekly. A, plants were sprayed with a dilute Tween control. Col-0 and *IGPD OE* show no significant difference. Unpaired T tests were performed on final measurements. A 0 µM p=0.5772 B, 25 µM p = 0.2074 C, 50 µM IGPD OE p = 0.5307 D, 100 µM p = 0.2999. n = 8

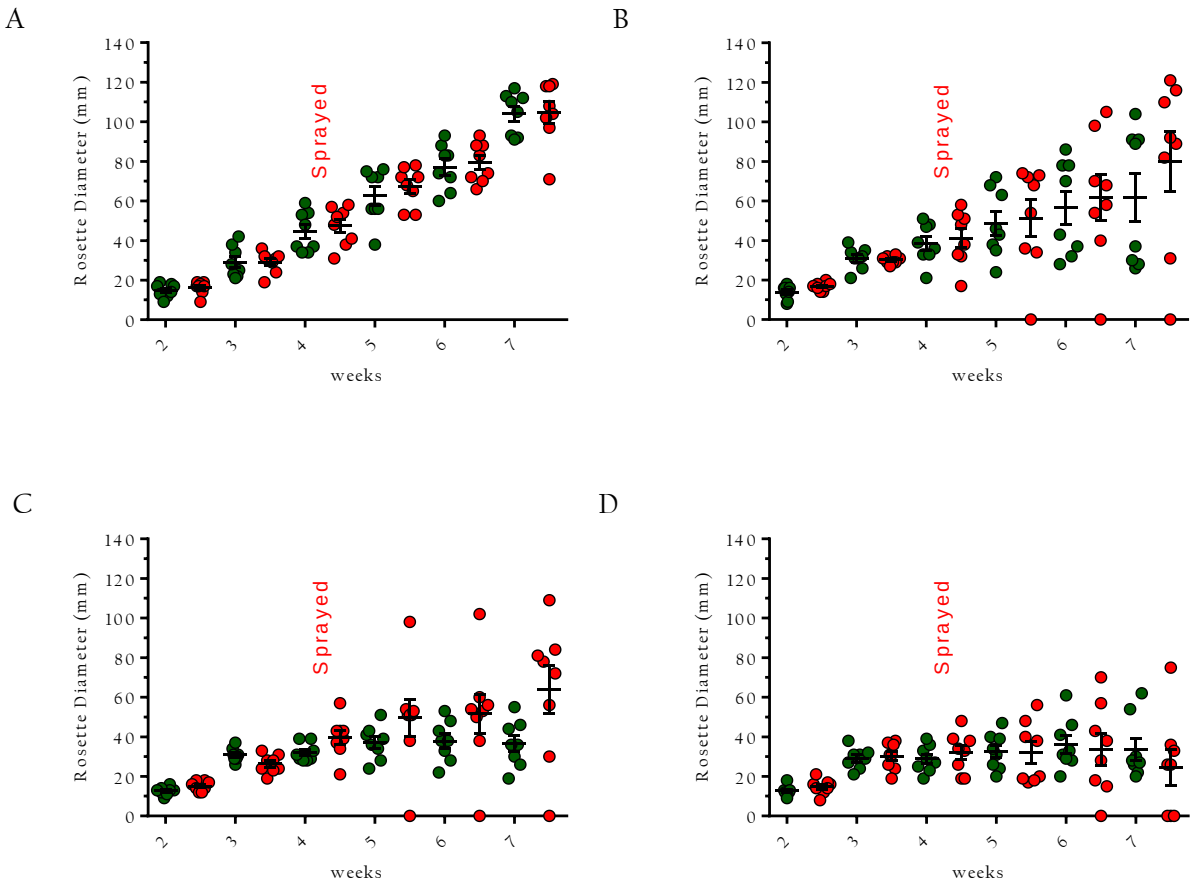


Figure 4-11 IGPD OE plants show large variation in their sensitivity to C348

Line 6.4 Individual dots represent individual plants. Green dots represent Col-0 plants and red dots *IGPD OE* plants. Plants were sprayed with different concentrations of C348 and measured weekly. A, plants were sprayed with a tween control. Col-0 and *IGPD OE* show no significant difference. Unpaired T tests were performed on final measurements. A 0 µM p =0.9421 B, 25 µM p = 0.3672 C, 50 µM p = 0.0522 D, 100 µM p = 0.4022 n = 8

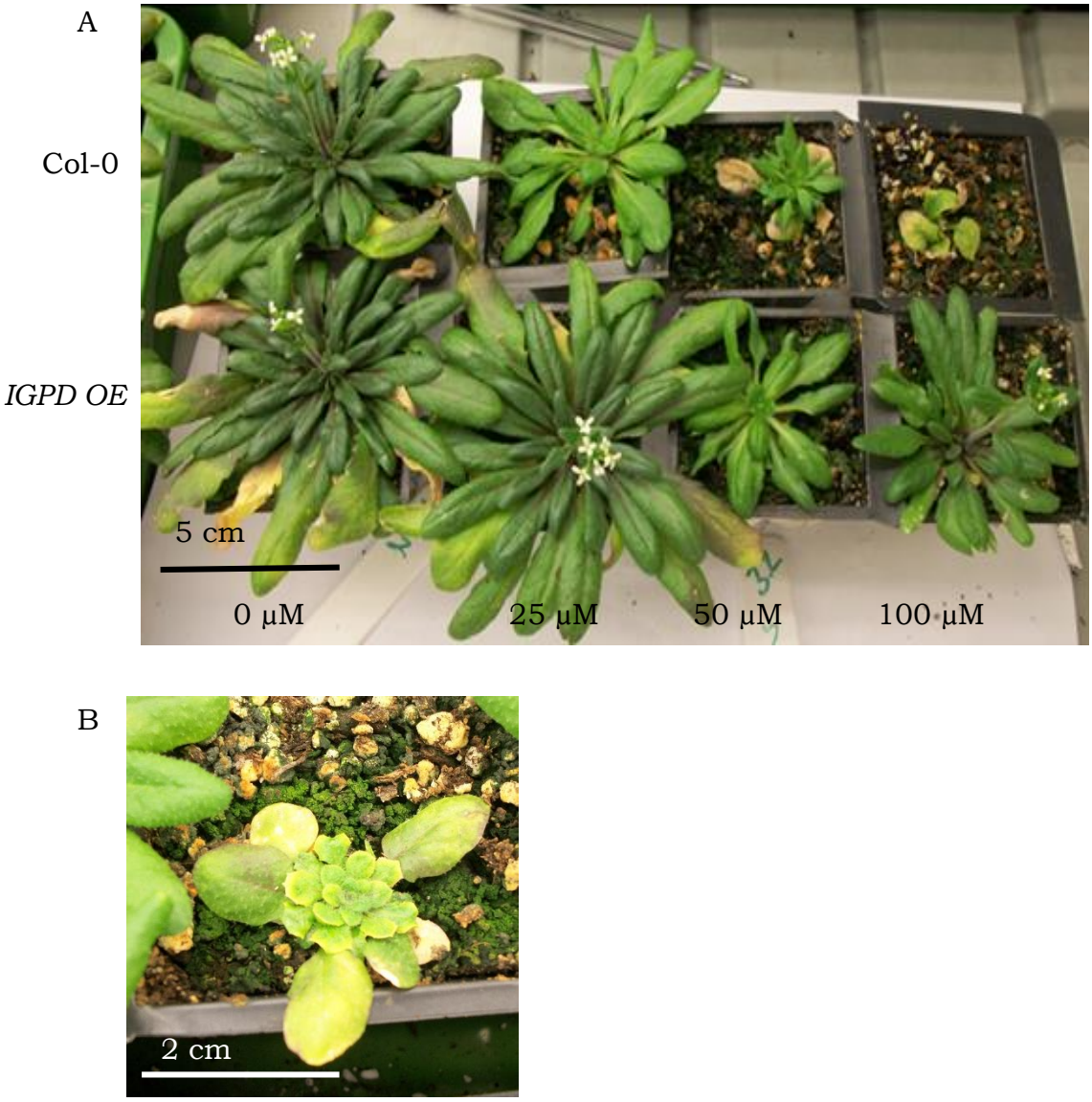


Figure 4-12 a subset of plants show an increased yellowing before sudden death

A, a photograph of representative plants at 7 weeks, 4 weeks after spraying of C348. Col-0 at the top of the photo are more susceptible to C348 in comparison to *IGPD OE* plants on the bottom row. The effect of C348 is dose dependent left 0 µM – 100 µM to the right. B, A representative photo of yellowing and grey spots on "spontaneous death" plants. *IGPD OE 4.4* plant two weeks after spraying 50 µM C348.

different, with grey and yellowing of leaves before death, Figure 4.13 B, A survey of the 'gardening' literature suggested that this leaf discolouration may be indicative of manganese deficiency. Manganese is crucial for many plant metabolic processes including photosynthesis and ATP synthesis as well as being a co-factor for many enzymes. Interestingly, manganese deficiency has recently been associated with a reduction in the length of the primary root of Arabidopsis plants grown on agar plates (Gruber et al., 2013).

Structural studies have shown that the active IGPD enzyme forms a 24mer protein which contains two manganese ions in the active site of each subunit. It is therefore apparent that the IGPD enzyme requires a considerable amount of manganese for activity. Indeed, it has been reported that *E. coli* genetically engineered to over express IGPD requires a medium supplemented with additional manganese to grow (Glynn et al., 2005).

The potentially high requirement for manganese ions, and the nutrient deficient phenotype raised the possibility that manganese deficiency could be the cause of the *IGPD OE* plant fatality. A manganese supplementation experiment was designed to investigate this further. Several preliminary experiments were performed with different concentrations of manganese, Appendix 1-2, until the concentration was determined that did not affect the growth of the Col-0 control plants.

In manganese supplementation experiments plants were transferred from plant agar to 3:1 soil to perlite 1 week after germination, sprayed with 1 ml 50 μM C348 tween mixture 4 weeks, after germination and measured

7 weeks after germination. Manganese supplemented plants were supplemented with 500 ml 0.5% manganese sulphate solution once a week for three weeks, one week before spraying and the two following weeks, Figure 4.14. Due to space constraints the experiment was limited to 2 independent lines of *IGPD OE* plus controls.

Manganese supplementation of 500 ml 0.5% manganese sulphate solution once a week for three weeks did not affect the rosette size of either Col-0 or *IGPD OE* plants growth in the absence of C348. In the absence of manganese supplementation, application of C348 by spraying 1 ml 50 µM

C348 tween mixture 4 weeks post germination caused higher rates of fatality in Col-0 than seen in the previous experiment (Figure 4.10-4.12) which may have been because a fresh preparation of C348 was supplied for this experiment. *IGPD OE* plants showed large variation in growth as observed previously; ranging from a subset of plants that died to plants that grew with no signs of sensitivity to C348. Interestingly with manganese supplementation fatality rate in both Col-0 and *IGPD OE* lines decreased. Col-0 showed some recovery from the effects of C348 and astonishingly *IGPD OE* plants showed a full recovery of growth. In the presence of additional manganese there was little difference between the growth of *IGPD OE* plants that had been sprayed with C348 and control plants which had not been sprayed.

4.3 Discussion

IGPD, an enzyme required for histidine biosynthesis was over-expressed under the control the strong CaMV 35S promoter in an attempt to produce

plants resistant to the potential herbicide C348 which is a potent inhibitor of IGPD activity. Despite the well-documented efficiency of this gene promoter, IGPD transcript levels were less than three times as high as wild-type, suggesting that higher levels of IGPD may have a detrimental effect on plant viability i.e. any plants expressing higher levels of IGPD may not have survived the original germination and selection process.

Although no detrimental effect on photo-chemistry could be detected by chlorophyll fluorescence, the *IGPD OE* plants did show phenotypic defects; the primary roots of seedlings were significantly stunted and a subset of older *IGPD OE* plants developed discoloured leaves and died. This 'spontaneous death phenotype' occurred across all experiments and initially was inexplicable.

Nevertheless, the majority of *IGPD OE* plants grew normally, and did show resistance to levels of C348 that inhibited the growth of wild-type plants. On agar plates wild-type root growth was abolished by 25 μM C348 but IGPD OE were able to grow roots relatively normally at 50 μM. Individual plants varied in their sensitivity to C348, however in all cases primary root length was affected more than leaf rosette diameter, perhaps because the root was in direct contact with C348 in the agar, or this may be related to the root growth defect observed in the absence of C348. When plants were grown in pots and treated with C348 many of the *IGPD OE* plants were able to grow relatively normally when sprayed with up to 50 μM C348 whereas the growth of all wild-type plants was restricted. However, the mean size of the treated IGPD OE plants was not significantly different to

that of wild-type because of the subset of *IGPD OE* plants that exhibited the 'spontaneous death phenotype'.

The *IGPD OE* plants that died spontaneously appeared to be suffering from a nutrient deficiency although they were unlikely to have insufficient supply of nutrients as the wild-type plants, and most of the *IGPD OE* plants, grew vigorously under the same conditions. Because the IGPD enzyme is known to bind manganese, and manganese deficiency has previously been shown to affect root morphology (Yang et al., 2008) experiments were designed to investigate whether *IGPD OE* plants required higher than normal levels of manganese. In the experiment shown in Figure 4.15 none of the *IGPD OE* plants spontaneously died in the absence of IGPD treatment. When IGPD was applied 5/16 IGPD OE plants died but when similar plants were supplemented with IGPD only 1/16 plants died suggesting that the *IGPD OE* plants were more likely to die from insufficient manganese availability than from inhibition of histidine biosynthesis by C348. Unexpectedly manganese supplementation also increased the viability of wild-type plants following treatment with C348 from 2/8 to 6/8 plants. This could perhaps be explained if the wild-type plants attempt to restore histidine biosynthesis in the presence of C348 by increasing IGPD levels, and hence also increasing their manganese requirement.

In summary, the results presented in this chapter demonstrate that resistance to the potential herbicide C348 can be engineered by overexpressing the target enzyme IGPD. However it appears that IGPD overexpression may be intrinsically detrimental to plant growth due to the

high requirement of this enzyme for manganese. Thus although C348 has herbicidal activity as demonstrated in Chapter 3, IGPD overexpression is probably not a suitable method for producing herbicide tolerant crops as plant could be susceptible to 'spontaneous death'. Nonetheless it remains a possibility that C348 resistant crops could be designed based on a mutant IGPD strategy as has been so successfully demonstrated in Roundup Ready glyphosate resistant crops.

Week 0	Germination	
Week 1	Transfer to soil	
Week 2		
Week 3		**Manganese supplementation**
Week 4	**C348 Application**	**Manganese supplementation**
Week 5		**Manganese supplementation**
Week 6		
Week 7	Measurements Taken	

Figure 4-13 Timeline of manganese supplementation experiment

Plants were germinated on agar and transferred to a 3:1 soil: perlite mixture 1 week after germination. Plants were supplemented with Manganese one week before C348 application and the following two weeks. Plants were sprayed with C348 4 weeks after germination. Measurements were taken 7 weeks after germination.

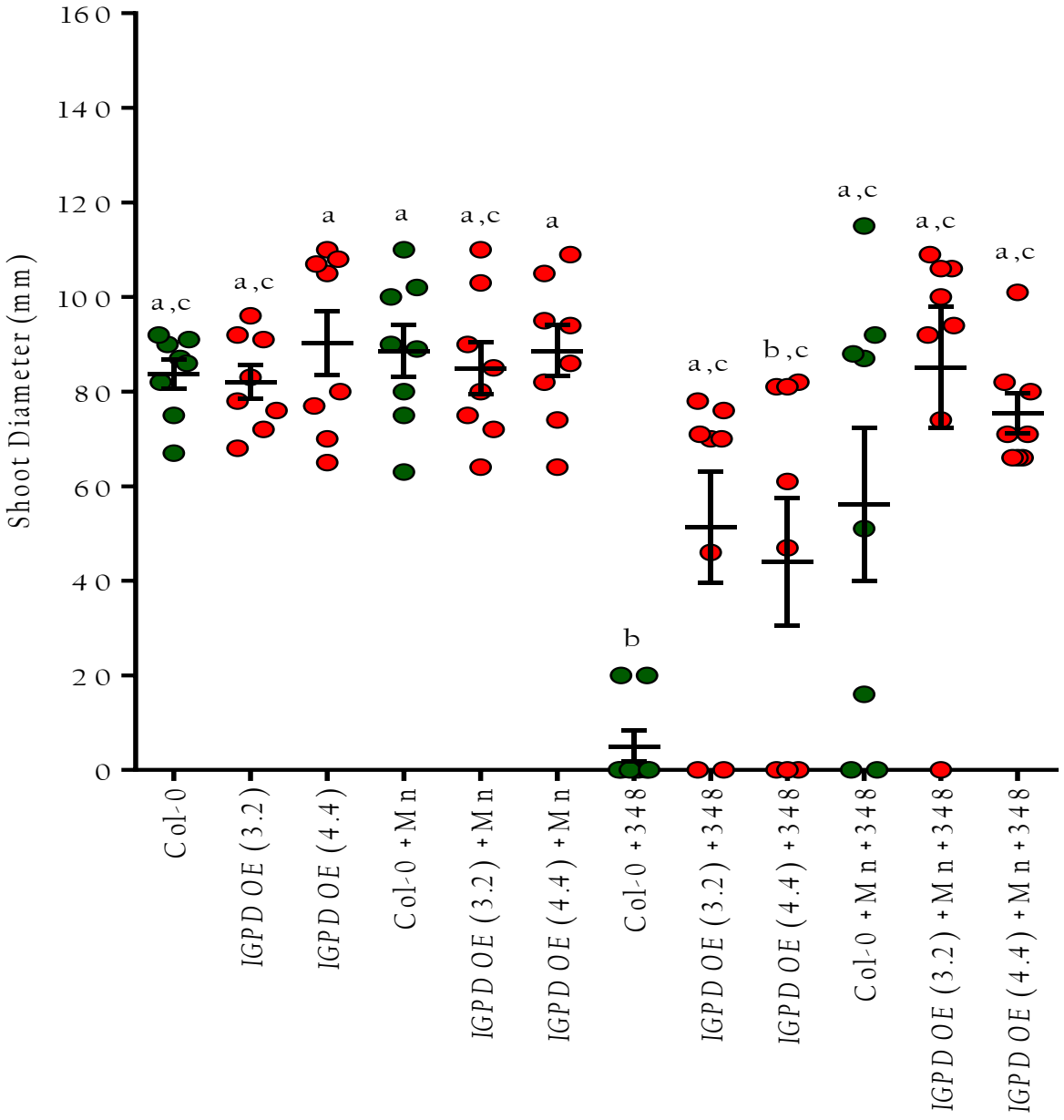


Figure 4-14 Manganese supplementation recovers IGPD OE fatality

Individual dots represent individual plants. Plants were sprayed with a 50 µM C348 tween solution and supplemented with a manganese solution and measured 3 weeks after the application. Manganese has no significant difference in control plants. Supplementation of manganese decreases sensitivity of C348 in Col-0 and recovers IGPD OE plants so there is no significant difference between them and the control group of plants. n = 8 one way ANOVA $p < 0.0001$

5. Chapter 5 - The effect of altered transpiration on root morphology.

5.1 Stomata

The main site for gaseous exchange in a plant is through its stomata. Stomata are microscopic pores on the surfaces of leaves created by a pair of guard cells that can adjust their turgor to increase or decrease the pore aperture, figure 5.1. As both the main entrance for carbon dioxide and the main site for water vapour loss stomata play a crucial role in plant water and carbon homeostasis. Stomata also play a significant role the regulation of water and carbon cycles on a global scale; for example it is estimated that twice the amount of water vapour content in the atmosphere passes through stomata each year (Hertherington and Woodward, 2003).

The acquisition of stomata on the aerial epidermis of plants is believed to have been a key evolutionary trait, allowing plants to regulate their water loss. The acquisition of stomata, along with the acquisition of roots and a waterproof cuticle, is believed to have facilitated the survival of early land dwelling plants and their successful colonisation of the land masses of the earth. This probably occurred about 0.5 billion years ago as primitive stomata are observed in early land plant fossils dating back over 450 million years.

Stomata play a pivotal role in balancing gaseous exchange while protecting the plant from water loss. They can respond to environmental cues both in the short term, by adjusting the aperture of the stomatal pore or over a longer period, by modulating the level of stomatal development on newly forming leaves. Environmental changes in atmospheric carbon dioxide concentrations, light intensity

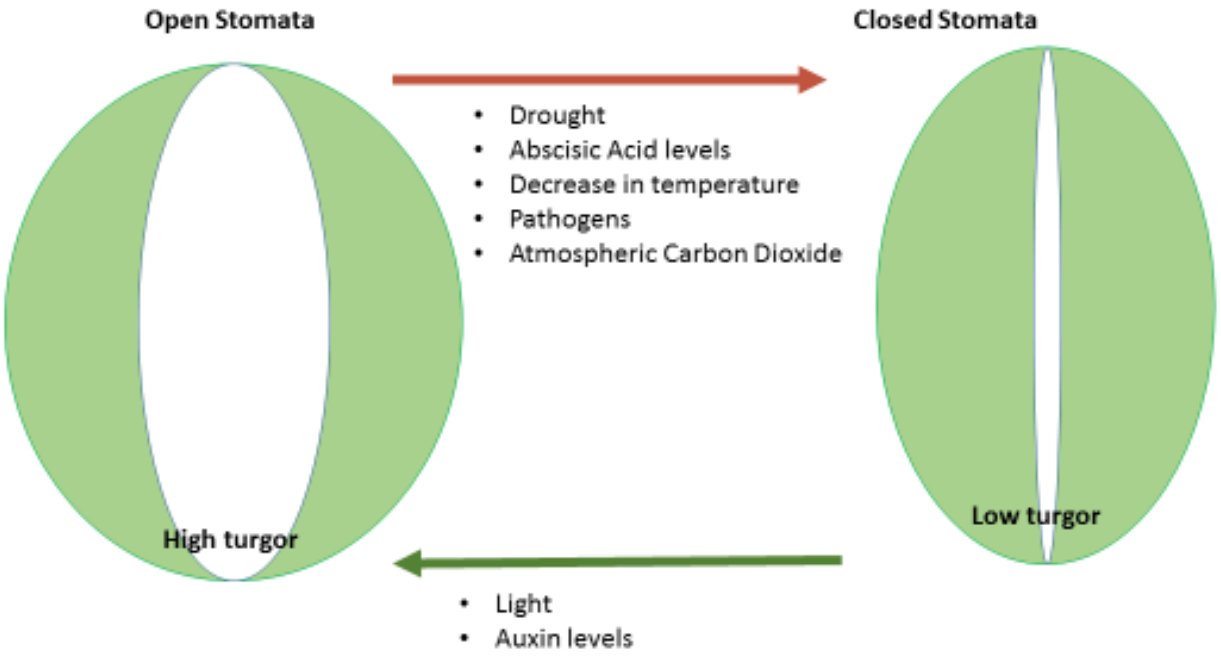


Figure 5-1 several environmental signals stimulate stomatal opening or closure.

Stomata are microscopic pores on the epidermis of plants formed by two guard cells. The turgidity of these guard cells regulates the aperture of the stomatal pore, with less turgid guard cells causing a decrease in aperture. Stomatal apertures are adjusted in response to several environmental cues. For example an increase in drought, abscisic acid, certain pathogens or atmospheric carbon dioxide concentration leads to stomatal closure. In comparison, an increase in light intensity or auxin levels are cues for stomatal opening.

and atmospheric humidity, as well as changes in specific phytohoromones have all been shown to have an effect on both the degree of stomatal opening and the degree of stomatal development. Adjustments in stomatal aperture are caused by a change in the turgor pressure of the two guard cells that surround each pore. As the two guard cells are joined at their ends, when guard cells are turgid they bend around their stiffened inner cell wall and the pore aperture opens and when they become flaccid the pore closes. Turgidity of guard cells is controlled by a signalling pathway which regulates the uptake and release (or metabolism) of intracellular osmolytes. Guard cell turgor is mainly controlled by the influx and efflux of potassium cations which are counterbalanced by chloride or malate anions (Hetherington, 2001). The drought hormone abscisic acid (ABA) has been shown to affect stomatal pore size and plays a key role in the activation and deactivation of ion channels and regulation of transcriptional events. As stomata play such a pivotal role in adaption to environmental change, and have such complex signalling pathways, they are of great interest for scientific investigation and have been relatively well-characterised.

5.2 Stomatal Development in *Arabidopsis thaliana*

Much of our understanding of the cell division patterns and molecular components regulating stomatal development come from studies of the model plant *Arabidopsis thaliana*. It has long been observed that stomata on the species *Arabidopsis thaliana* show a one-cell-spacing

pattern, meaning that stomata almost always have a non-stomatal cell between them and do not form next to each other. This stomatal one-cell spacing pattern was hypothesised to be created by cell-to-cell signals in 1991 (Sachs, 1991).

Since first hypothesised, the molecular pathway that creates the one-cell-spacing pattern has been one of the most extensively explored in plant science.

Stomatal development occurs in several steps, characterised by a tightly regulated series of cell divisions and transitions Figure 5.2. The initial step is characterised by the transition of a protodermal cell to a meristemoid mother cell (MMC) which has the capacity to undergo an asymmetric cell division to produce a stomatal precursor cell. This asymmetric 'entry' division is controlled by a basic helix-loop-helix transcription factor known as SPEECHLESS (SPCH). Mutant plants that lack SPCH expression (*spch*) form an epidermis entirely void of stomata (MacAlister et al., 2007, Pillitteri and Torii, 2007, Pillitteri et al., 2007). The SPCH-regulated 'entry' division produces two cells, a small triangular shaped meristemoid and a larger sister cell. The sister cell can enter the stomatal development pathway and like other meristemoid mother cells can divide asymmetrically to create further meristemoids. The meristemoid usually divides asymmetrically two to three times to create further pavement cell precursors before differentiating into an oval shaped guard mother cell (GMC) (Zhao and Sack, 1999).

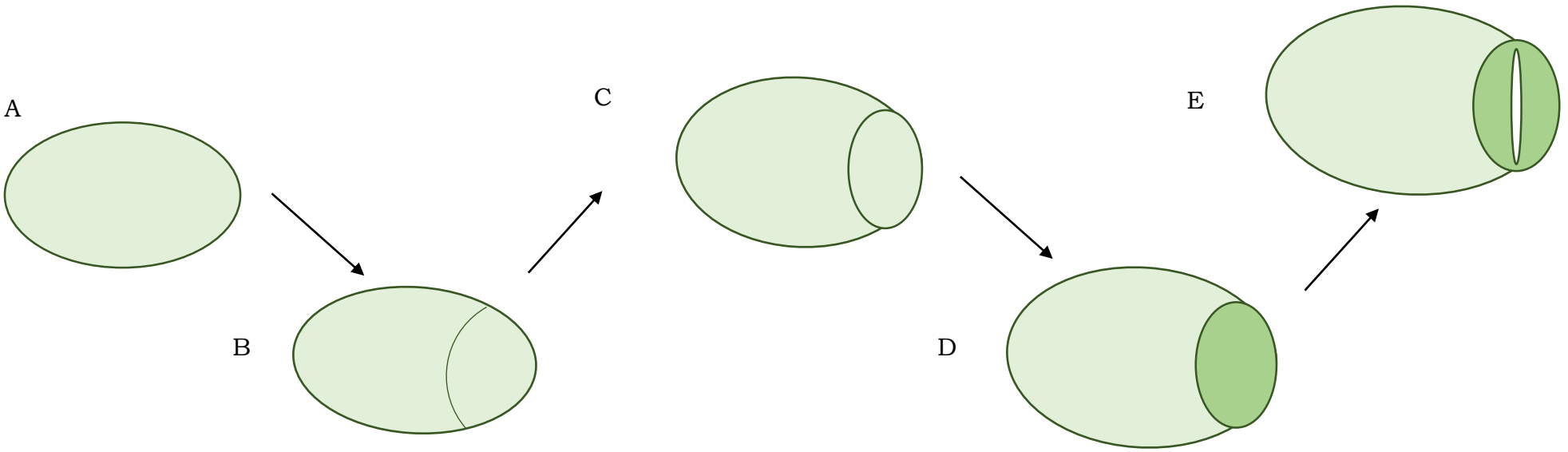


Figure 5-2 The pattern of epidermal cell divisions leading to stomatal development

Regulated cell divisions typically lead to the development of a stomate. A-B, A MMC divides asymmetrically to form a meristemoid and a larger sister cell. B-C, The triangular shaped meristemoid divides asymmetrically 0-3 times before differentiating to form an oval shaped GMC. D-E, The GMC divides symmetrically to form two cells which mature in to the two kidney shaped guard cells of a stomate. The larger sister cell may also divide asymmetrically to form an additional meristemoid which will undergo the above transitions to produce a satellite stomate.

This transition is controlled by another related bHLH transcription factor, MUTE (MacAlister et al., 2007, Pillitteri and Torii, 2007, Pillitteri et al., 2007). The final 'exit' division step is controlled by a third bHLH transcription factor, FAMA which induces the GMC to divide symmetrically only once to form the two guard cells

The transcription factors that control the regulated transitions of the stomatal lineage cells have been shown to be under the control of a mitogen activated protein kinase (MAPK) cascade which is activated by extracellular binding of secretory peptides. The peptides signals which are produced and secreted by stomatal precursor cells prevent stomata from forming adjacent to one another. In contrast, a related peptide produced by the developing mesophyll acts to stimulate stomatal development.

5.3 The Molecular Basis of Stomatal Development in *Arabidopsis thaliana*

Stomatal development is controlled by a family of secretory peptides known as Epidermal Patterning Factors (EPFs). There are 11 members of the Arabidopsis EPF family, all characterised by an N-terminal signal sequence which mediates secretion, and 6 cysteine residues found in conserved positions of the peptide (Rowe and Bergmann, 2010). These cysteine residues are thought to play a vital role in the

structure and function of the mature peptides by forming disulphide cross-bonds which have been confirmed by structural studies of EPF1 and EPFL9 (also known as STOMAGEN) (Kondo et al., 2010). So far several of the 11 EPFs have been characterised and found to affect stomatal development. EPF1 and EPF2, secreted exclusively from stomatal lineage cells, have been shown to negatively regulate stomatal development (Hara et al., 2007, Hunt and Gray, 2009), whereas EPFL9, secreted from the mesophyll, has been shown to positively regulate stomatal development (Kondo et al., 2010, Hunt et al., 2010). EPFL6 (also known as CHALLAH) is expressed in the hypocotyl and again is a negative regulator of stomatal development. The positive and negative regulatory peptides are believed to compete for binding to the same receptor components.

It is thought the most likely receptors for the EPF ligands are the ERECTA family of leucine rich repeat receptor kinases, specifically ERECTA (ER), ERECTA-LIKE 1 (ERL1), ERECTA-LIKE 2(ERL2) and intermolecular binding has been demonstrated. The receptor-like protein TOO MANY MOUTHS (TMM) which is also involved in EPF ligand perception is expressed exclusively in the stomatal lineage cells, and is encoded by one of the first genes identified to be involved in stomatal development. When TMM expression is perturbed by ethyl methanesulfonate mutagenesis stomata neglect the one-cell-spacing rule and mutants show clustering of stomata (Yang M and D, 1996).

In a *tmm* mutant the leaves exhibit increases in both stomatal density and clustering, however, the stems lack stomata. Interestingly unlike EPF1 and EPF2, that show no phenotype in a *tmm* background the over expression of EPFL6 in a *tmm* background has been shown to give an exaggerated phenotype (Abrash and Bergmann, 2010).

The TMM putative leucine rich receptor-like protein (LRR-LP) does not have a cytoplasmic domain and therefore is thought to work in coordination with the ERECTA family of leucine rich repeat- receptor like kinases (LRR-RK) (Shpak et al., 2005). Single and double mutants of ER, ERL1 and ERL2 show one-cell-spacing phenotypes consistent with the wild type, nevertheless, *er:erl1:erl2* triple mutants have multiply clustered stomata indicating the proteins function redundantly as a negative regulators of stomatal clustering. It is believed that TMM and the ERECTA family form components of a receptor which binds extracellular EPF1 or EPF2 and activates an intracellular MAPKK cascade which then leads to the phosphorylation of SPCH transcription factor and inhibition of stomatal fate. In contrast, EPFL9/STOMAGEN competes with the other EPF's for receptor binding thereby promoting stomatal development.

5.4 The Manipulation of Stomatal Densities in Pursuit of Increased Drought Tolerance.

Current forecasts of long-term climate patterns suggest an increase in extreme weather events, including longer lasting drought periods

(IPCC, 2007b). Although the screening of existing crop germplasm for drought tolerance has yielded differences in water use efficiency, this has only on rare occasions been translated through to the field (Condon, 2004). Several biotechnological solutions have also been proposed to increase crop drought tolerance such as manipulating the levels of transcription factors (Nelson et al., 2007), bacterial chaperones (Harrigan et al., 2009), dehydrins (Hassan et al., 2013) or the alteration of stomatal aperture control (Miura et al., 2013, Cominelli et al., 2005, Garcia and Lamattina, 2001). While these approaches have proved tractable the development of elite varieties has been slow.

Our rapidly improving molecular understanding of the components controlling stomatal development has recently facilitated studies of the physiological implications of altering stomatal density within plants of the same genetic background. Manipulating the level of expression of the epidermal patterning factors has produced *Arabidopsis thaliana* plant lines which have stomatal densities ranging from approximately 20% to 325% of normal levels, and has shown that plants with reduced stomatal density have reduced levels of transpiration, potentially enhanced water use efficiency, and are able to grow larger, especially under conditions of water restriction (Doheny-Adams et al., 2012, Dow et al., 2013). Together these findings suggest that plants with reduced stomatal density may be better able to survive drought conditions.

The increased size of leaf rosettes observed in a genotype manipulated to have a lower stomatal density in the Doheny-Adams *et al.* (Doheny-Adams et al., 2012) study has not been explained. In the current study it was explored whether this increase in above ground leaf area could be attributed to the plant being better able to conserve water and therefore putting less resource into an extensive root system, below ground. In this chapter I discuss the results of investigations into the impact of altering stomatal development and therefore transpiration rates, on a range of root parameters. This set of experiments focused on examining the roots of plants manipulated to have an increased or decreased level of stomatal development by manipulation of the expression levels of EPF1 and EPF2. These plants have been described previously (Hunt and Gray, 2009, Doheny-Adams et al., 2012, Dow et al., 2013). Both EPF1 and EPF2 are native regulators of stomatal development; affecting both stomatal density and stomatal clustering. EPF2 acts early in stomatal development and prevents too many cells entering the stomatal lineage, it also has a minor role in preventing stomatal clustering. EPF1 acts slightly later and principally regulated stomatal clustering but also has a minor role in supressing stomatal development.

5.4 Root hair development

Root hairs are specialised epidermal cells that elongate from their tip (Tanaka et al., 2014). It has long been suggested that root hairs play

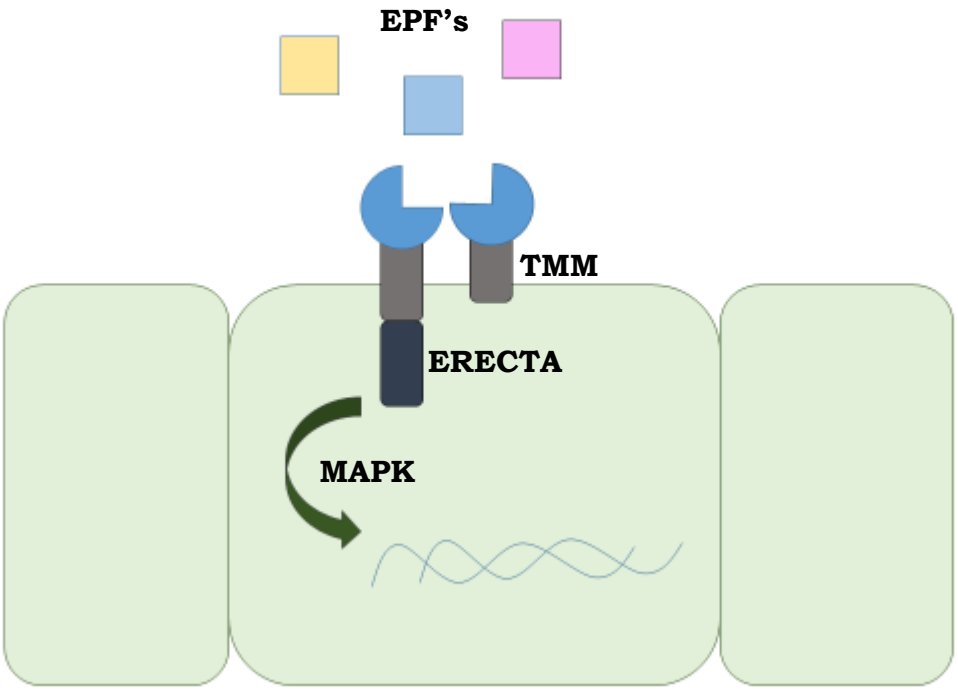


Figure 5-3 the molecular basis of stomatal development

Stomatal development is regulated by a relatively well characterised plant signalling pathway. Epidermal patterning factor secreted peptides (EPFs) compete for binding to a receptor complex involving TMM and members of the ERECTA family. In the case of EPF1 and EPF2, extracellular peptide binding leads to the activation of a MAPK cascade and the phosphorylation and destabilisation of the SPCH transcription factor. In the case of EPFL9/STOMAGEN, extracellular binding prevents EPF 1 or EPF2 binding, the MAPK cascade is not activated, SPCH remains active and can stimulate the asymmetric 'entry' division required to promote stomatal development.

important roles in anchorage, water uptake and nutrient uptake by increasing the surface area of the root (Datta et al., 2011). Root hairs in *A. thaliana* are positioned in a striped pattern alternating between root hair cells and non-root hair cells, this is controlled by a network of feedback loops (Libault et al., 2010).

In a similar way to stomatal development root hair development is regulated by a series of positive and negative molecules (Tominaga-Wada et al., 2011). The development of root hairs is often divided into three phases; the initiation phase, regulated by transcription factors, GTPases and cell wall enzymes. The bulge stage and the elongation stage, characterised by polarized cell growth (Datta et al., 2011). ROOT HAIR DEFECTIVE6-LIKE 4 (RSL4) is a basic helix loop helix transcription factor that controls root hair initiation. Similar to RHD6, ROOT HAIR DEFECTIVE 6-LIKE 1 (RSL1) is another bHLH transcription factor involved in root hair initiation, Mutants that have function of RHD6 and RSL1 knocked out do not develop any root hairs (Yi et al., 2010).

5.5 Results

First, the expression patterns of *EPF1* and *EPF2* were re-examined to determine whether these genes could be expressed in root tissues. Previous reports have shown that *EPF2* is principally expressed in meristemoids and guard mother cells, and *EPF1* is expressed in guard

mother cells and young guard cells (Hara et al., 2007, Hara et al., 2009, Hunt and Gray, 2009). This is consistent with the roles of the encoded signalling peptides in controlling the density and distribution of stomata during leaf development. However, the expression of *EPF1* and *EPF2* in below ground tissues, has not been previously examined. To investigate the possibility of gene expression in the roots, Arabidopsis *Col-0* plants modified to express the histological stain β-glucoronidase (GUS) under the control of the *EPF1* or *EPF2* 5'-DNA promoter region (Hunt and Gray, 2009) were stratified and sown on agar plates. Seedlings were removed and incubated with the X-gluc substrate at 7 days post germination to localise GUS activity. The results were clear. Neither the *pEPF1:GUS* nor the *pEPF2:GUS* seedlings showed any GUS expression in seedling roots indicating that neither of the encoded signalling peptides are expressed in below ground tissues. As expected, both genotypes showed GUS expression in stomatal lineage cells of the seedling leaves as previously reported (Figure 5.4).

In the next set of experiments it was investigated whether the growth and architecture of roots could be affected in plants with altered stomatal density. The Arabidopsis genotypes studied were *EPF2*OE which has been manipulated to ectopically overexpress EPF2 under the control of the CaMV35S promoter and exhibits a dramatic reduction in stomatal development; and *epf1epf2* which lacks

expression of both *EPF1* and *EPF2*, has much increased stomatal density, additional arrested stomatal lineage cells, and displays a low level of stomatal clustering (Doheny-Adams et al., 2012, Hunt and Gray, 2009, Dow et al., 2013). Two experimental set-ups were used; plants were grown in rhizotrons until leaves were fully expanded to study mature root size, or on agar plats to examine seedling root length, and also root hair length and density. For plants grown in rhizotrons, roots were supported by a glass fibre disc so that they grew in on a flat surface and 2 dimensional areas could be measured and was compared. Plants were harvested at 7 weeks after germination, when the leaf rosette was fully expanded. Root area was assessed by digitally scanning the glass fibre disc, and stomatal densities were recorded from mature leaves by taking dental putty impressions. A significant linear correlation between stomatal density and root area was observed (Figure 5.4). The *EPF2*OE plants which have a decreased stomatal density, also had a smaller root area in comparison to Col-0 background controls. *epf1epf2* mutants showed essentially the opposite phenotype, an increase in both stomatal density and root area.

Experiments were also conducted looking at early root development on agar plates. Measurements on primary root length 10 days after germination showed no significant differences between the length of *EPF2*OE, Col-0 or *epf1epf2* seedling roots (Figure 5.4). However, it was

clear that both the length and density of root hairs were enhanced in the plants with increased stomatal density (*epf1epf2*) when compared with wild-type controls, although the root hairs of plants with low stomatal density were not significantly altered (Figure 5.5). Root cell length, excluding root hairs, was significantly shorter in *EPF2*OE mutants compared to Col-0, however no significance was observed between *epf1epf2* and Col-0.

To examine in more detail whether the differences in root growth that are described above are specific to the EPF mutants examined, or are as a result of a wider phenomenon related to changes in stomatal conductance and water use, the root characteristics of a range of stomatal mutants were investigated. This included mutant genotypes that were affected in either stomatal development (e.g. *tmm*) or mutant genotypes defective in stomatal function, for example the *open stomata 1* (*ost1*) mutant which is unable to shut it's stomatal in response to ABA, and consequently exhibits high levels of stomatal conductance. Seedlings of each genotype were sown on agar plates alongside their appropriate background control. Similar alterations in root hair length and density to those described above were observed; genotypes that are likely to be associated with high levels of stomatal conductance were found to have either longer or denser root hair development, and those associated with low levels of stomatal conductance were found to have reduced root hair development, figure 5.8-5.9.

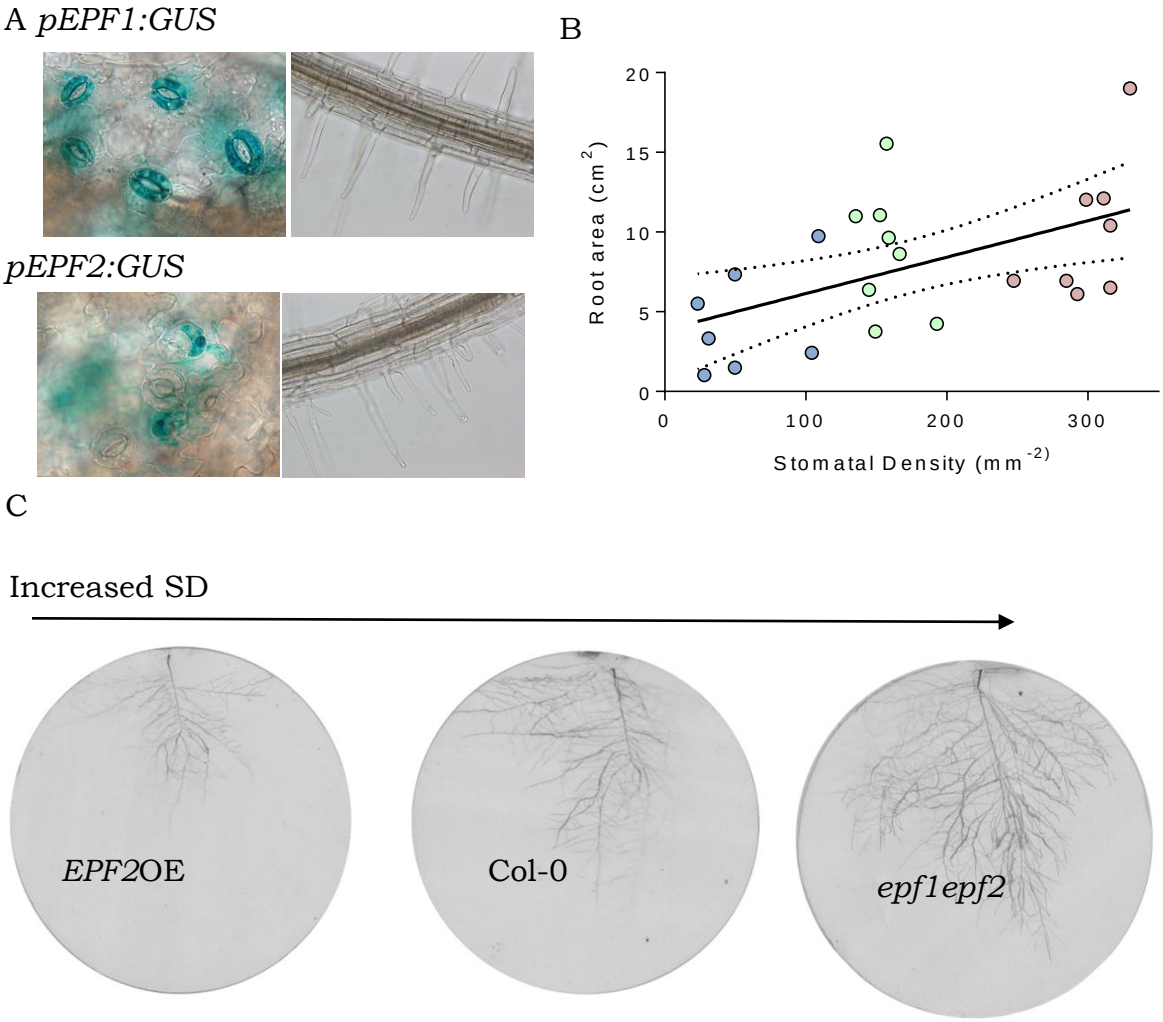


Figure 5-4 Plants with an increased stomatal density have an increase in root area when grown in rhizotrons.

A. Histochemical staining for β-glucoronidase (GUS) activity. Plants expressing GUS under the control of *EPF1* and *EPF2* gene promoters show no GUS activity in roots, but have activity in stomatal lineage cells. B. Size of root of plants grown in rhizotrons. Individual dots represent individual plants. Blue dots represent *EPF2*OE plants, green Col-0 and *epf1epf2* are represented with red dots. There is a significant linear relationship between stomatal density and root area. p= 0.0083 n= 8. C, representative scans of glass fibre paper from the rhizotrons with an increase in both stomatal density and root area from left to right.

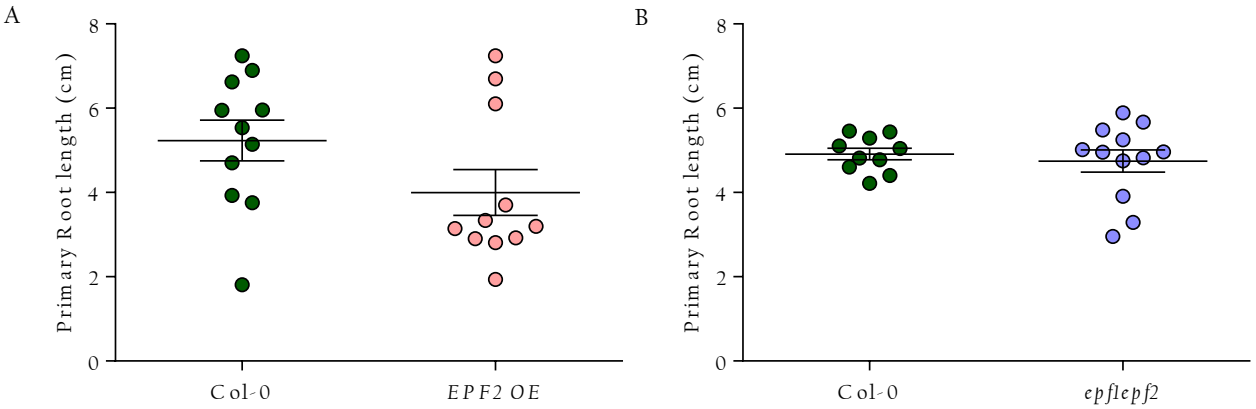


Figure 5-5 No significant differences are seen in primary root length on agar.

Primary root length was measured on agar 10 days after germination. No significant differences were seen between Col-0 and the stomatal genotypes. A) *EPF2 OE* plants, with a lower stomatal density, show no significant difference in primary root length when grown on agar. p = 0.1048 B) *epf1epf2* plants, with a higher stomatal density show no significant difference. p = 0.6016

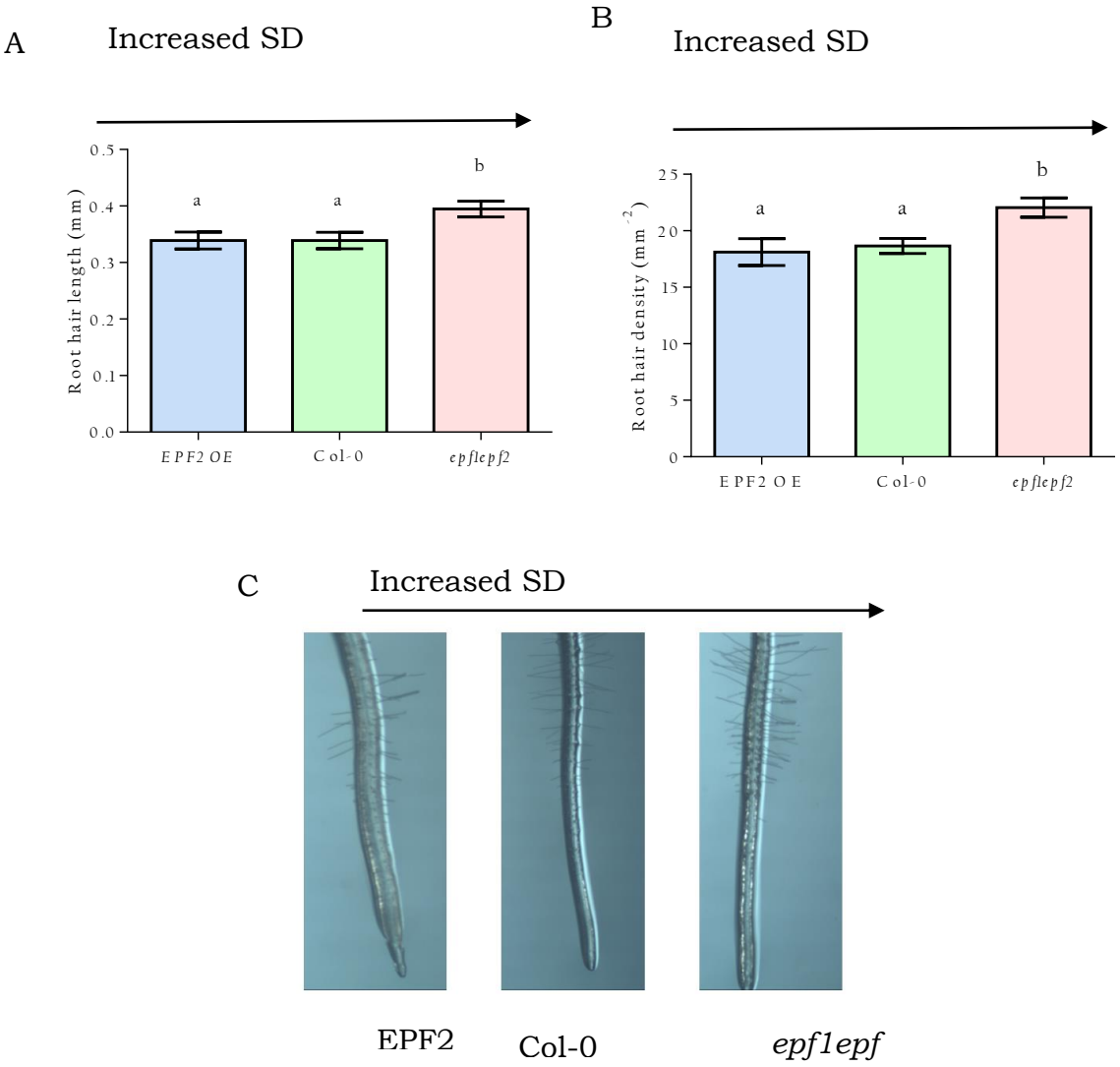


Figure 5-6 epf1epf2 mutants show an increase in root hair length and density.

A) epf1epf2 mutants showed an increase in root hair length. There is no significant difference observed between EPF2 OE and Col-0 plants. p= 0.0083 B) *epf1epf2* mutants showed an increase in root hair density. There is no significant difference observed between EPF2 OE and Col-0 plants p= 0.0128 C) representative micrographs of seedling roots.

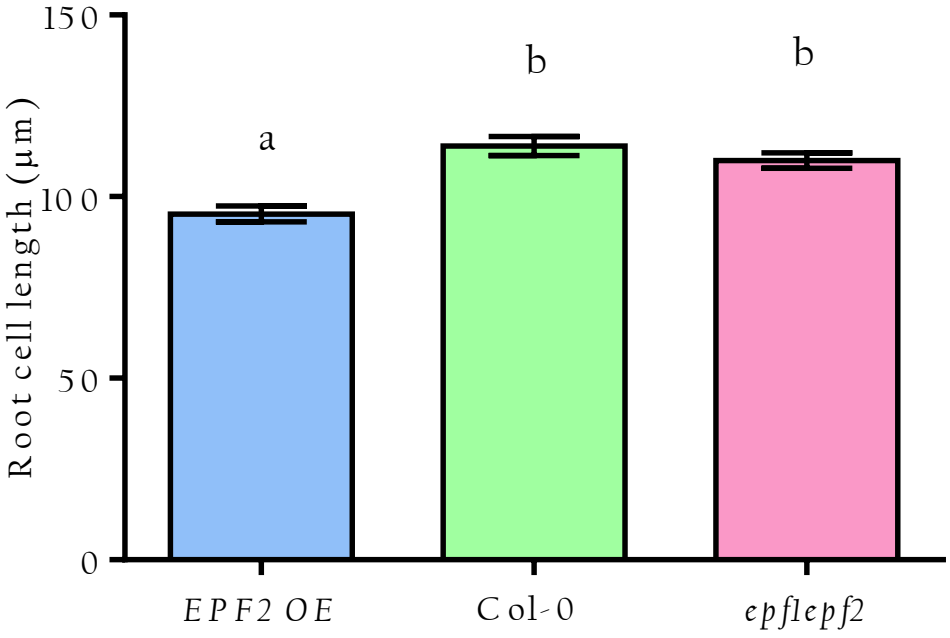


Figure 5-7 Root cell length is shorter in EPF2OE seedlings in comparison to Col-0 and epf1epf2

10 day old seedling roots show a significant shortening in root cell length in the *EPF2*OE mutant, but no significant changes between Col-0 and *epf1epf2.* One way ANNOVA p=0.019 n=8.

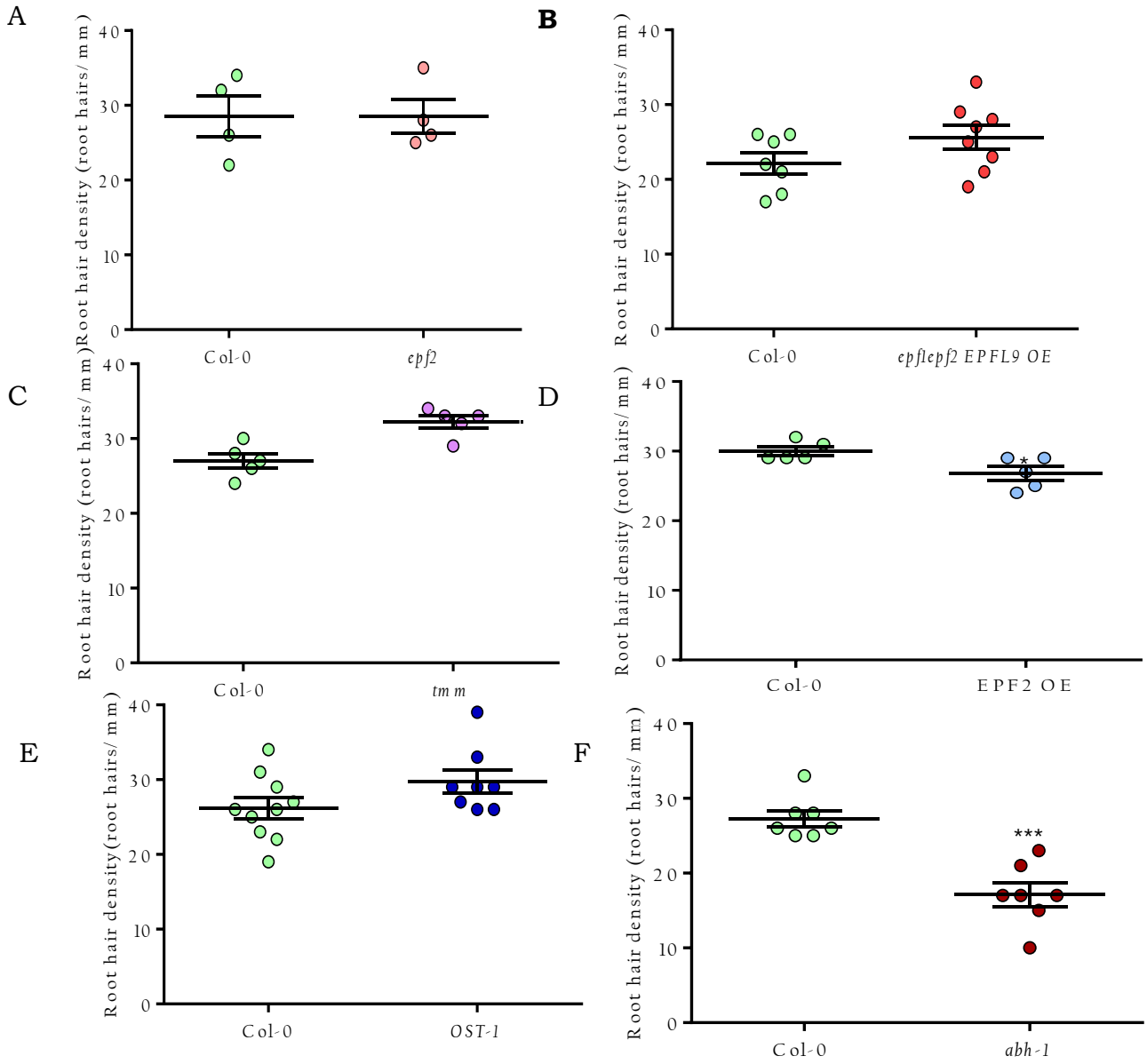


Figure 5-8 Root hair densities of stomatal mutants in comparison to Col-0

A-F, Root hair densities of stomatal mutants in comparison to Col-0. As seedlings are grown on different agar plates and therefore have different microenvironments each graph can be treated as an individual experiment. A, No difference in root hair density observed between Col-0 and epf2 n=4 p>0.999 B, No difference in root hair density observed between Col-0 *epf1epf2EPFL9OE* n= 8 p= 0.1327 C, *tmm* showed a significant increase in root hair density. n= 5 p=0.0043 D, *EPF2 OE* showed a significant decrease in root hair density. n= 5 p=0.0285 E, No difference in root hair density observed between Col-0 and Ost-1 n=8 p=0.1069 F) *abh-1* showed the largest decrease in root hair density. n= 7 p=0.0002.

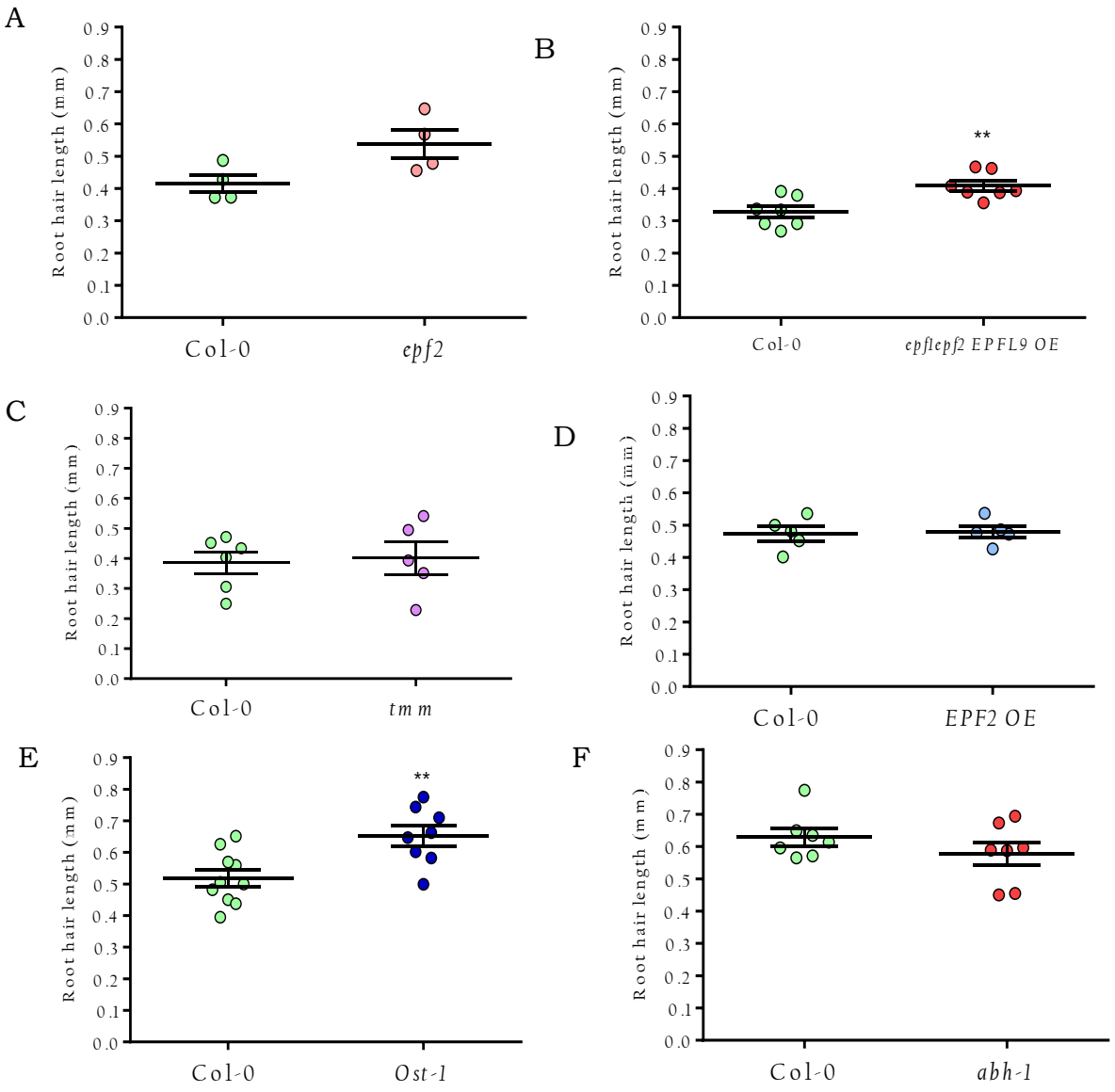


Figure 5-9 Root hair lengths of stomatal mutants in comparison to Col-0

A-F) Root hair lengths of stomatal mutants in comparison to Col-0. As seedlings are grown on different agar plates and therefore have different microenvironments each graph can be treated as an individual experiment. A) No difference in root hair length observed between Col-0 and epf2 n=4 p=0.0561 B) A significant increase in root hair length observed between Col-0 *epf1epf2EPFL9OE* was observed n= 8 p= 0.0.0045 C) No difference in root hair length observed between Col-0 and *tmm*. n= 5 p=0.8127 D) *EPF2 OE* showed no significant difference in root hair length. n= 5 p=0.8603 E) A significant increase in *OST-1* mutants in comparison to Col-0 n=8

p=0.0046 F) No difference in root hair length observed between Col-0 and *ahb-1*. n= 7 p=0.2790.

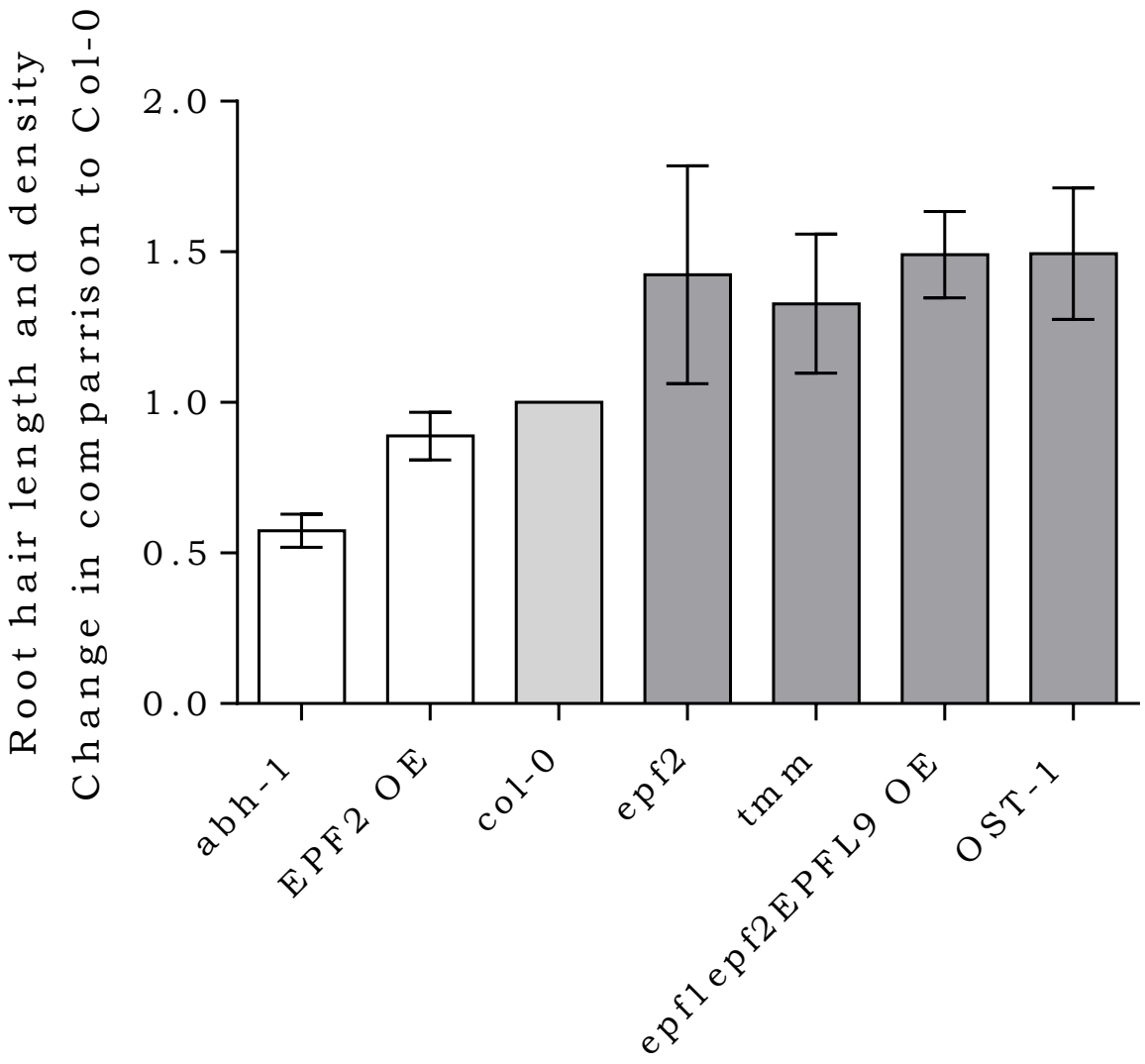


Figure 5-10 graph to compare the root hair length and density in stomatal mutants

A graph to compare the root hair length and density in stomatal mutants. Results have been standardised with Col-0 = 1 to allow for the micro-environments within the plates. White bars show the plants with a lower stomatal density or hyper-sensitive stomata and in darker grey plants with a higher stomatal density or less sensitive stomata.

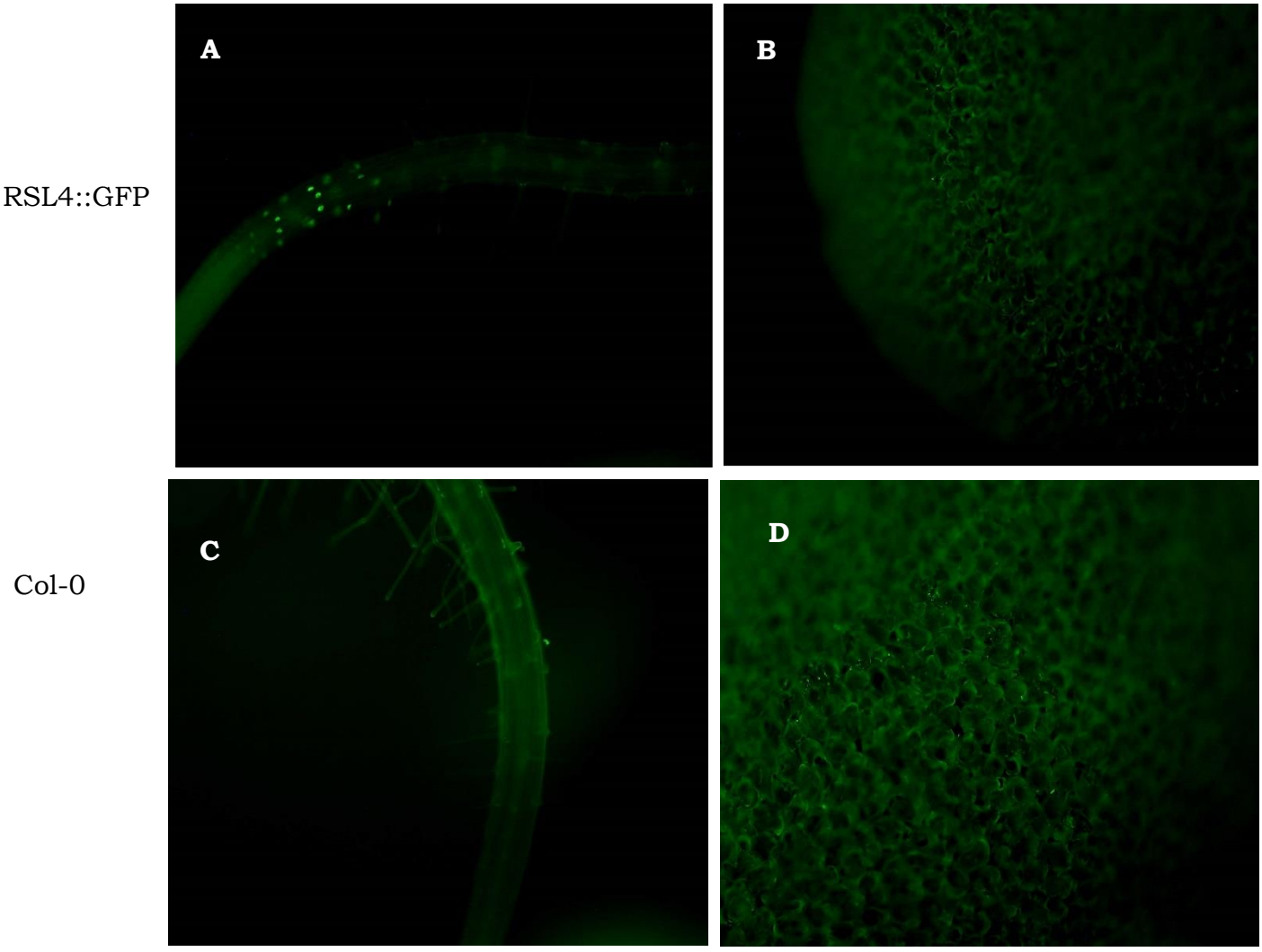


Figure 5-11 RSL4 is expressed specifically in root hairs.

A, Expression of GFP directed by the *RSL4* gene promoter region shows *RSL4* is expressed specifically at the ends of root hairs, where elongation takes place. B, C and D show background level of fluorescence in the *pRSL:GFP* promoter fusion leaf, and also in the root and leaf in a representative Col-0 plant respectively.

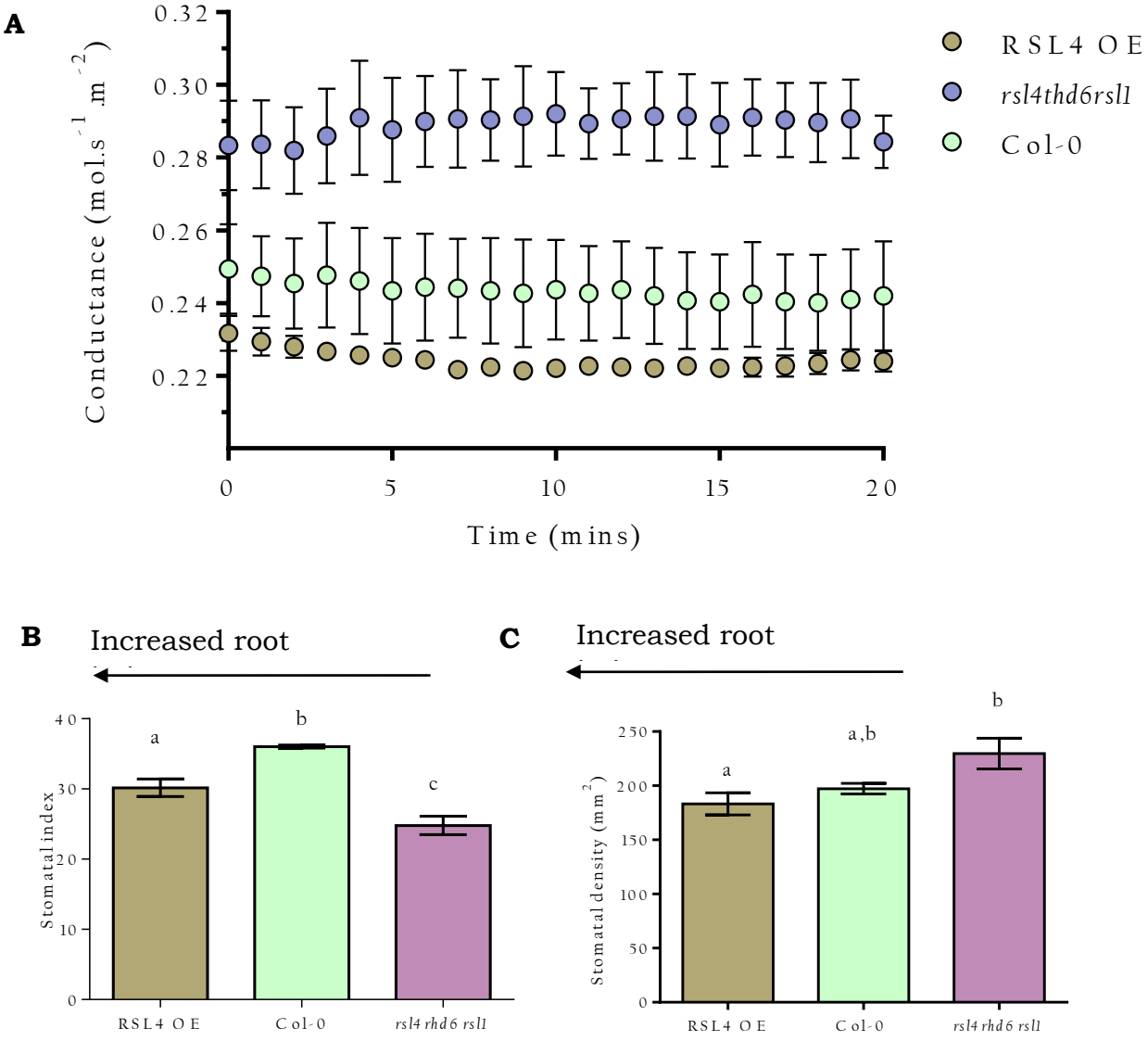


Figure 5-12 Root hair mutants show significant differences in stomatal conductance, index and density.

A. Stomatal conductance was measured on fully expanded leaves by IRGA at 65%-75% relative humidity, flow rate 500µmol s^{-1}, block temperature 20°C. Light intensity 1000 $\mu mol.m^{-2}.s^{-1}$ and CO2 set to 450ppm over 30 minutes. Plants with an increase in root hair length and density show a decrease in stomatal conductance. Plants with a decrease in root hair length and density show an increase in stomatal conductance. Final point stats t-test Col-0 v *rsl4thd6rsl1* p= 0.0477. Col-0 v *RSL4*OE p= 0.3407 *RSL4*OE v *rsl4thd6rsl1* p= 0.0041. n=3 B. Differences in stomatal index on fully expanded leaves are observed P=0.001. C Plants with an increase in root length and density, *RSL4*OE, have a decrease in stomatal density. Plants with a decrease in root hair length and density show an increase in stomatal density. p=0.031, n=3.

Having observed from the above experiments that stomatal conductance appears to influence root hair development, more experiments were carried out to further investigate any potential links between stomatal and root hair development. In what was essentially the reverse of the previous experiment, it was examined whether plants with altered root hair density exhibit any alteration in their stomatal development, and if so whether this affects their stomatal conductance. Arabidopsis plants with altered expression of RHD6, RSL1 and RSL4; were studied. These genotypes have been manipulated to have increased or decreased levels of RSL family transcription factors which are reported to be exclusively expressed in root hairs and their progenitor cells, and which regulate root hair initiation and growth (Yi et al., 2010, Masucci and Schiefelbein, 1994, Menand et al., 2007). To confirm that these root hair genes are not also expressed in stomatal precursor cells, plants expressing GFP under the control of an *RSL4* promoter were examined. As expected, GFP expression was observed specifically at the end of root hairs, where elongation takes place, and not in the leaf tissues (Figure 5.10).

The RSL family mutants were grown in pots and their mature leaf stomatal density and index (a measure of the proportion of epidermal cells that are stomata) examined. Differences in both stomatal index and stomatal density were observed between RSL family mutants. An increase in in root hair length and density in the *RSL4OE* have

decrease in stomatal density. Plants with a decrease in root hair length and density, *rsl4rhd6rsl1* mutants, show an increase in stomatal density.

To investigate whether the altered stomatal density observed in the RSL family mutants might also affect stomatal gas exchange, infrared gas analysis (IRGA) was carried out. Mature leaves of pot grown *RSL4*OE plants which have increased root hair density and decreased stomatal density, exhibited significantly decreased levels of stomatal conductance across 30 mins in the IRGA chamber (Figure 5.11), however the conductance at the final 30 min timepoint at consistent conditions was not significantly different to Col-0 controls, probably because of the wider variability in control plant conductances. The opposite was true of *rsl4thd6rsl1* which has decreased in root hair density and increased stomatal density. This genotype exhibited increased stomatal conductance in line with its reduced stomatal density. Thus it appears that plants with poor root development might be able to enhance their stomatal conductance by adjusting the level of stomatal development.

5.6 Discussion.

In this chapter results are presented showing that EPF family mutants which have altered levels of stomatal development also have altered root development, and that stomatal density is negatively correlated with root area. This alteration in root development could occur

indirectly in response to a change in plant water status due an alteration in stomatal conductance, or could occur as a direct effect of the change in level of EPF signalling peptides in the root. GUS staining confirmed that neither expression of *EPF1* nor *EPF2* are targeted to the roots, at least not by the promoter sequences used here. It is therefore unlikely that the lack of *EPF1* and *EPF2* expression in the *epf1epf2* root has a direct effect on root anatomy or physiology. However, it is possible the secreted EPF peptides may normally be transported extracellularly to the roots, or that the constitutive over-expression of EPF2 in the *EPF2*OE mutant has a direct effect on the development of roots. It has previously been observed that constitutive over expression of genes in roots, can lead to shortened root cells (Liam Dolan, personal communication). As *EPF2*OE root cells were found to be shorter in this study, this may account at least in part for the effect of over-expression of EPF2 on the root. However, the *EPF2*OE roots did not show a significantly altered root area, and the root phenotype was much more evident in *epf1epf2* plants where over-expression is not a consideration.

The findings that *EPF2*OE plants have a larger leaf rosette and a smaller root system suggest that these plants may invest less resources into their roots and more in to their shoots, perhaps because of their reduced stomatal conductance and improved water status. The results present here also suggest the opposite is true and the *epf1epf2*

which have high stomatal density may invest more in their roots and less into their shoots. These findings that the EPF mutants also have root development phenotypes may help to explain the observations of Doheny-Adams *et al.* who reported larger leaf rosettes in the *EPF2*OE mutants in comparison to Col-0, and smaller rosettes in the *epf1epf2* mutant again in comparison to Col-0 (Doheny-Adams et al., 2012). Together with the results in Figure 5.8-5.9, these data indicate that plants with high stomatal density and an increased capacity for transpiration also have an increased root size and root hair surface area. However, this negative relationship between stomatal density and rosette size was not observed by Tanaka et al. (Tanaka et al., 2013) using a different collection of stomatal density mutants. Therefore, the relationship between rosette size and stomatal density may either not be universal across genotypes, or may be sensitive to growth conditions.

As the results discussed above, indicated that manipulating the level of stomatal conductance either by changing stomatal density or stomatal aperture, could induce changes in root size and surface area, it was investigated whether the opposite could be true; whether changing root characteristics could alter stomatal development and conductance. Remarkably, changes in stomatal conductance, index and density were observed in RSL family mutants which have manipulated root hair length and density. Thus it appears that root

characteristics can induce changes in stomatal development. It appears to be unlikely that altered RSL transcription factor levels exert a direct effect on stomatal development because *RSL4* expression could not be detected in leaves using a GFP promoter reporter system.

Together the results in this chapter indicate that stomatal conductance exerts an indirect effect on root development, and conversely that root hair development could have an indirect effect on stomatal development and stomatal conductance. However the relationship between stomatal development and root development is not simple. An increase stomatal density is associated with an increase in root hair area, whereas a reduction in root hair area is associated with an increase in stomatal density.

The results presented above reveal a complex and previously undescribed plasticity in plant development that appears to modulate root and stomatal characteristic in an attempt to balance plant water uptake and loss. As illustrated in the model in Figure 5.12, the results presented in this chapter indicate that plants manipulated to have high levels of stomatal conductance put less of their resources into root development, whereas plants with reduced root development adjust their stomatal development to enhance their stomatal conductance. The mechanism by which plants transmit these developmental signals between roots and shoots is not known. However, ABA as a known modulator of stomatal development (Chater

et al., 2014) root hair development and water stress responses (Schnall and Quatrano, 1992, Chen et al., 2006) would be an obvious candidate.

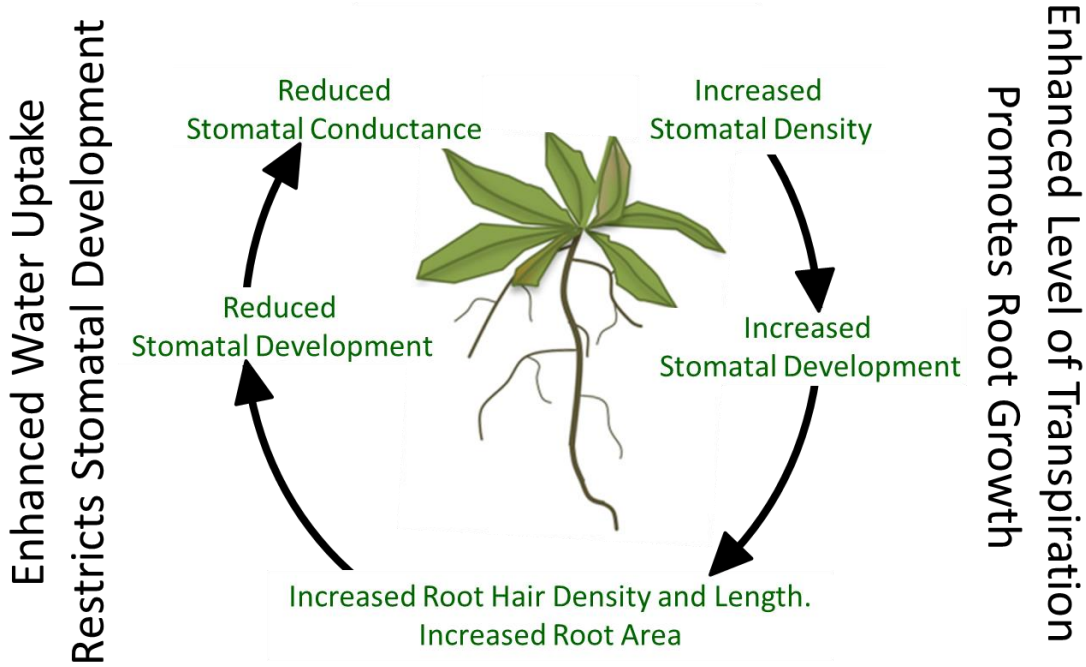


Figure 5-13 Model to illustrate the complex interplay between stomatal and root development

Model to illustrate the complex interplay between stomatal and root development revealed by the results presented in this chapter. It is proposed that plants are able (by an unknown mechanism) to monitor water loss via stomatal conductance and to respond by adjusting and root and stomatal development to maintain a viable balance between water uptake and water loss.

Chapter 6 – Discussion

6.1 Introduction

With an ever increasing population and an increase in severe weather conditions improving food security has become one of the major agendas for both the scientific and political community. Although often the western world is unaffected by food shortages due to agricultural failure, it is thought that around 30% of people globally are malnourished (FAO, Last accessed: 09/10/2014). With the population predicted to continue to increase until approximately 2050 this problem is not going to be easily solved. As a multifaceted problem solutions need to be found on several economic, political and agricultural levels; including investigating food waste, nutritional value of food and global agricultural knowledge transfer.

Throughout this thesis I have described a range of ways to improve food security by focusing on agricultural methods, to both make farming more sustainable and increase the possible areas for agricultural practices.

6.2 Developing novel herbicides using structural studies for design

Novel herbicides are of great commercial interest, particularly with the rise of resistance to the number one selling herbicide, glyphosate, in recent years. Herbicides often inhibit the active sites of enzymes that

are vital for a plant to live and are used to restrict the growth of unwanted plants in agricultural areas. In chapter three I investigated the potency of novel herbicides that had been designed to 'fit' potential inhibitory sites using 3-dimensional structures of an enzyme derived from X- diffraction studies of crystalline protein. These herbicides were designed to specifically target IGPD, an enzyme in the histidine biosynthesis pathway that is critical for plant vitality.

C348, a chemical previously published as having an inhibitory effect on the recombinant enzyme (Cox et al., 1997), showed the most potent herbicidal activity when applied to plants. C367, C368 and C358, also designed by Professor Dave Rice's research group to inhibit IGPD, showed some inhibitory effects on plant growth. However high concentrations of these inhibitors were required to restrict plant growth and therefore made the chemicals non-commercially viable as an herbicide.

Structurally designing inhibitors may help in designing potential herbicides, however my investigations showed that it is also important to use *in vivo* studies alongside the *in vitro* biochemical studies. Potency of chemicals in experiments with plants varied from the *in vitro* biochemical analysis which is likely to be due to the molecules having varied interactions and transportation within plant.

6.3 Developing herbicide resistant plants for no-till farming

Soil degradation is thought to be one of the major agricultural challenges of the 21st century. Having continuous crop cover on land is a way of protecting soil from degradation. No-till farming is a farming practice that helps to conserve soils often by utilising herbicides rather than ploughing to reduce growth of weeds. The recent availability of genetically modified herbicide resistant plants has made this practice more widespread and enabled post-emergence application of herbicides to ensure the ground has constant cover. Again in this system glyphosate is the most widely used herbicide and finding both a novel herbicide and herbicide resistant plant is of great interest. One way to enhance resistance to an inhibitor is to increase the levels of the target enzyme.

In this thesis I have shown that by over-expressing IGPD in Arabidopsis plants, by introducing an additional copy of the *IGPD* gene into the genome under the control of a strong constitutive gene promoter, it is possible to confer enhanced tolerance to the potential herbicidal chemical C348. However, I also observed some defects in the IGPD over-expressing plants including a "spontaneous death syndrome" which, although enhanced after the application of the herbicide, was present in all *IGPD* OE populations. Investigations showed that this was likely to be due to a manganese deficiency,

caused by the high demand of manganese ions for the additional IGPD enzyme, which is heavily manganese dependent (Glynn et al., 2005).

Overexpressing the *Arabidopsis thaliana* IGPD enzyme was effective in some individual plants which were able to withstand much higher levels of C348 than controls'. But this strategy for the herbicide resistance was not effective within a population of plants due to the unexpected defects caused by suspected, high levels of IGPD enzyme. Thus simply enhancing levels of the target enzyme IGPD is not a viable way of creating herbicide resistance. There are other ways to genetically enhance herbicide resistance, for example by expressing a version of the enzyme which has a reduced affinity for the inhibitor. It is clear that future studies should consider the possible problems caused by genetic modification, especially if using IGPD with its high manganese requirement.

6.4 The effect of altered transpiration on root morphology.

Alongside soil degradation another big agricultural threat is water use. Again a multi-facetted problem. It is thought 80-90% of fresh water used by human is utilised in agriculture (Morison et al., 2008). With an increasing population and diminishing supplies of fresh water it is important to find ways of increasing the amount of crop produced while decreasing the amount of water used. Plant water loss is regulated by microscopic valves on the plant aerial surfaces, known as

stomata. In this project experiments focused on the effect of altering plant water loss by manipulating the frequency of stomata.

Doheny-Adams *et al.* have previously demonstrated an increase in water use efficiency (WUE); amount of carbon dioxide assimilated per amount of water used, by genetically manipulating stomatal density (Doheny-Adams et al., 2012). Not only does the decrease in stomatal density allow the plant leaf rosettes to grow larger under water limiting conditions, the plants also have higher leaf biomass in well watered conditions. Experiments in this thesis have given a possible reason for the increase in biomass by investigating the above-below ground relationship.

Experiments showed distinct root changes in populations with altered stomatal densities. Although hard to distinguish between direct and in-direct effects of genetic modification, genes modified were not natively expressed in roots. This would indicate that knockout genotypes (plants lacking expression of the particular gene) are unlikely to have their root architecture directly affected, however where the particular gene was constitutively overexpressed any observed effects on the roots may have resulted from mis-expression of the gene product.

Genotypes that had a higher stomatal density tended to show an increase in both root area and root hair length and density. In

comparison, plants with a lower stomatal density (and larger leaf rosettes) showed a decrease in root area, but no significant changes in root hairs in comparison to wild type lines. Interestingly changes in root hair morphology were also observed in stomatal aperture mutants which would also be expected to have altered water loss. These results indicate that a change in transpiration and not just a change in stomatal density, may have a significant effect on root hair morphology. One of the most remarkable findings was that mutants with an increased root hair length and density showed a decrease in stomatal conductance, index and density.

The mechanism and potential signal behind the changes mentioned previously is unknown but would be of great interest. As the changes in root hair length and density were caused by both stomatal density mutants and stomatal opening mutants which have transpiration changes in common, it would be easy to speculate that this could be the trigger that leads to changes in a signal and then a change in physical characteristics.

The phytohormone abscisic acid (ABA) has long been known to affect stomatal aperture (Mittelheuser and Van, 1971). Root hairs have also shown to be affected by ABA, however, it was reported that ABA causes shortening and bulbous root hairs, not the phenotype seen in the plants with an increased transpiration rate (Schnall and Quatrano, 1992). The control of the development of root hairs has not been

completely elucidated, but it is clear auxin plays a key role in the pathway, particularly in the regulation of RSL4 (Lee and Cho, 2013). Moreover, it was recently demonstrated that auxin negatively regulates stomatal development through repression of EPFL9 (Zhang et al., 2014).

Results indicate a previously unknown complex relationship between root hair and stomatal development that would be interesting for further investigation. Results demonstrated the complexity of this relationship showing an increase in stomatal density leaded to an increase in root and root hair architecture, however an increase in root hair length and density caused a decrease in stomatal density. This would indicate there is some form of plasticity that modulates root and stomatal characteristics, possibly to balance water uptake and loss.

6.5 The application of research for future crop improvement.

From my previous conclusions it is clear that my research into IGPD and its inhibitors is not near to being transferred into crops and a lot more work to create new inhibitors and find a way to create inhibitor resistant plants is needed. However, the work investigating stomatal mutants has a lot of potential to move into a crop model. Crop model selection will have to consider a matrix of parameters. The work in this thesis and the work of Doheny Adams *et al., (Doheny-Adams et al., 2012)* has shown the

potential for *EPF2 OE* plants to have both an increase in biomass and an increase in drought tolerance.

The same thesis discussed moving the technology into barley and designed constructs of EPF-homologues (Doheny-Adams, 2013). However, other crops should be considered. Ethically a crop which utilises the technology to help the neediest would make the ideal candidate. UNICEF data estimates 13% of all Ethiopians are at immediate risk due to drought (UNICEF, Last accessed 15/02/2015). The highest produced crop in terms of yield (tonnes) in Ethiopia is maize, with over 6. 5 million tonnes grown in 2013 (STATS, Last accessed 15/02/2015). Making maize an interesting candidate for this technology.

Scientifically finding a suitable crop will have to consider the ease of genetic modification in that crop as well as the cross over in the genomes between *A. thaliana* and the potential crop. Maize can be transformed relatively easy using agrobacteria or particle bombardment, has been successfully modified and is one of the most extensively sold genetically modified crops globally (Prado et al., 2014). However, using BLAST software there are no sequences in the Maize genome with significant similarity to the EPF2 DNA sequence, therefore other crop models should be considered.

Just taking these parameters would be interesting to the scientific community, however, to take something from the research bench into the field would take a considerable amount of funding. Funding would be

needed for the extensive field studies and safety tests that are necessary for GM crops to be gown on farm (Prado et al., 2014). This would need to take into account who will be able to afford the end product, intellectual property and how widely grown it is expected the crop could be grown.

With the ongoing pressures to food security that have been discussed throughout this thesis it is likely the best solutions will be a combination of measures. Combining the technologies in this thesis could give a multidimensional technique to help with water retention. Decreasing tillage, alongside increasing the drought tolerance of a crop in combination could be a future technique in areas where soil is dry and there is a higher than average rate of soil loss.

6.6 Future studies.

Although some potency was shown by the potential novel herbicides tested future experiments are needed to produce a commercially viable compound. IGPD remains a target for future herbicides and further compounds are being synthesised taking into account transportation and interactions of the chemicals within the plant, alongside the direct interactions with the enzyme IGPD.

Future experiments to produce an herbicide tolerant plant for herbicides targeted at IGPD could include looking for an alternative IGPD to over-express. It is possible that a mutagenized version of the enzyme could be created that has lower affinity for IGPD, or that enzymes from different biological sources may have differing affinities

for C348, that could be valuable in the creation of herbicide resistant crops. For example using an IGPD enzyme from a bacterial species which have smaller IGPD enzymes could be explored. Furthermore it may be possible to do experiments looking at supplementation with an alternative, cheaper metal that may have the correct properties to replace manganese in IGPD, for example iron.

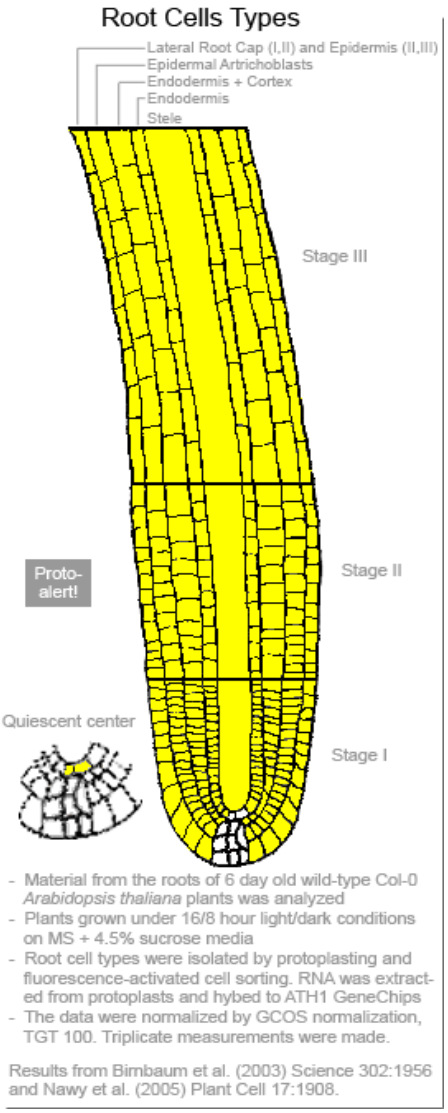
It is very difficult to distinguish between the direct and in-direct effects of manipulating stomatal density, particularly when over-expressing an epidermal patterning factor on a constitutive promoter. It would therefore be beneficial to express EPF genes under the control of promoters with a more specific expression pattern, for example on a green tissue promoter, like a cuticle expressing promoter like the promoter for the CUT1 gene. Preliminary analysis of the expression of these genes, Figure 6.1-6.2, would indicate CUT1 might be the most appropriate promoter to use for such studies as its expression is similar to EPF2 in roots (extremely low or absent), and much higher in guard cells.

It may also be interesting to look into the consequences of the decreased or increased root area associated with stomatal density changes. As roots are vital for not only accessing water, which is mainly what I have focused on in this thesis, they are also vital for the acquisition of nutrients. It may be most interesting to start by analysing phosphate uptake into plants as phosphate root hairs are

strongly associated with phosphate uptake and phosphate is a resource that is becomingly increasingly scarce (BIELENBERG et al., 2001, Peret et al., 2011, Wu, 2003). Clearly it would be of little advantage to create crops that have a reduced water requirement if their nutrient uptake capacity is impaired.

The experiments described in the proceeding chapters have explored opportunities that have arisen from in depth molecular studies, with the aim of creating plants with novel herbicide tolerance or improved water use traits. Both sets of experiments have revealed the complexities involved in manipulating plant traits with the aim of crop improvement. These studies highlight the need for extensive whole plant studies before embarking on experiments to translate laboratory findings into crop species and ultimately into the field.

A



B

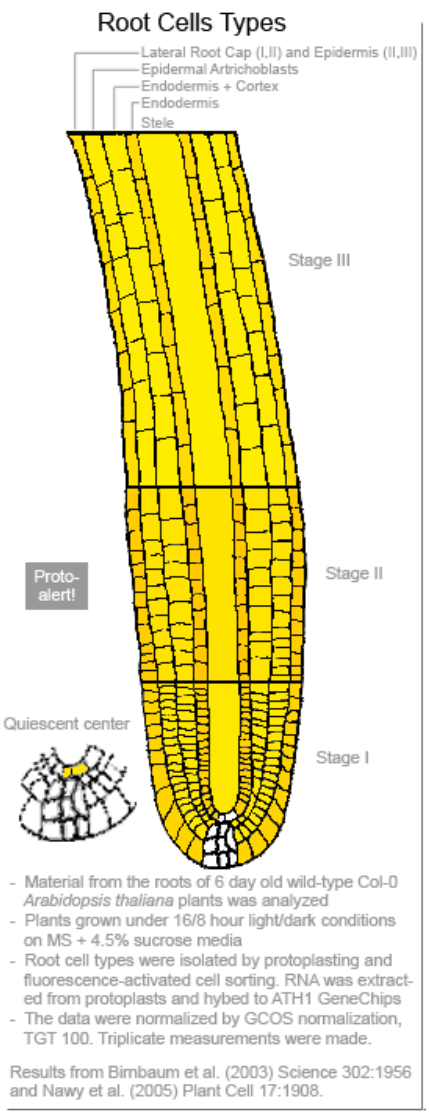
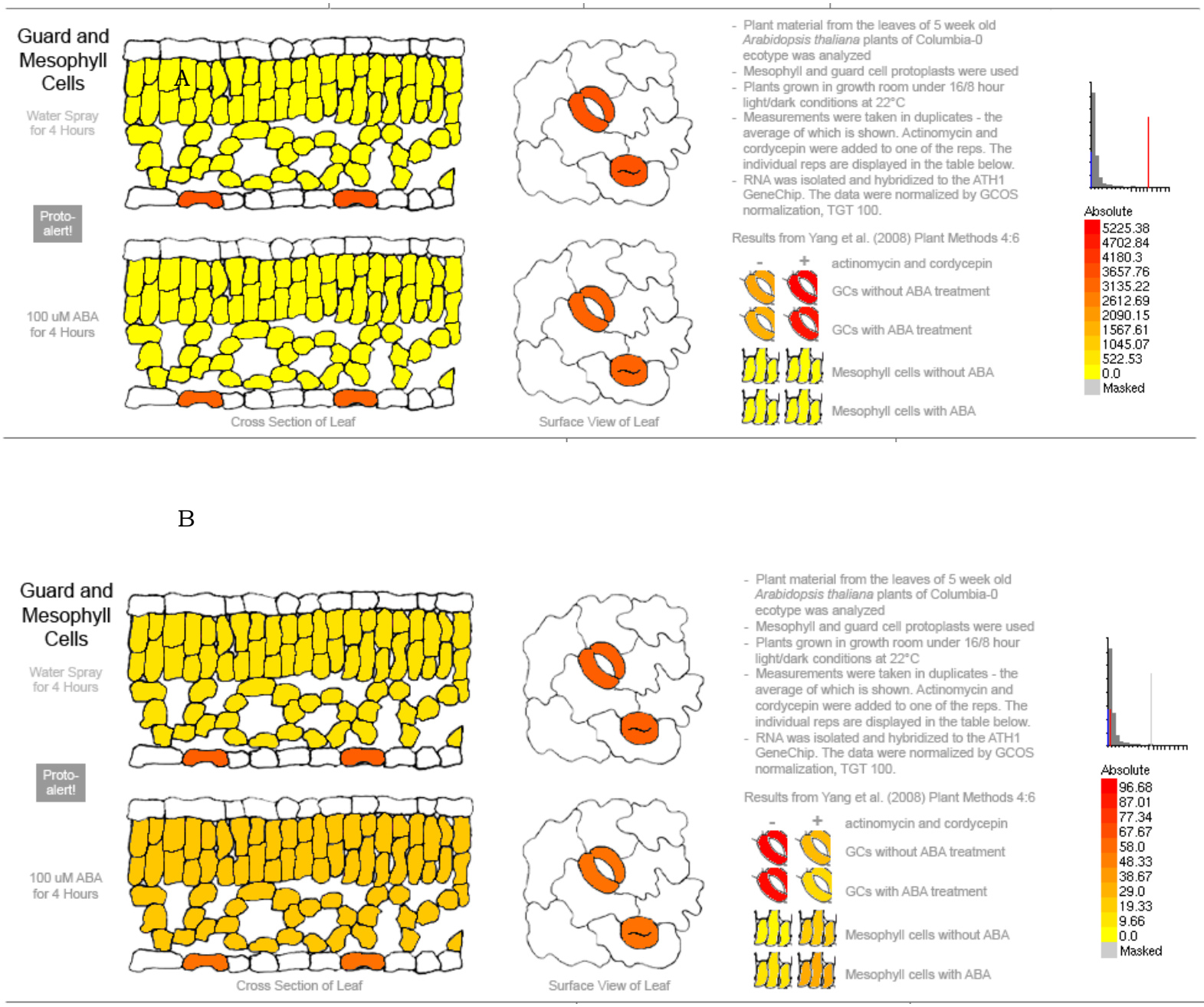


Figure 6.1

A, CUT1 expression levels in the root is very low/ not seen. B, EPF2 expression is similar to that of CUT1 being extremely low or non-existent. Affymetrix microarray experiment data from Arabidopsis eFP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi).

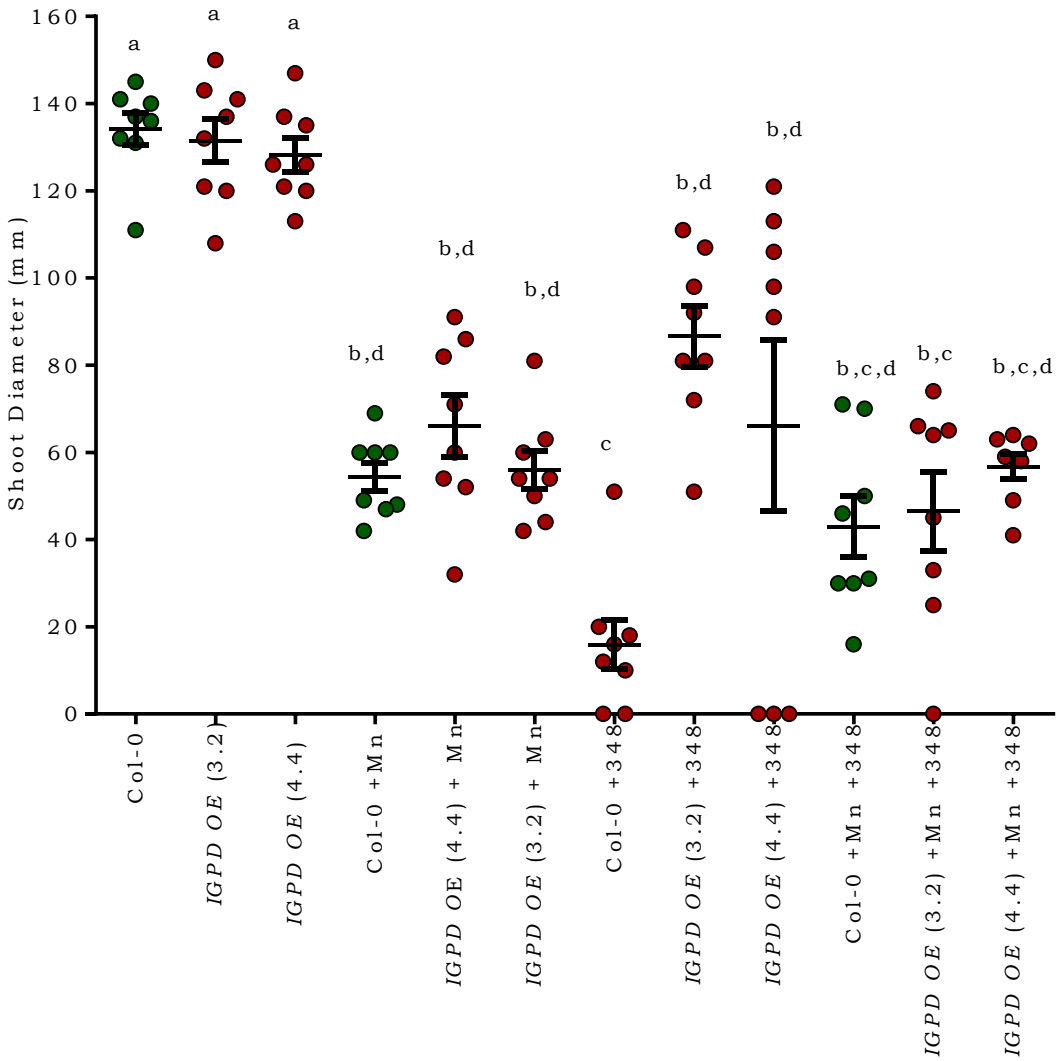


B

Figure 6.2

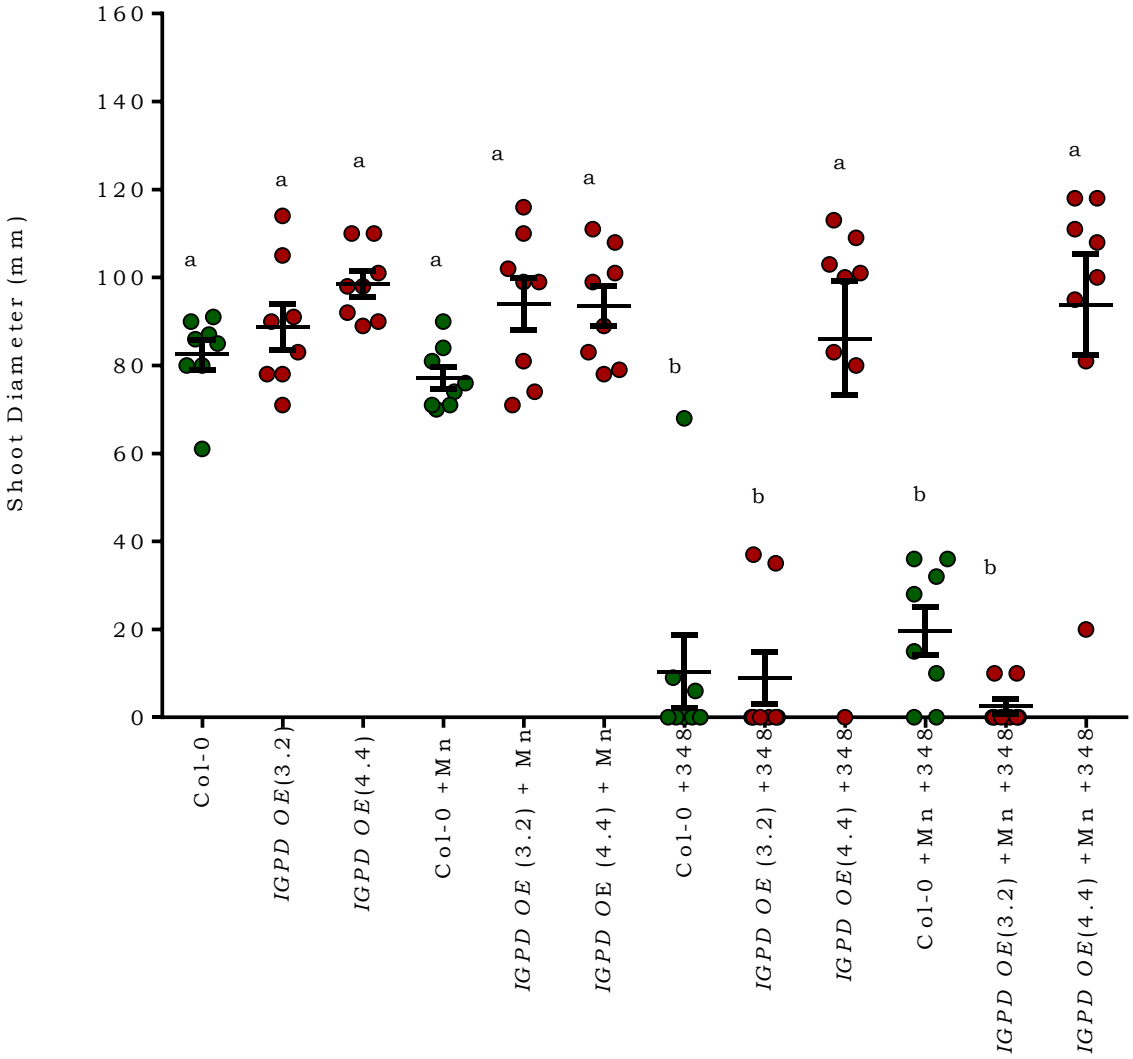
A, CUT1 expression levels in guard cells. B, EPF2 expression levels in guards cells. Genes show similar expression, but CUT1 has a slightly higher expression level. Affymetrix microarray experiment data from Arabidopsis eFP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi).

Appendices



Appendix 1

Individual dots represent individual plants. Plants were sprayed with a 50 µM C348 tween solution and supplemented with 500 ml 0.5% manganese sulphate weekly from germination. Manganese has a significant difference in control plants. n = 8 one way ANOVA $p < 0.0001$



Appendix 2

Individual dots represent individual plants. Plants were sprayed with a 50 µM C348 tween solution and supplemented with 500 ml 0.1% manganese sulphate weekly from germination. Manganese has no significant difference in control plants. The addition of manganese also shows no significant differences in any other group of plants, we therefore increased the manganese sulphate concentration for the final experiments. n = 8 one way ANOVA $p < 0.000$

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