Hormone Interactions and the Regulation of Seedling Growth

by

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DECLARATION

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ABSTRACT

An increased understanding in recent years of the biosynthesis and signal transduction pathways for various plant hormones, including gibberellin (GA), is providing an important tool for understanding interactions between these hormones and their role in plant physiology. In this thesis, interactions between auxin and gibberellin in regulating root growth, and between abscisic acid (ABA) and gibberellin in regulating shoot growth, are explored using the model species, pea.

The GA signalling pathway, in particular the involvement of "DELLA" proteins, has received much attention in the last decade. The GAs act by destabilising the growth inhibitory DELLAs; essentially, GA acts as an "inhibitor of an inhibitor". In Arabidopsis, DELLA proteins have been shown to promote the biosynthesis of active GAs, with the DELLA mutant *rga* displaying elevated expression of the biosynthesis gene *GA4*. The recently-sequenced pea DELLA genes *LA* and *CRY* are used in this thesis to show that in roots also, DELLA proteins effectively promote GA synthesis gene expression, including a new member of the pea GA 3-oxidase family which appears to play a major role in these organs. Furthermore, these DELLA mutants are used to investigate the role of GA signalling in the interactions with auxin and ABA to regulate growth.

Auxin has been shown to promote GA biosynthesis in the above-ground parts of pea (Ross et al., 2000). However, it cannot be assumed that the same interaction also occurs in pea roots. Indeed, another study indicates that auxin acts by enhancing the capacity of GA to destabilise DELLAs in roots of Arabidopsis (Fu and Harberd, 2003). According to the Fu and Harberd model, auxin would down-regulate GA synthesis, the opposite of the up-regulation found by Ross et al. (2000) in stems and consequently, the Ross et al. (2000) and Fu and Harberd (2003) models predict opposite effects of auxin on the expression of GA synthesis genes. Here, to understand the interactions between auxin and GAs in pea roots, wild-type pea roots were treated with the inhibitors of auxin action and auxin transport. These compounds generally down-regulated GA synthesis genes and up-regulated GA deactivation genes, and reduced the level of the bioactive GA_1, suggesting that in pea

roots, auxin at normal endogenous levels stimulates GA biosynthesis, agreeing with the Ross et al. (2000) model. It is also shown that supra-optimal levels of exogenous auxin reduce the endogenous level of bioactive GA in roots, although the effect appears too small to account for the strong growth-inhibitory effect of high auxin levels.

ABA is a known inhibitor of plant growth and historically, ABA and GA have generally been shown to act antagonistically. Previous evidence indicates that ABA inhibits GA synthesis while GA inhibits ABA synthesis. However, the evidence presented here suggests otherwise. GA synthesis gene expression and endogenous levels were not altered in ABA-treated shoots, indicating that, at least in pea, ABA does not regulate GA biosynthesis. The effects of GA deficiency on ABA levels were also investigated. Furthermore, ABA has been reported to act via the GA signalling pathway to inhibit root growth. However, there are conflicting reports on whether ABA acts on GA signalling via the DELLA proteins or downstream of these proteins. Here it is shown that ABA inhibits shoot growth in both the WT and pea DELLA mutants to a similar degree, suggesting that the DELLA proteins are not involved in the ABA-induced inhibition of pea shoot growth.

The results presented in this thesis clarify a number of conflicting reports on the auxin-GA and the ABA-GA interactions and how they influence the growth of the plant.

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CHAPTER 1

An Introduction to the Gibberellins (GAs)

1.1 Background

The plant hormones gibberellins (GAs) are naturally occurring tetracyclic diterpenoid acids with structures based on *ent*-gibberellane (Sponsel and Hedden, 2004; Figure 1.1). They were first discovered in 1935 by Tejiro Yabuta, who isolated a growth-active substance produced by the phytopathogenic fungus *Gibberella fujikuroi,* from which they derive their name (Sponsel and Hedden, 2004; Phinney, 1983).

Currently there are 136-fully characterised GAs, designated GA_1 through to GA_{136}. These compounds have been identified from 128 species of vascular plants, seven bacteria, and seven fungi (Sponsel and Hedden, 2004). Of these 136 GAs, only 6 are thought to act as the biologically-active compounds (i.e. directly responsible for developmental processes), with the others acting as precursors to these bioactive GAs and their resulting catabolites (Yamaguchi, 2008). The 6 known bioactive GAs are GA_1, GA_3, GA_4, GA_5, GA_6 and GA_7 – not surprisingly, these were among the first GAs to be discovered (Sponsel and Hedden, 2004). GA_1 is the most important bioactive gibberellin in vascular plants and is widely known to be responsible for stem elongation, induction of seed germination, fruit setting and growth, and pollen production (Davies, 2004). One exception to the rule is Arabidopsis which contains more GA_4 than GA_1 and is more sensitive to GA_4 than to GA_1 (Desgagne-Penix et al., 2005).

In this chapter, I will discuss the biosynthesis and signalling pathways of these growth-promoting hormones, mutants that have been vital in understanding the role of gibberellin in plant development processes, and how other plant hormones interact with gibberellin to regulate growth.

1.2 GA biosynthesis

Due to the large number of endogenous GAs found in higher plants and fungi, the elucidation of the GA biosynthesis pathway has been an arduous task. The beginning of our understanding of this pathway began with studies on *G. fujikuroi* followed by higher species, and involved the application of isotopically-labelled putative intermediates to plants or cell-free extracts and monitoring the subsequent products by GC-MS (MacMillan, 1997).

The discovery of biosynthesis mutants also played a crucial role in understanding the GA biosynthetic pathway and the role that GAs play in plant development. To date, GA biosynthesis mutants in pea are represented at five genetic loci, (*LS, LH, NA, LE* and *SLN;* Figure 1.2), and each affect various steps in the GA biosynthesis pathway. Each of the five mutations is recessive and results in both reduced elongation and GA content if located in the biosynthesis pathway, or increased elongation and GA content if located in the deactivation pathway as is the case with *sln* (Figure 1.2; Reid and Ross, 1993). Each of these pea GA biosynthesis mutants is discussed further below.

The GA biosynthesis pathway can be divided into three distinct parts: the synthesis of *ent*-kaurene in the plastid, the oxidation of *ent*-kaurene to the first GA (GA_{12}) in the endoplasmic reticulum, and the oxidation of GA_{12} to other C20-GAs and C19-GAs in the cytosol (Sponsel and Hedden, 2004). It is the last stage that is of particular interest in the present studies which will be discussed in more detail than the first two steps.

1.2.1 Synthesis of ent-*kaurene (Stage 1)*

The gibberellins are diterpenoid compounds synthesised from geranylgeranyl diphosphate (GGDP) via isopentenyl diphosphate (IPP) in young tissues of the shoot and developing seed (Sponsel and Hedden, 2004). IPP is the 5-carbon building block for all terpenoid/isoprenoid compounds (Sponsel and Hedden, 2004). The production of IPP can occur via two different pathways, the acetate/mevalonate (MVA) pathway which occurs in the cytosol and the methylerythritol phosphate (MEP) pathway which occurs in the plastid (Sponsel and Hedden, 2004; Figure 1.3).

The MVA pathway involves the synthesis of IPP from acetyl coenzyme A (acetyl CoA) which is used to build up the C_{20} GGDP molecule (Hopkins, 1999; Figure 1.3A). For the MEP pathway, IPP is synthesised from glyceraldehyde 3-phosphate and pyruvate with MEP acting as an important intermediate (Eisenreich et al. 2001). In Arabidopsis it has been shown that the majority of IPP is provided from the MEP pathway, with only a minor contribution being made from the MVA pathway (Kasahara et al., 2002).

Once IPP is synthesised, it is converted to GGDP. GGDP is a precursor of many diterpenoid compounds, including the phytol side chain of chlorophyll, and tetraterpenoids (40 carbons), including carotenoids. It is only after GGDP that, in most plants, the pathway is dedicated for GA biosynthesis (Sponsel and Hedden, 2004). GGDP is then converted to the tetracyclic hydrocarbon intermediate *ent*-kaurene, in a two-step process including the two enzymes copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS) (Figure 1.3; Sun and Kamiya, 1994; Yamaguchi 2008; Hedden and Proebsting, 1999).

In the *ls-1* pea mutant, *ent*-kaurene biosynthesis is blocked in the shoots and in the seed during early development (Ingram and Reid, 1987). Functional assays carried out on embryos homozygous for *ls-1* showed reduced ability to convert MVA and GGDP to *ent*-kaurene compared to WT embryos, demonstrating that *LS* encodes the enzyme CPS (Ait-Ali et al., 1997).

1.2.2 Oxidation of ent-*kaurene to* GA_{12} *(Stage 2)*

The second stage in the synthesis of gibberellins is the conversion of kaurene to GA_{12} (Hopkins, 1999). All metabolic steps from this point forward are oxidative in nature (Sponsel and Hedden, 2004).

Initially, *ent*-kaurene is converted by *ent*-kaurene oxidase (KO), a P450 moonoxygenase enzyme, to *ent*-kaurenoic acid which is oxidised by *ent*-kaurenoic acid oxidase (KAO), a second P450 enzyme, to GA_{12} – the common precursor for all GAs in higher plants (Figure 1.3; Thomas and Hedden, 2006; Yamaguchi, 2008;

Sponsel and Hedden, 2004; Figure 1.3). The two P450 enzymes involved in this process each catalyse three reactions, making this a six-step process (Sponsel and Hedden, 2004).

In pea, the *NA* gene encodes the KAO enzyme PsKAO1 (Davidson et al., 2003). When this gene is mutated (*na*) plants display a severely dwarfed shoot and root phenotype, shown to be the result of an equally severe GA deficiency (Potts and Reid, 1983; Yaxley et al., 2001). Interestingly, the seeds of these mutants do not show altered GA_1 levels and germinate normally, suggesting that the expression of this gene is tissue specific (Potts and Reid, 1983; Davidson et al., 2003).

Mutants at the *LH* locus of pea are GA deficient and their internodes are reduced to nearly one-third of their WT length (Reid, 1986). The *lh* mutation has been shown to block the 3-step conversion of *ent*-kaurene to *ent*-kaurenoic acid, suggesting it encodes the KO enzyme (Swain et al., 1997). This suggestion was later supported by Davidson et al. (2004) who isolated the pea homolog of the Arabidopsis *GA3* gene (an already known KO gene) and showed that the sequence of this gene was altered in the *lh* mutants *lh-1*, *lh-2*, and *lh-3*.

1.2.3 Oxidation of GA_{12} to bioactive GAs (Stage 3)

While the first two stages of the GA biosynthesis pathway are common for all plants, the final stage can vary substantially from genus to genus or even in different tissues in the same plant (Hopkins, 1999). Here, the final stage of the pathway will be described for pea, as this is the model species for the present studies.

Gibberellin A_{12} lies in a branch-point in the biosynthetic pathway where it either undergoes 20-oxidation to GA_9, or 13-hydroxylation to produce GA_{53} (Thomas and Hedden, 2006). For pea seedlings, GA biosynthesis occurs via the early 13-hydroxylation pathway (Kamiya and Graebe, 1983; Sponsel and Hedden, 2004). GA_{53} is then converted to the bioactive GAs through a series of oxidation steps requiring two 2-oxoglutarate-dependent dioxygenases (Yamaguchi, 2008). These dioxygenases are GA 20-oxidase and GA 3β-hydroxylase (more commonly known as GA 3-oxidase; Yamaguchi, 2008; Hedden and Proebsting, 1999). The GA 20-

oxidase converts C-20 from a methyl group to an aldehyde and then removes the C atom to form the characteristic γ-lactone of the C_{19} GAs (Hedden and Proebsting, 1999). Second, a hydroxyl group is introduced at the 3β position by GA 3-oxidase (Hedden and Proebsting, 1999).

In pea, there are two known GA 20-oxidase enzymes. They are encoded by *PsGA20ox1* (Martin et al., 1996) and *PsGA20ox2* (only reported to be present in developing seeds; Lester et al., 1996; Ait-Ali et al., 1997), and are responsible for the conversion of GA_{53} to GA_{44} to GA_{19} to GA_{20} (Figure 1.4). To date, no pea mutants have been identified that block GA 20-oxidation, and only one has been identified in Arabidopsis (*ga5*; Ross et al., 1997).

Prior to this study, there was only one known GA 3-oxidation enzyme present in pea, encoded by *PsGA3ox1* – more commonly referred to as *LE* (Lester et al., 1997; Martin et al., 1997) which is responsible for the conversion of GA_{20} to GA_1 (Figure 1.4). Interestingly, the *le* mutant is the most historically significant pea gibberellin mutant as it was studied by Gregor Mendel. Mendel examined seven pairs of traits in pea, one of which he referred to as the "difference in the length of the stem" – later termed *LE*, and found that the tall character dominated that of the dwarf (3:1).

1.2.4 Gibberellin deactivation

Deactivation of GAs is an important mechanism for regulating levels of the bioactive GAs. Deactivation can occur via two different mechanisms, 2β-hydroxylation and epoxidation. 2β-hydroxylation is a well-characterised mechanism and has been shown to occur in pea (among other species), whereas epoxidation, in regards to GA deactivation, is a relatively new mechanism and to date has only been demonstrated in Arabidopsis (Yamaguchi, 2008). Epoxidation will not be discussed further as a result.

2β-hydroxylation deactivation is catalysed by 2-oxoglutarate-dependent dioxygenase enzymes that add a hydroxyl group to the 2β position of the GA molecule (Hedden and Proebsting, 1999). There are two known GA 2-oxidase enzymes in pea, encoded by *PsGA2ox1* and *PsGA2ox2*. *PsGA2ox1* (also known as *SLN*) is thought to play a

major role in the deactivation of GA_{20} to GA_{29}, although it has also shown to be able to deactivate GA_1 to GA_8 (Figure 1.4; Ross et al., 1995; Lester et al., 1999b; Martin et al., 1999). Mutants of this gene (*sln*) display a slender phenotype in young seedlings as a result of the accumulation of the GA_1 precursor, GA_{20} in the dry seed (Ross et al., 1993). *PsGA2ox2* is believed to be important for the deactivation of GA_1 to GA_8 but no pea mutants blocking this step have been identified to date (Figure 1.4; Lester et al., 1999b; Martin et al., 1999).

1.3 DELLA proteins and GA signal transduction

In recent years there has been an immense amount of work undertaken to understand gibberellin signalling and how the transduction of this signal influences GA-responsive growth and development (Thomas and Hedden, 2006). Much of this research has revolved around the role of the growth-repressing 'DELLA' proteins. In this section the various stages of gibberellin signalling are discussed, focussing on the DELLA proteins, a topic that is further studied in the experimental parts of this thesis.

1.3.1 DELLA proteins are repressors of GA signalling

The DELLA proteins, whose name stems from their conserved N-terminal domain D-E-L-L-A, act to repress the GA signalling transduction cascade (Thomas and Hedden, 2006; Olszewski et al., 2002). DELLA proteins form the sub-group of GRAS (named after GAI, RGA, and SCR members) family of transcriptional regulators (Thomas and Hedden, 2006), and display conserved amino acid sequences among both dicot (Arabidopsis) and monocot (rice, wheat and barley) species (Silverstone et al., 1998; Gubler et al., 2002). However, the available evidence indicates greater redundancy in dicots compared with monocots (Ikeda et al., 2001; Thomas and Hedden, 2006). There have been five DELLA genes isolated from Arabidopsis (*GAI, RGA, RGL1, RGL2* and *RGL3*), yet only one in wheat (*RHT;* although a hexaploid), rice (*SLR1*), barley (*SLN1*), and maize (*D8*) (Peng et al., 1997; Silverstone et al., 1998; Peng et al., 1999; Ikeda et al., 2001; Chandler et al., 2002; Gubler et al., 2002), with the possibility of another DELLA gene in maize (*D9*;

Accession Number ABI84225). It should be noted, however, that DELLAs have been studied in fewer dicot model species than in grass species.

1.3.2 GA response mutants

There are two distinct types of GA response mutants: elongated slender mutants and GA-insensitive dwarf mutants. The recessive slender mutants phenotype is a result of a constitutively activated GA response pathway and resemble plants that have been treated with GA, whereas the GA-insensitive dwarfs are semi-dominant mutants that are comparable to GA-deficient plants but in fact have elevated levels of endogenous GA and do not respond to GA application (Thomas and Hedden, 2006; Sun and Gubler, 2004; Silverstone et al., 1998).

Early observations on the "slender" mutant of pea triggered the very early suggestion that GA acts an inhibitor of an inhibitor (Brian, 1957). The elongated slender mutant, conferred by the gene combination *la cry-s*, has long been considered to show constitutive GA signalling (Potts et al., 1985), but the exact nature and function of the *LA* and *CRY* genes had not been reported before the recent Weston et al. (2008) publication. This is the earliest example of a recessive, slender DELLA mutant.

Another recessive response mutant is that of Arabidopsis *spindly* (*spy*). This mutant was first identified when mutant seeds germinated in the presence of paclobutrazol, an inhibitor of GA biosynthesis (Jacobson and Olszewski, 1993). It was also later shown that the recessive *spy* alleles could partially rescue the dwarf phenotype of the GA-deficient *ga1-3* mutant, suggesting that SPY inhibits an early step in GA signalling (Sun and Gubler, 2004).

In most cases of the semi-dominant mutants, the mutations result in truncations, deletions or substitutions within the DELLA domain, indicating the importance of this domain in GA signalling (Thomas and Hedden, 2006). Peng et al. (1997) cloned *GAI* and showed through insertional mutagenesis that the *gai* allele is a semi-dominant gain-of-function, rather than loss-of-function mutant. A 17-amino-acid deletion in GAI results in a mutated protein (gai) that causes a reduction in GA responses and an increased resistance to paclobutrazol, demonstrating that GAI is

involved in GA signal-transduction and acts as a negative regulator (Peng et al., 1997). The cloning of RGA followed that of GAI in 1998 demonstrating the increased interest in unravelling the GA signalling pathway (Silverstone et al., 1998). Like GAI, RGA has also been shown to opereate as a negative regulator of GA signalling as the *rga* mutant was shown to be able to overcome the phenotypic effects of the Arabidopsis GA biosynthesis mutant *ga1-3* (Silverstone et al., 1998).

1.3.3 Gibberellins promote the degradation of the DELLA proteins

Gibberellins promote plant growth and development by leading to increased degradation of the growth-inhibiting DELLA proteins, essentially acting as an "inhibitor of an inhibitor" (Figure 1.5; Harberd et al., 1998). This has been demonstrated in transgenic Arabidopsis containing a GFP:RGA fusion protein, whereby the application of GA to roots resulted in a rapid reduction in the levels of the fusion protein and conversely the application of paclobutrazol resulted in an increase in levels of the fusion protein (Silverstone et al., 2001). This phenomenon appears to be conserved across all angiosperms having been also demonstrated in rice and barley (Gubler et al., 2002; Itoh et al., 2002).

The gain-of-function mutants mentioned above have played an important role in elucidating the mechanism required for the GA-induced degradation of the DELLA proteins (Thomas and Hedden, 2006). For example, the *gai-1* mutant is the result of a 17 amino acid deletion from the DELLA domain, and produces a constitutively active repressor of GA signalling that cannot be degraded by the application of GA (Peng et al., 1997). Loss-of-function mutants have also been important for gaining understanding of this mechanism. Gubler et al. (2002) and Dill et al. (2004) demonstrated that loss-of-function mutations in the GRAS domain of the DELLA genes *SLN1* in barley and *RGA* in Arabidopsis, also result in the inability of GA to promote the degradation of the mutant DELLA proteins.

1.4 Regulation of the GA biosynthesis pathway

The GA biosynthetic pathway is regulated by homeostatic (feedback), developmental, and environmental factors (light and temperature). Other hormones

have also been shown to regulate the GA biosynthesis pathway, but this is discussed further in Chapter 1.5. As the present studies revolve around peas, regulatory examples for this species will be discussed (unless otherwise stated).

1.4.1 Homeostatic regulation

Studies involving GA mutants have provided evidence that bioactive GAs negatively regulate their own levels, by modulating the expression of GA biosynthesis genes (Croker et al., 1990; Scott, 1990; Ross et al., 2003; Hedden and Phillips, 2000). Interestingly, this feedback phenomenon appears to only affect the later stages of GA biosynthesis, as applied *ent*-kaurene was shown to have relatively little effect on plant growth (Otsuka et al., 2004). When bioactive GAs (GA_1 and GA_3) levels are increased in pea shoots, significant reductions in the transcript levels of *PsGA20ox1* and *PsGA3ox1* (Martin et al., 1996; van Huizen et al., 1997; Ayele et al., 2006), and significant increases in those of *PsGA2ox1* and *PsGA2ox2* are observed (Elliott et al., 2001; Ayele et al., 2006).

The GA signalling transduction cascade has also been shown to be required for this homeostatic mechanism to occur, as GA response mutants display disrupted feedback regulation. For example, the Arabidopsis gain-of-function mutant *rga* exhibits a high expression level of *AtGA3ox1* that is unaffected by the application of GA (Dill et al., 2001), suggesting that DELLA proteins are positive regulators of 3-oxidation (Thomas and Hedden, 2006). This mechanism has not been shown to occur in roots prior to this study.

1.4.2 Developmental regulation

The GA biosynthesis pathway is also regulated by developmental processes (Ross et al. 2003, Hedden and Phillips, 2000). For example, mRNA transcript levels of *PsCPS1*, the gene that encodes CPS in the GA biosynthetic pathway, were present from the maturation phase of seed development through to the maturation of pea embryos, with fluctuations in expression seen throughout this process (Ayele et al., 2006). Upon imbibition, *PsCPS1* transcript levels decreased and remained low for

the following 6 days, then increased in the actively growing embryo axis, and the shoots and roots of the young seedlings (Ayele et al., 2006).

1.4.3 Environmental regulation

When pea seedlings are grown in the dark, a number of phenotypic responses occur, including an etiolated white stem with yellow leaves (Reid et al., 2002). This last phenotype triggered early researchers to suspect that GA may be playing a role in this response as this is the phenotype seen when light-grown pea seedlings are treated with GA. Later, however, when endogenous GA quantification was possible, it was found that the GA levels were not elevated in the dark grown pea seedlings, suggesting that GAs were not regulated by light (Weller et al., 1994). Then in the late 1990s, several studies showed that when pea seedlings are transferred from the dark into the light, an inhibition of growth and a rapid transient drop in GA_1 is seen (e.g. Ait-Ali et al., 1999; O'Neill et al., 2000). Yet, although it appears that light does regulate GA biosynthesis, the signalling mechanism for pea is still largely unknown. However, the recent discovery of a new pea mutant, *long1*, which has a light-dependent elongated phenotype, has provided evidence that the *LONG1* gene plays a major role in mediating light regulation of GA biosynthesis in de-etiolating seedlings, providing some insight into the mechanism (Weller et al., 2009).

Temperature has also been shown to be an environmental factor in the regulation of the GA biosynthesis pathway. For example, when pea seedlings growing in light conditions are subjected to a short, moderate temperature drop, the rate of stem elongation is shown to decrease, and a corresponding increase in the transcript levels of *PsGA2ox2* and reduction in GA_1 levels are seen (Stavang et al., 2007). This study indicates that GA biosynthesis is an important player in the acclimation of plant growth to changes in ambient temperature (Stavang et al., 2007).

1.5 GAs and their interactions with other hormones

Many plant hormones have overlapping roles in plant development processes; however, in the majority of cases they are unable to substitute for one another (Kuppusamy et al., 2009). For example, the application of any one of the hormones

auxin, gibberellins, or brassinosteroids can result in the elongation of cells in the seedling stem. If the action of any of these hormones is inhibited, so too is the growth, indicating that a complex interaction (or cross-talk) between the hormone's biosynthesis and signalling pathways is required to regulate this one physiological response (Kuppusamy et al., 2009). An increased understanding in recent years of biosynthesis and signal transduction pathways of various plant hormones, including gibberellin, is providing an important tool for understanding the interaction mechanisms of these hormones and their role in plant physiology (Weiss and Ori, 2007). In this chapter I discuss interactions between gibberellin and other hormones that have not been studied here. However, the interactions between gibberellin and auxin and abscisic acid is discussed further in Chapters 5 and 6.

1.5.1 Gibberellins and brassinosteroids

Brassinosteroids (BRs) collectively refer to a large number (over 60) of naturally-occurring plant steroids that can manipulate growth at very low concentrations (Davies, 2004). Both GAs and BRs have been shown to play important roles in shoot elongation in plants (Davies, 2004; Nemhauser and Chory, 2004). BR-deficient plants are dwarf in stature, as are those plants deficient in GA. This observation suggested that it is possible that these two hormones are interacting with each other to cause this developmental response.

Indeed, Bouquin et al. (2001) showed that the application of BR to Arabidopsis seedlings results in increased transcript levels of the GA 20-oxidase gene *GA5*, and that these transcript levels were reduced in the BR response mutant *bri1-201* and the BR deficient mutant *cpd*. As a result of these findings, Bouquin et al. (2001) concluded that BR appears to act as a positive regulator of GA synthesis in Arabidopsis.

However, conflicting evidence has been demonstrated in both tomato and pea. Early studies on the BR-GA interaction in tomato demonstrated that GA_{20} levels are increased in the shoots and leaves of the extreme BR-deficient d^x (Nadhimov et al., 1988). Similarly in pea, Jager et al. (2005) showed that BR mutants have elevated GA_{20} levels and the application of BR reduced levels to those comparable to WT

plants. Unlike the other studies, however, Jager et al. (2005) took their studies further and measured GA_1 levels in these BR-deficient mutants. They found that the GA_1 content was not reduced in the BR-deficient mutants as was expected on the basis of Bouquin et al. (2001); in fact on occasion, the levels were found to be elevated, suggesting that the dwarf stature of BR-deficient plants is a direct result of BR deficiency and not mediated via GA (Jager et al., 2005).

Conversely, it was also possible that GA acts on BR. This suggestion was negated by Jager et al. (2005) who showed that GA-deficient plants did not display reduced levels of BR. However, it has been shown that the rice *SPINDLY (SPY)* gene, a negative regulator of gibberellin signalling, is responsible for modulating BR biosynthesis (Shimada et al., 2006). Shimada et al. (2006) showed that *SPY* knockdown plants (RNAi and antisense) exhibited increased lamina joint bending (a phenotype associated with BRs and not GAs), slightly higher BR content than WT plants, and reduced expression of genes shown to be negatively regulated by BR, indicating that SPY may not only play a role in the GA signalling pathway but also the BR pathway.

1.5.2 Gibberellins and ethylene

The interaction between GAs and ethylene appears to be quite complex as both positive and negative effects are seen depending on the developmental and environmental circumstances of the plant. A further complication is that these interactions have been shown to operate on both the biosynthesis and signal transduction levels (Weiss and Ori, 2007).

Ethylene is a hormone that is responsible for the characteristic “triple response” seen in emerging seedlings: exaggeration of the apical hook, shortening and thickening of the hypocotyls, and inhibition of root growth (Davies, 2004). It has been suggested for Arabidopsis that, at least in part, this response is the result of ethylene modulating the growth-inhibitory DELLA proteins RGA and GAI (Achard et al., 2003). For example, the application of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) to Arabidopsis plants results in reduced root elongation and the addition of GA can overcome this reduction, although it has little effect on root

elongation in the absence of ACC (Achard et al., 2003). Furthermore, the DELLA mutants *gai-t6* (lacks GAI) and *rga-24* (lacks RGA) treated with ACC both display longer roots than WT plants grown in the absence of ACC, and the double mutant *gai-t6 rga-24* display longer roots again, suggesting that GAI and RGA are involved in the ethylene-induced inhibition of root elongation (Achard et al., 2003). Ethylene treatment also inhibited the GA-mediated RGA degradation in root cell nuclei (Achard et al., 2003). Hence, in contrast to GA, the role of ethylene appears to be to stabilise the DELLA proteins rather than degrade them.

An interaction between ethylene and GA has also been shown to be important for floral transition. The activation of ethylene signalling using the constitutive *ctr1-1* mutant results in a reduction in GA levels (and hence an accumulation of DELLAs as a result of feedback) and flower initiation under short-day conditions (Achard et al., 2007). In addition, the DELLAs are shown to be directly responsible for the delayed flowering as in the *ctr1-1 gai-t6 rga-24* mutant the lack of GAI and RGA largely suppresses the late flowering seen in the *ctr1-1* mutant (Achard et al., 2007). Lastly, Achard et al. (2007) show that the DELLAs delay flowering by repressing the expression of the floral meristem-identity genes *LEAFY (LFY)* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1)*.

1.5.3 Gibberellins and cytokinin

Cytokinins (CKs) are responsible for a number of plant development processes including the promotion of cell division, the counteraction of senescence, the regulation of apical dominance and the transmission of nutritional signals (Sakakibara, 2004). GA and cytokinin (CK) predominantly act in an antagonistic manner for a number of developmental processes including shoot and root elongation, cell differentiation, shoot regeneration in culture, and shoot apical meristem (SAM) activity (Greenboim-Wainberg et al., 2005; Jasinski et al., 2005). This interaction has been shown to act at both the biosynthesis and signal transduction levels (Weiss and Ori, 2007).

Brenner et al. (2005) undertook genome-wide expression profiling on young Arabidopsis seedlings that had been treated with CK. They found that CK application

resulted in inhibited expression of the GA 20-oxidase and GA 3-oxidase (*GA4*) genes, and promoted the expression of the DELLA genes *GAI* and *RGA* (Brenner et al., 2005). Brenner et al. (2005) suggest that CK may reduce GA activity by repressing GA biosynthesis gene expression and upregulation of repressors of GA signalling genes (Brenner et al., 2005). Further support for this model comes from a study that found that when GA signalling is repressed in Arabidopsis, there is a promotion of SAM activity by the KNOTTED1-like homeobox (KNOXI) protein as a result of elevated CK synthesis (Hay et al., 2002), inhibited GA biosynthesis (Sakamoto et al., 2001; Hay et al., 2002), and enhanced GA deactivation (Jasinski et al., 2005).

In Arabidopsis, SPY has been demonstrated to act not only as a repressor of GA signalling, but also as a positive regulator of CK signalling (Greenboim-Wainberg et al., 2005). The *spy* mutant displayed phenotypes that are not seen in GA-treated plants, and assays showed that this mutant is in fact resistant to applied CK. In addition, the application of GA to WT plants resulted in repression of CK responses normally seen in the plant (Greenboim-Wainberg et al., 2005).

1.6 Aims of thesis

An increased understanding in recent years of biosynthesis and signal transduction pathways of various plant hormones, including gibberellin (GA), is providing an important tool for understanding interactions between these hormones and their role in plant physiology. In this thesis, interactions between auxin and gibberellin in regulating root growth, and between abscisic acid (ABA) and gibberellin in regulating shoot growth, are explored using the model species, pea.

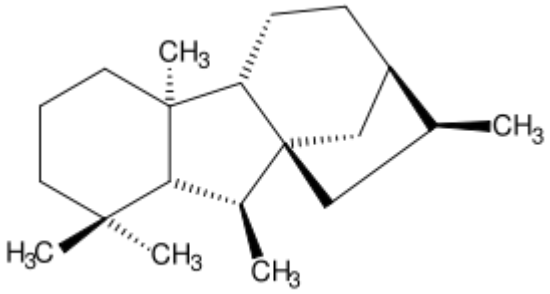


Figure 1.1 *ent*-gibberellane structure common to the gibberellins. This structure is first seen in the intermediate GA_{12}-7-aldehyde during the second stage of GA biosynthesis. All GA compounds from this stage forward display this structure.

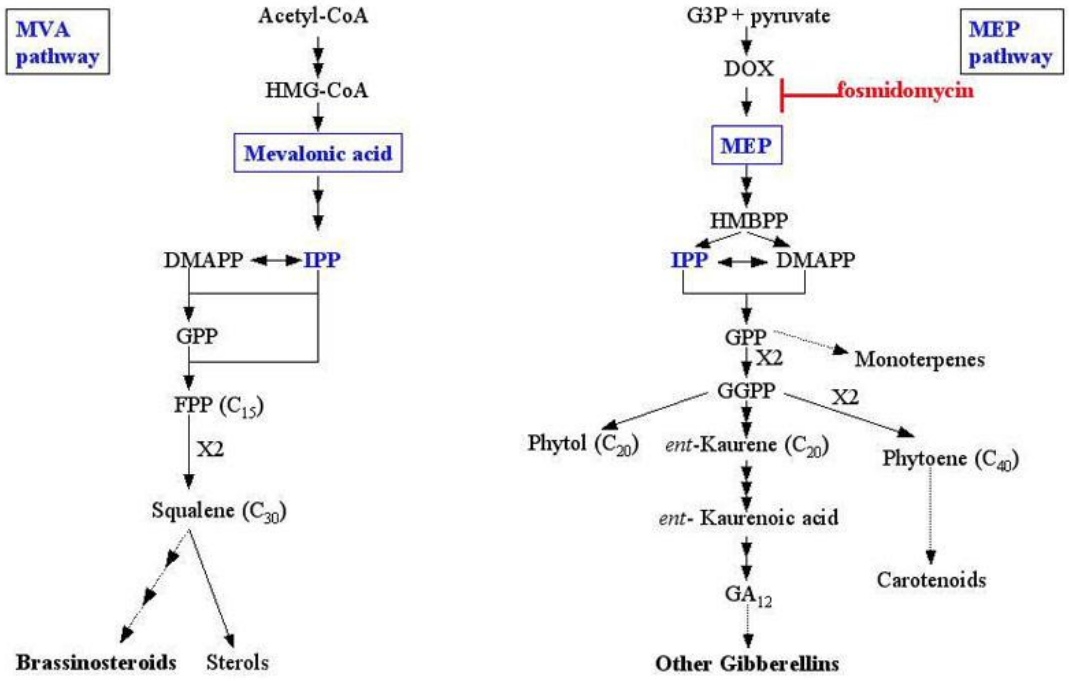


Figure 1.2 First and second stage of the GA biosynthesis pathway. Shown are the two known pathways for the synthesis of IPP, an intermediate required for the synthesis of *ent*-kaurene: *left* is the acetate/mevalonate (MVA) pathway, *right* is the methylerythritol phosphate (MEP) pathway. Also shown is the conversion of *ent*-kaurene to GA_{12}. *Figure taken from* *http://4e.plantphys.net/article.php?ch=t&id=366*

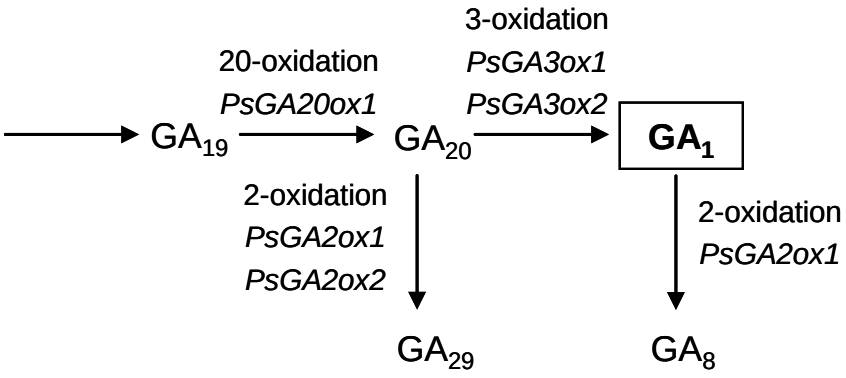


Figure 1.3 Last stage of the GA biosynthesis pathway in vegetative pea plants.

Figure 1.4 Photo of GA biosynthesis mutants at 2 weeks. Left to right: WT (L107) and *le-3* (L5839) (A), WT (L107) and *lh-1* (L511) (B), WT (L107) and *ls-1* (L181) (C), WT (L205+) and *na* (L205-) (D), and WT (L309+) and *sln* (L309-) (E).

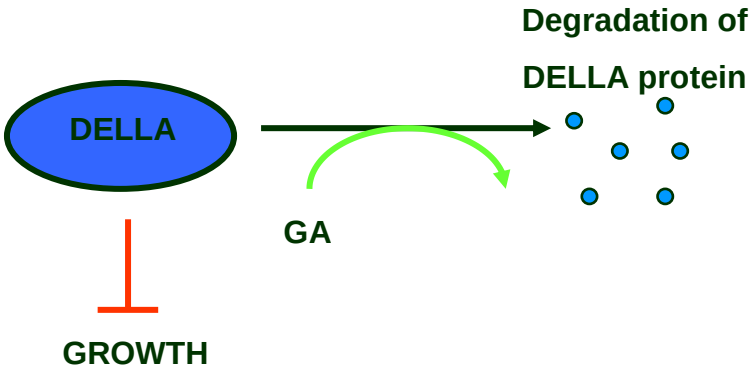


Figure 1.5 Model of how GA increases the degradation of the DELLA proteins to promote growth, essentially acting as an "inhibitor of an inhibitor".

CHAPTER 2

Materials and Methods

2.1 Plant material and growing conditions

2.1.1 Plant material

The pure lines of *Pisum sativum* L. used in these studies were obtained from the collection held by the School of Plant Science, University of Tasmania. A variety of lines were used in these studies and are outlined in each chapter.

2.1.2 Growing conditions

Seeds were sown in pots of 10 cm diameter at a density of between 2 to 5 per pot, depending on the age of the plant at harvest. The growing medium consisted of either 100% potting mix, or a 50:50 vermiculite/gravel mix topped with a layer of potting mix. Unless otherwise stated, plants were grown in an 18 h photoperiod in a heated glasshouse. Glasshouse temperatures generally ranged from 13 to 21°C during the coolest month and 17 to 35°C during the warmest month. The average daily maximum temperature was 25°C.

2.2 Quantification of endogenous IAA, GA and ABA levels

2.2.1 Hormone extraction

The root tissue was homogenized and hormones extracted at 3ºC for 24 h, before being filtered using Whatman no.1 filter paper in a Buchner apparatus, with 3 x 80% methanol washes of the sample beaker. The volume of the filtered product was then recorded for each sample. The following internal standards were then added to the filtered samples as required: $^{13}C_6$ IAA, ^{3}H GA_{20}, $^{2}H_2$ GA_1, $^{2}H_2$ GA_8, $^{2}H_2$ GA_{19}, $^{2}H_2$ GA_{20}, $^{2}H_2$ GA_{29}, $^{2}H_2$ GA_{53} and $^{2}H_2$ GA_{81}, $^{2}H_4$ ABA.

2.2.2 Sep-Pak purification

Half the volume of each sample was taken and dried down on a rotary evaporator (Buchi Rotavapor; Buchi Labortechnik AG, Switzerland) under vacuum at a temperature of 35ºC until approximately 1 mL of sample remained. Each sample was re-suspended in 1 mL of 0.4% acetic acid in distilled water. A Vac-RC C-18 Sep-Pak cartridge was preconditioned with a 15 mL wash of 100% methanol followed by 15 mL of 0.4% acetic acid in distilled water. The re-suspended sample was passed through the Sep-Pak cartridge, followed by 2 x 1 mL washes of the sample with 0.4% acetic acid in distilled water. The hormones were eluted from the column into a round-bottom flask with 15 mL of 70% methanol in 0.4% acetic acid for GAs, and 45% methanol in 0.4% acetic acid for ABA. The elutate was reduced to dryness under vacuum at 35ºC and transferred to a glass scintillation vial with 2 x 1 mL washes of 100% methanol. These samples were dried under gaseous nitrogen.

2.2.3 High Performance Liquid Chromatograhy (HPLC) to separate the various GAs

The HPLC system consists of two Waters M45-Solvent Delivery Systems (Waters Assoc., MA, USA), a Model U6K Universal Liquid Chromatograph Injector (sample loading loop volume = 2 mL), a Waters Model 660 Solvent Programmer, a Z-module Radial Compression Separation system, a 10 mL Radial-Pak A cartridge C18 column and a Waters Model 440 variable wavelength absorbance detector. The solvent program was a linear gradient from 20-75% MeOH in 0.4% acetic acid over 35 min with a flow rate of 2mL.min^{-1}. The samples were re-suspended in 2 x 1 mL washes of 20% MeOH in 0.4% acetic acid, filtered through a 25 mm PTFE 0.45 µm filter (Waters Assoc., MA, USA) and injected into the HPLC injector. A Frac-100 Fraction Collector (Pharmacia Corp., NJ, USA) was used to collect fractions at one min intervals over the entire period of the run. After collection 100 µL aliquots were taken into 4 mL of Ready-Safe liquid scintillation counting cocktail (Beckman Instruments Inc., CA, USA) and counted for radioactivity on the Beckman LS 6500 liquid scintillation counter. Fractions containing various GAs were determined based on the retention time of the ^{3}H GA_{20} internal standard. The appropriate fractions containing the hormone of interest (i.e. GA_{20}, GA_{1}, GA_{19}, GA_{29}, GA_{8}, GA_{81}, and GA_{53}) were grouped and dried down using a sample concentrator. Note: This step

was not taken for all GA analysis. When this method was not undertaken, the samples proceeded directly to Chapter 2.2.4.

2.2.4 Methylation, ether partitioning and derivatisation

Samples were methylated with 400 µL 100% methanol and 1.5 mL of diazomethane and left to stand for five minutes before drying under gaseous nitrogen. As a final purification step, the samples were re-suspended in 1 mL of distilled water then partitioned against 3 x 400 µL of diethyl ether. In later experiments, an ether transfer method was used whereby 300 µL was added to the dried sample and transferred to a GC-MS vial. The ether fraction was transferred to a new vial and reduced to dryness under gaseous nitrogen. To prepare ABA samples for GC-MS, the dried samples were resuspended in approximately 30 µL chloroform and transferred to an insert vial. To prepare GA and IAA samples for GC-MS analysis, the methylated samples were silylated twice, firstly with 10 µL pyridine and 20 µL N,O-bis(trimethylsilyl)trifluoro-acetamide (BSTFA) at 80ºC for 20 min, then after drying under gaseous nitrogen, with 15 µL of BSTFA at 80ºC for 15 min.

2.2.5 GC-MS-SIM quantification of hormones

GC-MS-SIM quantification of GAs was performed using a Hewlett-Packard 5890 Series II gas chromatograph linked via a direct inlet into a Kratos Concept ISQ mass spectrometer, controlled by a Mach 3 data system (Sun Microsystems Inc., CA, USA). Approximately 0.8 µL of sample was injected in splitless mode at 60ºC onto a 25 m long x 0.32 mm inner diameter HP1 colums with a 0.17 µm film thickness (Hewlett Packard). The carrier gas used was helium at an internal flow rate of 2 $mL.min^{-1}$ at 60ºC under a pressure of 190kPa. Perfluorokerosene was used as the reference compound to provide lock masses. For quantification of GAs, the temperature was increased from 60ºC to 230ºC at 30ºC per minute, then a bake off at 290ºC (Jager, 2006).

For IAA and ABA samples, GC-MS-MS analysis was performed with a Varian 8400 Autosampler and a Varian 3800 GC, coupled to a Varian 1200 triple quadrupole MS. An 1177 split/splitless injector was used to make splitless injections of 1 µL onto a

30 m long x 0.25 mm inner diameter Varian FactorFour VF-5ms column with a 0.25 µM film thickness. Helium was used as a gas carrier at a constant flow rate of 1.4 mL min^{-1}. The initial temperature of the column oven was maintained at 50ºC for 2 min, then increased to 190ºC at 30ºC min^{-1} and then to 270ºC at 10ºC min^{-1} and held for 5 min. The system was operated in MS/MS selected reaction monitoring mode (Jager, 2006).

Hormones were identified on the basis of retention times and by monitoring selected pairs of ions characteristic of the hormone to be studied (Table 2.1). Quantification of endogenous hormones was achieved by comparing the peak areas of ion pairs derived from the endogenous hormone and a corresponding stable-isotope-labelled internal standard, as determined by GC-MS. Due to the addition of internal standards, there is a contribution of incomplete labelled standards to the endogenous ion peak (Lawrence et al., 1992). To compensate for this, pre-determined correction factors were applied when calculating hormone levels obtained for the Kratos system. (Table 2.2). The endogenous hormone level ($ng.g(FW)^{-1}$) is calculated as follows:

$$\text{endogenous level} = \frac{\text{corrected endogenous peak area}}{\text{corrected internal standard peak area}} \times \frac{\text{internal standard (ng)}}{\text{fresh weight (g)}}$$

2.2.6 Analysis of results

A standard student's t-test was used to calculate statistical significance of all experiments unless otherwise specified.

2.3 Gene expression studies

2.3.1 RNA extraction

The harvested tissue was ground to a fine powder in a pre-cooled (using liquid nitrogen) mortar and pestle. Approximately 100 mg of this powder was placed into an eppendorf tube and RNA extraction was undertaken using the RNeasy Plant Mini

Kit (Qiagen, VIC, Australia) and optional on-column DNase digestion set using the Qiagen RNase-Free DNase Set, according to the manufacturer's instructions.

To estimate the RNA concentration of each sample, either a spectrophotometer or fluorometer was used. For the spectrophotometer method, a 1 in 30 dilution of RNA was made. RNase-free water was placed into a quartz container and 'blanked' on a spectrophotometer to calibrate the machine. The sample solution was then placed into the quartz container and the A_{260} and concentration were tested and recorded. For the fluorometer method, a 1 in 15 dilution of RNA was made and RNA concentration was determined using the Picofluor fluorometer (Turner Biosystems, USA), as per manufacturers instructions.

To determine whether the RNA has been extracted effectively, the samples were run on a 1% agarose gel. To make the 1% agarose gel, 0.5 g of agarose and 50 mL of water was dissolved in the microwave until agarose had fully dissolved. To the molten agarose, 1 µL GelRed was added and mixed by gently swirling the mixture. The liquid was then poured into a gel mould and left to set for approximately 30 min. To keep the RNA quantity consistent, 1 or 2 µg of RNA sample was placed into each well (calculated from the spectrophotometer or flurometer reading).

2.3.2 cDNA synthesis

One to 2 µg of total RNA was used to synthesise single-strand cDNA using the QuantiTect Reverse Transcription kit (Qiagen, VIC, Australia). For each set of samples a NRT (no reverse transcriptase added) was made. cDNA samples were diluted to 1 in 15 ready for quantitative real-time PCR (qRT-PCR).

2.3.3 Quantitative Real Time PCR (qRT-PCR)

The sequences of the primers used for qRT-PCR reactions are given in Table 2.3, and were acquired from Geneworks (Adelaide, SA, Australia). Two microlitres of cDNA was used for qRT-PCR using BioRad iQ Sybr master mix (BioRad) following the manufacturer's recommendations. Duplicates of each sample were made, including for the no template control (NTC). Reactions were run on a Rotorgene

2000 Dual Channel machine (Corbett Research, NSW, Australia). The qRT-PCR program used was: 50 cycles of 95ºC for 5 seconds, 58ºC for 40 seconds and 79ºC for 5 seconds. A melt curve was produced by ramping from 60°C to 99°C at 1°C per step, waiting for 30 seconds on the first step and 5 seconds for every step thereafter. Analysis was undertaken using the support software for the Rotor-Gene 6 machine. Mean expression levels of the gene of interest were calculated relative to the expression of actin.

2.3.4 Analysis of results

Actin was used as a 'house-keeping' gene as a means to normalise the transcription levels of the gene of interest. Standard curves for each of the genes that were tested (shown in Figures 2.1 – 2.6) were used as a means to determine the relative transcription level of the corresponding gene in the experimental samples. The values obtained from the three replicate runs of duplicates were averaged and the means of the sample replicates were calculated. For each sample, the values obtained from the gene of interest were adjusted according to the expression level of the 'house-keeping' gene (Actin), to give expression levels relative to the amount of total RNA. In ABA-GA experiments, standard curves for each gene were run again.

A standard student's t-test was used to calculate statistical significance of all experiments.

Table 2.1 Ion pairs of derivatised endogenous plant hormones and their respective internal standards, monitored by GC-MS-SIM (using the Kratos system).

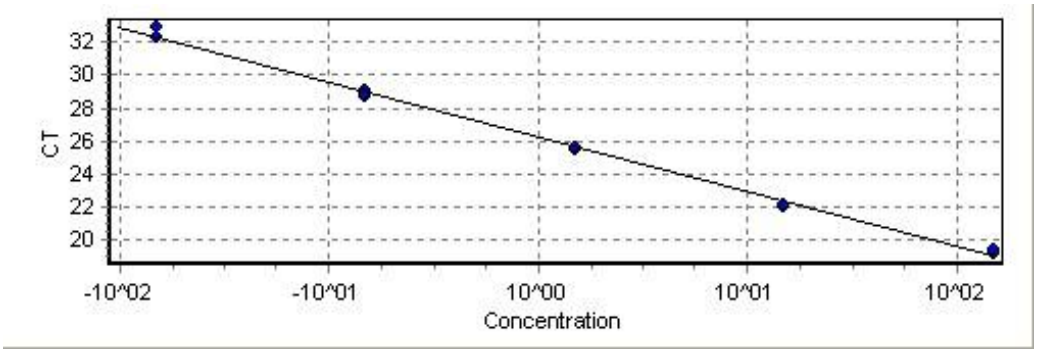
Endogenous compound / internal standard	Ion pair	Additional ions
GA_1 / $[^2H_2]GA_1$	506.2519 / 508.2645	491.2284
GA_8 / $[^2H_2]GA_8$	594.2863 / 596.2988	
GA_{19} / $[^2H_2]GA_{19}$	434.2488 / 436.2614	374.2278 / 376.2404
GA_{20} / $[^2H_2]GA_{20}$	418.2176 / 420.2301	375.1628 / 377.1754
GA_{29} / $[^2H_2]GA_{29}$	506.2519 / 508.2645	
GA_{29}-cat / $[^2H_2]GA_{29}$-cat	446.2124 / 448.2250	518.2519 / 520.2645
GA_{53} / $[^2H_2]GA_{53}$	448.2644 / 450.2769	389.2511 / 391.2636

Table 2.3 Correction factors for GAs and ABA for the Kratos GC-MS, resolution 3,000.

GA_1	Corrected 506 = total 506 – 0.004 x total 508 Corrected 508 = total 508 – 0.14 x corrected 506
GA_{29}	Corrected 506 = total 506 – 0.005 x total 508 Corrected 508 = total 508 – 0.14 x corrected 506
GA_8	Corrected 594 = total 594 – 0.023 x total 596 Corrected 596 = total 596 – 0.20 x corrected 594
GA_{20}	Corrected 418 = total 418 – 0.009 x total 420 Corrected 420 = total 420 – 0.08 x corrected 418
GA_{19}	Corrected 434 = total 434 – 0.11 x total 436 Corrected 436 = total 436 – 0.08 x corrected 434
ABA	Corrected 194 = total 194 – 0.008 x total 190

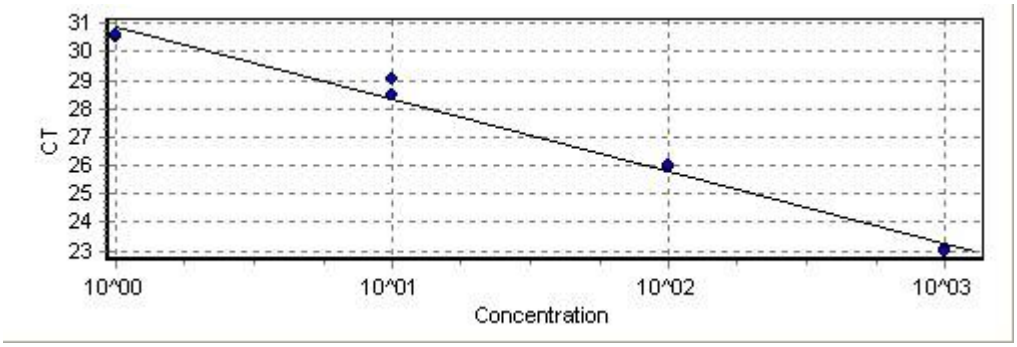
Table 2.3 Sequences of primers used for qRT-PCR.

Genes	Primer	Sequence (5' -3')
PsGA20ox1	Ps20ox1-fwd	CATTCCATTAGGCCAAATTTCAAT
	Ps20ox1-rev	CTGCCCTATGTAAACAACTCTTGTATCT
PsGA3ox1	PsLE87-fwd	TTCGAGAACTCTGGCCTCAAG
	PsLE87-rev	ATGTTCCTGCTAACTTTTTCATGGTT
PsGA3ox2	Ps3ox2-fwd	ATCATGGGGTCACCGTCTAA
	Ps3ox2-rev	GCTAGTGTCTTCATTTGCTTTTGA
PsGA2ox1	PsSLN-fwd	CACAACCAATCAAGAACACAATTTC
	PsSLN-rev	CCCTTCTGCCATCAAATCAAG
PsGA2ox2	Ps2ox2-fwd	CCCTCCTGACCCCAGTGAAT
	Ps2ox2-rev	CTCACACTCACAAATCTTCCATTTG
Actin	PsActin-fwd	GTGTCTGGATTGGAGGATCAATC
	PsActin-rev	GGCCACGCTCATCATATTCA
18S	Ps18S-fwd	ACGTCCCTGCCCTTTGTACA
	Ps18S-rev	CACTTCACCGGACCATTCAAT



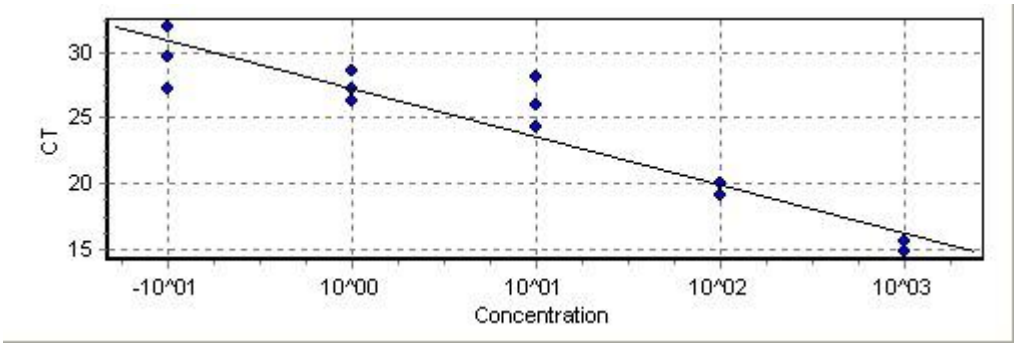
No.	Colour	Name	Ct	Given Conc ($/ul)	Calc Conc ($/ul)	Rep. Ct
1		*PsActin* Std Dilution 1	19.17	150.0000	134.4355	19.31
2		*PsActin* Std Dilution 1	19.40	150.0000	115.1787	
3		*PsActin* Std Dilution 1	19.35	150.0000	119.3454	
4		*PsActin* Std Dilution 2	22.05	15.0000	18.3182	22.05
5		*PsActin* Std Dilution 2	22.00	15.0000	18.9809	
6		*PsActin* Std Dilution 2	22.12	15.0000	17.4690	
7		*PsActin* Std Dilution 3	25.65	1.5000	1.5083	25.55
8		*PsActin* Std Dilution 3	25.48	1.5000	1.6880	
9		*PsActin* Std Dilution 3	25.54	1.5000	1.6274	
10		*PsActin* Std Dilution 4	29.08	0.1500	0.1388	28.86
11		*PsActin* Std Dilution 4	28.64	0.1500	0.1885	
12		*PsActin* Std Dilution 4	28.85	0.1500	0.1634	
13		*PsActin* Std Dilution 5	32.28	0.0150	0.0151	32.50
14		*PsActin* Std Dilution 5	32.90	0.0150	0.0098	
15		*PsActin* Std Dilution 5	32.31	0.0150	0.0148	

Figure 2.1 Quantitation Report for the standard curve of the *PsActin* housekeeping gene used for auxin-GA studies was generated by Rotor-Gene Q 1.7.94 © Qiagen 2003-2009.



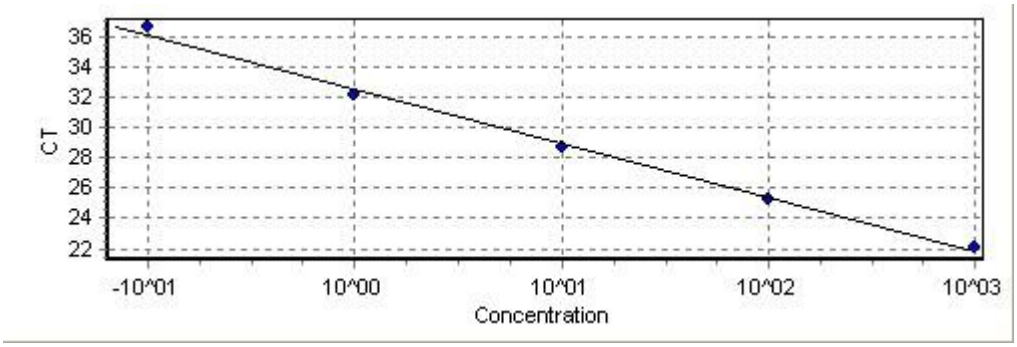
No.	Colour	Name	Ct	Given Conc (Copies)	Calc Conc (Copies)	Rep. Ct
1		*20ox1* Std Dilution 1	23.04	1,000.0	1,220.0	23.01
2		*20ox1* Std Dilution 1	22.98	1,000.0	1,294.8	
3		*20ox1* Std Dilution 2	25.97	100.0	86.1	25.97
4		*20ox1* Std Dilution 2	25.98	100.0	84.6	
5		*20ox1* Std Dilution 3	28.46	10.0	8.9	28.75
6		*20ox1* Std Dilution 3	29.03	10.0	5.4	
7		*20ox1* Std Dilution 4	30.49	1.0	1.4	30.55
8		*20ox1* Std Dilution 4	30.61	1.0	1.3	

Figure 2.2 Quantitation Report for the standard curve of the *PsGA20ox1* gene used for auxin-GA studies was generated by Rotor-Gene Q 1.7.94 © Qiagen 2003-2009.



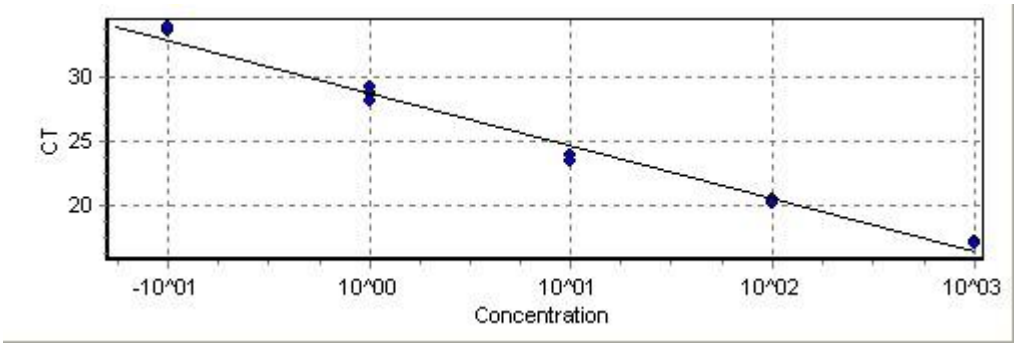
No.	Colour	Name	Ct	Given Conc (Copies)	Calc Conc (Copies)	Rep. Ct
1		*3ox1* Std Dilution 1	15.61	1,000.0	1,427.1	15.06
2		*3ox1* Std Dilution 1	14.78	1,000.0	2,397.5	
3		*3ox1* Std Dilution 1	14.80	1,000.0	2,366.2	
4		*3ox1* Std Dilution 2	19.04	100.0	167.2	19.48
5		*3ox1* Std Dilution 2	19.93	100.0	95.4	
6		*3ox1* Std Dilution 2		100.0		
7		*3ox1* Std Dilution 3	25.91	10.0	2.3	26.07
8		*3ox1* Std Dilution 3	28.11	10.0	.6	
9		*3ox1* Std Dilution 3	24.20	10.0	6.6	
10		*3ox1* Std Dilution 4	27.21	1.0	1.0	27.35
11		*3ox1* Std Dilution 4	28.52	1.0	.4	
12		*3ox1* Std Dilution 4	26.32	1.0	1.8	
13		*3ox1* Std Dilution 5	29.64	.1	.2	29.59
14		*3ox1* Std Dilution 5	27.21	.1	1.0	
15		*3ox1* Std Dilution 5	31.92	.1	.1	

Figure 2.3 Quantitation report for the standard curve of the *PsGA3ox1* gene used for auxin-GA studies was generated by Rotor-Gene Q 1.7.94 © Qiagen 2003-2009.



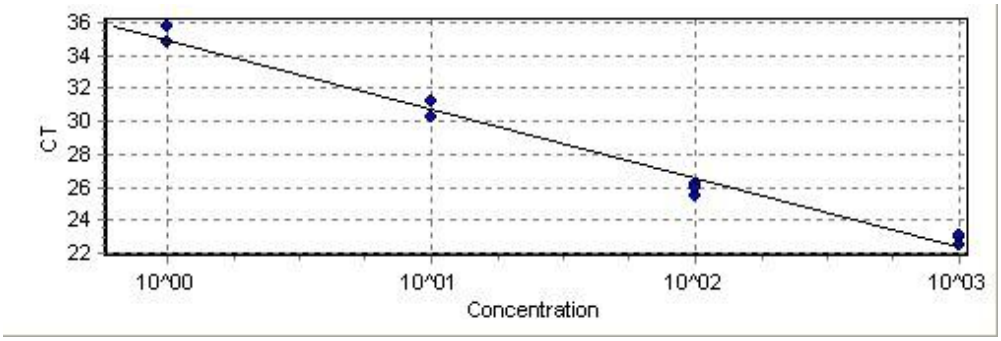
No.	Colour	Name	Ct	Given Conc (Copies)	Calc Conc (Copies)	Rep. Ct
A1		*3ox2* Std Dilution 1	22.10	1,000.0	827.7	22.07
A2		*3ox2* Std Dilution 1	22.04	1,000.0	861.9	
A3		*3ox2* Std Dilution 2	25.25	100.0	108.4	25.24
A4		*3ox2* Std Dilution 2	25.24	100.0	108.5	
A5		*3ox2* Std Dilution 3	28.67	10.0	11.8	28.67
A6		*3ox2* Std Dilution 3	28.67	10.0	11.8	
A7		*3ox2* Std Dilution 4	32.18	1.0	1.2	32.18
A8		*3ox2* Std Dilution 5	36.60	.1	.1	36.60

Figure 2.4 Quantitation report for the standard curve of the *PsGA3ox2* gene used for auxin-GA studies was generated by Rotor-Gene Q 1.7.94 © Qiagen 2003-2009.



No.	Colour	Name	Ct	Given Conc (Copies)	Calc Conc (Copies)	Rep. Ct
1		*2ox1* Std Dilution 1	17.16	1,000.00	647.75	17.10
2		*2ox1* Std Dilution 1	17.05	1,000.00	687.31	
3		*2ox1* Std Dilution 1	17.08	1,000.00	675.37	
4		*2ox1* Std Dilution 2	20.10	100.00	125.09	20.24
5		*2ox1* Std Dilution 2	20.52	100.00	98.72	
6		*2ox1* Std Dilution 2	20.08	100.00	126.35	
7		*2ox1* Std Dilution 3	23.84	10.00	15.52	23.59
8		*2ox1* Std Dilution 3	23.42	10.00	19.64	
9		*2ox1* Std Dilution 3	23.50	10.00	18.74	
10		*2ox1* Std Dilution 4	29.27	1.00	.75	28.76
11		*2ox1* Std Dilution 4	28.79	1.00	.98	
12		*2ox1* Std Dilution 4	28.21	1.00	1.36	
13		*2ox1* Std Dilution 5	33.55	.10	.07	33.74
14		*2ox1* Std Dilution 5	33.94	.10	.06	

Figure 2.5 Quantitation report for the standard curve of the *PsGA2ox1* gene used for auxin-GA studies was generated by Rotor-Gene Q 1.7.94 © Qiagen 2003-2009.



No.	Colour	Name	Ct	Given Conc (Copies)	Calc Conc (Copies)	Rep. Ct
1		*2ox2* Std Dilution 1	22.46	1,000.0	953.9	22.84
2		*2ox2* Std Dilution 1	22.96	1,000.0	723.5	
3		*2ox2* Std Dilution 1	23.11	1,000.0	666.6	
4		*2ox2* Std Dilution 2	25.49	100.0	180.4	25.84
5		*2ox2* Std Dilution 2	26.14	100.0	125.7	
6		*2ox2* Std Dilution 2	25.90	100.0	143.4	
7		*2ox2* Std Dilution 3	30.27	10.0	12.9	30.77
8		*2ox2* Std Dilution 3	31.26	10.0	7.5	
9		*2ox2* Std Dilution 4	34.77	1.0	1.1	35.27
10		*2ox2* Std Dilution 4	35.76	1.0	.6	

Figure 2.6 Quantitation report for standard curve of *PsGA2ox2* gene used for auxin-GA studies was generated by Rotor-Gene Q 1.7.94 © Qiagen 2003-2009.

CHAPTER 3

Characterisation of a New GA Synthesis Gene, *PsGA3ox2*

Parts of this chapter have been published in:
Weston DE, Elliott RC, Lester DR, Rameau C, Reid JB, Murfet IC, Ross JJ (2008) The pea DELLA proteins LA and CRY are important regulators of gibberellin synthesis and root growth. *Plant Physiology* **147**, 199-205.

3.1 Introduction

In 1866, Gregor Mendel established the laws of inheritance, now a core principle in current-day genetics (Mendel, 1866). These laws resulted from studies on seven different clearly-distinguishable phenotypes in the common garden pea, one of which was the length of the stems - later assigned the symbols *LE*(tall)*/le*(dwarf) (Griffiths et al., 2000).

Mendel's *LE* gene was first linked to gibberellins (GAs) in a study by Brian and Hemming (1955) who found that the application of GA_3 to pea seedlings stimulated stem elongation in dwarf peas. This connection was further enhanced when *LE* was found to be responsible for the conversion of GA_{20} to GA_1 (Ingram et al., 1984). Ingram et al. (1984) fed radiolabelled GA_{20} to *LE* and *le-1* plants and found radiolabelled GA_1 was produced at a much reduced quantity in the *le-1* mutant plants than the wild-type (WT) *LE* plants. Finally, in 1997 Mendel's *LE* gene was cloned and found to encode a GA 3-oxidase responsible for the conversion of GA_{20} to the bioactive GA_1 (Figure 1.1; Lester et al., 1997; Martin et al., 1997).

When further phenotypic analysis was undertaken on *LE* and the *le-1* mutant it was found that while *le-1* displayed a dwarf shoot phenotype, the root phenotype was comparable to the roots of the wild-type *LE* plants (Figure 3.1; Tanimoto, 1990; Yaxley et al., 2001). Furthermore, when the levels of GA_{20} and GA_1 were measured in the *le-1* root tips, the levels were found to be comparable to those of the wild-type roots (Yaxley et al., 2001). This might have resulted from *le-1* being a 'leaky' mutant, thus allowing GA_1 to be produced in the roots at sufficient levels to alter root

elongation (Tanimoto, 1990). However, when Yaxley et al. (2001) analysed the root phenotype and GA levels in the biochemically null mutant *le-2*, they were comparable to those of the *le-1* mutant, dispelling the idea of a lower threshold for growth in the roots compared with the shoots.

The lack of phenotypic and GA content change in the roots of the mutants *le-1* and *le-2* did, however, support the notion that another GA 3-oxidase could be primarily expressed in pea roots and compensating for the lack of *LE* activity in the mutants (Yaxley et al. 2001). The results described here show another GA 3-oxidase is present in peas (*PsGA3ox2*), with functional assays demonstrating its role in the conversion of GA_{20} to GA_1 (see also Weston et al., 2008). Using this newly-discovered pea GA 3-oxidase, the hypothesis was tested that this gene is responsible for the normal root phenotype of *le-1* plants.

3.2 Materials and methods

3.2.1 Plant material

Experiments monitoring GA biosynthesis and catabolism gene expression were conducted with the isogenic tall (WT) Hobart line HL205+ and dwarf (*le-1*) line HL205-.

3.2.2 Plant growth and chemical treatments

Plants were grown in 100% potting mix for 4-5 d in a heated glasshouse at 15 - 25˚C, by which time they had typically just begun to emerge. Entire shoot and root tissue was harvested and immediately immersed in liquid nitrogen and stored in a -70ºC freezer.

3.2.3 RNA extraction, cDNA synthesis and quantitative RT-PCR

RNA extraction, cDNA synthesis and qRT-PCR were carried out as in Chapter 2.2.

3.2.4 Cloning of PsGA3ox2

The initial portion of *PsGA3ox2* was isolated using primers based on a partial *Medicago* BAC sequence (gi89514974). The 3' end of the *PsGA3ox2* sequence was isolated by 3' RACE (Frohman et al., 1988). The partial sequence thereby obtained was used as a probe to screen approximately 350,000 clones of a pea seedling shoot library. A single clone containing the 5' end of the gene was isolated, sequenced, and then ligated into pGem-T Easy (Clonetech, USA) and expressed in *Escherichia coli*. The functional activity of the expression product was tested as before (Lester et al., 1997), using [14C]GA_{20} as a substrate.

3.2.5 Functional assay of PsGA3ox2

Functional assay was carried out as per the procedure used by Lester et al., (1997). Enzyme preparations were incubated at 30°C with GA substrates in 200 µL of 100 mM Tris, pH 7.8, containing 4 mM DTT, 4 mM ascorbate, 4 mM 2-oxoglutarate, 0.5 mM FeSO, and 1 mg/mL catalase. The ability of the LE and le cDNA products to metabolize GA, was determined by using a [^{14}C]GA_{20} substrate. Reactions were stopped after 1 hour by adding 25 µL of glacial acetic acid, and samples were then purified using Sep-Pak (Waters Associates, Milford, MA) C18 cartridges. Products from [^{14}C]GA_{20} were fractionated using HPLC: the HPLC program was 20 to 75% methanol (in 0.4% acetic acid) over 25 min (linear) at a flow rate of 2 mL/min. One-minute fractions were collected, and 50% aliquots were radiocounted. Fractions containing radioactivity at the retention time of GA_1 were dried, methylated, and derivatised (refer to Chapter 2.1.4).

3.3 Results

3.3.1 Cloning of a new GA 3-oxidase gene, PsGA3ox2

Roots of the GA biosynthesis mutants *le-1* and *le-2* (null) are phenotypically similar to WT and contain similar levels of endogenous GA_{20} and GA_1 to WT, in contrast to their dwarf shoot phenotype (Figure 3.1; Yaxley et al., 2001). It was therefore expected that another GA 3-oxidase must carry out substantial 3-oxidation in the roots (Yaxley et al., 2001). A previously unidentified pea GA 3-oxidase gene,

PsGA3ox2 (Figure 3.2), was isolated using PCR primers based on *Medicago* sequence, 3' RACE and cDNA clones.

3.3.2 PsGA3ox2 can convert GA_{20} to GA_1 in pea

To determine whether the newly-identified *PsGA3ox2* gene (Figure 3.2) encodes a GA 3-oxidase enzyme, a functional assay was carried out using the substrate [^{14}C]GA_{20}. When [^{14}C]GA_{20} substrate was added to the *PsGA3ox2* expression product, [^{14}C]GA_1 was recovered (fractions 13 and 14), demonstrating the role of *PsGA3ox2* as a GA 3-oxidase gene that converts GA_{20} to GA_1 in pea (Figure 3.3). GC-MS-SIM also verified that the expression product produced GA_1 from [^{14}C]GA_{20} (Figure 3.4).

3.3.3 PsGA3ox2 is expressed more highly in root than shoot tissue in LE and le-1 seedlings

As the roots of the *LE* and *le-1* and *le-2* mutants are shown to be phenotypically similar and contain comparable levels of GA_1 (Yaxley et al., 2001), it was hypothesised that another GA 3-oxidase gene must compensate for the reduction of *PsGA3ox1* activity. To test this hypothesis, the relative expression levels of the 3-oxidase genes *PsGA3ox1* (Lester et al., 1997; Martin et al., 1997) and *PsGA3ox2* were measured in the shoot and root tissue of 6-day-old *LE* and *le-1* pea seedlings using real-time PCR. In both the *LE* and *le-1* seedlings, *PsGA3ox1* was more highly expressed in the shoot compared with root tissue, with an approximate 2.5-fold difference in LE ($P < 0.001$, Figure 3.5) and 2-fold difference in *le-1* ($P < 0.02$, Figure 3.6). Conversely, *PsGA3ox2* showed approximately 2-fold higher expression in the root tissue than the shoot tissue in the LE ($P < 0.02$, Fig. 3.5) and *le-1* (although not quite statistically significant $P=0.058$; Figure 3.6).

Figure 3.1 Photograph showing the root and shoot phenotypes of left, WT (205+) and right, le-1 (205-). (Taken from Yaxley et al., 2001).

Figure 3.2 cDNA coding frame sequence of *PsGA3ox2* (in colour) with its corresponding amino acid sequence (in black).

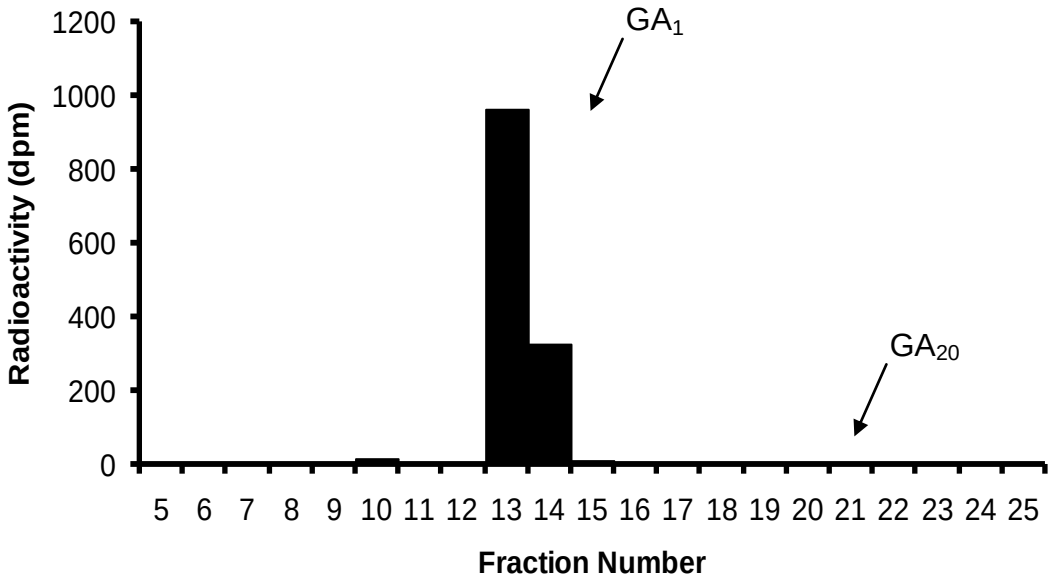


Figure 3.3 Functional assay of PsGA3ox2. PsGA3ox2 was transformed into E. coli and fed [^{14}C]GA_{20} in a functional assay.

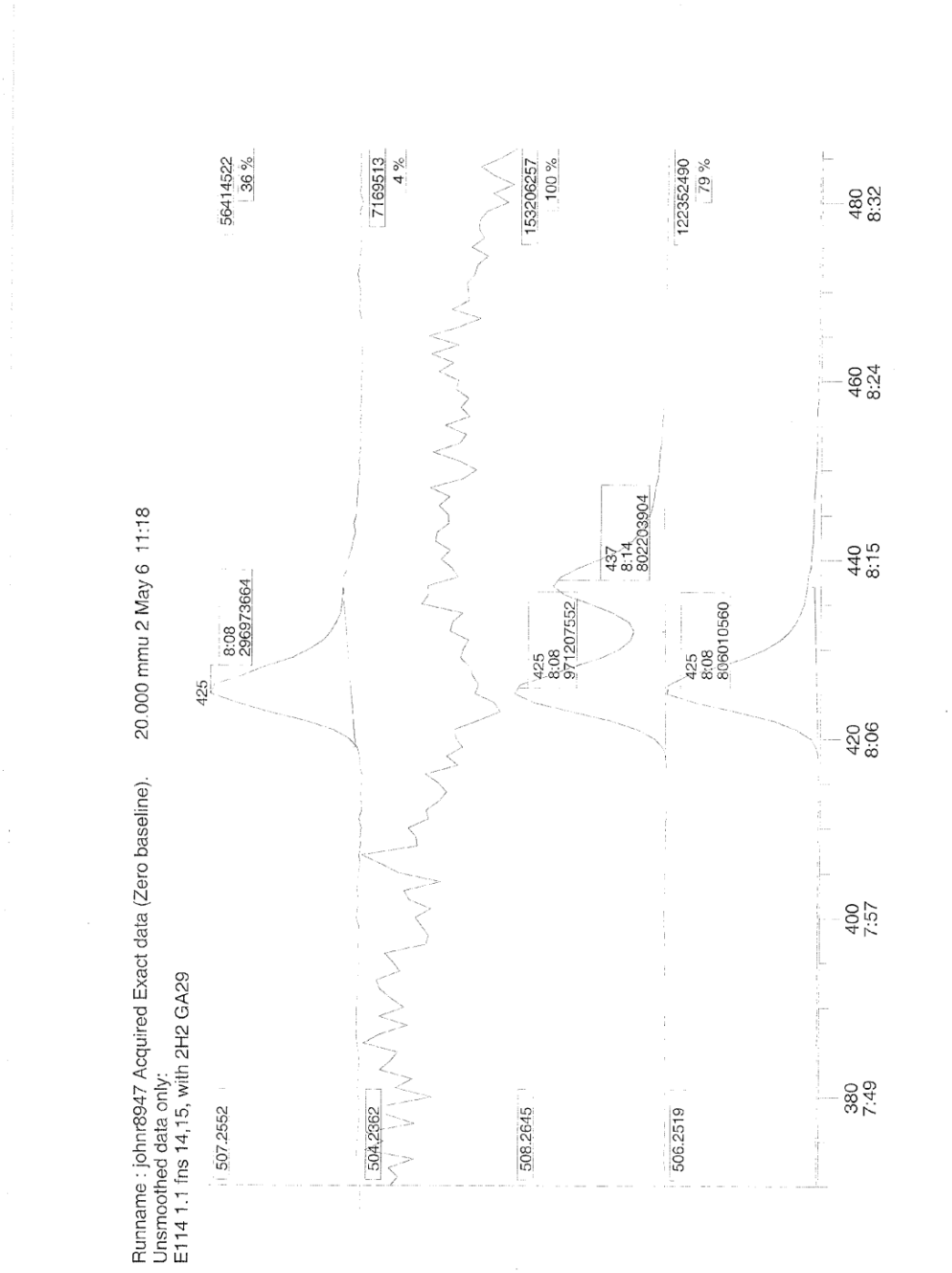


Figure 3.4 Verification of the conversion of GA_{20} to GA_1 by PsGA3ox2 by GC-MS-SIM. HPLC was used to separate GAs, and fractions 14 and 15 were used to identify GA_1. The 508 peak seen at 8:08 minutes corresponds to the $^2H_2$ GA_1 internal standard and the 506 peak at the same retention time corresponds to the endogenous GA_1 present after the expression product was fed [^{14}C]GA_{20}. The 508 peak at 8:14 minutes corresponds to the $^2H_2$ GA_{29} internal standard.

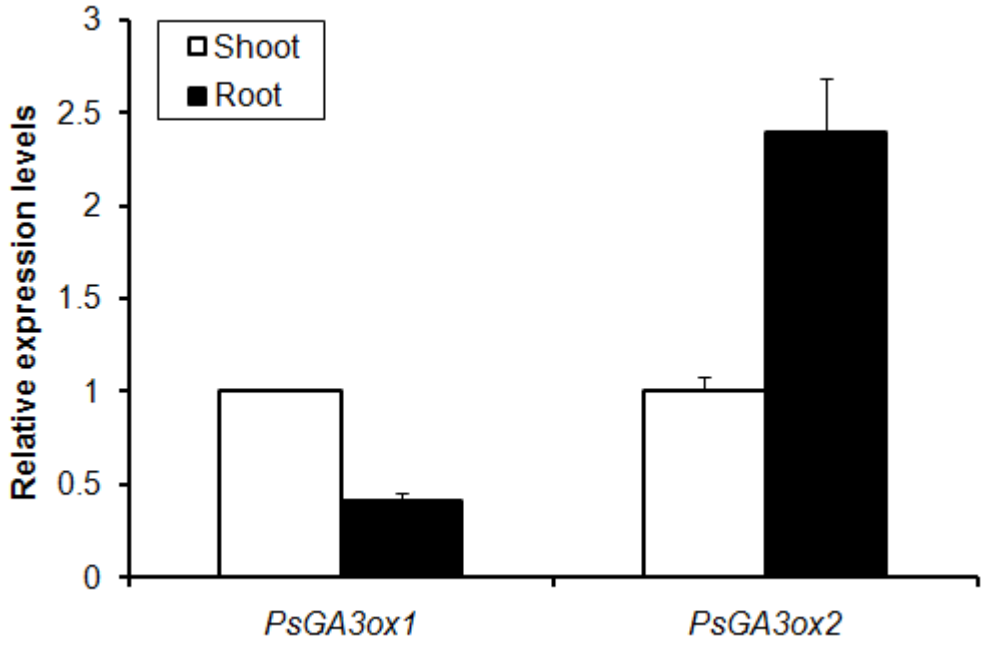


Figure. 3.5 Transcript levels of GA 3-oxidase genes in shoot and root tissue on *LE* seedlings. Transcript levels of *PsGA3ox1* (*LE*) and *PsGA3ox2* in root and shoot tissue of 5-d-old seedlings (line 205+) measured by quantitative real-time PCR. Shown are means with SE (n = 3). For each gene, the transcript level in shoot tissue was set to 1, and the level in the root tissue was calculated relative to the shoot tissue value. It should be noted that direct comparison between the expression levels of the two genes is not valid.

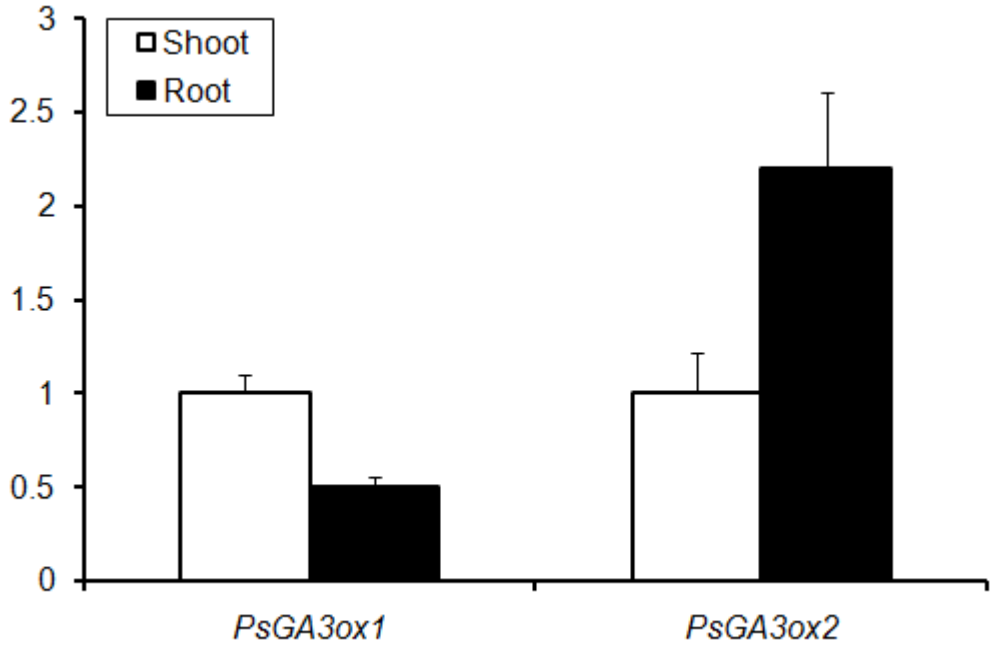


Figure. 3.6 Transcript levels of GA 3-oxidase genes in shoot and root tissue on *le-1* seedlings. Transcript levels of *PsGA3ox1* (*LE*) and *PsGA3ox2* in root and shoot tissue of 5-d-old seedlings (line 205-) measured by quantitative real-time PCR. Shown are means with SE (n = 3). For each gene, the transcript level in shoot tissue was set to 1, and the level in the root tissue was calculated relative to the shoot tissue value. It should be noted that direct comparison between the expression levels of the two genes is not valid.

3.4 Discussion

A key GA biosynthesis gene in pea is Mendel's *LE*, also referred to as the GA 3-oxidase gene *PsGA3ox1* (Lester et al., 1997; Martin et al., 1997). Mendel exploited the dwarf stature of mutant *le-1* shoots in his original genetics experiments, but it is interesting to note that the roots of *le-1* plants (and of other *le* mutants) are indistinguishable from the WT, and contain normal GA levels suggesting another GA 3-oxidase is present in the roots and able to compensate for the reduced PsGA3ox1 activity (Figure 3.1; Yaxley et al., 2001).

Indeed, a second candidate GA 3-oxidase gene (*PsGA3ox2*) from pea was recently discovered (Figure 3.2) and here we show that it is responsible for the conversion of GA_{20} to GA_1 (Figures 3.3 and 3.4). A protein blast (blastp) of the amino acid sequence for PsGA3ox2 was found to have 66% identical residue composition to that of PsGA3ox1 (LE; Lester et al., 1997), further supporting its role as a GA 3-oxidase. It was also shown that *PsGA3ox2* is relatively strongly expressed in roots compared to the shoots of both *LE* and *le-1* (Figures 3.5 and 3.6), suggesting that it may compensate for the reduction in PsGA3ox1 activity in *le-1* roots, and even for the complete loss of that activity in roots of the null mutant *le-2* (Martin et al., 1997; Lester et al., 1999a), but cannot compensate for the reduced PsGA3ox1 activity in the shoots (Figure 3.3), and consequently *le-1* and *le-2* shoots are dwarfed. However, when the relative expression of *PsGA3ox2* between the roots of the two genotypes was compared, *PsGA3ox2* expression was similar (Figures 3.5 and 3.6), suggesting that the reduction of PsGA3ox1 activity does not lead to enhanced PsGA3ox2 activity in the *le-1* mutant. Of course, transcript levels are not necessarily reflective of the enzyme levels or activity that the respective gene encodes.

Further unravelling of roles that *PsGA3ox2* may play in pea development, a recent study has found that *PsGA3ox2* plays little or no role in pea pericarp development (Ozga et al. 2009). Ozga et al. (2009) showed that there was little to no mRNA abundance of *PsGA3ox2* detected in young developing pericarp tissue, and that the application of IAA or GA_3 (which have been shown to regulate GA biosynthesis) also had no effect on *PsGA3ox2* expression.

Results presented here provide support for the theory (Yaxley et al., 2001) that another GA 3-oxidase could be primarily expressed in pea roots and compensating for the lack of LE activity, with the newly-discovered gene *PsGA3ox2* shown to be more highly-expressed in the roots than in shoot tissue of young *LE* and *le-1* pea seedlings. This result finally provides a possible explanation for the comparable root phenotypes of *LE* and the *le* mutant described previously by both Tanimoto (1990) and Yaxley et al. (2001).

CHAPTER 4

DELLA Proteins Regulate GA synthesis in Roots

Parts of this chapter have been published in:

Weston DE, Elliott RC, Lester DR, Rameau C, Reid JB, Murfet IC, Ross JJ (2008) The pea DELLA proteins LA and CRY are important regulators of gibberellin synthesis and root growth. *Plant Physiology* **147**, 199-205.

4.1 Introduction

It is well known that the levels of endogenous GAs are maintained through the process of feed-back and feed-forward regulation (Croker et al., 1990; Scott, 1990; Ross et al., 2003; Hedden and Phillips, 2000; Elliott et al., 2001). In pea, feed-back regulation was demonstrated in the GA-deficient mutant *na*, with the conversion of GA_{20} to GA_1 being promoted compared to the WT (Ross et al., 1999). Furthermore, the transcript levels of *PsGA3ox1* were shown to be up-regulated in *na* plants (Ross et al., 1999). Elliot et al. (2001) then demonstrated that in the *na* mutant, expression of the 2-oxidase genes *PsGA2ox1* and *PsGA2ox2* was reduced, compared with the associated wild type, thus demonstrating feed-forward regulation.

More recently, the GA signalling components involved in these homeostatic mechanisms have been identified. The GAs act by destabilising the growth inhibitory DELLA proteins, proteins which play an important role in gibberlelin signalling (Peng et al., 1997; Harberd et al., 1998; Silverstone et al., 2001; Alvey and Harberd, 2005). In other words, GA acts as an "inhibitor of an inhibitor" (Harberd et al., 1998). Interestingly, there is also evidence that DELLA proteins promote the biosynthesis of active GAs. For example, in the Arabidopsis DELLA mutant *rga*, the expression of the biosynthesis gene *GA4* is elevated, indicating that high DELLA protein levels are associated with an up-regulation of GA synthesis genes (Silverstone et al., 2001). More recently Zentella et al. (2007) provided evidence that the Arabidopsis GA synthesis genes *AtGA3ox1* (*GA4*) and *AtGA20ox2* are direct DELLA targets. Evidence also suggests that in Arabidopsis shoots, DELLA proteins

feed-back regulate the GA biosynthesis genes *AtGA3ox1* and *AtGA20ox1* (Dill and Sun, 2001; King et al., 2001; Silverstone et al., 2001).

Until recently, DELLA-encoding genes from pea had not been reported, although observations on the "slender" mutant of pea triggered the early suggestion that GA acts an inhibitor of an inhibitor (Brian, 1957). The elongated slender mutant, conferred by the gene combination *la cry-s*, has long been considered to show constitutive GA signalling (Potts et al., 1985), but the exact nature and function of the *LA* and *CRY* genes remained unknown. Weston et al. (2008) recently showed that *LA* and *CRY* encode DELLA proteins. Importantly, it was shown that the mutant alleles *la* and *cry-s* both appear to encode non-functional proteins, and the stem elongation of *la cry-s* plants is similar to that of WT plants given a saturating dose of bioactive GA (Brian, 1957; Potts et al., 1985), suggesting that, at least with respect to shoot elongation, *LA* and *CRY* are the only DELLA-encoding genes in pea.

Weston et al. (2008) also demonstrated the importance of the pea DELLA proteins in root and shoot development. They showed that *LA* is the main functioning DELLA protein in the roots of pea. When *la* and/or *cry-s* were recombined with the GA-deficient dwarf *na-1* mutant, *la* was shown to largely rescue the root phenotype, whereas *cry-s* alone could not (Figure 4.1; Weston et al. 2008). This is in agreement to the previous observations by de Hann (1927) and Reid et al. (1983) that *LA* is a more effective inhibitor of shoot elongation than *CRY*.

With the recent identification of the two pea DELLA genes (*LA* and *CRY*) it was possible to measure the expression of key GA synthesis and deactivation genes in the roots of the pea DELLA WT (*LA cry-s* and *la CRY*) and slender mutant (*la cry-s*). Here we show that DELLAs are important regulators of GA synthesis and catabolism in the roots of pea. This is the first time that DELLA regulation of the GA synthesis pathway has been studied in roots.

4.2 Materials and Methods

4.2.1 Plant material

The plant material used in these studies included the tall (WT) Hobart line HL205+ (*LA CRY*; see Ross and Reid, 1989). Progenies segregating for *LA/la* and/or *CRY/cry-s* were derived from the following crosses: HL133 (*la cry-s NA*) X (NGB1766 (*LA CRY na-1*; Potts et al., 1985); HL6 (*LA cry-s wa*) X HL73 (*la CRY WA*); and HL^ (*LA cry-s wa*) X line K524 (*LA CRY WA*). Genotyples *LA cry-s* and *la cry-s* were derived from the same F4 plant from cross HL133 X NGB1766. Other genotypes were selected from a cross between HL107 (*LA CRY NA*) and HL188 (*la cry-s na-1*; HL188 was selected from cross HL133 X NGB1766) (Weston et al., 2008). The foundation lines HL2 (Lamm line 2), HL6 (Lamm line 6), HL7 (Lamm line 7), and HL8 (Lamme line 8a) were kindly provided in 1957 by Dr. Robert Lamm.

4.2.2 Plant growth and chemical treatments

Plants were grown in 100% potting mix for 4-5 d in a heated glasshouse at 15 - 25˚C, by which time they had typically just begun to emerge. Gene expression material was immediately immersed in liquid nitrogen and stored in a -70ºC freezer.

4.2.3 RNA extraction, cDNA synthesis and quantitative RT-PCR

RNA extraction, cDNA synthesis and qRT-PCR were carried out as in Chapter 2.2.

4.3 Results

4.3.1 Pea DELLA proteins promote the expression of GA synthesis genes and inhibit that of GA deactivation genes

To investigate whether the pea DELLA proteins are involved in the feed-back regulation of key GA biosynthesis genes in pea roots, we first performed qRT-PCR on *LA* and the mutant *la* on a *cry-s* background. In the *la* mutant, there was a 4-fold and 6-fold down-regulation of *PsGA3ox1* ($P < 0.001$) and *PsGA3ox2* ($P < 0.05$) respectively (Figure 4.2A). The greatest effect on gene expression was seen for *PsGA20ox1*, which was down-regulated 14-fold in the mutant ($P < 0.01$, Figure

4.2A). In contrast, a more than 2-fold up-regulation of the 2-oxidase genes *PsGA2ox1* ($P < 0.02$, Figure 4.2B) and *PsGA2ox2* ($P < 0.01$, Figure 4.2B) was observed in the mutant roots.

Similar results were obtained for *CRY* and the mutant *cry-s* on a *la* background. In the mutant *la cry-s* a 19-fold down-regulation of *PsGA3ox1* ($P < 0.001$) and a 6-fold down-regulation of *PsGA3ox2* ($P < 0.05$) was observed respectively (Figure 4.3A). The greatest effect on gene expression in this comparison was again on *PsGA20ox1*, which was down-regulated 24-fold ($P < 0.05$, Figure 4.3A). The 2-oxidase genes *PsGA2ox1* and *PsGA2ox2* were up-regulated by 3-fold ($P < 0.001$) and 2-fold ($P < 0.05$), respectively (Figure 4.3B), very similar to the results from the first comparison.

Figure 4.1 Shoot and root phenotypes of pea genotypes. Left to right: *NA LA* and/or *CRY*; *na-1 LA cry-s*; *na-1 la CRY/cry-s*; *na-1 la cry-s*; *NA la cry-s*. The *na-1* mutation on a *LA* background gives rise to the characteristic nana phenotype (second from left), with a very short shoot and shortened roots. The homozygous presence of cry-s does not rescue the root phenotype, whereas la largely does (Taken from Weston et al., 2008).

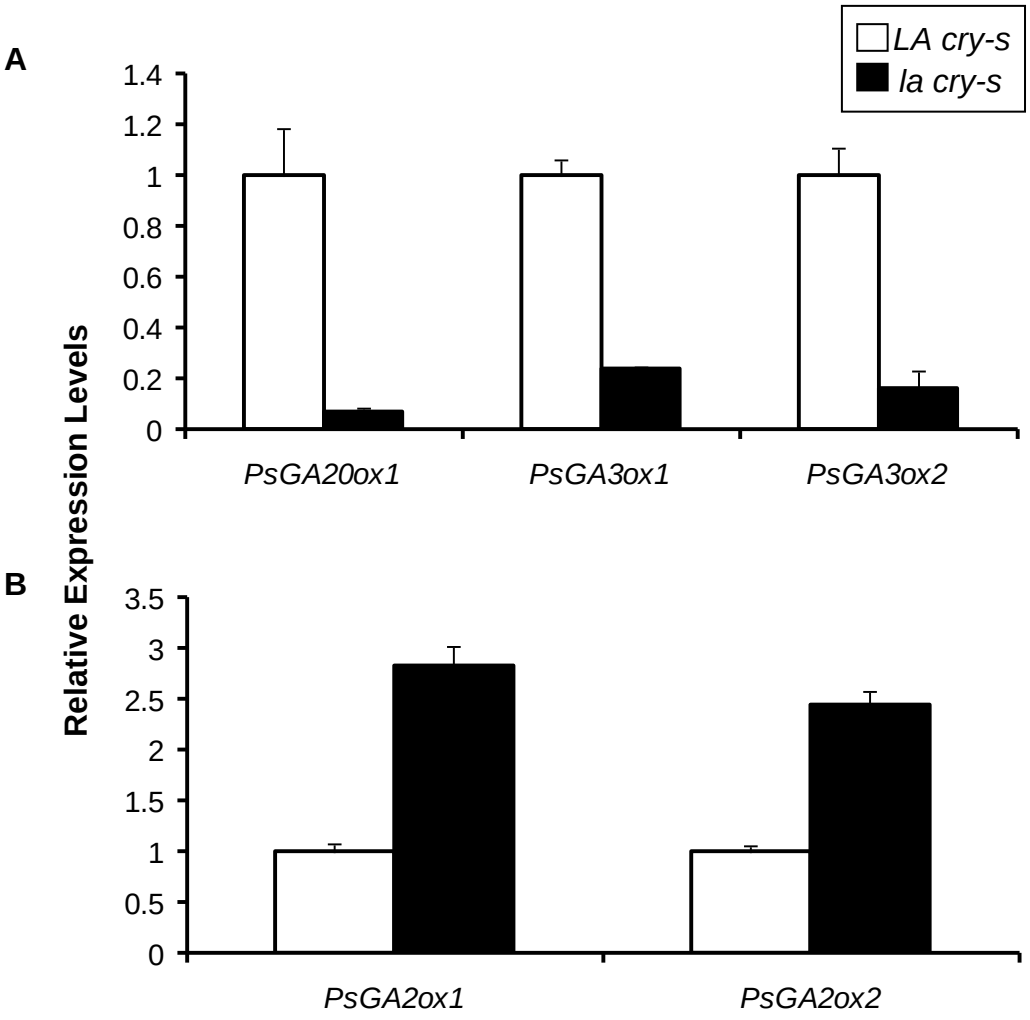


Figure 4.2 Effects of la on expression of GA genes. Transcript levels of GA biosynthesis (A) and deactivation (B) genes in *LA cry-s* and *la cry-s* pea roots, as measured by quantitative real-time PCR. Shown are means with SE (n = 3). For each gene, the transcript level in *LA* plants was set to 1, and the level in the la mutant was calculated relative to the *LA* value.

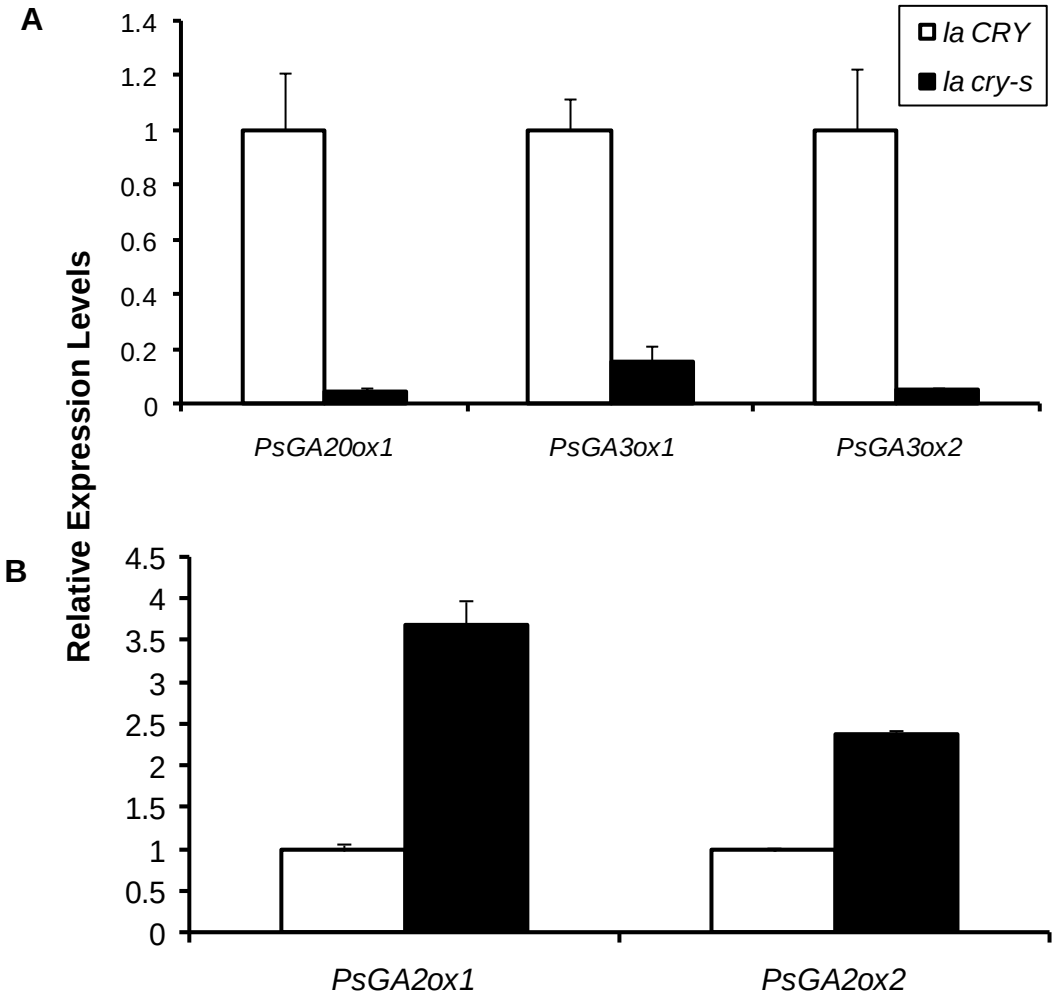


Figure 4.3 Effects of *cry* on expression of GA genes. Transcript levels of GA biosynthesis (A) and deactivation (B) genes in *la CRY* and *la cry-s* pea roots, as measured by quantitative real-time PCR. Shown are means with SE (n = 3). For each gene, the transcript level in *CRY* plants was set to 1, and the level in the *cry-s* mutant was calculated relative to the *CRY* value.

4.4 Discussion

It has been known for some time that GA homeostasis is achieved by the feed-back and feed-forward regulation of endogenous GAs. But the involvement of the GA signalling DELLA proteins had not been demonstrated until more recent times. To date, GA synthesis has been shown to be reduced in the shoots of the DELLA slender mutants *rga* and *gai* in Arabidopsis (Silverstone et al., 2001; Zentella et al., 2007), *sln* in barley (Chandler et al., 2002), *slr1* in rice (Itoh et al., 2002), and *la cry-s* in pea (Martin et al., 1996). It is shown here for the first time that DELLA proteins also regulate GA synthesis and catabolism genes in roots.

With the recent identification of *LA* and *CRY* as DELLA-encoding genes (Weston et al., 2008), it was possible to study the importance of DELLAs in the regulation of GA synthesis in roots. When both DELLA genes were null (*la cry-s*), strong reductions in the expression of *PsGA20ox1*, *PsGA3ox1* and *PsGA3ox2*, and strong promotions of *PsGA2ox1* and *PsGA2ox2* expression were seen, compared with *LA cry-s* (Figure 4.2) and *la CRY* plants (Figure 4.3). Therefore, DELLA proteins promote the expression of GA synthesis genes and inhibit that of GA deactivation genes, indicating that in roots, DELLAs are an integral part of the feed-back and feed-forward phenomena, whereby bioactive GA reduces GA synthesis and speeds up GA deactivation (Dill et al., 2001).

However, these proteins have also recently been shown to play an equally important role in the inhibition of root elongation (Weston et al., 2008). This poses an interesting paradox; how can DELLA proteins promote the synthesis of the bioactive GA_1, but still result in inhibited growth? It is possible that the DELLA proteins are not only an important homeostatic mechanism for GAs to regulate their own levels, but also an important mechanism for root elongation to regulate its rate of elongation, although further research is required to validate this hypothesis. However, there is evidence for developmental feedback regulation in relation to shoot branching. The shoot branching gene *RMS2* has been shown to feedback regulate strigolactone (a newly discovered inhibitory branching hormone; Gomez-Roldan et al., 2008; Umehara et al., 2008) biosynthesis (Beveridge, 2006).

The use of the *la* and *cry-s* mutations has shown for the first time that DELLA proteins can be viewed as positive regulators of the expression of GA biosynthesis genes in roots. However, these proteins have also been shown to inhibit root growth, a developmental response contradictory with increased GA levels. It is therefore postulated that the DELLA proteins are important for maintaining a homeostatic root elongation response. This hypothesis will require further study.

CHAPTER 5

Auxin is Required for GA Biosynthesis in the Roots

Parts of this chapter have been published in:
Weston DE, Reid JB, Ross JJ (2009) Auxin regulation of gibberellin biosynthesis in the roots of pea (*Pisum sativum). Functional Plant Biology* **36**, 362-369.

5.1 Introduction

It is well known that primary root growth is strongly influenced by the plant hormones auxin and gibberellin (GA; Davies 2004). For instance, applied auxin typically inhibits root elongation at high concentrations but can be promotory at low concentrations (Evans *et al.*, 1994; Fu and Harberd, 2003; Silva and Davies, 2007).

The application of bioactive GA to roots treated with the growth inhibitor ancymidol completely restored growth to that of the untreated plants (Tanimoto, 1990). Yaxley et al. (2001) further established the importance of GAs for root growth in peas by using GA-deficient mutant plants. In the *na-1* mutant, for example, root GA_1 levels, and root elongation, were significantly reduced compared with WT plants, and when the GA_1 content was restored to WT levels, so too was root elongation. The *na-1* root phenotype can also be rescued by the "slender" gene combination *la cry-s* (Weston et al., 2008). It was shown recently that *LA* and *CRY* encode DELLA proteins, which are inhibitors of stem and root growth. A second function of the DELLAs in pea roots, as in other systems (Zentella et al., 2007), is to promote the expression of GA synthesis genes and to inhibit that of GA deactivation (2-oxidation) genes (Weston et al., 2008). Bioactive GAs destabilise DELLAs, thereby promoting growth and inhibiting GA synthesis; the latter effect is part of a homeostasis mechanism by which GA regulates its own levels (Silverstone et al., 2001).

The aim of this study was to investigate how auxin and gibberellin interact (if at all) in roots. In pea shoots auxin promotes GA biosynthesis, by up-regulating the GA 3-oxidation gene *PsGA3ox1* (Ross et al., 2000). Auxin also inhibits GA deactivation in pea shoots (O'Neill and Ross, 2002), by down-regulating the GA 2-oxidation gene

PsGA2ox1. The overall effect of auxin is to maintain high levels of the bioactive GA, GA_1, in pea internodes, and we have presented evidence that this accounts for at least some growth-promotory effects of auxin (Ross et al., 2003). Auxin also promotes GA biosynthesis in tobacco (Wolbang and Ross, 2001) and in barley (Wolbang et al., 2004). Interestingly, in tobacco, auxin appears to promote GA 20-oxidation to a greater extent than 3-oxidation (Wolbang and Ross 2001). In pea fruits, the auxin 4Cl-IAA up-regulates both GA 20-oxidation and GA 3-oxidation (van Huizen et al., 1997; Ozga et al., 2003). However, despite the significance of Arabidopsis as a model species, evidence that auxin may promote GA biosynthesis in this species has been published only recently. Frigerio et al. (2006) found that auxin up-regulates the expression of two key GA biosynthesis genes in (whole) Arabidopsis seedlings, although the levels of GAs were not monitored. The effects (if any) of auxin on GA synthesis and deactivation in roots have not been reported previously.

It should not be assumed that endogenous auxin promotes the accumulation of bioactive GAs in roots as it does in stems. Indeed, a current model for auxin-GA interactions in roots (Fu and Harberd, 2003) proposes that auxin enhances the capacity of GA to destabilise DELLAs; hence, auxin would according to this model, down-regulate GA synthesis. This down-regulation would be the opposite of the up-regulation found by Ross et al. (2000) in stems and consequently, the Ross et al. (2000) and Fu and Harberd (2003) models predict opposite effects of auxin on the expression of GA synthesis genes. In fact, in examining the relative roles of the two models, it is more informative to monitor transcript levels of GA synthesis (and deactivation) genes than to monitor DELLA protein levels, since an accumulation of DELLAs after a disruption of auxin signalling is predicted by both models.

For this reason we measured the transcript levels of a range of GA synthesis and deactivation genes after treatment of pea roots with auxin action and transport inhibitors. The genes measured included a key GA 20-oxidase expressed in vegetative tissues (*PsGA20ox1*), two GA 3-oxidases, *PsGA3ox1* (Mendel's *LE* gene; Lester et al., 1997) and *PsGA3ox2* (which is expressed at a greater level in young roots than in young shoots; Weston et al., 2008) and the two well established GA 2-oxidases, *PsGA2ox1* and *PsGA2ox2* (Lester et al., 1999b). We also measured

endogenous GA levels in roots treated with an auxin action inhibitor to determine the overall effect of changes in gene expression.

The auxin action inhibitors *p*-chlorophenoxyisobutyric acid (PCIB; Oono et al., 2003; Biswas et al., 2007) and Yokonolide B (YkB; Hayashi et al., 2003) and the auxin transport inhibitor 1-naphthylphthalamic acid (NPA) were used for these studies. The auxin action inhibitors are reported to act by stabilising key Aux/IAA signalling proteins, which are destabilised by auxin (Hayashi et al, 2003; Oono et al., 2003; Biswas et al., 2007.). Both compounds inhibit root elongation (Oono et al., 2003; Hayashi et al., 2003), consistent with evidence that at normal levels, endogenous auxin promotes root elongation (Fu and Harberd, 2003). Both compounds inhibit root elongation to a smaller extent in auxin signalling mutants than in WT, providing further evidence that they act by affecting auxin signalling (Oono et al., 2003; Hayashi et al. 2003). NPA has been shown to block the basipetal movement of indole-3-acetic acid (IAA) from its site of synthesis in the shoot apex to sites of action further down the stem (Ross et al., 1998).

Although the promotion of GA synthesis by IAA has been verified in a number of species and tissues, evidence (although fewer) has been presented that suggests that GA regulates IAA synthesis. Paleg (1965) reviews some of the earlier studies that showed the application of gibberellic acid (GA_3) enhanced IAA levels using simple assay techniques. Following from these early studies, further reports of this interaction occurring have been provided by David Law who studied this phenomenon extensively. In 1984, Law and Hamilton showed that the application of GA_3 to dwarf pea seedlings (Little Marvel) resulted in increased stem length and elevated free IAA levels in leaves and elongating regions of the stem. This study was followed up in 1987, whereby GA_3 was applied to peas that had also been treated with radiolabelled tryptophan, a precursor of IAA (Law, 1987). Law (1987) showed that the labelled tryptophan converted to free auxin and its conjugate 3-fold greater than in plants not treated with GA. In 1989, Law and Hamilton then applied the gibberellin biosynthesis inhibitor uniconazol to pea and showed that both internode elongation and IAA levels were reduced compared to untreated plants. More recently, Barratt and Davies (1997) showed that pea early-expansion stem segments

treated with GA displayed enhanced elongation as well as increased IAA levels. Due to the extensive study by Law and others (1984, 1987, 1989) and the more recent study by Barratt and Davies (1997), the reverse interaction (that GA promotes IAA synthesis) is also explored by quantifying IAA levels in the GA-deficient mutant *nana*.

The results indicate that the promotion of GA biosynthesis by endogenous auxin is a strong interaction in roots, but GA is not a regulator of IAA synthesis.

5.2 Materials and methods

5.2.1 Plant material

The plant material used in these studies included tall (WT) Hobart line HL205+ (*LA CRY*; see Ross and Reid, 1989), the GA-deficient *nana* line, and genotypes *LA cry-s* and *la cry-s* derived from the same F_4 plant from cross HL133 x NGB1766 (see Weston *et al.*, 2008).

5.2.2 Plant growth and chemical treatments

For agar-based experiments, plants were grown in 100% potting mix for 4-5 d in a heated glasshouse at 15 - 25˚C, by which time they had typically just begun to emerge. Plants to be treated with chemicals were then transferred to glass 16 mm x 150 mm test tubes containing 1.3% sterilised agar with MS-salts, containing *p*-chlorophenoxyisobutyric acid (PCIB; Sigma Aldrich, USA), napthylphthalamic acid (NPA; Alltech Association Australia), or indole-3-acetic acid (IAA; Sigma Aldrich, USA). Chemicals were added to molten agar (<60˚C). Plants were placed in a 20˚C dark room for 24 h. After 24 h of treatment (unless otherwise specified), material for gene expression and endogenous hormone quantification was harvested. Gene expression material was immediately immersed in liquid nitrogen and stored in a -70ºC freezer. Material for GA analysis was immediately immersed in cold (-20ºC) 80% methanol, containing butylated hydroxytoluene (Sigma, 250 mgl^{-1}), and stored in a freezer (-20ºC).

For IAA quantification experiments, *nana* plants were grown in 100% pasteurised potting mix for 9 d in a growth cabinet set to a 22°C day/17°C night 18 hr photoperiod. The apical meristem of control plants were then treated with 25µL 0.1% Tween-20 solution and GA-treated plants with 6.2µg GA_3 in 0.1% Tween-20. After 24 h, the 3cm root tips of the seedling were harvested and immediately immersed in cold (-20ºC) 80% methanol, containing butylated hydroxytoluene (Sigma, 250mgl^{-1}), and stored in a freezer (-20ºC).

5.2.3 RNA extraction, cDNA synthesis and quantitative RT-PCR

RNA extraction, cDNA synthesis and qRT-PCR was carried out as in Chapter 2.2.

5.2.4 Quantification of endogenous IAA and GAs

Endogenous IAA and GA quantification analysis was undertaken as described in Chapter 2.1.

5.2.5 Statistical analysis

Differences between means were analysed using Students' t-test. The significance of interactions were tested using log transformed data, and one-tailed ANOVA (JMP 4.0, SAS Institute Inc., Cary, North Carolina, 2000).

5.3 Results

5.3.1 Transferring soil-germinated seedlings to 1.3% agar medium is optimal method for applying hormones and inhibitors to the root

Previously (in Honours), hormones and inhibitors were applied to plants that were immersed in an aerated aqueous solution containing these chemicals. However, this does not represent 'normal' growing conditions and plants tended to display a water-logging phenotype, so another improved method was required which still allowed the easy visualisation of root growth. It was decided that an agar medium would be a preferential medium as it is widely used in these types of studies, particularly with the small Arabidopsis seedlings.

At first, I tried germinating 205+ seeds in test tubes containing a 1% agar solution with MS-media. At first, this method appeared to be promising, with seeds displaying signs of imbibition after 24 hrs and an emerging radicle after 48 hrs. However, growth of the emerging seedlings appeared to cease at this stage and the agar appeared to have lost some of its solid properties (data not shown).

Next, I tried a higher agar concentration of 1.3% agar with MS-media and tried once again to germinate the seeds on the agar. As occurred previously, the seeds imbibed well and a radicle emerged, but in most cases, no further growth was seen (data not shown). It was decided that it might be worthwhile germinating the seeds in soil before transplanting to test tubes containing the 1.3% agar solution. After sowing seeds in 100% soil, I left them to germinate for 3 days before transplanting them to the agar solution which hadn't quite fully set, allowing the root to penetrate the agar easily. Seedlings showed healthy and even growth, with no contamination seen in the agar for a short period of time (generally up to 5 days; data not shown). An example of healthy seedlings growing using this method can be seen in Figure 5.1.

A limitation of this experimental system is that the root elongation does diminish after approximately 5-d. To take this into account, experiments using this system were generally undertaken within the first 24 to 48 h.

5.3.2 Inhibiting auxin action and transport reduced root elongation in young pea seedlings

To establish the effect of the auxin action inhibitor PCIB on root elongation over 48 hrs, 50 μM PCIB was applied to the roots of young pea seedlings using the above mentioned agar system. After 24 hrs, the roots of the PCIB-treated plants were approximately 37% shorter compared to un-treated plants ($P < 0.001$; Figure 5.2). After 48 hrs, the difference in lengths between the treated and un-treated plants had grown to 78% ($P < 0.001$; Figure 5.2).

NPA is a well-documented compound and has been shown to block the basipetal movement of indole-3-acetic acid (IAA) from its site of synthesis in the shoot apex (Ross et al., 1998). To ensure these effects will be seen when NPA is applied to

transferred seedlings in agar a dose response curve was undertaken for this inhibitor. NPA was applied to young seedlings at concentrations of 0, 0.1 μM, 1.0 μM, 10 μM, and 50 μM using the agar method. No significant difference in root elongation was seen for plants treated with 0.1 μM and 1.0 μM after 24 hrs and 48 hrs (Figure 5.3). The application of 10 μM and 50 μM however, resulted in a significant reduction of 50% for both treatments after both 24 and 48 hrs ($P < 0.001$, Figure 5.3). After 144 h, a clear phenotypic difference in root lengths can be seen over the varying doses of NPA (Figure 5.3).

5.3.3 Auxin action inhibitors inhibit GA biosynthesis in roots of pea

Transcript levels of key biosynthesis and deactivation genes were measured in roots that were treated with 50 μM PCIB, 0.5 μM YkB, or left untreated. From the dose-response study shown in Figure 5.2, we determined that 50 μM PCIB strongly inhibited primary root elongation, consistent with the findings of Oono et al. (2003). For an appropriate concentration of YkB, we relied on a previous report due to the limited supply of this compound (Hayashi et al., 2003). PCIB and YkB both reduced *PsGA20ox1* mRNA levels in roots, compared with controls, by approximately 10-fold ($P < 0.02$; Figure 5.4 A) and 2-fold ($P < 0.001$; Figure 5.4 B), respectively. Both inhibitors also reduced *PsGA3ox2* mRNA levels, ($P < 0.001$; Figures 5.4A and 5.4B). YkB reduced the levels of *PsGA3ox1* mRNA ($P < 0.01$; Figure 5.4B), while the effects of PCIB on this gene were variable. In the experiment shown in Figure 5.4A, PCIB actually increased *PsGA3ox1* mRNA levels, while the opposite was observed in another experiment (data not shown). However, metabolism studies undertaken in my Honours year with [^{14}C]GA_{20} consistently showed reduced GA 3-oxidation in both PCIB- and YkB-treated roots, as indicated by the levels of GA_8 and particularly of GA_8-catabolite (both products of 3-oxidation; Figure 5.5).

The GA deactivation genes were up-regulated by both PCIB and YkB. In PCIB-treated roots, the level of *PsGA2ox1* mRNA was approximately 8-fold greater ($P < 0.001$, Figure 5.4A), and that of *PsGA2ox2* approximately 3-fold greater ($P < 0.001$, Figure 5.4A) than in the control. Similarly, in the YkB-treated roots, *PsGA2ox1* and *PsGA2ox2* mRNA levels was approximately 5-fold greater

(P < 0.001, Figure 5.4B) and 14-fold greater (P < 0.01, Figure 5.4B), respectively, than in the control.

5.3.4 Inhibiting auxin transport down-regulates GA 20-oxidation and up-regulates GA 2-oxidation in mature portions of roots

In experiments with NPA, we divided the root into mature and young (tip) regions, and results are presented for the mature section. We reasoned that since root tips are thought to synthesise IAA (Ljung et al., 2005) the mature section of NPA-treated roots would become auxin-depleted because of reduced transport into that part from the root tip and possibly from the shoot. To check that prediction, we measured IAA levels in the mature region of NPA-treated roots. Treatment with this compound resulted in a 2-fold reduction in IAA levels (P < 0.01; Figure 5.6).

In agreement with these previous results obtained for the auxin action inhibitors PCIB and YkB, the application of 25 μM NPA resulted in a reduction of *PsGA20ox1* mRNA levels, by nearly 2-fold (P < 0.05, Figure 5.7A), and an increase of *PsGA2ox2* mRNA levels, by 2-fold (P < 0.01, Figure 5.7B), in the roots. However, NPA treatment did not affect the expression of *PsGA3ox1*, *PsGA3ox2*, nor *PsGA2ox1* (Figure 5.7).

5.3.5 Evidence that auxin acts both via GAs and independently of GAs

To determine whether IAA is required for the GA growth response as suggested by both Ross et al. (2000) and Fu and Harberd (2003), GA_3 was applied to PCIB-treated roots to see if it was capable of restoring the inhibited root growth seen in PCIB-treated plants. After 48 hrs, PCIB-treated plants showed a significant reduction in elongation, similar to the results seen in Figure 5.2. However, GA_3-treated plants did not display significant increased elongation as expected (Figure 5.8), suggesting that the plants already had optimal endogenous GA present. To see if this problem could be overcome, GA_3 was applied to the GA-deficient *nana* mutants. When 10 μM GA3 was applied, elongation is approximately 25% greater in the treated compared to the control after 24 hrs (P < 0.005, Figure 5.9) but after 48 hrs no statistical difference was seen (Figure 5.9). This suggested that the physiological response caused by the

GA_3 treatment is unable to be sustained for 48 hrs, so this is not a suitable method for showing whether IAA is required for a GA growth response.

After further consideration, the notion that if auxin promotes elongation via the GAs, factors that impede auxin signalling should be less inhibitory to elongation in the case of DELLA mutants, in which GA-dependent elongation is constitutive, compared with the WT. To test this possibility, we applied PCIB to *LA* and DELLA-deficient *la* roots (on a *cry-s* background) and monitored elongation. PCIB significantly inhibited growth, by 56% in *LA cry-s* ($P < 0.05$, Figure 5.10) and 29% in *la cry-s* ($P < 0.05$, Figure 5.10). Inhibition of the WT root growth by PCIB was greater than in the *la* mutant ($P < 0.05$, one-tailed ANOVA, Figure 5.10). The difference between the WT and mutant should represent the GA-dependent auxin action, but in itself does not distinguish between an effect of auxin on GA biosynthesis and an effect on the response pathway.

5.3.6 Supra-optimal levels of IAA reduce GA_1 levels and promote 2-oxidase expression

There is substantial evidence that IAA inhibits the growth of roots at high concentrations (Evans *et al.*, 1994; Silva and Davies, 2007). To determine whether the reduction in elongation at supra-optimal auxin levels may involve a reduction in GA_1 content, endogenous GA levels were measured. The application of 10 µM IAA to the roots resulted in an approximately 38% reduction in GA_1 levels ($P < 0.01$, Table 5.1), and a 30% reduction in GA_8 levels ($P < 0.05$, Table 5.1). Even at 1 µM IAA, the inhibition of root growth in just 24 h was very strong, resulting in a 75% reduction in our conditions ($P < 0.001$, Figure 5.11).

To determine whether supra-optimal auxin caused a more substantial reduction in GA_1 content at a time earlier than 24 h, we measured GA_1 levels in control and auxin-treated (10 µM) roots at 0, 3, 6 and 12 h. The largest reduction in GA_1 content at these time points was 21% (Figure 5.12). It should also be noted that the GA_1 levels in the control samples showed the same decreasing-trend over time as the IAA-treated plants, although not to the same degree. These results indicate that in general, supra-optimal IAA levels cause only moderate reductions in GA_1 content.

When GA gene transcript levels were analysed in roots treated with 10 µM IAA, it was found that levels of GA 2-oxidase mRNA were increased, with *PsGA2ox1* 1.5-fold greater ($P < 0.01$, Figure 5.13) and *PsGA2ox2* 1.8-fold greater ($P < 0.001$, Figure 5.13) than in the control roots. This result could explain the IAA-induced reductions in GA_1 and GA_8 content.

5.3.7 GA_3 application does not alter IAA levels

Previous studies have shown that GA can also regulate IAA synthesis in pea (Law and Hamilton, 1984; Law, 1987; Law and Hamilton, 1989; Barratt and Davies, 1997). To validate these claims, endogenous IAA was quantified in the GA-deficient mutant *nana*. If GA can regulate IAA synthesis we would expect the IAA levels in the untreated GA-deficient mutant to be substantially less than the GA_3-treated mutant. However, no significant difference in endogenous IAA levels between the control and GA_3-treated *nana* seedlings was seen, contrary to these previous studies mentioned (Figure 5.14).

Table 5.1 Effects of supra-optimal IAA levels on endogenous GA levels. Shown are mean levels of endogenous GA_1 and GA_8 ($ng.gFW^{-1}$) present in the 30 mm root tip of line 205+ roots immersed in aerated dH_2O containing 0 or 10 µM IAA for 24 h (n = 3). HPLC was used to separate GAs.

	GA_1	**GA_8**
Control	0.21 ± 0.01	5.7 ± 0.5
IAA	0.13 ± 0.01	4.0 ± 0.1

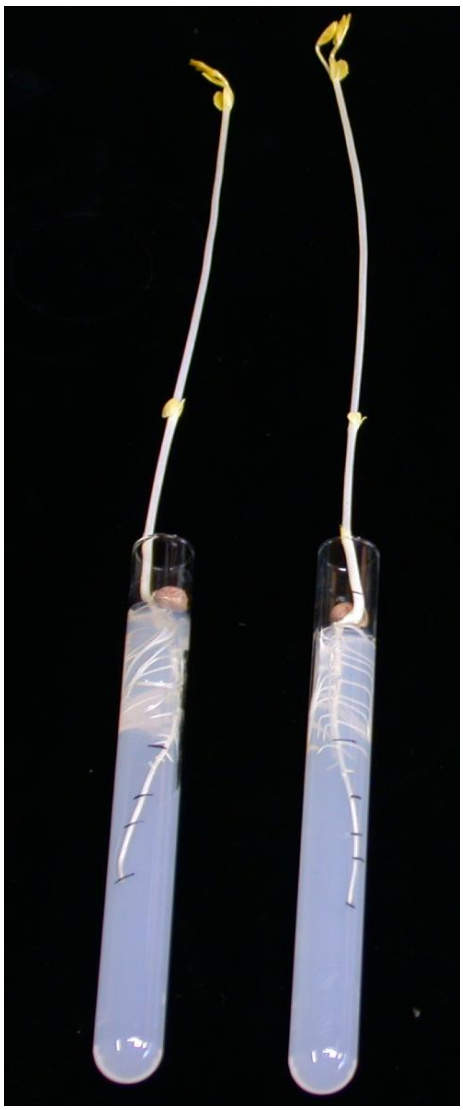


Figure 5.1 Photo showing healthy growth of dark-grown seedlings in test tubes containing 1.3% agar + MS media following germination in 100% soil for 3 d.

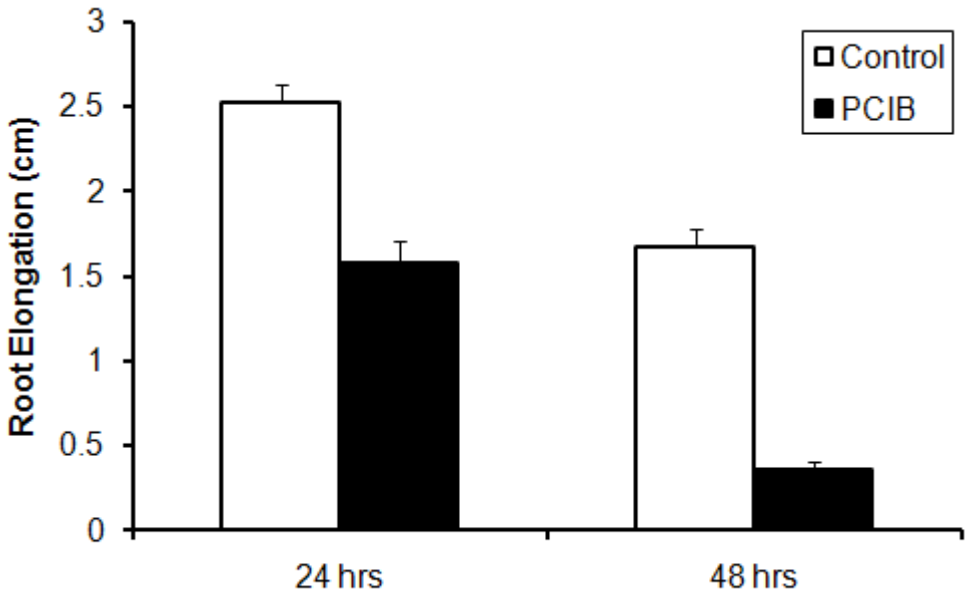
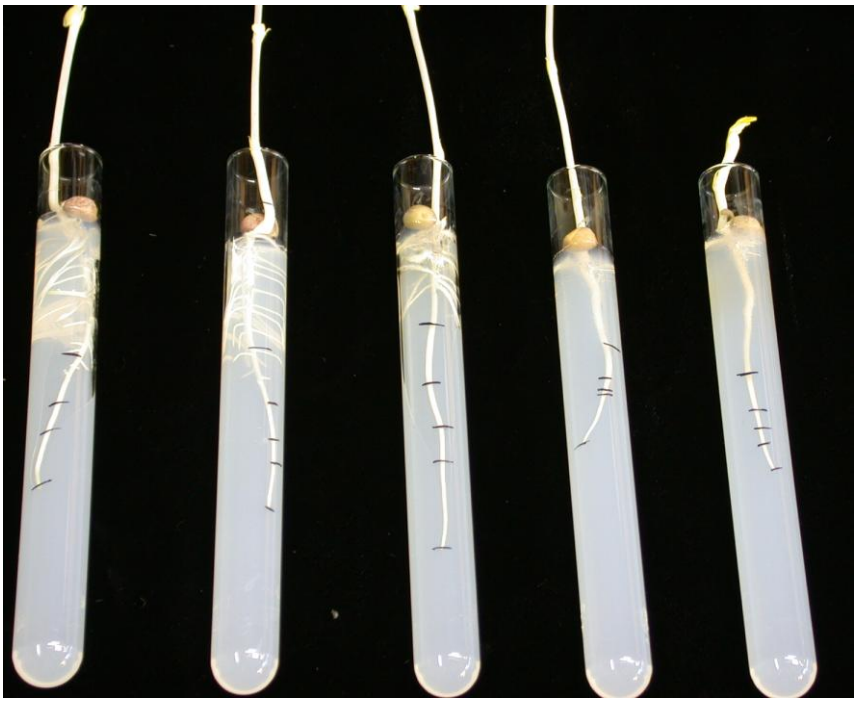


Figure 5.2 Effect of 50µM PCIB on root elongation over 48 hours. Plants (L205+) were grown in pasteurised potting mix for 5 d, and then transferred into agar medium (with or without PCIB). Shown are the means with SE of the root elongation (cm) every 24 h up to 48 h (n=10).

A



B

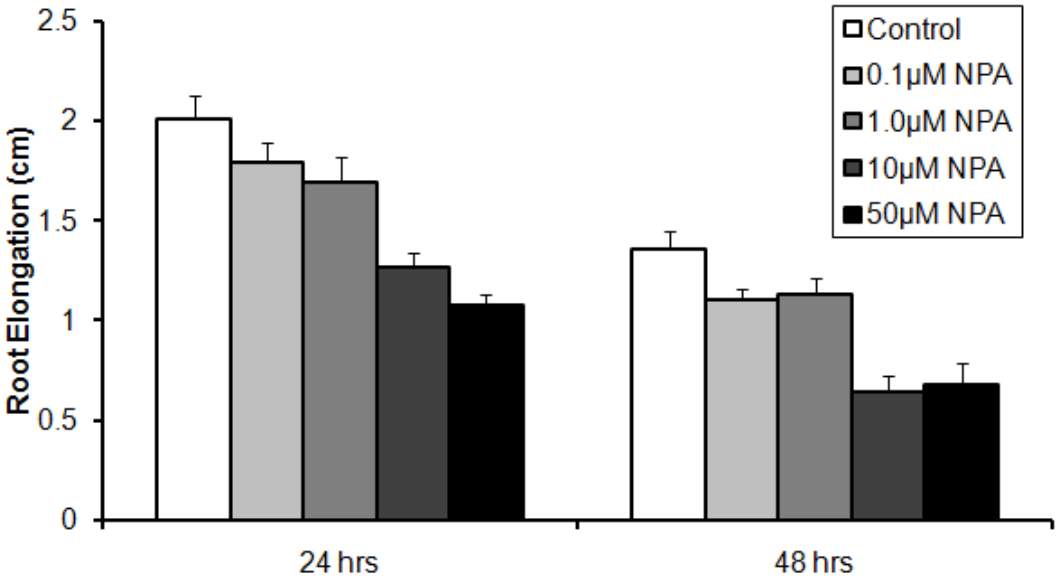


Figure 5.3 Dose response curve after 0, 0.1 µM, 1.0 µM, 10 µM, and 50 µM treatments with NPA **(A)** Photo showing the root physiological response after 144 h (from left: 0, 0.1 µM, 1.0 µM, 10 µM, and 50 µM NPA). Marks on test tubes indicate the time points, from top: 0 h, 24 h, 48 h, 72 h, and 144 h. **(B)** Mean with SE of the root elongation (cm) every 24 h up to 48 h (n=10).

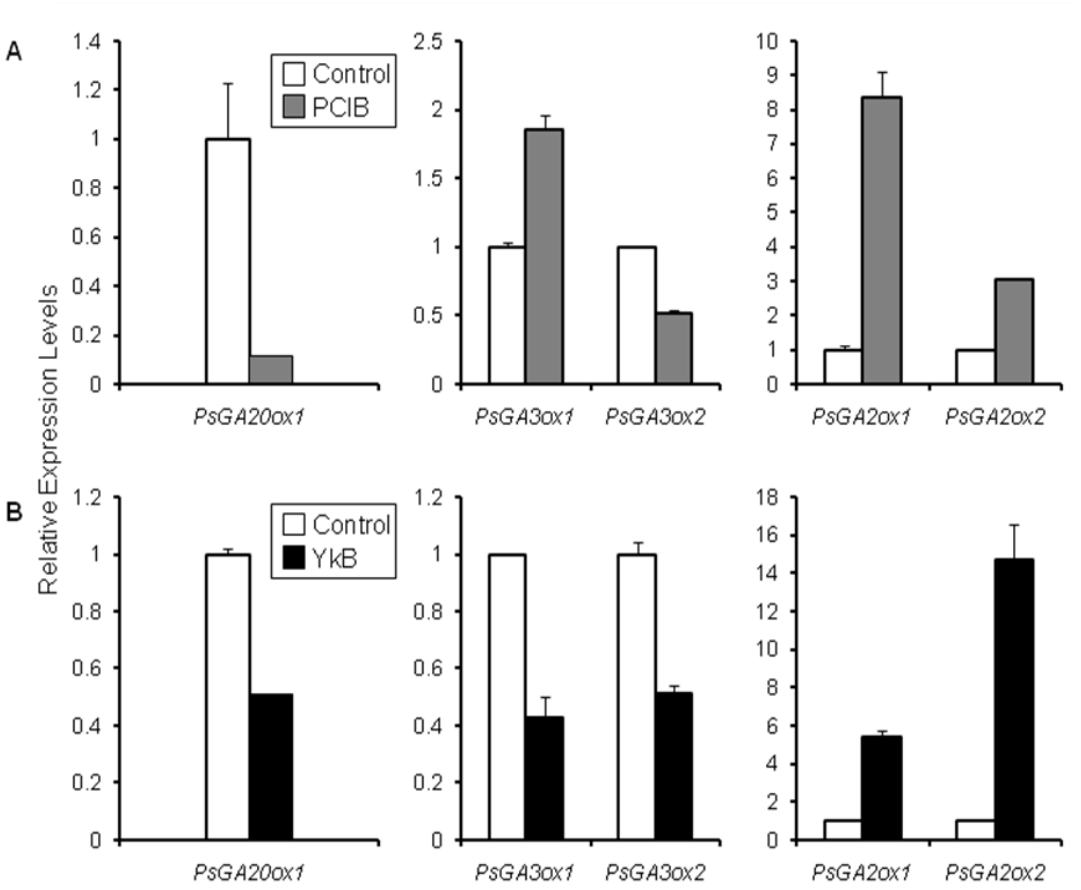


Figure 5.4 Effects of auxin action inhibitors on expression of GA genes. Transcript levels of GA biosynthesis and deactivation genes in control and 50 µM PCIB-treated pea roots **(A)**, and control and 0.5 µM YkB-treated roots **(B)**, measured by quantitative real-time PCR. Plants (line 205+) were grown in pasteurised potting mix for 5 d, and then transferred into agar medium (with or without the inhibitor) for 24 h. For each gene, the control value was set to 1, and the level in the treated sample was calculated relative to the corresponding control value. Shown are means with SE (n = 3). Note: PCIB and YkB results were obtained from separate experiments.

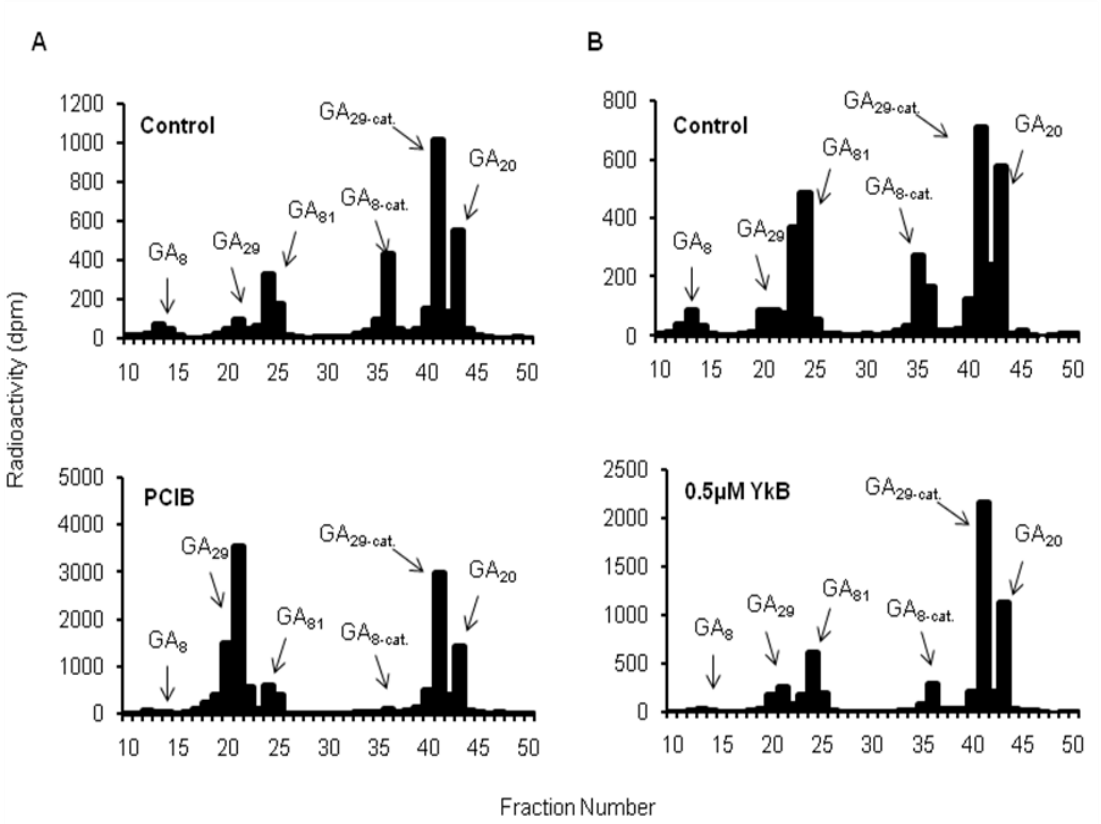


Figure 5.5 Effect of PCIB **(A)** and YkB **(B)** on GA 3-oxidation. Plants (line 205+) were grown in pasteurised potting mix for 5 d then transferred to aerated distilled water containing [^{14}C]GA_{20} (10,000 dpm.mL^{-1}), with and without 50 µM PCIB (A) or 0.5 µM YkB for 24 h. Metabolites were analysed by HPLC as methyl esters; retention times are shown on the chromatograms. Products were identified by GC-MS-SIM. The main 3-oxidation products detected were [^{14}C]GA_8 and its metabolite, [^{14}C]GA_8-catabolite; [^{14}C]GA_1 did not accumulate. The main 2-oxidation products were [^{14}C] GA_{29} and its metabolite, [^{14}C]GA_{29}-catabolite. Similar results were obtained in replicate experiments. (Figure taken from Weston et al., 2008).

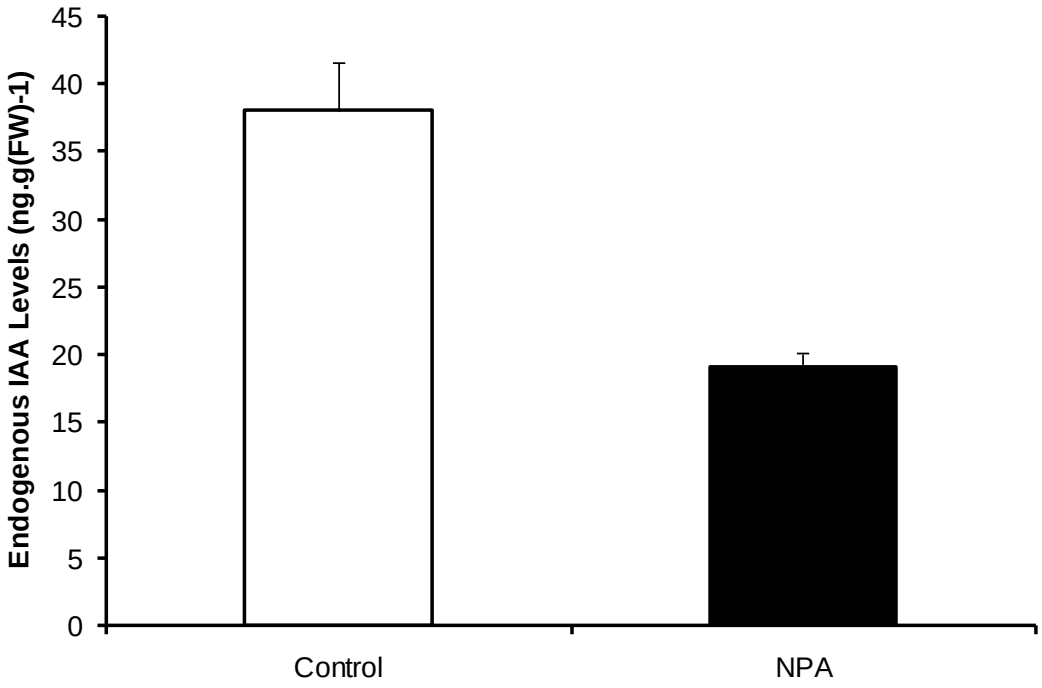


Figure 5.6 Effects of the auxin transport inhibitor NPA on endogenous IAA levels in the mature root. Plants (line 205+) were grown in pasteurised potting mix for 5 d, and then transferred into agar medium (with or without the inhibitor) and harvested after 24 h. Endogenous IAA levels were quantified using GC-MS (n=3).

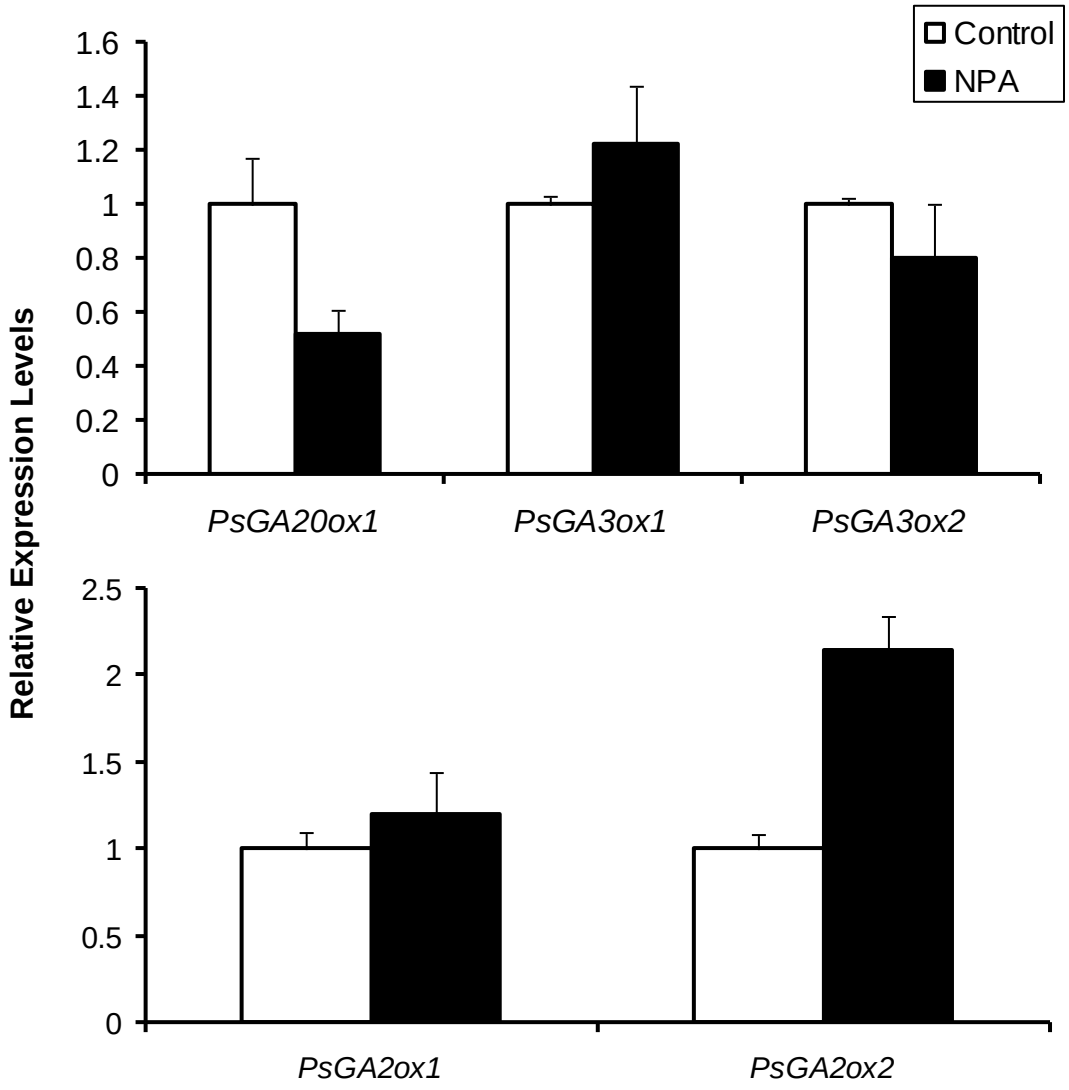


Figure 5.7 Effects of the auxin transport inhibitor NPA on expression of GA genes in the mature root. Transcript levels of GA biosynthesis and deactivation genes in control and 50 µM NPA-treated pea roots measured by quantitative real-time PCR. Plants (line 205+) were grown in pasteurised potting mix for 5 d, and then transferred into agar medium (with or without the inhibitor) for 24 h. For each gene, the control value was set to 1, and the level in the treated sample was calculated relative to the corresponding control value. Shown are means with SE (n = 3).

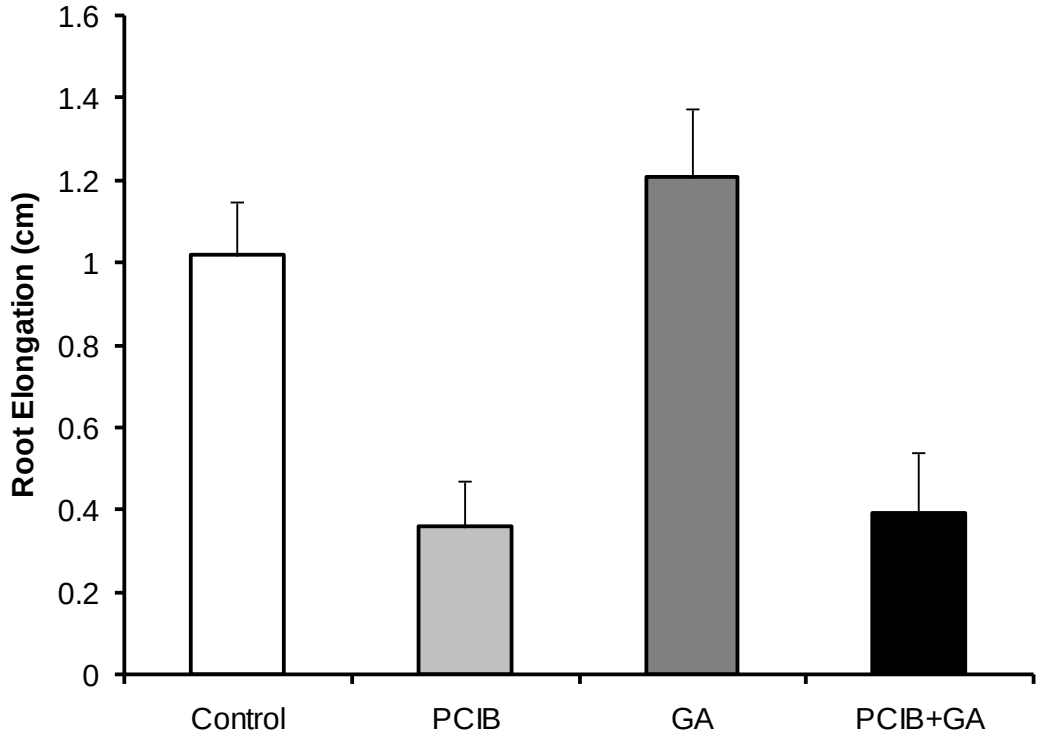


Figure 5.8 Root elongation of plants treated with 50μM PCIB, 0.1 μM GA_3, and 50μM PCIB + 0.1μM GA_3 after 24 hrs (n=12). Plants (L205+) were grown in pasteurised potting mix for 5 d, and then transferred into agar medium (with or without PCIB and/or GA). The control value was set to 1, and the elongation in the treated sample was calculated relative to the corresponding control value.

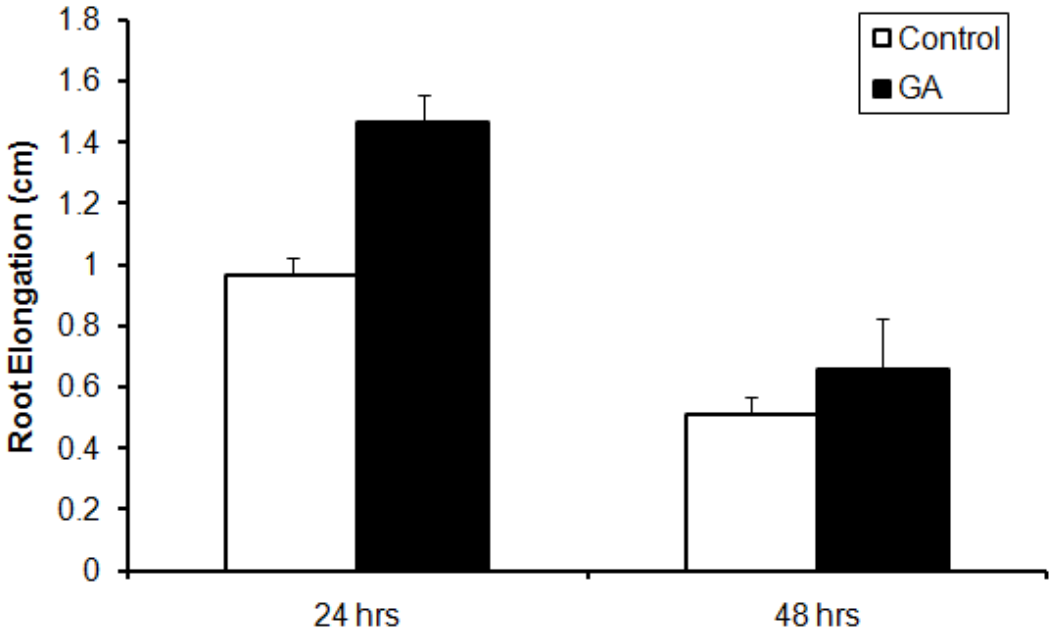


Figure 5.9 Root elongation of the GA-deficient *nana* plants treated with 10 µM GA_3, after 0, 24, and 48 h. Plants were grown in pasteurised potting mix for 5 d, and then transferred into agar medium (with or without GA_3). Shown are the means with SE of the root elongation (cm) every 24 h up to 48 h (n=8).

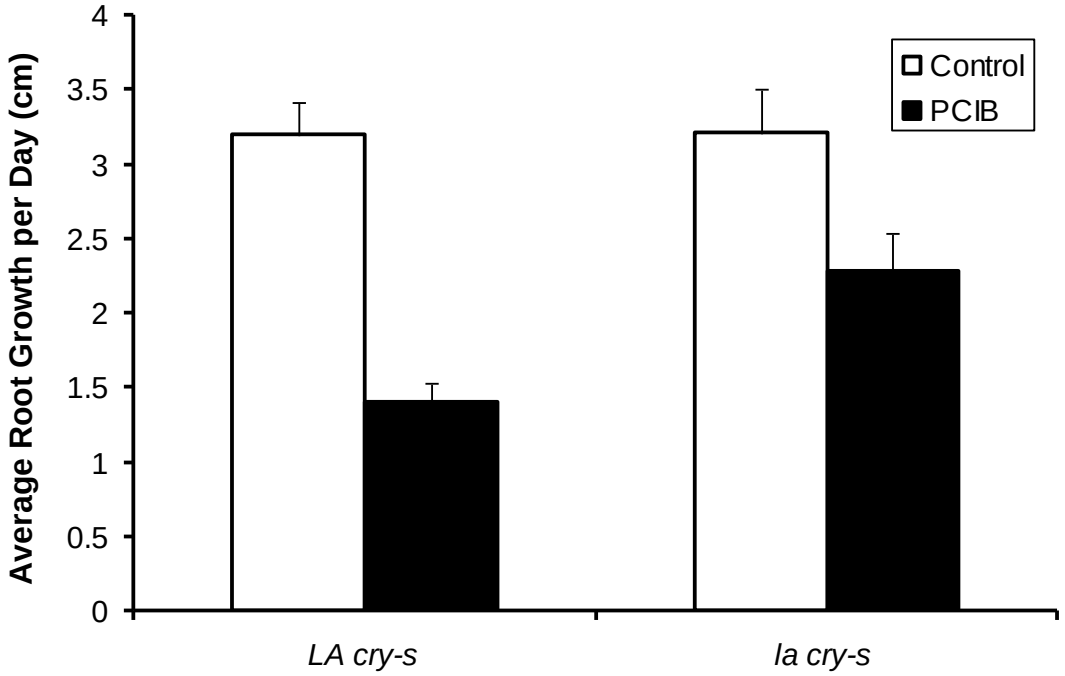


Figure 5.10 Effect of PCIB on root length of DELLA mutants. Plants were grown in pasteurised potting mix for 5 d, and then transferred into agar medium (with and without 50 µM PCIB). Shown are the means with SE of the subsequent root elongation over 3 d of *LA cry-s* and *la cry-s* seedlings. Differences between means and interactions were tested using log transformed data, and one-tailed ANOVA (JMP 4.0, SAS Institute Inc., Cary, North Carolina, 2000).

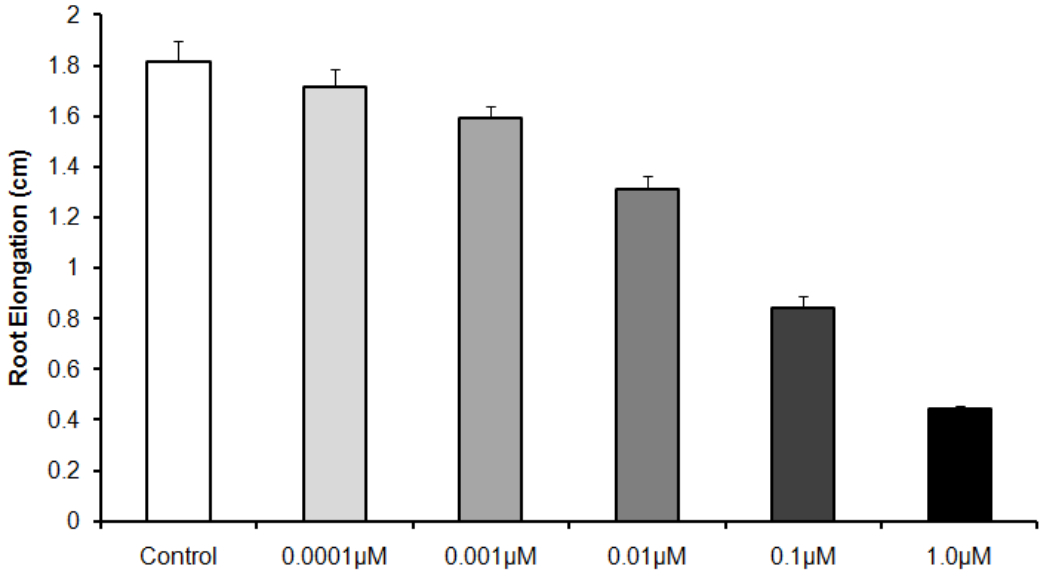


Figure 5.11 Root elongation rate in 205+ plants treated with varying concentrations of IAA after 24 h. Plants were grown in pasteurised potting mix for 5 d, and then transferred into agar medium (with and without IAA). Shown are the means with SE of the subsequent root elongation after 24 h.

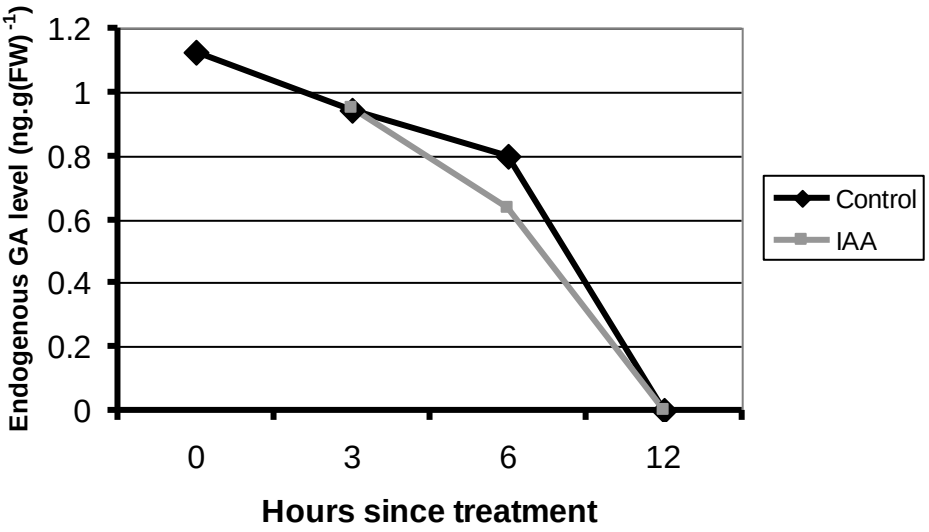


Figure 5.12 IAA application time-course study. Plants (line 205+) were grown in pasteurised potting mix for 5 d, then transferred to agar with and without 10 µM IAA. The 30-mm root tips were harvested at 0, 3, 6 and 12 h. Shown are the mean levels of endogenous GA_1 (ng.gFW-1) present in the 30-mm root tip at each time point (n = 3), as determined by GC-MS-SIM.

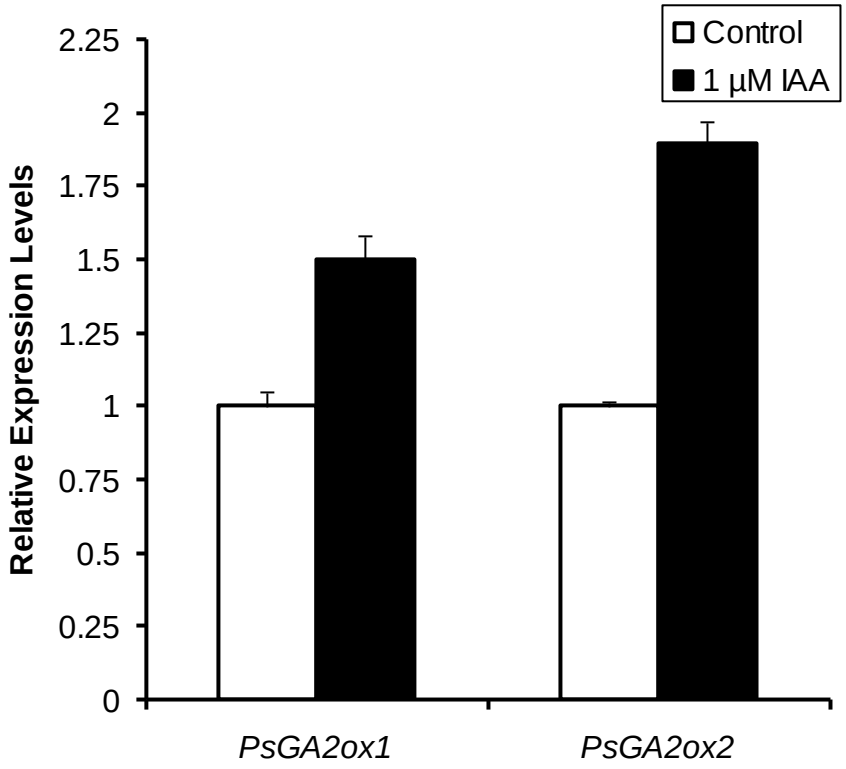


Figure 5.13 Expression of GA 2-oxidase genes in pea seedlings treated with IAA. Transcript levels of the deactivation genes *PsGA2ox1* and *PsGA2ox2* in control and 10 µM IAA-treated roots, monitored by quantitative real-time PCR. For each gene, the control value was set to 1, and the level in the treated sample was calculated relative to the corresponding control value. Shown are means with SE (n = 3).

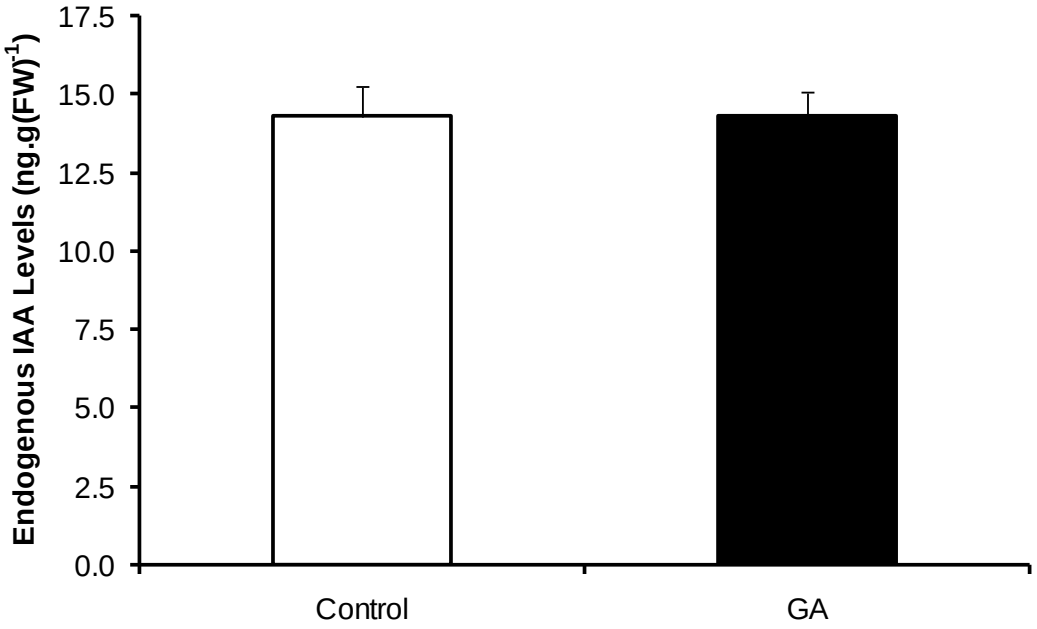


Figure 5.14 Effects of the application of GA_3 on endogenous IAA levels in root tips. Plants (*nana*) were grown in pasteurised potting mix for 8 d before 0.1% Tween-20 solution (with or without GA_3) was applied to the apical shoot meristem. Endogenous IAA levels were quantified 24 h after treatment using GC-MS (n=3).

5.4 Discussion

The present results indicate that in roots, endogenous auxin promotes GA synthesis and inhibits GA deactivation. This conclusion is based on the observation that when either of the auxin action inhibitors PCIB or yokonolide B, or the auxin transport inhibitor NPA, was applied to pea roots, transcript levels of *PsGA20ox1* and *PsGA3ox2* were reduced, and either or both of the deactivation genes *PsGA2ox1* and *PsGA2ox2* were increased, while *PsGA3ox1* showed variable changes in levels. This result was further supported by quantification of endogenous GAs, which showed that the levels of GA_{20} and of GA_1 were significantly reduced by PCIB (Weston et al., 2009). The finding that NPA reduced *PsGA20ox1* transcript levels (Figure 5.7) is noteworthy, because for *Arabidopsis*, it has been reported that this compound increased *AtGA20ox1* expression (Desgagne-Penix and Sponsel, 2008). It should be realised, however, that in the *Arabidopsis* experiments, NPA caused an accumulation of auxin in parts of the shoot (as indicated by *DR5::GUS* reporter gene studies), rather than an auxin depletion as occurred in the mature pea root tissue in our study (Figure 5.8).

While our findings indicate that endogenous IAA acts to reduce the expression of GA 2-oxidation (deactivation) genes, exogenous IAA (at the concentrations used here) had the reverse effect: transcript levels were elevated (Figure 5.14). In other words, there appears to be an optimal IAA level for minimising 2-oxidation and therefore for maximising GA_1 content. This might explain why Frigerio et al. (2006), who used a high and possibly supra-optimal concentration of exogenous auxin (50 µM 1-naphthaleneacetic acid (NAA)), found increased 2-oxidation gene mRNA levels after this treatment.

It is well known that auxin can be supra-optimal for the elongation of roots (Evans *et al.*, 1994; Silva and Davies, 2007), and the question now arises: is the inhibition of root elongation by exogenous auxin mediated by a reduction in bioactive GA_1 content? The answer appears to be that while there is a small reduction, (Table 5.1), it is insufficient to account for more than a small part of the growth inhibition caused by added auxin. Furthermore, the application of GA to PCIB-treated roots could not restore root elongation to the length seen in untreated plants (Figure 5.8), suggesting

that auxin, in-part, as well as GA, can directly affect root elongation. On the other hand, GAs may well mediate a substantial part of the promotion of elongation by endogenous auxin, as indicated by the reduced effect of PCIB on root elongation in the slender mutant.

It is interesting that GA 20-oxidation is strongly auxin-dependent in the roots of pea, but apparently not in the shoot (Ross et al., 2000; O'Neill and Ross 2002). Possibly in the internodes an effect of auxin content is counteracted by the feedback mechanism. Feedback might also explain how, in the experiment shown in Figure 5.5A, PCIB treatment led to increased levels of*PsGA3ox1* mRNA while that of *PsGA3ox2* was reduced. The reduced GA_1 content of PCIB-treated roots might have led to the increased expression of *PsGA3ox1,* although this apparently failed to offset the reduction in *PsGA3ox2* expression, since the overall effect of PCIB was a decrease in 3-oxidation (Figure 5.6A). These observations are consistent with evidence that *PsGA3ox2* is a major 3-oxidase gene in pea roots (Weston et al 2008; Yaxley et al 2001).

In summary, our previous experiments with PCIB and YkB, and recent experiments with NPA indicate that auxin promotes the accumulation of bioactive GA in pea roots. Our findings do not entirely rule out the Fu and Harberd (2003) suggestion that auxin also facilitates the destabilisation of DELLAs by GAs, but it does appear that this interaction plays a relatively minor role in pea roots. If it played a major role, the disruption of auxin signalling would be predicted to promote GA synthesis (due to the expected accumulation of DELLAs), the opposite to the effect reported here.

CHAPTER 6

An Investigation of Possible ABA – GA Interactions in the Regulation of Shoot Growth

6.1 Introduction

Abscisic acid (ABA) is a terpenoid compound derived from isopentenyl diphosphate (IPP) and so shares a common early biosynthetic pathway with the GAs (Nambara and Marion-Poll, 2005). Many studies have focussed on the involvement of ABA in response to stressed conditions, such as drought and exposure to salt, particularly through its role in regulating stomatal closure. However, ABA has also been shown to inhibit shoot growth, induce storage protein synthesis in seeds, and counteract the effect of GA on α-amylase synthesis in cereal grains (Davies, 2004). Historically, ABA and GA have generally been shown to cause opposite physiological effects on plants; in other words to act antagonistically (Norman et al., 1983). For example, GA is known to promote germination, growth and flowering whereas ABA is shown to inhibit these developmental processes (Weiss and Ori, 2007). The question is, are these antagonistic physiological responses a result of ABA and GA interacting or acting directly?

Early studies used GA biosynthesis inhibitors to examine the possible interaction between GA and ABA in regulating growth. For example, the application of ancymidol results in decreased rates of root elongation (Moore and Dickey, 1985) as well as an inhibition of both GA and ABA biosynthesis (Norman et al., 1983). Paclobutrazol has also been shown to inhibit ABA biosynthesis effectively at 0.1 and 1-µM in the fungus *Cercospora rosicola* (Norman et al., 1986). Contrary to this, more recent studies found that the seeds of the GA-deficient mutant *ga1-3* reportedly have high levels of ABA (Oh et al., 2007), and similarly, GA application to tomato ovaries was shown to reduce the transcript levels of the ABA synthesis gene *LeNCD1* (Nitsch et al., 2009). On the other hand, young seedlings of the pea GA biosynthesis mutant *lh-2* displayed unchanged ABA levels (Batge et al., 1999). Studies on the reverse interaction have indicated also that in the Arabidopsis ABA-deficient mutant *aba2,* GA biosynthesis was enhanced (Seo et al., 2006).

ABA has also been reported to interact with GA via the GA signalling pathway. In Arabidopsis, GA promotes and ABA suppresses root growth, a process that appears to be mediated by the DELLA proteins (Achard et al., 2006). ABA application was reported to block the GA-induced degradation of the GFP-RGA fusion protein (Achard et al., 2006). In addition, the Arabidopsis quadruple DELLA mutant (loss of *GAI, RGA, RGL1,* and *RGL2)* is relatively resistant to the growth-inhibitory effects associated with ABA application, compared with WT plants (Achard et al., 2006). This suggests that the inhibition of root growth by ABA acts via the DELLA proteins.

Conversely to the suggestion of Achard et al. (2006), other evidence indicates that ABA interacts with the GA signalling pathway downstream of the DELLAs. When Zentella et al. (2007) monitored endogenous RGA protein in whole Arabidopsis seedlings treated with ABA they found no change in RGA accumulation, whereas Achard et al. (2006) had found the opposite using the GFP method. Furthermore, the results of Zentella et al. (2007) are in agreement with previous studies on rice plants. In both WT and *slender* (*slr1*; a constitutive GA signalling response mutant) genotypes of rice, the application of ABA results in a comparable reduction in shoot length, suggesting that the ABA sensitivity is similar to WT and therefore that ABA is likely to act downstream of SLR1 in the rice GA signalling pathway (Ikeda et al., 2002). When GA and ABA levels in the shoots of the *slr1* mutant were measured, GA levels were found to be reduced (Ikeda et al., 2001) and ABA levels markedly elevated (Ikeda et al., 2002), compared to WT plants. However, when GA was applied to WT plants at saturating levels, ABA content is not increased to the same level as seen in the *slr1* mutant, suggesting that GA signalling, not levels, is closely associated with the regulation of endogenous ABA biosynthesis (Ikeda et al., 2002).

So the question that is raised here is: how does ABA interact with GA in pea – via the biosynthesis pathway or the signalling pathway or not at all? Furthermore, if ABA is acting via the GA signalling pathway, does this occur at the level of the DELLA proteins or downstream of the DELLAs? To answer these questions, the growth of young pea seedlings was monitored after ABA application to determine

how ABA influences growth. GA biosynthesis and catabolism genes were monitored and endogenous GA levels quantified in ABA-treated seedlings to determine whether ABA regulates growth by modulating GA biosynthesis. The reverse interaction was studied also, with ABA levels quantified in a GA-deficient mutant. To finish, ABA levels were quantified in the pea DELLA mutant *la cry-s* and DELLA protein expression monitored in ABA-treated seedlings to determine whether ABA is interacting with GA via the DELLA proteins.

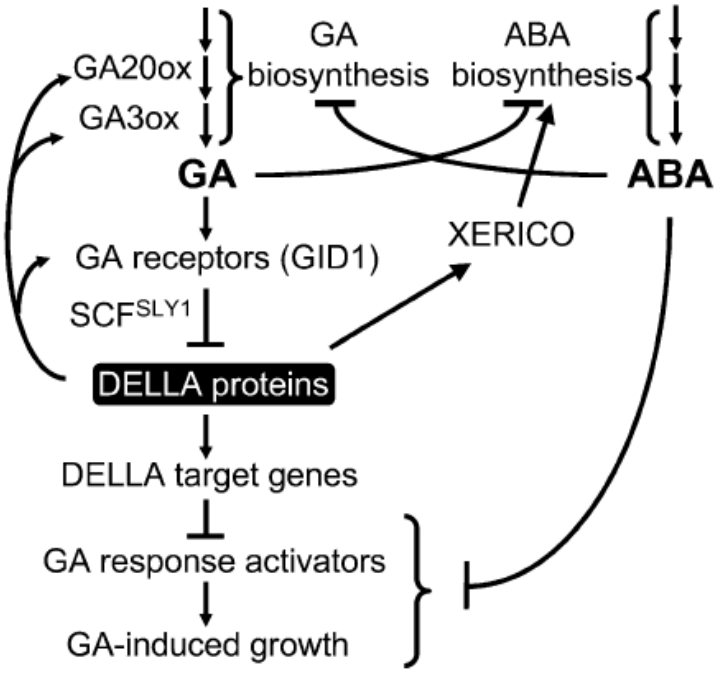


Figure 6.1 Diagram taken from Zentella et al. (2007) showing their proposed interaction of the GA and ABA pathways in Arabidopsis.

6.2 Materials and methods

6.2.1 Plant Material

The plant material used in these studies included the tall (WT) L107 (*LE*) and the isogenic GA mutant lines L5839 (*le-3*), L181 (*ls-1*). DELLA studies were undertaken on the isogenic *la CRY* and *la cry-s* lines, derived from the same F_4 plant from cross HL107 (*LA CRY*) and 188 (*la cry-s*).

6.2.2 Plant Growth and Chemical Treatments

Plants to be transplanted to agar for chemical treatment were grown in 100% potting mix for up to 5 d in a heated glasshouse at 15 - 25˚C, then transferred to glass 16 mm x 150 mm test tubes containing 1.3% sterilised agar with MS-salts containing 25 μM ABA (Sigma Aldrich, USA). Chemicals were added to molten agar (<60˚C). Plants were placed in a 20˚C dark room for up to 7 d. Plants that were not subjected to hormone or inhibitor application were grown in a 50% gravel/vermiculite mix topped with soil in a heated glasshouse at 12 - 25˚C for up to 14 d.

After ABA treatment, plant material (as specified in results) for gene expression and endogenous hormone quantification was harvested. Gene expression material was immediately immersed in liquid nitrogen and stored in a -70ºC freezer. Material for GA and ABA analysis was immediately immersed in cold (-20ºC) 80% methanol, containing butylated hydroxytoluene (Sigma, 250 mgl^{-1}), and stored in a freezer (-20ºC).

6.2.3 RNA extraction, cDNA synthesis and Quantitative RT-PCR

RNA extraction, cDNA synthesis and quantitative RT-PCR was carried out as in Chapter 2.2.

6.2.4 Quantification of endogenous GAs and ABA

GA and ABA analysis was undertaken as described in Chapter 2.1.

6.3 Results

6.3.1 ABA application reduces primary root growth, lateral root growth and shoot growth in pea

To determine the levels of ABA that have a clear phenotypic effect on pea shoots and roots, young seedlings were transferred to agar test tubes containing 0, 1 µM, 10 µM and 25 µM ABA. As can be seen in Figure 6.1, 25 µM had the strongest effect on pea, with reduced shoot and root length and a reduced number of lateral roots compared with control plants.

When the root growth rate was analysed further for 25 µM-treated plants, a significant reduction of approximately 30% was seen after 24 h ($P < 0.05$, Figure 6.2), but by 48 h no significant reductions in root growth rate was seen (Figure 6.2). In terms of root development, it is interesting to note that the number of lateral roots in 25 µM-treated plants was significantly reduced, by approximately 50%, over a period of 6 d ($P < 0.001$, Figure 6.3).

The strongest phenotypic effect was seen in the young shoots. The 25 µM-treated plants showed a 40% reduction in shoot length after 6 d ($P < 0.001$, Figure 6.4). In a separate experiment, the shoot lengths of the GA-deficient pea (L5839) and 25 µM-treated ABA plants were shown to be comparable, possibly suggesting that GA levels are reduced by ABA (Figure 6.5).

6.3.2 ABA application results in an increase in endogenous levels of GA 2-oxidation products but does not alter GA catabolism genes in the shoot

As there was no clear long-term effect of ABA on root elongation, it is unlikely that an ABA-GA interaction is regulating this growth. It was for this reason that studies on ABA-GA interactions were pursued for the shoot only.

The application of 25 µM ABA to pea seedlings using the agar method results in a dramatic 20-fold increase in ABA levels in the shoot compared to the control ($P < 0.001$, Table 6.1). Furthermore, the final shoot length of the plants harvested was found to be inhibited by approximately 33% ($P < 0.001$; Table 6.1).

When GA levels were measured in 25 µM ABA-treated pea shoots, no significant change in the levels of the GA 3-oxidation product GA_1 (bioactive) was seen. However, there was a significant increase of approximately 100% in the levels of the GA 2-oxidation product GA_{29} compared to the control ($P < 0.001$, Table 6.1). We also observed the same trend for the 2-oxidation product GA_8 although the difference in endogenous content was not found to be significantly different in this case.

As ABA application was found to result in elevated 2-oxidation products endogenously, it was expected that the GA 2-oxidase genes *PsGA2ox1* and *PsGA2ox2* would be up-regulated in young shoots. However, it was found that the expression of the GA 2-oxidase genes was not significantly altered in ABA-treated shoots (Figure 6.6). We also monitored *PsGA3ox1* and *PsGA20ox1* mRNA levels in this study, but found, likewise, that the expression of these genes was not altered by ABA treatment (Figure 6.6).

6.3.3 ABA content was reduced in the leaves of GA-deficient plants

Previous studies in the fungus *Cercospora rosicola* have shown that the application of the GA biosynthesis inhibitors ancymidol and paclobutrazol result in not only a decrease in GA biosynthesis, but also of ABA biosynthesis, possibly due to the shared early steps in their biosynthetic pathways (Norman et al., 1983; Norman et al., 1986). However, the opposite was seen in Arabidopsis shoots, with the application of the GA biosynthesis inhibitor uniconazole-P resulting in increased levels of ABA (Saito et al., 2006). As a result of these conflicting reports, the aim of this study was to determine whether GA levels affect ABA biosynthesis, by quantifying endogenous ABA levels in the shoots and leaves of the GA-deficient plants *le-3* (L5839) and *ls-1* (L181).

In the leaves of 14 d-old *le-3* and *ls-1* mutants, ABA levels were reduced by 63% ($P < 0.05$; Figure 6.7) and 59% ($P < 0.05$; Figure 6.7), respectively, compared with the WT. In a separate experiment, the apical portion of the shoot was harvested in the WT and *le-3* seedlings, and likewise, the ABA levels were 50% lower in the mutant

compared with the WT ($P < 0.001$; Figure 6.8). These results suggest that GA promotes ABA biosynthesis in young plants.

6.3.4 ABA levels are elevated in the pea DELLA mutant

Ikeda et al. (2002) reported that in the rice DELLA mutant *slr1*, ABA levels were elevated compared to WT plants, again suggesting that GA signalling plays an important role in ABA biosynthesis. To test whether this is true for pea also, endogenous ABA content was measured in the leaves and apical internode tissue of the pea DELLA mutants *la CRY* (WT phenotype), and *la cry-s*. In the leaves, ABA levels were elevated in the double mutant (*la cry-s*) by 52% compared to the WT ($P < 0.01$; Figure 6.9). In the apical internode tissue, ABA levels were elevated by 28% in the double mutant compared to the WT ($P < 0.05$; Figure 6.10). Thus it appears that GA signalling inhibits ABA biosynthesis. These are in agreement with the reports made by Ikeda et al. (2002), although the degree of elevation is a lot lower in our studies.

6.3.5 ABA-treated DELLA mutants display comparable shoot growth inhibition to WT

Achard et al. (2006) reported that ABA acts via the DELLAs to inhibit growth, because the roots of the Arabidopsis quadruple-DELLA mutant treated with ABA were relatively resistant to the usual growth inhibition seen in WT after ABA application. To determine whether the shoots of pea DELLA mutants are less sensitive to ABA than WT plants, 25 μM ABA was applied to *la CRY* and *la cry-s* seedlings. ABA significantly inhibited shoot growth by approximately 46% in *la CRY* seedlings ($P < 0.01$; Figure 6.11) and 40% in la cry-s seedlings ($P < 0.001$; Figure 6.11) after 72 h. However, the degree of inhibition of the WT shoot growth by ABA was not significantly different to the inhibition seen in the mutant ($P = 0.62$, two-tailed ANOVA; Figure 6.11).

Table 6.1 Endogenous levels (ng.g(FW)$^{-1}$) of ABA, the GA 3-oxidation product GA_1, and the GA 2-oxidation products GA_{29} and GA_8 in control (L107), GA deficient *le-3* (L5839) and 25µM ABA-treated (L107) shoots (n=3). Plants were grown in pasteurised potting mix for 4 d, and then transferred into agar medium (with or without ABA). Shown also is the mean final shoot length (mm) before harvest (n=29).

	ABA	**Final Shoot Length**	**GA_1**	**GA_{29}**	**GA_8**
Control	30.7 ± 2.2	63.8 ± 0.3	11.6 ± 1.0	7.7 ± 1.1	14.1 ± 0.3
le-3	25.8 ± 1.4	45.5 ± 0.3	5.0 ± 0.5	37.5 ± 1.7	8.2 ± 0.5
25 µM ABA	662.9 ± 112.1	42.5 ± 0.2	12.0 ± 1.4	14.4 ± 0.9	19.4 ± 2.0

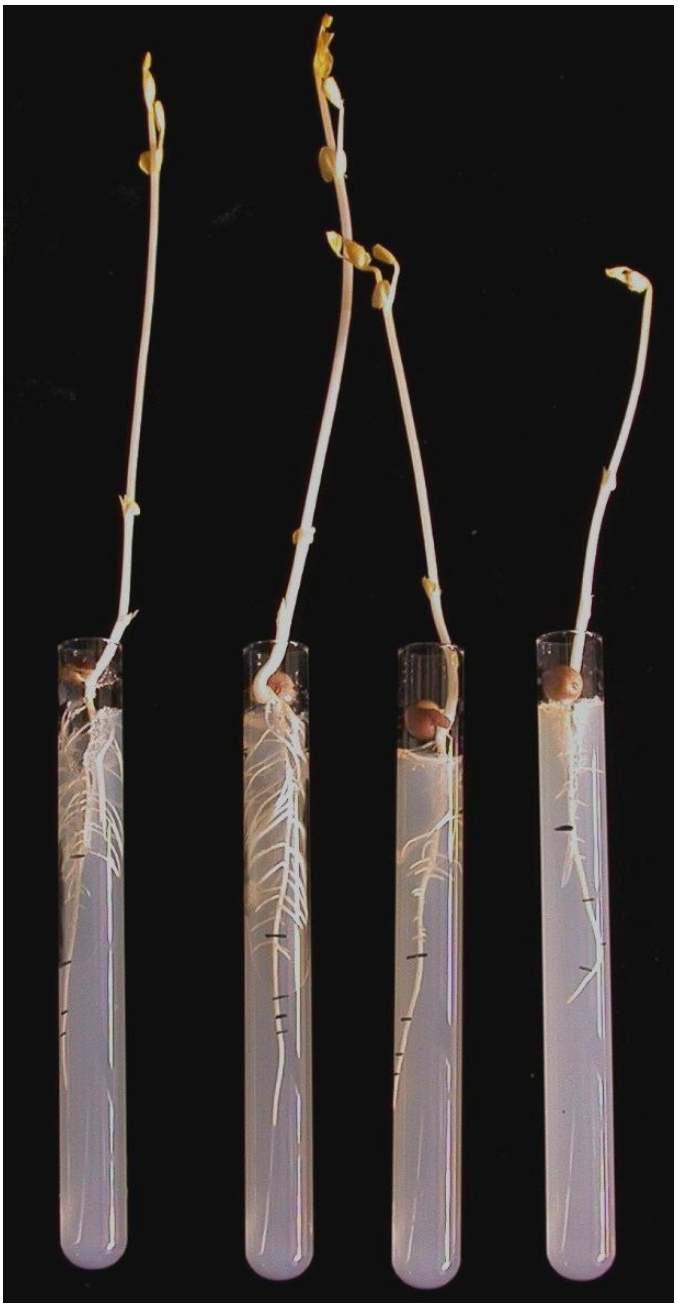


Figure 6.1 Photograph showing the effect of varying levels of exogenous ABA on shoot, root and lateral root growth in dark-grown seedlings. From left: 0 µM, 1 µM, 10 µM, and 25 µM ABA. Plants (L205+) were grown in pasteurised potting mix for 4 d, and then transferred into agar medium (with or without ABA). Lines on the test-tubes indicate the measurements taken initially, then after every 24 h.

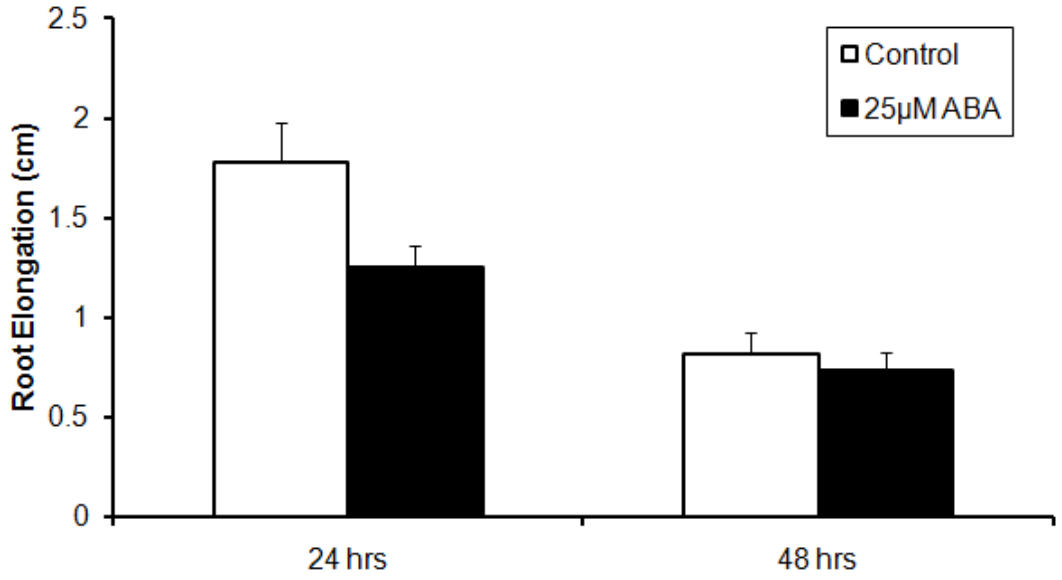


Figure 6.2 Effect of 25µM ABA on root growth over 48 hours. Plants (L205+) were grown in pasteurised potting mix for 4 d, and then transferred into agar medium (with or without ABA). Shown are the means with SE of the subsequent root elongation every 24 h up to 48 h (n=8).

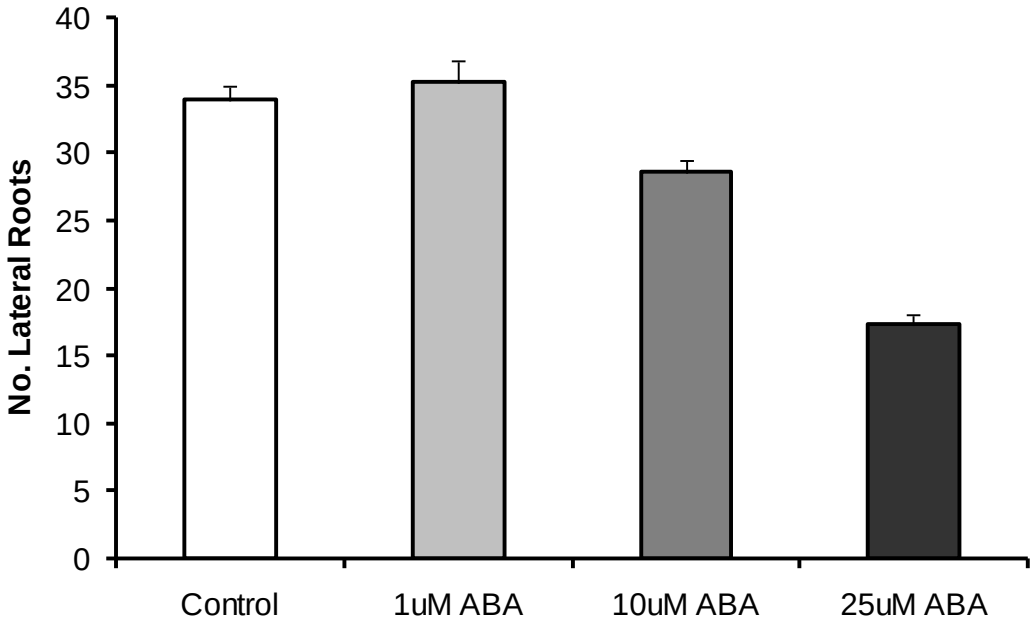


Figure 6.3 Effect of various exogenous ABA application on the number of lateral roots after 6 d. Plants (L205+) were grown in pasteurised potting mix for 4 d, and then transferred into agar medium (with or without ABA). Shown are the means with SE of the total number of later roots quantified after 6 d-exposure to ABA in agar (n=8).

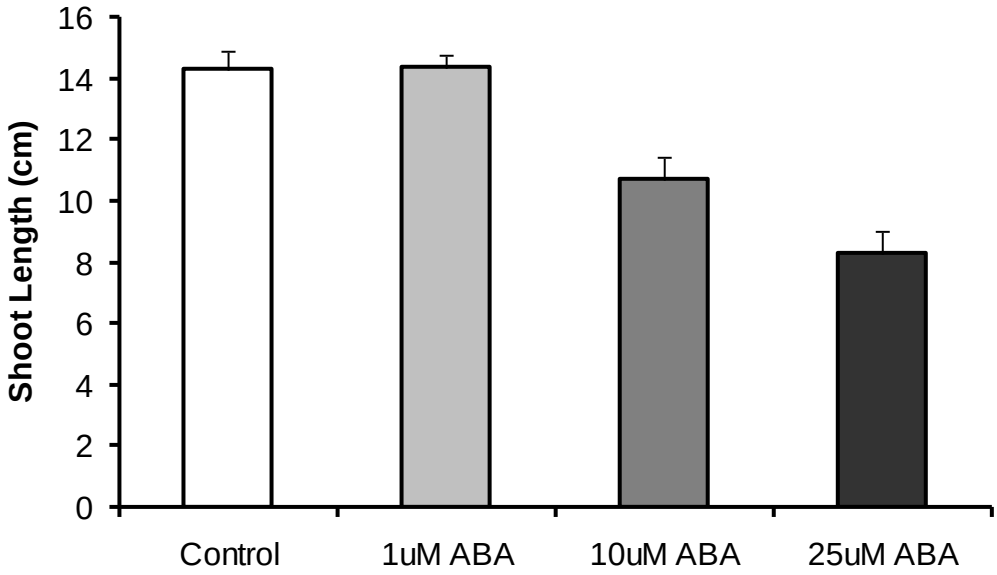


Figure 6.4 Effect of various levels of exogenous ABA on total shoot length 6 d after transferring to agar. Plants (L205+) were grown in pasteurised potting mix for 4 d, and then transferred into agar medium (with or without ABA). Shown are the means with SE of the subsequent shoot length measured after 6 d-exposure to ABA in agar (n=8).

Figure 6.5 Comparison of shoot phenotypes of *LE* (Control; L107), *le-3* (L5839) and 25 µM ABA-treated *LE* seedlings after 7 d. Plants were grown in pasteurised potting mix for 4 d, and then transferred into agar medium (with or without ABA).

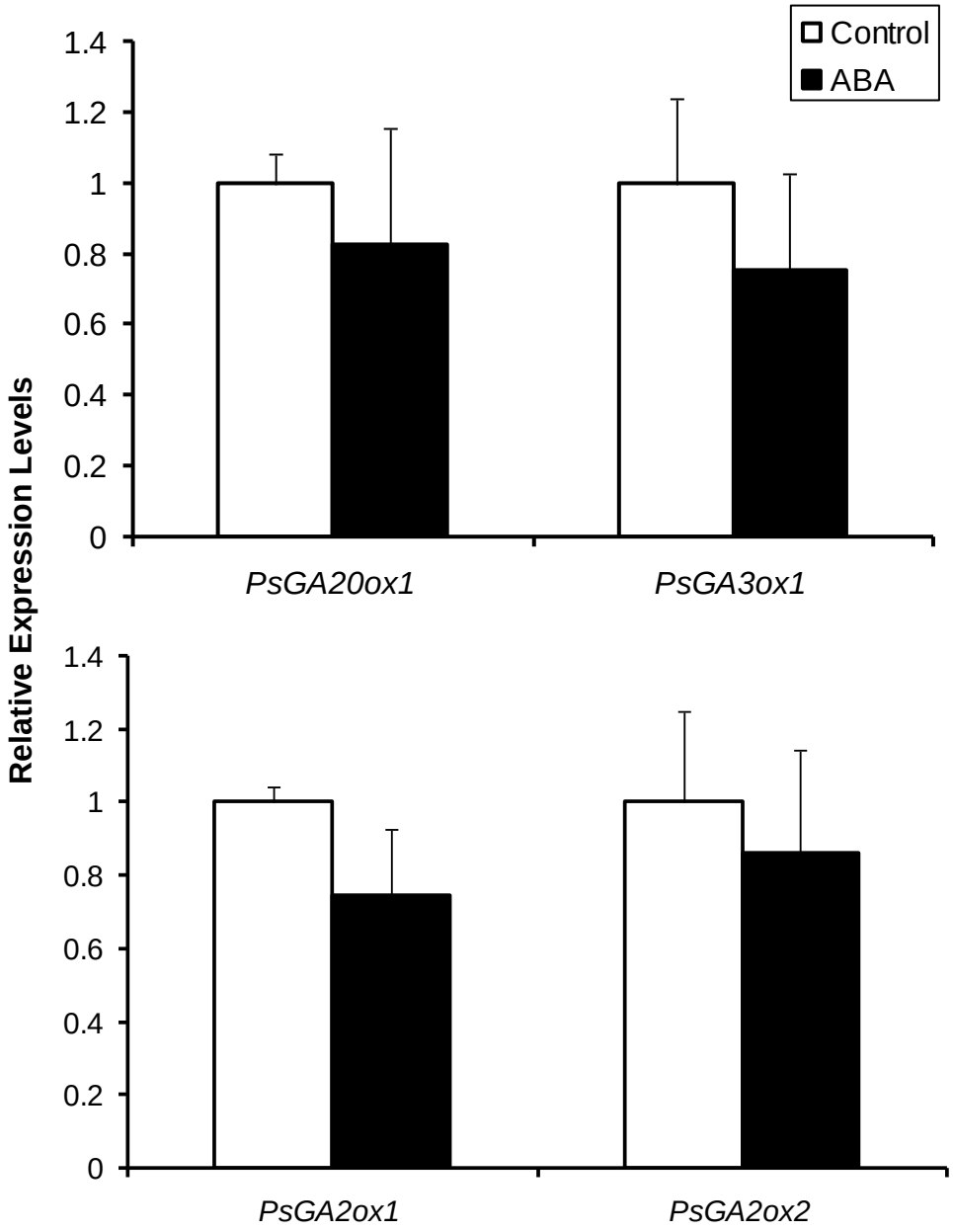


Figure 6.6 Effects of ABA on expression of GA genes. Transcript levels of GA biosynthesis and deactivation genes in control and 25 µM ABA-treated pea shoots measured by quantitative real-time PCR. Plants were grown in pasteurised potting mix for 5 d, and then transferred into agar medium (with or without ABA) for 4 d. For each gene, the control value was set to 1, and the level in the treated sample was calculated relative to the corresponding control value. Shown are means with SE (n = 3).

Note: See Supplemental Figures 6.1 - 6.6 for Quantitation Reports of each gene monitored by qRT-PCR.

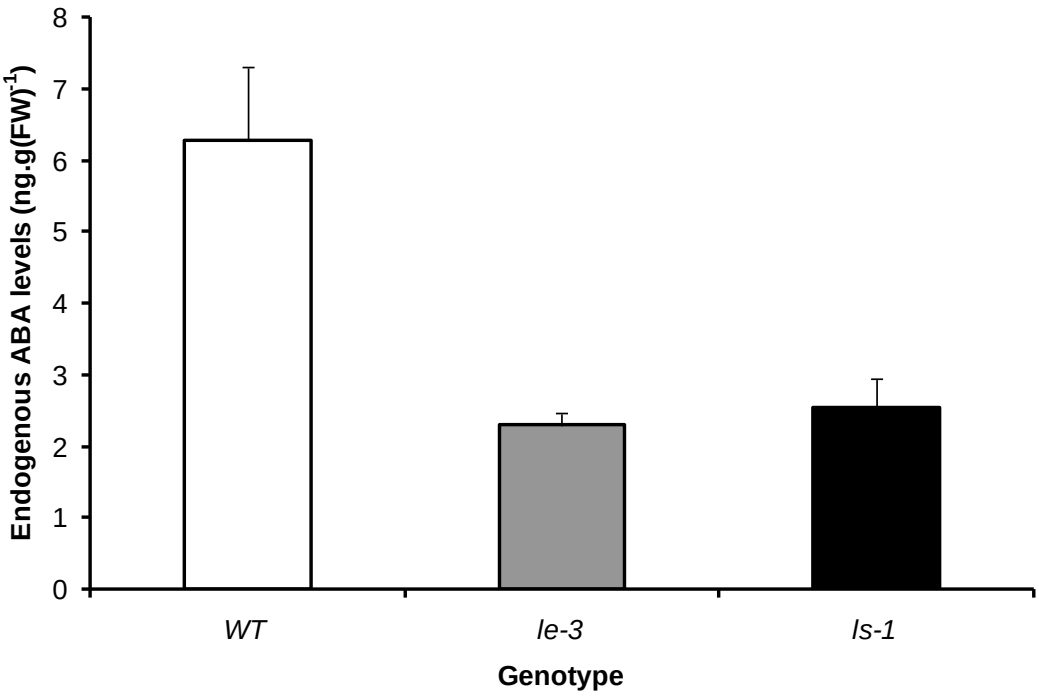


Figure 6.7 Shown are mean levels of endogenous ABA (ng.gFW^{-1}) present in the leaves of genotypes *LE* (L107), *le-3* (L5839), and *ls-1* (L181) (n = 3). Plants were grown in a 50% vermiculite/gravel mix topped with pasteurised potting mix for 14 d. The uppermost expanded leaf of each plant was harvested.

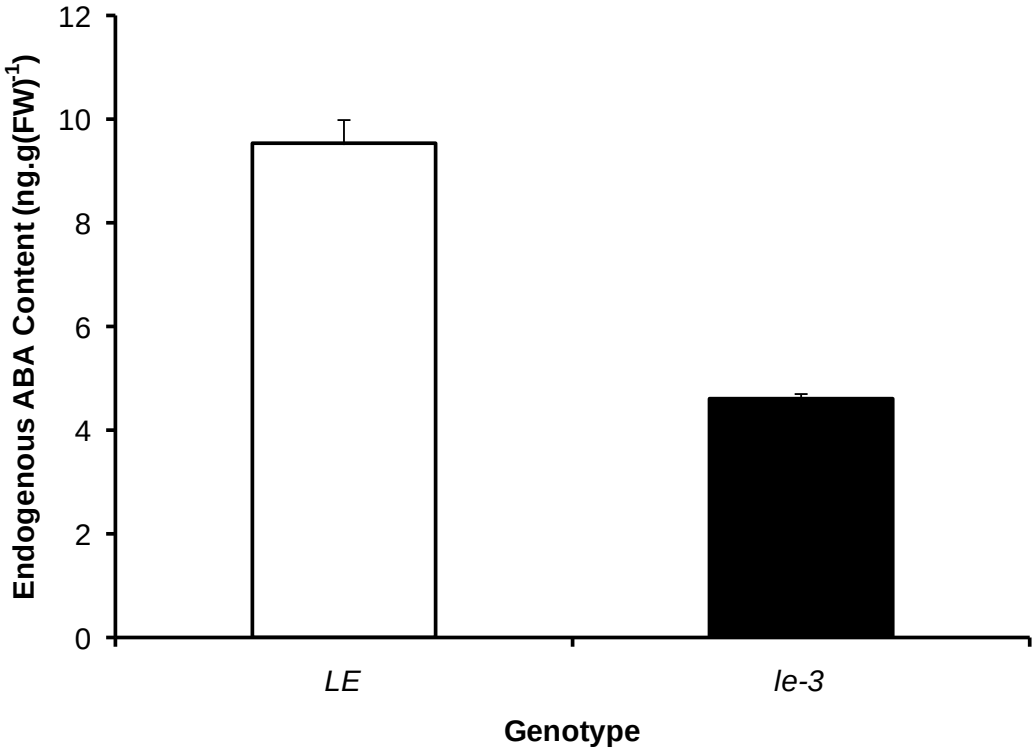


Figure 6.8 Shown are mean levels of endogenous ABA (ng.gFW^{-1}) present in the apical tissue of genotypes *LE* (L107) and *le-3* (L5839) (n = 3). Plants were grown in a 50% vermiculite/gravel mix topped with pasteurised potting mix for 14 d. The apical tissue (harvested from the uppermost expanded leaf) of each plant was harvested.

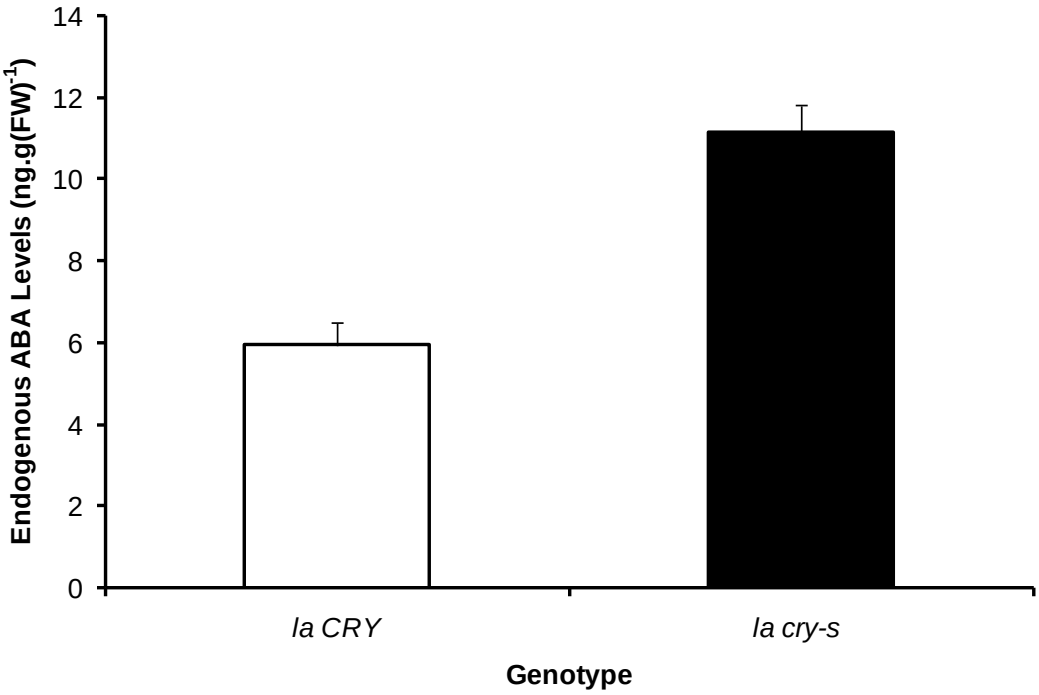


Figure 6.9 Shown are mean levels of endogenous ABA (ng.gFW^{-1}) present in the leaves of genotypes *la CRY* and *la cry-s* (n = 3). Plants were grown in a 50% vermiculite/gravel mix topped with pasteurised potting mix for 13 d. The uppermost expanded leaf of each plant was harvested.

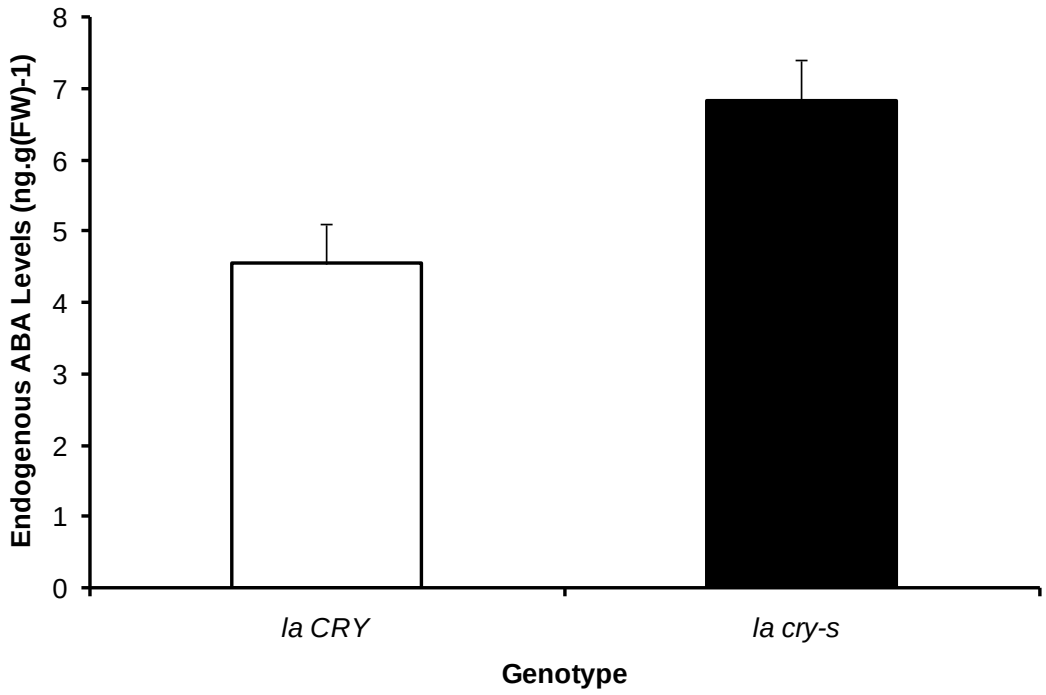


Figure 6.10 Shown are mean levels of endogenous ABA (ng.gFW^{-1}) present in the apical internodes of genotypes *la CRY* and *la cry-s* (n = 3). Plants were grown in a 50% vermiculite/gravel mix topped with pasteurised potting mix for 14 d. The apical tissue (harvested from the uppermost expanded leaf) of each plant was harvested.

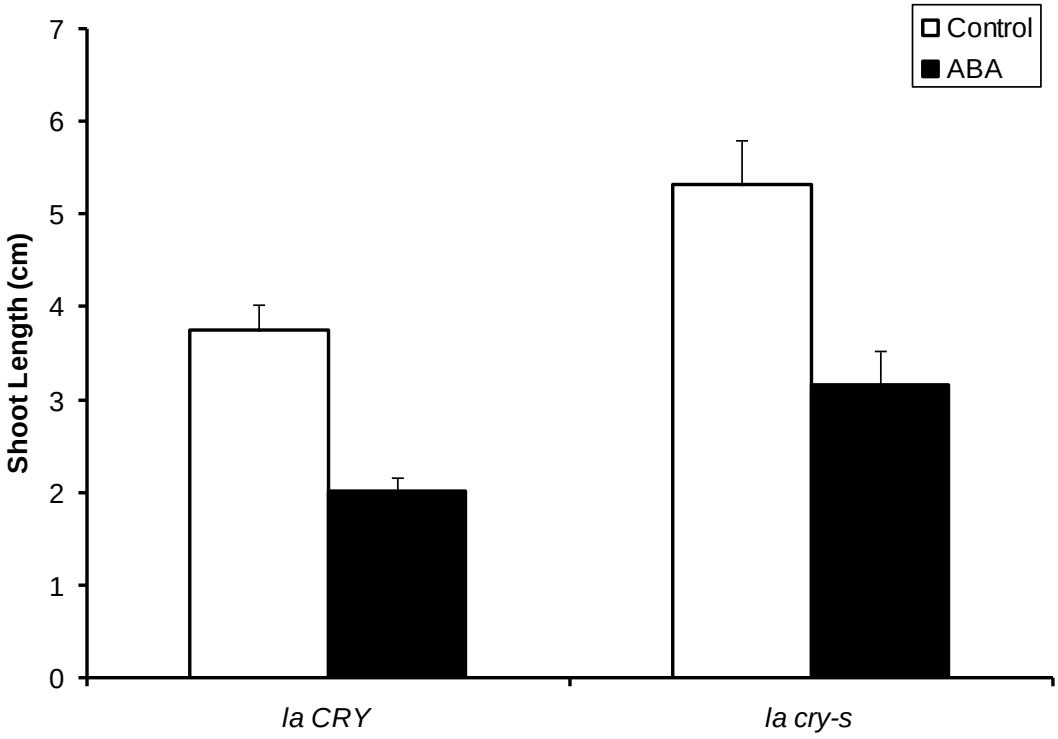


Figure 6.11 Effect of ABA on shoot elongation of DELLA mutants. Plants were grown in pasteurised potting mix for 3 d, and then transferred into agar medium (with and without 25 µM ABA). Shown are the means with SE of the subsequent shoot length after 3 d, of *la CRY* and *la cry-s* seedlings (n=9).

6.4 Discussion

There have been few studies that have examined the interaction between ABA and GA in regulating plant growth that have not been in relation to stress conditions or seed germination, and those that have been undertaken have not provided consistent results in relation to the interaction that is occurring. For example, Achard et al. (2006) reported that in Arabidopsis, the suppression of root growth by ABA acts via the DELLA proteins, but studies in both Arabidopsis and rice have suggested that ABA regulates growth by interacting with GA downstream of the DELLA proteins (Zentella et al., 2007; Ikeda et al., 2002). Adding further complexity to the story, it has also been reported that ABA acts via the GA biosynthesis pathway to regulate seed germination (Seo et al., 2006; Oh et al., 2007). In this study, the ABA-GA interaction was studied in young pea seedlings to determine whether ABA interacts via the GA biosynthesis pathway or via the GA signalling pathway. The reverse interaction was also studied.

Here it is shown for the first time that normal GA levels are required for the maintenance of "normal" ABA levels in shoots of pea. In the leaves of GA-deficient *le-3* and *ls-1* plants, ABA content is reduced by over half compared with the WT (Figure 6.8). This is not in agreement with the previous study by Batge et al. (1999) that reported no change in ABA content in the 10 d-old pea GA-deficient *lh-2* seedlings, or by Nitsch et al. (2009) and Oh et al. (2007) that report that GA is an inhibitor of ABA biosynthesis. Although early studies have shown that ABA levels are reduced in fungi treated with GA-biosynthesis inhibitors (Norman et al., 1983; Normal et al., 1986), this is the first time this phenomenon has been reported for higher-plant species. Strangely, however, ABA application was shown to reduce shoot elongation in pea by approximately 40% (Figure 6.4) - in agreement with previous reports (Davies, 2004; Achard et al., 2006) - but raises a paradox: if ABA is an inhibitor of growth and GA a promoter, how can GA promote ABA biosynthesis? This can be explained by the fact that ABA and GA share the early steps of their biosynthetic pathways (Nambara and Marion-Poll, 2005). The same phenomenon was also seen by Norman et al. (1983 and 1986) and they also concluded that the shared biosynthetic pathway was the plausible reason for these unusual results.

It is also interesting to note that in contrast to the present finding that ABA inhibits shoot growth in pea (Figure 6.4), in the tomato ABA-deficient mutants *flc* and *not*, shoot growth was inhibited, indicating that in that species, ABA may have a different effect on growth (Sharp et al. 2000).

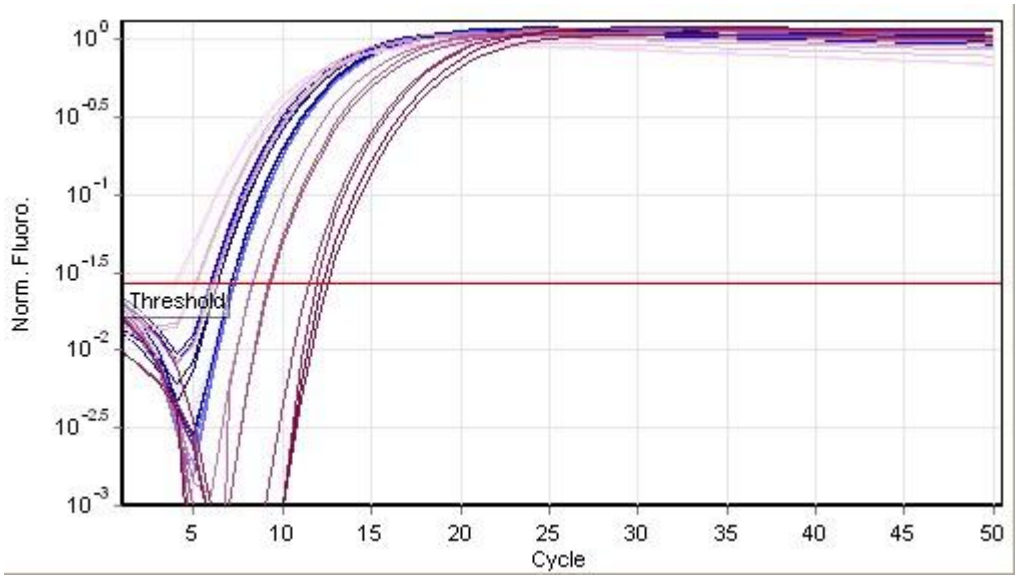
Endogenous ABA levels in the pea DELLA double mutant *la cry-s* were also found to be elevated in both leaf and internode tissue (Figures 6.9 and 6.10), and is in agreement to previous reports that found that the seeds of the rice DELLA mutant *slr1* contained higher levels of ABA compared to the WT (Ikeda et al., 2002). Due to the fact that DELLA proteins have been shown to feedback regulate GA biosynthesis, it is possible that there is a complex homeostatic mechanism operating between GA biosynthesis and signalling to regulate ABA production.

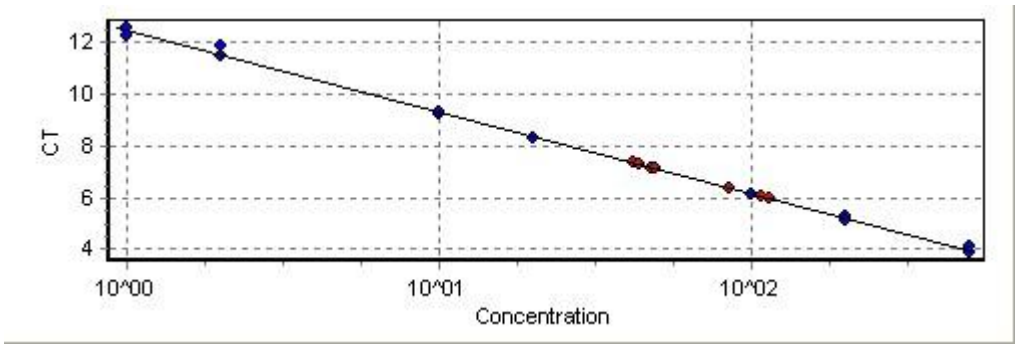
To explore the role of ABA and the DELLAs further, ABA was applied to *la CRY* and *la cry-s* seedlings and shoot growth monitored. It was found that the shoot growth was inhibited significantly in both genotypes (Figure 6.11). However, the difference between the extents of inhibition was not significant, indicating ABA does not act through the DELLAs to inhibit growth. This is not in agreement with the Achard et al. (2006) study that reported that the roots of the Arabidopsis quadruple-DELLA mutant treated with ABA were relatively resistant to the usual growth inhibition seen in WT after ABA application. However, it is possible that ABA interacts with GA signalling downstream from the DELLAs, in agreement with the study by Zentella et al. (2007).

There have also been reports of ABA regulating GA biosynthesis. In the seeds of the Arabidopsis ABA-deficient mutant *aba2* the expression of GA biosynthesis genes were found to be enhanced (Seo et al., 2006). However, when the expression of GA biosynthesis genes were monitored in ABA-treated pea shoots, no significant difference in expression was seen for any of the genes in comparison to the untreated shoots (Figure 6.6). Likewise, when GA levels were quantified, there was no significant difference in GA_1 levels, although there was an increase in the GA 2-oxidation product GA_{29} (Table 6.1). In pea shoots, at least, ABA does not regulate GA biosynthesis although it does inhibit growth.

In conclusion, it has been shown for the first time that GA and the DELLA proteins are regulators of ABA biosynthesis in shoots, but there is no evidence for ABA to act through the GA biosynthetic pathway or via the DELLA proteins to regulate shoot growth in pea.

6.5 Supplemental figures

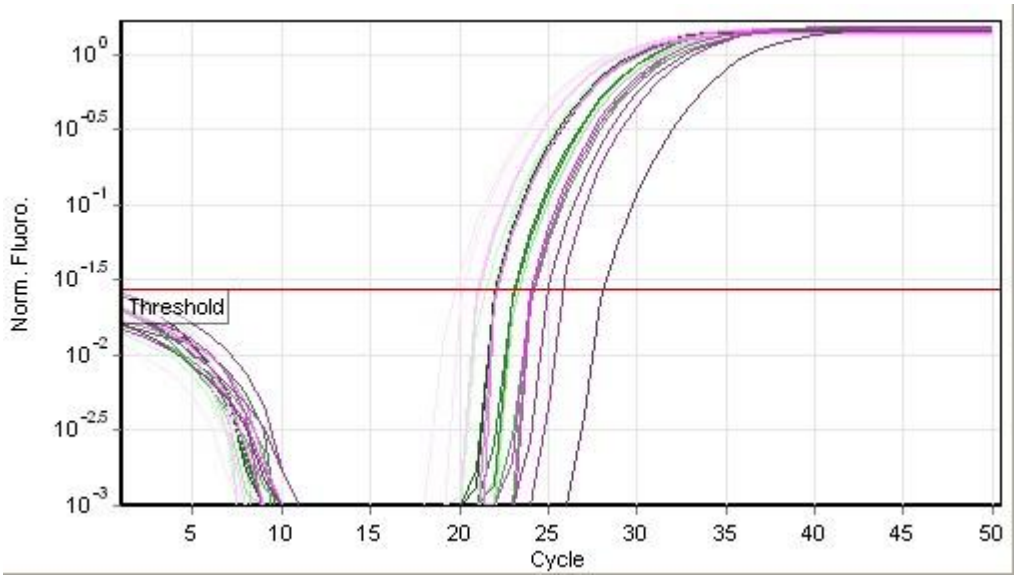


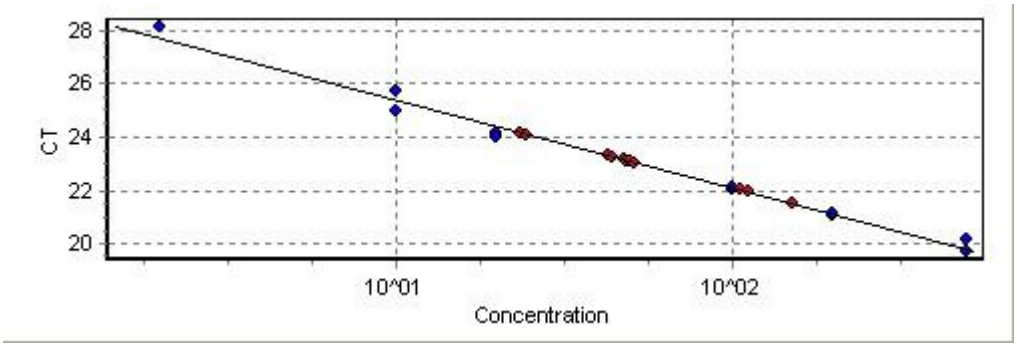


No.	Colour	Name	Type	Ct	Given Conc (ng/reaction)	Calc Conc (ng/reaction)	Rep. Ct
1		*18S* Control R1	Unknown	7.10		49.5232	7.12
2		*18S* Control R1	Unknown	7.15		47.9482	
3		*18S* Control R2	Unknown	7.26		44.1899	7.26
4		*18S* Control R2	Unknown	7.26		44.2505	
5		*18S* Control R3	Unknown	7.32		42.2488	7.29
6		*18S* Control R3	Unknown	7.26		44.2262	
7		*18S* ABA R1	Unknown	6.04		107.4235	6.00
8		*18S* ABA R1	Unknown	5.95		115.2265	
9		*18S* ABA R2	Unknown	7.10		49.7271	7.10

No.	Colour	Name	Type	Ct	Given Conc (ng/reaction)	Calc Conc (ng/reaction)	Rep. Ct
10		*18S* ABA R2	Unknown	7.11		49.2122	
11		*18S* ABA R3	Unknown	6.36		85.5318	6.36
12		*18S* ABA R3	Unknown	6.36		85.1578	
S1		*18S* 1/2	Standard	4.12	500.0000	441.2429	4.00
S2		*18S* 1/2	Standard	3.88	500.0000	524.9238	
S3		*18S* 1/5	Standard	5.24	200.0000	193.6842	5.19
S4		*18S* 1/5	Standard	5.15	200.0000	207.3549	
S5		*18S* 1/10	Standard	6.15	100.0000	99.3838	6.12
S6		*18S* 1/10	Standard	6.08	100.0000	104.5783	
S7		*18S* 1/50	Standard	8.30	20.0000	20.6472	8.29
S8		*18S* 1/50	Standard	8.28	20.0000	20.8976	
S9		*18S* 1/100	Standard	9.18	10.0000	10.8263	9.23
S10		*18S* 1/100	Standard	9.28	10.0000	10.0628	
S11		*18S* 1/500	Standard	11.42	2.0000	2.0980	11.63
S12		*18S* 1/500	Standard	11.84	2.0000	1.5387	
S13		*18S* 1/1000	Standard	12.52	1.0000	0.9331	12.37
S14		*18S* 1/1000	Standard	12.21	1.0000	1.1687	

Supplemental Figure 6.1 Quantitation Report of the standard curve of the *18S* gene and the *18S* transcript levels in control and 25 µM ABA-treated pea roots was generated by Rotor-Gene Q 1.7.94 © Qiagen 2003-2009.

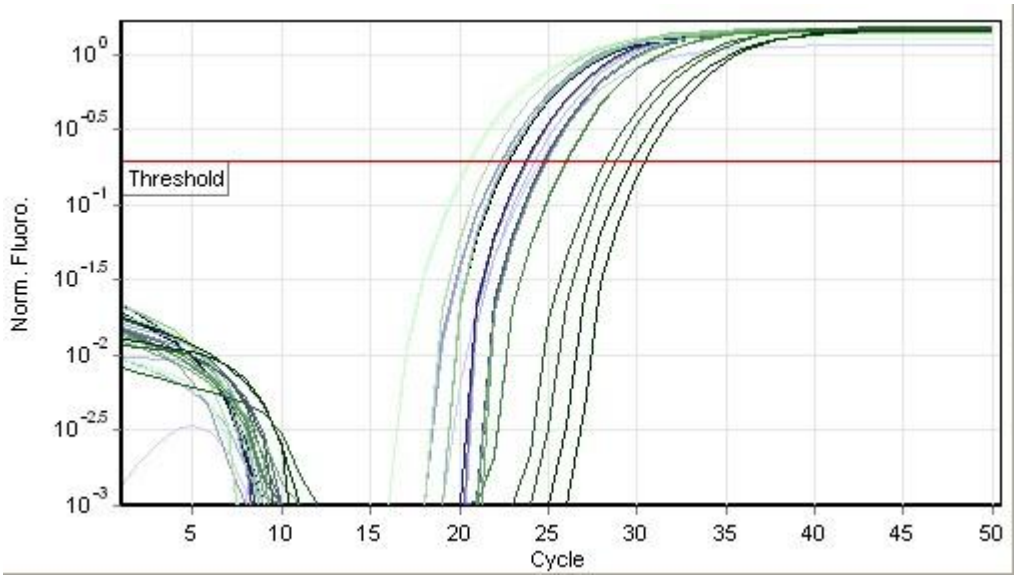


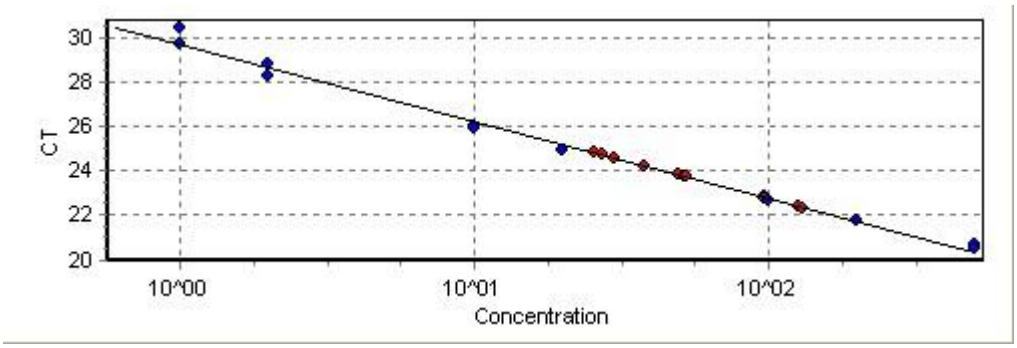


No.	Colour	Name	Type	Ct	Given Conc (ng/reaction)	Calc Conc (ng/reaction)	Rep. Ct
1		*20ox1* Control R1	Unknown	23.15		47.5457	23.20
2		*20ox1* Control R1	Unknown	23.25		44.3217	
3		*20ox1* Control R2	Unknown	21.93		111.7824	21.71
4		*20ox1* Control R2	Unknown	21.50		151.2332	
5		*20ox1* Control R3	Unknown	23.31		42.5532	23.20
6		*20ox1* Control R3	Unknown	23.09		49.8069	
7		*20ox1* ABA R1	Unknown	24.11		24.3950	24.14
8		*20ox1* ABA R1	Unknown	24.16		23.4736	

No.	Colour	Name	Type	Ct	Given Conc (ng/reaction)	Calc Conc (ng/reaction)	Rep. Ct
9		*20ox1* ABA R2	Unknown	23.12		48.4983	23.09
10		*20ox1* ABA R2	Unknown	23.05		51.1642	
11		*20ox1* ABA R3	Unknown	22.10		99.5238	22.06
12		*20ox1* ABA R3	Unknown	22.02		105.2689	
S1		*20ox1* 1/2	Standard	19.71	500.0000	525.9603	19.93
S2		*20ox1* 1/2	Standard	20.15	500.0000	386.9356	
S3		*20ox1* 1/5	Standard	21.04	200.0000	207.4033	21.08
S4		*20ox1* 1/5	Standard	21.12	200.0000	196.0331	
S5		*20ox1* 1/10	Standard	22.08	100.0000	100.8312	22.10
S6		*20ox1* 1/10	Standard	22.13	100.0000	97.4162	
S7		*20ox1* 1/50	Standard	24.15	20.0000	23.6674	24.08
S8		*20ox1* 1/50	Standard	24.01	20.0000	26.1524	
S9		*20ox1* 1/100	Standard	24.94	10.0000	13.6872	25.33
S10		*20ox1* 1/100	Standard	25.72	10.0000	7.8990	
S11		*20ox1* 1/500	Standard	28.13	2.0000	1.4709	28.13

Supplemental Figure 6.2 Quantitation Report of the standard curve of the *PsGA20ox1* gene and the *PsGA20ox1* transcript levels in control and 25 µM ABA-treated pea roots was generated by Rotor-Gene Q 1.7.94 © Qiagen 2003-2009.

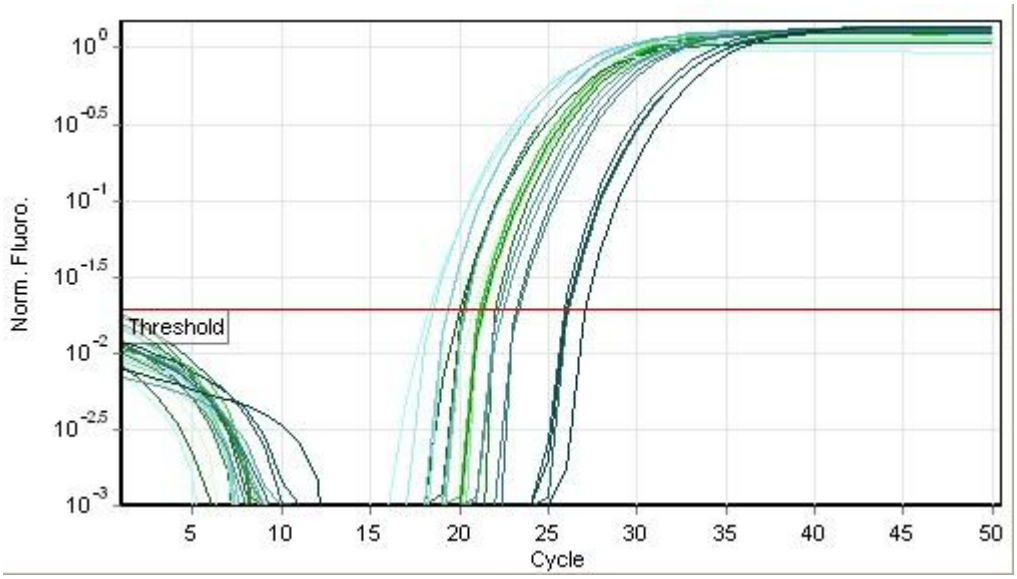


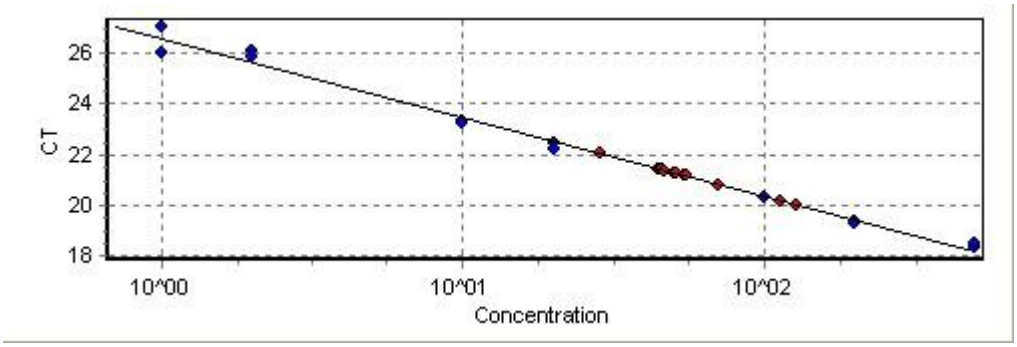


No.	Colour	Name	Type	Ct	Given Conc (ng/reaction)	Calc Conc (ng/reaction)	Rep. Ct
1		*3ox1* Control R1	Unknown	24.58		29.7134	24.41
2		*3ox1* Control R1	Unknown	24.23		37.6163	
3		*3ox1* Control R2	Unknown	22.42		125.1267	22.39
4		*3ox1* Control R2	Unknown	22.36		129.8850	
5		*3ox1* Control R3	Unknown	23.82		49.1296	23.77
6		*3ox1* Control R3	Unknown	23.72		52.6590	
7		*3ox1* ABA R1	Unknown	24.80		25.6610	24.76
8		*3ox1* ABA R1	Unknown	24.72		27.0865	
9		*3ox1* ABA R2	Unknown	23.74		51.9328	23.73

No.	Colour	Name	Type	Ct	Given Conc (ng/reaction)	Calc Conc (ng/reaction)	Rep. Ct
10		*3ox1* ABA R2	Unknown	23.73		52.4277	
11		*3ox1* ABA R3	Unknown	22.82		95.8810	22.81
12		*3ox1* ABA R3	Unknown	22.80		97.1556	
S1		*3ox1* 1/2	Standard	20.65	500.0000	403.7915	20.59
S2		*3ox1* 1/2	Standard	20.52	500.0000	439.4235	
S3		*3ox1* 1/5	Standard	21.75	200.0000	194.9363	21.75
S4		*3ox1* 1/5	Standard	21.74	200.0000	195.2386	
S5		*3ox1* 1/10	Standard	22.66	100.0000	106.1164	22.68
S6		*3ox1* 1/10	Standard	22.70	100.0000	103.6970	
S7		*3ox1* 1/50	Standard	24.90	20.0000	24.0900	24.90
S8		*3ox1* 1/50	Standard	24.90	20.0000	24.1274	
S9		*3ox1* 1/100	Standard	26.00	10.0000	11.5816	25.98
S10		*3ox1* 1/100	Standard	25.96	10.0000	11.9403	
S11		*3ox1* 1/500	Standard	28.25	2.0000	2.6091	28.56
S12		*3ox1* 1/500	Standard	28.86	2.0000	1.7401	
S13		*3ox1* 1/1000	Standard	30.45	1.0000	0.6048	30.09
S14		*3ox1* 1/1000	Standard	29.73	1.0000	0.9756	

Supplemental Figure 6.3 Quantitation Report of the standard curve of the *PsGA3ox1* gene and the *PsGA3ox1* transcript levels in control and 25 µM ABA-treated pea roots was generated by Rotor-Gene Q 1.7.94 © Qiagen 2003-2009.

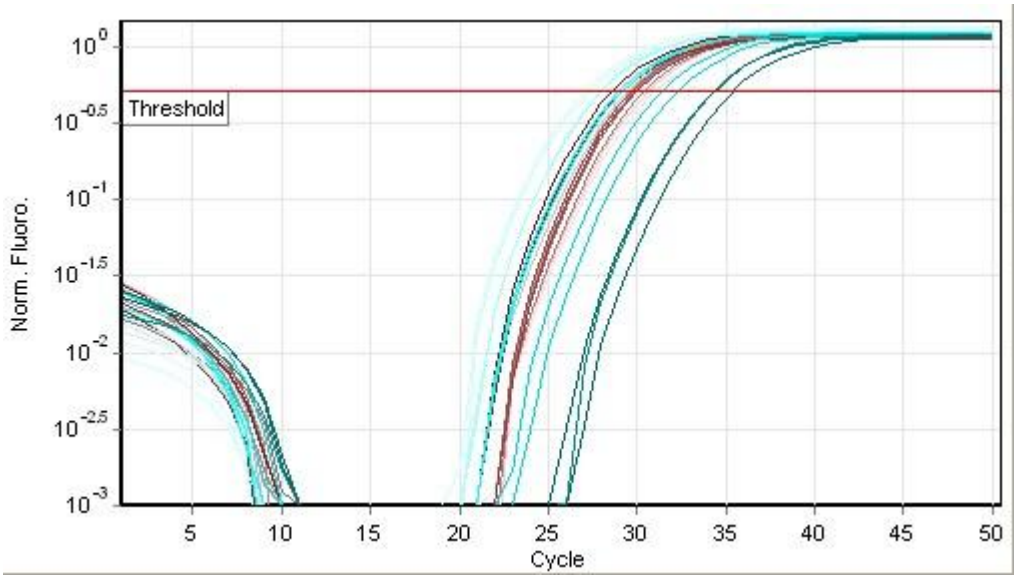


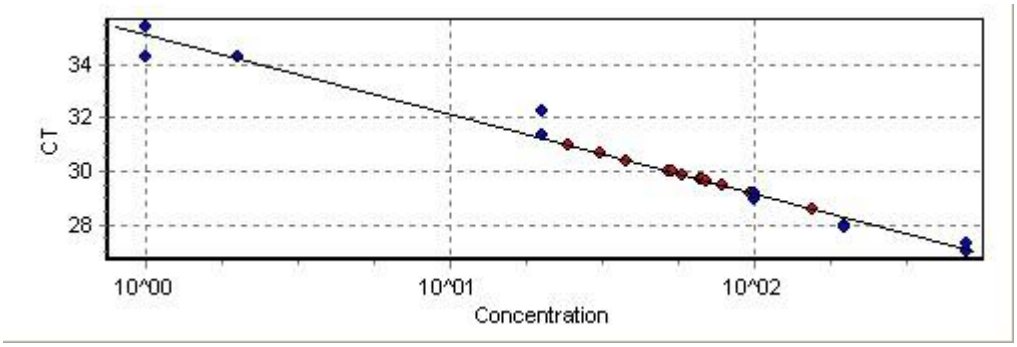


No.	Colour	Name	Type	Ct	Given Conc (ng/reaction)	Calc Conc (ng/reaction)	Rep. Ct
1		*2ox1* Control R1	Unknown	20.80		71.0185	20.97
2		*2ox1* Control R1	Unknown	21.14		55.1066	
3		*2ox1* Control R2	Unknown	21.44		44.3413	21.35
4		*2ox1* Control R2	Unknown	21.25		50.7269	
5		*2ox1* Control R3	Unknown	21.40		45.7164	21.40
6		*2ox1* Control R3	Unknown	21.41		45.3281	
7		*2ox1* ABA R1	Unknown	21.25		51.0068	21.20
8		*2ox1* ABA R1	Unknown	21.15		54.7741	
9		*2ox1* ABA R2	Unknown	21.36		46.8496	21.70
10		*2ox1* ABA R2	Unknown	22.03		28.6853	

No.	Colour	Name	Type	Ct	Given Conc (ng/reaction)	Calc Conc (ng/reaction)	Rep. Ct
11		*2ox1* ABA R3	Unknown	19.99		128.1716	20.08
12		*2ox1* ABA R3	Unknown	20.16		113.2458	
S1		*2ox1* 1/2	Standard	18.27	500.0000	457.0288	18.38
S2		*2ox1* 1/2	Standard	18.49	500.0000	388.2285	
S3		*2ox1* 1/5	Standard	19.31	200.0000	212.5249	19.29
S4		*2ox1* 1/5	Standard	19.28	200.0000	216.7768	
S5		*2ox1* 1/10	Standard	20.26	100.0000	105.1672	20.28
S6		*2ox1* 1/10	Standard	20.30	100.0000	102.0601	
S7		*2ox1* 1/50	Standard	22.48	20.0000	20.6193	22.35
S8		*2ox1* 1/50	Standard	22.23	20.0000	24.7863	
S9		*2ox1* 1/100	Standard	23.33	10.0000	11.0266	23.27
S10		*2ox1* 1/100	Standard	23.21	10.0000	12.0282	
S11		*2ox1* 1/500	Standard	26.10	2.0000	1.4301	25.97
S12		*2ox1* 1/500	Standard	25.83	2.0000	1.7424	
S13		*2ox1* 1/1000	Standard	26.01	1.0000	1.5315	26.54
S14		*2ox1* 1/1000	Standard	27.06	1.0000	0.7050	

Supplemental Figure 6.4 Quantitation Report of the standard curve of the *PsGA2ox1* gene and the *PsGA2ox1* transcript levels in control and 25 µM ABA-treated pea roots was generated by Rotor-Gene Q 1.7.94 © Qiagen 2003-2009.





No.	Colour	Name	Type	Ct	Given Conc (ng/reaction)	Calc Conc (ng/reaction)	Rep. Ct
1		*2ox2* Control R1	Unknown	30.99		24.4071	30.83
2		*2ox2* Control R1	Unknown	30.67		31.3270	
3		*2ox2* Control R2	Unknown	29.49		78.7515	29.59
4		*2ox2* Control R2	Unknown	29.69		67.3915	
5		*2ox2* Control R3	Unknown	29.65		69.5926	29.76
6		*2ox2* Control R3	Unknown	29.87		58.5800	
7		*2ox2* ABA R1	Unknown	30.42		38.0006	30.22
8		*2ox2* ABA R1	Unknown	30.02		52.1984	
9		*2ox2* ABA R2	Unknown	29.70		66.8370	29.84

No.	Colour	Name	Type	Ct	Given Conc (ng/reaction)	Calc Conc (ng/reaction)	Rep. Ct
10		*2ox2* ABA R2	Unknown	29.98		53.5844	
11		*2ox2* ABA R3	Unknown	29.21		97.6262	28.91
12		*2ox2* ABA R3	Unknown	28.61		155.4906	
S1		*2ox2* 1/2	Standard	27.03	500.0000	532.6981	27.17
S2		*2ox2* 1/2	Standard	27.31	500.0000	428.5365	
S3		*2ox2* 1/5	Standard	28.00	200.0000	250.9641	27.97
S4		*2ox2* 1/5	Standard	27.94	200.0000	263.4594	
S5		*2ox2* 1/10	Standard	29.01	100.0000	114.5320	29.10
S6		*2ox2* 1/10	Standard	29.20	100.0000	98.8922	
S7		*2ox2* 1/50	Standard	32.25	20.0000	9.1395	31.81
S8		*2ox2* 1/50	Standard	31.37	20.0000	18.1752	
S9		*2ox2* 1/500	Standard	34.28	2.0000	1.8909	34.28
S10		*2ox2* 1/1000	Standard	34.29	1.0000	1.8678	34.84
S11		*2ox2* 1/1000	Standard	35.38	1.0000	0.7976	

Supplemental Figure 6.5 Quantitation Report of the standard curve of the *PsGA2ox2* gene and the *PsGA2ox2* transcript levels in control and 25 µM ABA-treated pea roots was generated by Rotor-Gene Q 1.7.94 © Qiagen 2003-2009.

CHAPTER 7

Conclusion

Despite the relatively small number of hormones present in higher plants, these compounds are able to regulate a vast number of plant developmental, environmental, and metabolic responses (Kuppusamy et al., 2009; Bennett et al., 2005). Even more interesting is the ability of a number of hormones to regulate the same response, such as shoot growth, although they are usually unable to compensate for the loss of another hormone (Kuppusamy et al., 2009; Bennett et al., 2005). This diversity is due in part to the capacity of the hormones to interact, or cross-talk, in particular ways. An increased understanding in recent years of biosynthesis and signal transduction pathways of various plant hormones, including GA, is providing an important tool for understanding the interactions between these hormones and their role in plant physiology. In this thesis, interactions between auxin and GA in regulating root growth, and between ABA and GA in regulating shoot growth, were explored using the model species, pea.

A study by Yaxley et al. (2001) reported that the roots of the GA-deficient *le-1* mutant had similar root phenotypes and comparable GA_1 levels to the WT, and suggested that another GA 3-oxidase gene may be primarily expressed in the roots. Validating this suggestion, here it is revealed that indeed, another GA 3-oxidase gene (*PsGA3ox2*) is present in pea, with functional assays providing evidence for the ability of the protein to convert GA_{20} to GA_1. Furthermore, *PsGA3ox2* was shown to be more highly expressed in the root compared to shoot tissue in pea seedlings. The identification of this gene has provided a more detailed view on the genes involved in the GA biosynthetic pathway, and will allow more thorough investigations on future GA biosynthesis studies in pea.

In this thesis, evidence has been presented to show that auxin promotes GA biosynthesis in pea roots, as the application of the auxin action inhibitor PCIB results in downregulation of *PsGA20ox1, PsGA3ox1*, *PsGA3ox2* and the upregulation of *PsGA2ox1* and *PsGA2ox2*. This is the first time that this interaction has been demonstrated in roots, although previous studies have shown that it occurs in above-

ground parts of the plant. In pea stems, auxin has been shown to promote bioactive GA biosynthesis and to inhibit its catabolism, although 20-oxidation was not found to be regulated, unlike in the roots (Ross et al., 2000; O'Neill and Ross, 2003). However, this has been shown to be the same in tobacco (Wolbang and Ross, 2001) and barley (Wolbang et al., 2004). The new information reported here prompts a re-examination of the situation in the shoot. Given that GA 20-oxidation is strongly auxin-dependent in the root, why does shoot decapitation (and therefore auxin depletion) fail to substantially reduce 20-oxidation in internodes (Ross et al., 2000)? Alternatively, given that decapitated internodes become deficient in GA_1, why does this not lead, as a result of feedback, to a substantial increase in 20-oxidation? A likely explanation is that the opposing effects of auxin and feedback regulation are of approximately equal strength in pea internodes, and that these two effects "cancel" each other out after decapitation (O'Neill and Ross, 2002).

Contrary to the finding reported here that auxin at normal levels promotes GA synthesis, another study has reported that in roots auxin is required for the facilitation of the GA-degradation of DELLA proteins (Fu and Harberd, 2003). DELLA proteins are inhibitors of growth that are degraded by GA; thus GA acts as an inhibitor of an inhibitor. Here it is shown that in pea roots DELLA proteins up-regulate the expression of GA biosynthesis genes and inhibit that of GA deactivation genes; in other words, feedback and feed-forward regulate GA levels in roots. The finding that DELLAs promote the expression of GA synthesis genes in roots can be used to compare the "Hobart" and Fu and Harberd models on how auxin and GA interact to regulate growth. According to the Fu and Harberd theory, auxin (at optimal levels for growth) would be expected to inhibit GA biosynthesis; in other words, auxin would enhance the negative feedback of bioactive GA on its own biosynthesis. We would expect, then, that an auxin action inhibitor would have the opposite effects to auxin, promoting GA synthesis and reducing deactivation. However, this was not found. When PCIB or yokonolide B was applied to pea roots, the expression of *PsGA20ox1* was down-regulated and the deactivation genes *PsGA2ox1* and *PsGA2ox2* were up-regulated. Similar results were also obtained using the auxin-transport inhibitor NPA. This result was further supported by previous GA quantification results, which showed that the levels of GA_{20} and of GA_1 were significantly reduced by PCIB

(Weston et al., 2009). The down-regulation of GA biosynthesis by the inhibitors of auxin action and transport indicates that auxin (at normal levels) positively regulates GA synthesis in roots, as it does in shoots. This promotion of GA biosynthesis by auxin appears to override any inhibition of GA synthesis that might result from the capacity of auxin to facilitate GA-induced destabilisation of DELLA proteins (which positively regulates GA synthesis).

Furthermore, on closer inspection of the GFP:RGA results published by Fu and Harberd (2003), a discrepancy is seen. It is important to note that the theory put forth by Fu and Harberd (2003) stipulates that auxin acts by enhancing the capacity of GA to destabilize DELLA proteins, rather than by up-regulating GA levels (which would then destabilize DELLAs). The Fu and Harberd theory is based on evidence that a reduction in auxin content (or action) impedes the DELLA-destabilising ability of GA supplied exogenously, in non-limiting amounts. Thus, if the Fu and Harberd model is correct, then the GFP:RGA present in the decapitated GA-treated roots should be comparable to that seen for the decapitated control roots, as the decapitation reduces IAA content, and so should not be able to facilitate the GA-induced RGA degradation. This is not seen (Figure 7.1); in fact, GA strongly destabilised the GFP-DELLA protein, even though IAA levels were (presumably) low. Thus the figure more strongly supports the model of auxin-GA interaction presented here. For this model, it would be expected that the expression of GFP:RGA would decrease when the roots are treated with GA, as GA biosynthesis is by-passed with the application of the bioactive GA used, and indeed, this is what is observed.

According to both the Fu and Harberd (2003) model, and the model presented here, auxin action on root elongation is mediated, at least in part, by GAs. The finding that PCIB was less effective at retarding elongation in the DELLA mutant *la cry-s* than in the WT provides further evidence that GAs do indeed mediate auxin action, although this result in itself does not differentiate between an affect of auxin on GA synthesis and an effect on GA signalling. Interestingly, in *la cry-s* plants an inhibition in growth by PCIB was still observed, indicating that auxin reduces root elongation to some extent independently of GAs. Fu and Harberd (2003) also observed some effect

of NPA on the elongation of *gai-t6 rga-24* roots, which show GA-saturated growth, indicating that in Arabidopsis also, auxin acts independently of GAs to some extent.

It is well known that supra-optimal levels of auxin result in an inhibition of root elongation. Although this effect has been known for some time, its physiological basis has remained elusive. Here it is shown that the application of 10 µM IAA to the roots results in a small but significant reduction in GA_1 and GA_8 content. These results indicate that the reduction in root elongation (Eliasson et al., 1989) may in part be a result of the reduced GA_1. In addition, this result provides a potential explanation for the promotion by auxin of GA 2-oxidase (deactivation) genes in Arabidopsis observed by Frigerio et al. (2006). It is possible that the concentration of auxin used in that study (50 µM NAA) may have been supra-optimal for growth and for other processes, at least in the roots. In this study, it was seen that levels of exogenous auxin that are supra-optimal for growth upregulate GA 2-oxidase (deactivation) genes. It is possible that the down-regulation of GA biosynthesis by supra-optimal auxin levels is mediated by ethylene, as recent evidence indicates that ethylene can down-regulate GA synthesis (Foo et al., 2006; Achard et al., 2007).

The majority of researchers who have studied ABA-GA interactions in higher plants have reported that GA is an inhibitor of ABA biosynthesis. Contrary to these studies, however, evidence provided here shows consistently that in pea shoots and leaves, normal GA levels are required for the promotion of ABA biosynthesis. ABA levels were quantified in the leaves of both the *le-3* and *ls-1* GA-deficient mutants and were found to be reduced by more than half compared with the leaves of the WT. A separate experiment that quantified ABA in the apical portion of the shoot of *le-3* seedlings also provided similar results. Interestingly, the results presented here create a paradox: if ABA is an inhibitor of growth and GA a promoter, how can GA promote ABA biosynthesis? This can be explained by the fact that ABA and GA share the early steps of their biosynthetic pathways (Nambara and Marion-Poll, 2005). The same phenomenon was also seen by Norman et al. (1983 and 1986) and they also concluded that the shared biosynthetic pathway was the plausible reason for these unusual results. Furthermore, it could be that the rise in ABA levels in the mutants is not enough to be biologically significant, as it was shown here that a 20-

fold increase in ABA levels only resulted in a 33% inhibition of shoot growth compared to control plants, a small change considering the dramatic increase in ABA levels.

Previous reports have also suggested that the opposite interaction, that ABA regulates GA biosynthesis, can occur. In the seeds of the Arabidopsis ABA-deficient *aba2*, GA biosynthesis was found to be upregulated (Seo et al., 2006). However, no evidence of this interaction was found from the shoots of pea. No significant changes were seen in the expression of GA synthesis or catabolism genes in the shoots after ABA application. In addition, there was no significant change in the levels of the bioactive GA, GA_1. It is possible, however, that this interaction may occur in different developmental stages, such as seed germination, as reported previously.

It has been suggested that ABA may act through the GA signalling pathway to inhibit growth. Currently, there are two suggestions on how this may occur: Achard et al. (2006) suggest that ABA acts through the DELLAs to inhibit root growth in Arabidopsis, whereas Zentella et al. (2007) suggests that ABA interacts with GA signalling downstream of the DELLAs. The results presented in this thesis are not in agreement with the Achard et al. (2006) model. When ABA was applied to *la CRY* and *la cry-s* seedlings shoot growth was inhibited, but the difference between the extents of inhibition was not significant, indicating ABA does not act through the DELLAs to inhibit growth. Although no evidence has been provided to support the Zentella et al. (2007) model, no evidence is provided to negate it, so it is possible that ABA interacts with GA signalling downstream from the DELLAs.

One recurring theme that has been seen throughout both the auxin-GA and ABA-GA interaction studies is the differing reports on the interactions occurring not only across species, but also between tissues in the same plant. For example, in Arabidopsis it has been reported that auxin facilitates the GA-degradation to regulate root growth (Fu and Harberd, 2003), but we found no evidence to suggest that this interaction occurs in roots of pea. Furthermore, previous studies on the auxin-GA interaction in pea found that *PsGA20ox1* did not seem to be regulated by auxin in pea shoots (Ross et al., 2000), although it is in the roots. In regards to the ABA-GA

interactions, there were a number of contrasting reports in relation to how these two hormones interact. For instance, Achard et al. (2006) found that the roots of an Arabidopsis quadruple DELLA mutant are more resistant to ABA than the WT, but in pea shoots, this is not the case. These studies show that hormone interactions are largely variable and thus care must be taken when assuming the interactions are consistent for all other species and tissues.

In conclusion, the results presented in this thesis have clarified a number of issues on auxin-GA and ABA-GA interactions and how they influence the growth of young pea seedlings, as summarised in Figure 7.2.

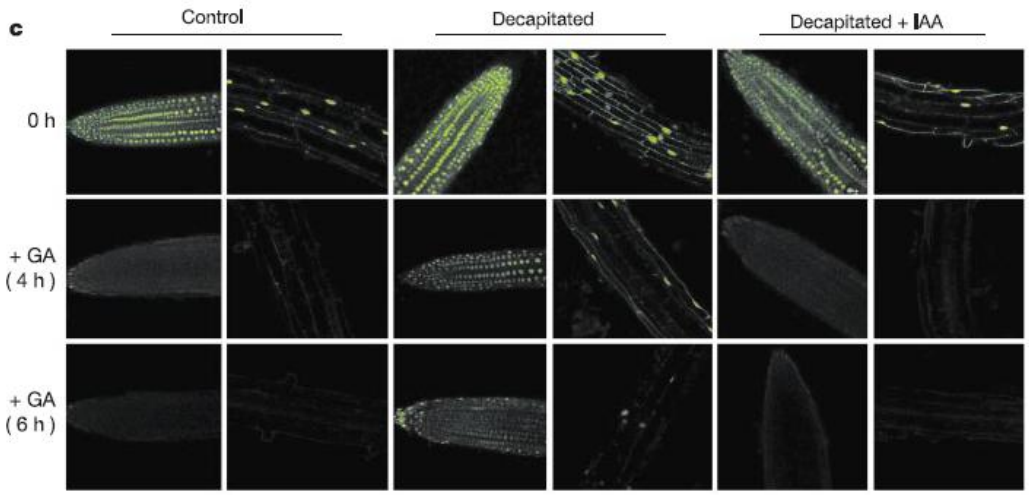


Figure 7.1 Results taken from Fu and Harberd (2003) showing GFP:RGA expression of GA-treated (1mM GA3) pRGA:GFP-RGA Arabidopsis seedling root tips (left) and the elongation zone (right).

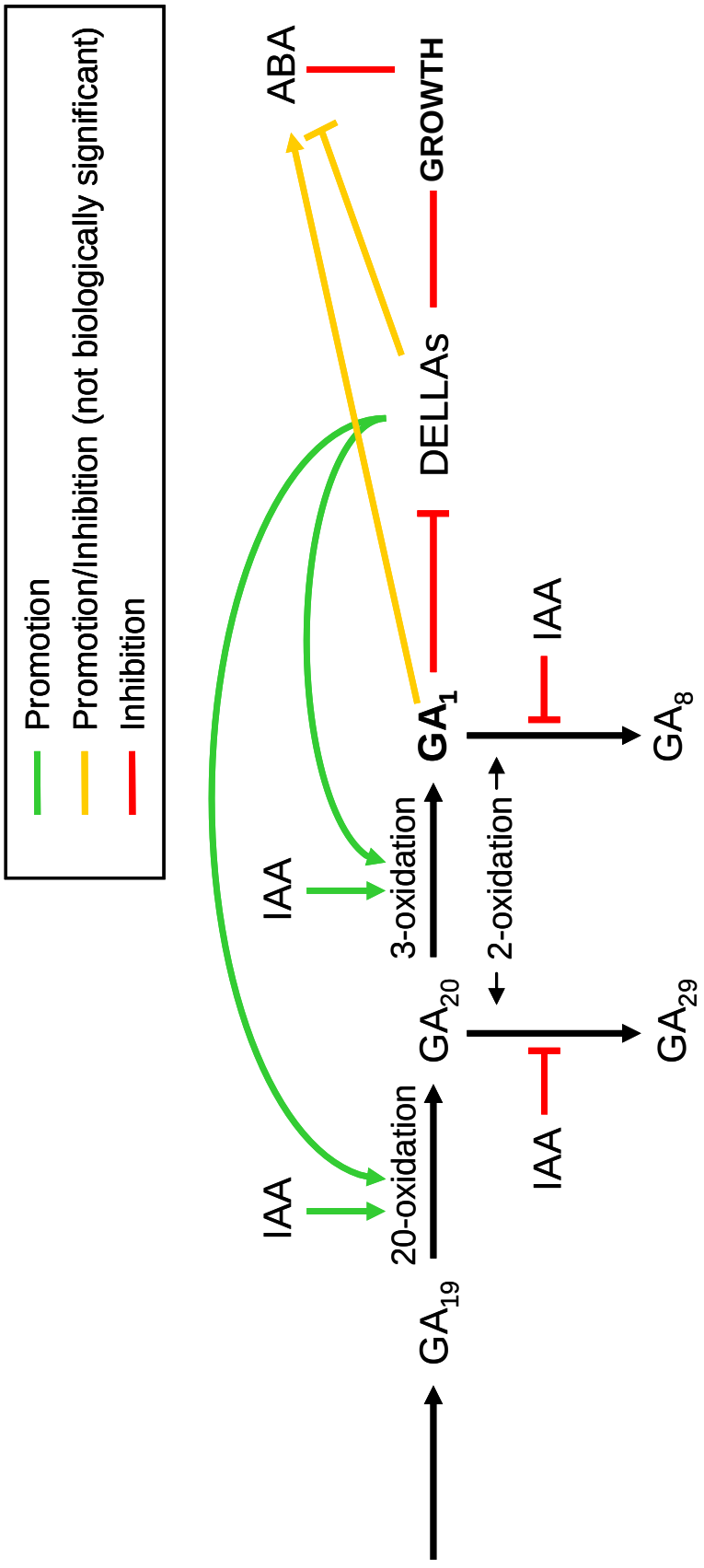


Figure 7.2 Diagram of hormone interactions studied in relation to the regulation of the GA biosynthesis pathway and growth.

REFERENCES

Achard P, Baghour M, Chapple A, Hedden P, Van Der Straeten D, Genschik P, Moritz T, Harberd NP (2007) The plant stress hormone ethylene controls floral transition via DELLA-dependent regulation of floral meristem-identify genes. *PNAS* **104**, 6484-6489.

Achard P, Cheng H, De Grauwe L, Decat J, Schoutteten H, Moritz T, Van Der Straeten D, Peng J, Harberd NP (2006) Integration of plant responses to environmentally activated phytohormonal signals. *Science* **311**, 91-94.

Achard P, Vriezen WH, Van Der Straeten D, Harberd NP (2003) Ethylene regulates *Arabidopsis* development via the modulation of DELLA protein growth repressor function. *The Plant Cell* **15**, 2816-2825.

Ait-Ali T, Frances S, Weller JL, Reid JB, Kendrick RE, Kamiya Y (1999) Regulation of gibberellin 20-oxidase and 3β-hydroxylase transcript accumulation during de-etiolation of pea seedling. *Plant Physiology* **121**, 783-791.

Ait-Ali T, Swain SM, Reid JB, Sun TP, Kamiya Y (1997) The *LS* locus of pea encodes the gibberellin biosynthesis enzyme ent-kaurene synthase A. *The Plant Journal* **11**, 443-454.

Alvey L, Harberd NP (2005) DELLA proteins: integrators of multiple plant regulatory inputs? *Physiologia Plantarum* **123**, 153-160.

Ayele BT, Ozga JA, Reinecke DM (2006) Regulation of GA biosynthesis genes during germination and young seedling growth of pea (*Pisum sativum* L.). *Journal of Plant Growth Regulation* **25**, 219-232.

Barratt NM, Davies PJ (1997) Developmental changes in the gibberellin-induced growth response in stem segments of light-grown pea genotypes. *Plant Growth Regulation* **21**, 127-134.

Batge SL, Ross JJ, Reid JB (1999) Abscisic acid levels in seeds of the gibberellin-deficient mutant *lh-2* of pea (*Pisum sativum*). *Physiologia plantarum* **105**, 485-490.

Bennett M, Bellini C, Van Der Straeten D (2005) Integrative biology: dissecting cross-talk between plant signalling pathways. *Physiologia Plantarum* **123**, 109.

Beveridge C (2006) Advances in the control of axillary bud outgrowth: sending a message. *Current Opinion in Plant Biology* **9**, 35-40.

Biswas KK, Ooura C, Higuchi K, Miyazaki Y, Van Nguyen V, Rahman A, Uchimiya H, Kiyosue T, Koshiba T, Tanaka A, Narumi I, Oono Y (2007) Genetic characterization of mutants resistant to the antiauxin *p*-chlorophenoxyisobutyric acid (PCIB) reveals that *AAR3*, a gene encoding a DCN1-like protein, regulates responses to the synthetic auxin 2,4-dichlorophenoxyacetic acid in Arabidopsis roots. *Plant Physiology* **145**, 773-785.

Bouquin T, Meier C, Foster R, E. NM, Mundy J (2001) Control of specific gene expression by gibberellin and brassinosteroid. *Plant Physiology* **127**, 450-458.

Brenner WG, Romanov GA, Köllmer I, Bürkle L, Schmülling T (2005) Immediate-early and delayed cytokinin response genes of *Arabidopsis thaliana* identified by genome-wide expression profiling reveal novel cytokinin-sensitive processes and suggest cytokinin action through transcriptional cascades. *The Plant Journal* **44**, 314-333.

Brian P, Hemming H (1955) The effect of gibberellic acid on shoot growth of pea seedlings. *Physiologia plantarum* **8**, 669-681.

Brian PW (1957) The effects of some microbial metabolic products on plant growth. *Symposia of the Society for Experimental Biology* **11**, 166-181.

Chandler PM, Marion-Poll A, Ellis M, Gubler F (2002) Mutants at the *Slender1* locus of barley cv Himalaya. Molecular and physiological characterization. *Plant Physiology* **129**, 181-190.

Croker SJ, Hedden P, Lenton JR, Stoddart JL (1990) Comparison of gibberellins in normal and slender barley seedlings. *Plant Physiology* **94**, 194-200.

Davidson SE, Elliott RC, Helliwell CA, Poole AT, Reid JB (2003) The pea gene *NA* encodes *ent*-kaurenoic acid oxidase. *Plant Physiology* **131**, 335-344.

Davidson SE, Smith JJ, Helliwell CA, Poole AT, Reid JB (2004) The pea gene *LH* encodes *ent*-kaurene oxidase. *Plant Physiology* **134**, 1123-1134.

Davies PJ (2004) (Ed.)^(Eds) 'Plant Hormones - Biosynthesis, signal transduction, action! (3rd edn).' (Kluwer Academic Publishers: The Netherlands)

de Haan H (1927) Length-factors in *Pisum*. *Genetica* **9**, 481-498.

Desgagné-Penix I, Eakanunkul S, Coles JP, Phillips AL, Hedden P, Sponsel VM (2005) The auxin *transport inhibitor response 3* (tir3) allele of *BIG* and auxin transport inhibitors affect the gibberellin status of *Arabidopsis*. *The Plant Journal* **41**, 231-242.

Desgagné-Penix I, Sponsel VM (2008) Expression of *gibberellin 20-oxidase1* (*AtGA20ox1*) in *Arabidopsis* seedlings with altered auxin status is regulated at multiple levels. *Journal of Experimental Botany* **59**, 2057-2070.

Dill A, Jung H, Sun T (2001) The DELLA motif is essential for gibberellin-induced degredation of RGA. *PNAS* **98**, 14162-14167.

Dill A, Sun T (2001) Synergistic depression of gibberellin signaling by removing RGA and GAI function in *Arabidopsis thaliana*. *Genetics* **159**, 777-785.

Dill A, Thomas SG, Hu JH, Steber CM, Sun TP (2004) The *Arabidopsis* F-box protein SLEEPY1 targets gibberellin signaling repressors for gibberellin-induced degradation. *The Plant Cell* **16**, 1392-1405.

Eisenreich W, Rohdich F, Bacher A (2001) Deoxyxylulose phosphate pathway to terpenoids. *Trends in Plant Science* **6**, 78-84.

Elliott RC, Ross JJ, Smith JJ, Lester DR, Reid JB (2001) Feed-forward regulation of gibberellin deactivation in pea. *Journal of Plant Growth Regulation* **20**, 87-94.

Evans ML, Ishikawa H, Estelle MA (1994) Responses of *Arabidopsis* roots to auxin studied with high temporal resolution: comparison of wild type and auxin-response mutants. *Planta* **194**, 215-222.

Frigerio M, Alabadí D, Pérez-Gómez J, García-Cárcel L, Phillips AL, Hedden P, Blázquez MA (2006) Transcriptional regulation of gibberellin metabolism genes by auxin signaling in Arabidopsis. *Plant Physiology* **142**, 553-563.

Frohman MA, Dush MK, Martin GR (1988) Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proceedings of the National Academy of Sciences* **85**, 8998-9002.

Fu X, Harberd NP (2003) Auxin promotes *Arabidopsis* root growth by modulating gibberellin response. *Nature* **421**, 740-743.

Gomez-Roldan V, Fermas S, PB B, Puech-Pagès V, Dun E, Pillot J, Letisse F, Matusova R, Danoun S, Portais J, Bouwmeester H, Bécard G, Beveridge C, Rameau C, SF R (2008) Strigolactone inhibition of shoot branching. *Nature* **455**, 189-194.

Greenboim-Wainberg Y, Maymon I, Borochov R, Alvarez J, Olszewski N, Ori N, Eshed Y, Weiss D (2005) Cross talk between gibberellin and cytokinin: the Arabidopsis GA response inhibitor SPINDLY plays a positive role in cytokinin signaling. *The Plant Cell* **17**, 92-102.

Griffiths A, Miller J, Suzuki D, Lewontin R, Gelbart W (2000) 'An introduction to genetics analysis.' (W. H. Freeman and Company: New York)

Gubler F, Chandler PM, White RG, Llewellyn DJ, Jacobsen JV (2002) Gibberellin signaling in barley aleurone cells. Control of SLN1 and GAMYB expression. *Plant Physiology* **129**, 191-200.

Harberd NP, King KE, Carol P, Cowling RJ, Peng J, Richards DE (1998) Gibberellin: inhibitor of an inhibitor of...? *BioEssays* **20**, 1001-1008.

Hay A, Kaur H, Phillips A, Hedden P, Hake S, Tsiantis M (2002) The gibberellin pathway mediates KNOTTED1-Type homeobox function in plants with different body plans. *Current Biology* **12**, 1557-1565.

Hayashi K, Jones AM, Ogino K, Yamazoe A, Oono Y, Inoguchi M, Kondo H, Nozaki H (2003) Yokonolide B, a novel inhibitor of auxin action, blocks degredation of AUX/IAA factors. *The Journal of Biological Chemistry* **278**, 23797-23806.

Hedden P, Phillips AL (2000) Gibberellin metabolism: new insights revealed by the genes. *Trends in Plant Science* **5**, 523-530.

Hedden P, Proebsting WM (1999) Genetic analysis of gibberellin biosynthesis. *Plant Physiology* **119**, 365-370.

Hopkins WG (1999) 'Introduction to plant physiology.' (John Wiley & Sons, Inc.: Brisbane)

Ikeda A, Sonoda Y, Vernieri P, Perata P, Hirochika H, Yamaguchi J (2002) The *slender* rice mutant, with constitutively activated gibberellin signal transduction, has enhanced capacity for abscisic acid level. *Plant and Cell Physiology* **43**, 974-979.

Ikeda A, Ueguchi-Tanaka M, Sonoda Y, Kitano H, Koshioka M, Futsuhara Y, Matsuoka M, Yamaguchi J (2001) *slender* rice, a constitutive gibberellin response mutant, is caused by a null mutation of the *SLR1* gene, an ortholog of the height-regulating gene *GAI/RGA/RHT/D8*. *The Plant Cell* **13**, 999-1010.

Ingram T, Reid JB (1987) Internode length in *Pisum*. Gene *na* may block gibberellin synthesis between *ent*-7α-hydroxykaurenoic acid and gibberellin A_{12}-aldehyde. *Plant Physiology* **83**, 1048-1053.

Ingram T, Reid JB, Murfet IC, Gaskin P, Willis CL, MacMillan J (1984) Internode length in *Pisum*: the *Le* gene controls the 3β-hydroxylation of gibberellin A20 to gibberellin A1. *Planta* **160**, 455-463.

Itoh H, Ueguchi-Tanaka M, Sato Y, Ashikari M, Matsuoka M (2002) The gibberellin signaling pathway is regulated by the appearance and disappearance of SLENDER RICE1 in nuclei. *The Plant Cell* **14**, 57-70.

Jacobson SE, Olszewski NE (1993) Mutations at the *SPINDLY* locus of *Arabidopsis* alter gibberellin signal transduction. *The Plant Cell* **5**, 887-896.

Jager CE (2006) Brassinosteroids and hormone interactions in pea. University of Tasmania.

Jager CE, Symons GM, Ross JJ, Smith JJ, Reid JB (2005) The brassinosteroid growth response in pea is not mediated by changes in gibberellin content. *Planta* **221**, 141-148.

Jasinski S, Piazza P, Craft J, Hay A, Woolley L, Rieu I, Phillips A, Hedden P, Tsiantis M (2005) KNOX action in *Arabidopsis* is mediated by coordinate regulation of cytokinin and gibberellin activities. *Current Biology* **15**, 1-6.

Kamiya Y, Graebe JE (1983) The biosynthesis of all major pea gibberellins in a cell-free system from *Pisum sativum*. *Phytochemistry* **22**, 681-689.

Kasahara H, Hanada A, Kuzuyama T, Takagi M, Kamiya Y, Yamaguchi S (2002) Contribution of the mevalonate and methylerythritol phosphate pathways to the biosynthesis of gibberellins in *Arabidopsis*. *Journal of Biological Chemistry* **277**, 45188-45194.

King KE, Moritz T, Harberd NP (2001) Gibberellins are not required for normal stem growth in *Arabidopsis thaliana* in the absence of GAI and RGA. *Genetics* **159**, 767-776.

Kuppusamy KT, Walcher CL, Nemhauser JL (2009) Cross-regulatory mechanisms in hormone signaling. *Plant Molecular Biology* **69**, 375-381.

Law DM (1987) Gibberellin-enhanced indole-3-acetic acid biosynthesis: D-Tryptophan as the precursor of indole-3-acetic acid. *Physiologia plantarum* **70**, 626-632.

Law DM, Hamilton RH (1984) Effects of gibberellic acid on endogenous indole-3-acetic acid and indoleacetyl aspartic acid levels in a dwarf pea. *Plant Physiology* **75**, 255-256.

Law DM, Hamilton RH (1989) Reduction in the free indole-3-acetic acid levels in Alaska pea by the gibberellin biosynthesis inhibitor uniconazol. *Physiologia plantarum* **76**, 535-538.

Lawrence NL, Ross JJ, Mander LN, Reid JB (1992) Internode length in *Pisum.* Mutants *lk, lka* and *lkb* do not accumulate gibberellins. *Journal of Plant Growth Regulation* **11**, 1-3.

Lester DR, MacKenzie-Hose AK, Davies PJ, Ross JJ, Reid JB (1999a) The influence of the null *le-2* mutation on gibberellin levels in developing pea seeds. *Plant Growth Regulation* **27**, 83-89.

Lester DR, Ross JJ, AitAli T, Martin DN, Reid JB (1996) A gibberellin 20-oxidase cDNA (Accession No. U58830) from pea seed (PGR 96-050). *Plant Physiology* **111**, 1353.

Lester DR, Ross JJ, Davies PJ, Reid JB (1997) Mendel's stem length gene (*Le*) encodes a gibberellin 3ß-hydroxylase. *The Plant Cell* **9**, 1435-1443.

Lester DR, Ross JJ, Smith JJ, Elliott RC, Reid JB (1999b) Gibberellin 2-oxidation and the *SLN* gene of Pisum sativum. *The Plant Journal* **19**, 65-73.

Ljung K, Hull AK, Celenza J, Yamada M, Estelle M, Normanly J, Sandberg G (2005) Sites and regulation of auxin biosynthesis in *Arabidopsis* roots. *The Plant Cell* **17**, 1090-1104.

MacMillan J (1997) Biosynthesis of the gibberellin plant hormones. *Natural Product Reports* **14**, 221-244.

Martin DN, Proebsting WM, Hedden P (1997) Mendel's dwarfing gene: cDNAs from the Le alleles and function of the expressed proteins. *PNAS* **94**, 8907-8911.

Martin DN, Proebsting WM, Hedden P (1999) The *SLENDER* gene of pea encodes a gibberellin 2-oxidase. *Plant Physiology* **121**, 775-781.

Martin DN, Proebsting WM, Parks TD, Dougherty WG, Lange T, Lewis MJ, Gaskin P, Hedden P (1996) Feed-back regulation of gibberellin biosynthesis and gene expression in *Pisum sativum* L. *Planta* **200**, 159-166.

Mendel G (1866) Veruche über Pflazen-hybriden. *Verh. Natforsch. Ver. Brünn* **4**, 3-47. (For the English version, see: Druery, C. T and William Bateson (1901) Experiments in plant hybridization. *Journal of the Royal Horticultural Society* **26**, 1-32 http://www.esp.org/foundations/genetics/classical/gm-65.pdf)

Moore R, Dickey K (1985) Growth and graviresponsiveness of primary roots of *Zea mays* seedlings deficient in abscisic acid and gibberellic acid. *Journal of Experimental Botany* **36**, 1793-1798.

Nadhzimov UK, Jupe SC, Jones MG, Scott IM (1988) Growth and gibberellin relations of the extreme dwarf d^x tomato mutant. *Physiologia plantarum* **73**, 252-256.

Nambara E, Marion-Poll A (2005) Abscisic acid biosynthesis and catabolism. *Annual Review of Plant Biology* **56**, 165-185.

Namhauser JL, Chory J (2004) BRing it on: new insights into the mechanism of brassinosteroid action. *Journal of Experimental Botany* **55**, 265-270.

Nitsch LMC, Oplaat C, Feron R, Ma Q, Wolters-Arts M, Hedden P, Mariani C, Vriezen WH (2009) Abscisic acid levels in tomato ovaries are regulated by *LeNCED1* and *SlCYP707A1*. *Planta* **229**, 1335-1346.

Norman SM, Bennett RD, Poling SM, Maier VP, Nelson MD (1986) Paclobutrazol inhibits abscisic acid biosynthesis in *Cercospora rosicola*. *Plant Physiology* **80**, 122-125.

Norman SM, Poling SM, Maier VP, Orme ED (1983) Inhibition of abscisic acid biosynthesis in *Cercospora rosicola* by inhibitors of gibberellin biosynthesis and plant growth retardants. *Plant Physiology* **71**, 15-18.

Oh E, Yamaguchi S, Hu J, Yusuke J, Jung B, Paik I, Lee H, Sun T, Kamiya Y, Choi G (2007) PIL5, a phytochrome-interacting bHLH protein, regulates gibberellin responsiveness by binding directly to the *GAI* and *RGA* promoters in *Arabidopsis* seeds. *The Plant Cell* **19**, 1192-1208.

Olszewski N, Sun T, Gubler F (2002) Gibberellin signalling: biosynthesis, catabolism, and response pathways. *The Plant Cell* **Supplement**, S61-S80.

O'Neill DP, Ross JJ (2002) Auxin regulation of the gibberellin pathway in pea. *Plant Physiology* **130**, 1974-1982.

O'Neill DP, Ross JJ, Reid JB (2000) Changes in gibberellin A1 levels and response during de-etiolation of pea seedlings. *Plant Physiology* **124**, 805-812.

Oono Y, Ooura C, Rahman A, Aspuria ET, Hayashi K, Tanaka A, Uchimiya H (2003) *p*-Chlorophenoxyisobutyric acid impairs auxin response in *Arabidopsis* root. *Plant Physiology* **133**, 1135-1147.

Otsuka M, Kenmoku H, Ogawa M, Okada K, Mitsuhashi W, Sassa T, Kamiya Y, Toyomasu T, Yamaguchi S (2004) Emission of *ent*-kaurene, a diterpenoid hydrocarbon precursor for gibberellins, into the headspace from plants. *Plant and Cell Physiology* **45**, 1129-1138.

Ozga JA, Reinecke DM, Ayele BT, Ngo P, Nadeau C, Wickramarathna A (2009) Developmental and hormonal regulation of gibberellin biosynthesis and catabolism in pea fruit. *Plant Physiology* **150**, 448-462.

Ozga JA, Yu J, Reinecke DM (2003) Pollination-, development-, and auxin specific regulation of gibberellin 3ß-hydroxylase gene expression in pea fruits and seeds. *Plant Physiology* **131**, 1137-1146.

Paleg LG (1965) Physiological effects of gibberellins. *Annual Review of Plant Physiology* **16**, 291-322.

Peng J, Carol P, Richards DE, King KE, Cowling RJ, Murphy GP, Harberd NP (1997) The *Arabidopsis GAI* gene defines a signaling pathway that negatively regulates gibberellin responses. *Genes and Development* **11**, 3194-3205.

Peng J, Richards DE, Hartley NM, Murphy GP, Devos KM, Flintham JE, Beales J, Fish LJ, Worland AJ, Pelica F, Sudhakar D, Christou P, Snape JW, Gale MD, Harberd NP (1999) 'Green revolution' genes encode mutant gibberellin response modulators. *Nature* **400**, 256-261.

Phinney BO (1983) The history of gibberellins. In 'The biochemistry and physiology of gibberellins'. (Ed. A Crozier) pp. 15-52. (Praeger: New York)

Potts WC, Reid JB (1983) Internode length in *Pisum:* III. The effect and interaction of the *Na/na* and *Le/le* gene differences on endogenous gibberellin-like substances. *Plant Physiology* **57**, 448-454.

Potts WC, Reid JB, Murfet IC (1985) Internode length in *Pisum.* Gibberellins and the slender phenotype. *Physiologia Plantarum* **63**, 357-364.

Reid JB (1986) Internode length in *Pisum.* Three further loci, lh, ls and lk. *Annals of Botany* **57**, 577-592.

Reid JB, Botwright NA, Smith JJ, O'Neill DP, Kerckhoffs LHJ (2002) Control of gibberellin levels and gene expression during de-etiolation in pea. *Plant Physiology* **128**, 734-741.

Reid JB, Murfet IC, Potts WC (1983) Internode length in *Pisum*. II. Additional information on the relationship and action of loci *Le, La, Cry, Na* and *Lm*. *Journal of Experimental Botany* **34**, 349-364.

Reid JB, Ross JJ (1993) A mutant approach, using *Pisum sativum*, to understanding plant growth. *International Journal of Plant Sciences* **154**, 22-34.

Ross JJ (1998) Effects of auxin transport inhibitors on gibberellins in pea. *Journal of Plant Growth Regulation* **17**, 141-146.

Ross JJ, MacKenzie-Hose AK, Davies PJ, Lester DR, Twitchin B, Reid JB (1999) Further evidence for feedback regulation of gibberellin biosynthesis in pea. *Physiologia Plantarum* **105**, 532-538.

Ross JJ, Murfet IC, Reid JB (1997) Gibberellin mutants. *Physiologia Plantarum* **100**, 550-560.

Ross JJ, O'Neill DP, Rathbone DA (2003) Auxin-gibberellin interactions in pea: integrating the old with the new. *Journal of Plant Growth Regulation* **22**, 99-108.

Ross JJ, O'Neill DP, Smith JJ, Kerckhoffs LHJ, Elliott RC (2000) Evidence that auxin promotes gibberellin A_1 biosynthesis in pea. *The Plant Journal* **21**, 547-552.

Ross JJ, Reid JB (1989) Intemode length in *Pisum*. Biochemical expression of the *le* gene in darkness. *Physiologia Plantarum* **76**, 164-172.

Ross JJ, Reid JB, Swain SM (1993) Control of stem elongation by gibberellin A_1: evidence from genetic studies including the slender mutant *sln*. *Australian Journal of Plant Physiology* **20**, 585-599.

Ross JJ, Reid JB, Swain SM, Hasan O, Poole AT, Hedden P, Willis CL (1995) Genetic regulation of gibberellin deactivation in *Pisum*. *The Plant Journal* **7**, 513-523.

Saito S, Okamoto M, Shinoda S, Kushiro T, Koshiba T, Kamiya Y, Hirai N, Todoroki Y, Sakata K, Nambara E, Mizutani M (2006) A plant growth retardant, uniconazole, is a potent inhibitor of ABA catabolism in *Arabidopsis*. *Bioscience, Biotechnology, and Biochemistry* **70**, 1731-1739.

Sakakibara H (2004) Cytokinin biosynthesis and metabolism. In 'Plant Hormones - Biosynthesis, signal transduction, action!'. (Ed. PJ Davies) pp. 95-114. (Kluwer Academic Publishers: The Netherlands)

Sakamoto T, Kamiya N, Ueguchi-Tanaka M, Iwahori S, Matsuoka M (2001) KNOX homeodomain protein directly suppresses the expression of a gibberellin biosynthetic gene in the tobacco shoot apical meristem. *Genes and Development* **15**, 581-590.

Scott IM (1990) Plant hormone response mutants. *Physiologia plantarum* **78**, 147-152.

Seo M, Hanada A, Kuwahara A, Endo A, Okamoto M, Yamauchi Y, North H, Marion-Poll A, Sun T, Koshiba T, Kamiya Y, Yamaguchi S, Nambara E (2006) Regulation of hormone metabolism in Arabidopsis seeds: phytochrome regulation of abscisic acid metabolism and abscisic acid regulation of gibberellin metabolism. *The Plant Journal* **48**, 354-366.

Sharp RE, LeNoble ME, Else MA, Thorne ET, Gherardi, F (2000) Endogenous ABA maintains shoot growth in tomato independently of effects on plant water

balance: evidence for an interaction with ethylene. *Journal of Experimental Botany* **51**, 1575-1584.

Shimada A, Ueguchi-Tanaka M, Sakamoto T, Fujioka S, Takatsuto S, Yoshida S, Sazuka T, Ashikari M, Matsuoka M (2006) The rice *SPINDLY* gene functions as a negative regulator of gibberellin signaling by controlling the suppressive function of the DELLA protein, SLR1, and modulating brassinosteroid biosynthesis. *The Plant Journal* **48**, 390-402.

Silva T, Davies PJ (2007) Hormone growth responses of roots of shoot height mutants of pea. *Physiologia Plantarum* **129**, 813-821.

Silverstone AL, Ciampaglio CN, Sun T (1998) The Arabidopsis *RGA* gene encodes a transcriptional regulator repressing the gibberellin signal transduction pathway. *The Plant Cell* **10**, 155-169.

Silverstone AL, Jung H, Dill A, Kawaide H, Kamiya Y, Sun T (2001) Repressing a repressor: gibberellin-induced rapid reduction of the RGA protein in *Arabidopsis*. *The Plant Cell* **13**, 1555-1565.

Sponsel VM, Hedden P (2004) Gibberellin biosynthesis and inactivation. In 'Plant Hormones - Biosynthesis, signal transduction, action!'. (Ed. PJ Davies) pp. 63-94. (Kluwer Academic Publishers: The Netherlands)

Stavang JA, Junttila O, Moe R, Olsen JE (2007) Differential temperature regulation of GA metabolism in light and darkness in pea. *Journal of Experimental Botany* **58**, 3061-3069.

Sun T, Gubler F (2004) Molecular mechanisms of gibberellin signaling in plants. *Annual Review of Plant Biology* **55**, 197-223.

Sun T, Kamiya Y (1994) The *Arabidopsis GA1* locus encodes the cyclase *ent*-kaurene synthetase A of gibberellin biosynthesis. *The Plant Cell* **6**, 1509-1518.

Swain SM, Reid JB, Kamiya Y (1997) Gibberellins are required for embryo growth and seed development in pea. *The Plant Journal* **12**, 1329-1338.

Tanimoto E (1990) Gibberellin requirement for the normal growth of roots. In 'Gibberellins'. (Eds N Takahashi, B Phinney and J MacMillan) pp. 229-240. (Springer-Verlag: New York)

Thomas SG, Hedden P (2006) Gibberellin metabolism and signal transduction. In 'Plant hormone signaling'. (Eds P Hedden and SG Thomas) pp. 147-185. (Blackwell Publishing: Australia)

Umehara M, Hanada A, Yoshida S, Akiyama K, Arite T, Takeda-Kamiya N, Magome H, Kamiya Y, Shirasu K, Yoneyama K, Kyozuka J, Yamaguchi S (2008) Inhibition of shoot branching by new terpenoid plant hormones. *Nature* **455**, 195-201.

van Huizen R, Ozga JA, Reinecke DM (1997) Seed and hormonal regulation of gibberellin 20-oxidase expression in pea pericarp. *Plant Physiology* **115**, 123-128.

Weiss D, Ori N (2007) Mechanisms of cross talk between gibberellin and other hormones. *Plant Physiology* **144**, 1240-1246.

Weller JL, Hecht V, Vander Schoor JK, Davidson SE, Ross JJ (2009) Light regulation of gibberellin biosynthesis in pea is mediated through the COP1/HY5 pathway. *The Plant Cell* **21**, 800-813.

Weller JL, Ross JJ, Reid JB (1994) Gibberellins and phytochrome regulation of stem elongation in pea. *Planta* **192**, 489-496.

Weston DE, Elliott RC, Lester DR, Rameau C, Reid JB, Murfet IC, Ross JJ (2008) The pea DELLA proteins LA and CRY are important regulators of gibberellin synthesis and root growth. *Plant Physiology* **147**, 199-205.

Weston DE, Reid JB, Ross JJ (2009) Auxin regulation of gibberellin biosynthesis in the roots of pea (*Pisum sativum*). *Functional Plant Biology* **36**, 362-369.

Wolbang CM, Chandler PM, Smith JJ, Ross JJ (2004) Auxin from the developing inflorescence is required for the biosynthesis of active gibberellins in barley stems. *Plant Physiology* **134**, 769-776.

Wolbang CM, Ross JJ (2001) Auxin promotes gibberellin biosynthesis in decapitated tobacco plants. *Planta* **214**, 153-157.

Yamaguchi S (2008) Gibberellin metabolism and its regulation. *Annual Review of Plant Biology* **59**, 225-251.

Yaxley JR, Ross JJ, Sherriff LJ, Reid JB (2001) Gibberellin biosynthesis mutations and root development in pea. *Plant Physiology* **125**, 627-633.

Zentella R, Zhang Z, Park M, Thomas SG, Endo A, Murase K, Fleet CM, Jikumaru Y, Nambara E, Kamiya Y, Sun T (2007) Global analysis of DELLA direct targets in early gibberellin signaling in *Arabidopsis*. *The Plant Cell* **19**, 3037-3057.

PUBLICATIONS

Weston DE, Elliott RC, Lester DR, Rameau C, Reid JB, Murfet IC, Ross JJ

(2008)

The pea DELLA proteins LA and CRY are important regulators of gibberellin synthesis and root growth

Plant Physiology 147, 199-205

Ross JJ, Reid JB, Murfet, IC, Weston DE, Elliott RC

(2008)

The slender phenotype of pea is deficient in DELLA proteins

Plant Signaling and Behavior 3, 590-592

Weston DE, Reid JB, Ross JJ

(2009)

Auxin regulation of gibberellin biosynthesis in the roots of pea (*Pisum sativum*)

Functional Plant Biology 36, 362-369