Molecular genetics and regulation of gibberellin biosynthesis in pea

by

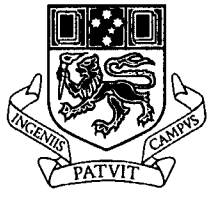
Sandra Elizabeth Davidson

B.Sc(Hons), M.Sc

Submitted in the fulfilment of the requirements for

the degree of

Doctor of Philosophy

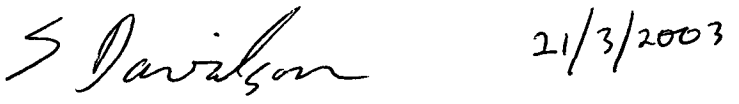


UNIVERSITY OF TASMANIA

March 2003

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any tertiary institution, and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text.



21/3/2003

Sandra E. Davidson

Access Statement

This thesis may be made available for loan and limited copying in accordance with the *Copyright Act 1968.*

Abstract

The molecular identity of the final two classical gibberellin (GA) biosynthesis mutants of *Pisum sativum*, *na* and *lh*, have been determined. *NA* and *LH* encode adjacent cytochrome P450 monooxygenases in the GA biosynthesis pathway and together oxidise the six steps from *ent*-kaurene to GA_{12}.

The GA deficient dwarf *na* mutant in pea has severely reduced internode elongation, reduced root growth, and decreased leaflet size. However, the seeds develop normally. Two genes, *PsKAO1* and *PsKAO2*, encoding cytochrome P450 monooxygenases of the subfamily CYP88A were isolated. Both PsKAO1 and PsKAO2 had *ent*-kaurenoic acid oxidase (KAO) activity; catalysing the 3 steps of the GA biosynthetic pathway from *ent*-kaurenoic acid to GA_{12} when expressed in yeast.

The *NA* gene encodes PsKAO1, since in two independent mutant alleles, *na-1* and *na-2*, *PsKAO1* had altered sequences and the 5 base deletion in *PsKAO1* associated with the *na-1* allele co-segregated with the dwarf *na* phenotype. *PsKAO1* was expressed in the stem, apical bud, leaf, pod and root, organs in which GA levels have previously been shown to be reduced in *na* plants. *PsKAO2* was expressed only in seeds and this may explain the normal seed development and normal GA biosynthesis in seeds of *na* plants.

The pea gene associated with the GA deficient *lh* dwarf mutant was isolated and found to encode a cytochrome P450 monooxygenase, of the subfamily CYP701A. This gene had high similarity to genes encoding *ent*-kaurene oxidases in *Arabidopsis* and pumpkin, *AtKO1* and *CmKO1* respectively. The *PsKO1* gene sequence was altered in all three of the dwarf pea mutant alleles, *lh-1, lh-2* and *lh-3*.

This confirmed previous work that showed that the *lh-2* mutant blocked the 3 step metabolism of *ent*-kaurene to *ent*-kaurenoic acid. The phenotypes of the *lh-1* and *lh-3* alleles differ from *lh-2* in a tissue specific manner. In the seed, the *lh-1* and *lh-3* alleles had little effect while the *lh-2* allele phenotype had smaller seeds and decreased seed survival. However, in the vegetative parts of the plants all three alleles are of similar dwarf stature. However, the *lh-2* mutant is up to 30 times more sensitive to the KO specific inhibitor paclobutrazol than is the *lh-1* or *lh-3* alleles. The different phenotypes appear to reflect the different natures of the aberrant proteins produced by the different alleles.

With the isolation of genes encoding the two major cytochrome P450 monooxygenases from the GA biosynthesis pathway in pea, control of the early sections of the pathway was investigated. Northern blot analysis was used to measure the mRNA transcript levels of genes encoding enzymes at three steps including the first committed step of GA biosynthesis, cyclization of GGDP to copalyl diphosphate (CPS), and the two monooxygenases which oxidise *ent*-kaurene, (KO), and *ent*-kaurenoic acid, (KAOs).

ent-kaurenoic acid oxidase activity is encoded by a 2 membered gene family with tissue specific expression patterns. *PsKAO2* is only expressed in developing seed and therefore potentially allows differential regulation of GA biosynthesis in seed. In contrast, the single copy *ent*-kaurene oxidase, *PsKO1*, is expressed at high levels in all tissue and developmental stages tested. Although copalyl diphosphate synthase, *PsCPS*, was expressed at low levels in all tissues tested, it had higher expression in seed and stem tissue.

There is evidence of developmental regulation of the genes in seeds at the transcript level. All four genes examined, *PsCPS*, *PsKO1*, *PsKAO1* and *PsKAO2*, have a transcript level peak around the time of contact point coinciding with a peak of GA_{20} accumulation in maturing seeds. *PsKO1* expression levels were also high in young seeds (11 days after anthesis) coinciding with the tail end of the peak of GA_1 content in young seeds. However, genes for these steps appear unaffected at the

transcript level by maturation of the pea shoot. *PsCPS*, *PsKO1* and *PsKAO1* were expressed in mature stem and leaf tissue, even though the mature tissue contained less bioactive GA_1 and had ceased expanding.

Unlike some genes later in the GA biosynthesis pathway, those early in the pathway, *PsCPS*, *PsKO1*, *PsKAO1* and *PsKAO2*, are not under feed-back regulation by GA_1 activity. Expression levels were unaltered in samples from plants with reduced stature and decreased bioactive GA levels as in GA deficient mutants or plants treated with the GA biosynthesis inhibitor, paclobutrazol.

Acknowledgements

I wish to thank the many people that provided support and encouragement during the last three and a half years. I wish to thank my supervisor Prof Jim Reid for his scientific insight and enthusiasm to develop an excellent topic and the strategic advice he provided throughout this project; Dr Bob Elliott for guidance with all the molecular biology theory and methods and always offering at least two ways to approach every problem; Dr Huub Kerckhoffs for passing on his careful RNase free and library screening techniques; Jenny Smith for getting me into the lab on my first day and showing me the basics of molecular biology; Dr Adam Smolenski for getting me started with sequencing and use of CEQ2000 gel scanner; Ian Cummings and Tracey Jackson for technical support in the glasshouse. Dr John Ross, Dr Stephen Swain, Prof Ian Murfet, Dr Shona Batge, Damien Platten, Dr Greg Symons for many helpful discussions.

I wish to gratefully acknowledge Dr Chris Helliwell and Andrew Poole (CSIRO Plant Industry, Canberra) for transforming my constructs into the yeast and then analysing the products of the expression studies (Chapter 2) and Dr Bob Elliott for the initial screening of the cDNA libraries with a *D3*-like probe and preparation of a Southern blot gel (Chapter 2).

I especially would like to thank Neil Davidson who encouraged me to start, gave excellent support throughout and even did all the household duties in the last year. Also many thanks to my parents, Harry and Cathy Jamison, Kath Medlock and Carol Bishop for child care without which I would not have been able to complete. Thanks also to Emily, Matthew and Claire for managing to continue to grow up into nice people despite their mum doing a PhD.

Table of Contents

Chapter 1

General Introduction

Gibberellins (GAs) are important plant growth hormones that regulate many aspects of plant growth including stem, petiole, pollen tube and root elongation, leaf expansion and the growth of seeds and fruit (Reid and Ross, 1993; Hooley, 1994; Yaxley et al., 2001; Singh et al., 2002). They are also involved in seed germination and seed development (Hooley, 1994; Swain et al., 1997; Yamaguchi and Kamiya, 2002).

The gibberellin biosynthetic pathway (Fig. 1.1) can be divided into 3 sections (Hedden and Kamiya, 1997; Hedden and Phillips, 2000). The first section, catalysed by terpene cyclases, involves the cyclisation of geranylgeranyl diphosphate (GGDP) to *ent*-kaurene in the plastid stroma. In the second section, the hydrophobic *ent*-kaurene is oxidized to GA_{12} by membrane bound cytochrome P450 monooxygenases. Similarly, GA_{12} may be further converted to GA_{53} by the introduction of a 13-hydroxyl group. The third section consists of further oxidation, removing the C-20 then introducing a 3β-hydroxyl group, to form the bioactive GA_1 by soluble 2-oxoglutarate dependent dioxygenases in the cell cytosol. The bioactive GAs may be deactivated by similar soluble 2-oxoglutarate dependent dioxygenases introducing a 2β-hydroxyl group. The early sections of the pathway are common to all the plant species investigated so far (Hedden and Phillips, 2000). However, the wide range of dioxygenases allow variation in the pathway after GA_{12} in different species and tissues (Phillips et al., 1995; Garcia-Martinez et al., 1997; Yamaguchi et al., 1998a). The early 13-hydroxylase pathway predominates in vegetative tissue of

pea, producing bioactive GA_1 (Ingram et al., 1986; Reid and Ross, 1993; Poole et al., 1995). However, the non-13 hydroxylated GA_4 appears to be the main active product in *Arabidopsis* (Talon et al., 1990; Sponsel et al., 1997).

Many of the GA biosynthetic genes encoding enzymes of the first and third sections of the GA biosynthetic pathway have been cloned in a range of species (Hedden and Phillips, 2000). In the pea these include: a terpene cyclase, copalyl diphosphate synthase (*CPS*)(Ait-Ali et al., 1997), the dioxygenases including two GA 20-oxidases (Lester et al., 1996; Martin et al., 1996; Garcia-Martinez et al., 1997), a 3ß-hydroxylase (GA 3-oxidase) (Lester et al., 1997; Martin et al., 1997) as well as two catabolic enzymes, GA 2-oxidases (Lester et al., 1999b; Martin et al., 1999). Until recently, the biosynthetic pathway from *ent*-kaurene via *ent*-kaurenoic acid to GA_{12} or GA_{53} had been less extensively studied (Fig. 1.1).

Since *ent*-kaurene and its intermediates are hydrophobic, the enzymes involved are associated with membranes. They are generally assumed to be cytochrome P450 monooxygenases (Murphy and West, 1969; Hedden, 1997). Cytochrome P450 enzymes are numerous in plants, animals, bacteria and fungi (Nelson et al., 1993) (http://drnelson.utmem.edu/CytochromeP450.html). There are 272 genes encoding cytochrome P450s (including 26 pseudogenes) in the *Arabidopsis* genome with diverse biological functions (Werck-Reichhart et al., 2002). Cytochrome P450 enzymes all share a common catalytic center of a haem group (Kalb and Loper, 1988). Iron is bound equatorially to 4 nitrogens in the haem protoporphyrin IX prosthetic group leaving 2 axial iron binding sites available (Poulos et al., 1985; 1986). The 5th ligand, below the plane of the haem ring is bond to a thiolate (S^-) anion provided by a conserved cysteine at the haem binding active site of the enzyme. The remaining 6th ligand binding site is occupied by water and later oxygen during the reaction (Porter and Coon, 1991; Hedden, 1997). Cytochrome P450 enzymes fold into a triangular prism form (Poulos et al., 1985;

1986) and are anchored to membranes through a short hydrophobic segment at the N-terminus (Chen and Kemper, 1996; Werck-Reichhart et al., 2002).

The cytochrome P450 monoxygenase reactions are usually based on activation of molecular oxygen with the insertion of one oxygen atom in the substrate and reduction of the other to form water (Porter and Coon, 1991). The Cytochrome P450 monooxygenases of the GA biosynthesis pathway require the co-enzyme NADPH-cytochrome P450 reductase, NADPH and oxygen (Hedden, 1997). Since, bacteria do not use this coenzyme (Porter and Coon, 1991) it has been difficult to express the cDNAs of plant monooxygenases in heterologous systems (Hedden, 1997; 1999). However, the enzymes have been successfully expressed in yeast, although the expression of the *Arabidopsis* and barley *ent*-kaurenoic acid oxidases, of interest in Chapter 2, required modified yeast engineered to express an *Arabidopsis* NADPH-cytochrome P450 reductase (Pompon et al., 1996; Helliwell et al., 2000).

GA_{12} and GA_{12}-aldehyde are of intermediate polarity and are more hydrophilic. Therefore, they can be substrates for 7-oxidases and 13-hydroxylases belonging to both the membrane bound monooxygenase and the soluble dioxygenase classes (Kamiya and Graebe, 1983; Gilmour et al., 1986; Hedden, 1997). Pumpkin, in addition to the monooxygenase 7- oxidase activity, contains a 2-oxoglutarate-dependent dioxygenase, GA 7-oxidase, which can oxidise GA_{12}-aldehyde to GA_{12} (Lange, 1997). However, this dioxygenase has not been found in other species (Hedden et al., 2002).

The range of GA synthesis mutants has proved to be a very effective tool to illuminate the action of GAs (Reid, 1986a; Reid and Ross, 1993). The pea GA deficient mutant, *na*, showed that GAs are directly involved in stem internode elongation (Reid et al., 1983) and root elongation (Yaxley et al., 2001) while the *lh-2* mutant demonstrated that an early GA peak is important for pea seed development (Swain et al., 1997). Mutants have been used in other species such as *Arabidopsis* to

show that GAs are essential for seed germination and pollen tube growth (Koornneef and van der Veen, 1980; Singh et al., 2002). Pea mutants, also have been useful to investigate the GA biosynthetic and catabolic pathway in peas (Ross et al., 1997; Hedden and Proebsting, 1999). For example endogenous GA_1 was identified as the bioactive GA in pea, from the quantitative relationship between the decreasing GA_1 levels and reduced internode elongation in the *LE*, *le-1* and *le-2* allelic series (Ross et al., 1989). In addition, grafting studies utilizing the *lh*, *na*, and *le* mutants have demonstrated which GAs and their precursors may be transported around the plant (Reid et al., 1983; Reid and Potts, 1986; Proebsting et al., 1992). The feed-back phenomenon whereby bioactive GAs appear to regulate their own biosynthesis was confirmed by up-regulation of GA 20-oxidation and GA 3-oxidation at the transcriptional stage in pea GA deficient mutants (Martin et al., 1996; 1997; Ross et al., 1999). Similarly *Arabidopsis* GA deficient mutants demonstrated feed-back regulation in *Arabidopsis* (Chiang et al., 1995; Phillips et al., 1995). The feed-forward phenomenon where bioactive GAs regulate their catabolism was investigated using mutant peas (Elliott et al., 2001). Furthermore, once the *LE* and *SLN* genes were cloned (Lester et al., 1997; 1999b; Martin et al., 1997; 1999) the regulation of these genes by auxin was revealed (Ross et al., 2000; 2002).

In pea, some of the genes associated with the GA biosynthetic and catabolic mutants have been cloned including *LS* (CPS, Ait-Ali et al., 1997), *LE* (3-oxidase, Lester et al., 1997; Martin et al., 1997), and *SLN* (2-oxidase Lester et al., 1999b; Martin et al., 1999) (Fig. 1.1). However the genes associated with the GA deficient dwarf pea mutants *na* and *lh* have not previously been isolated and this is addressed in this thesis.

Previous work indicates that *na* and *lh* mutants are likely to have blocks in the second section of the GA biosynthesis pathway (Ingram and Reid, 1987; Swain et al., 1997) and therefore encode cytochrome P450 monooxygenases. At the commencement of this thesis the *Arabidopsis GA3* (*AtKO1*) gene had recently been

cloned and shown to catalyse the three steps from *ent*-kaurene to *ent*-kaurenoic acid (Helliwell et al., 1998; 1999). These were the same steps blocked in the *lh* mutant (Swain et al., 1997; Fig. 1.1). Therefore the pea homologue of the *Arabidopsis GA3* gene, based on feeding studies, was likely to be *LH* (Chapter 3).

However, the biochemical genetics of the next four steps was ambiguous. At commencement of this thesis, the maize *D3* gene was the only other gene cloned in the second section of the GA biosynthesis pathway (Winkler and Helentjaris, 1995). The maize *D3* gene was shown to encode a cytochrome P450 monooxygenase of a new subfamily CYP88A, although which of the 4 remaining steps blocked in the GA responsive dwarf maize *d3* mutant was uncertain (Winkler and Helentjaris, 1995). Fujioka et al. (1988a) suggested that one or both of the 2 steps from GA_{12}-aldehyde to GA_{53} was most likely when combined with the bioassay data of Phinney and Spray (1982). However the biochemical data was also consistent with a block anywhere between *ent*-kaurene and GA_{53} (Fujioka et al., 1988a). Although feeding studies suggest that the block in the *na* mutant is prior to GA_{12}-aldehyde (Ingram and Reid, 1987), the maize *D3* gene may be a candidate homologue of *NA*. Therefore a maize *D3*-like EST probe from soybean was used to screen a pea cDNA library in an attempt to clone another gene encoding a cytochrome P450 monooxygenase from the second section of the GA biosynthesis pathway with the possibility that it may cosegregate with the dwarf *na* mutant (Chapter 2).

In this thesis the *NA* (Chapter 2) and *LH* (Chapter 3) genes are cloned in pea. Then the regulation of the early 2 sections of the GA biosynthesis pathway, from GGDP to GA_{12}, were investigated in relation to GA biosynthesis as a whole (Chapter 4).

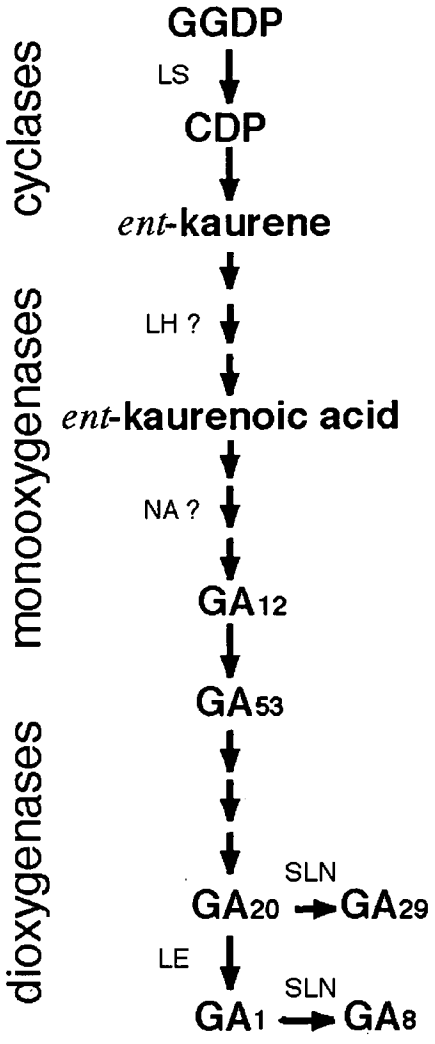


Figure 1.1

A simplified gibberellin (GA) biosynthetic pathway in pea. The pathway is divided into the first section (cyclase), second section (monooxygenase) and third section (diooxygenase) enzyme classes. The pea genes associated with the mutations *LS, LE* and *SLN* and possible sites of *LH* and *NA* are indicated.

Chapter 2

The pea gene *NA* encodes *ent*-kaurenoic acid oxidase

The majority of the results presented in this chapter have been published as follows:

The pea gene NA encodes ent-kaurenoic acid oxidase

Sandra E. Davidson, Robert C. Elliott, Chris A. Helliwell, Andrew T. Poole, and James B. Reid. *Plant Physiology,* January 2003, vol. 131, pp 335-344

INTRODUCTION

The gibberellin (GA) responsive mutant *na* has an extreme dwarf or "nana" phenotype (Potts and Reid, 1983). There are two mutant alleles, *na-1* and *na-2* (Fig. 2.1). The *na* seedlings have extremely short internodes with a dramatic decrease in cell length of the epidermal and outer cortical cells as well as a reduction in the total number of these cells in the internode (Reid et al., 1983). The *na* mutants have small darkly coloured foliage with reduced area of individual leaflets, decreased stipule size and petiole length (Reid and Ross, 1993). The root growth is also altered with taproot length reduced by 50% (Yaxley et al., 2001). The vegetative part of the *na* pea plant is severely deficient in endogenous gibberellins. It was not possible to show the presence of any C-19 GAs by dilution of [$^{13}C^{3}H$]GA_{20} metabolites by endogenous [^{12}C]GAs using GC-MS techniques (Ingram et al., 1984). The *na* mutation markedly reduces the production of GAs, including the predominant bioactive GA_1 , in shoots and stems, (Potts and Reid, 1983), leaves (Reid and Ross,

1993), roots (Yaxley et al., 2001) and pods (Potts and Reid, 1983; Potts, 1986). However, the effect of the *na* mutation is tissue specific and this was amongst the earliest information suggesting that alternative enzymes (or gene families) may be involved in GA biosynthesis (Reid, 1986a). In contrast to the vegetative part of the *na* plant, where the level of GA_1 in expanding tissue was reduced to 2% of the wild-type (Proebsting et al., 1992), the developing seeds contain similar gibberellin levels to those found in the seeds of wild-type *NA* plants (Potts and Reid, 1983; Potts, 1986). In addition the seeds of *na* plants develop normally (Potts and Reid, 1983).

The *na* mutation appears to block GA biosynthesis prior to GA_{12}-aldehyde (Fig. 2.2). There are two lines of evidence to support this supposition. The *na* plants did not respond to the application of precursors prior to GA_{12}-aldehyde such as *ent*-kaurene, *ent*-kaurenoic acid and *ent*-7α-hydroxy-kaurenoic acid but showed marked stem elongation in response to GA_{12}-aldehyde. Also, $[^2H]GA_{12}$-aldehyde was metabolised to C-19 GAs such as GA_{20}, GA_{29}, GA_1 and GA_8 by *na* plants but these plants do not metabolize *ent*-$[^3H_2]$kaurenoic acid to these GAs even though wild-type plants appear to do so (Ingram and Reid, 1987).

The section of the GA biosynthetic pathway from *ent*-kaurene via *ent*-kaurenoic acid to GA_{12} or GA_{53} (Fig. 2.2) is generally assumed to involve cytochrome P450 monooxygenases and require the co-enzyme NADPH-cytochrome P450 reductase, NADPH and oxygen (Hedden, 1997). The CYP88A family of cytochrome P450s have recently been shown to encode *ent*-kaurenoic acid oxidase, which catalyzes the three steps from *ent*-kaurenoic acid to GA_{12} via *ent*-7α-hydroxy-kaurenoic acid and GA_{12}-aldehyde, in *Arabidopsis* and barley (*Hordeum vulgare*)(Helliwell et al., 2001a; Fig. 2.2). Other CYP88A cytochrome P450 genes from pumpkin (*Cucurbita maxima*)(Helliwell et al., 2000), and maize (*Zea mays*) (Winkler and Helentjaris, 1995) have also been isolated. A *Gibberella fujikuroi* gene encoding a cytochrome P450 (CYP68) has been isolated and shown to encode an *ent*-kaurenoic acid oxidase activity (Rojas et al., 2001). The *G. fujikuroi* gene is not

closely related to the plant *ent*-kaurenoic acid oxidases. When this gene was expressed in *G. fujikuroi* lacking the GA biosynthetic gene cluster it catalysed the conversion of *ent*-kaurenoic acid to GA_{12} and also GA_{14} as well as a number of putatively identified side products (Rojas et al., 2001).

Arabidopsis does not have a mutant affecting *ent*-kaurenoic acid oxidation presumably due to redundancy because the two *Arabidopsis* genes, *AtKAO1* and *AtKAO2*, have similar expression patterns throughout the plant (Helliwell et al., 2001a). The GA-responsive maize *d3* mutants have defects in a CYP88A gene (Winkler and Helentjaris, 1995). The barley GA responsive dwarf mutant, *grd5*, accumulates *ent*-kaurenoic acid in developing grains (Helliwell et al., 2001a). Mutations were found in the barley *HvKAO1*, in each of three independent mutant alleles of the barley dwarf *grd5* (Helliwell et al., 2001a).

In this chapter genes encoding *ent*-kaurenoic acid oxidase activity are identified in pea by screening a cDNA library using a maize *D3*-like EST probe from soybean (*Glycine max.*). I show that one of these genes is *NA* and then explain the tissue specific nature of the *na* mutation.

Figure 2.1

The phenotype of 21 day old seedlings of wild -type *NA* (WL1769) and two independent mutants, *na-1* (WL1766) and *na-2* (L81).

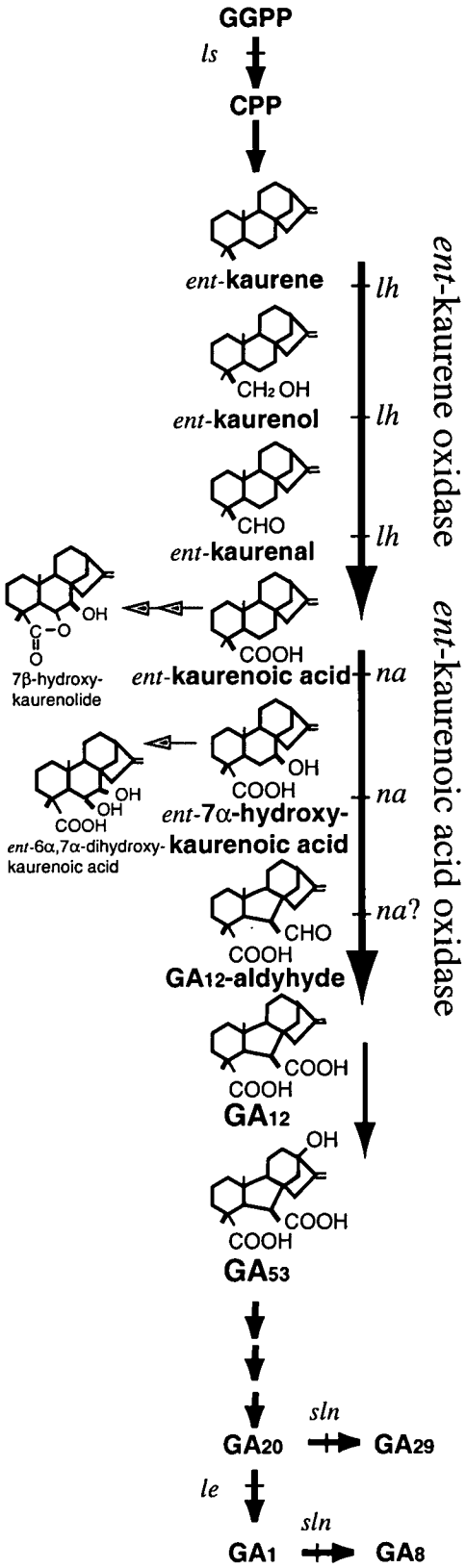


Figure 2.2.

The gibberellin (GA) biosynthetic pathway in pea. Product structures are represented for the cytochrome P450 monooxygenase mediated steps showing the steps catalysed by *ent*-kaurene oxidase (KO) and *ent*-kaurenoic acid oxidase (KAO). The steps blocked by the pea mutants (*ls, lh, na, le, and sln)* are indicated. Putative side products of PsKAO1 and PsKAO2 activity are indicated.

RESULTS

Isolation of two CYP88A genes from pea

Two genes encoding cytochrome P450s of the subfamily CYP88A were isolated by library screening using a maize *D3*-like EST from soybean as probe. The initial screening was performed by Dr RC Elliott. A partial cDNA of *PsKAO1* was obtained from a pea (cv. Alaska) apical bud cDNA library. The longest clone (500bp) was extended by 3' and 5' RACE using cDNA prepared from wild-type *NA* (WL1769) as template (Frohman et al., 1988). A full-length cDNA, *PsKAO2*, was obtained from a pea seed library (cv.Torsdag).

PsKAO1 (*CYP88A6*, GenBank accession no. AF537321 sequenced from *NA* WL1769) and *PsKAO2* (*CYP88A7*, GenBank accession no. AF537322) showed close homology at the nucleotide level (60-100 bit score, 82-93% identities) to *AtKAO1* and *AtKAO2* (BLASTN, Altschul et al., 1997). At the amino acid level, the full length putative proteins PsKAO1 and PsKAO2 are similar to AtKAO1, AtKAO2 and CmKAO1 (644-661 bit score, 63-65% identities, 79-81% positives; NCBI Blast 2 sequences). Over the full length *PsKAO1* is similar to *PsKAO2* at the nucleotide (462 bit score, 78% identity) and protein level (756 bit score, 74% identity, 86% positive; NCBI Blast 2 sequences). This is comparable with the *Arabidopsis* KAO putative proteins where AtKAO1 is 76% identical to AtKAO2 (Fig. 2.3).

In genomic Southern blots at high stringency, bands associated with each of the *PsKAO1* and *PsKAO2* genes can clearly be seen (Fig. 2.4). However, at lower stringency the two genes cross-hybridise. Additional bands were revealed at low stringency by the *PsKAO2* probe. This suggests the possibility that there may be a third member in the gene family.

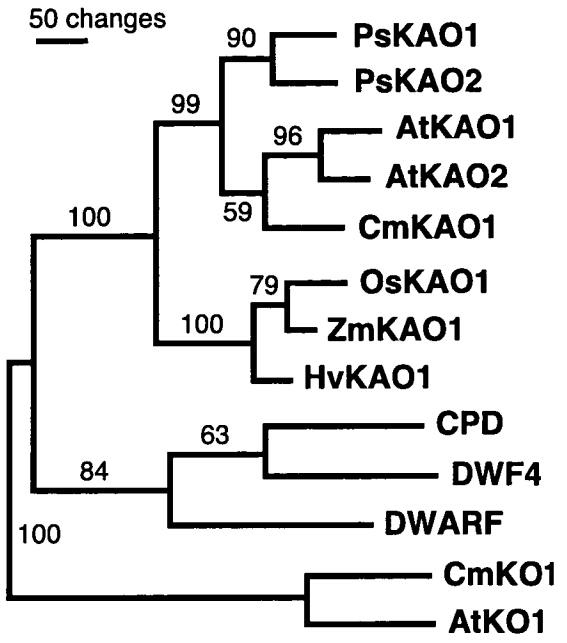


Figure 2.3

Inferred phylogenetic relationship of *ent*-kaurenoic acid oxidases [CYP88A] and representatives of related cytochrome P450 enzymes. Numbers shown represent the bootstrap support values (%).
The phylogram was generated by PAUP 4.8b8 (Swofford, 1999) using putative amino acids of full-length genes (excluding gaps) with CYP701A as the outgroup. *ent*-kaurenoic acid oxidase proteins used in addition to the pea PsKAO1 and PsKAO2 were from *Arabidopsis* (AtKAO1 and AtKAO2; Helliwell et al., 2001a), pumpkin (CmKAO1; Helliwell et al., 2000), rice (OsKAO1,GenBank accession no.AP000616), maize (*D3* : ZmKAO1; Winkler and Helentjaris, 1995), and barley (*Grd5* :HvKAO1; Helliwell et al., 2001a). The related brassinosteroid biosynthetic enzymes used include *Arabidopsis CPD* [CYP90A1](Szekeres et al., 1996) and *DWF4* [CYP90B1](Choe et al., 1998) and tomato *DWARF* [CYP85A1] (Bishop et al., 1999). The outgroup consisted of the cytochrome P450 monooxygenase *ent*-kaurene oxidases [CYP701A] from pumpkin CmKO1 (Helliwell et al., 2000) and *Arabidopsis GA3* : AtKO1 (Helliwell et al., 1998) of the GA biosynthetic pathway.

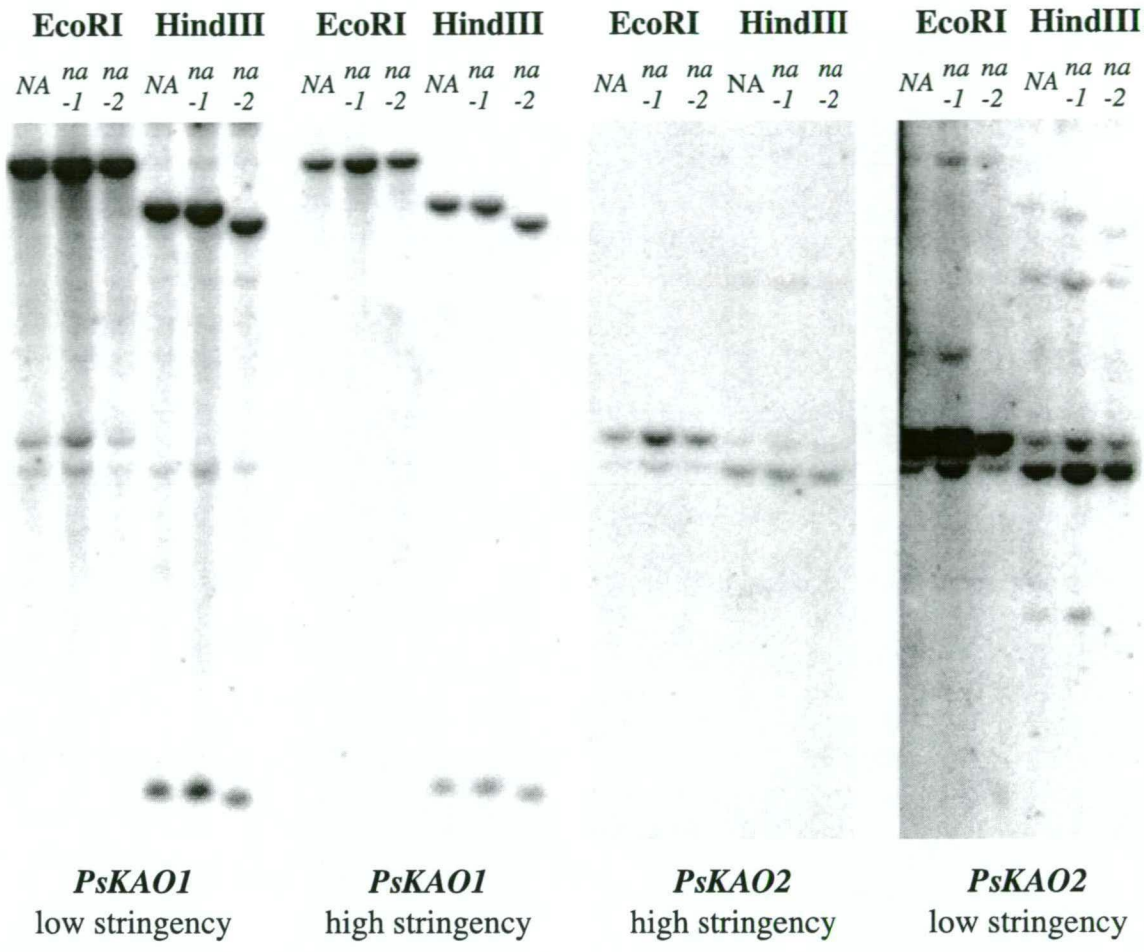


Figure 2.4

Genomic DNA Southern blot analysis of the pea *ent*-kaurenoic acid oxidase family. DNA from *NA* (WL1769), *na-1* (WL1766) and *na-2* (L81) plants was digested with restriction enzymes EcoRI and HindIII and probed with either *PsKAO1* or *PsKAO2* and then washed at either high or low stringency.

PsKAO1 is mutated in the *na-1* and *na-2* mutants

The *PsKAO1* cDNA from the *na-1* line WL1766 contained a 5 base deletion when compared to *NA* (WL1769). This would change the reading frame for the encoded protein leading to a premature stop codon (Fig. 2.5). The predicted protein would be 194 amino acids long, which is much smaller than the expected 485 amino acid length of the putative PsKAO1 enzyme. The predicted protein would be truncated before the catalytic domains (Kalb and Loper, 1988), including the active haem-binding site common to all cytochrome P450 enzymes. There was less *PsKAO1* mRNA measured in the *na-1* mutant tissue than the isogenic wild-type *NA* (Fig. 2.8 C). This may be due to the instability of mRNA with a premature stop codon (Gutierrez et al., 1999).

The *PsKAO1* from the *na-2* line L81 also is altered. Initially, PCR of the cDNA obtained from RNA of *na-2* stems produced 3 bands (Fig. 2.6). When gel purified the largest band (W) was found to have 48 base pairs incorrectly spliced out and the smallest band (Z) to have 364 base pairs incorrectly spliced out (Fig. 2.6). The band Y could not be gel purified. However, if the products W and Z were combined, melted and annealed the original 3-band PCR pattern reappeared. This suggests that the Y band represents a duplex between the W and Z bands (Fig. 2.6). Genomic DNA sequence data was then used to further define the *PsKAO1 na-2* mutation. The genomic sequence of *na-2* revealed a 25 base pair deletion (5bp from the 3' end of a large intron and 20bp of exon sequence) compared to the wild-type. The AG that is required for the positioning of splicing (Brown, 1996) was lost from the intron. Hence, splicing did not occur in the same place as in the wild type. Therefore this mutation leads to aberrant processing of the resultant pre-RNA. The 2nd and 18th AG following the deletion were used as 3' splicing points for the RNA that produced the W and Z PCR bands respectively. Splicing of the following intron was not affected (Fig. 2.6).

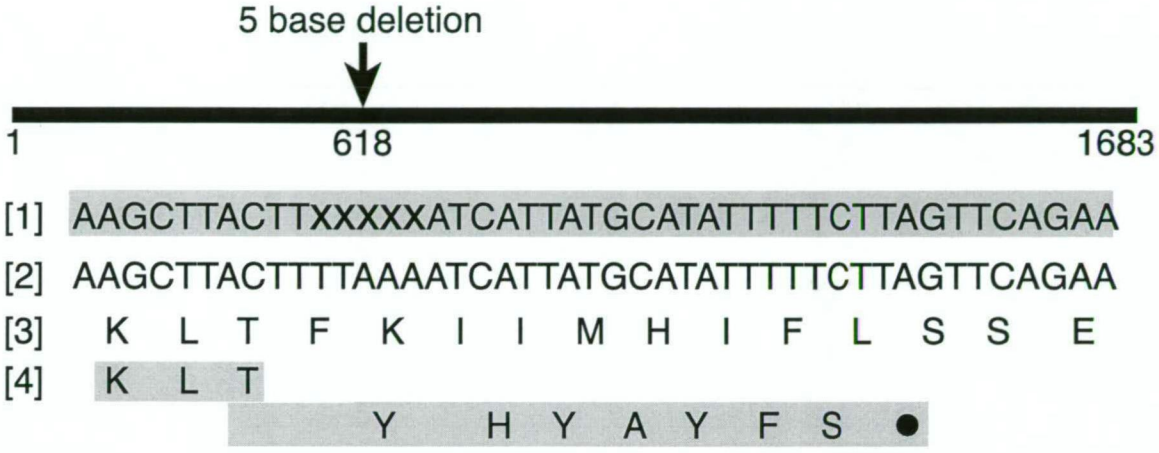
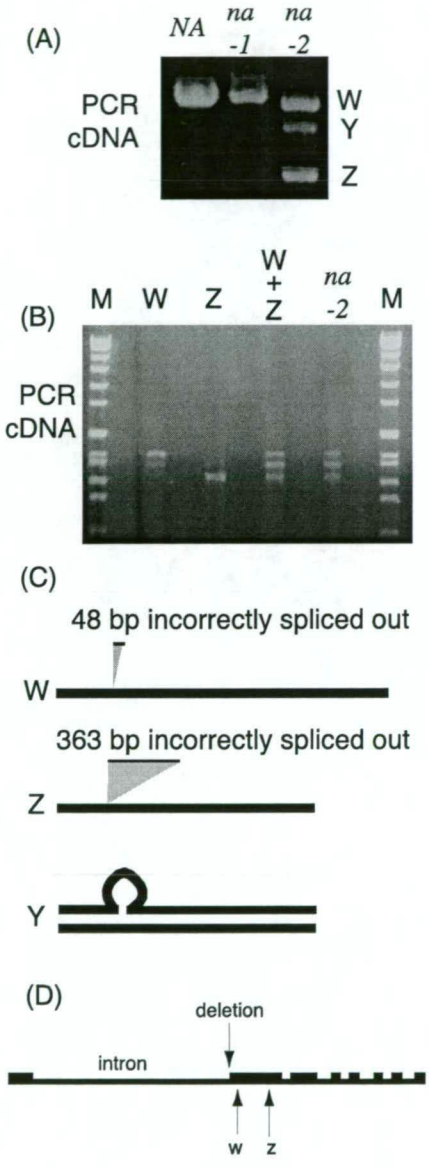


Figure 2.5

Schematic diagram and *PsKAO1* cDNA sequence surrounding the 5 bp deletion (xxxxx) in the mutant *na-1* (WL1766).
[1] cDNA sequence obtained from the mutant *na-1*,
[2] cDNA sequence obtained from the wild-type *NA*,
[3] wild-type *NA* amino acid translation,
[4] mutant *na-1* amino acid translation indicating the reading frame shift and premature stop codon ●.

Figure 2.6.



(**A**) The cDNA PCR products of *PsKAO1* from wild-type *NA* (WL1769) and mutants *na-1* (WL1766) and *na-2* (L81) using the same specific primers run on 1% agarose/TAE gel containing EtBr at 80V for 45 minutes. The *na-2* bands have been labeled W, Y and Z.

(**B**) Lanes W and Z contain previously gel purified PCR product bands W and Z from *na-2* mutant cDNA (see (A)). Lane W+Z is the product formed when the gel purified products W and Z were combined, melted and annealed (3 cycles of melting 95^{0}C and annealing 55^{0}C then 70^{0}C extension). Lane *na-2* is PCR product of *na-2* mutant cDNA as seen in (A) and Lane M is the SPPI-EcoRI size marker run on 1% agarose/TAE gel containing EtBr.

(**C**) Schematic diagram explanations of the cDNA bands W, Z and Y of (A) from sequence and experimental data, (B).

(**D**) Schematic diagram of genomic DNA of *PsKAO1* from sequence data. The 25 bp deletion site as well as the W and Z cryptic splice sites of the *na-2* mutation are indicated.

Figure 2.7

A Photograph of F_2 seedlings from one of the segregating families of cross *NA* (WL1769) with *na-1* (WL1766) plants.
B Co-segregation analysis of the *PsKAO1* gene in segregating families of cross *NA* (WL1769) with *na-1* (WL1766) plants. The *PsKAO1* PCR products identified the 5 base deletion of the *na-1* mutant gene when run on a denaturing gel. The phenotype of the individual plant from which the DNA was obtained is indicated as tall (T) or dwarf (D). The size marker (m) indicated the number of bases.

The mutation in *na-1* cosegregates with the *na* mutant phenotype

DNA was extracted from individual plants from 4 segregating F9 families from a cross between *NA* (WL1769) and *na-1* (WL1766) plants. The HEX-labeled PCR products (243bp in the wild-type) identified the 5 base deletion of the *na-1* mutant gene when run on denaturing gel. The 5 base deletion of *PsKAO1* cosegregates with the dwarf phenotype of *na* plants (Fig. 2.7). Of the 50 individuals in the 4 families: 12 were homozygous tall (*NA NA*); 26 were heterozygous tall (*NA na*); and 12 were homozygous dwarf (*na na*) in agreement with expected (χ^2_2=0.08, P>0.95). Therefore, the *na-1* phenotype co-segregates with the mutation in the *PsKAO1* gene.

Yeast expression

Yeast strains WAT11 and WAT21 were transformed with *PsKAO1* and *PsKAO2* expression constructs and yeast strains expressing PsKAO1 and PsKAO2 were identified by RNA gel blots (C.A. Helliwell). Both yeast strains expressing PsKAO1 and PsKAO2 converted *ent*-kaurenoic acid through to GA_{12} at a greater rate than yeast expressing the *Arabidopsis* AtKAO1 (C.A. Helliwell and A.T. Poole) (Table 2.1). The intermediates *ent*-7α hydroxykaurenoic acid and GA_{12}-aldehyde as well as the final product GA_{12} were detected (Table 2.1) and confirmed to be authentic by comparison to known standards (C.A. Helliwell and A.T. Poole) (Table 2.2). The PsKAO2 construct expressed in both yeast strains appeared more effective than the PsKAO1 and converted all the *ent*-kaurenoic acid substrate provided. Direct comparison may not be possible since the PsKAO1 construct may have 4 extra amino acids in the 5'untranslated region as there were 2 possible start codons in the *PsKAO1* sequence. GA_{53} and GA_{14} were not detected in any of the samples (Table 2.1). WAT21 yeast transformed with PsKAO1 and PsKAO2 expression constructs fed with intermediates *ent*-7α hydroxy kaurenoic acid or GA_{12}-aldehyde converted

these substrates to GA_{12} although at a lower rate than expected from the *ent*-kaurenoic acid feed data (data not shown). A substrate earlier in the GA biosynthetic pathway, *ent*-kaurene, was not metabolized by yeast expressing PsKAO1 or PsKAO2 (data not shown). Untransformed wild-type (WT) yeast did not metabolize *ent*-kaurenoic acid to intermediates in the GA biosynthetic pathway (Table 2.1).

The compound 7β-hydroxy-kaurenolide was detected and confirmed against authentic standard (C.A. Helliwell and A.T. Poole) (Table 2.2) in the samples with PsKAO1 and PsKAO2 activity after *ent*-kaurenoic acid feeds but not when the intermediates *ent*-7α-hydroxykaurenoic acid or GA_{12}-aldehyde were used as substrates. *ent*-6α,7α-dihydroxy-kaurenoic acid was detected (Table 2.1) and tentatively identified based on comparison with published spectra (Gaskin and MacMillan, 1991) (Table 2.2) in yeast with PsKAO1, PsKAO2 or AtKAO1 activity when fed with kaurenoic acid. However, this product was also present following feeds of *ent*-7α-hydroxykaurenoic acid (data not shown). Further conversion to fujenoic acid was not observed. Neither 7β-hydroxy-kaurenolide or *ent*-6α,7α-dihydroxy-kaurenoic acid were detected in the WT untransformed yeast samples and appeared to be a result of the KAO activity. However, in the WT untransformed yeast and yeast expressing AtKAO1 and PsKAO1 samples, the C/D ring-rearranged compounds, stachenoic acid and trachylobanic acid were present in significant amounts. However, neither compound was present in the more active PsKAO2 expressing samples which appeared to metabolize these compounds to the *ent*-7α hydroxy and *ent*-6α,7α-dihydroxy derivatives and also through to GA_{12}-like derivatives (C.A. Helliwell and A.T. Poole) (Table 2.3).

Table 2.1

Putative products from WAT21 and WAT11 yeast strains expressing CYP88A cytochrome P450s

The GC-MS total ion current (TIC) areas were measured for *ent*-7α hydroxykaurenoic acid (*ent*-7α-OH KA), GA_{12}-aldehyde, GA_{12}, GA_{53}, GA_{14}, 7β-hydroxy kaurenolide, and *ent*-6α,7α-dihydroxykaurenoic acid after *ent*-kaurenoic acid (KA) feeds. Data provided by C.A. Helliwell and A.T. Poole.

Enzyme	Yeast	KA TIC ($x10^6$)	*ent*-7α-OH KA TIC ($x10^6$)	GA_{12}-aldehyde TIC ($x10^6$)	GA_{12} TIC ($x10^6$)	GA_{53} or GA_{14}	7β-OH kaurenolide TIC ($x10^6$)	*ent*-6α,7α-diOH KA TIC ($x10^6$)
WT yeast	W21	1131	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
AtKAO1	W21	1204	49	15	≈2.5[a]	n.d.	n.d.	18
PsKAO1	W21	702	414	22	84	n.d.	≈5[a]	39
PsKAO2	W21	n.d.	520	108	265	n.d.	≈54[a]	96
PsKAO1	W11	921	437	23	95	n.d.	≈5[a]	42
PsKAO2	W11	n.d.	480	131	265	n.d.	≈49[1]	79

[1] Approximate TIC area value where baseline too high or peak contaminated, calculated from the uncontaminated major ion and a conversion factor based on the appropriate standard.

n.d. not detected

Table 2.2

Authentication of GA biosynthetic intermediates and additional products in yeast extracts

GC-MS relative ion abundances and Kovats' retention index (KRI) were compared to authentic standards. Samples are methyl ester (ME), trimethyl silyl (TMS), or methyl ester trimethyl silyl (METMS) derivatives. Data provided by C.A. Helliwell and A.T. Poole.

Reference or Putative Compounds	KRI	Characteristic Ions - Relative abundance as % of Base Peak								
		404(M+)	**389**	**314**	**299**	**255**	**254**	**239**	**223**	**199**
ent-7α-hydroxy KA METMS Standard	2359	22	8	100	32	69	59	43	19	22
ent-7α- hydroxy KA METMS	2360	35	13	100	33	67	60	45	19	23
		330(M+)	**287**	**270**	**255**	**242**	**241**	**239**	**227**	**199**
GA_{12} aldehyde ME Standard	2337	12	14	40	14	24	100	24	14	10
GA_{12} aldehyde ME	2336	11	14	40	15	25	100	24	15	11
		360(M+)	**328**	**300**	**285**	**241**	**240**	**225**	**185**	
GA_{12} ME Standard	2315	1	17	100	24	35	29	20	12	
GA_{12} ME	2316	1	17	100	22	35	31	26	15	
		388(M+)	**345**	**298**	**283**	**270**	**255**	**227**	**163**	**137**
7β-hydroxykaurenolide TMS standard	2457	2	9	100	15	17	12	10	12	58
7β-hydroxykaurenolide TMS	2452	1	10	100	17	22	14	13	27	95
		492(M+)	**477**	**402**	**387**	**343**	**327**	**269**	**253**	**209**
ent-6α,7α-dihydroxy KA METMS[a]	2493	<1	92	16	5	4	8	100	16	50

[a] Tentative identification based on comparison with published spectra (Gaskin and MacMillan, 1991).

Table 2.3

Putative C/D ring rearranged products in untransformed wild-type (WT) yeast and yeasts expressing KAOs fed with *ent*-kaurenoic acid (KA)

GC-MS relative ion abundances and Kovats' retention index (KRI) are indicated. Data provided by C.A. Helliwell and A.T. Poole.

Putative Compound	Present following feed of KA to	KRI	Characteristic Ions - (Relative abundance as % of Base Peak)
Stachenoic acid ME	WT yeast, *AtKAO1*, *PsKAO1*	2124	316(75), 301(8), 273(5), 257(22), 256(11), 241(11), 194(27), 181(24), 159(20), 148(39), 135(100)
Trachylobanic acid ME	WT yeast, *AtKAO1*, *PsKAO1*	2178	316(77),301(27), 273(5), 260(100), 257(58), 256(19), 245(45), 241(46), 201(30), 200(27)
ent-7α-hydroxy Stachenoic acid METMS[a]	*PsKAO1*, *PsKA02*	2244	404(25), 389(7), 314(51), 301(18), 299(15), 286(76), 283(63), 255(54), 254(44), 239(32), 223(42), 193(24), 181(46), 133(97), 73(100)
ent-7α-hydroxy Trachylobanic acid METMS[a]	*PsKAO1*, *PsKA02*	2299	404(2), 389(4), 314(100), 299(17), 255(55), 254(60), 239(41), 209(31), 185(43), 157(65)
ent-6α, 7α-dihydroxy Stachenoic acid METMS	*PsKA02*	2371	492(<1), 477(57), 402(18), 387(4), 343(4), 327(11), 269(100), 253(15), 209(45)
ent-6α, 7α-dihydroxy Trachylobanic acid METMS	*PsKA02*	2431	492(<1), 477(18), , 402(12), 387(3), 343(2), 327(4), 269(100), 253(17), 209(40)
Stacheno-GA_{12} ME[b]	*PsKA02*	2199	360(4), 328(47), 300(63), 285(22), 240(34), 225(53), 119(100)
Trachylo-GA_{12} ME	*PsKA02*	2260	360(4), 328(21), 300(100), 285(84), 241(73), 240(29), 225(31), 164(68), 119(24)

[a] Tentatively identified as *ent*-7α-hydroxy Stachenoic acid METMS and *ent*-7α-hydroxy Trachylobanic acid METMS based on the similarities in their mass spectra with *ent*-7α-hydroxy KA METMS, and similar relative KRI values of *ent*-7α-stachenoic (putative) / *ent*-7α-trachylobanic (putative) / *ent*-7α-kaurenoic acid METMS compared to the stachenoic / trachylobanic / kaurenoic acid ME KRI values.

[b] Tentatively identified as 'stacheno-GA_{12} ME' because of the similarity of its spectra to GA_{12} ME and Trachylo-GA_{12} ME, and similar relative KRI values to the two groups of three above.

Northern blot expression studies

The members of the pea kaurenoic acid oxidase (KAO) gene family are differentially expressed. *PsKAO1* is expressed in the stem and to a lesser extent in the leaf, root, apical bud, pod and seed while *PsKAO2* is only expressed in the seed (Fig. 2.8 A). *PsKAO2* is expressed most strongly around the time of contact point when the embryo just fills the testa and the liquid endosperm is all consumed (Fig. 2.8 B). This coincides with the rapid build up of GA levels in maturing seeds (Frydman et al., 1974; Swain et al., 1993).

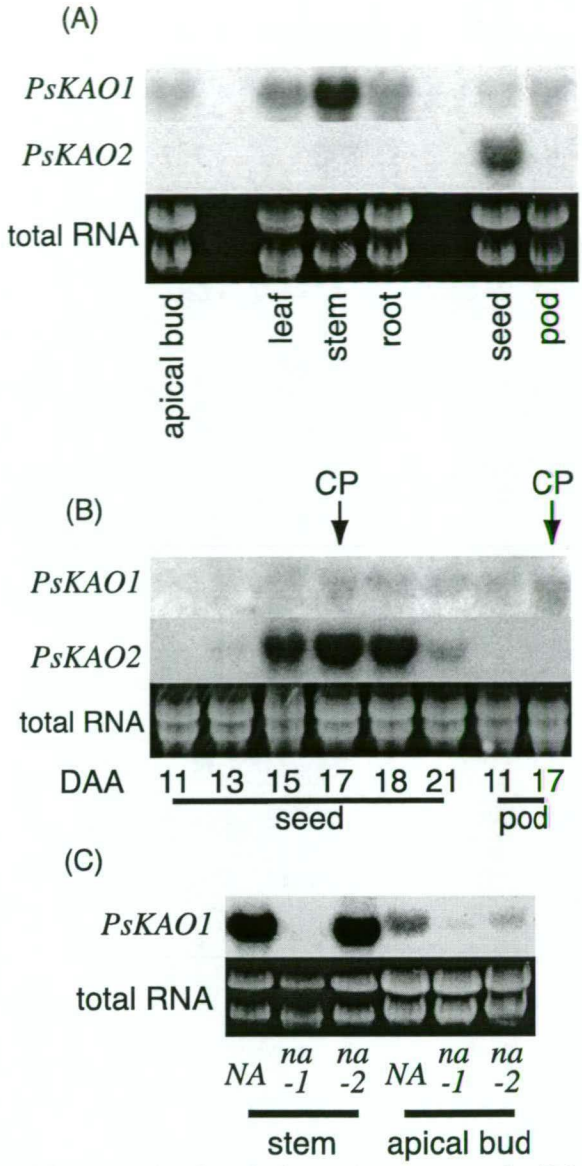


Figure 2.8

(A) *PsKAO1* and *PsKAO2* transcript levels in various parts of wild-type pea (L107). 5μg total RNA from the apical bud (all material above the uppermost fully expanded leaf), leaf (the uppermost fully expanded leaf), stem (internode immediately below the uppermost fully expanded leaf, the internode was 80% to 100% fully expanded), and root (50mm off the end of the tap and lateral roots) of 19 day old seedlings; also 5μg total RNA from seeds (3 days after contact point) and pods (that originally contained these seeds) from mature plants were loaded on the gel.

(B) *PsKAO1* and*PsKAO2* transcript levels in wild-type pea (L107) seeds at various developmental ages. Total RNA was extracted from whole seeds and their pods between 11 and 21 days after anthesis (DAA). Contact point (CP, the first day that no liquid endosperm remained in seeds) occurred at 17 days after anthesis.

(C) Northern blot analysis of *PsKAO1* in wild-type *NA* (WL1769) and mutants *na-1* (WL1766) and *na-2* (L81) from the apical bud (all tissue above the uppermost fully expanded leaf) or fully expanded stem tissue of 18 day old pea seedlings.

The EtBr stained total RNA is included as a loading control for each northern analysis.

DISCUSSION

PsKAO1 and *PsKAO2* encode CYP88A cytochrome P450 monooxygenases

Two genes, *PsKAO1* and *PsKAO2*, encoding cytochrome P450 monooxygenases from the subfamily CYP88A were identified in pea using a maize *D3*-like EST from soybean as probe. They have high similarity to the recently cloned genes, *AtKAO1* and *AtKAO2* of *Arabidopsis* (Helliwell et al., 2001a) and *CmKAO1* of pumpkin (Helliwell et al., 2000). They are grouped with the GA biosynthetic *ent*-kaurenoic acid oxidase (KAO) enzymes in the subfamily CYP88A as defined by the maize *D3* enzyme (Winkler and Helentjaris, 1995). The KAOs from the dicotelydons (pea, *Arabidopsis* and pumpkin) are grouped separately from those of the monocotyledons (rice, maize and barley) (Fig. 2.3). In addition, the two enzymes of pea are grouped, as are those of *Arabidopsis,* suggesting that gene duplication occurred late in the evolutionary process. The KAO deduced proteins contain the 4 catalytic domains A, B, C and D common to eukaryotic cytochrome P450s (Kalb and Loper, 1988). However, a critical conserved threonine of the A domain which was shown in the crystal structure of P450cam to form a hydrogen bond with glycine to produce an oxygen binding pocket (Poulos et al., 1985) and may also be involved in oxygen transfer (Imai et al., 1989), is replaced by serine in all the KAOs sequenced so far. In P450cam this threonine could be replaced by serine in site directed mutagenesis without altering the monooxygenase activity, whereas replacement with amino acids without the hydroxyl group uncoupled the oxygen consumption of the enzyme (Imai et al., 1989).

Pea *NA* encodes PsKAO1

The evidence shows that the pea *NA* gene encodes PsKAO1. Firstly, *PsKAO1* from both the *na-1* and *na-2* GA responsive dwarf mutants had altered sequences (Fig. 2.5 and 2.6). The mutations *na-1* and *na-2* are allelic and produced by independent mutational events (Reid et al., 1983). Furthermore, the 5 base deletion in *PsKAO1* associated with the *na-1* mutation co-segregated with the dwarf phenotype (Fig. 2.7). *PsKAO1* is expressed in the tissues where the *na* mutant phenotype is expressed. The mutant *na* plant has dwarfed stature, reduced taproot length, and reduced leaflet area (Reid et al., 1983; Reid and Ross, 1993; Yaxley et al., 2001). The GA_1 levels are reduced in the shoot, roots, leaves and pods (Potts and Reid, 1983; Ingram et al., 1984; Potts, 1986; Yaxley et al., 2001). *PsKAO1* was expressed in stems, roots, leaves, apical buds, pods and seeds (Fig. 2.8). However the other pea CYP88A gene, *PsKAO2* was only expressed in developing seeds and not any of the other tissues tested (Fig. 2.8). Since *PsKAO2* was expressed in seeds KAO activity could be expected in the seeds of the *na* mutant. This would explain the observation that mutant *na* plants have normal seed development and the same GA content in their seeds as wild-type pea seeds (Potts and Reid, 1983). In contrast the two *Arabidopsis* genes have similar expression patterns. Probably due to this redundancy, no mutants have been found in Arabidopsis (Helliwell et al., 2001a).

There is a possibility that there may be another pea gene similar to *PsKAO1* and *PsKAO2* (Fig. 2.4). One would not expect this gene to be expressed at a high level in the tissues that display a marked *na* mutant phenotype. Thus, *PsKAO1* is probably the predominant KAO activity expected to be expressed in apical, stem, root and leaf tissue. The extra bands in the genomic southern blot were observed only when the *PsKAO2* probe was used suggesting a greater similarity to the seed expressed gene. However, one cannot discount the possibility that the extra bands may indicate cross-hybridisation with another closely related cytochrome P450 subfamily.

The *na* mutants are dwarfs with extreme reduction in internode lengths. The differences in internode lengths between WL1766 and L81 (Fig. 2.1) arise from differences in their genetic background because genetic analysis could not distinguish between the two alleles at the phenotypic level (Reid et al., 1983). The putative proteins from the mutants (*na-1* and *na-2*) are severely altered from wild-type PsKAO1 (Fig. 2.5 and 2.6). However, the double recessive with other GA biosynthetic mutants (*na lh* and *na ls*) are shorter than the single *na* mutant (Reid, 1986b). This could be because of limited activity by other KAO enzymes in pea shoots because extra bands were observed on the genomic Southern blots at low stringency (Fig. 2.4) as discussed above. Alternatively there may be movement of intermediates from other tissues where the *PsKAO2* gene is expressed. The latter is possible as the effect of the *NA* (*PsKAO1*) gene is graft transmissible (Reid et al., 1983; Proebsting et al., 1992) and mature pea seeds at sowing contain GA_{20} (Ross et al., 1993). The other *KAO* gene (*PsKAO2*) is expressed in the seed (Fig. 2.8 A and B) and the seeds of *na* mutant plants develop normally (Potts and Reid, 1983; Potts, 1986). This may provide a limited amount of GA precursors in the stem of the mutant *na* seedlings allowing the epicotyl to develop almost normally before the extreme dwarfism sets in (Reid et al., 1983). Furthermore, the phenotype of double mutant seedlings appear to reflect the accumulation of GAs in the seed. For example the *na sln* double mutant accumulates abnormally large amounts of GA_{20} in their mature seeds and the seedlings initially have elongated internodes until they revert to the short internode *na* phenotype (Reid et al., 1992; Ross et al., 1995). The *le* mutation has no effect on seed GA content (Lester et al., 1999a) and therefore double mutant *na le* seedlings are similar in stature to *na LE* seedlings (Reid et al., 1983). In contrast, both the *ls* and *lh* mutations cause a decrease in GA levels in maturing seeds (Swain et al., 1995; Sun and Kamiya, 1997) and the *na ls* and *na lh* double mutants have such short internodes that the plants appear rosette-like (Reid, 1986b).

PsKAO1 and PsKAO2 catalyse *ent*-kaurenoic acid to GA_{12}

PsKAO1 and PsKAO2 when expressed in yeast catalysed the 3 steps from *ent*-kaurenoic acid to *ent*-7α-hydroxykaurenoic acid to GA_{12}-aldehyde to GA_{12} (Table 2.1, Fig. 2.2). This was observed previously with *AtKAO1* and *AtKAO2* from *Arabidopsis* and *HvKAO1* of barley (Helliwell et al., 2001a). Many of the enzymes in the GA biosynthetic pathway are multifunctional (Hedden, 1997). This occurs normally where the enzyme catalyses multiple oxidations at the same carbon position. For example the GA-20 oxidases catalyse the 3 step oxidation from GA_{53} to GA_{20} at the C-20 position (Phillips et al., 1995; Xu et al., 1995). Similarly the monooxygenase immediately preceding KAO in the GA biosynthetic pathway, *ent*-kaurene oxidase, catalyses *ent*- kaurene to *ent*-kaurenoic acid in three oxidation steps at the C-19 position (Fig. 2.2; (Helliwell et al., 1999). Likewise all the oxidations from *ent*-kaurenoic acid to GA_{12} are at the C-7 position of the GA skeleton (Hedden, 1997; MacMillan, 1997; Helliwell et al., 2001a).

The *na* mutation blocks GA biosynthesis prior to GA_{12}-aldehyde

To see if the 3-step oxidation of *ent*-kaurenoic acid observed in the yeast expression studies is demonstrated in the plant we can look at the corresponding mutants. The pea *na-1* and *na-2* mutants, which have a defective *PsKAO1* gene, do not metabolize [^{3}H] *ent*-kaurenoic acid to substances co-eluting with GA_{20}, GA_1 or GA_8 even though *NA* plants do carry out this conversion (Ingram and Reid, 1987). This supports the yeast expression data (Table 2.1) and the finding that the barley mutant (*grd5*) accumulates *ent*-kaurenoic acid in its seed (Helliwell et al., 2001a). However, metabolism studies show that *na* plants can convert [^{2}H] GA_{12}-aldehyde to C_{19}-GAs such as GA_{20}, GA_{29}, GA_1 and GA_8. In addition, application studies show that although *na* plants do not respond to precursors prior to GA_{12}-aldehyde, including *ent*-kaurenoic acid and *ent*-7α-hydroxy-kaurenoic acid, they readily

convert labeled GA_{12}-aldehyde to GA_1 (Ingram and Reid, 1987). These results were confirmed using another source of precursor ([6-$^3H_1$]GA_{12}-aldehyde instead of [18-$^2H_1$]GA_{12}-aldehyde to minimize the chance of substrate contamination (Ingram and Reid, 1987). The *na* mutation therefore appears to block the first two biosynthetic steps but not the final GA_{12}-aldehyde to GA_{12} step in the plant. This could occur if the nature of the *na* mutation altered the specificity of the PsKAO1 enzyme for the substrates. However this is not likely as the *na-1* (WL1766) is a null mutation and is expected to produce a severely truncated protein which does not even contain the catalytic domains (Kalb and Loper, 1988) including the active haem binding site common to cytochrome P450 enzymes (Fig. 2.5; Nebert and Gonzalez, 1987; Winkler and Helentjaris, 1995). Also, there is markedly less mRNA expressed in the stems of the *na-1* mutant than in the wild-type (Fig. 2.8 C). This may be due to the instability of mRNA with a premature stop codon (Gutierrez et al., 1999).

Alternatively, there may be other specific or non-specific enzymes present in the plant that can catalyse the last step but not the earlier steps catalysed by PsKAO1 and PsKAO2 when expressed in yeast. Application of either GA_{12}-aldehyde or GA_{53}-aldehyde to wild type or *na* seedlings produced a stem elongation response (Ingram and Reid, 1987). Both of these aldehydes would require oxidation at the carbon-7 position before the formation of active GAs. GA_{12}-aldehyde and GA_{12} are more hydrophilic than *ent*-kaurenoic acid and *ent*-7α-hydroxykaurenoic acid and can act as substrates for enzymes belonging to both the membrane-bound cytochrome P450 monooxygenases or the soluble 2-oxoglutarate dependent dioxygenases (Hedden, 1997). A GA 7-oxidase dioxygenase has been found in pumpkin (Lange, 1997) in addition to the monooxygenase 7-oxygenase activity (Hedden et al., 1984). This GA 7-oxidase dioxygenase catalyses the oxidation of GA_{12}-aldehyde to GA_{12} and GA_{14}-aldehyde to GA_{14} (Lange, 1997) but has not yet been found in other species (Hedden and Phillips, 2000).

Perhaps the most likely explanation is that non-specific activity may be involved since multigene families of aldehyde oxidases have been cloned from maize and *Arabidopsis* (Sekimoto et al., 1997; 1998) and some of these oxidise a wide range of aldehydes (Seo et al., 1998).

In barley embryos a cell free fraction was able to convert GA_{12}-aldehyde to GA_{12} but further metabolism to GA_{53} did not occur. However, a microsomal fraction could convert GA_{12}-aldehyde to GA_{12} then GA_{53} and GA_{12} to GA_{53} (Groβelindemann et al., 1992). PsKAO1 and PsKAO2 when expressed in yeast did not produce GA_{53} from any of the precursors provided, although the early 13-hydroxylation GA biosynthetic pathway predominates in pea (Ingram et al., 1986; Reid and Ross, 1993; Poole et al., 1995). In immature pea embryos, the formation of GA_{53} from GA_{12} was associated with the microsomal fraction and required NADPH and oxygen (Kamiya and Graebe, 1983), suggesting that there may be another membrane-bound cytochrome P450 monooxygenase in pea catalysing GA_{12} to GA_{53}.

Additional products of PsKAO activity

In addition to the main products of *ent*-7α hydroxykaurenoic acid, GA_{12}-aldehyde and GA_{12} several side products of PsKAO1 and PsKAO2 activity were identified in the yeast expression studies of Chris Helliwell and Andrew Poole. After either *ent*-kaurenoic acid (KA) or *ent*-7α-hydroxykaurenoic acid feeds, the byproduct *ent*-6α,7α-dihydroxykaurenoic acid on a branch pathway from *ent*-7α-hydroxykaurenoic acid was detected (Fig. 2.2). This compound was also detected as a product of AtKAO1 activity. The compound, *ent*-6α,7α-dihydroxykaurenoic acid was noted in pumpkin (Hedden, 1997; MacMillan, 1997) and related products were previously detected in pea (Ingram and Reid, 1987). The product 7β-hydroxykaurenolide was detected after KA (but not *ent*-7α-hydroxykaurenoic acid) feeds in yeast expressing PsKAOs and is presumably a side product of the formation

of the double bond and epoxide from *ent*-kaurenoic acid via *ent*-kauradienoic acid (Hedden, 1997). The P450-1 enzyme of *Gibberella fujikuroi* which catalyses the 4 steps from *ent*-kaurenoic acid to GA_{14} , also produced 7β-hydroxykaurenolide and *ent*-6α,7α-dihydroxykaurenoic acid (Rojas et al., 2001). Fungi and higher plants appear to have evolved their GA biosynthetic pathway independently and P450-1 belongs to a different cytochrome P450 subfamily (CYP68) with low sequence homology to higher plant KAOs of subfamily CYP88A (Hedden et al., 2002). Since the additional products are common to both enzymes they may be inevitable consequences of the reactions rather than specific products of the respective enzymes. In line with the expected difference between the fungi and higher plant KAO enzymes, the compound GA_{14} was not detected from PsKAO activity. It was interesting to note however that the C/D ring rearranged stachenoic acid and trachylobanic acid can act as substrate for the PsKAO activity.

CONCLUSION

The two *CYP88A* genes in pea, *PsKAO1* and *PsKAO2*, were cloned. Both of these genes catalyse the 3 steps from *ent*-kaurenoic acid to GA_{12} when expressed in yeast. The genes have distinct expression patterns. *PsKAO1* is the pea *NA* gene and is expressed in the stem, apical bud, root, leaf, pod and seed. Mutation in the *PsKAO1* gene results in the extreme dwarf *na* phenotype. *PsKAO2* is expressed in developing seeds explaining the normal seed GA levels and seed development of *na* plants.

MATERIALS AND METHODS

Plant material and growing conditions

Two independent mutational events in *Pisum sativum* L. resulted in the alleles *na-1* and *na-2* (Reid et al., 1983). The *na-1* fast neutron induced recessive mutant is from the Weibullsholm line WL1766 (genotype *na-1 LE LH LS*) and the *na-2* mutation is the Hobart line L81 (genotype *na-2 le LH LS*). The tall *NA* WL1769 (genotype *NA LE LH LS*) was used as wild-type and contains the progenitor sequence for the *na-1* mutation. The *na-1* and *NA* plants used were isogenic as a result of 8 generations of single plant selection after a cross between the closely related lines WL1766 (*na-1*) and WL1769 (*NA*). Another wild-type Hobart line L107 (genotype *NA LE LH LS*) derived from cv.Torsdag was used for some northern analyses.

Plants were grown 2 per pot in a heated glasshouse under an 18h photoperiod (Beveridge and Murfet, 1996).

UNIVERSITY OF TAS LIBRARY

Library screening

Dr Robert Elliott did the initial screening of both the seed and shoot cDNA libraries. The seed cDNA library was constructed in Lambda ZAPII (Stratagene) with cDNA prepared from L107 cv. Torsdag pea seeds at "contact point" (Ait-Ali et al., 1997). The library screening and the isolation of clones were according to methods recommended by the manufacturer (Stratagene). The shoot cDNA library was in Lambda gt11 prepared from pea cv. Alaska apical buds (CLONTECH). The library screening method was similar to above, however I obtained inserts directly by PCR with nested vector primers from original pure clones.

The probe used was 339 nucleotide fragment from maize-*D3* like EST from soybean. The conserved 3' end of soybean gi9483278 (BE657386) nearly identical to gi6915567 (AW397097) was ^{32}P labeled using the Decalabel DNA labeling kit (MBI Fermentas).

Genomic Southern Blots

Genomic DNA was isolated (Dellaporta et al., 1983), digested and run on a 0.7% agarose/TAE gel and blotted to Genesceen Plus (Dupont/NEN) in 2xSSC by Dr RC Elliott. I then hybridised the blots at 55°C in a hybridisation solution (0.5M sodium phosphate pH7.2, 1mM EDTA, 7% SDS). The initial washes were at 60°C with 2xSSC and 0.1%SDS. Then the membrane was washed at either high stringency (65°C, 0.2xSSC and 0.1% SDS) or low stringency (60°C, 1xSSC and 0.1%SDS).

A gel purified cDNA fragment of *PsKAO1* or *PsKAO2* (1.1kb from WL1769 stem or 0.9kb from L107 seed respectively) was labeled with ^{32}P using the Decalabel DNA labeling kit (MBI Fermentas) and used as probe for the Southern and northern blots.

Northern Blot Analysis

Total RNA was extracted using either the Phenol/SDS Method (Ausubel et al., 1994) Fig. 2.8 A); or RNeasy Plant Kit (QIAGEN; Fig. 2.8 B and C) consistent within the blot. The RNA (5μg per lane) was fractionated in 1.5% agarose gel containing formaldehyde and transferred to Genescreen Plus hybridisation transfer membrane (Dupont/NEN) using

10xSSC. The membrane was hybridised at 42°C in 5xSSC, 5xDenhardts, 50% formamide, 1% SDS, and 200μg ml^{-1} salmon sperm with a cDNA ^{32}P probe (see

above). The membrane was washed in 2xSSC, 0.1% SDS then 0.2xSSC, 0.1% SDS at 65°C and exposed to Kodak biomax X-ray film at -70°C.

Co-segregation analysis

DNA was extracted from the leaves of 50 individuals of 4 segregating families from the F9 generation of cross WL1766 (*na-1*) x WL1769 (*NA*). The genomic *PsKAO1* PCR products (243bp in the wild-type) were visualised using a 5' primer labeled with the fluorescent dye, hexachloro fluorescein (HEX). The PCR fragment encompassed the 5-base deletion of the *na-1* mutant gene. The PCR products were denatured (94° C for 3 min) in loading buffer containing deionised formamide and bromophenol blue then placed on ice prior to loading on a denaturing gel (5% Acrylamide gel in 0.6xTBE containing 7M urea) in the Gel-Scan 2000 (CORBETT Research).

Yeast Expression

The constructs were prepared in the pYEDP60 plasmid vector (Pompon et al., 1996). Oligonucleotide primers with restriction sites incorporated at the 5' end were designed and checked with the aid of the Oligo version 6.74 Primer Analysis Software (Wojciech and Rychlik © 1989-2001). The *PsKAO1* and *PsKAO2* cDNA were prepared from RNA extracted from WL1769 stems or L107 seeds respectively. The cDNA PCR products encompassed the putative protein-coding sequence with the 5'-UTR region as short as possible and were amplified using Pfu Turbo® DNA polymerase (Stratagene). These PCR products were cloned into pGEM-T vector (Promega) and sequenced to check for PCR generated mutations. Selected clones were digested using restriction enzymes corresponding to the sites introduced in the PCR primers and ligated into pYEDP60 vector in the sense orientation with

reference to the GAL10-CYC1 promoter (Pompon et al., 1996). These constructs were sent to Dr Chris Helliwell (CSIRO, Canberra) to transform into yeast. An *Arabidopsis* AtKAO1 construct was used for comparison (Helliwell et al., 2001a). The WAT11 and WAT21 yeast lines which are modified to express *Arabidopsis* NADPH-cytochrome P450 reductases, ATR1 and ATR2-1 respectively (Pompon et al., 1996; Urban et al., 1997), were transformed with the construct plasmids (Cullin and Pompon, 1988). The transformed yeasts and untransformed yeast as a control were incubated with 10μg of the substrates (*ent*-kaurenoic acid, *ent*-7α hydroxy kaurenoic acid, GA_{12}-aldehyde or *ent*-kaurene) for 2 h at 28°C (Helliwell et al., 1999). The products were then analysed by Andrew Poole and Chris Helliwell. In preparation for GC-MS analysis, methylation or trimethylsilylation was required. Extracts in ca. 2ml hexane/EtOAc were dried almost completely by speed vacuum then to completion under nitrogen. Methylation was in the same test tubes by addition of 50μL MeOH and 400μL of ethereal diazomethane. Samples were left for 15 minutes, dried as before, then transferred to reactivials using 4 x 50μL EtOAc. These were dried and then trimethylsilylated using 5μL pyridine and 5μL of bis(TMS)trifluoroactetamide + 1% trimetylchlorosilane which was heated at 90°C for 30 minutes (Helliwell et al., 1999). Injections were of 1μL samples with 0.1μL parafilm standard. The Kovats' retention indices (KRI) were calculated using hydrocarbon peaks from the co-injected parafilm standard. Identities of products were confirmed by GC-MS comparison of spectra and KRI to authentic standards where possible. Alternatively some side products were tentatively identified based on comparison with published spectra (Gaskin and MacMillan, 1991) and relative KRI values.

Chapter 3

The pea gene *LH* encodes *ent*-kaurene oxidase

INTRODUCTION

The *lh-1* and *lh-2* mutants are GA deficient dwarfs. Early internode lengths are reduced to nearly one-third (Reid, 1986b) the length of the wild-type (Fig. 3.1). They have a pronounced elongation response to applied GA_1 (Reid and Potts, 1986) and GA_3 (Swain and Reid, 1992). The normal tall plant phenotype can be restored by application of 10 μg GA_1 (Reid and Potts, 1986). Conversely, treatment of wild-type cv Torsdag plants with the GA biosynthesis inhibitor, paclobutazol, decreased their internode lengths to produce a *lh*-like phenotype (Swain and Reid, 1992).

The *lh* mutation blocks the 3-step oxidation of *ent*-kaurene to *ent*-kaurenoic acid (Swain et al., 1997). Extracts from developing embryos of the *lh-2* mutant were unable to metabolise [^{14}C] labeled *ent*-kaurene, *ent*-kaurenol, and *ent*-kaurenal to labeled products. In contrast, the wild type extracts readily metabolized *ent*-kaurene and all the intermediates (Fig. 3.2; Swain et al., 1997). Both the *lh-2* mutant and wild-type were able to form *ent*-kaurene from the precursors, mevalonic acid (MVA), geranylgeranyl diphosphate (GGDP) or copalyl diphosphate (CDP) and could metabolize *ent*-kaurenoic acid (Swain et al., 1997). This confirmed the earlier enzyme kinetic studies in wild cucumber (*Marah macrocarpus*) that showed inhibition of each of the three steps, *ent*-kaurene to *ent*-kaurenoic acid, by ancymidol. The similar inhibition kinetics of these steps suggested that all three were

catalysed by the same enzyme. However, the next step in the GA biosynthesis pathway, *ent*-kaurenoic acid to *ent*-7α-hydroxykaurenoic acid, had different kinetics and was not significantly inhibited by ancymidol (Coolbaugh et al., 1978).

An *Arabidopsis ent*-kaurene oxidase (AtKO1) was cloned (Helliwell et al., 1998) and found to be a cytochrome P450 monooxygenase of the subfamily CYP701A. When AtKO1was expressed in yeast it catalysed the 3 steps *ent*-kaurene to *ent*-kaurenoic acid (Fig. 3.2; Helliwell et al., 1999). Similarly two pumpkin *ent*-kaurene oxidases (CYP701A1 and CYP701A2) were isolated (Helliwell et al., 2000) and CmKO1 (Cyp701A1) expressed in yeast, catalysed *ent*-kaurene to *ent*-kaurenoic acid (Helliwell et al., 2001a).

Besides the *lh* mutant in pea, there are two other known *ent*-kaurene oxidase mutants, *ga3* in *Arabidopsis* (Koornneef and van der Veen, 1980) and *dx* in rice (Ogawa et al., 1996). In *Arabidopsis* the two alleles, *ga3-1* and *ga3-2* are recessive GA responsive dwarfs. They accumulate *ent*-kaurene and show a growth response to applied *ent*-kaurenoic acid but not *ent*-kaurene (Helliwell et al., 1998). The *ga3-1* allele is a more severe dwarf and requires exogenous GA for good germination (Helliwell et al., 1998). The pumpkin CYP701A1 complemented the *Arabidopsis* *ga3-2* mutant (Helliwell et al., 2000).

The two pea mutant alleles, *lh-1* and *lh-2* phenotypes differ in a tissue-specific manner. In the shoot the *lh-1* mutant is slightly more severe with a greater reduction in GA_1 and GA_{20} levels (Swain and Reid, 1992) and slightly shorter seedling stature than the *lh-2* mutant. There is a good correlation between internode length (nodes 4 to 6) and the log of endogenous GA_1 levels for the *lh-1, lh-2, LH* allelic series (Swain and Reid, 1992).

However, in the seed, the *lh-1* allele has only a mild effect on seed abortion and transient effect on embryo growth (Swain et al., 1997). In contrast, the *lh-2* allele phenotype has a large effect on seeds. The seeds are smaller, have delayed development (ie. take up to 6 days longer to reach contact point) (Swain et al., 1993)

and have decreased seed survival (less than 50% survived compared to the wild type) (Swain et al., 1993; 1997). Again the phenotypic differences between the alleles reflect the GA content. In young seeds the GA_1 levels of the *lh-1* mutant were reduced by only 50% whereas those of the *lh-2* allele were reduced by 90% compared with the wild-type (Swain et al., 1995). The GA_1, GA_3, GA_8, GA_{20}, GA_{29} levels were all reduced in seeds carrying the *lh-2* allele relative to the wild -type seeds (Swain et al., 1993; 1995). The *lh-2* seeds did not show an early GA peak around 7days after anthesis, although this was evident in the *lh-1* mutant and wild-type seeds. Swain et al. (1995) suggested that because the *lh-2* mutant was the only mutation with a phenotype affecting seed survival and the only mutation that altered the GA peak early in seed development, then this early peak in GA levels was essential for the development of the seed. At contact point (the first day that no liquid endosperm remained in seeds), there is a second peak in GA levels in normal developing seeds (Frydman et al., 1974; Swain et al., 1993). Both *lh-1* and *lh-2* alleles had decreased GA_{20} and GA_{29} levels at contact point compared with the wild-type (Swain et al., 1995). Swain et al. (1997) showed using genetic analyses that the seed phenotype is conferred directly by the seed genotype and the decrease in survival of *lh-2* seeds is not primarily due to changes in the assimilation of nutrients. The homozygous *lh-2 lh-2* seeds were smaller and lighter than both the *LHlh-2* and *lh-1lh-2* heterozygous seeds throughout development, when hand pollinated on the same maternal background. Further, 62% of *lh-2lh-2* seeds aborted compared to 5% of *LHlh-2* seeds on the *lh-2lh-2* maternal background (Swain et al., 1997). In the seed, the *lh-2* allele is recessive to both *lh-1* and wild-type (*LH*) (Swain et al., 1997). In the vegetative part of the plant, the *lh-1* allele has partial dominance over the *lh-2* allele with the *lh-1lh-2* crosses more closely resembling the slightly shorter stature of the *lh-1lh-1* seedlings (Swain and Reid, 1992).

Another marked difference in the *lh-1 and lh-2* alleles is their response to the specific *ent*-kaurene oxidase inhibitor, paclobutrazol (Sugavanam, 1984; Hedden and Graebe, 1985). *lh-2* seedlings are 30 times more sensitive to paclobutrazol than *lh-1*

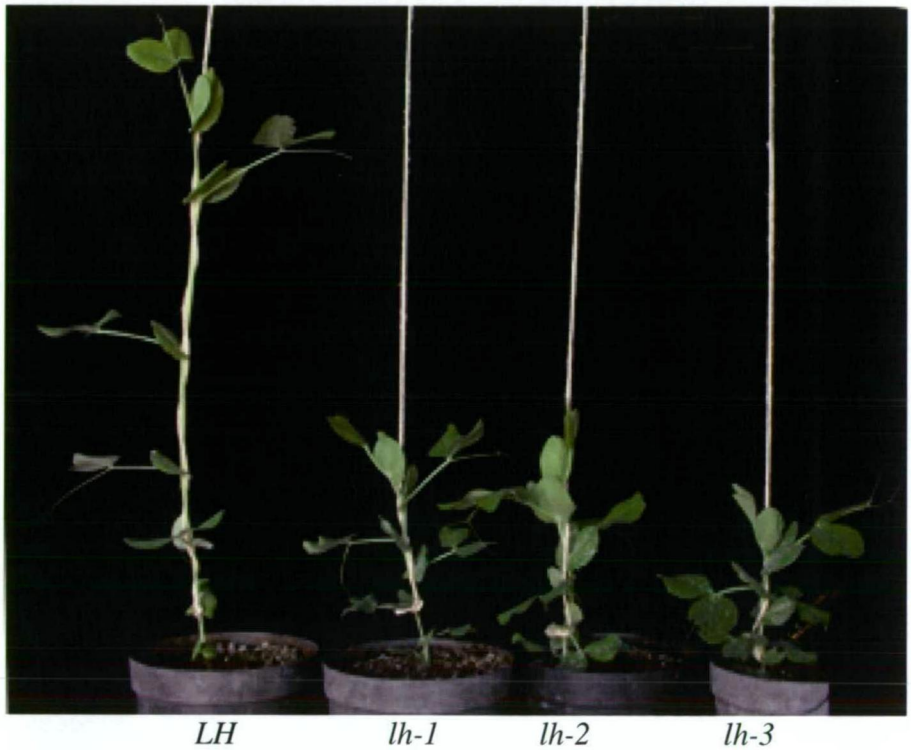
seedlings (Fig. 3.3; Swain et al., 1997). This inhibition appears to be directly related to GA biosynthesis, as GA_3 rescues *lh-1* and *lh-2* from the dwarfing effect of paclobutrazol (Swain and Reid, 1992).

The *ent*-kaurene oxidase enzyme is thought to play an important role in linking the plastid and endoplasmic reticulum located steps of gibberellin biosynthesis (Helliwell et al., 2001b). The highly hydrophobic *ent*-kaurene is produced in the plastid stroma (Sun and Kamiya, 1994; Aach et al., 1995) and is likely to be partitioned into membranes (Hedden, 1997). The *Arabidopsis* AtKO1 protein is directed to the outer envelope membrane of the chloroplast and therefore may channel the less hydrophobic product *ent*-kaurenoic acid to the *ent*-kaurenoic acid oxidase enzymes located in the endoplasmic reticulum (Helliwell et al., 2001b).

In this chapter, the pea homologue of the *Arabidopsis GA3* is isolated and shown to encode *LH*. Then an attempt is made to explain the previously perplexing differences between the *lh-1* and *lh-2* mutant phenotypes including the difference between the seed and shoot phenotypes, and the increased sensitivity of the *lh-2* mutant to triazole inhibitors. In addition a third allele *lh-3* is characterised.

LH *lh-1* *lh-2* *lh-3*

16 day old pea seedlings with leaf at fifth node fully expanded



LH *lh-1* *lh-2* *lh-3*

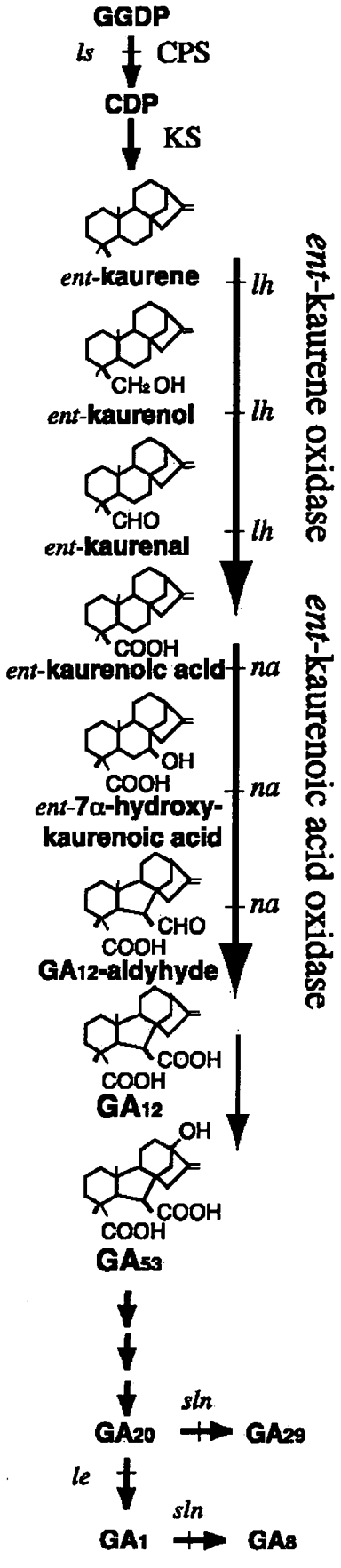
27 day old pea seedlings with leaf at seventh or eighth node fully expanded

LH *lh-1* *lh-2*

Mature flowering pea plants

Figure 3.1

Figure 3.2



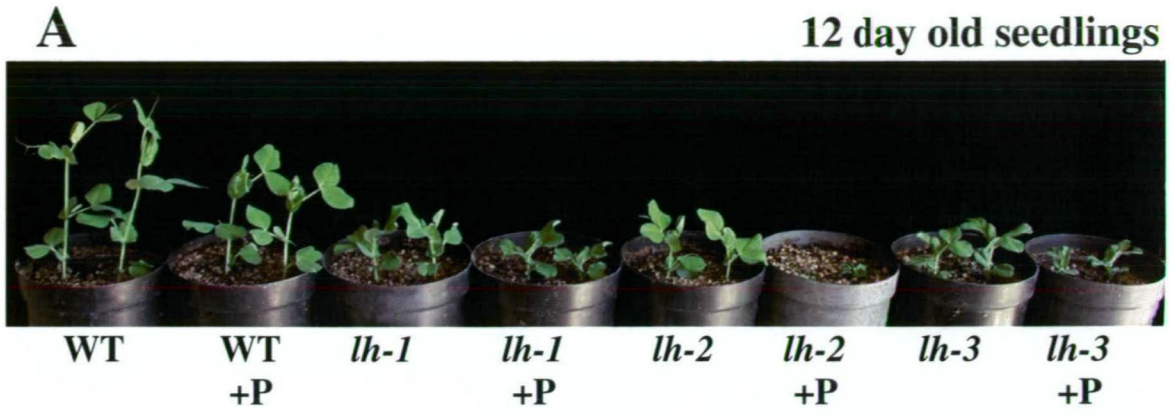
The gibberellin (GA) biosynthetic pathway in pea. Product structures are represented for the cytochrome P450 monooxygenase mediated steps showing the steps catalysed by *ent*-kaurene oxidase (KO) and *ent*-kaurenoic acid oxidase (KAO). The steps blocked by the pea mutants (*ls, lh, na, le, and sln)* are indicated. Steps catalysed by copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS) are also indicated.

RESULTS

Characterisation of the *lh-3* allele

The mutant *lh-3* (ABO4) is allelic to *lh-1* (K511) (Dr. J.L. Weller, personal communication). *lh-3* seedlings are of similar dwarf stature to the other mutants (*lh-1* and *lh-2*) and have internode lengths reduced to approximately 40% of the isogenic wild-type cv Torsdag (Fig. 3.1 and 3.3C). The seed survival rates of *lh-3* seeds (<5% seed abortions) were similar to that of the wild-type and the *lh-1* mutant ($\chi^2_1 = 3.76$; $P > 0.05$) but different from the *lh-2* mutant seeds of which 37% aborted ($\chi^2_1 = 47$; $P<0.001$)(Table 3.1). Similarly the seed weight of *lh-3* was similar to the wild-type and *lh-1* but greater than seeds of *lh-2* ($P<0.001$)(Table 3.1). To further compare the *lh-1* and *lh-3* mutants, the F_2 plants from a *lh-1* x *lh-3* cross was analysed. This was done to randomise any genetic background difference between the pure lines. The genotypes were determined by restriction digest with BfaI of PCR amplified fragments of DNA extracted from each seedling since the *lh-3* lesion removed a BfaI site present in the *PsKO1* gene of the *lh-1*. Genotyped *lh-3lh-3* seedlings were similar in stature (t-test on lengths between nodes 1 and 6; $P>0.5$) and had similar seed survival ($\chi^2_1 = 1.2$; $P>0.25$) to *lh-1lh-1* (Fig. 3.4).

The *lh-2* mutant is extremely sensitive to the specific GA biosynthesis inhibitor, paclobutrazol , and even at the low dose of 1 µg/seed paclobutrazol caused a 90% reduction in the internode length between nodes 1 and 6 in the *lh-2* mutant seedlings (Fig. 3.3; Swain et al., 1997). In contrast, the dwarfing response to paclobutrazol (1 µg/seed) of the *lh-3* plants was similar to that of the wild-type cv Torsdag and mutant *lh-1* within the range of a 20% to 40% reduction in internode length (Fig. 3.3).



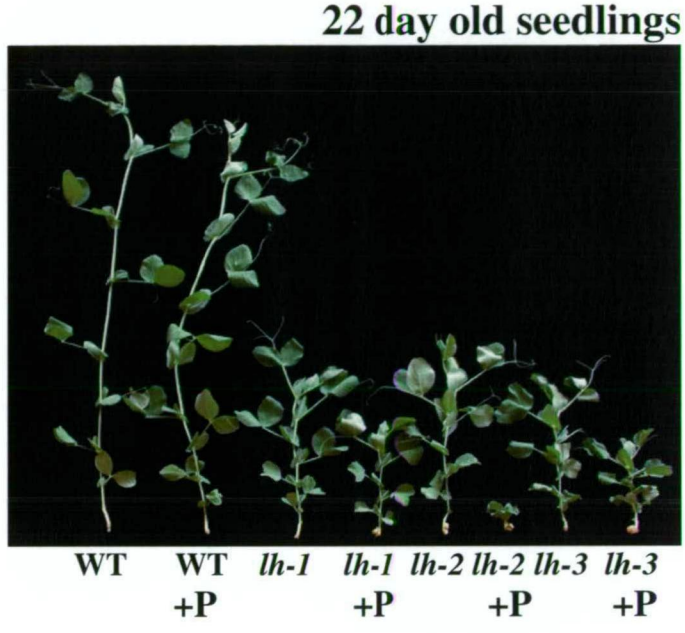


Figure 3.3

Wild-type cv Torsdag L107 (**WT**), and GA deficient mutants ***lh-1***, ***lh-2*** and ***lh-3*** were untreated controls or treated (**+P**) with the specific GA biosynthesis inhibitor paclobutrazol PP333 (1µg/seed) before planting.

C

Graph of internode length between node 4 and 6 of the controls (▨) and paclobutrazol treated (□) plants.

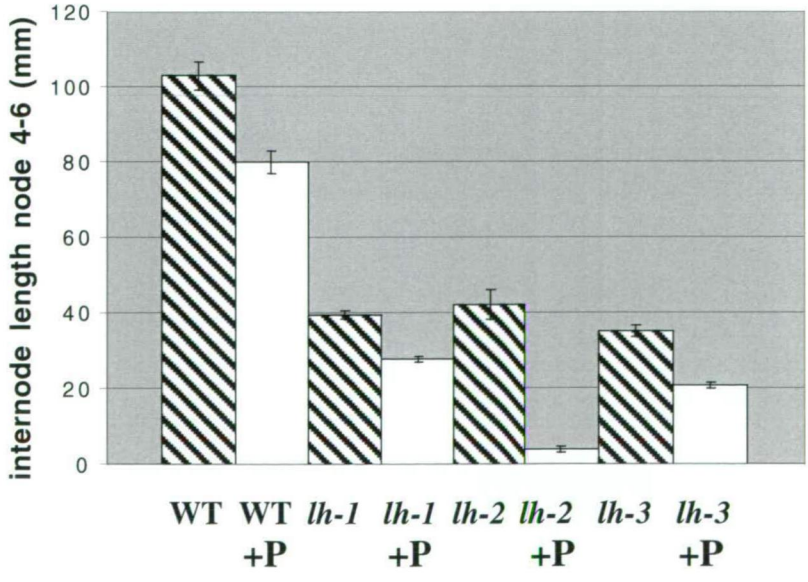
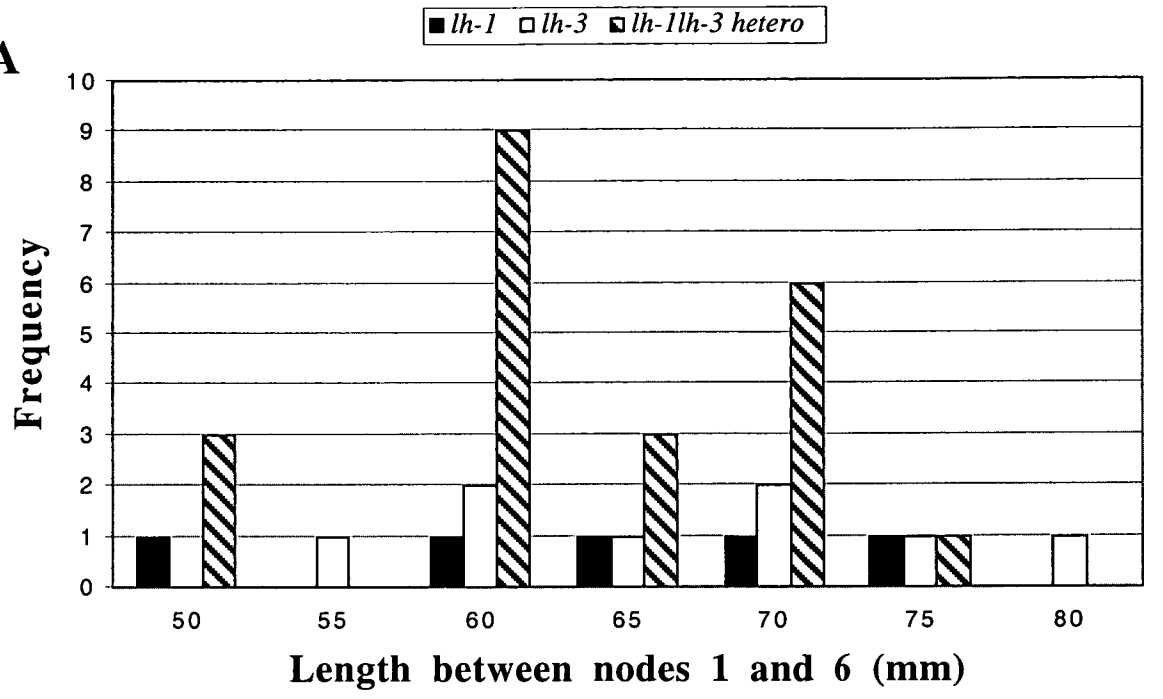


Table 3.1

Seed weight of healthy seeds and the number of seed abortions for seeds of wild-type (*LH*), *lh-1*, *lh-2* and *lh-3*.
Seeds were harvested on the same day for the aborted seed data and over 2 weeks for the seed weight data. Batches of 30 healthy dried seeds were weighed.

genotype	healthy seed weight (g/seed ± SE)	healthy seeds (number)	aborted seeds (number)	% seed abortions
wild-type (L107)	0.242 ± 0.005	110	0	0
lh-1 (K511)	0.262 ± 0.013	210	9	4
lh-2 (NBG5843)	0.198 ± 0.002	197	116	37
lh-3 (ABO4)	0.255 ± 0.005	90	0	0

A



B

F_2 products of *lh-1* x *lh-3* cross

genotype	plants	healthy seeds	aborted seeds	% seed abortions
lh-1lh-1	5	99	1	1
lh-3lh-3	9	125	4	3
lh-1lh-3	22	413	7	2

Figure 3.4

A Frequency graph of internode lengths between nodes 1 and 6 of homozygous *lh-1*, *lh-3* and heterozygous *lh-1lh-3* of F2 seedlings from the *lh-1* x *lh-3* cross. The genotypes were determined by restriction digest with BfaI of PCR amplified DNA extracted from each seedling since the *lh-3* lesion removed a BfaI site present in the *PsKO1* gene of the *lh-1*.

B Table of seeds from F_2 plants of *lh-1* x *lh-3* cross.

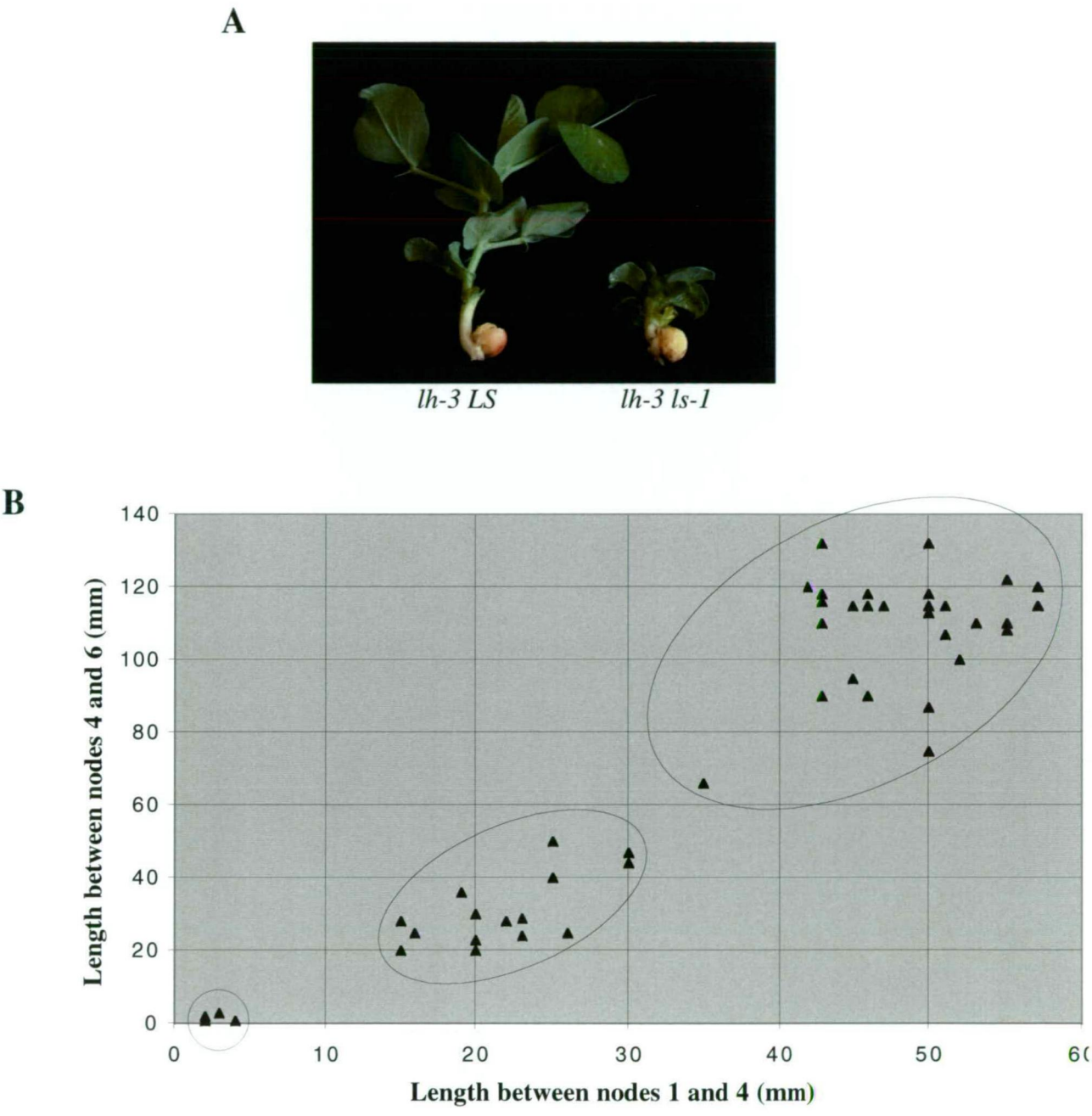


Figure 3.5

A Photo of double mutant (*lh-3lh-3 ls-1ls-1*) compared with single (*lh-3lh-3*) mutant. The seedlings were photographed 17 days after planting.

B Graph of internode lengths between nodes 1 and 4 plotted against lengths between nodes 4 and 6 of F_2 seedlings of the *lh-3* x *ls-1* cross. The seedlings group into tall (*LH--, LS--*), dwarf (*lh-3lh-3, LS--* or *LH--, ls-1ls-1*) and rosette-like dwarf double mutant (*lh-3lh-3, ls-1ls-1*).

The double mutant *lh-3 ls-1* is a rosette-like dwarf with markedly reduced stature compared to the single *lh-3* mutant (Fig. 3.5 previous page). The 54 F_2 seedlings from 4 families of the *lh-3* x *ls-1* cross divided into 30 tall (*LH--, LS--*), 19 dwarf (*lh-3lh-3, LS--* or *LH--, ls-1ls-1*) and 5 rosette-like dwarf double mutants (*lh-3lh-3, ls-1ls-1*) in agreement with the expected 9:6:1 segregation (χ^2_2= 0.86, P>0.5).

Isolation of pea *CYP701A*, the pea homologue of *Arabidopsis GA3*

The pea homologue of the *Arabidopsis GA3* gene was isolated by screening a pea seed cDNA library (cv. Torsdag) using the full length *GA3* cDNA as probe. The nearly full-length (1795bp) clone obtained was extended by 5' RACE using cDNA prepared from wild-type (cv. Torsdag) as template (Frohman et al., 1988).

The pea gene isolated, *PsKO1* (*CYP701A10* [a]; GenBank accession number AY245442) showed close homology at the nucleotide level (82 bit score, 84-90% identities) to the *Arabidopsis GA3 gene, AtKO1* (BLASTN, (Altschul et al., 1997). At the amino acid level, the full length putative protein PsKO1 is similar to the pumpkin *ent*-kaurene oxidase, CmKO1 (641 bit score, 65% identities, 80% positives) and the *Arabidopsis ent*-kaurene oxidase, AtKO1 (592 bit score, 61% identities, 77% positives; NCBI Blast 2 sequences) (Fig. 3.6).

PsKO1 is grouped with the other members of the cytochrome P450 monooxygenase subfamily CYP701A in the plant A-type cytochrome P450s and away from the other GA biosynthesis *ent*-kaurenoic acid oxidases, the brassinosteroid biosynthesis and the fungal GA biosynthesis cytochrome P450s (Fig. 3.7). This was confirmed in a phylogram when representatives from the fungal B

---

[a] Rice sequences recently released: CYP701A6 (AP00502.1a), CYP701A7 (AC087597.1b), CYP701A8 (AP005302.1c) and CYP701A9 (AP005302.1d).

cytochrome P450 clan were used as the outgroup (Fig. 3.8). Note that as expected CYP94A1, a representative of the 86-clan is separate from the 85-clan members (CYP88As (KAOs), 707As, 85As, 90As and 90Bs) and the plant A-type clan (Fig. 3.8) (Method Tables 3.3 and 3.4).

No other *CYP701A* clones were isolated from the pea seed library after a further 125,000 pfu were screened using a 1.3kb PCR fragment of *PsKO1* as probe.

PsKO1 sequence was altered in all three mutant alleles, *lh-1*, *lh-2* and *lh-3*.

lh-1 mutation

The PsKO1 sequence of the *lh-1* mutant plants had a single base change of guanine to adenine, which translates to a change from serine to asparagine in the putative protein (Fig. 3.9). These are both amino acids with uncharged polar side chains, however the serine hydroxyl group can be available for hydrogen bonding and also can be associated with enzyme active sites (Imai et al., 1989). This serine is in a region highly conserved in the *ent*-kaurene oxidase enzymes but not conserved in the cytochrome P450 plant clan A except in some members of the CYP71A and CYP71D subfamilies (Fig. 3.9). The serine is not conserved in other cytochrome P450 enzymes including the GA biosynthesis *ent*-kaurenoic acid oxidases and brassinosteroid biosynthesis enzymes.

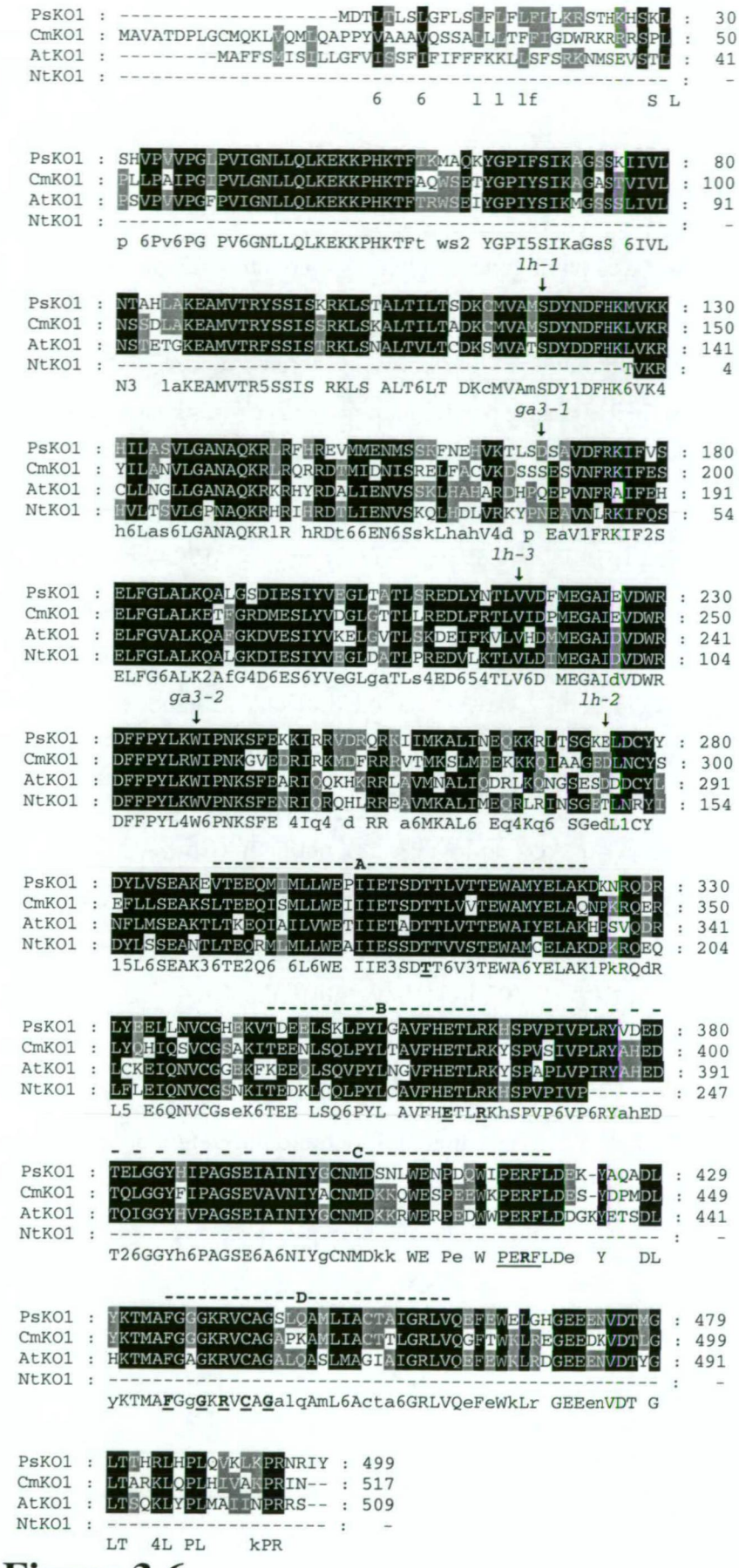

PsKO1 : ----------MDTLILSLGFLSFLFLFLLKRSTHKHSKL : 30
CmKO1 : MAVATDPLGCMQKLVQMLQAPPYVAAAVQSSALLTFFIGDWRKRRRSPL : 50
AtKO1 : ---------MAFFSMISILLGFVISSFIFIFFFKKLLSFSRKNMSEVSTL : 41
NtKO1 : ---------- : -
6 6 1 1 1f S L
PsKO1 : SHVPVVPGLPVIGNLLQLKEKKPHKTFTKMAQKYGPIFSIKAGSSKIIVL : 80
CmKO1 : ELLPAIPGIPVLGNLLQLKEKKPHKTFAQWSETYGPIYSIKAGASTVIVL : 100
AtKO1 : ESVPVVPGFPVIGNLLQLKEKKPHKTFTRWSETYGPIYSIKMGSSSLIVL : 91
NtKO1 : ---------- : -
p 6Pv6PG PV6GNLLQLKEKKPHKTFt ws2 YGPI5SIKaGsS 6IVL
1h-1
PsKO1 : NTAHLAKEAMVTRYSSISKRKLSTALTILTSDKCMVAMSDYNDFHKMVKK : 130
CmKO1 : NSSDLAKEAMVTRYSSISSRKLSKALTILTADKCMVAMSDYNDFHKLVKR : 150
AtKO1 : NSTETGKEAMVTRFSSISTRKLSNALTVLTCDKSMVATSDYDDFHKLVKR : 141
NtKO1 : ----------TVKR : 4
N3 laKEAMVTR5SSIS RKLS ALT6LT DKcMVAmSDY1DFHK6VK4
ga3-1
PsKO1 : HILASVLGANAQKRLRFHREVMMENMSSKFNEHVKTLSDSAVDFRKIFVS : 180
CmKO1 : YILANVLGANAQKRLRQRRDIMIDNISRELFACVKDSSSESVNFRKIFES : 200
AtKO1 : CLLNGLLGANAQKRKRHYRDALIENVSSKLHAHARDHPQEPVNFRAIFEH : 191
NtKO1 : HVLTSVLGPNAQKRHRIHRDTLIENVSKQLHDLVRKYPNEAVNLRKIFQS : 54
h6Las6LGANAQKRlR hRDt66EN6SskLhahV4d p EaV1FRKIF2S
1h-3
PsKO1 : ELFGLALKQALGSDIESIYVEGLTATLSREDLYNTLVVDFMEGAIEVDWR : 230
CmKO1 : ELFGLALKETFGRDMESLYVDGLGTTLLREDLFRTLVIDFMEGAIEVDWR : 250
AtKO1 : ELFGVALKQAFGKDVESIYVKELGVTLSKDEIFKVLVHDMMEGAIDVDWR : 241
NtKO1 : ELFGLALKQALGKDIESIYVEGLDATLPREDVLKTLVLDTMEGAIDVDWR : 104
ELFG6ALK2AfG4D6ES6YVeGLgaTLs4ED654TLV6D MEGAIdVDWR
ga3-2 1h-2
PsKO1 : DFFPYLKWIPNKSFEKKIRRVDRQRKIIMKALINEQKKRLTSGKELDCYY : 280
CmKO1 : DFFPYLRWIPNKGVEDRIRKMDFRRRVTMKSLMEEKKKQIAAGEDLNCYS : 300
AtKO1 : DFFPYLKWIPNKSFEARIQQKHKRRLAVMNALIQDRLKQNGSESDDCYL : 291
NtKO1 : DFFPYLKWVPNKSFENRIQRQHLRREAVMKALIMEQRLRINSGETLNRYI : 154
DFFPYL4W6PNKSFE 4Iq4 d RR a6MKAL6 Eq4Kq6 SGedL1CY
A
PsKO1 : DYLVSEAKEVTEEQMIMLLWEPIIETSDTTLVTTEWAMYELAKDKNRQDR : 330
CmKO1 : EFLLSEAKSLTEEQISMLLWEIIIETSDTTLVVTEWAMYELAQNPKRQER : 350
AtKO1 : NFLMSEAKTLTKEQIAILVWETIIETADTTLVTTEWAIYELAKHPSVQDR : 341
NtKO1 : DYLSSEANTLTEQRMLMLLWEAIIESSDTTVVSTEWAMCELAKDPKRQEQ : 204
15L6SEAK36TE2Q6 6L6WE IIE3SDTT6V3TEWA6YELAK1PkRQdR
B
PsKO1 : LYEELLNVCGHEKVTDEELSKLPYLGAVFHETLRKHSPVPIVPLRYVDED : 380
CmKO1 : LYDHIQSVCGSAKITEENLSQLPYLTAVFHETLRKYSPVSIVPLRYAHED : 400
AtKO1 : LCKEIQNVCGGEKFKEEQLSQVPYLNGVFHETLRKYSPAPLVPIRYAHED : 391
NtKO1 : LFLEIQNVCGSNKITEDKLCQLPYLCAVFHETLRKHSPVPIVP------- : 247
L5 E6QNVCGseK6TEE LSQ6PYL AVFHETLRKhSPVP6VP6RYahED
C
PsKO1 : TELGGYHIPAGSEIAINIYGCNMDSNLWENPDQWIPERFLDEK-YAQADL : 429
CmKO1 : TQLGGYFIPAGSEVAVNIYACNMDKKQWESPEEWKPERFLDES-YDPMDL : 449
AtKO1 : TQIGGYHVPAGSEIAINIYGCNMDKKRWERPEDWWPERFLDDGKYETSDL : 441
NtKO1 : ---------- : -
T26GGYh6PAGSE6A6NIYgCNMDkk WE Pe W PERFLDe Y DL
D
PsKO1 : YKTMAFGGGKRVCAGSLQAMLIACTAIGRLVQEFEWELGHGEEENVDTMG : 479
CmKO1 : YKTMAFGGGKRVCAGAPKAMLIACTTLGRLVQGFTWKLREGEEDKVDTLG : 499
AtKO1 : HKTMAFGAGKRVCAGALQASLMAGIAIGRLVQEFEWKLRDGEEENVDTYG : 491
NtKO1 : ---------- : -
yKTMAFGgGKRVCAGalqAmL6Acta6GRLVQeFeWkLr GEEenVDT G
PsKO1 : LTTHRLHPLQVKLKPRNRIY : 499
CmKO1 : LTARKLQPLHIVAKPRIN-- : 517
AtKO1 : LTSQKLYPLMAIINPRRS-- : 509
NtKO1 : ---------- : -
LT 4L PL kPR

Figure 3.6

Figure 3.7

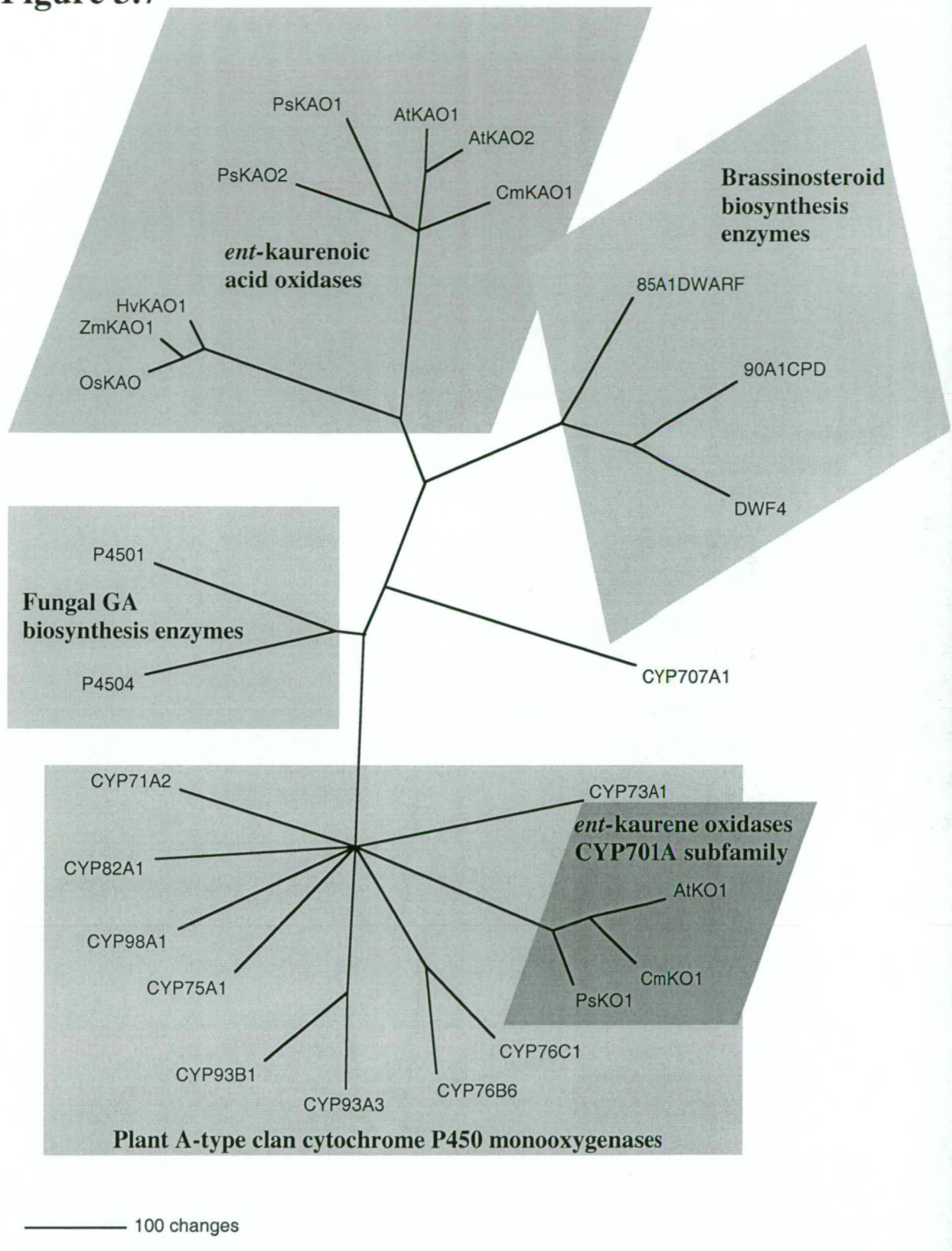
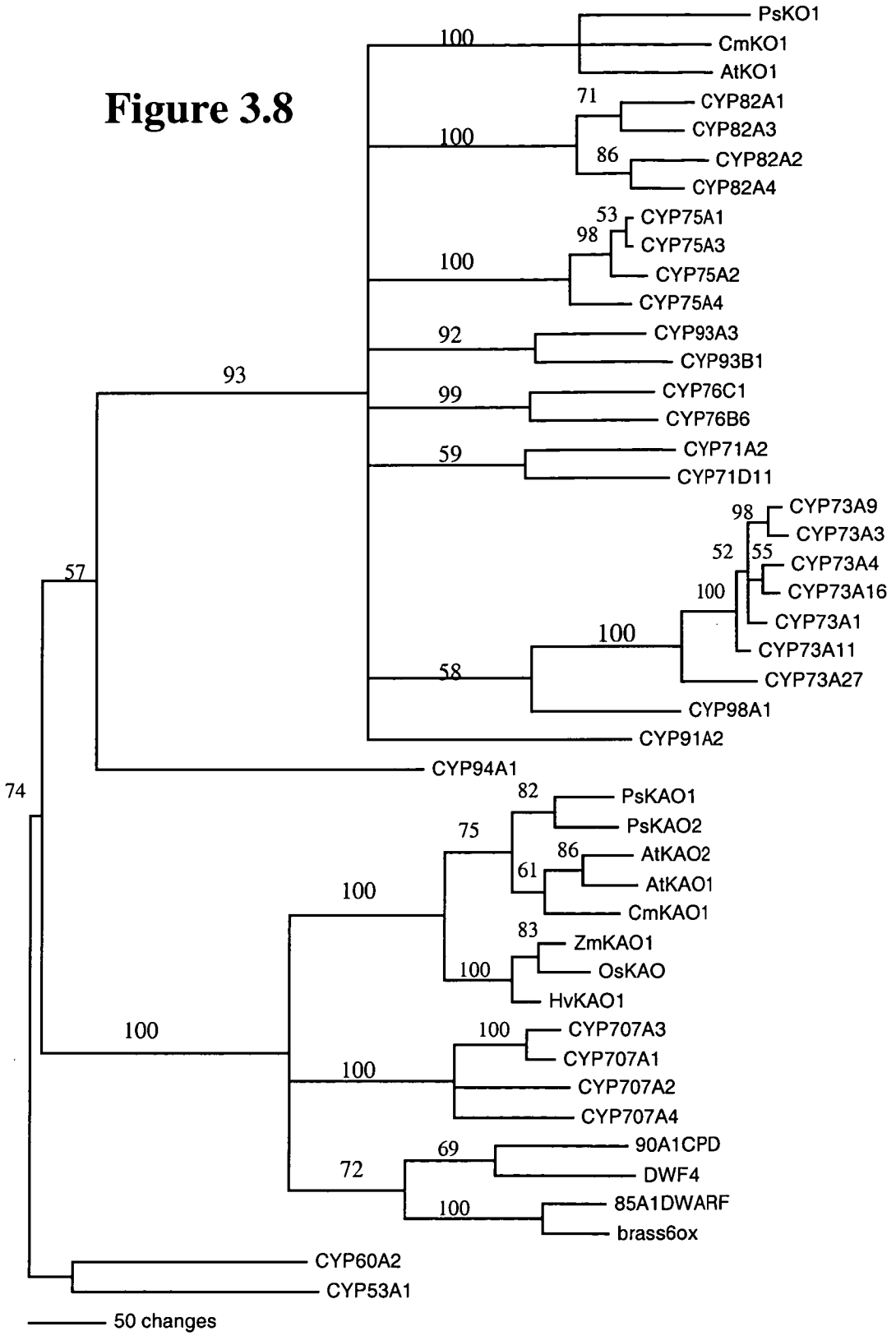

PsKAO1
AtKAO1
AtKAO2
PsKAO2
CmKAO1
ent-kaurenoic acid oxidases
HvKAO1
ZmKAO1
OsKAO
Brassinosteroid biosynthesis enzymes
85A1DWARF
90A1CPD
DWF4
P4501
Fungal GA biosynthesis enzymes
P4504
CYP707A1
CYP71A2
CYP73A1
ent-kaurene oxidases CYP701A subfamily
CYP82A1
AtKO1
CYP98A1
CmKO1
CYP75A1
PsKO1
CYP76C1
CYP93B1
CYP76B6
CYP93A3
Plant A-type clan cytochrome P450 monooxygenases
100 changes

Figure 3.8

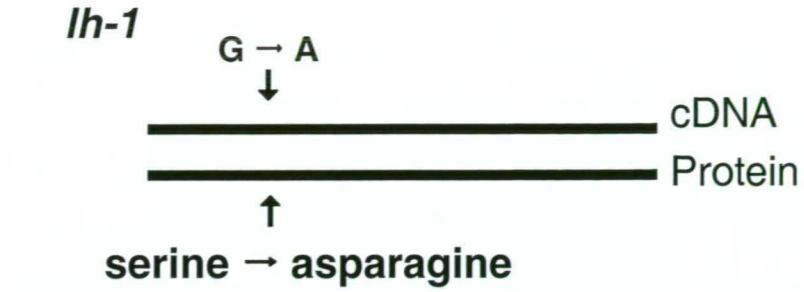

PsKO1
CmKO1
AtKO1
CYP82A1
CYP82A3
CYP82A2
CYP82A4
CYP75A1
CYP75A3
CYP75A2
CYP75A4
CYP93A3
CYP93B1
CYP76C1
CYP76B6
CYP71A2
CYP71D11
CYP73A9
CYP73A3
CYP73A4
CYP73A16
CYP73A1
CYP73A11
CYP73A27
CYP98A1
CYP91A2
CYP94A1
PsKAO1
PsKAO2
AtKAO2
AtKAO1
CmKAO1
ZmKAO1
OsKAO
HvKAO1
CYP707A3
CYP707A1
CYP707A2
CYP707A4
90A1CPD
DWF4
85A1DWARF
brass6ox
CYP60A2
CYP53A1
50 changes

lh-2 mutation

Initially the cDNA PCR fragment from *lh-2* mutant apical bud tissue was larger than the equivalent wild-type and *lh-1* while the genomic PCRs using the same primers had the same sized fragments (Fig. 3.10A). Then the *PsKO1* genomic sequence was found to have a single base substitution of adenine for guanine at the beginning of an intron. This removed the highly conserved G at the beginning of the intron important for forming a lariat and correct pre-mRNA splicing (Fig. 3.10C) (Brown, 1996; Brown et al., 1996). The whole 83 base intron was retained which included a stop codon in the reading frame (Fig. 3.10B and C). This would result in a 275 amino acid truncated putative protein which would not contain the active catalytic domains A, B, C and D nor the active haem binding site common to cytochrome P450s (Fig. 3.6) (Kalb and Loper, 1988) and therefore would be expected to be a null mutation.

lh-3 mutation

The *PsKO1* sequence of the *lh-3* mutant plants had a single base change of guanine to adenine, which translates to a change from valine to methionine in the putative protein (Fig. 3.11). Both valine and methionine have nonpolar side chains. This replaced valine is conserved in all 4 *ent*-kaurene oxidases with sequences available (Fig. 3.6) and is conserved in many members of the cytochrome P450 plant group A clan (Fig. 3.11) (www.drnelson.utmem.edu/CytochromeP450.html; www.biobase.dk/P450/p450.shtml) and is second degree boxshade highlighted in cytochrome P450 A-type *Arabidopsis* alignment of Paquette et al. (2000) (www.biobase.dk/P450/p450.shtml). This valine is not conserved in the GA biosynthesis *ent*-kaurenoic acid oxidases or brassinosteroid biosynthesis enzymes (Fig. 3.11).



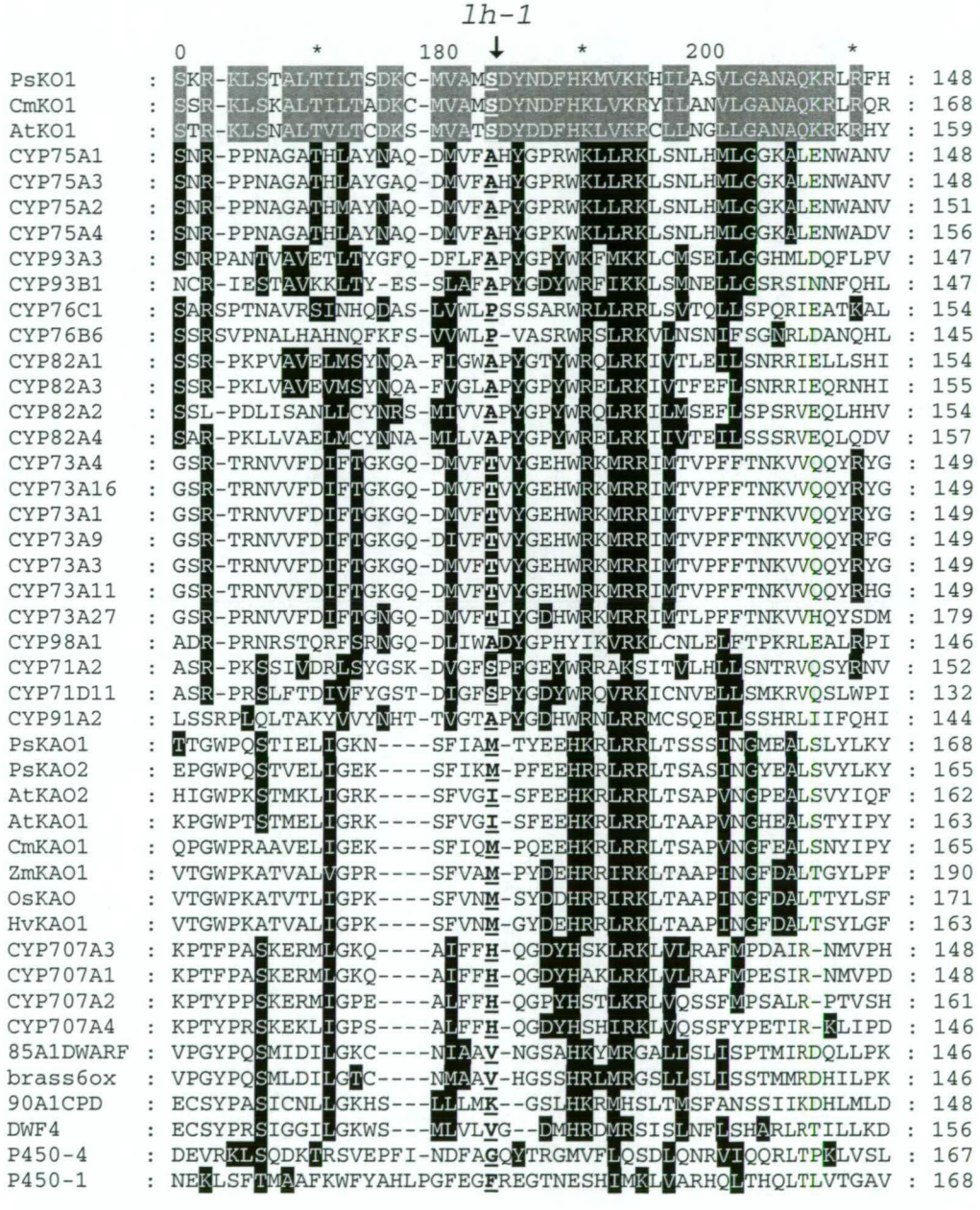
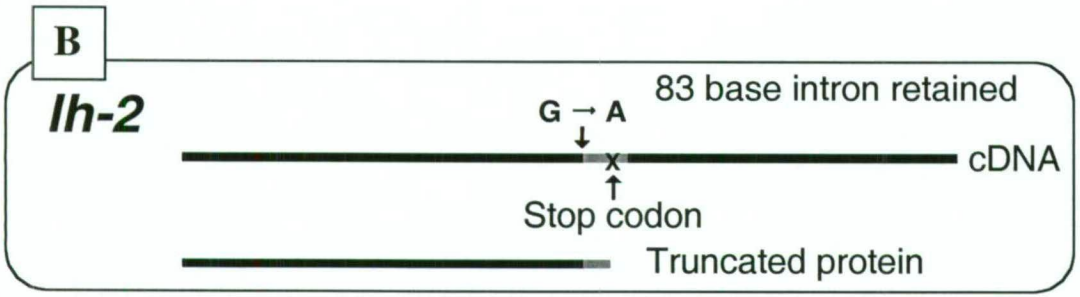


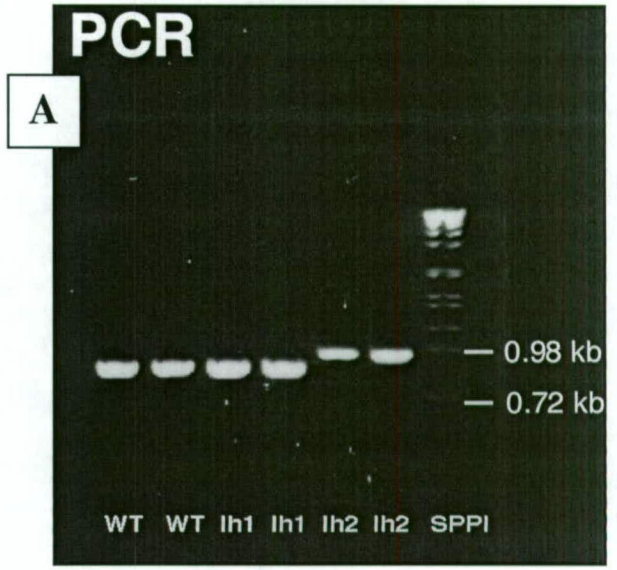
Figure 3.9

Schematic diagram of the *lh-1* mutation and GeneDoc display of relevant cytochrome P450s (Method Tables 3.3 and 3.4) around the *lh-1* mutation site (S).

Figure 3.10

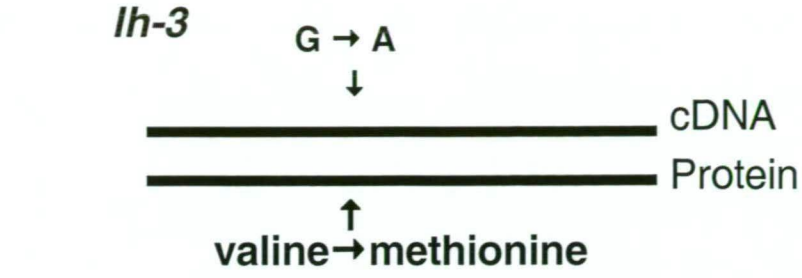
A. 2%agarose/TAE electophoresis gel of PCR products with the same primers of wild-type, *lh-1* and *lh-2* cDNA.
B. Schematic diagram of *lh-2* mutation.
C. A comparison of genomic and cDNA sequence from wild-type and *lh-2* to illustrate the *lh-2* genetic lesion.





C

```
WT genomic DNA   TTGACATCAGGAAAAGTAAGTCTTTCTTGTTTGCAATTTCAAAATTATGCATAAACCATGATAGAGTTATCTTAACTTGATTGTTTGGGATGGAACAGGAATTAGATTGTTA
WT cDNA          TTGACATCAGG AAAA----------------------------------------------------------------------------------GAATTAGATTGTTA
lh-2 cDNA        TTGACATCAGGAAAAATAAGTCTTTCTTGTTTGCAATTTCAAAATTATGCATAAACCATGATAGAGTTATCTTAACTTGATTGTTTGGGATGGAACAGGAATTAGATTGTTA
lh-2 genomic DNA TTGACATCAGGAAAAATAAGTCTTTCTTGTTTGCAATTTCAAAATTATGCATAAACCATGATAGAGTTATCTTAACTTGATTGTTTGGGATGGAACAGGAATTAGATTGTTA
                      ↑                                                                                    ↑
                   mutation                                                                          stop codon
                                                                                                        TAA
```



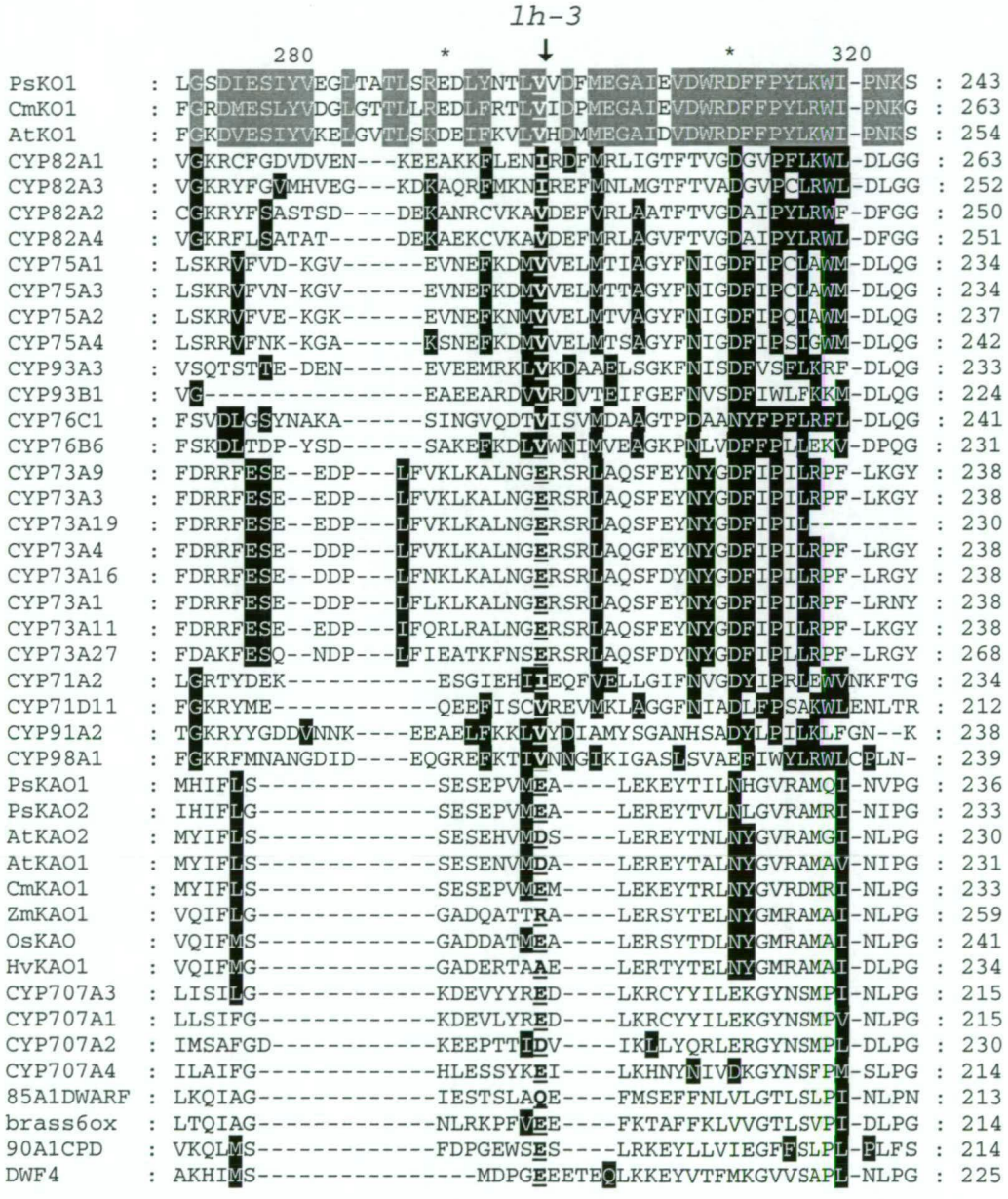


Figure 3.11

Schematic diagram of *lh-3* mutation and GeneDoc identity presentation of clustal W alignment with relevant cytochrome P450s surrounding the *lh-3* mutation site (V).

Northern blot Analysis

The *PsKO1* gene was expressed in all pea organs tested including apical bud, stem, leaf, root, seed and pod (Fig. 3.12) although there was less mRNA observed in the leaf than other tissues. The *lh-2 PsKO1* mRNA runs slower consistent with the expected larger mRNA due to the retention of the 83 base intron; giving confidence in the specificity of the probe.

PsKO1 is expressed at all stages of seed development tested (Figs. 3.13 and 3.14A). *PsKO1* was expressed more strongly at an early stage of pea seed development and then again at contact point (Figs. 3.13 and 3.14A). This pattern was consistently observed in different harvests and with different methods of RNA extraction. The *PsKO1* expression in young 11 DAA seeds was more pronounced in the homozygous mutant seeds (*lh-1* and *lh-2*)(Figs. 3.13 and 3.14A). Seeds from the GA deficient dwarf mutant, *ls-1*, with impaired copalyl diphosphate synthase (*CPS*) earlier in the GA biosynthesis pathway (Fig. 3.2), also had slightly stronger *PsKO1* expression than the wild-type at 11 days after anthesis (Fig. 3.13). This possibly may reflect delayed development and initially smaller seeds of the mutant phenotypes if we are at the tail end of this expression peak as suggested by the *PsCPS* (*LS*) expression peak observed by (Ait-Ali et al., 1997) at 4 days after anthesis. The expression patterns of other GA biosynthetic genes such as *PsKAO2* and *PsCPS* will be discussed in chapter 4.

Alternate splicing of the mutant *lh-2* mRNA

The *PsKO1* probe (prepared from a 750bp template 5' of the *lh-2* lesion) revealed two bands associated with the altered mRNA of the *lh-2* mutant seed when expression levels were high (Figs. 3.13). This was observed using a different method

of RNA extraction (Figs. 3.14A and B). One band was larger than wild-type and was also seen in *lh-2* apical buds (Fig. 3.12) and is expected to be due to the retention of the intron containing the lesion associated with the *lh-2* mutant. The other *PsKO1* band was smaller than wild-type and was only clearly noted in *lh-2* seeds when expression levels were high (Figs. 3.13 and 3.14A). The smaller extra band was not observed in the wild-type seed with similar expression levels, age and weight of seeds (Fig. 3.14B) and is approximately 1kb (between the 0.2 and 1.3kb RNA ladder markers) consistent with being smaller than the small ribosomal band (Fig. 3.15C). When a probe prepared from a 925bp template spanning the *lh-2* lesion (425bp 5' and 500bp 3' of the *lh-2* lesion) was used another band of intermediate size (perhaps near 1.3kb) was noted in the seed (Fig. 3.15A) and apical bud tissue (Fig. 3.15C) in addition to the 2 bands observed with the 5' probe. This band is associated with the region 3' of the *lh-2* lesion and was not observed in the wild-type or *lh-1* samples (Fig. 3.15C).

Since alternate splicing is occurring in the *lh-2* mutant mRNA, PCR was used to test if any correct splicing occurred in the *lh-2* producing a wild-type mRNA which could encode an enzyme with normal activity. PCR using a primer spanning the intron/exon boundry (of the intron containing the *lh-2* lesion) revealed a band of the same size as the wild-type in the *lh-2* samples (Fig. 3.16). For similar intensity on the gel, a larger volume of PCR product from *lh-2* and controls was loaded on the gel than wild-type samples, suggesting that markedly less correctly spliced product was present in the *lh-2* cDNA than the wild-type (cv Torsdag) cDNA.

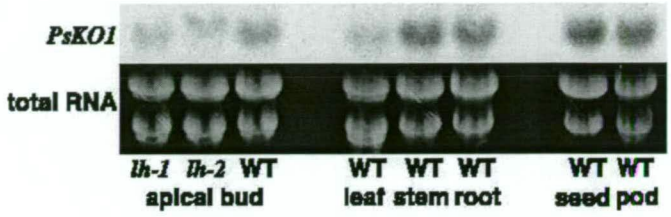


Figure 3.12

PsKO1 transcript level in various parts of wild-type pea (L107) and the apical bud from *lh-1* (K511) and *lh-2* (NGB5843) mutants. 5μg total RNA from the apical bud (all material above the uppermost fully expanded leaf), leaf (the uppermost fully expanded leaf), stem (internode immediately below the uppermost fully expanded leaf, the internode was 80% to 100% fully expanded), and root (50mm off the end of the tap and lateral roots) of 19 day old seedlings; also 5μg total RNA from seeds (3 days after contact point) and pods (that originally contained these seeds) from mature plants were loaded on the gel.

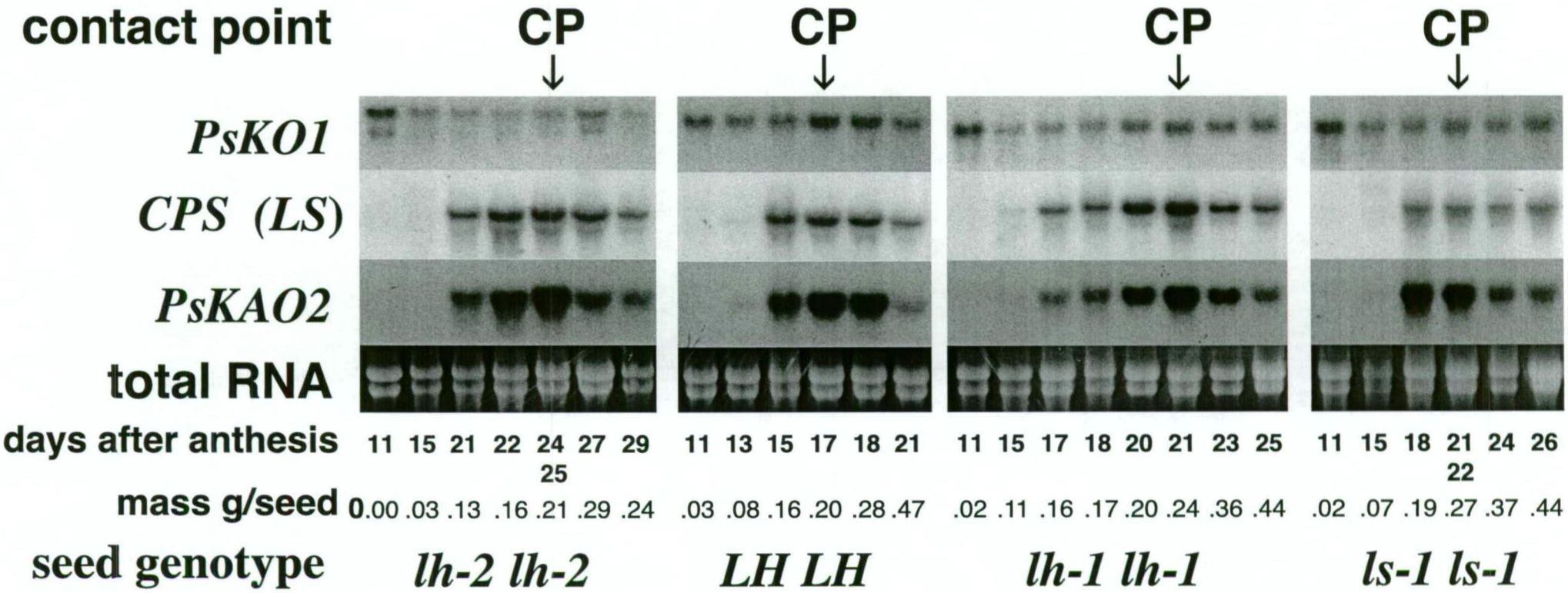


Figure 3.13

Northern blot analysis of seeds at various ages and weights during development from homozygous wild-type (*LH*), and dwarf mutant *lh-1*, *lh-2* and *ls-1* plants. Total RNA (5mg) from whole seeds at various ages (days after anthesis) were loaded on the gel. *PsKO1* (ent-kaurene oxidase), *CPS* (*LS*, copalyl diphosphate synthase) and *PsKAO2* (ent-kaurenoic acid oxidase) transcript levels were measured. (Note this figure is repeated with additional information in Chapter 4)
CP Contact point (the first day that no liquid endosperm remained in seeds) is indicated.

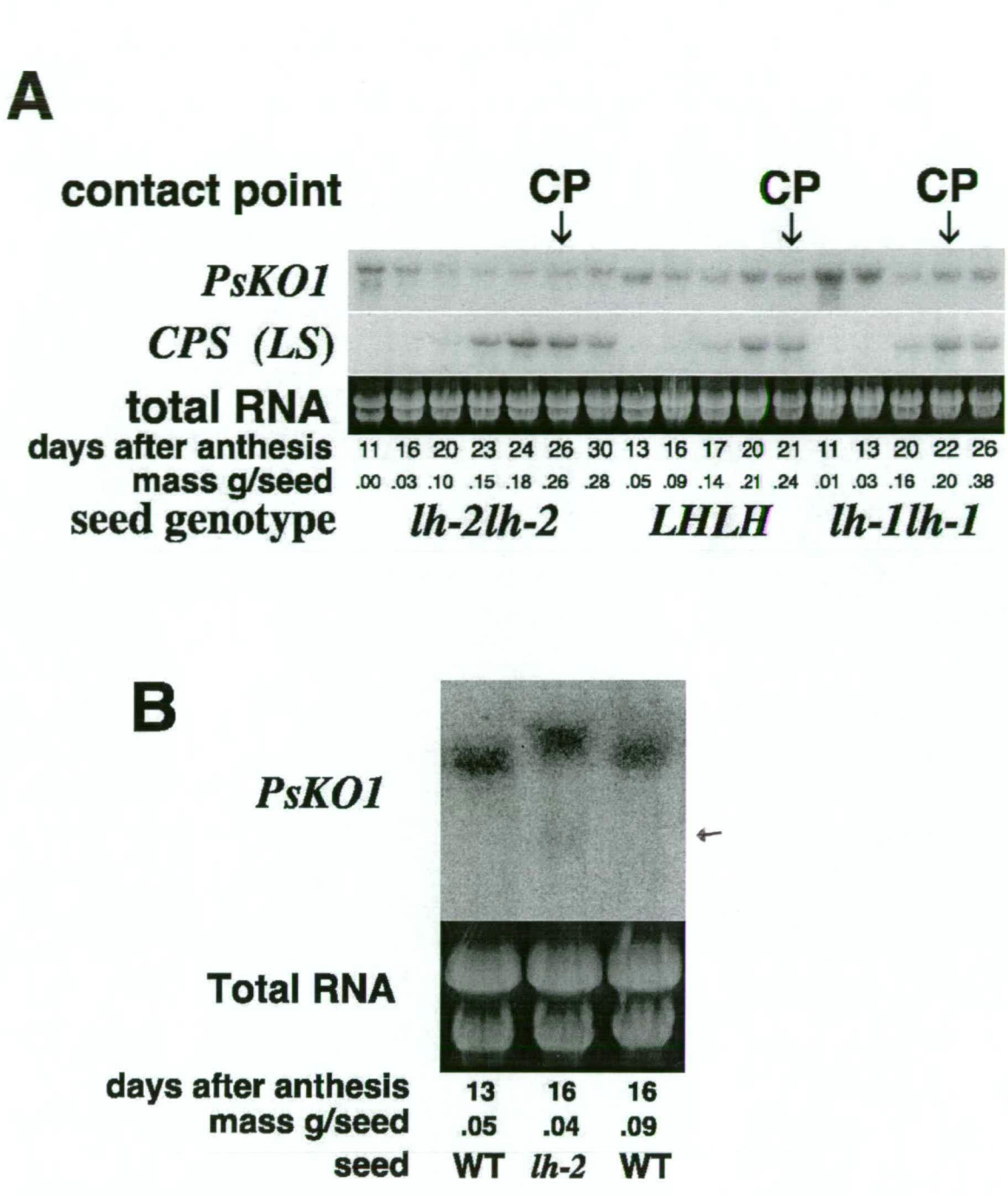


Figure 3.14

A Northern blot analysis of seeds at various ages and weights during development from homozygous wild-type (*LH*), and dwarf mutant *lh-1* and *lh-2* plants. Total RNA (5µg) from whole seeds at various ages (days after anthesis) were loaded on the gel. *PsKO1* (*ent*-kaurene oxidase) and *CPS* (*LS*, copalyl diphosphate synthase) transcript levels were measured. (Note **A** is repeated with additional information in Chapter 4)
B Northern blot using the 5' *PsKO1* probe to compare the band pattern of ***lh-2*** with wild-type (cv. Torsdag)(**WT**) seeds with similar *PsKO1* transcript levels at the same age (16 days after anthesis) and similar weight (g/seed). Total RNA (5µg) was loaded on the gel.

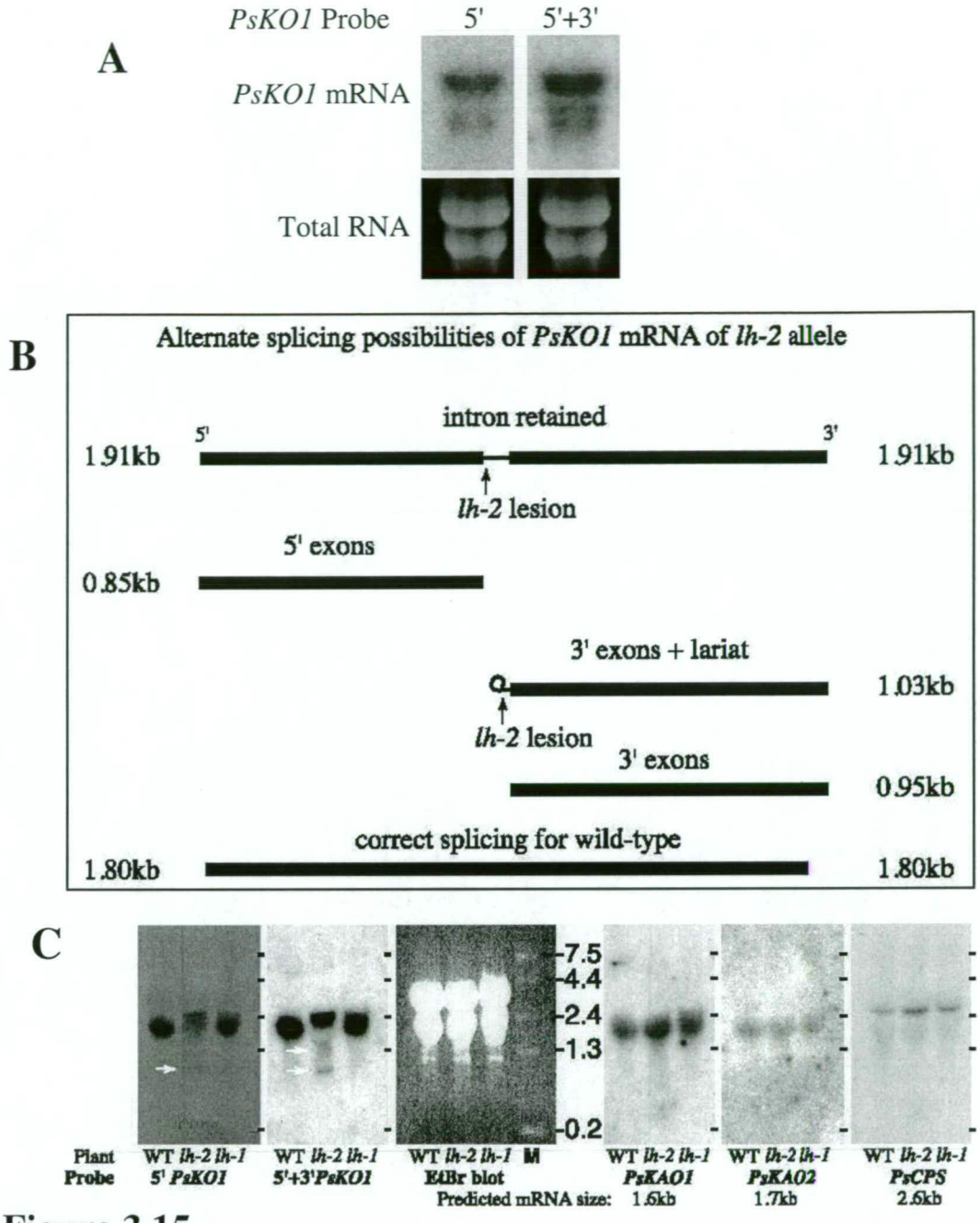


Figure 3.15

A Northern blot analysis of *lh-2* seed probed with ^{32}P labeled 5' *PsKO1* probe (prepared from 750bp template 5' of the *lh-2* lesion) or 5'+3' *PsKO1* probe prepared from a 925bp template spanning the *lh-2* lesion (425bp 5' and 500bp 3' of the *lh-2* lesion).

B Diagram of theoretically possible alternate splicing products of *PsKO1* mRNA of the *lh-2* allele.

C Northern blot analysis of *lh-2* apical bud tissue probed with 5' *PsKO1* probe or 5'+3' *PsKO1* probe (described above in **A**) and compared with the Ethidium bromide (EtBr) stained ribosomal bands and RNA ladder of the EtBr blot. The same blot was probed with *PsKAO1*, *PsKAO2* and *PsCPS* also for size comparison. Gel **C** ran longer than **A**.

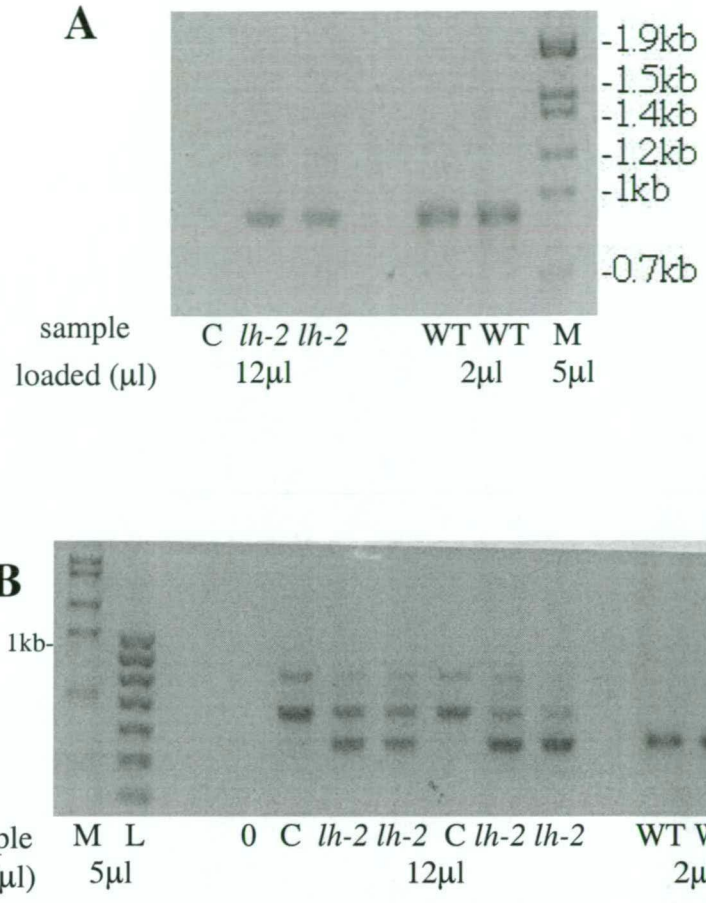


Figure 3.16

Ethidium bromide stained 2% agarose gels of PCR products after amplification with a forward primer spanning the 5' intron boundary containing the *lh-2* lesion in such a way that only correctly spliced wild-type cDNA would be amplified. Two different reverse primers are used giving a product of expected size 900b in **A** and 540b in **B.** The extra bands in gel B are present also in the no reverse transcriptase controls (C) and are assumed to be amplifed from the small amount of genomic DNA present in the RNA extraction at time of cDNA production. This indicates that the test is sensitive and there is no cDNA contamination from wild-type cDNA at time of PCR amplification. With the reverse primer of gel **A** no mispriming occurred.
12µl of PCR product from ***lh-2*** cDNA templates each from different harvests and different cDNA preparations and no reverse transcriptase controls (**C**), while only 2µl of the PCR product from the wild-type (**WT**) cDNA template was loaded on the gel.
0 is a no template control.
M is the SPP-1 DNA cut with EcoRI size marker.
L is 100-1000 bp ladder in 100bp increments.

Genomic southern blot analysis

At high stringency the *PsKO1* gene appears to be a single copy gene (Fig. 3.17). XhoI was not effective at cutting the pea genomic DNA. The other seven restriction enzymes at high stringency revealed one or two bands indicating hybridization with the *PsKO1* probe. The two bands observed when cut by HindIII, SacI, BamHI and BglII were expected as those restriction sites are present in the cDNA sequence. Note that the second band when cut by BglII is weak and although not clearly seen on the scan was observed on two different genomic Southern blots. The 2 bands observed when cut by EcoRI or XbaI may be a consequence of restriction sites present in introns not yet sequenced at both the 5' and 3' ends of the gene. At low stringency additional bands were noted (Fig. 3.17).

Prediction of plastid targeting sequence

ChoroP program (www.cbs.dtu.dk/services/ChloroP; (Emanuelsson et al., 1999) used a neural network based method to identify potential chloroplast transit peptides (Table 3.2). The *ent*-kaurene oxidases all have chloroplast transit peptides (cTP) scores close to the 0.5 cut off. Although the AtKO1 (Helliwell et al., 2001b) cTP score was above that cut off, it has a very low cleavage site score (Table 3.2). The pea copalyl diphosphate synthase PsCPS (LS) and pea ribulose 1.5 bisphosphate carboxylase S (RbcS) as expected were predicted to have chloroplast transit peptides and high cleavage site scores. The other GA biosynthetic or catabolic enzymes were not predicted to have chloroplast transit peptides and had low cTP scores since *ent*-kaurenoic acid oxidases (KAOs) are predominantly located in the endoplasmic reticulum (ER) (Helliwell et al., 2001b) and the dioxygenases are soluble enzymes present in the cytoplasm (Kamiya and Graebe, 1983).

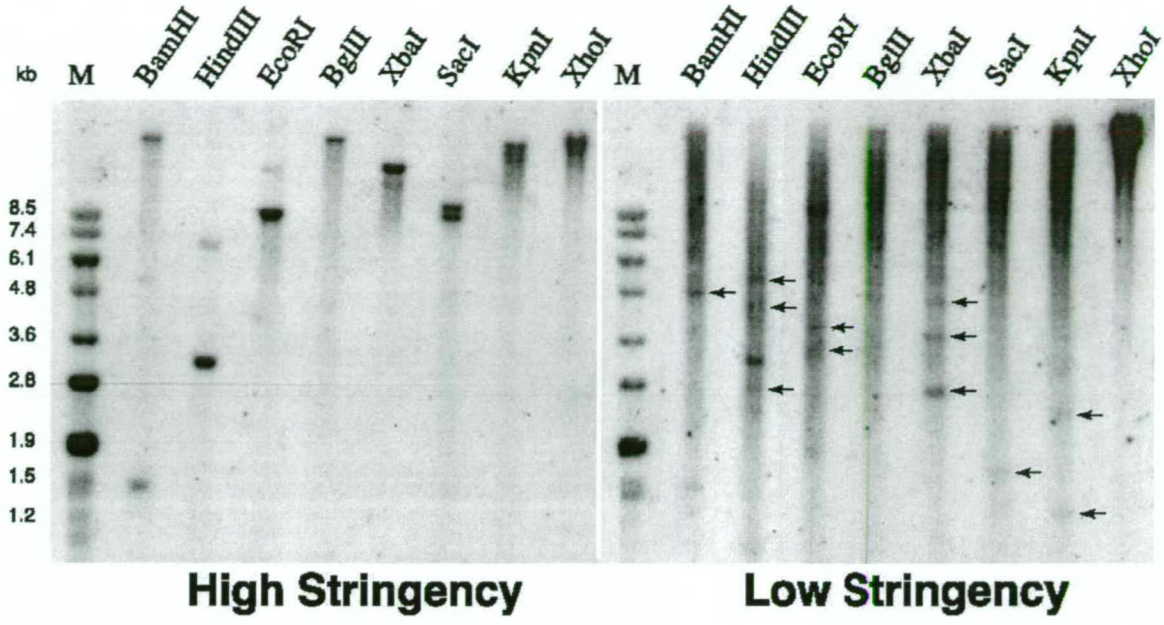


Figure 3.17

Genomic DNA Southern blot analysis of the pea *ent*-kaurene oxidase, *PsKO1*. DNA from *Pisum sativum* cv. Torsdag plants was digested with restriction enzymes BamHI, HindIII, EcoRI, BglII, XbaI, SacI, KpnI, or XhoI and probed with *PsKO1* at either high or low stringency hybridisation and washing conditions. Extra bands visible at low stringency are indicated with arrows. The size marker, M, is SPPI.

Table 3.2

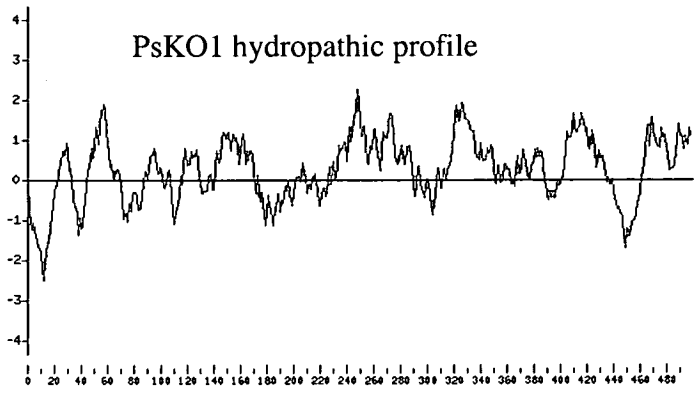
Summary of ChloroP predictions for enzymes of the GA biosynthetic pathway.

Protein	Genbank accession number	Amino Acids	cTP score	Prediction	Cleavage Site Score	Predicted Cleavage Site
PsCPS (LS)	U63652	801	0.56	cTP	4.36	44-45
PsKO1		499	0.48	no cTP		
AtKO1	AF047719	509	0.54	cTP	-0.89	28-29
CmKO1	AF212990	517	0.49	no cTP		
PsKAO1(NA)	AF537321	484	0.44	no cTP		
PsKAO2	AF537322	490	0.44	no cTP		
PsGA20ox1	X91658	380	0.44	no cTP		
PsGA20ox2	U58830	379	0.46	no cTP		
PsGA3ox1(LE)	U85045	374	0.43	no cTP		
PsGA2ox1(SLN)	AF100955	309	0.44	no cTP		
PsGA2ox2	AF100954	345	0.46	no cTP		
RbcS (pea)	X04333	180	0.58	cTP	11.17	56-57

The chloroplast transit peptides (cTP) score, the prediction of whether the protein contains a cTP and if applicable the cleavage site score and location of the predicted cleavage site is shown for each of the protein sequences analysed.

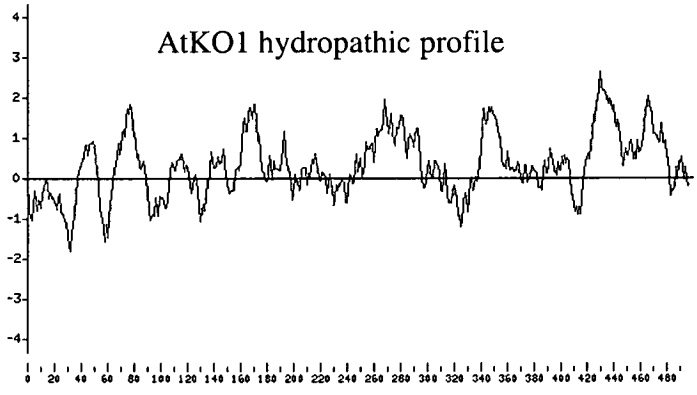
Prediction of hydrophobic potential transmembrane regions

Possible transmembrane helixes were predicted for all three ent-kaurene oxidase enzymes within 40 amino acids from the N-terminal (TMpred, Fig. 3.18) and this was supported by their Kyte-Doolittle hydropathic profiles (Fig. 3.18). The hydropathic profiles of the *lh-1* and *lh-3* putative aberrant proteins were similar to the wild-type. The truncated *lh-2* putative protein still had the similar hydropathic profile over the first 250 N-terminal amino acids (Fig. 3.19).



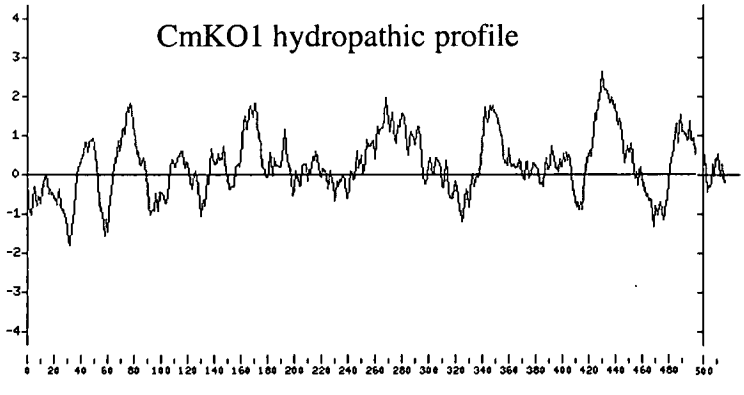
PsKO1

Possible transmembrane helixes. TMpred					
inside to outside			outside to inside		
from	to	score	from	to	score
1	20	1955	4	20	2152
441	460	1233	441	463	657



AtKO1

Possible transmembrane helixes. TMpred					
inside to outside			outside to inside		
from	to	score	from	to	score
6	24	3062	6	24	3259
			38	57	576
453	472	1121	453	475	900



CmKO1

Possible transmembrane helixes. TMpred					
inside to outside			outside to inside		
from	to	score	from	to	score
24	40	1359	22	40	1424
			50	66	562
461	483	698	461	483	684

Figure 3.18
The hydropathic profiles of the PsKO1, AtKO1 and CmKO1 putative proteins using the Kyte-Doolittle method for calculating hydrophilicity over a window length of 17 amino acids (http://bioinformatics.weizmann.ac.il/hydroph). The tables indicate the possible transmembrane helixes predicted (www.ch.embnet.org/software/TMPRED_form.html) for the putative proteins PsKO1, AtKO1 and CmKO1. The amino acid, start and finish position for the predicted span and a score value for both directions are indicated. Score values under 500 are considered insignificant and therefore are not shown.

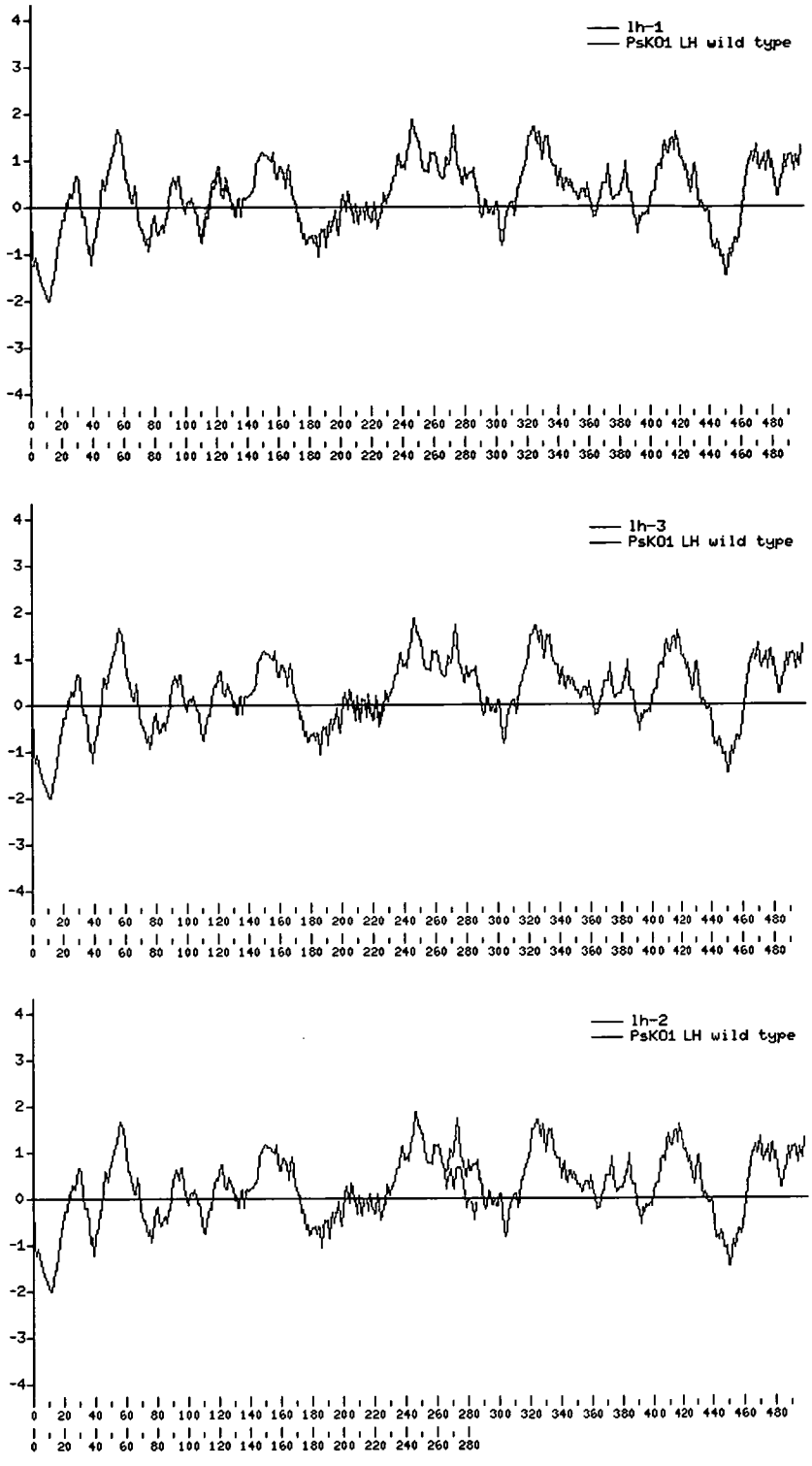


Figure 3.19
A comparison of the mutant *lh-1*, *lh-3* and truncated *lh-2* putative PsKO1 proteins' hydropathic profiles (red) with that of the wild-type LH (blue). The Kyte-Doolittle method was used to calculate hydrophilicity over a window length of 19 amino acids (http://bioinformatics.weizmann.ac.il/hydroph).

DISCUSSION

Isolation of the pea homologue of *Arabidopsis GA3*

PsKO1, the pea homologue of the *Arabidopsis GA3*gene, was isolated. This gene had high similarity to *AtKO1*, *CmKO1* and *NtKO1* of *Arabidopsis*, pumpkin and tobacco respectively (Fig. 3.6). It is grouped with the *ent*-kaurene oxidases (*AtKO1* and *CmKO1*) in the cytochrome P450 CYP701A subfamily. The CYP701As are found within the plant A-type clan and away from the other cytochrome P450 GA and brassinosteroid biosynthetic enzymes (Figs. 3.7 and 3.8). This coincides with the cytochrome P450 alignment of D.R. Nelson (www.drnelson.utmem.edu/CytochromeP450.html) and the *Arabidopsis* alignments (Paquette et al., 2000) (www.biobase.dk/P450/p450.shtml).

The pea *LH* encodes PsKO1

The evidence shows that the pea *LH* gene encodes PsKO1. Firstly, the *PsKO1* sequence is altered compared to the wild-type progenitor, cv. Torsdag (L107) in tissue from all three GA responsive dwarf mutants, *lh-1, lh-2* and *lh-3* (Figs. 3.9, 3.10 and 3.11). The mutations *lh-1, lh-2* and *lh-3* are allelic (Swain and Reid, 1992; Dr. J.L. Weller personal communication) and produced by independent mutational events. Also the predominant mRNA of the *lh-2* mutant seen on northern blots is larger consistent with the predicted larger size due to retention of an 83 base intron (Figs. 3.12 and 3.14; Chapter 4).

Secondly, *PsKO1* is expressed in the organs where an *lh* phenotype is observed. *PsKO1* was expressed in the shoot including the apical bud, stem and leaf (Fig. 3.12) and all three *lh* alleles had decreased stature compared to the wild type (Fig. 3.1). Also, the GA_1 levels in the shoot were reduced in the *lh-1* and *lh-2* mutants (Swain and Reid, 1992). *PsKO1* is expressed in the roots (Fig. 3.12) and the roots of *lh-2* mutant seedlings have decreased tap root length (Batge et al., 1999; Yaxley et al., 2001) and lateral root length (Yaxley et al., 2001) coinciding with reduced GA_1 and GA_{19} levels in the root (Yaxley et al., 2001). *PsKO1* is also expressed in the pod (Fig. 3.12) and *lh-2* allele had a reduced pod GA_1, GA_{20}, GA_{29}, and GA_{19} levels (Swain et al., 1993; MacKenzie-Hose et al., 1998) unrelated to the seed phenotype (Swain et al., 1993). However the observed reduction in pod elongation containing *lh-2* seeds may be predominantly a consequence of the decreased seed size and survival (Ozga et al., 1992; MacKenzie-Hose et al., 1998). *PsKO1* is expressed in the seed (Fig. 3.12) at all developmental stages tested with peaks during the early stage of seed development and again around the time of contact point (Figs. 3.13 and 3.14A). Both *lh-1* and *lh-2* mutant seeds had reduced GA_1 levels in young seeds and reduced GA_{20} and GA_{29} levels at contact point (Swain et al., 1995). The *lh-1* mutant has a transient effect on seed growth and development (Swain et al., 1997) and the *lh-2* mutant has decreased seed size and survival (Swain et al., 1993).

Thirdly, extracts from *lh-2* plants were previously shown to be unable to metabolise *ent*-kaurene, *ent*-kaurenol or *ent*-kaurenal (Swain et al., 1997). Therefore the *lh* mutation blocked the same three-step oxidation of *ent*-kaurene to *ent*-kaurenoic acid catalysed by other CYP701A (*ent*-kaurene oxidase) enzymes such as *Arabidopsis* AtKO1 (GA3) (Helliwell et al., 1999) and pumpkin CmKO1 (Helliwell et al., 2001a; Fig. 3.2).

Now that the *LH* gene has been cloned and the nature of the genetic lesions of the three *lh* alleles are known, perhaps we can explain some of the previously perplexing phenotypic differences between plants carrying the *lh-1* and *lh-2* alleles.

Sensitivity to paclobutrazol

One dramatic difference between the *lh-1* and *lh-2* seedlings is in their response to triazole inhibitors (Swain et al., 1997). The *lh-2* mutant is 30 times more sensitive to the *ent*-kaurene oxidase specific inhibitor, paclobutrazol, than the *lh-1* mutant or the LH wild-type (Swain et al., 1997). Fig. 3.3 shows that *lh-3* seedlings have similar sensitivity to paclobutrazol as *lh-1* seedlings. Swain et al. (1997) proposed "the changed structure [of the *lh-2* gene product] both decreases enzyme activity and increases the affinity for inhibitors, possibly by altering the active site." However, evidence presented here shows that the putative *lh-2* aberrant protein is truncated (Fig. 3.10) before the cytochrome P450 catalytic domains (Fig. 3.6) and would not be expected to have an active site. Therefore there must be another explanation. Since paclobutrazol is a competitive inhibitor specific for *ent*-kaurene oxidase (Sugavanam, 1984; Hedden and Graebe, 1985), the *lh-1* and *lh-3* aberrant protein potentially have a substrate-binding site (Fig. 3.6, 3.9 and 3.11). Therefore, the *lh-1* and *lh-3* aberrant protein may bind paclobutrazol and therefore dilute the effect of the inhibitor on residual or alternate KO activity and thus have a similar sensitivity to paclobutrazol as the wild-type. The *lh-2* aberrant protein however is truncated before the cytochrome P450 substrate binding sites (Fig. 3.6 and 3.10) leaving all the applied inhibitor free to affect a small quantity of alternative splice products or another *ent*-kaurene oxidase activity.

Since *ent*-kaurene and *ent*-kaurenoic acid are part of the GA biosynthesis pathway common to all plant species and tissues (Hedden and Phillips, 2000) some form of KO activity is expected in the *lh-1*, *lh-2* and *lh-3* mutants since they germinate, grow to one-third the height of the wild-type and set seed. A mutation causing a plant to be devoid of GAs may be expected to be lethal (Koornneef and

van der Veen, 1980; Hooley, 1994; Swain et al., 1997; Yamaguchi and Kamiya, 2002) or at least very much shorter than *lh-1*, *lh-2* and *lh-3* plants.

The *ent*-kaurene oxidation step of GA biosynthesis appears to be "leaky". Firstly, although the putative protein from the *lh-1* and *lh-3* mutants are altered from wild-type PsKO1 (Figs. 3.9 and 3.11) the GA biosythetic inhibitor, paclobutrazol, further reduced the internode lengths of the *lh-1* and *lh-3* mutants (Fig. 3.3) (Swain et al., 1997). Similarly, other GA biosynthetic inhibitors including a copalyl diphosphate synthase (CPS) inhibitor (AMO-1618) and a 2-oxoglutarate-dependent dioxygenase inhibitor (BX-112) further reduced the stature of the *lh-1* mutant (Swain et al., 1997). Secondly, the *lh-3 ls-1* double mutant seedlings are rosette-like with stature reduced relative to the single *lh-3* mutants (Fig. 3.5). Similarly, the *lh-1* double recessive with other GA biosynthetic mutants (*ls lh-1* and *na lh-1*) have such short internodes that the plants appear rosette-like (Reid, 1986b; Reid and Ross, 1993) and *le lh-1* have the extremely short "nana" phenotype and thus also are shorter than the single *lh-1* mutant (Swain, 1994). Thirdly, (Ingram and Reid, 1987) found that the *lh-1* mutant still responded to added *ent*-kaurene when endogenous production of *ent*-kaurene was prevented by the inhibitor AMO-1618, although the elongation response was less than that achieved by the *ls* mutant or wild type (Torsdag) plants.

It cannot be ruled out that the "leaky" nature of *ent*-kaurene oxidation step may be due to limited activity by other KO enzymes in pea shoots. However, *PsKO1* appears to be a single copy gene in the genomic restriction enzyme Southern blot at high stringency (Fig. 3.17) and additional library screening with *PsKO1,* as probe did not reveal another CYP701A gene. The extra bands present in the Southern blot analysis at low stringency may indicate some similar genes but probably indicate cross-hybridisation with another closely related cytochrome P450 subfamily (Fig. 3.17). *PsKO1* is expressed in all the tissue tested so far (Fig. 3.12) and all the developmental stages so far investigated (Figs. 3.13 and 3.14; Chapter 4) so it is

possible for it to be the sole *ent*-kaurene oxidase activity in pea. Pumpkin has two *ent*-kaurene oxidases (CYP701A1 and CYP701A2) (Helliwell et al., 2000) while *Arabidopsis* has a single copy gene (Helliwell et al., 1998).

The *lh-1* and *lh-3* putative protein is full-length and contains the substrate binding and catalytic domains common to cytochrome P450 monooxygenases (Kalb and Loper, 1988; Fig. 3.6) and therefore may have some residual *ent*-kaurene oxidase activity.

However, the *lh-2* mutant with the severely truncated putative protein (Fig. 3.10) also had an extremely short double mutant *ls lh-2* phenotype (Swain et al., 1995) and the *lh-2* mutant was further reduced in stature by the application of GA biosynthesis inhibitors (paclobutrazol, AMO-1618 and BX-112) (Swain et al., 1997). The *lh-2* mutant putative protein is truncated and would not be expected to contain any of the catalytic domains (Kalb and Loper, 1988) and would not even be expected to contain the haem binding active site common to all cytochrome P450 monooxygenases (Poulos et al., 1985; Nebert and Gonzalez, 1987; Fig. 3.6). Therefore no residual enzymatic activity would be expected in the truncated protein.

The premature stop codon associated with the *lh-2* allele resulted from incorrect splicing with retention of an intron (Fig. 3.10). Ait-Ali et al. (1997) found that the *ls-1* mutant with a base substitution at the 3' acceptor splice site produced a series of alternate spliced products. Some of these products may have some activity as a more severe dwarf allele *ls-3* has been found suggesting that the *ls-1* mutation was not null. The *ga3-2* mutant allele of the *Arabidopsis AtKO1* has a less severe phenotype than the *ga3-1* mutant and therefore is expected to contain some activity although they both have single base substitutions that translate to in-frame premature stop codons before the catalytic domains (Helliwell et al., 1998; Fig. 3.6). The wild-type *AtKO1* had some evidence of alternate splicing unrelated to the *ga3* lesions. Helliwell et al. (1998) suggested that one possible way to explain some *ga3-2* activity would be for occasional aberrant splicing to remove the exon containing the

premature stop codon allowing the production of a protein, although altered, that would contain the catalytic domains.

Evidence of intron retention in mRNA from the *lh-2* mutant is presented in *PsKO1* northern blot analysis. The predominant band in *lh-2* mutant tissue is larger than wild-type, consistent with intron retention (Figs. 3.12, 3.13 and 3.14). If some small percentage of the mutant RNA splicing is not prevented and occurs normally then protein prepared from this correctly spliced mRNA would be full length with catalytic domains and have enzymatic activity. A band corresponding in size to the wild-type *LH* mRNA was not observed (Figs. 3.12, 3.13 and 3.14). Similarly, cryptic splicing may occur but again there is no band near wild-type size observed on the northern blot and no obvious in-frame cryptic sites that may encode a protein with the catalytic domains. However, in the seed, where LH expression levels are higher, there is another band smaller than the wild type mRNA present (approximately 1kb compared with 1.8kb wild-type) (Figs. 3.13, 3.14 and 3.15). This may be the product of a different splicing event, perhaps skipping the mutated 5' splice site and removing several exons to attain the observed size (Fig. 3.15). Alternatively, it may be due to impaired splicing. Normal removal of introns is a two-step cleavage-ligation reaction. The first step involves cleavage of the 5' slice site with the formation of intron lariat with the G (changed to A in the *lh-2* mutant; Fig. 3.10) binding to a branch point in the intron. In the second step the 3'splice site is cleaved, the exons ligate and the intron lariat is released to be debranched and degraded (Brown et al., 1996). The GT to AT mutation at the 5' intron splicing site in genes in Arabidopsis (Orozco et al., 1993; Bradley et al., 1995) and maize (Lal et al., 1999) formed a lariat but did not proceed to the second step and the 5' exons and an intermediate (consisting of the intron lariat and 3'exons) were detected. The smaller band of *lh-2* mRNA (Fig. 3.13 and 3.14) could correspond to the cleaved off 5'exons, since it is approximately the correct size and hybridized with a *PsKO1* probe prepared from the template 5' of the *lh-2* genetic lesion. Another *PsKO1* probe template spanning the *lh-2* lesion revealed a minor additional band of intermediate

size to the bands detected by the probe 5' of the *lh-2* lesion (Fig. 3.15). This band is a little larger than predicted for the 3'+lariat mRNA (Fig. 3.15B). However it is associated with mRNA 3' of the *lh-2* lesion and the lariat irreversibility bound to its branch point may alter the movement of this mRNA through the gel. This suggests that the first step of intron removal has occurred on some occasions. Liu and Filipowicz (1996) found that a small percentage (10%) of the lariat-exon intermediates of a synthetic gene with the GT to AT mutation could go on to complete the second step. If any *lh-2* product was correctly spliced then it would have *ent*-kaurene oxidase activity.

Northern blot analysis of *lh-2* may not be sensitive enough to show a small percentage of wild-type mRNA and PCR may be a more sensitive tool. PCR amplification with a primer spanning the intron/ exon boundry (of the intron containing the *lh-2* lesion) detected a small percentage of wild-type (correctly spliced) product in the *lh-2* samples (Fig. 3.16). Preliminary real-time PCR (qPCR) results indicate that *lh-2* apical bud samples contained approximately 0.5% correctly spliced mRNA compared to wild-type apical buds using primers of my design (J.J. Smith personal communication).

The increased sensitivity of the *lh-2* mutant to paclobutrazol, therefore, may occur if the small quantity of potential residual activity provided by correct splicing in the *lh-2* mutant was exposed to all the applied paclobutrazol.

Phenotypes of *lh-1*, *lh-3* and *lh-2* differ in a tissue specific manner

In the seed the *lh-2* allele has a more severe phenotype than *lh-1* and *lh-3*, while the shoot phenotype of all three alleles is similar (Figs. 3.1; 3.3 and 3.4). Perhaps the different putative aberrant proteins of the alleles can explain this.

Seed phenotype

The *lh-2* mutant had a severe decrease in survival of seeds. Up to 62% of seeds abort and those that continue to develop have delayed development taking up to 6 days longer to achieve contact point. Then the mature *lh-2* seeds are smaller and lighter than wild-type and *lh-1* seeds (Swain et al., 1993). In contrast the *lh-1* allele has only a transient effect on embryo and seed growth and very mild increase in seed abortions (Swain et al., 1997). The *lh-3* allele seed survival was similar to *lh-1* (Fig. 3.4; Table 3.1).

The *lh-2* allele seeds had decreased GA levels in young seeds and seeds at contact point. The *lh-2* allele did not show an early GA peak although this was evident in the *lh-1* mutant and wild-type seeds. Swain et al. (1995) suggested that because the *lh-2* mutant was the only pea GA biosynthesis mutation with a phenotype affecting pea seed survival and the only mutation that altered the GA peak early in seed development then this early GA level peak may be essential for the development of seeds. In contrast to the other pea GA biosynthetic genes (*PsKAO2* and *PsCPS*), *PsKO1* is expressed at all stages of seed development tested (Figs. 3.13 and 3.14). *PsKO1* was expressed more strongly at an early stage of pea seed development and then again at contact point (Figs. 3.13 and 3.14). This coincided with the observed 2 peaks in GA levels during the development of normal seeds (Frydman et al., 1974; Swain et al., 1993). The expression pattern of *PsKO1* (*LH*) compared to the other early GA biosynthetic genes lends support to the importance of the early GA production peak to seed survival and development (Figs. 3.13 and 3.14). Previous work has shown that seed survival and growth is directly related to the GA_1 levels in the young seed (embryo and endosperm) (Swain et al., 1995; 1997; MacKenzie-Hose et al., 1998) and not primarily associated with GAs imported from

the pod (Swain et al., 1995), assimilation of nutrients (Swain et al., 1997) or the effect of other hormones such as abscisic acid (ABA) (Batge et al., 1999).

In contrast to the shoot phenotype (discussed below), the differences in *lh-2* and both *lh-1* and *lh-3* seed phenotypes follow the differences in severity of the genetic lesion. The young seeds of the *lh-2* mutant, which is expected to have a severely truncated PsKO1 putative protein, had GA_1 levels reduced to 10% of the wild-type value (Swain et al., 1993; 1995). However the GA_1 levels were only reduced to approximately 50% in young *lh-1* seeds (Swain et al., 1993) with a full-length putative protein containing only a single base substitution. A log-linear relationship exists between endogenous GA_1 levels and internode elongation in pea shoots (Ingram et al., 1986; Ross et al., 1989) and if a similar relationship is present in seeds then perhaps only a 90% reduction in GA_1 levels may cause an observable phenotype while 50% reduction produces a transient effect. Also *ls* and *le* mutants with normal seed development do not have reduced GA_1 levels in their young seeds (Swain et al., 1995). Therefore, the difference between *lh-2* and both *lh-1* and *lh-3* in seed phenotype and *lh-2* and *lh-1* GA_1 levels of young seeds (Swain et al., 1997) reflects the severity of the genetic lesion.

Shoot phenotype

In contrast to the large difference in the phenotypes of *lh-2* and both *lh-1* and *lh-3* in the seed, all three alleles are dwarfs of similar stature and have an approximate 40% reduction in stature from the wild type (Figs. 3.1 and 3.3C). The environment presented to the *ent*-kaurene oxidase enzyme within the cells of the seeds and vegetative tissue may differ. *Arabidopsis ent*-kaurene oxidase (AtKO1) is targeted to the outer chloroplast envelope membrane (Helliwell et al., 2001b). The *ent*-kaurene oxidases of pea (PsKO1), *Arabidopsis* (AtKO1) and pumpkin (CmKO1) all had ChoroP potential chloroplast transit peptide prediction values close to the cut off value (Table 3.2). This may indicate a greater affinity for the plastid membranes than the endoplasmic reticulum (ER) directed pea *ent*-kaurenoic acid enzymes

(PsKAO1 and PsKAO2) or the pea cytoplasmic GA biosynthetic enzymes (Table 3.2). N-terminal cleavage sites were not expected for the *ent*-kaurene oxidases (Table 3.2) consistent with no reduction in size of AtKO1 during the pea chloroplast import assay (Helliwell et al., 2001b). This suggests that the N-terminal leader is not processed. Many of the chloroplast outer envelope membrane targeted proteins do not have the cleavable targeting sequence common to stroma or thylakoid directed proteins (Keegstra and Cline, 1999). Instead, the targeting information is contained in the protein and is associated with a hydrophobic segment (Cline and Henry, 1996; Li and Chen, 1996). PsKO1 and the other *ent*-kaurene oxidases have a hydrophobic region consistent with a possible transmembrane helix within the first 40 N-terminal amino acids (Fig. 3.18). This is consistent with the data of (Helliwell et al., 2001b) where the N-terminal 100 amino acids of AtKO1were sufficient to target GRF to tobacco leaf chloroplasts or isolated pea chloroplasts. Other outer envelope membrane proteins have the targeting and integration signal contained in the first 30 amino acids of (Li and Chen, 1996). The *lh-1* and *lh-3* mutations do not appear to affect the protein's hydropathic profile (Fig. 3.19). Also, although the putative protein of the *lh-2* mutant would be truncated it would not be expected to disrupt the targeting hydrophobic N-terminal domain (Fig. 3.19). Therefore all three aberrant proteins are still expected to target and attach to the outer envelope membrane of the plastid.

ent-kaurene oxidase, located in the plastid outer envelope membrane, is ideally positioned to link the *ent*-kaurene produced in the plastid stroma (Sun and Kamiya, 1994; Aach et al., 1995) and the *ent*-kaurenoic acid oxidases located in the endoplasmic reticulum (ER) (Helliwell et al., 2001b). Therefore differences in the cellular environment between the shoot and seed may be important especially when the enzyme is operating sub-optimally as in *lh* mutant plants. Since proplastids differentiate into different plastids depending on the tissue (Harrak et al., 1995), differences between the plastid quality and quantity in seed and vegetative tissue may be expected.

Slight differences in stature of the *lh-1*, *lh-2* and *lh-3* seedlings (Fig. 3.1) and GA_1 levels in *lh-1* and *lh-2* seedlings (Swain and Reid, 1992) are not easily explained by differences in their putative aberrant proteins and may be due to differences in their general genetic background although they all share the same progenitor (cv.Torsdag). When background was standardised by the F_2 seedlings of a *lh-1* X *lh-3* cross, no difference in *lh-1* and *lh-3* internode lengths were observed (Fig. 3.4).

CONCLUSION

The pea *LH* gene encodes *ent*-kaurene oxidase. *PsKO1* is expressed in all tissues tested including apical bud, stem, leaf, root, seed and pod. Also the level of expression of *PsKO1* coincides with the 2 GA peaks characteristic of seed development. This contrasts with the tissue specific expression of most other GA biosynthetic genes examined. The *PsKO1* gene sequence was altered in all three dwarf pea mutant alleles, *lh-1*, *lh-2* and *lh-3*. The phenotypes of *lh-1*, *lh-2* and *lh-3* alleles differ in a tissue specific manner and this possibly may be explained by differences in the putative aberrant proteins.

Differences in the sensitivity of the *lh* mutant alleles to paclobutrazol inhibitor, where *lh-2* is far more sensitive than both *lh-1* and *lh-3*, reflect differences in the aberrant proteins. Paclobutrazol is a competitive inhibitor specifically inhibiting the same step in the GA biosynthesis pathway as PsKO1. The *PsKO1* of the *lh-1* and *lh-3* alleles have full-length predicted aberrant proteins with single amino acid changes and contain the substrate binding and catalytic domains so can act to dilute the effect of paclobutrazol on their limited activity. In contrast the putative protein of the *lh-2* allele is predicted to be severely truncated and would not

contain the catalytic domains. Residual activity is provided in the *lh-2* mutant by a small percentage of correctly spliced and therefore wild-type like enzyme which would be severely affected by the inhibitor.

MATERIALS AND METHODS

Plant material and growing conditions

Three independent mutational events in *Pisum sativum* L. cv.Torsdag resulted in the alleles *lh-1*, *lh-2* and *lh-3*. The *lh-1* mutant line K511 (genotype *lh-1 NA LE LS*) was ethylmethane sulphonate (EMS) induced and *lh-2* mutant line NGB5843 (genotype *lh-2 NA LE LS*) (formally lh^i) was ethyleneimine (EI) induced by Dr K.K. Sidorova (Novosibirsk, Russia) (Reid, 1986b; Swain and Reid, 1992). The *lh-3* mutant line ABO4 was EMS induced from cv Torsdag by Dr J.L. Weller (Hobart, Australia) (Weller et al., 1997). The essentially isogenic Hobart L107 (genotype *LH NA LE LS*) derived from cv.Torsdag was used as wild-type.

Plants were grown 2 per pot in a heated glasshouse under an 18h photoperiod (Beveridge and Murfet, 1996).

Paclobutrazol treatment

Seeds of wild-type (L107), *lh-1* (K511), *lh-2* (NBG5843) and *lh-3* (ABO4) were treated with the GA inhibitor, paclobutrazol (1μg/seed), prior to planting. The seed testa was nicked and 5μl ethanol (control) or 5μl paclobutrazol PP333 (0.2μg

PP333/μl ethanol) was applied and allowed to dry under the testa of each seed before planting.

Library screening

A total of 280,000 plaque forming units (pfu) were screened from pea seed cDNA library to isolate the pea homologue of the *Arabidopsis GA3* gene. A further 125,000 pfu were screened from that library in an attempt to find other genes similar to the pea *CYP701A* gene initially isolated.

The seed cDNA library used was constructed in Lambda ZAPII (Stratagene) with cDNA prepared from L107 cv. Torsdag pea seeds at contact point (Ait-Ali et al., 1997). The library screening and the isolation of clones was according to methods recommended by manufacturer (Stratagene).

The probe template for the initial middle stringency screening (65^{o}C hybridization, 65^{o}C washes with 2xSSC + 0.1% SDS) was the full-length (1.5 kb) *Arabidopsis GA3* cDNA supplied by E.S. Dennis, CSIRO Plant Industry, Canberra (Helliwell et al., 1998). It was digested out of a pYE22msc vector with salI and BamHI then gel purified from 1% agarose/TAE gel (QIAquick gel extraction kit; QIAGEN) and then ^{32}P labeled (Gigaprime DNA labeling kit; Bresatec). The probe template for the subsequent low stringency screening (50^{o}C hybridization, 60^{o}C washes with 2xSSC + 0.1% SDS) was a 1.3 kb PCR product amplified from cDNA prepared from L107 apical buds using specific primers of the *PsKO1* sequence cDNA obtained from the initial library screening. The PCR product was gel purified on 1%Agarose/TAE (QIAquick gel extraction kit, QIAGEN), then gel filtered (CENTISPIN 10; PRICETON SEPARATIONS), then was ^{32}P labeled as above.

Genotyping F_2 seedlings from the *lh-3* x *lh-1* cross

Crude DNA was extracted from each seedling. Liquid N_2 frozen leaf tissue was ground and incubated in tris/EDTA extraction buffer containing sodium bisulphite and 1% Sarkosyl, then DNA was precipitated with ammonium acetate in isopropanol, then washed in 70% ethanol. The genotypes were determined by restriction digest with BfaI of PCR amplified fragments of the extracted DNA by comparison of the size patterns when digest products were run on agarose gel with known *lh-3* and *lh-1* samples. The *lh-3* lesion removed a BfaI site present in the *PsKO1* gene of *lh-1*. The genotyping was checked by digests of PCR fragments generated by two different primer pairs.

Genomic Southern Blots

Genomic DNA was isolated (Ellis, 1994), digested and run on a 0.7% agarose/TAE gel for 24.5 h at 20 V. This was blotted to Genesceen Plus (Dupont/NEN) in 2xSSC and hybridised in a hybridisation solution (0.5M sodium phosphate pH7.2, 1mM EDTA, 7% SDS)(Ausubel et al., 1994). For high stringency the membrane was hybridised at 65°C with 65°C (0.2xSSC and 0.1% SDS) washes. The same blots were then hybridized at low stringency (50°C) with initial washes at 50°C then final washes at 60°C with 2xSSC and 0.1%SDS. The *PsKO1* cDNA (1.8kb) obtained from the library screening (above) was cut out of the Lambda ZapII vector with XhoI and EcoRI then gel purified and labeled with ^{32}P (Gigaprime DNA labeling kit; Bresatec) to use as the probe.

Northern Blot Analysis

Total RNA was extracted using either the Phenol/SDS Method (Ausubel et al., 1994); Fig. 3.12A), or RNeasy Plant Kit (QIAGEN; Figs. 3.13 and 3.15) or Tri

Reagent™ RNA extraction (SIGMA®; Fig. 3.14) consistent within the blot. The RNA (5μg per lane) was fractionated in 1.5% agarose gel containing formaldehyde and transferred to Genescreen Plus hybridisation transfer membrane (Dupont/NEN) using 10xSSC. The membrane was hybridised at 42°C in 5xSSC, 5xDenhardts, 50% formamide, 1% SDS, and 200μg ml^{-1} salmon sperm with a cDNA ^{32}P probe (see above). The membrane was washed in 2xSSC, 0.1% SDS then 0.2xSSC, 0.1% SDS at 65°C and exposed to Kodak biomax X-ray film at -70°C.

The *PsKO1*probe template was a PCR fragment amplified from the clone isolated by the library screening (above) using vector primers and then nested with *PsKO1* specific primers to give a 750bp fragment covering the 5' region of the gene. The fragment, 5' of the lh-2 genetic lesion was chosen as template to allow direct comparison of the *lh-1*, *lh-2* and wild type expression patterns. In addition another *PsKO1* probe (5'+3' *PsKO1*) was prepared from a 925bp template spanning the *lh-2* lesion (425bp 5' and 500bp 3' of the *lh-2* lesion) initially PCR amplified from the clone isolated by the library screening (above) using vector primers and then 2 times nested with *PsKO1* specific primers.

Other probe templates used include the gel purified 0.9kb *PsKAO2* template cDNA fragment from L107 seed (Chapter 2) and the 2X gel purified cDNA 1.7kb *PsCPS* (*LS*) template generated by two rounds PCR using nested specific primers for the *LS* gene (Ait-Ali et al. 1997) using RNA from wild-type (L107) stem tissue. The probes were labeled with ^{32}P using the Decalabel DNA labeling kit (MBI Fermentas).

PCR of correctly spliced wild-type in *lh-2* mutant tissue

34 cycle PCR amplification with a 25 base forward primer spanning the intron/exon boundary of the intron containing the *lh-2* lesion (designed in such a way that only correctly spliced wild-type cDNA would be amplified) with two different

reverse primers. RNA was extracted using Phenol/SDS Method (Ausubel et al., 1994) and cDNA prepared with superscript™ (Life Technologies).

Hydropathic profiles and transmembrane predictions

The hydropathic profile of the putative proteins were performed on the web site (http://bioinformatics.weizmann.ac.il/hydroph) of the Bioinformatics Unit, Weizmann Institute of Science, Israel using the Kyte-Doolittle method for calculating hydrophilicity over a window length of 17 amino acids (Kyte and Doolittle, 1982).

The possible transmembrane helixes were predicted using the website (www.ch.embnet.org/software/TMPRED_form.html) of the Swiss Institute for Cancer Research (ISREC) using an algorithm based on the statistical analysis of a database of naturally occuring transmembrane proteins (TMbase) (Hofmann and Stoffel, 1993).

Sequence analysis

The putative amino acids of full-length genes were aligned using Clustal W (searchlauncher.bcm.tmc.edu/multi-align) (Thompson et al., 1994) and then presented in GeneDoc (http://www.psc.edu/biomed/genedoc/) (Nicholas et al., 1997) or analysed by PAUP 4.0b10 analysis (Swofford, 1999). The PAUP analysis used the bootstrap method (1000 replicates) with heuristic search on the aligned sequences with gaps excluded. CYP701A sequences used in addition to the pea PsKO1, included AtKO1(*GA3*, GenBank accession number AAC39507) (Helliwell et al., 1998) from *Arabidopsis thaliana* and CmKO1(AAG41776) (Helliwell et al., 2000)

from *Cucurbita maxima*. Details of other sequences used for comparison are listed below (Method Tables 3.3 and 3.4).

DNA primer sequences

LhF01[a]	AGCGACTCCGTTTTCACAGA
LhR01[a,b]	TCAACATGGCCTGAAGAGAC
LhR02[a]	TTTCTTTGGCGATCGACTCT
Lh INEX F5[b]	TGACATCAGGAAAA[v]GAATAGATTG
LhKpn1 R6[b]	GGGGTACCAAAACTAACTAGCCTCTCTT

[a] genotyping
[b] correct wild-type splicing
[v] intron-exon boundary

Method Table 3.3

Representatives of Plant A-type clan				
CYP	genebank accession number	enzyme	species	reference
82A1	AAG09208	wound-inducible P450 hydroxylase	*Pisum sativum*	(Whitbred and Schuler, 2000)
82A2	O81972	elicitor-induced cytochrome P450s	*Glycine max*	(Schopfer and Ebel, 1998)
82A3	O49858	P450 CP6	*Glycine max*	(Schopfer and Ebel, 1998)
82A4	O49859	elicitor-induced cytochrome P450s	*Glycine max*	(Schopfer and Ebel, 1998)
93A3	O81973	P450 CP5	*Glycine max*	(Schopfer and Ebel, 1998)
93B1	P93149	flavone synthase II	*Glycyrrhiza echinata*	(Akashi et al., 1998)
76C1	O64636		*Arabidopsis thaliana*	(Mizutani et al., 1998)
76B6	CAC80883	geraniol 10-hydroxylase	*Catharanthus roseus*	(Collu et al., 2001)
71A2	P37118		*Solanum melongena*	(Umemoto et al., 1993)
71D11	O22307	nodule-specific gene	*Lotus japonicus*	(Szczyglowski et al., 1997)
73A1	Q04468	Trans-cinnamate 4-monooxygenase	*Helianthus tuberosus*	(Teutsch et al., 1993)
73A3	P37114	Trans-cinnamate 4-monooxygenase	*Medicago sativa*	(Fahrendorf and Dixon, 1993)
73A4	P48522	Trans-cinnamate 4-monooxygenase	*Catharanthus roseus*	(Hotze et al., 1995)
73A9	Q43067	Trans-cinnamate 4-monooxygenase	*Pisum sativum*	(Whitbred and Schuler, 2000)
73A11	Q42797	Trans-cinnamate 4-monooxygenase	*Glycine max*	(Schopfer and Ebel, 1998)
73A16	Q43054	Trans-cinnamate 4-monooxygenase	*Populus kitakamiensis*	(Kawai et al., 1996)
73A19	O81928	Trans-cinnamate 4-monooxygenase	*Cicer arietinum*	(Overkamp et al., 2000)
73A27	AAK62344	elicitor-inducible cytochrome P450	*Nicotiana tabacum*	(Ralston et al., 2001)
98A1	O48956		*Sorghum bicolor*	(Bak et al., 1998)
91A2	BAA28539		*Arabidopsis thaliana*	(Mizutani et al., 1998)

Method Table 3.4

name	genebank accession number	comment	species	reference
GA biosythesis *ent*-kaurenoic acid oxidases				
PsKAO1	AF537321	CYP88A6 *NA*	*Pisum sativum*	Chapter 2
PsKAO2	AF537322	CYP88A7	*Pisum sativum*	Chapter 2
AtKAO1	AAK11564	CYP88A3	*Arabidopsis thaliana*	(Helliwell et al., 2001)
AtKAO2	AAK11565	CYP88A4	*Arabidopsis thaliana*	(Helliwell et al., 2001)
CmKAO1	AAG41777	CYP88A2	*Cucurbita maxima*	(Helliwell et al., 2000)
ZmKAO1	Q43246	CYP88A1 *D3*	*Zea-mays*	(Winkler and Helentjaris, 1995)
OsKAO	AP000616	CYP88A5	*Oryza sativa*	
HvKAO1	AAK11616	*Grd5*	*Hordeum vulgare*	(Helliwell et al., 2001)
Brassinosteroid biosynthesis enzymes				
85A1DWARF	Q43147	CYP85A1 *DWARF*	*Lycopersicon esculentum*	(Bishop et al., 1999)
brass6ox	BAB60858	brassinosteroid-6-oxidase	*Arabidopsis thaliana*	
90A1CPD	NP_196188	CYP90A1 AtCPD	*Arabidopsis thaliana*	(Szekeres et al., 1996)
DWF4	NP_190635	CYP90B1 steroid 22-alpha-hydroxylase	*Arabidopsis thaliana*	(Choe et al., 1998)
other representative plant cytochrome P450 enzymes				
CYP707A1	T04444		*Arabidopsis thaliana*	
CYP707A2	NP_180473		*Arabidopsis thaliana*	
CYP707A3	NP_199347		*Arabidopsis thaliana*	
CYP707A4	BAB02968		*Arabidopsis thaliana*	
CYP94A1	O81117	representative of 86 clan fatty acid omega-hydroxylase	*Vicia sativa*	(Tijet et al., 1998)
Fungal GA biosynthesis enzymes				
P450-1	Y15277	Fungal KAO (KA to GA_{14})	*Gibberella fujikuroi*	(Tudzynski and Holter, 1998)
P450-4	Y17243	Fungal KO	*Gibberella fujikuroi*	(Tudzynski et al., 2001)
outgroup Fungal B cytochrome P450 enzymes				
CYP60A2	U34740	sterigmatatocystin synthesis stcF	*Aspergillus nidulans*	(Brown et al., 1996)
CYP53A1	X52521	benzoate-para-hydroxylase bphA	*Aspergillus niger*	(van Gorcom et al., 1990)

Chapter 4

Regulation of the early GA biosynthesis pathway

INTRODUCTION

Gibberellins (GA) have multiple essential roles in plant growth and development including stem and petiole elongation, leaf expansion, seed development and germination (Reid and Ross, 1993; Hooley, 1994; Singh et al., 2002). Therefore the regulation of GA biosynthesis is expected to be complex. There are a large number of enzymes and genes involved in GA biosynthesis, many of which form gene families that catalyse the same step or steps but have different tissue specific expression patterns. This is especially noticeable with the dioxygenase enzymes of the later part of the GA biosynthesis pathway (Phillips et al., 1995; Garcia-Martinez et al., 1997; Yamaguchi et al., 1998a; Coles et al., 1999; Lester et al., 1999a).

The interaction of environmental factors with the tissue type or developmental stage of the plant appears important for regulating the biosynthesis of bioactive GAs. The principal environmental signal regulating GA biosynthesis is light (Garcia-Martinez and Gil, 2002; Hedden et al., 2002). White, red or blue light reduce GA_1 levels compared with pea seedlings grown in the dark by decreasing the expression level of *PsGA3ox1* (*LE*) and increasing the expression of mRNA

encoding the catalytic enzyme, *PsGA2ox2* (Gil and Garcia-Martinez, 2000; Reid et al., 2002). In germinating lettuce and *Arabidopsis* seeds red light increases bioactive GAs and the expression of GA 3-oxidases (Toyomasu et al., 1998; Yamaguchi et al., 1998a). Photoperiod may also influence GA biosynthesis. Transferring *Arabidopsis* from short to long days increased the GA 20-oxidase transcript levels (Xu et al., 1995). Furthermore, the effect of light intensity depends on the time of day pea seedlings are irradiated. Thus GA 20-oxidase transcript levels decrease with increasing light intensity at the end of a 16 h day but increase with increased light intensity at the end 8 h dark period (Garcia-Martinez and Gil, 2002).

Another environmental factor affecting GA biosynthesis in some species is temperature. Plant species with a bolting habit after vernalization such as *Thlapsi arvense* and *Brassica napus* have cold induced stem elongation and flowering, with increased metabolism of *ent*-kaurenoic acid. This is consistent with the regulation of *ent*-kaurenoic acid oxidase and to a lesser extent *ent*-kaurene oxidase by cold treatment (Metzger, 1985; Hazebroek et al., 1993). However a GA signal does not appear to be involved in the cold induced flowering of *Tulipa gesneriana* (Rebers et al., 1995) or *Pisum sativum* (Reid et al., 1996).

Other plant growth hormones such as auxin and brassinosteroids influence GA biosynthesis. Auxin promotes GA biosynthesis in decapitated pea seedlings by up-regulating *PsGA3ox1* (*LE*) in pea and decreases catabolism of bioactive GA_1 by down-regulating *PsGA2ox1* (Ross et al., 2000). In tobacco, auxin up-regulates GA 20-oxidase and to a lesser extent GA 3-oxidase (Wolbang and Ross, 2001). Brassinosteroids are reported to up-regulate GA 20-oxidase in *Arabidopsis* (Bouquin et al., 2001).

A possible molecular mechanism of control by these environmental signals and endogenous developmental programs are transcriptional regulators. There is evidence that some transcriptional regulators are involved in GA biosynthesis regulation. KNOTTED-type homeobox genes including *NtH15* of tobacco and *OsH1*

of rice can act as negative transcriptional regulators of GA biosynthesis. Overexpressed *NTH15* or *OSH1* produce a dwarf phenotype with reduced GA_{20} and GA_1 and increased GA_{19} levels by decreasing the GA 20-oxidase expression level (Kusaba et al., 1998; Tanaka-Ueguchi et al., 1998). This may be involved in control of the tissue distribution of GA biosynthesis, since *in situ* hybidisation indicates that GA 20-oxidase mRNA is present in the rib meristem but not where NTH15 is localized in the tunica, corpus and procambium tissues. Thus NTH15 may suppress GA 20-oxidase expression in these cells (Tanaka-Ueguchi et al., 1998). Furthermore, the KNOTTED1-like homeobox protein SHOOTMERISTEMLESS (STM) excludes the transcription of *AtGA20ox1* from the *Arabidopsis* shoot apical meristem allowing normal meristem function (Hay et al., 2002).

Another transcription factor, RSG, which is a transcriptional activator with a basic leucine zipper (bZip) domain, binds and activates the promoter of *Arabidopsis ent*-kaurene oxidase (AtKO; *GA3*)(Fukazawa et al., 2000). In addition, in transgenic tobacco expressing a dominant-negative form of RSG, the reduced expression of *NtKO* coincides with lower GA_1 levels and a dwarf phenotype (Fukazawa et al., 2000). In turn the signaling protein 14:3:3 regulates shuttling RSG out of the nucleus perhaps depending on environmental or developmental signals (Igarashi et al., 2001).

Overlaid on the environmental and developmental regulation of GA biosynthesis are homeostatic mechanisms to regulate the levels of bioactive GAs. The feed-back phenomenon whereby bioactive GAs appear to regulate their own biosynthesis and the feed-forward phenomenon where bioactive GAs regulate their catabolism have been observed. Work of Martin et al. (1996) suggested a strong feedback effect on the oxidation of GA_{20} to GA_1 and a weaker effect on the oxidation of GA_{19} to GA_{20}. Ross et al. (1999) using GC-MS methods showed that in GA deficient peas $[^{13}C^3H]GA_{20}$ was converted to $[^{13}C^3H]GA_1$ at a greater rate than in wild-type plants. Both GA 20-oxidase and GA 3-oxidase transcript levels are increased in GA deficient mutants of pea (Martin et al., 1997; Ross et al., 1999) and

Arabidopsis (Chiang et al., 1995; Phillips et al., 1995; Cowling et al., 1998). In addition, transcript levels of *Os20oxidase* were elevated in GA deficient mutants of rice (Toyomasu et al., 1997). Decreasing the GA content with chemical inhibitors of GA biosynthesis, such as Paclobutrazol and uniconazole-P, also increased the transcript level of GA 3-oxidase or GA 20-oxidase in treated wild-type plants (Toyomasu et al., 1997; Cowling et al., 1998). Conversely application of bioactive GAs, including GA_3 and GA_4, to GA deficient mutants decreased the GA 3-oxidase and GA 20-oxidase transcripts levels and decreased the metabolism of GA_{19} and GA_{20} (Chiang et al., 1995; Phillips et al., 1995; Xu et al., 1995; Martin et al., 1996; 1997; Toyomasu et al., 1997; Cowling et al., 1998). The deactivation enzyme GA 2-oxidase shows a feed-forward effect at the mRNA level. *PsGA2ox1* and *PsGA2ox2* are down-regulated in GA deficient mutants of pea and the application of bioactive GA_1 to these mutants increased the *PsGA2ox1* transcript level (Elliott et al., 2001). Similarly, application of bioactive GA_3 to a GA-deficient *Arabidopsis* mutant up-regulated GA 2-oxidase (Thomas et al., 1999).

The factor controlling the feedback is downstream of the bioactive GAs. In GA insensitive dwarf mutants of many species, paradoxically there was increased GA 20-oxidase and GA 3-oxidase expression with elevated levels of the bioactive GA_1 or GA_4 (Fujioka et al., 1988b; Scott, 1990; Talon et al., 1990; Appleford and Lenton, 1991; Cowling et al., 1998; Ueguchi-Tanaka et al., 2000). Conversely response mutants that appear as if saturated with GAs although they have low bioactive GA levels (e.g. pea *la cry*^{s}, *Arabidopsis rga gai* and barley *sln)* (Potts et al., 1985; Croker et al., 1990; Martin et al., 1996) have low GA 20-oxidase transcript levels (Dill and Sun, 2001; Silverstone et al., 2001). Only the GAs which had a bioactive growth response, in that species, caused the feed-back effect (Cowling et al., 1998). However the feed-back response (decrease in transcript levels) occurs rapidly, 1-3h after application of exogenous bioactive GAs; long before the growth response is evident (Hedden and Kamiya, 1997). Therefore the feed-back regulation may share at least part of the same signal transduction pathway as the growth

responses to GA (Cowling et al., 1998; Yamaguchi and Kamiya, 2000). Some signaling components have been suggested. These candidates include the negative GA response regulator RGA (Silverstone et al., 1998; 2001; Dill and Sun, 2001) and the regulator factors altered in the *Arabidopsis shi* (Fridborg et al., 1999) spy-7 and *gar2-1* mutants (Peng et al., 1999; Yamaguchi and Kamiya, 2000).

The feed-back/ feed-forward mechanisms are effective at maintaining the bioactive GAs within a narrow range. However, during development different optimal GA levels may be required at different stages. An example of how the homeostatic control may be selectively circumvented is presented during *Arabidopsis* seed germination (Yamaguchi and Kamiya, 2000; 2002). The two AtGA3oxidases are co-localized in the same cell types (Yamaguchi et al., 2001) however only the *AtGA3ox1* gene is under negative feedback control leaving *AtGA3ox2* free to increase the GA levels above the homeostatic range during germination (Yamaguchi et al., 1998a).

GA biosynthesis may be regulated during seed development as levels of GAs alter during the development and growth of pea seeds. There are two peaks of GAs in pea seeds as they develop. The second peak does not include bioactive GA_1 (Frydman et al., 1974; Garcia-Martinez et al., 1991; Swain et al., 1993). GA levels vary during development of seeds of other species including maize (White et al., 2000), pumpkin (Lange et al., 1997) and walnut (Tadeo et al., 1994).

In the vegetative part of the plant, developmental control of GA biosynthesis is also likely, as there is more GA_{20} and bioactive GA_1 in the actively expanding and developing apical bud of pea than in mature fully expanded tissue (Proebsting et al., 1992; Smith et al., 1992; Ross, 1998; O'Neill et al., 2000). Expression of the GA biosynthesis gene, *AtKO1*, was greater in young *Arabidopsis* seedlings than older seedlings (Helliwell et al., 1998) and *CmCPS1* and *CmCPS2* were expressed more strongly in immature apical tissue than mature pumpkin tissue (Smith et al., 1998). In addition the *Arabidopsis AtCPS* promoter directed the expression of a β-

glucuronidase (GUS)-reporter gene fusion to rapidly growing *Arabidopsis* shoot apices although expression was also observed in veins of mature leaves. (Silverstone et al., 1997). Gibberellins are also involved in the bolting process in *Thlaspi arvense* (Metzger, 1985) and other species (Davies, 2002) and the *AtCPS* promoter is active in the inflorescence meristem after bolting (Silverstone et al., 1997). In wheat, with the typical monocot growth habit, *ent*-kaurene synthesis activity was greatest in the node immediately below a rapidly expanding internode (Aach et al., 1997). Moreover, in wheat at the fourth node after the internode reached its final length, the *ent*-kaurene synthesis activity declined then increased again as the tiller developed (Aach et al., 1997).

In this chapter, the control of the early GA biosynthesis pathway will be investigated in pea using northern blot analysis of copalyl diphosphate synthase (*PsCPS*), *ent*-kaurene oxidase (*PsKO1*) and the *ent*-kaurenoic acid oxidases (*PsKAO1* and *PsKAO2*). The aspects considered include tissue specificity, the existence of gene families, developmental regulation during seed development, GA biosynthesis in mature vegetative tissue and feed-back on GA biosynthesis by GA activity.

For the convenience of arrangement and discussion, some data is duplicated in this chapter. Figures 4.2, 4.3 and 4.4 present, for confirmation, similar data from different harvests and RNA extraction methods. Figure 4.4 is a repeat of Figure 3.13 with some additional data and part of Figure 4.12 is derived from the same northern blot as Figure 4.3 to aid discussion.

RESULTS

Tissue specificity

The four genes investigated in this chapter have different expression patterns, which need to be taken into account when investigating the developmental control and feedback regulation of GA biosynthesis. As mentioned in previous chapters *PsKO1* (*LH*) is expressed in all tissues tested (Chapter 3) as is *PsKAO1* (*NA*) although the latter gene is expressed most strongly in the stem and only weakly in the seed (Fig. 4.1; Chapter 2). In contrast, *PsKAO2* is only expressed in the seed (Chapter 2). *PsCPS* is expressed strongly in the seed, to a lesser extent in the stem and only weakly in other tissues including apical bud, root and pod (Fig. 4.1). *PsCPS* is only very weakly expressed compared to the other genes tested.

Expression during seed and pod development

The mRNA levels of the 4 genes were measured by northern blot analysis of whole seeds at various ages after anthesis. The *PsKAO2* and *PsCPS* (*LS*) were expressed strongly around the time of contact point (Fig. 4.2). Contact point is the stage in pea seed development where the embryo just fills the whole seed and all the endosperm is just consumed. The strong *PsKAO2* and *PsCPS* expression in seeds around contact point was confirmed with different blots and harvests, different RNA extraction methods, and for the GA deficient mutants *lh-1*, *lh-2*, and *ls-1* as well as the wild type cv. Torsdag (Figs. 4.3 and 4.4). Both *PsKAO2* and *PsCPS* expression peaks start to buildup 3 days before contact point, peak at contact point and then start to tail off 4 days after contact point (Figs. 4.3 and 4.4). The expression level appears to be more closely related to the weight of the seeds and therefore the developmental stage of the different mutant phenotypes than the time in days after anthesis (Fig. 4.2). Note that *lh-2* mutant has delayed seed development (Chapter 3). Also,

although *PsKAO1* (*NA*) was weakly expressed in seeds, there is an expression peak around the time of contact point (Fig. 4.3). In contrast, as discussed previously (Chapter 3), *PsKO1* (*LH*) is expressed at all stages of seed development with two peaks of expression. One peak in the young 11 to 13 day old seeds which weigh less than 30mg and another peak around contact point (Figs. 4.2; 4.3 and 4.4). Note that a larger *PsKO1* band and, at higher expression levels, a second band associated with the *lh-2* lesion (Chapter 3) were observed, as expected, in *lh-2* mutant tissue samples.

Northern blot analysis was also used to compare the gene expression levels in pods (with seeds removed at harvest) at two different developmental stages. Young pods (11 or 13 days after anthesis) and pods originally containing seeds at contact point were compared (Fig. 4.5). *PsKO1* (*LH*) had similar expression levels in pods at both stages. The older pods had higher *PsKAO1* (*NA*) expression than the younger pods and although the *PsCPS* expression levels were generally very low, the younger pods appear to have slightly higher levels.

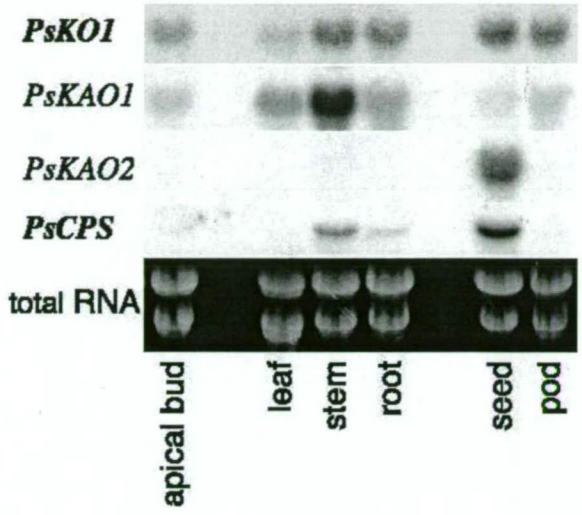


Figure 4.1

PsKO1, PsKAO1, PsKAO2 and *PsCPS* transcript levels in various parts of wild-type pea (L107). 5μg total RNA from the apical bud (all material above the uppermost fully expanded leaf), leaf (the uppermost fully expanded leaf), stem (internode immediately below the uppermost fully expanded leaf, the internode was 80% to 100% fully expanded), and root (50mm off the end of the tap and lateral roots) of 19 day old seedlings; also 5μg total RNA from seeds (3 days after contact point) and pods (that originally contained these seeds) from mature plants were loaded on the gel. X-ray film exposure times were *PsKO1*(1 day), *PsKAO1*(2 days), *PsKAO2* (2 days) and *PsCPS* (3 days).

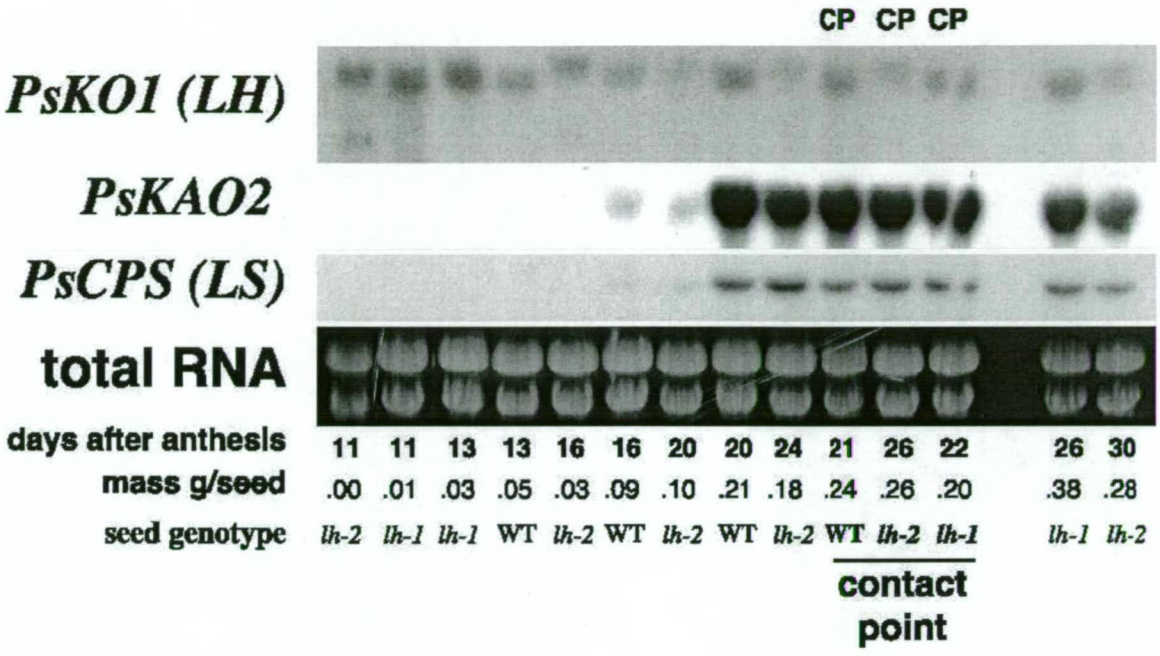


Figure 4.2

PsKO1, PsKAO2 and *PsCPS* transcript levels of seeds during development arranged in increasing age (days after anthesis) and weight (g) from various genotypes, wild-type L107 (WT) or homozygous *lh-1* or *lh-2*. Total RNA (5μg) from whole seeds at various ages was loaded on the gel. **CP** Contact point (the first day that no liquid endosperm remained in seeds) is indicated. RNA was extracted using Tri Reagent™ (SIGMA®).

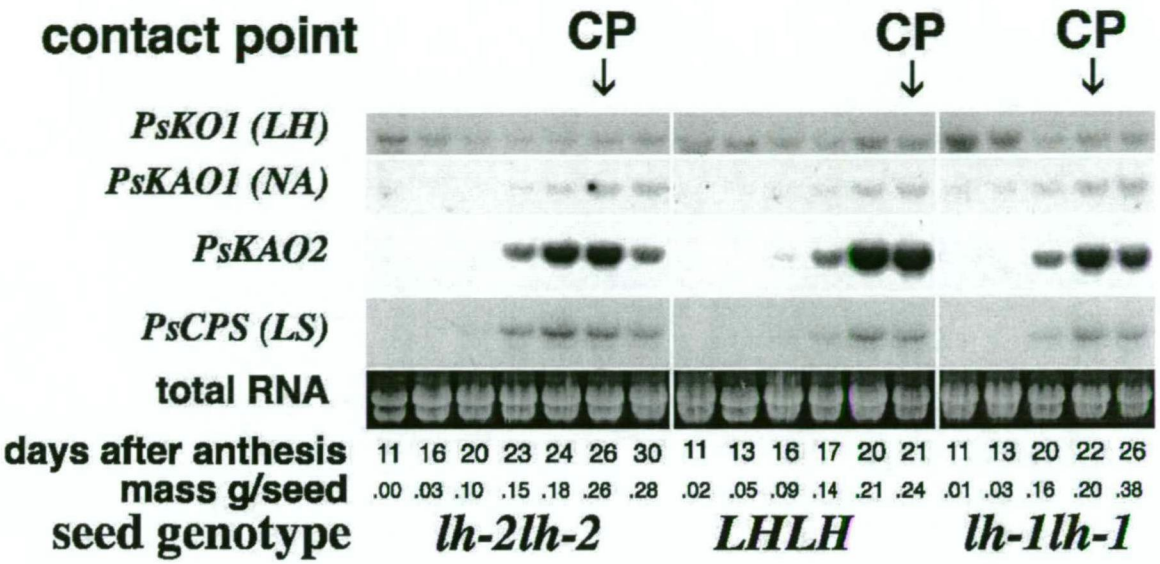


Figure 4.3

PsKO1, PsKAO1, PsKAO2 and *PsCPS* transcript levels of seeds at various ages and weights during development from homozygous wild-type (*LH*), and dwarf mutant *lh-1* and *lh-2* plants. Total RNA (5µg) from whole seeds at various ages (days after anthesis) were loaded on the gel. **CP** Contact point (the first day that no liquid endosperm remained in seeds) is indicated. RNA was extracted using Tri Reagent™ (SIGMA®).

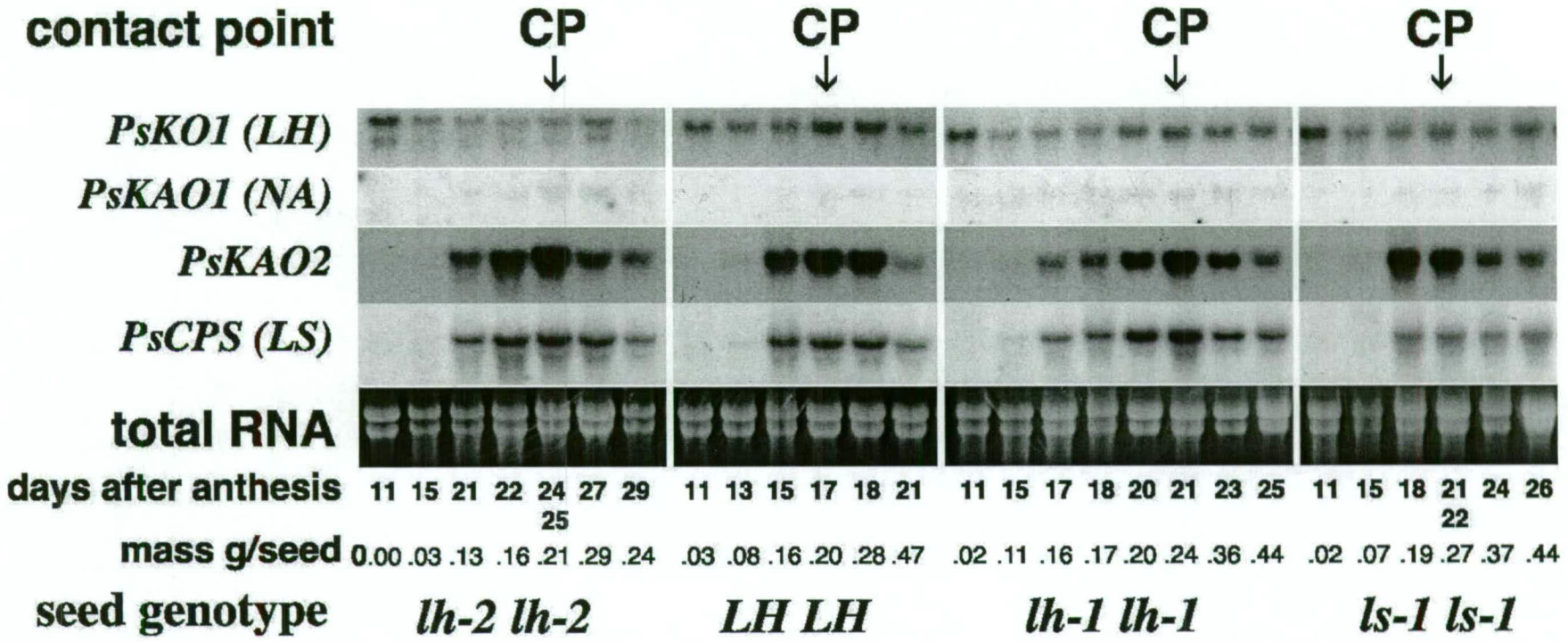
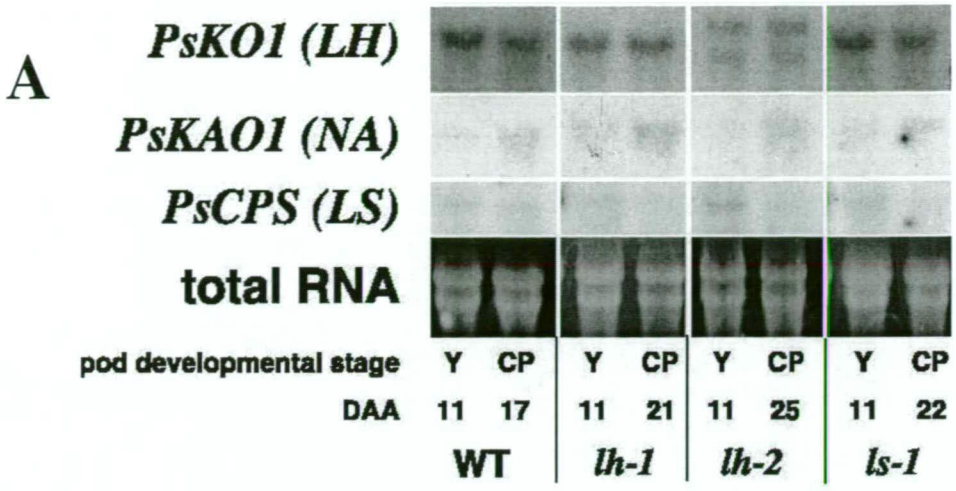


Figure 4.4

PsKO1, PsKAO1, PsKAO2 and *PsCPS* transcript levels of seeds at various ages and weights during development from homozygous wild-type (*LH*), and dwarf mutant *lh-1*, *lh-2* and *ls-1* plants. Total RNA (5μg) from whole seeds at various ages (days after anthesis) were loaded on the gel. RNA was extracted using RNeasy Plant Kit with RLC buffer (QIAGEN).
CP Contact point (the first day that no liquid endosperm remained in seeds) is indicated.



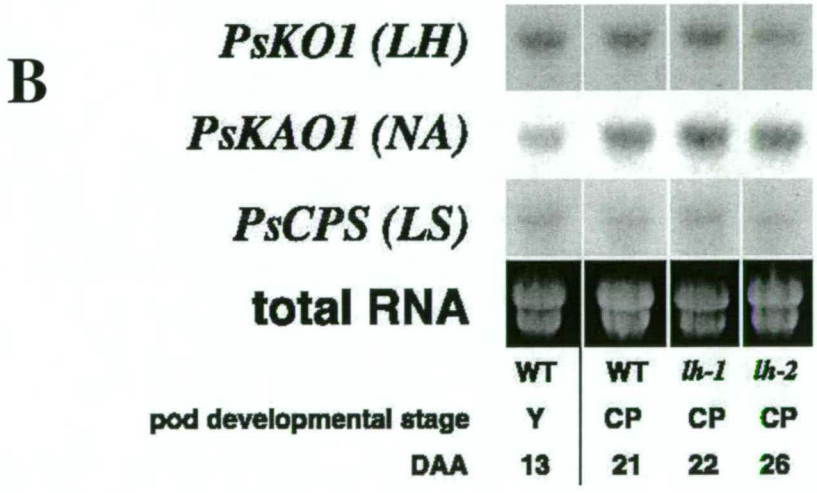


Figure 4.5

A *PsKO1, PsKAO1* and *PsCPS* transcript levels in young (**Y**) pods and pods from which seeds at contact point were removed (**CP**) from wild-type (**WT**) L107 and GA deficient dwarf mutants, ***lh-1, lh-2*** and ***ls-1***. RNA was extracted using RNeasy Plant Kit with RLC buffer (QIAGEN). Note that 2 *PsKO1* bands are observed at high expression levels for the *lh-2* mutant because of the nature of the lesion (Chapter 3).

B *PsKO1, PsKAO1* and *PsCPS* transcript levels in pods, from which seeds at contact point were removed, (**CP**) from wild-type (**WT**) L107, and GA deficient dwarf mutants ***lh-1*** and ***lh-2***. Wild-type young pods (Y) from which seeds, 13 days after anthesis (DAA), were removed, were also sampled. RNA was extracted using Tri Reagent™ (SIGMA®).

Expression in mature and developing vegetative tissue

Pea seedlings were partitioned into the apical bud (all the material above the uppermost just fully expanded leaf), young still expanding stem tissue (internode immediately below the uppermost fully expanded leaf), mature non-expanding stem tissue, uppermost just fully expanded leaf, and mature leaf (Fig. 4.6).

PsKO1 (*LH*), *PsKAO1* (*NA*) and *PsCPS* (*LS*) were consistently expressed in mature non-expanding stem tissue as well as young still expanding stem tissue (Figs. 4.7, 4.8 and 4.9). This was evident for different harvests, plantings at different times of the year, different wild type lines (L107 and WL1769), and GA deficient mutants (*lh-1*, *lh-2*, *ls-1*, *na-1*) (Figs. 4.7, 4.8 and 4.9). Even mature stem tissue 7 nodes below the apical bud relatively strongly expressed all three genes (Fig. 4.8). Note that *PsKAO1* (*NA*) probe was unable to give meaningful results with *na-1* mutant tissue because of the nature of the lesion (Chapter 2) and likewise probing *ls-1* tissue with the *PsCPS* (*LS*) probe was problematic (Ait-Ali et al., 1997). Both *PsKAO1* (*NA*) and *PsCPS* (*LS*) expression was greater in mature stem than apical bud tissue (Figs. 4.7, 4.8 and 4.9). In addition, mature leaf tissue, even 4 nodes below the apical bud, expressed *PsKO1* (*LH*) and *PsKAO1* (*NA*) as strongly as the leaf immediately below the apical bud and the apical bud itself (Fig. 4.7). The apical bud is made up of a mixture of leaf and stem tissue in the process of expanding and developing and *PsKAO1* and *PsCPS* expression may be somewhat diluted by the leaf component (Fig. 4.6). However, the apical bud is still a measure of the actively growing and developing tip of the pea seedling.

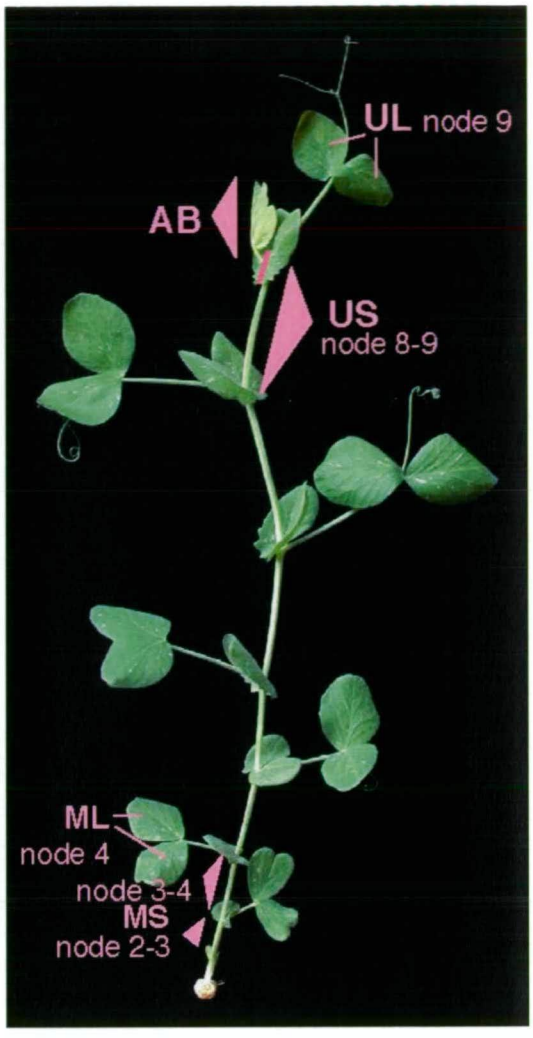


Figure 4.6

Harvest division of a pea seedling with the leaf at node 9 just fully expanded. The seedlings were partitioned into the **AB** apical bud (all the material above the uppermost just fully expanded leaf), **US** young still expanding stem tissue (internode immediately below the upper fully expanded leaf), **MS** mature non-expanding stem tissue (ranging from 2 to 7 internodes below the apical bud depending on the experiment), **UL** uppermost just fully expanded leaf, and **ML** mature leaf (3 or 4 nodes below the apical bud depending on the experiment)

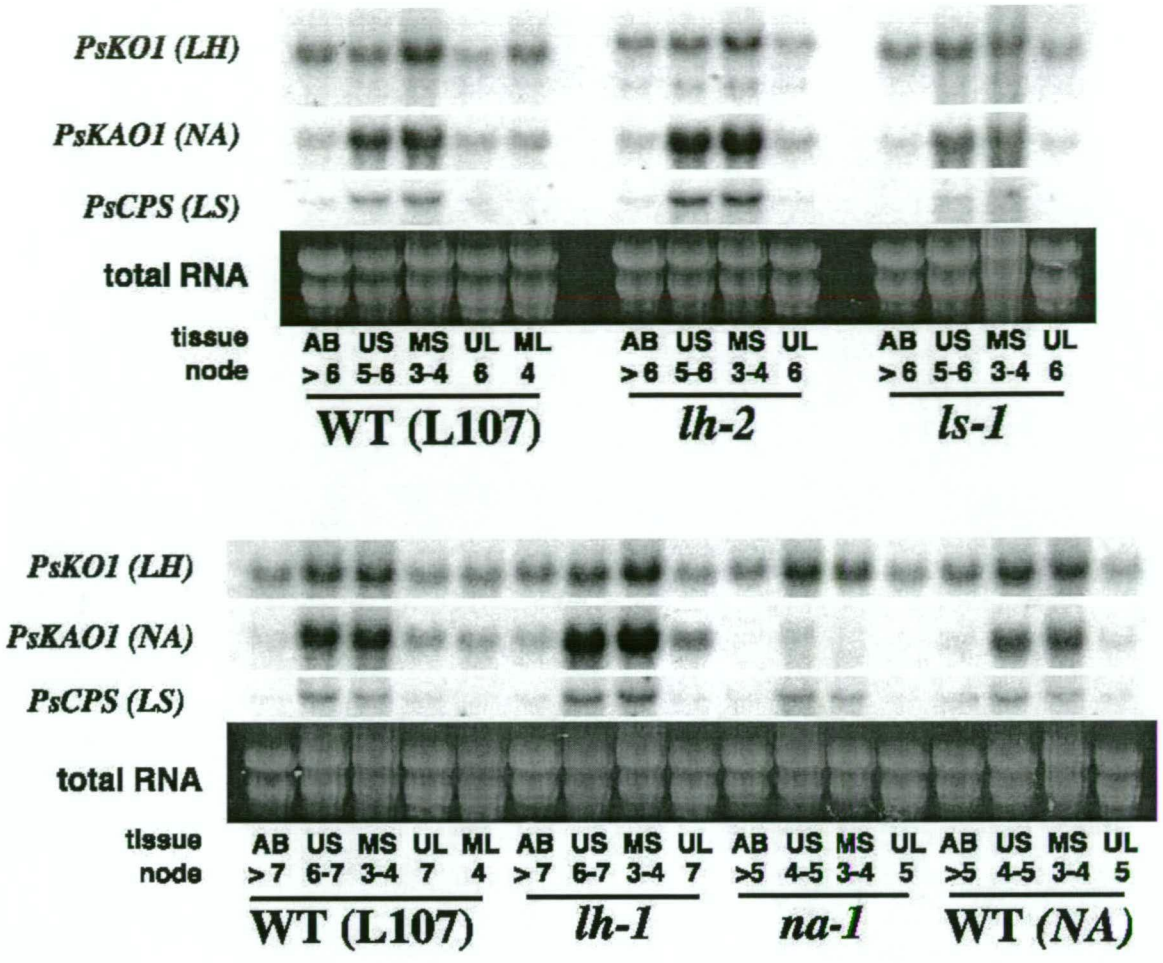


Figure 4.7

PsKO1, PsKAO1 and *PsCPS* transcript levels in various parts of the GA deficient mutants *lh-1*, *lh-2*, *ls-1* and their relevant wild-type cv.Torsdag (L107); and GA deficient mutant *na-1* and its isogenic wild-type *NA* (WL1769). This was 5μg total RNA from the **AB** apical bud (all material above the uppermost fully expanded leaf), **US** upper stem (internode immediately below the uppermost fully expanded leaf, the internode was 75% to 100% fully expanded), **MS** mature stem (fully elongated internode between nodes 3 and 4), **UL** upper leaf (the uppermost fully expanded leaf), and **ML** mature leaf (fully expanded leaf at node 4) from 18 day old seedlings were loaded on the gel in two rows. All samples were harvested on the same day and were hybridised on the same two row membrane blot. Note that some wild-type L107 and WL1769 were planted two days later so that they would be at the same developmental stage when harvested as the mutants with delayed development (*lh-2*, *ls-1* and *na-1*). Note that the 2 bands observed when *PsKO1* is expressed strongly in the *lh-2* mutant tissue are expected because of the nature of the lesion (Chapter 3).

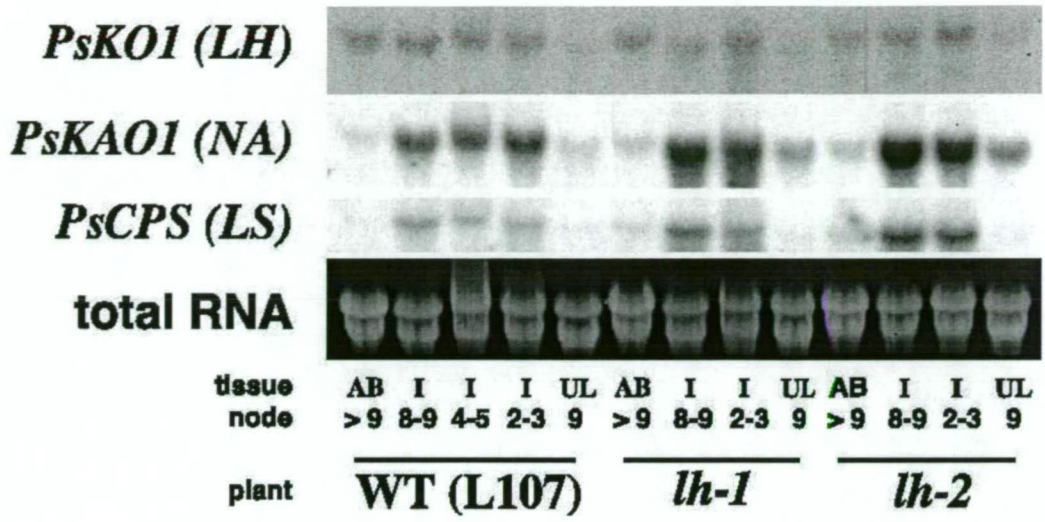


Figure 4.8

PsKO1, PsKAO1 and *PsCPS* transcript levels in various parts of the GA deficient mutants *lh-1*, *lh-2* and their relevant wild-type cv.Torsdag (L107). 5µg total RNA from the **AB** apical bud (all material above the uppermost fully expanded leaf, above node 9), **I8-9** (internode immediately below the uppermost fully expanded leaf; the internode was 75% to 95% fully expanded), **I4-5** (mature fully elongated stem between nodes 4 and 5), **I2-3** (mature fully elongated stem between nodes 2 and 3) **UL** upper leaf (the uppermost fully expanded leaf at node 9) were loaded on the gel.

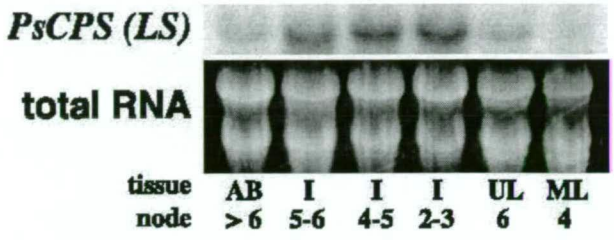


Figure 4.9

PsCPS transcript levels in various parts of wild-type pea (L107). RNA was extracted from 7 (15 day old) Torsdag L107 plants harvested into the portions: AB (apical bud above node 6); I nd5-6, I nd4-5, and I nd2-3 (stem between nodes 5 to 6,4 to 5 and 2 to 3 respectively); UL (upper fully expanded leaf at node 6); and ML (mature fully expanded leaf at node 4) and 5µg total RNA was loaded on the gel.

Feed-back

Gene expression levels in GA deficient mutant tissue

To ascertain feedback of GA activity on the GA biosynthesis genes *PsKO1* (*LH*), *PsKAO1* (*NA*), *PsKAO2* and *PsCPS* (*LS*) their transcript levels were measured in mutants with decreased GA levels due to blockages in the GA biosynthesis pathway (Fig. 4.10) at *PsCPS* (*ls-1*; Ait-Ali et al., 1997), *PsKO1* (*lh-1* and *lh-2*; Chapter 3) and *PsKAO1* (*na-1*; Chapter 2). Feed-back regulation of a gene would be indicated by an increase in transcript level in the GA deficient mutants compared with the wild-type.

PsKO1 (LH)

PsKO1 showed no indication of feed-back by GA activity. *PsKO1* (*LH*) expression is similar in the apical buds of the GA deficient mutants, *lh-1*, *lh-2*, *ls-1* and *na-1* and their respective wild-types (Figs. 4.7, 4.8 and 4.11). This was also observed for stem and leaf tissue (Figs. 4.7 and 4.8). *PsKO1* is also expressed in seeds at contact point which is the stage in pea seed development where the embryo just fills the whole seed and all the endosperm is just consumed. Since this is a distinguishable stage in seed development, comparisons could be made of expression levels between GA deficient mutants and the wild-type. Seeds at contact point (Figs. 4.2 and 4.12) and pods (Fig. 4.5) from GA deficient mutants, *lh-1*, *lh-2*, and *ls-1* also did not have increased *PsKO1* expression compared to the wild-type.

PsKAO1 (NA)

The *PsKAO1* (*NA*) expression levels in the *lh-2* mutant stem tissue was slightly greater than the expression of these genes in the corresponding wild-type harvested at the same time and same developmental stage, although this may partially be explained by slight differences in loading total RNA on the gel (Fig. 4.7).

This small increase was confirmed in a different planting and harvest with older plants harvested when they reached the 9 expanded leaf stage (Fig. 4.8). There was a small difference in the *PsKAO1* (*NA*) expression levels of stem tissue from different wild-types, L107 (cv Torsdag) and WL1769 although this was not noted when the same blot hybridized with *PsCPS* (*LS*) or *PsKO1* (*LH*) probes (Fig. 4.7). The *PsKAO1* expression appeared also slightly greater in the *lh-1* mutant compared to the corresponding wild-type harvest (Fig. 4.7) but this was not obvious with other harvests (Fig. 4.8). However, *PsKAO1* expression was not increased in another GA deficient mutant, *ls-1* mutant stem tissue compared to the corresponding wild-type harvest (Fig. 4.7) and as expected the *na-1* mutant tissue had decreased mRNA due to the instability of the mRNA with that lesion (Chapter 2). The *PsKAO1* (*NA*) expression levels tend to show the same trends in apical bud and leaf tissue as in the stem tissue (Figs. 4.7 and 4.8).

PsKAO2

PsKAO2 is only expressed in seed (Fig. 4.1; Chapter 2). No feedback was indicated by the similar expression levels of the wild-type and GA deficient mutants, *lh-1*, *lh-2*, *and ls-1* in seeds at contact point (Figs. 4.2 and 4.12).

PsCPS (LS)

PsCPS (*LS*) expression levels in the *lh-2* mutant stem tissue appeared slightly greater than the expression of this gene in the corresponding wild-type harvested at the same time and same developmental stage, although this may partially be explained by slight differences in the loading total RNA on the gel (Fig. 4.7). This small difference was confirmed in a different planting and harvest with older plants (Fig. 4.8). However, *PsCPS* expression levels in other GA deficient mutants, *lh-1*, *ls-1* and *na-1* were similar to their relevant wild-type stem tissue (Figs. 4.7 and 4.8). *PsCPS* (*LS*) is also strongly expressed in the seeds around the time of contact point, which is a distinguishable developmental stage useful for comparison between

genotypes (Fig. 4.1). *PsCPS* (*LS*) expression was similar in seeds at contact point in GA deficient mutants (*lh-2* and *lh-1*) and wild-type (Figs. 4.2 and 4.12). The expression level of *ls-1* seed was, as expected, less than wild-type (Fig. 4.12B) because of the nature of the lesion (Ait-Ali et al., 1997). Furthermore although the *PsCPS* expression levels in the apical bud is low there was no noticeable increase in *PsCPS* expression in the GA deficient mutants, *lh-1*, *lh-2*, and *na-1* compared to their relevant wild-type (Fig. 4.11).

Gene expression levels in a mutant with high GA content

A blot originally prepared by Dr. R.C. Elliott was probed with *PsCPS* (*LS*) and showed transcript levels were similar in the GA accumulating mutant, *sln,* as the wild-type (Fig. 4.13). Note that the wild-type cv.Torsdag (L107) and *sln* (Lester et al., 1999b; Fig. 4.10) are not isogenic therefore there may be general background differences. However feedback was previously demonstrated for GA 20-oxidase and GA 3-oxidase with these blots (personal communication Dr. R.C. Elliott). Expression levels in the *ls-1* mutant are low due to the nature of the lesion (Ait-Ali et al., 1997).

Treatment with paclobutrazol

The GA content of wild-type or already GA deficient dwarf mutants was decreased by application of the GA biosynthesis inhibitor, paclobutrazol. This inhibitor specifically inhibits the oxidation of *ent*-kaurene to *ent*-kaurenoic acid and therefore blocks the same site as the *lh* mutation (Fig. 4.10; Chapter 3). The paclobutrazol treated wild-type cv. Torsdag was harvested when it was of shorter stature than the untreated mutants and therefore would be expected to have an even lower GA content without any general background differences (Fig. 4.14).

PsKO1 (LH)

Again, there is no indication of feedback by GA activity. Paclobutrazol treated wild-type cv. Torsdag seedlings were dwarfed but had similar *PsKO1* expression levels in the apical bud and stem tissue as untreated controls (Fig. 4.15). This was so whether seedlings were at the 4 or 9 expanded leaf stage (Fig. 4.15A). In addition, further decreasing the GA content of the GA deficient mutants, *lh-1*, *lh-2*, *ls-1* with paclobutrazol had no affect the expression of *PsKO1* (Fig. 4.15A and B).

PsKAO1 (NA)

Decreasing the GA content by paclobutrazol inhibition had no effect on the expression of *PsKAO1* in stem, apical bud and leaf tissue in wild-type cv Torsdag seedlings. In addition, *PsKAO1* expression levels in the apical bud of the GA deficient mutants, *lh-1* and *lh-2* were similar in paclobutrazol treated and untreated seedlings (Fig. 4.15B).

PsCPS (LS)

PsCPS expression in the apical bud or stem was similar for paclobutrazol treated and control wild-type seedlings when harvested in plants either at the 4 or 9 fully expanded leaf stage (Fig. 4.15). Paclobutrazol treatment of the GA deficient mutants *lh-1* and *lh-2* also did not alter the apical bud *PsCPS* expression (Fig. 4.15A).

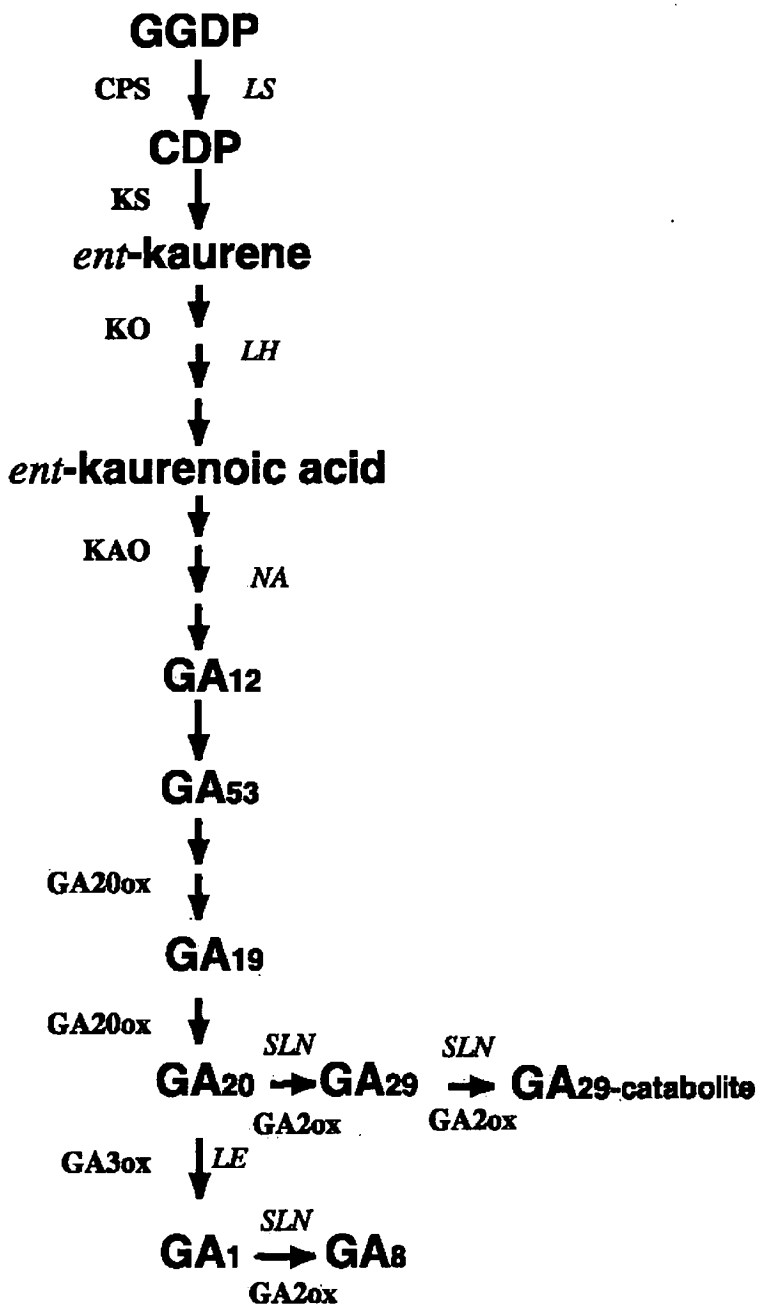


Figure 4.10

A simplified gibberellin GA biosynthesis pathway in pea. The GA biosynthesis enzymes copalyl diphosphate synthase (CPS), *ent*-kaurene synthase (KS), *ent*-kaurene oxidase (KO), *ent*-kaurenoic acid oxidases (KAO), GA 20-oxidase (GA20ox), GA 3-oxidase (GA3ox) and GA deactivation enzyme GA 2-oxidase (GA2ox) are indicated. Genes with mutant alleles in pea, *LS, LH, NA, LE,* and *SLN* are indicated.

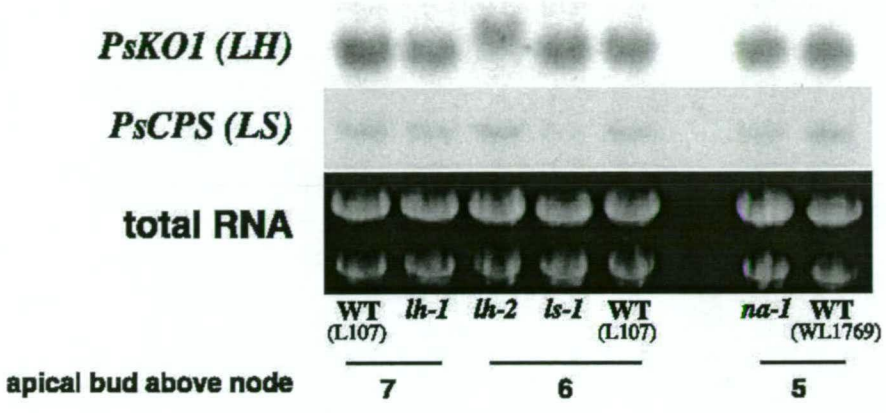


Figure 4.11

PsKO1 and *PsCPS* transcript levels in the apical bud (all the material above the uppermost just fully expanded leaf) of the GA deficient mutants *lh-1*, *lh-2*, *ls-1* and their relevant wild-type cv.Torsdag (L107); and GA deficient mutant *na-1* and its isogenic wild-type *NA* (WL1769). The apical buds were harvested on the same day, at the same developmental stage but from above either node 7, 6 or 5. Total RNA (5μg) was loaded on the gel. X-ray film exposure times: *PsKO1* (1.5 days) and *PsCPS* (5 days). Note that the *PsKO1* band is larger in lh-2 sample as expected because of the nature of the lesion (chapter 3).

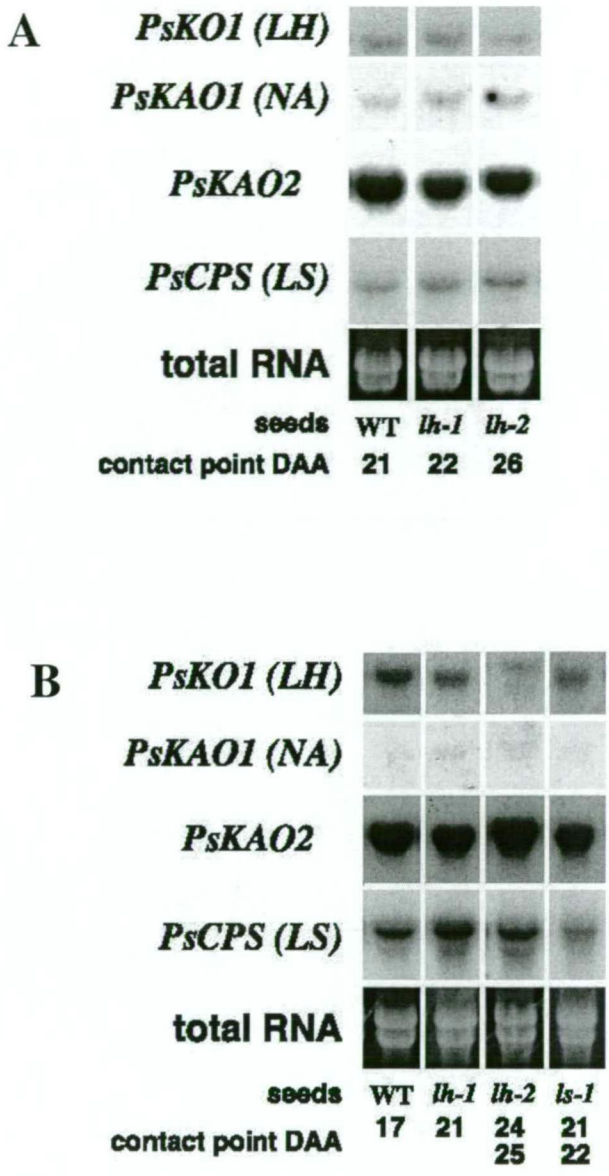


Figure 4.12

PsKO1, PsKAO1, PsKAO2 and *PsCPS* transcript levels in seeds at contact point of wild-type L107 (**WT**) and GA deficient dwarf mutants, ***lh-1, lh-2*** and ***ls-1***. Contact point (**CP**) is a developmentally destinguishable stage where the embryo just fills the whole seed and all the endosperm is just consumed. RNA was extracted from the different harvests using Tri Reagent™ (SIGMA®) (**A**) or RNeasy Plant Kit with RLC buffer (QIAGEN) (**B**).

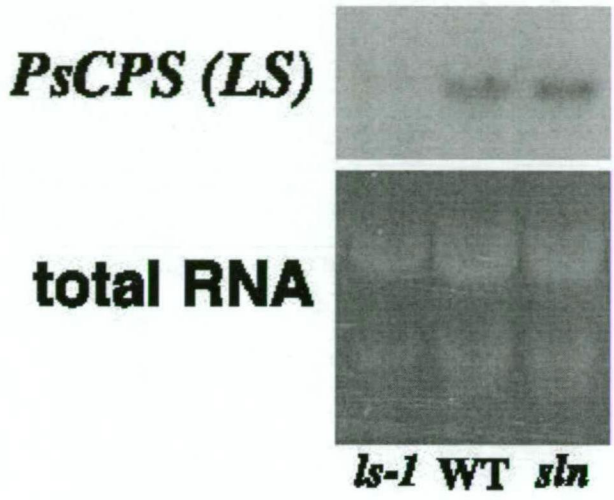


Figure 4.13

PsCPS transcript levels in wild-type (L107) (**WT**), GA deficient mutant (***ls-1***) and GA accumulating mutant (***sln***). This blot was originally prepared by Dr. R.C. Elliott (Elliott et al., 2001) then stripped and reprobed with *PsCPS*. 5μg of upper stem (internode, immediately below the uppermost fully expanded leaf) was loaded on the gel.

	WT	*lh-1*	*lh-2*	WT +P	*lh-1* +P	*lh-2* +P
dev. to node	9	8	6	7	7	6

Figure 4.14

4 week old pea seedlings of wild-type (**WT**) and GA deficient dwarf mutants, ***lh-1*** and ***lh-2***.
Treated seedlings (**+P**) had a large dose of paclobutrazol, PP333, (5µg/seed) was applied to seeds prior to planting. Control plants had ethanol applied.
The node with the uppermost fully expanded leaf at time of photograph, is indicated for each plant.

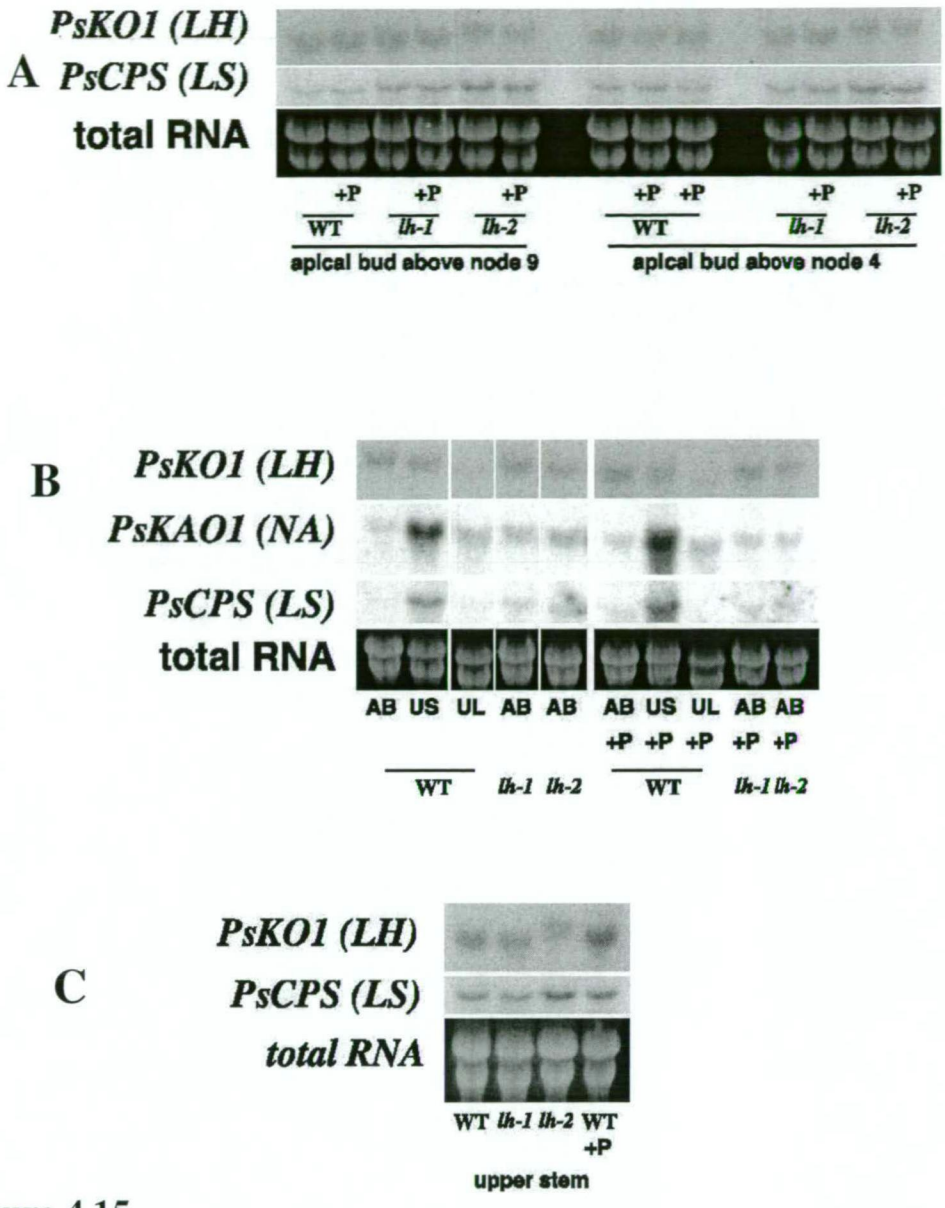


Figure 4.15

PsKO1, PsKAO1 and *PsCPS* transcript levels of untreated and, paclobutrazol (5µg/seed) treated (**+P**) wild-type (L107) or GA deficient mutants *lh-1* or *lh-2* seedlings. 5µg/seed paclobutrazol GA biosynthesis inhibitor treatment reduced the stature of the wild-type to less than the untreated GA deficient mutants.

A Total RNA (5µg/lane) from apical buds (all the material above the uppermost just fully expanded leaf) either above node 4 (2 week old seedlings) or node 9 (seedlings older than 4 weeks)

B 5µg total RNA from the **AB** apical bud (all material above the uppermost fully expanded leaf, above node 9), **US** upper stem (internode, 8-9, immediately below the uppermost fully expanded leaf; the internode was 75% to 95% fully expanded) and **UL** upper leaf (the uppermost fully expanded leaf at node 9)

C 5µg of total RNA extracted from stem tissue (between nodes 3-4) immediately below the uppermost fully expanded leaf was loaded on the gel.

DISCUSSION

Tissue specificity and gene families

There are at least two genes encoding *ent*-kaurenoic acid oxidase activity in pea (Chapter 2). They are differentially expressed with *PsKAO1* expressed in all tissues tested while *PsKAO2* is expressed only in seed (Chapter 2 and Fig. 4.1). The importance of this different expression pattern for the phenotype of the pea *na-1* mutant phenotype was discussed in Chapter 2. The presence of a gene family with different tissue specificity enables differential control of that step of the pathway in different tissues. There are also two KAO genes in *Arabidopsis* but they have similar expression patterns (Helliwell et al., 2001a). However, the only pumpkin *ent*-kaurenoic acid oxidase gene cloned, *CmKAO1*, was obtained from a pumpkin seed library and is only expressed in the seed (Helliwell et al., 2000). Since GA biosynthesis occurs in the vegetative parts of the pumpkin seedling (Lange et al., 1997; Smith et al., 1998) another gene encoding KAO is expected to be expressed in other tissue types suggesting possible similar tissue specific regulation in pumpkin to that demonstrated in pea.

In contrast, pea *PsKO1* is a single copy gene (Chapter 3). *PsKO1* is expressed in all tissue types tested and all the seed and vegetative developmental stages tested (Figs. 4.1; 4.3 and 4.7) and therefore may not be important in tissue specific regulation. There is also only one gene encoding *ent*-kaurene oxidase in *Arabidopsis* (Helliwell et al., 1998). *Arabidopsis AtKO1* expression levels seem to vary more between different organs (Helliwell et al., 1998) than did the pea *PsKO1*. Of the two KOs in pumpkin, *CmKO1* is expressed in the seed and leaf but not in other tissues

tested and the expression pattern of the other pumpkin KO gene has not been reported (Helliwell et al., 2000). The transcriptional activator, RSG, binds and activates the *ent*-kaurene oxidase promoter of *AtKO* and *NtKO* in *Arabidopsis* and tobacco respectively (Fukazawa et al., 2000). In addition, the core binding site of RSG is present in the promoters of *AtKAO1* and *AtKAO2* (Helliwell et al., 2001a). It would be interesting to see if RSG also binds to the promoters of these genes in pea and to speculate on the importance of this regulator on genes which otherwise seem poorly positioned as sites of GA biosynthesis regulation.

In pea there is one functional copy with CPS activity, *PsCPS* (*LS*), and a pseudogene (with no mRNA detected even by RT-PCR in any tissue) (Ait-Ali et al., 1997). *PsCPS* has low transcript levels with more mRNA accumulating in the seed than stem and extremely low levels in the apical bud, leaf, root and pod (Fig. 4.1). In *Arabidopsis*, *AtCPS1* is also expressed at extremely low levels and is considered to play a part in GA biosynthesis regulation by tissue specific mRNA expression (Silverstone et al., 1997). However, overexpression of *AtCPS* did not increase the levels of bioactive GAs in *Arabidopsis* (Sun and Kamiya, 1994). Pumpkin has two genes encoding CPSs with differing expression patterns and they also are considered to be tissue developmentally regulated (Smith et al., 1998). Note that the *ent*-kaurene synthase (*KS*) has not yet been cloned in pea but *KS* is a single copy gene in pumpkin and *Arabidopsis* (Yamaguchi et al., 1996; 1998b). Therefore gene families do not appear to be important among the genes encoding the cyclase enzymes (CPS and KS) although developmental regulation of *CPS* is probable in some species.

Gene families with differing expression patterns are common amongst the genes encoding enzymes in the latter part of the GA biosynthesis pathway including GA 20-oxidases (Phillips et al., 1995; Garcia-Martinez et al., 1997; Coles et al., 1999), GA 3-oxidases (Yamaguchi et al., 1998a; Lester et al., 1999a) and the GA deactivation enzymes, GA 2-oxidases (Elliott et al., 2001). In contrast, KAO appears to be the only gene in the early GA biosynthesis pathway to have a second gene with

tissue specific expression in pea. Gene families are also rare in this part of the pathway in other species and if present appear to be limited to two genes (Yamaguchi et al., 1996; 1998b; Helliwell et al., 1998; 2000; 2001a; Smith et al., 1998).

Developmental regulation of *PsKO1* (*LH*), *PsKAO1* (*NA*), *PsKAO2* and *PsCPS* (*LS*) expression during pea seed development.

PsKO1 is expressed at all stages of seed development tested but was expressed more strongly at an early stage of pea seed development and then again at contact point (Figs. 4.2, 4.3 and 4.4). This coincided with the observed 2 peaks in GA levels during the development of normal seeds (Frydman et al., 1974; Swain et al., 1993). Swain et al. (1995) suggested that the early GA_1 level peak may be essential for the development of seeds because the *lh-2* mutant is the only mutant so far to have a severely affected seed phenotype and also the only mutant without an early GA peak. As discussed previously in Chapter 3, the *PsKO1* (LH) expression pattern during seed development supports this proposition.

In contrast *PsCPS* and *PsKAO2* were only expressed around the time of contact point and not in the younger seeds (Figs. 4.2, 4.3 and 4.4). Although *PsKAO1* expression levels are very low and *PsKAO2* is likely to be the major *ent*-kaurenoic acid oxidase activity in the seed, *PsKAO1* expression also peaks around contact point (Figs. 4.3 and 4.4). This *PsCPS* and *PsKAO2* expression peak around contact point coincided with the second GA peak observed in developing pea seeds (Frydman et al., 1974; Swain et al., 1993; 1995). The physiological importance of these elevated levels of GA_{20} are as yet unknown since GA_1 is not detected and the GA_{20} levels gradually decrease to lower levels in the dry seed (Reid et al., 1992; Ross et al., 1993; Swain et al., 1993). However the expression levels do not merely correlate with an increase in cotyledon or embryo size as transcript levels decrease 2

to 4 days after contact point (Figs. 4.2, 4.3 and 4.4) and therefore appear to indicate some developmental regulation at the transcript level in developing seeds.

Previously (Ait-Ali et al., 1997) found that *PsCPS* (LS) was also expressed in even less developed seeds at 4 days after anthesis as well as at contact point. Perhaps the GA biosynthesis gene expression associated with the first GA peak begins before sampling commenced at 11 days after anthesis.

No Feedback response evident for *PsCPS, PsKO1, PsKAO1, PsKAO2*

ent-kaurene oxidase, *PsKO1* (LH), is not subject to feedback regulation by GA activity. Decreased GA content of GA deficient mutants, *lh-1, lh-2, ls-1, na-1* with blocks in the GA biosynthesis pathway prior, at or after *LH* (Fig. 4.10) had no effect on the expression of *PsKO1*. This was so for all tissues tested, including stem, leaf, apical bud (Figs. 4.7, 4.8 and 4.11), seed and pod (Figs. 4.2, 4.5 and 4.12). Furthermore, decreasing the GA content and stature of wild type and mutants (*lh-1*, *lh-2* and *ls-1*) with the GA inhibitor, paclobutrazol, (Fig. 4.14) had no effect on the expression of *PsKO1* (Fig. 4.15). Helliwell et al. (1998) found that there was no evidence of feedback regulation of *Arabidopsis ent*-kaurene oxidase since *AtKO1* expression in the GA deficient mutants, *ga1-3*, *ga4-1* and *ga5-1*, was similar to wild-type and the application of GA_3 did not alter expression levels.

Although the copalyl diphosphate synthase, *PsCPS*, (*LS*) transcript level is consistently slightly higher in stem tissue from the GA deficient *lh-2* mutant (Figs. 4.7 and 4.8), there does not appear to be a feedback response. There is no noticeable increase in expression of *PsCPS* in the other GA deficient mutant *lh-1* compared to wild type Torsdag plants (Figs. 4.7 and 4.8). The more severe *na-1* mutant which has even more reduced GA_1 levels compared to its wild type than *lh-2* (Potts and Reid, 1983) did not show any increase in *PsCPS* expression (Fig. 4.7). Furthermore no increase in *PsCPS* expression was observed when the GA biosynthesis was inhibited

by paclobutrazol (Fig. 4.15). Conversely, *PsCPS* expression was not reduced in the GA accumulating mutant, *sln* (Fig. 4.13) with decreased GA deactivation due to a lesion in *PsGA2ox1* (Lester et al., 1999b). The small increase in *PsCPS* expression in the *lh-2* mutant stem tissue as measured by northern gel blot analysis may be due to background differences. Possible differences in background between *lh-2* and *lh-1* have already been suggested (Chapter 3). Similarly in *Arabidopsis*, no feedback inhibition of CPS (*GA1*) expression was observed in *Arabidopsis* seedlings grown on medium containing GA_3 (Silverstone et al., 1997).

PsKAO1 (*NA*), transcript levels as measured by northern blot analysis appeared to vary slightly in different wild types, L107 compared to WL1769 (Fig. 4.7). *PsKAO1* expression appeared a little elevated in the *lh-2* mutant and occasionally in *lh-1* mutant tissue compared to the corresponding wild-type (Figs. 4.7 and 4.8). This variation was not observed when the same blots were stripped and reprobed with *PsKO1* or *PsCPS* probes (Figs. 4.7 and 4.8). This may reflect possible differences in genetic background. None the less, there does not appear to be consistent evidence of feedback by GA activity because tissue from the *ls-1* mutant had similar expression levels to that of the corresponding wild-type (Fig. 4.7). In addition decreasing the GA content by paclobutrazol treatment did not alter the *PsKAO1* expression levels in the apical bud or stem (Fig. 4.15B). Likewise, the other *ent*-kaurenoic acid oxidase, *PsKAO2*, is not under feedback regulation as its expression levels, in seeds at contact point, are similar in GA deficient mutants and wild-type (Figs. 4.2 and 4.12).

Northern blot analyses of the expression levels of the genes early in the GA biosynthesis pathway, therefore, indicate that *PsCPS*, *PsKO1*, *PsKAO1* and *PsKAO2* are not under feed-back regulation by GA activity. The other early GA biosynthesis gene, *ent*-kaurene synthase (*KS*), is abundantly expressed in all the tissue types tested so far in *Arabidopsis* and pumpkin (Yamaguchi et al., 1996; 1998b). Although translation rates and the efficiency of enzyme activity is unknown *KS* is not

considered to be a likely candidate for regulation of the synthesis *ent*-kaurene (Sun and Kamiya, 1997; Olszewski et al., 2002). Therefore, there is no feedback regulation by GA activity on the genes encoding enzymes early in GA biosynthesis from GGDP to GA_{12} in pea. Feed-back (and feed-forward) homeostasis mechanisms appear to be confined to the dioxygenases. The GA biosynthesis dioxygenases GA 3-oxidases and to a lesser extent GA 20-oxidases are down regulated by GA activity (Martin et al., 1996; 1997; Ross et al., 1999). Conversely the GA deactivating dioxygenases GA 2-oxidases were up-regulated by increased GA activity in pea (Elliott et al., 2001) and *Arabidopsis* (Thomas et al., 1999).

In general, changes in activity of rate limiting enzymes have a relatively large effect on flux through biosynthetic pathways. Overproduction of CPS does not have any dramatic effect on the growth or development of transgenic *Arabidopsis* (Sun and Kamiya, 1994). However, the overexpression of GA 20-oxidase genes also using the CaMV-35s promoter in transgenic *Arabidopsis* increased the bioactive GA content and growth rate to produce the GA overdose phenotype (Huang et al., 1998; Coles et al., 1999). Conversely over-expression of a GA deactivation enzyme, bean GA 2-oxidase, produced extreme dwarfs in *Arabidopsis* and wheat transgenic plants (Hedden and Phillips, 2000). Due to their relatively large effect on flux, rate-limiting enzymes are strong candidates for points of regulation. GA_{20} accumulates in pea (Proebsting et al., 1992) suggesting that the GA 3-oxidation of GA_{20} to GA_1 is the rate limiting step in pea and this is also the major site of feedback control in this species (Martin et al., 1996; 1997; Ross et al., 1999). In addition, GA 20-oxidase, catalysing the previous steps in the GA biosynthetic pathway, is also down regulated in pea (Martin et al., 1996; Ross et al., 1999). In monocots the rate limiting step is usually GA 20-oxidation with the accumulation of GA_{19} and this is the major site subject to feedback regulation in barley and wheat (Croker et al., 1990; Appleford and Lenton, 1991).

In the species studied so far, the GA biosynthetic step most affected by auxin is the rate limiting step. In pea, *PsGA3ox1* is up-regulated by IAA (Ross et al., 2000; 2002). In tobacco, GA_{19} to GA_{20} is the rate limiting step (Nilsson et al., 1993) and IAA up-regulates GA 20-oxidase to markedly greater extent than GA 3-oxidase (Wolbang and Ross, 2001). In addition IAA down-regulates the deactivation enzyme, *PsGA2ox1* (Ross et al., 2000), thus further increasing the GA_1 level in response to auxin.

The rate limiting enzymes in the GA biosynthetic pathway are targets for environmental control by light. In the germinating seed of lettuce and *Arabidopsis* irradiation by red light increased the transcript levels of the GA 3-oxidases while imbibition alone was necessary to elevate GA 20-oxidases (Toyomasu et al., 1998; Yamaguchi et al., 1998a; Yamaguchi and Kamiya, 2002). In addition, one of the *Arabidopsis* GA 20-oxidases is modulated by photoperiod in the stem (Xu et al., 1995).

The homeostatic control of the GA production by feed-back and feed-forward appears to target the enzymes (GA 3-oxidase, GA 20-oxidase and GA 2-oxidase) which not only are rate limiting steps but are also close in the pathway to the bioactive product GA_1 or GA_4. Similarly control by environmental factors such as light (Toyomasu et al., 1998; Yamaguchi et al., 1998a; Yamaguchi and Kamiya, 2002) and activation by other hormones including auxin and brassinosteroids (Ross et al., 2000; Bouquin et al., 2001; Wolbang and Ross, 2001) target some or all these same enzymes. Targeting the enzymes close to the desired bioactive product may increase the effectiveness of the control because it avoids the side-steps in the pathway. There are many branches in the GA biosynthesis pathway. GGDP is a common precursor for other diterpenes and even *PsKAO1* and *PsKAO2* were found to produce side products including 7β-hydroxy-kaurenolide (Chapter 2). Most notable are the multiple dioxygenases, each catalysing several steps with broad substrate specificities to create a complex network of paths in many species (Martin

et al., 1996; Lange et al., 1997; Phillips, 1998). Therefore, the latter part of the GA biosynthesis pathway in *Arabidopsis* divides into up to three interconnected branches (Talon et al., 1990) and similarly 21 different GAs have been detected in pea (MacMillan, 2002). Therefore regulating enzymes close to the bioactive product may ensure precise control of the bioactive GA. This may also enhance the speed of response and elicit rapid control of bioactive GA levels as seen during de-etiolation (Reid et al., 2002).

PsKO1 (*LH*), *PsKAO1* (*NA*) and *PsCPS* (*LS*) are expressed in mature vegetative tissue

PsKO1 (LH) and *PsKAO1 (NA)* are expressed as strongly in mature fully expanded stem tissue as in still expanding stem tissue (Figs. 4.7 and 4.8). Even mature stem tissue 7 nodes below the apical bud expressed these genes (Fig. 4.8) and in fact, *PsKAO1 (NA)* expression was greater in mature stem than apical bud tissue (Figs. 4.7 and 4.8). In addition, mature leaf tissue, even 4 nodes below the apical bud, expressed *PsKO1 (LH)* and *PsKAO1 (NA)* as strongly as the leaf immediately below the apical bud and the apical bud itself (Fig. 4.7). Although the levels of total RNA decline in mature tissue, potentially over-estimating expression levels in mature tissue when measured by northern blot analysis based on equal total RNA loading on the gel, there are difficulties in measuring the expression levels relative to the weight of tissue because RNA yields are variable. Furthermore, expression levels do not necessarily directly represent the enzyme activity because of post mRNA influences including translational rates, enzyme efficiencies and compartmentalization, but often offer a good guide especially when comparing the same gene in similar tissue types in the same plant and are useful for looking at regulatory mechanisms directly affecting RNA. Mature cells have the same proportion of *PsKO1* or *PsKAO1* relative to the total RNA, therefore transcript

regulation of *PsKO1* and *PsKAO1* at the promoter level is unlikely as the cell matures. Also, the cells from the mature stem still express the genes *PsKO1* and *PsKAO1* so GAs still may be produced in mature tissue if the other genes of GA pathway are also still active, as suggested by (Ross et al., 2003).

The expression of *KO* and *KAO* in mature and developing vegetative tissue was not specifically investigated in other species. However, the expression of *AtKO1* in *Arabidopsis* seedlings decreased with age of the seedling when 5 day old seedlings were compared to 19 day old *Arabidopsis* seedlings (Helliwell et al., 1998). This may be the result of the mix of leaf and stem tissue in the different aged seedlings since *AtKO1* is expressed at a higher level in stem than leaf tissue (Helliwell et al., 1998).

The abundant *PsKO1* and *PsKAO1* mRNA in mature pea tissue was unexpected as mature tissue contains much less GA_1 and GA_{20} than young, expanding tissue (Proebsting et al., 1992; Smith et al., 1992; Ross, 1998; O'Neill et al., 2000). Generally it is now becoming widely held in reviews that GA biosynthesis in peas is confined to actively expanding tissue (Hedden and Kamiya, 1997; Davies, 2002). Perhaps *PsKO1* and *PsKAO1* genes are expressed and their enzymes are present in mature tissue without any substrate (*ent*-kaurene) and therefore no GA biosynthesis is achieved. In wheat and pea, *ent*-kaurene synthesis is associated with the stroma of proplastids (developing chloroplasts) or leucoplasts and not with mature chloroplasts (Aach et al., 1995; 1997). Of the two enzymes (*CPS* and *KS*) (Fig. 4.10) required to synthesize *ent*-kaurene, *CPS* is considered most likely to be regulated. *CPS* catalyses the first committed step of GA biosynthesis. Its substrate GGDP is a precursor for the biosynthesis of other diterpenes including carotenoids and the phytol chain of chlorophyll (Silverstone et al., 1997; Yamaguchi and Kamiya, 2000; Sponsel, 2002). *CPS* has an extremely low mRNA abundance in *Arabidopsis* (Sun et al., 1992; Silverstone et al., 1997) and has a specific tissue type and developmentally regulated expression pattern in *Arabidopsis* (Silverstone et al.,

1997) and pumpkin (Smith et al., 1998). *AtCPS* also has at least 4 regulatory elements in its promoter and first 3 introns and therefore appears highly regulated at the molecular level (Chang and Sun, 2002). Conversely, *KS* is expressed at a higher level in *Arabidopsis* and pumpkin, shows no tissue specificity and fluctuates less during development than *CPS* in these species (Yamaguchi et al., 1996; 1998b; Smith et al., 1998). The promoter of the *Arabidopsis AtCPS* gene directed the β-glucuronidase (GUS)-reporter gene fusion to rapidly growing *Arabidopsis* tissue including shoot apices, root tips, developing flowers and seeds (Silverstone et al., 1997). However, *AtCPS* promoter activity was also high in the vascular tissue of mature expanded leaves (Silverstone et al., 1997). Although in pea there is a low abundance of *PsCPS* (*LS*) mRNA in pea vegetative tissue (Fig. 4.1), *PsCPS* is consistently expressed in mature non-expanding stem tissue as strongly as in young expanding tissue (Figs. 4.7, 4.8 and 4.9). Mature stem tissue up to 7 nodes below the apical buds had similar *PsCPS* expression as the young stem tissue (Figs. 4.7 and 4.8). Northern blot analysis also demonstrated that there was more *PsCPS* mRNA in pea mature stem than the apical bud (Figs. 4.7, 4.8 and 4.9). Therefore, *ent*-kaurene is likely to be available for oxidation by *PsKO1* then *PsKAO1* in mature vegetative stem tissue.

Genes encoding enzymes necessary for the subsequent steps in the GA biosynthesis pathway, GA 20-oxidase and GA 3-oxidase (Fig. 4.10), are also expressed in mature tissue in pea (Garcia-Martinez et al., 1997; Ross et al., 2003). Furthermore, there are substantial amounts of some GAs (eg. GA_{19}) in mature tissue (Proebsting et al., 1992; Ross et al., 2003). Moreover, grafting studies in pea give strong evidence that mature tissue is capable of synthesising GAs (Reid et al., 1983; Proebsting et al., 1992). Reid et al. (1983) grafted wild type mature leaf and stem tissue as inter-stock in the severe dwarf *na-1* with a lesion in the *PsKAO1* gene (Chapter 2) and products from this mature tissue restored normal growth to the *na* scion. The GA_1 content of *na* scions were normalized when grafted on wild type (*NA*) rootstocks (Proebsting et al., 1992) although GA_1 does not appear to be

transported across the graft boundary (Reid et al., 1983; Proebsting et al., 1992). Instead it is suggested that GA_{20} is the major GA transported in pea (Proebsting et al., 1992). The grafting studies do not exclude the possibility that GAs may be initially synthesised in wild type immature tissue and then stored as that tissue matures. However, Ross et al. (2003) have demonstrated that mature tissue can maintain GA_{19} content without importing either GA_{19} or its precursors from other tissue and that [^{14}C]GA_{19} was rapidly converted to GA_{20} and bioactive GA_1 in mature tissue in pea. Never the less, there is less GA_{20} and GA_1 present in mature tissue than young expanding tissue (Proebsting et al., 1992; Smith et al., 1992; Ross, 1998; O'Neill et al., 2000). Ross et al. (2003) suggest that more rapid deactivation of GA_{20} and GA_1 in mature tissue removes these GAs from mature tissue. Mature tissue has relatively high *PsGA2ox1* expression (Ross et al., 2003). This GA 2-oxidase converts GA_{20} to GA_{29} to GA_{29}-catabolite and GA_1 to GA_8 (Lester et al., 1999b) and there may be other, as yet, uncharacterised GA 2-oxidases in pea (Elliott et al., 2001) that also may have increased expression levels in mature tissue. Mature tissue rapidly converted [^{14}C]GA_{19} to [^{14}C]-labeled GA_{29}, GA_8 and GA_{29}-catabolite (Ross et al., 2003). Mature tissue converted a higher proportion of added [^{3}H]GA_{20} to [^{3}H]-labeled GA_{29} (Ingram et al., 1985) and added [^{14}C]GA_{20} to [^{14}C]GA_{29} than did immature tissue (Ross et al., 2003).

Therefore, it appears that the early steps in the GA biosynthetic pathway are not the major sites of control of bioactive GA levels in pea vegetative tissue as it matures. Instead, GAs are produced in mature tissue and the levels of GA_{20} and GA_1 are then controlled by GA 2-oxidase deactivation. This directs the control of GA biosynthesis close to the bioactive product itself. As previously discussed, this appears to hold also for feed-back regulation of GA biosynthesis. Hormones, in general, do not need to be produced in large quantity, therefore production of GA precursors is probably not wasteful of raw materials or energy, and control near the bioactive product may allow for a more rapid and controlled response.

CONCLUSION

Unlike the dioxygenases, where there are families of genes at each step with potential differential regulation in different tissues, the early GA biosynthesis genes appear to have only 1 or 2 genes encoding enzymes at each step. The small (2 membered) *ent*-kaurenoic acid oxidase gene family in pea, with *PsKAO2* expression restricted to seeds, provides potential tissue differential control early in the biosynthesis of GAs. In contrast, the single copy *PsKO1* is uniformly expressed at high levels in all tissues and may maintain a steady flux of precursors in all tissues to be regulated by KAOs or later in the pathway.

There is evidence of marked developmental regulation during seed development by all the early GA biosynthesis genes tested, (*PsCPS*, *PsKO1*, *PsKAO1* and *PsKAO2*). Apart from during the development of the seed, regulation at the transcript level of GA biosynthesis by the genes early in the pathway appear to have little importance. There is no feed-back regulation by GA activity and transcript levels do not alter with shoot maturation. Feed-back regulation and control of the GA levels in non-expanding mature tissue is largely left to the dioxygenases later in the GA biosynthesis pathway.

MATERIALS AND METHODS

Plant materials and growing conditions

The tall Hobart line L107 (genotype *LH NA LE LS*) derived from cv.Torsdag was used as wild-type and is essentially isogenic to the *lh-1* mutant line K511 (genotype *lh-1 NA LE LS*) (Chapter 3), *lh-2* mutant line NGB5843 (genotype *lh-2 NA LE LS*) (Chapter 3) and *ls-1* mutant line Hobart line L181 (genotype *LH NA LE ls-1*)(Reid and Potts, 1986; Ait-Ali et al., 1997).

The tall *NA* Weibullsholm line WL1769 (genotype *LH NA LE LS*) was also used as wild-type and is isogenic to the *na-1* mutant WL1766 (genotype *LH na-1 LE LS*). The *na-1* and *NA* plants used were isogenic as a result of 8 generations of single plant selection after a cross between the closely related lines WL1766 (*na-1*) and WL1769 (*NA*) (Chapter 2).

Plants were grown 2 per pot in a heated glasshouse under an 18h photoperiod (Beveridge and Murfet, 1996)

Harvest

Pea seedlings were partitioned into the apical bud (all the material above the uppermost just fully expanded leaf), young still expanding stem tissue (internode immediately below the upper fully expanded leaf), mature non-expanding stem tissue (ranging from 2 to 7 internodes below the apical bud depending on the experiment), uppermost just fully expanded leaf, and mature leaf (3 or 4 nodes below the apical bud depending on the experiment) (Fig. 4.6). Samples at similar developmental stage were combined from between 5 to 9 similar plants.

Flowers were tagged and date recorded at time of anthesis. At various numbers of days after anthesis, 8 to 12 (usually 10) seeds from at least 3 different pods were combined and frozen in liquid nitrogen ready for RNA extraction. Contact point was determined by cutting a representative seed from each pod to see if the embryo completely filled the whole seed yet was still moist.

Paclobutrazol treatment

Seeds of wild-type (L107), *lh-1* (K511) and *lh-2* (NBG5843) were treated with the GA inhibitor, paclobutrazol, prior to planting. The seed testa was nicked and 5µl ethanol (control) or 5µl paclobutrazol PP333 (1µg PP333/µl ethanol) was applied and allowed to dry under the testa of each seed before planting. The plants were then either harvested at the 4th node or 9th node leaf expanded stage (Fig. 4.15). Note that a few samples with the 9th node leaf expanded stage only had samples from 3 or 4 plants combined because of the difficulty in obtaining material at uniform developmental stage. Samples from the 4th node leaf expanded stage always consisted of 5 or more plants combined.

Northern Blot Analysis

Total RNA was extracted using either the Phenol/SDS Method (Ausubel et al., 1994; Fig. 4.1), or RNeasy Plant Kit with RLT buffer (QIAGEN; Figs. 4.7, 4.8, 4.9, 4.11 and 4.15A), or RNeasy Plant Kit with RLC buffer (QIAGEN; Figs. 4.4 and 4.5A) or Tri Reagent™ RNA extraction (SIGMA®; Figs 4.2, 4.3 and 4.5B) consistent within the blot. The total RNA was quantified and purity assessed by UV spectroscopy. The RNA (5µg per lane) was fractionated in 1.5% agarose gel containing formaldehyde and transferred to Genescreen Plus hybridisation transfer membrane (Dupont/NEN) using 10xSSC. The membrane was hybridised at 42°C in

5xSSC, 5xDenhardts, 50% formamide, 1% SDS, and 200μg ml^{-1} salmon sperm with a cDNA ^{32}P probe (see below). The membrane was washed in 2xSSC, 0.1% SDS then 0.2xSSC, 0.1% SDS at 65°C and exposed to Kodak biomax X-ray film at -70°C. The ethidium bromide (EtBr)-stained total RNA is included as a loading control for each northern analysis. Blot membranes were stripped and reprobed with other gene probes in the series.

The *PsKO1*probe template was a PCR fragment amplified from the clone isolated by the library screening (Chapter 3) using vector primers and then nested with *PsKO1* specific primers to give a 750bp fragment covering the 5' region of the gene. The fragment, 5' of the *lh-2* genetic lesion was chosen as template to allow direct comparison of the *lh-2, lh-1* and wild type expression patterns. The *lh-2* mutant tissue had a larger *PsKO1* band (due to retention of an intron) and at higher expression levels a second band also associated with the *lh-2* lesion (Chapter 3) giving confidence in the accuracy of the *PsKO1* probe. The gel purified 1.1kb *PsKAO1* template was PCR amplified using nested *PsKAO1* specific primers on cDNA prepared from RNA extracted from WL1769 (*NA*) stem tissue (Chapter 2). The *PsKAO2* probe template was a PCR fragment amplified from the clone isolated by the library screening from a cv Torsdag (L107) seed cDNA library (Chapter 2) using vector primers and then nested with *PsKAO2* specific primers to give a 0.9kb cDNA fragment which was then gel purified. The 2 times gel purified cDNA 1.7kb *PsCPS* (LS) template was generated by two rounds of PCR with nested specific primers for the *LS* gene (Ait-Ali et al. 1997) using RNA from wild-type cv Torsdag (L107) stem tissue. The probes were labeled with ^{32}P using the Decalabel DNA labeling kit (MBI Fermentas).

Chapter 5

Concluding Discussion

With the cloning of *LH* and *NA* the identity of all the gibberellin (GA) biosynthesis mutants of pea have been determined. Both *LH* and *NA* encode enzymes that catalyse 3 steps each at the same carbon. *LH* encodes *ent*-kaurene oxidase (KO) which catalyses the oxidation of *ent*-kaurene to *ent*-kaurenoic acid by sequential oxidation at the C-19 methyl group (Chapter 3) similar to *GA3* in *Arabidopsis* (Helliwell et al., 1999). *NA* encodes *ent*-kaurenoic acid oxidase (KAO) which catalyses *ent*-kaurenoic acid to GA_{12} in 3 oxidation steps at the C-7 position of the GA skeleton (Davidson et al., 2003; Chapter 2) similar to the KAOs in *Arabidopsis* and barley (Helliwell et al., 2001a). Therefore there are fewer genes (with only 2 or 3 genes) involved in the second section of the GA biosynthesis pathway from *ent*-kaurene to GA_{12} or GA_{53} than at first expected from the number of intermediates involved.

With the determination of the genetic lesions of the *lh* and *na* mutants their previously perplexing phenotypes can be clearly explained. The two membered *ent*-kaureneoic acid oxidase (KAO) gene family in pea were differentially expressed with the *na* genetic lesion in the *PsKAO1* gene which was expressed throughout the plant including the shoot and the other gene family member, *PsKAO2*, expressed only in the seed. This provides an explanation for the severely reduced stature yet normal seed development of the *na* mutant (Chapter 2). The identity of the genetic lesions of the *lh* alleles can explain the differing responses to the inhibitor paclobutrazol. The *lh-1* and *lh-3* mutants have single base changes in *ent*-kaurene

oxidase (*PsKO1*) and therefore still contain the substrate binding and catalytic domains available to bind the competitive inhibitor and therefore have similar sensitivity to paclobutrazol as wild-type. In contrast, the most abundant transcript of the *lh-2* allele is predicted to produce a severely truncated protein. However, a small quantity is correctly spliced and may code for normal KO activity but the small number of substrate binding sites available would be severely inhibited by paclobutrazol, a competitive inhibitor of KO (Chapter 3).

With two major genes cloned in the second section of the GA biosynthesis pathway, some aspects of regulation and control of the GA biosynthesis pathway as a whole were investigated. Previously GA biosynthesis control had only been investigated in the first and third sections of the pathway. Although there appears to be developmental regulation at the transcript level of genes in the early sections of the pathway, feedback regulation by GA activity and regulation during maturation of the vegetative parts of the plant appear to be left mainly to the dioxygenases, catalysing steps close to the bioactive product, of the third section of the pathway.

Genes from every step, except two, of the GA biosynthesis pathway have now been cloned from pea. The two enzymes yet to be cloned are copalyl diphosphate (CDP) to *ent*-kaurene (catalysed by *ent*-kaurene synthase, *KS*, in other species (Yamaguchi et al., 1996; 1998b)) and the monooxygenase catalysing GA_{12} to GA_{53} which has not yet been cloned in any species. *KS* is considered an unlikely candidate for major regulation and control of *ent*-kaurene synthesis from GGDP based on work in *Arabidopsis* (Sun and Kamiya, 1997; Olszewski et al., 2002). The earlier step catalysed by copalyl diphosphate synthase (*CPS*) is considered the most likely site of control of the first section of the GA biosynthesis pathway based on work on *Arabidopsis* and pumpkin (Silverstone et al., 1997; Smith et al., 1998; Chang and Sun, 2002). The other, as yet uncloned, gene is associated with the step, GA_{12} to GA_{53}. The hydroxylation of GA_{12} at C-13 to form GA_{53} is thought to be catalysed by a cytochrome P450 monooxygenase (Kamiya and Graebe, 1983;

Groβelindemann et al., 1992). This gene is presumed present in many species including pea where the early 13-hydroxylation GA biosynthesis pathway predominates (Reid and Ross, 1993; Poole et al., 1995; MacMillan, 1997) but less important in species such as *Arabidopsis* where the non-early 13-hydroxylation pathway leading to the GA_4 predominate (Talon et al., 1990; Sponsel et al., 1997; Phillips, 1998). This demonstrates the importance of investigating GA biosynthesis in species other than *Arabidopsis*. Pea has proved a good model species with its caulescent growth habit making it very suitable for the study of internode lengths (Reid and Ross, 1993). Its large, easy to work with seeds and the mutant *lh-2* with a seed phenotype (Chapter 3) have proved useful when investigating the importance of GAs on seed development and survival (Swain et al., 1995). In addition, there are well defined GA biosynthesis and catabolism mutants with many members of the gene families cloned (Lester et al., 1996; 1997; 1999b; Martin et al., 1996; 1997; 1999; Garcia-Martinez et al., 1997). *Pisum sativum*, therefore may be a suitable species to isolate the gene from the remaining step in the GA biosynthesis pathway (GA_{12} to GA_{53}) and further investigate the regulation of GA biosynthesis by environmental factors (eg. light) and the interaction of other hormones, such as auxin and brassinosteroids, on GA biosynthesis.

Literature Cited

Aach H, Bode H, Robinson DG, Graebe JE (1997) *ent*-kaurene synthase is located in proplastids of meristematic shoot tissues. Planta, **202**: 211-219

Aach H, Bose G, Graebe JE (1995) *ent*-Kaurene Biosynthesis in a Cell-Free System from Wheat (*Triticum-Aestivum* L) Seedlings and the Localization of *Ent*-Kaurene Synthetase in Plastids of 3 Species. Planta, **197**: 333-342

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. Plant Journal, **11**: 443-454

Akashi T, Aoki T, Ayabe S (1998) Identification of a cytochrome P450 cDNA encoding (2S)-flavanone 2-hydroxylase of licorice (*Glycyrrhiza echinata* L.; Fabaceae) which represents licodione synthase and flavone synthase II. FEBS Lett., **431**: 287-290

Altschul SF, Madden TL, Schaffer AA, Zhang JH, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research, **25**: 3389-3402

Appleford NEJ, Lenton JR (1991) Gibberellins and leaf expansion in near-isogenic wheat lines containing *Rht1* and *Rht3* dwarfing alleles. Planta, **183**: 229-236

Atkins WM, Sligar SG (1988) The roles of active site hydrogen bonding in cytochrome P-450cam as revealed by site-directed mutagenesis. Journal of Biological Chemistry, **263**: 18842-18849

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JG, Struhl K (1994) Current Protocols in Molecular Biology **1**. Wiley Interscience, New York.

Bak S, Kahn RA, Nielsen HL, Moller BL, Halkier BA (1998) Cloning of three A-type cytochromes P450, CYP71E1, CYP98, and CYP99 from *Sorghum bicolor* (L.) Moench by a PCR approach and identification by expression in *Escherichia coli* of CYP71E1 as a multifunctional cytochrome P450 in the biosynthesis of the cyanogenic glucoside dhurrin. Plant Mol. Biol., **36**: 393-405

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

Beveridge CA, Murfet IC (1996) The *gigas* mutant in pea is deficient in the floral stimulus. Physiologia Plantarum, **96**: 637-645

Bishop GJ, Nomura T, Yokota T, Harrison K, Noguchi T, Fujioka S, Takatsuto S, Jones JDG, Kamiya Y (1999) The tomato DWARF enzyme catalyses C-6 oxidation in brassinosteroid biosynthesis. Proceedings of the National Academy of Sciences of the United States of America, **96**: 1761-1766

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

Bradley JM, Whitelam GC, Harberd NP (1995) Impaired Splicing of Phytochrome-B Pre-Messenger-RNA in a Novel Phyb Mutant of *Arabidopsis*. Plant Molecular Biology, **27**: 1133-1142

Brown JWS (1996) *Arabidopsis* intron mutations and pre-mRNA splicing. Plant Journal, **10**: 771-780

Brown JWS, Smith P, Simpson CG (1996) *Arabidopsis* consensus intron sequences. Plant Molecular Biology, **32**: 531-535

Brown DW, Yu JH, Kelkar HS, Fernandes M, Nesbitt TC, Keller NP, Adams TH, Leonard TJ (1996) Twenty-five coregulated transcripts define a sterigmatocystin gene cluster in *Aspergillus nidulans*. Proceedings of the National Academy of Sciences of the United States of America, **93**: 1418-1422

Chang CW, Sun TP (2002) characterization of *cis*-regulatory regions responsible for developmental regulation of the gibberellin biosynthetic gene *GA1* in *Arabidopsis thaliana*. Plant Molecular Biology, **49**: 579-589

Chen CD, Kemper B (1996) Different structural requirements at specific proline residue positions in the conserved proline-rich region of cytochrome P450 2C2. Journal of Biological Chemistry, **271**: 28607-28611

Chiang HH, Hwang I, Goodman HM (1995) Isolation of the *Arabidopsis Ga4* Locus. Plant Cell, **7**: 195-201

Choe SW, Dilkes BP, Fujioka S, Takatsuto S, Sakurai A, Feldmann KA (1998) The *DWF4* gene of *Arabidopsis* encodes a cytochrome P450 that mediates multiple 22 alpha-hydroxylation steps in brassinosteroid biosynthesis. Plant Cell, **10**: 231-243

Cline K, Henry R (1996) Import and routing of nucleus-encoded chloroplast proteins. Annual Review of Cell and Developmental Biology, **12**: 1-26

Coles JP, Phillips AL, Croker SJ, Garcia-Lepe R, Lewis MJ, Hedden P (1999) Modification of gibberellin production and plant development in *Arabidopsis* by sense and antisense expression of gibberellin 20-oxidase genes. Plant Journal, **17**: 547-556

Collu G, Unver N, Peltenburg-Looman AM, van der Heijden R, Verpoorte R, Memelink J (2001) Geraniol 10-hydroxylase, a cytochrome P450 enzyme involved interpenoid indole alkaloid biosynthesis. FEBS Lett., **508**: 215-220

Coolbaugh RC, Hirano SS, West CA (1978) Studies on the Specificity and site of action of α-cyclopropyl-α-[*p*-methoxyphenyl]-5-pyrimidine methyl alcohol (Ancymidol), a plant growth regulator. Plant Physiology, **62**: 571-576

Cowling RJ, Kamiya Y, Seto H, Harberd NP (1998) Gibberellin dose-response regulation of *GA4* gene transcript levels in *Arabidopsis*. Plant Physiology, **117**: 1195-1203

Croker SJ, Hedden P, Lenton JR, Stoddart JL (1990) Comparison of Gibberellins in Normal and Slender Barley Seedlings. Plant Physiology, **94**: 194-200

Cullin C, Pompon D (1988) Synthesis of functional mouse cytochromes P-450 P1 and chimeric P-450 P3-1 in the yeast *Saccharomyces cerevisiae*. Gene., **65**: 203-217

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

Davies PJ (2002) Gibberellins: regulators of plant height. In L Taiz, E zeiger, eds, Plant Physiology. Sinauer Associates, Sunderland, Massachusetts, pp 461-492

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA miniprepreparation: version 2. Plant Mol. Biol. Rep, **1**: 19-22

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

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

Ellis THN (1994) Approaches to the genetic mapping of pea. In HF Linskens, JF Jackson, eds, Modern methods of plant analysis, Vol 16 Vegetables and vegetable products. Springer Verlag, Berlin, pp 117-160

Emanuelsson O, Nielsen H, von Heijne G (1999) ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. Protein Science, **8**: 978-984

Fahrendorf T, Dixon RA (1993) Stress responses in alfalfa (*Medicago sativa* L.). XVIII: Molecular cloning and expression of the elicitor-inducible cinnamic acid 4-hydroxylase cytochrome P450. Arch. Biochem. Biophys., **305**: 509-515

Fridborg I, Kuusk S, Moritz T, Sundberg E (1999) The *Arabidopsis* dwarf mutant shi exhibits reduced gibberellin responses conferred by overexpression of a new putative zinc finger protein. Plant Cell, **11**: 1019-1031

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 of the United States of America, **85**: 8998-9002

Frydman VM, Gaskin P, MacMillan J (1974) Qualitative and quantitative analyses of gibberellins throughout seed maturation in *Pisum sativum* cv. Progress no.9. Planta, **118**: 123-132

Fujioka S, Yamane H, Spray CR, Gaskin P, MacMillan J, Phinney BO, Takahashi H (1988a) Qualitative and quantitative analyses of gibberellins in vegetative shoots of normal, *dwarf-1*, *dwarf-2, dwarf-3,* and *dwarf-5* seedlings of *Zea mays* L. Plant Physiology, **88**: 1367-1372

Fujioka S, Yamane H, Spray CR, Katsumi M, Phinney BO, Gaskin P, MacMillan J, Takahashi N (1988b) The dominant non-gibberellin-responding dwarf mutant (*d8*) of maize accumulates native gibberellins. Proc. Natl. Acad. Sci. U.S.A., **85**: 9031-9035

Fukazawa J, Sakai F, Ishida S, Yamaguchi I, Kamiya Y, Takahashi Y (2000) Repression of shoot growth, a bZIP transcriptional activator, regulates cell elongation by controlling the level of gibberellins. The Plant Cell, **12**: 901-915

Garcia-Martinez JL, Gil J (2002) Light regulation of gibberellin biosynthesis and mode of action. Journal of Plant Growth Regulation, **20**: 354-368

Garcia-Martinez JL, Lopezdiaz I, Sanchezbeltran MJ, Phillips AL, Ward DA, Gaskin P, Hedden P (1997) Isolation and transcript analysis of gibberellin 20-oxidase genes in pea and bean in relation to fruit development. Plant Molecular Biology, **33**: 1073-1084

Garcia-Martinez JL, Santes C, Croker SJ, Hedden P (1991) Identification, quantification and distribution of gibberellins in fruits of *Pisum sativum* L. cv. Alaska during pod development. Planta, **184**: 53-60

Gaskin P, MacMillan J (1991) GC-MS of the gibberellins and related compounds: methodology and a library of spectra. University of Bristol, Cantock's Enterprises, Bristol.

Gil J, Garcia-Martinez JL (2000) Light regulation of gibberellin A_1 content and expression of genes coding for GA 20-oxidase and GA 3 beta-hydroxylase in etiolated pea seedlings. Physiologia Plantarum, **108**: 223-229

Gilmour SJ, Zeevaart JA, Schwenen L, Graebe JE (1986) Gibberellin metabolism in cell-free extracts from spinach leaves in relation to photoperiod. Plant Physiology, **82**: 190-195

Groβelindemann E, Lewis MJ, Hedden P, Graebe JE (1992) Gibberellin Biosynthesis from Gibberellin A_{12}-Aldehyde in a Cell-Free System from Germinating Barley (*Hordeum-vulgare* L, Cv Himalaya) Embryos. Planta, **188**: 252-257

Gutierrez RA, MacIntosh GC, Green PJ (1999) Current perspectives on mRNA stability in plants: multiple levels and mechanisms of control. Trends in Plant Science, **4**: 429-438

Harrak H, Lagrange T, Bisanz-Seyer C, Lerbs-Mache S, Mache R (1995) The Expression of Nuclear Genes Encoding Plastid Ribosomal Proteins Precedes the Expression of Chloroplast Genes during Early Phases of Chloroplast Development. Plant Physiol., **108**: 685-692

Hasemann CA, Kurumbail RG, Boddupalli SS, Peterson JA, Deisenhofer J (1995) Structure and Function of Cytochromes-P450 - a Comparative- Analysis of 3 Crystal-Structures. Structure, **3**: 41-62

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

Hazebroek JP, Metzger JD, Mansager ER (1993) Thermoinductive Regulation of Gibberellin Metabolism in *Thlaspi-Arvense* L .2. Cold Induction of Enzymes in Gibberellin Biosynthesis. Plant Physiology, **102**: 547-552

Hedden P (1997) The oxidases of gibberellin biosynthesis: Their function and mechanism. Physiologia Plantarum, **101**: 709-719

Hedden P (1999) Recent advances in gibberellin biosynthesis. Journal of Experimental Botany, **50**: 553-563

Hedden P, Graebe JE (1985) Inhibition of gibberellin biosynthesis by paclobutrazol in cell-free homogenates of *Cucurbita maxima* endosperm and *Malus pumila* embryos. Plant Growth Regulation, **4**: 111-122

Hedden P, Graebe JE, Beale MH, Gaskin P, MacMillan J (1984) The biosynthesis of 12α–hydroxylated gibberellins in a cell-free system from *Cucurbita maxima* endosperm. Phytochemistry, **23**: 569-574

Hedden P, Kamiya Y (1997) Gibberellin Biosynthesis: Enzymes, Genes and Their Regulation. Annual Review of Plant Physiology and Plant Molecular Biology, **48**: 431-460

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

Hedden P, Phillips AL, Rojas MC, Carrera E, Tudzynski B (2002) Gibberellin biosynthesis in plants and fungi: A case of convergent evolution? Journal of Plant Growth Regulation, **20**: 319-331

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

Helliwell CA, Chandler PM, Poole A, Dennis ES, Peacock WJ (2001a) The CYP88A cytochrome P450, *ent*-kaurenoic acid oxidase, catalyzes three steps of the gibberellin biosynthesis pathway. Proceedings of the National Academy of Sciences of the United States of America, **98**: 2065-2070

Helliwell CA, Olive MR, Gebbie L, Forster R, Peacock WJ, Dennis ES (2000) Isolation of an *ent*-kaurene oxidase cDNA from *Cucurbita maxima*. Australian Journal of Plant Physiology, **27**: 1141-1149

Helliwell CA, Poole A, Peacock WJ, Dennis ES (1999) *Arabidopsis ent*-kaurene oxidase catalyzes three steps of gibberellin biosynthesis. Plant Physiology, **119**: 507-510

Helliwell CA, Sheldon CC, Olive MR, Walker AR, Zeevaart JAD, Peacock WJ, Dennis ES (1998) Cloning of the *Arabidopsis ent*-kaurene oxidase gene *GA3*. Proceedings of the National Academy of Sciences of the United States of America, **95**: 9019-9024

Helliwell CA, Sullivan JA, Mould RM, Gray JC, Peacock WJ, Dennis ES (2001b) A plastid envelope location of *Arabidopsis ent*-kaurene oxidase links the plastid and endoplasmic reticulum steps of the gibberellin biosynthesis pathway. Plant Journal, **28**: 201-208

Hofmann K, Stoffel W (1993) TMbase - A database of membrane spanning proteins segments. Biol. Chem. Hoppe-Seyler, **374**: 166

Hooley R (1994) Gibberellins - perception, transduction and responses. Plant Molecular Biology, **26**: 1529-1555

Hotze M, Schroder G, Schroder J (1995) Cinnamate 4-hydroxylase from *Catharanthus roseus*, and a strategy for the functional expression of plant cytochrome P450 proteins as translational fusions with P450 reductase in *Escherichia coli.* FEBS Lett., **374**: 345-350

Huang S, Raman AS, Ream JE, Fujiwara H, Cerny RE, Brown SM (1998) Overexpression of 20-Oxidase Confers a Gibberellin-Overproduction Phenotype in *Arabidopsis*. Plant Physiology, **118**: 773-781

Igarashi D, Ishida S, Fukazawa J, Takahashi Y (2001) 14-3-3 proteins regulate intracellular localization of the bZIP transcriptional activator RSG. Plant Cell, **13**: 2483-2497

Imai M, Shimada H, Watanabe Y, Matsushima-Hibiya Y, Makino R, Koga H, Horiuchi T, Ishimura Y (1989) Uncoupling of the cytochrome P-450cam monooxygenase reaction by a single mutation, threonine-252 to alanine or valine: possible role of the hydroxy amino acid in oxygen activation. Proceedings of the National Academy of Sciences of the United States of America, **86**: 7823-7827

Ingram TJ, Reid JB (1987) Internode length in *Pisum* 1. Gene *na* may block gibberellin synthesis between *ent-7-alpha*-hydroxykaurenoic acid and gibberellin A_{12}-aldehyde. Plant Physiology, **83**: 1048-1053

Ingram TJ, Reid JB, MacMillan J (1985) Internode length in *Pisum sativum* L. The kinetics of growth and [^{3}H]gibberellin A_{20} metabolism in genotype *naLE*. Planta, **164**: 429-438

Ingram TJ, Reid JB, MacMillian J (1986) The quantitative relationship between gibberellin A_1 and internode growth in *Pisum sativum* L. Planta, **168**: 414-420

Ingram TJ, Reid JB, Murfet IC, Gaskin P, Willis CL, MacMillian J (1984) Internode Length in *Pisum*. Planta, **160**: 455-463

Kalb VF, Loper JC (1988) Proteins from eight eukaryotic cytochrome P-450 families share a segmented region of sequence similarity. Proceedings of the National Academy of Sciences of the United States of America, **85**: 7221-7225

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

Kawai S, Mori A, Shiokawa T, Kajita S, Katayama Y, Morohoshi N (1996) Isolation and analysis of cinnamic acid 4-hydroxylase homologous genes from a hybrid aspen, *Populus kitakamiensis*. Biosci. Biotechnol. Biochem., **60**: 1586-1597

Keegstra K, Cline K (1999) Protein import and routing systems of chloroplasts. Plant Cell, **11**: 557-570

Koornneef M, van der Veen JH (1980) Induction and analysis of gibberellin sensitive mutants in *Arabidopsis thaliana* (L.) Heynh. Theoretical and Applied Genetics, **58**: 257-263

Kusaba S, Fukumoto M, Honda C, Yamaguchi I, Sakamoto T, Kano-Murakami Y (1998) Decreased GA(1) content caused by the overexpression of OSH1 is accompanied by suppression of GA 20-oxidase gene expression. Plant Physiology, **117**: 1179-1184

Kyte J, Doolittle RF (1982) A Simple Method for Displaying the Hydropathic Character of a Protein. Journal of Molecular Biology, **157**: 105-142

Lal SL, Choi JH, Shaw JR, Hannah LC (1999) A splice site mutant of maize activates cryptic splice sites, elicits intron inclusion and exon exclusion, and permits branch point elucidation. Plant Physiology, **121**: 411-418

Lange T (1997) Cloning gibberellin dioxygenase genes from pumpkin endosperm by heterologous expression off enzyme activities in *Escherichia coli*. Proceedings of the National Academy of Sciences of the United States of America, **94**: 6553-6558

Lange T, Robatzek S, Frisse A (1997) Cloning and expression of a gibberellin 2 beta, 3 beta-hydroxylase cDNA from pumpkin endosperm. Plant Cell, **9**: 1459-1467

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, Ait-Ali T, Martin DN, Reid JB (1996) A gibberellin 20-oxidase cDNA (accession no. U58830) from pea (*Pisum sativum* L.) seed (PGR96-050). Plant Physiology, **111**: 1353

Lester DR, Ross JJ, Davies PJ, Reid JB (1997) Mendels stem length gene (*Le*) encodes a gibberellin 3-beta-hydroxylase. 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*. Plant Journal, **19**: 65-73

Li HM, Chen LJ (1996) Protein targeting and integration signal for the chloroplastic outer envelope membrane. Plant Cell, **8**: 2117-2126

Liu HX, Filipowicz W (1996) Mapping of branchpoint nucleotides in mutant pre-mRNAs expressed in plant cells. Plant Journal, **9**: 381-389

MacKenzie-Hose AK, Ross JJ, Davies NW, Swain SM (1998) Expression of Gibberellin Mutations in Fruits of *Pisum sativum* L. Planta, **204**: 397-403

MacMillan J (1997) Biosynthesis of the gibberellin plant hormones. Natural Product Reports, **14**: 221-243

MacMillan J (2002) Occurrence of gibberellins in vascular plants, fungi, and bacteria. Journal of Plant Growth Regulation, **20**: 387-442

Martin DN, Proebsting WM, Hedden P (1997) Mendels dwarfing gene: cDNAs from the *Le* alleles and function of the expressed proteins. Proceedings of the National Academy of Sciences of the United States of America, **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

Metzger JD (1985) Role of gibberellins in the enviromental control of stem growth in *Thlaspi arvense*. Plant Physiology, **78**: 8-13

Mizutani M, Ward E, Ohta D (1998) Cytochrome p450 superfamily in *Arabidopsis thaliana*: isolation of cDNAs, differential expression, and RFLP mapping of multiple cytochromes p450. Plant Molecular Biology, **37**: 39-52

Murphy PJ, West CA (1969) The role of mixed function oxidases in kaurene metabolism in *Echinocystis macrocarpa* Greene endosperm. Archives of Biochemistry and Biophysics, **133**: 395-407

Nebert DW, Gonzalez FJ (1987) P450 genes: structure, evolution, and regulation. Annual Review of Biochemistry, **56**: 945-993

Nelson DR, Kamataki T, Waxman DJ, Guengerich FP, Estabrook RW, Feyereisen R, Gonzalez FJ, Coon MJ, Gunsalus IC, Gotoh O, Okuda K, Nebert DW (1993) The P450 Superfamily - Update on New Sequences, Gene-Mapping, Accession Numbers, Early Trivial Names of Enzymes, and Nomenclature. DNA and Cell Biology, **12**: 1-51

Nicholas KB, Jr. NHB, Deerfield DWI (1997) GeneDoc: Analysis and Visualization of Genetic Variation. EMBNEW.NEWS, **4**: 14

Nilsson O, Moritz T, Imbault N, Sandberg G, Olsson O (1993) Hormonal characterization of transgenic tobacco plants expressing the *rolC* gene of *Agrobacterium rhizogenes* TL-DNA1. Plant Physiology, **102**: 363-371

O'Neill DP, Ross JJ, Reid JB (2000) Changes in gibberellin A(1) levels and response during de-etiolation of pea seedlings. Plant Physiology, **124**: 805-812

Ogawa S, Toyomasu T, Yamane H, Murofushi N, Ikeda R, Morimoto Y, Nishimura Y, Omori T (1996) A step in the biosynthesis of gibberellins that is controlled by the mutation in the semi-dwarf rice cultivar Tan-ginbozu. Plant and Cell Physiology, **37**: 363-368

Olszewski N, Sun TP, Gubler F (2002) Gibberellin signaling: Biosynthesis, catabolism, and response pathways. Plant Cell, **14**: S61-S80

Orozco BM, McClung CR, Werneke JM, Ogren WL (1993) Molecular-Basis of the Ribulose-1,5-Bisphosphate Carboxylase Oxygenase Activase Mutation in *Arabidopsis-Thaliana* Is a Guanine-to-Adenine Transition at the 5'-Splice Junction of Intron 3. Plant Physiology, **102**: 227-232

Overkamp S, Hein F, Barz W (2000) Cloning and characterization of eight cytochrome P450 cDNAs from chickpea (*Cicer arietinum* L.) cell suspension cultures. Plant Sci., **155**: 101-108

Ozga JA, Brenner ML, Reinecke DM (1992) Seed Effects on Gibberellin Metabolism in Pea Pericarp. Plant Physiology, **100**: 88-94

Paquette SM, Bak S, Feyereisen R (2000) Intron-exon organization and phylogeny in a large superfamily, the paralogous cytochrome P450 genes of *Arabidopsis thaliana*. DNA and Cell Biology, **19**: 307-317

Peng JR, Richards DE, Moritz T, Cano-Delgado A, Harberd NP (1999) Extragenic suppressors of the *Arabidopsis gai* mutation alter the dose-response relationship of diverse gibberellin responses. Plant Physiology, **119**: 1199-1207

Phillips AL (1998) Gibberellins in *Arabidopsis*. Plant Physiology and Biochemistry, **36**: 115-124

Phillips AL, Ward DA, Uknes S, Appleford NEJ, Lange T, Huttly AK, Gaskin P, Graebe JE, Hedden P (1995) Isolation and Expression of 3 Gibberellin 20-Oxidase cDNA Clones from *Arabidopsis*. Plant Physiology, **108**: 1049-1057

Phinney BO, Spray C (1982) Chemical genetics and the gibberellin pathway in *Zea mays* L. In PF Wareing, eds, Plant Growth Substances. Academic press, New York, pp 101-110

Pompon D, Louerat B, Bronine A, Urban P (1996) Yeast expression of animal and plant P450s in optimized redox environments. Methods in Enzymology, **272**: 51-64

Poole AT, Ross JJ, Lawrence NL, Reid JB (1995) Identification of gibberellin A_4 in *Pisum sativum* L. and the effects of applied gibberellins A_9, A_4, A_5 and A_3 on the *le* mutant. Plant Growth Regulation, **16**: 257-262

Porter TD, Coon MJ (1991) Cytochrome-P-450 - Multiplicity of Isoforms, Substrates, and Catalytic and Regulatory Mechanisms. Journal of Biological Chemistry, **266**: 13469-13472

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

Potts WC (1986) Gibberellins in light-grown shoots of *Pisum sativum* L. and the influence of reproductive development. Plant Cell Physiology, **27**: 997-1003

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. Physiologia Plantarum, **57**: 448-454

Poulos TL, Finzel BC, Gunsalus IC, Wagner GC, Kraut J (1985) The 2.6-A crystal structure of *Pseudomonas putida* cytochrome P-450. Journal of Biological Chemistry, **260**: 16122-16130

Poulos TL, Finzel BC, Howard AJ (1986) Crystal structure of substrate-free *Pseudomonas putida* cytochrome P-450. Biochemistry, **25**: 5314-5322

Proebsting WM, Hedden P, Lewis MJ, Croker SJ, Proebsting LN (1992) Gibberellin Concentration and Transport in Genetic Lines of Pea - Effects of Grafting. Plant Physiology, **100**: 1354-1360

Ralston L, Kwon ST, Schoenbeck M, Ralston J, Schenk DJ, Coates RM, Chappell J (2001) Cloning, heterologous expression, and functional characterizationof 5-epi-aristolochene-1,3-dihydroxylase from tobacco (*Nicotiana tabacum*). Arch. Biochem. Biophys., **393**: 222-235

Rebers M, Vermeer E, Knegt E, Shelton CJ, Vanderplas LHW (1995) Gibberellin Levels and Cold-Induced Floral Stalk Elongation in Tulip. Physiologia Plantarum, **94**: 687-691

Reid JB (1986a) Gibberellin Mutants. In PJ King, AD Blonstein, eds, Plant Gene Research: A Genetic Approach to Plant Biochemistry, Vol 3. Springer-Verlag, New York, pp 1-34

Reid JB (1986b) 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, Murfet IC, Singer SR, Weller JL (1996) Physiological-genetics of flowering in *Pisum*. Seminars in Cell & Developmental Biology, **7**: 455-463

Reid JB, Potts BM (1986) Internode length in *Pisum*. Two further mutants, *lh* and *ls*, with reduced gibberellin synthesis, and a gibberellin insensitive mutant, *lk*. Plant Physiology, **66**: 417-426

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

Reid JB, Ross JJ, Swain SM (1992) Internode length in *Pisum*. A new, slender mutant with elevated levels of C_{19} gibberellins. Planta, **188**: 462-467

Rojas MC, Hedden P, Gaskin P, Tudzynki B (2001) The *P450-1* gene of *Gibberella fujikuroi* encodes a multifunctional enzyme in gibberellin biosynthesis. Proceedings of the National Academy of Sciences of the United States of America, **98**: 5838-5843

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

Ross JJ, Davidson SE, Wolbang CM, Bayly-Stark E, Smith JJ, Reid JB (2003) Developmental regulation of the gibberellin pathway in pea shoots. Functional Plant Biology, **30**: 83-89

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, Smith JJ, Kerckhoffs LHJ, Elliott RC (2000) Evidence that auxin promotes gibberellin A(1) biosynthesis in pea. Plant Journal, **21**: 547-552

Ross JJ, O'Neill DP, Wolbang CM, Symons GM, Reid JB (2002) Auxin-gibberellin interactions and their role in plant growth. Journal of Plant Growth Regulation, **20**: 346-353

Ross JJ, Reid JB, Gaskin P, MacMillian J (1989) Internode length in *Pisum*. Estimation of GA_1 levels in genotypes *Le, le* and le^d. Physiologia Plantarum, **76**: 173-176

Ross JJ, Reid JB, Swain SM (1993) Control of stem elongation by gibberellin A1 - 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*. Plant Journal, **7**: 513-523

Schopfer CR, Ebel J (1998) Identification of elicitor-induced cytochrome P450s of soybean (*Glycine max* L.) using differential display of mRNA. Mol. Gen. Genet., **258**: 315-322

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

Sekimoto H, Seo M, Dohmae N, Takio K, Kamiya Y, Koshiba T (1997) Cloning and molecular characterization of plant aldehyde oxidase. Journal of Biological Chemistry, **272**: 15280-15285

Sekimoto H, Seo M, Kawakami N, Komano T, Desloire S, Liotenberg S, Marion-Poll A, Caboche M, Kamiya Y, Koshiba T (1998) Molecular cloning and characterization of aldehyde oxidases in *Arabidopsis thaliana*. Plant and Cell Physiology, **39**: 433-442

Seo M, Akaba S, Oritani T, Delarue M, Bellini C, Caboche M, Koshiba T (1998) Higher activity of an aldehyde oxidase in the auxin- overproducing superroot1 mutant of *Arabidopsis thaliana*. Plant Physiology, **116**: 687-693

Silverstone AL, Chang CW, Krol E, Sun TP (1997) Developmental regulation of the gibberellin biosynthetic gene *ga1* in *Arabidopsis thaliana*. Plant Journal, **12**: 9-19

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

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

Singh DP, Jermakow AM, Swain SM (2002) Gibberellins are required for seed development and pollen tube growth in *Arabidopsis*. Plant Cell, **14**: 3133-3147

Smith MW, Yamaguchi S, Ait-Ali T, Kamiya Y (1998) The first step of gibberellin biosynthesis in pumpkin is catalyzed by at least two copalyl diphosphate synthases encoded by differentially regulated genes. Plant Physiology, **118**: 1411-1419

Smith VA, Knatt CJ, Gaskin P, Reid JB (1992) The distribution of gibberellins in vegetative tissue of *Pisum sativum* L. Plant Physiology, **99**: 368-371

Sponsel VM (2002) The deoxyxylulose phosphate pathway for the biosynthesis of plastidic isoprenoids: Early days in our understanding of the early stages of gibberellin biosynthesis. Journal of Plant Growth Regulation, **20**: 332-345

Sponsel VM, Schmidt FW, Porter SG, Nakayama M, Kohlstruk S, Estelle M (1997) Characterization of new gibberellin-responsive semidwarf mutants of *Arabidopsis*. Plant Physiology, **115**: 1009-1020

Sugavanam B (1984) Diastereoisomers and enantiomers of paclobutrazol: their preparation and biological activity. Pestic. Sci., **15**: 296-302

Sun TP, Goodman HM, Ausubel FM (1992) Cloning the *Arabidopsis GA1* locus by genomic subtraction. Plant Cell, **4**: 119-128

Sun TP, Kamiya Y (1994) The *Arabidopsis ga1* locus encodes the cyclase *ent*-kaurene synthetase A of gibberellin biosynthesis. Plant Cell, **6**: 1509-1518

Sun TP, Kamiya Y (1997) Regulation and cellular localization of *ent*-kaurene synthesis. Physiologia Plantarum, **101**: 701-708

Swain SM (1994) Gibberellins and seed development in *Pisum*. PhD, University of Tasmania.

Swain SM, Reid JB (1992) Internode length in *Pisum*. A new allele at the *Lh* locus. Physiologia Plantarum, **86**: 124-130

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

Swain SM, Reid JB, Ross JJ (1993) Seed development in *Pisum* - the lh^i allele reduces gibberellin levels in developing seeds, and increases seed abortion. Planta, **191**: 482-488

Swain SM, Ross JJ, Reid JB, Kamiya Y (1995) Gibberellins and pea seed development - expression of the lh^i, *ls* and le^{5839} mutations. Planta, **195**: 426-433

Swofford DL (1999) PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Szczyglowski K, Hamburger D, Kapranov P, de Bruijn FJ (1997) Construction of a *Lotus japonicus* late nodulin expressed sequence tag library and identification of novel nodule-specific genes. Plant Physiol., **114**: 1335-1346

Szekeres M, Nemeth K, KonczKalman Z, Mathur J, Kauschmann A, Altmann T, Redei GP, Nagy F, Schell J, Koncz C (1996) Brassinosteroids rescue the deficiency of CYP90, a cytochrome P450, controlling cell elongation and de-etiolation in *Arabidopsis*. Cell, **85**: 171-182

Tadeo FR, Talon M, germain E, Dosba F (1994) Embryo sac development and endogenous gibberellins in pollinated and unpollinated ovaries of walnut (*Juglans regia*). Physiologia Plantarum, **91**: 37-44

Talon M, Koornneef M, Zeevaart JAD (1990) Endogenous gibberellins in *Arabidopsis thaliana* and possible steps blocked in the biosynthetic pathways of the semidwarf *ga4* and *ga5* mutants. Proceedings of the National Academy of Sciences of the United States of America, **87**: 7983-7987

Tanaka-Ueguchi M, Itoh H, Oyama N, Koshioka M, Matsuoka M (1998) Over-expression of a tobacco homeobox gene, *NTH15*, decreases the expression of a gibberellin biosynthetic gene encoding GA 20-oxidase. Plant Journal, **15**: 391-400

Teutsch HG, Hasenfratz MP, Lesot A, Stoltz C, Garnier JM, Jeltsch JM, Durst F, Werck-Reichhart D (1993) Isolation and sequence of a cDNA encoding the Jerusalem artichoke cinnamate 4-hydroxylase, a major plant cytochrome P450 involved inthe general phenylpropanoid pathway. Proc. Natl. Acad. Sci. U.S.A., **90**: 4102-4106

Thomas SG, Phillips AL, Hedden P (1999) Molecular cloning and functional expression of gibberellin 2- oxidases, multifunctional enzymes involved in gibberellin deactivation. Proceedings of the National Academy of Sciences of the United States of America, **96**: 4698-4703

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Research, **22**: 4673-4680

Tijet N, Helvig C, Pinot F, Le Bouquin R, Lesot A, Durst F, Salaun JP, Benveniste I (1998) Functional expression in yeast and characterization of a clofibrate-inducible plant cytochrome P-450 (CYP94A1) involved in cutin monomers synthesis. Biochem. J., **332**: 583-589

Toyomasu T, Kawaide H, Mitsuhashi W, Inoue Y, Kamiya Y (1998) Phytochrome regulates gibberellin biosynthesis during germination of photoblastic lettuce seedlings. Plant Physiology, **118**: 1517-1523

Toyomasu T, Kawaide H, Sekimoto H, vonNumers C, Phillips AL, Hedden P, Kamiya Y (1997) Cloning and characterization of a cDNA encoding gibberellin 20-oxidase from rice (*Oryza sativa*) seedlings. Physiologia Plantarum, **99**: 111-118

Tudzynski B, Hedden P, Carrera E, Gaskin P (2001) The *P450-4* gene of *Gibberella fujikuroi* encodes *ent*-kaurene oxidase in the gibberellin biosynthesis pathway. Applied and Environmental Microbiology, **67**: 3514-3522

Tudzynski B, Holter K (1998) Gibberellin biosynthetic pathway in *Gibberella fujikuroi*: Evidence for a gene cluster. Fungal Genetics and Biology, **25**: 157-170

Ueguchi-Tanaka M, Fujisawa Y, Kobayashi M, Ashikari M, Iwasaki Y, Kitano H, Matsuoka M (2000) Rice dwarf mutant *d1*, which is defective in the alpha subunit of the heterotrimeric G protein, affects gibberellin signal transduction. Proceedings of the National Academy of Sciences of the United States of America, **97**: 11638-11643

Umemoto N, Kobayashi O, Ishizaki-Nishizawa O, Toguri T (1993) cDNAs sequences encoding cytochrome P450 (CYP71 family) from eggplant seedlings. FEBS Lett., **330**: 169-173

Urban P, Mignotte C, Kazmaier M, Delorme F, Pompon D (1997) Cloning, yeast expression, and characterization of the coupling of two distantly related *Arabidopsis thaliana* NADPH-Cytochrome P450 reductases with P450 CYP73A5. Journal of Biological Chemistry, **272**: 19176-19186

van Gorcom RF, Boschloo JG, Kuijvenhoven A, Lange J, van Vark AJ, Bos CJ, van Balken JA, Pouwels PH, van den Hondel CA (1990) Isolation and molecular characterisation of the benzoate-para-hydroxylase gene (bphA) of *Aspergillus niger*: a member of a new gene family of the cytochrome P450 superfamily. Molecular & General Genetics, **223**: 192-197

Weller JL, Murfet IC, Reid JB (1997) Pea mutants with reduced sensitivity to far-red light define an important role for phytochrome A in day-length detection. Plant Physiology, **114**: 1225-1236

Werck-Reichhart D, Bak S, Paquette S (2002) Cytochromes P450. In C.R. Somerville, EM Meyerowitz, eds, The *Arabidopsis* Book doi/10.1199/tab.0028. American Society of Plant Biologists, Rockville, MD

Whitbred JM, Schuler MA (2000) Molecular characterization of *CYP73A9* and *CYP82A1* P450 genes involved in plant defense in pea. Plant Physiology, **124**: 47-58

White CN, Proebsting WM, Hedden P, Rivin CJ (2000) Gibberellins and seed development in maize. I. Evidence that gibberellin/abscisic acid balance governs germination versus maturation pathways. Plant Physiology, **122**: 1081-1088

Winkler RG, Helentjaris T (1995) The maize *Dwarf3* gene encodes a cytochrome P450-mediated early step in gibberellin biosynthesis. Plant Cell, **7**: 1307-1317

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

Xu YL, Li L, Wu KQ, Peeters AJM, Gage DA, Zeevaart JAD (1995) The *Ga5* locus of *Arabidopsis-thaliana* encodes a multifunctional gibberellin 20-oxidase - molecular-cloning and functional expression. Proceedings of the National Academy of Sciences of the United States of America, **92**: 6640-6644

Yamaguchi S, Kamiya Y (2000) Gibberellin biosynthesis: Its regulation by endogenous and environmental signals. Plant and Cell Physiology, **41**: 251-257

Yamaguchi S, Kamiya Y (2002) Gibberellins and light-stimulated seed germination. Journal of Plant Growth Regulation, **20**: 369-376

Yamaguchi S, Kamiya Y, Sun TP (2001) Distinct cell-specific expression patterns of early and late gibberellin biosynthetic genes during *Arabidopsis* seed germination. Plant Journal, **28**: 443-453

Yamaguchi S, Saito T, Abe H, Yamane H, Murofushi N, Kamiya Y (1996) Molecular cloning and characterization of a cDNA encoding the gibberellin biosynthetic enzyme *ent*-kaurene synthase B from pumpkin (*Cucurbita maxima* L). Plant Journal, **10**: 203-213

Yamaguchi S, Smith MW, Brown RGS, Kamiya Y, Sun TP (1998a) Phytochrome regulation and differential expression of gibberellin 3 beta-hydroxylase genes in germinating *Arabidopsis* seeds. Plant Cell, **10**: 2115-2126

Yamaguchi S, Sun TP, Kawaide H, Kamiya Y (1998b) The *GA2* locus of *Arabidopsis thaliana* encodes *ent*-kaurene synthase of gibberellin biosynthesis. Plant Physiology, **116**: 1271-1278

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

Table of the major genes discussed in this thesis

name	genebank accession number	comment	GA biosythesis	species	reference
PsKAO1	AF537321	CYP88A6 *NA*	***ent*-kaurenoic acid oxidases**	*Pisum sativum*	Chapter 2
PsKAO2	AF537322	CYP88A7		*Pisum sativum*	Chapter 2
AtKAO1	AAK11564	CYP88A3		*Arabidopsis thaliana*	(Helliwell et al., 2001)
AtKAO2	AAK11565	CYP88A4		*Arabidopsis thaliana*	(Helliwell et al., 2001)
CmKAO1	AAG41777	CYP88A2		*Cucurbita maxima*	(Helliwell et al., 2000)
ZmKAO1	Q43246	CYP88A1 ***D3***		*Zea-mays*	(Winkler and Helentjaris, 1995)
OsKAO	AP000616	CYP88A5		*Oryza sativa*	
HvKAO1	AAK11616	*Grd5*		*Hordeum vulgare*	(Helliwell et al., 2001)
PsCPS	U63652	***LS***	**Copalyl diphosphate synthase**	*Pisum sativum*	(Ait-Ali et al., 1997)
PsKO1	AY245442	CYP701A10 ***LH***	***ent*-kaurene oxidases**	*Pisum sativum*	Chapter 3
AtKO1	AF047719	***GA3***		*Arabidopsis thaliana*	(Helliwell et al.,1998)
CmKO1	AF212990	CYP701A1 CYP701A2		*Cucurbita maxima*	(Helliwell et al., 2000)
PsGA20ox1	X91658		**20-oxidases**	*Pisum sativum*	
PsGA20ox2	U58830			*Pisum sativum*	
PsGA3ox1	U85045	***LE***	**3-oxidase**	*Pisum sativum*	(Lester et al., 1997)
PsGA2ox1	AF100955	***SLN***	**2-oxidases**	*Pisum sativum*	(Lester et al., 1999b)
PsGA2ox2	AF100954			*Pisum sativum*	

The Pea Gene *NA* Encodes *ent*-Kaurenoic Acid Oxidase[1]

Sandra E. Davidson, Robert C. Elliott, Chris A. Helliwell, Andrew T. Poole, and James B. Reid*

School of Plant Science, University of Tasmania, G.P.O. Box 252–55, Hobart, Tasmania, 7001, Australia (S.E.D., R.C.E., J.B.R.); and Commonwealth Scientific and Industrial Research Organization, Plant Industry, G.P.O. Box 1600, Canberra, Australian Capitol Territory 2601, Australia (C.A.H., A.T.P.)

The gibberellin (GA)-deficient dwarf *na* mutant in pea (*Pisum sativum*) has severely reduced internode elongation, reduced root growth, and decreased leaflet size. However, the seeds develop normally. Two genes, *PsKAO1* and *PsKAO2*, encoding cytochrome P450 monooxygenases of the subfamily CYP88A were isolated. Both PsKAO1 and PsKAO2 had *ent*-kaurenoic acid oxidase (KAO) activity, catalyzing the three steps of the GA biosynthetic pathway from *ent*-kaurenoic acid to GA_{12} when expressed in yeast (*Saccharomyces cerevisiae*). In addition to the intermediates *ent*-7α-hydroxykaurenoic acid and GA_{12}-aldehyde, some additional products of the pea KAO activity were detected, including *ent*-6α,7α-dihydroxykaurenoic acid and 7β-hydroxykaurenolide. The *NA* gene encodes PsKAO1, because in two independent mutant alleles, *na-1* and *na-2*, *PsKAO1* had altered sequences and the five-base deletion in *PsKAO1* associated with the *na-1* allele cosegregated with the dwarf *na* phenotype. *PsKAO1* was expressed in the stem, apical bud, leaf, pod, and root, organs in which GA levels have previously been shown to be reduced in *na* plants. *PsKAO2* was expressed only in seeds and this may explain the normal seed development and normal GA biosynthesis in seeds of *na* plants.

GAs are important plant growth hormones that regulate many aspects of plant growth including stem and petiole elongation, leaf expansion, and the growth of seeds and fruit (Reid and Ross, 1993; Hooley, 1994). They are also involved in seed germination and seed development (Hooley, 1994; Swain et al., 1997). Mutants have been useful in the elucidation of these actions and of the GA biosynthetic pathway (Ross et al., 1997; Hedden and Proebsting, 1999). In pea (*Pisum sativum*), most of the genes associated with the GA biosynthetic mutants have been cloned, including *LS* (copalyl diphosphate synthase; Ait-Ali et al., 1997), *LE* (3-oxidase; Lester et al., 1997; Martin et al., 1997), and *SLN* (2-oxidase; Lester et al., 1999b; Martin et al., 1999; Fig. 1).

However, this is not the case for the GA-responsive mutant *na*, which has an extreme dwarf or "nana" phenotype (Fig. 2; Potts and Reid, 1983). It has extremely short internodes with a dramatic decrease in cell length of the epidermal and outer cortical cells as well as a reduction in the total number of these cells in the internode (Reid et al., 1983). The *na* mutants have small, darkly colored foliage with reduced area of individual leaflets, decreased stipule size, and petiole length (Reid and Ross, 1993). The root growth is also altered with taproot length reduced by 50% (Yaxley et al., 2001). The vegetative part of the *na* pea plant is severely deficient in endogenous GAs. It was not possible to show the presence of any C-19 GAs by dilution of $[^{13}C^{3}H]GA_{20}$ metabolites by endogenous $[^{12}C]$GAs using gas chromatography-mass spectrometry (GC-MS) techniques (Ingram et al., 1984). The *na* mutation markedly reduces the production of GAs, including the predominant bioactive GA_1, in shoots and stems (Potts and Reid, 1983), leaves (Reid and Ross, 1993), roots (Yaxley et al., 2001), and pods (Potts and Reid, 1983; Potts, 1986). However, the effect of the *na* mutation is tissue specific and this was among the earliest information suggesting that alternative enzymes (or gene families) may be involved in GA biosynthesis (Reid, 1986a). In contrast to the vegetative part of the *na* plant, where the level of GA_1 in expanding tissue was reduced to 2% of the wild type (Proebsting et al., 1992), the developing seeds contain similar GA levels to those found in the seeds of wild-type *NA* plants (Potts and Reid, 1983; Potts, 1986). In addition, the seeds of *na* plants develop normally (Potts and Reid, 1983).

The *na* mutation appears to block GA biosynthesis before GA_{12}-aldehyde. There are two lines of evidence to support this supposition. The *na* plants did not respond to the application of precursors before GA_{12}-aldehyde such as *ent*-kaurene, *ent*-kaurenoic acid (KA), and *ent*-7α-hydroxy-kaurenoic acid, but showed marked stem elongation in response to GA_{12}-aldehyde. Also, $[^{2}H]GA_{12}$-aldehyde was metabolized to C-19 GAs such as GA_{20}, GA_{29}, GA_1, and GA_8 by *na* plants, but these plants do not metabolize *ent*-$[^{3}H_2]$kaurenoic acid to these GAs even though wild-type plants appear to do so (Ingram and Reid, 1987).

The GA biosynthetic pathway (Fig. 1) can be divided into three sections (Hedden and Kamiya, 1997; Hedden and Phillips, 2000). The first section, cata-

[1] This work was supported by the Australian Research Council (grants to J.B.R.). S.E.D. was the recipient of an Australian Postgraduate Award.

* Corresponding author; e-mail Jim.Reid@utas.edu.au; fax 61–3–6226–2698.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.012963.

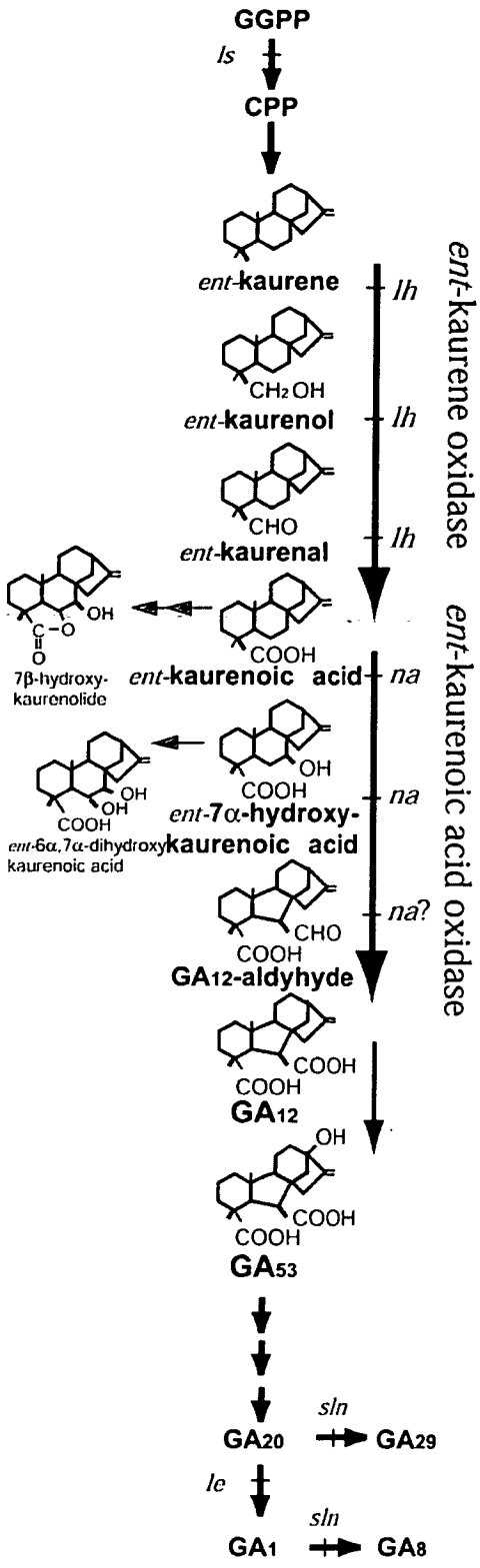


Figure 1. The GA biosynthetic pathway in pea. Product structures are represented for the cytochrome P450 monooxygenase-mediated steps showing the steps catalyzed by *ent*-kaurene oxidase (KO) and *ent*-kaurenoic acid oxidase (KAO). The steps blocked by the pea mutants (*ls*, *lh*, *na*, *le*, and *sln*) are indicated. Putative side products of PsKAO1 and PsKAO2 activity are indicated.

lyzed by terpene cyclases, involves the cyclization of geranylgeranyl diphosphate to *ent*-kaurene. In the second section, the hydrophobic *ent*-kaurene is oxidized to GA_{12} or GA_{53} by membrane-bound cytochrome P450 monooxygenases. The third section consists of further oxidation to form the bioactive GA_1 or GA_4 by soluble 2-oxoglutarate dependent dioxygenases. The early sections of the pathway are common to all the plant species investigated so far (Hedden and Phillips, 2000). However, the wide range of dioxygenases allows variation in the pathway after GA_{12} in different species and tissues. The early 13-hydroxylase pathway predominates in vegetative tissue of pea, producing bioactive GA_1 (Ingram et al., 1986; Reid and Ross, 1993; Poole et al., 1995). However, GA_4 appears to be the main active product in Arabidopsis (Talon et al., 1990; Sponsel et al., 1997).

The second section of the GA biosynthetic pathway from *ent*-kaurene via KA to GA_{12} or GA_{53} is generally assumed to involve cytochrome P450 monooxygenases and requires the coenzyme NADPH-cytochrome P450 reductase, NADPH, and oxygen (Hedden, 1997). The CYP88A family of cytochrome P450s have recently been shown to encode KAO, which catalyzes the three steps from KA to GA_{12} via *ent*-7α-hydroxykaurenoic acid and GA_{12}-aldehyde, in Arabidopsis and barley (*Hordeum vulgare*; Helliwell et al., 2001; Fig. 1). Other CYP88A cytochrome P450 genes from pumpkin (*Cucurbita maxima*; Helliwell et al., 2000) and maize (*Zea mays*) have also been isolated (Winkler and Helentjaris, 1995).

Arabidopsis does not have a mutant affecting KA oxidation presumably because of redundancy because the two Arabidopsis genes, *AtKAO1* and *AtKAO2*, have similar expression patterns throughout the plant (Helliwell et al., 2001). The GA-responsive maize *d3* mutants have defects in a CYP88A gene (Winkler and Helentjaris, 1995). The barley GA-responsive dwarf mutant, *grd5*, accumulates KA in developing grains (Helliwell et al., 2001). Mutations were found in the barley *HvKAO1*, in each of three independent mutant alleles of the barley dwarf *grd5* (Helliwell et al., 2001).

In this paper, we identify genes encoding KAO activity in pea by screening a cDNA library using a maize *D3*-like expressed sequence tag (EST) probe from soybean (*Glycine max*). We show that one of these genes is *NA* and then explain the tissue-specific nature of the *na* mutation.

RESULTS

Isolation of Two CYP88A Genes from Pea

Two genes encoding cytochrome P450s of the subfamily CYP88A were isolated by library screening using a maize *D3*-like EST from soybean as probe. A partial cDNA of *PsKAO1* was obtained from a pea cv Alaska apical bud cDNA library. The longest clone (500 bp) was extended by 3′- and 5-′ RACE using cDNA prepared from wild-type *NA* (WL1769) as

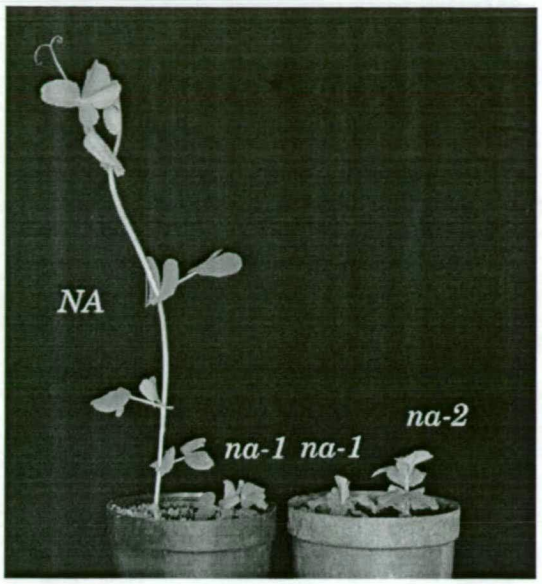


Figure 2. The phenotype of 21-d-old seedlings of wild-type *NA* (WL1769) and two independent mutants, *na-1* (WL1766) and *na-2* (L81).

template (Frohman et al., 1988). A full-length cDNA, *PsKAO2*, was obtained from a pea cv Torsdag seed library.

PsKAO1 (*CYP88A6*, GenBank accession no. AF537321 sequenced from *NA* WL1769) and *PsKAO2* (*CYP88A7*, GenBank accession no. AF537322) showed close homology at the nucleotide level (60–100-bit score, 82%–93% identities) to *AtKAO1* and *AtKAO2* (BLASTN; Altschul et al., 1997). At the amino acid level, the full-length putative proteins PsKAO1 and PsKAO2 are similar to AtKAO1, AtKAO2, and CmKAO1 (644–661-bit score, 63%–65% identities, 79%–81% positives; National Center for Biotechnology Information Blast 2 sequences). Over the full-length *PsKAO1* is similar to *PsKAO2* at the nucleotide (462-bit score, 78% identity) and protein level (756-bit score, 74% identity, 86% positive; National Center for Biotechnology Information Blast 2 sequences). This is comparable with the Arabidopsis KAO putative proteins, where AtKAO1 is 76% identical to AtKAO2 (Fig. 3).

Northern-Blot Expression Studies

The members of the pea kaurenoic acid oxidase (KAO) gene family are differentially expressed. *PsKAO1* is expressed in the stem and to a lesser extent in the leaf, root, apical bud, pod, and seed, whereas *PsKAO2* is only expressed in the seed (Fig. 4A). *PsKAO2* is expressed most strongly around the time of contact point when the embryo just fills the testa and the liquid endosperm is all consumed (Fig. 4B). This coincides with the rapid buildup of GA levels in maturing seeds (Frydman et al., 1974; Swain et al., 1993).

PsKAO1 Is Mutated in the *na-1* and *na-2* Mutants

The *PsKAO1* cDNA from the *na-1* line WL1766 contained a five-base deletion when compared with *NA* (WL1769). This would change the reading frame for the encoded protein leading to a premature stop codon. The predicted protein would be 194 amino acids long, which is much smaller than the expected 485-amino acid length of the putative PsKAO1 enzyme. The predicted protein would be truncated before the catalytic domains (Kalb and Loper, 1988), including the active haem-binding site common to all cytochrome P450 enzymes. There was markedly less *PsKAO1* mRNA measured in the *na-1* mutant tissue than the isogenic wild-type *NA* (Fig. 4C). This may be because of the instability of mRNA with a premature stop codon (Gutierrez et al., 1999).

The *PsKAO1* from the *na-2* line L81 also is altered compared with *NA* (WL1769). Initially, PCR of the cDNA obtained from RNA of *na-2* stems produced three bands (Fig. 5). When gel purified, the largest band (W) was found to have 48 bp incorrectly spliced out and the smallest band (Z) was found to have 364 bp incorrectly spliced out (Fig. 5). The band Y could not be gel purified. However, if the products W and Z were combined, melted, and annealed, the original three-band PCR pattern reappeared. This suggests that the Y band represents a duplex between the W and Z bands (Fig. 5). Genomic DNA sequence data was then used to further define the *PsKAO1 na-2* mutation. The genomic sequence of *na-2* revealed a 25-bp deletion (5 bp from the 3′ end of a large intron and 20 bp of exon sequence) compared with the wild type. The AG that is required for the positioning of splicing (Brown, 1996) was lost from the intron. Hence, splicing did not occur in the same place as in the wild type. Therefore, this mutation leads to aberrant processing of the resultant pre-RNA. The second and 18th AG after the deletion were used as 3′-splicing points for the RNA that produced the W and Z PCR bands, respectively. Splicing of the following intron was not affected (Fig. 5).

The Mutation in *na-1* Cosegregates with the *na* Mutant Phenotype

DNA was extracted from individual plants from four segregating F_9 families from a cross between *NA* (WL1769) and *na-1* (WL1766) plants (Fig. 2). The hexachloro fluorescein-labeled PCR products (243 bp in the wild type) identified the five-base deletion of the *na-1* mutant gene when run on denaturing gel. The five-base deletion of *PsKAO1* cosegregates with the dwarf phenotype of *na* plants (data not shown). Of the 50 individuals in the four families, 12 were homozygous tall (*NA NA*), 26 were heterozygous tall (*NA na*), and 12 were homozygous dwarf (*na na*) in agreement with expected ($\chi^2_2 = 0.08$; $P > 0.9$). Therefore, the *na-1* phenotype cosegregates with the mutation in the *PsKAO1* gene.

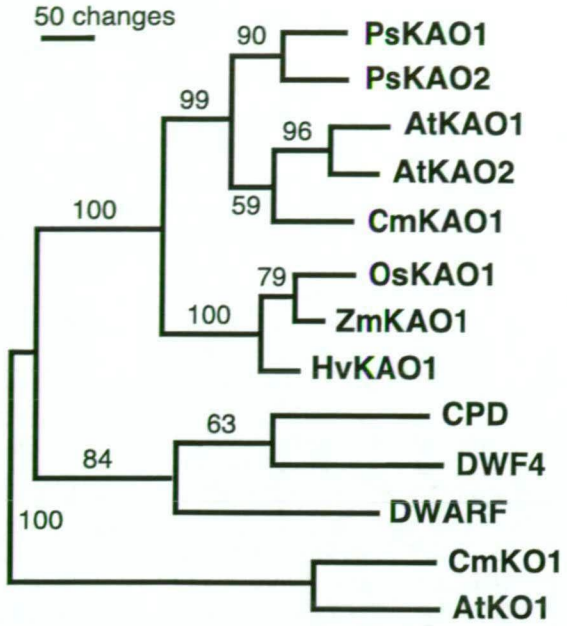


Figure 3. Inferred phylogenetic relationship of KAOs [CYP88A] and representatives of related cytochrome P450 enzymes. Numbers shown represent the bootstrap support values (%). The phylogram was generated by PAUP 4.8b8 (Swofford, 1999) using putative amino acids of full-length genes (excluding gaps) with CYP701A as the outgroup. KAO proteins used in addition to the pea PsKAO1 and PsKAO2 were from Arabidopsis (AtKAO1 and AtKAO2, Helliwell et al., 2001), pumpkin (CmKAO1, Helliwell et al., 2000), rice (*Oryza sativa*; OsKAO1, GenBank accession no. AP000616), maize (*D3*, ZmKAO1, Winkler and Helentjaris, 1995), and barley (*Grd5*, HvKAO1, Helliwell et al., 2001). The related brassinosteroid biosynthetic enzymes used include Arabidopsis *CPD* (CYP90A1, Szekeres et al., 1996) and *DWF4* (CYP90B1, Choe et al., 1998) and tomato (*Lycopersicon esculentum*) *DWARF* (CYP85A1, Bishop et al., 1999). The outgroup consisted of the cytochrome P450 monooxygenase *ent*-kaurene oxidases (CYP701A) from pumpkin CmKO1 (Helliwell et al., 2000) and Arabidopsis *GA3* (AtKO1, Helliwell et al., 1998) of the GA biosynthetic pathway.

Yeast (*Saccharomyces cerevisiae*) Expression

Yeast strains WAT11 and WAT21 were transformed with *PsKAO1* and *PsKAO2* expression constructs and yeast strains expressing PsKAO1 and PsKAO2 were identified by RNA gel blots. Both yeast strains expressing PsKAO1 and PsKAO2 converted KA through to GA_{12} at a greater rate than yeast expressing the Arabidopsis AtKAO1 (Table I). The intermediates *ent*-7α-hydroxykaurenoic acid and GA_{12}-aldehyde, as well as the final product GA_{12}, were detected (Table I) and confirmed to be authentic by comparison to known standards (Table II). The PsKAO2 construct expressed in both yeast strains appeared more effective than the PsKAO1 and converted all the KA substrate provided. Direct comparison may not be possible because the PsKAO1 construct may have four extra amino acids in the 5′-untranslated region because there were two possible start codons in the *PsKAO1* sequence. GA_{53} and GA_{14} were not detected in any of the samples (Table I). WAT21 yeast transformed with PsKAO1 and PsKAO2 expression constructs fed with intermediates *ent*-7α hydroxy kaurenoic acid or GA_{12}-aldehyde

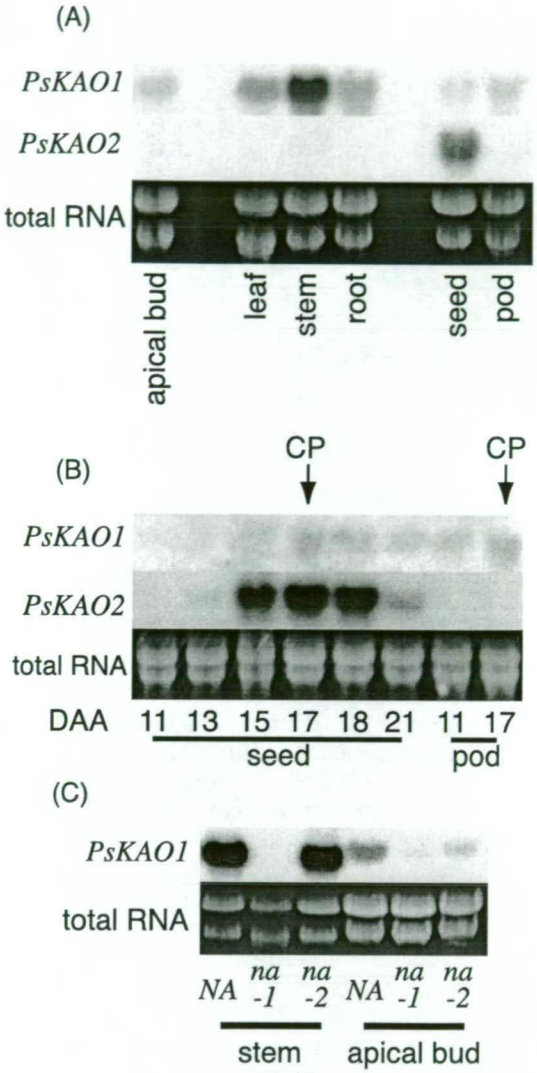


Figure 4. A, *PsKAO1* and *PsKAO2* transcript levels in various parts of wild-type pea (L107). Five micrograms of total RNA from the apical bud (all material above the uppermost fully expanded leaf), leaf (the uppermost fully expanded leaf), stem (internode immediately below the uppermost fully expanded leaf, the internode was 80%–100% fully expanded), and root (50 mm off the end of the tap and lateral roots) of 19-d-old seedlings; also, 5 μg of total RNA from seeds (3 d after contact point) and pods (that originally contained these seeds) from mature plants were loaded on the gel. B, *PsKAO1* and *PsKAO2* transcript levels in wild-type pea (L107) seeds at various developmental ages. Total RNA was extracted from whole seeds and their pods between 11 and 21 d after anthesis. Contact point (CP, the 1st d that no liquid endosperm remained in seeds) occurred at 17 d after anthesis. C, *PsKAO1* transcript levels in wild-type *NA* (WL1769) and mutants *na-1* (WL1766) and *na-2* (L81) from the apical bud (all tissue above the uppermost fully expanded leaf) or fully expanded stem tissue of 18-d-old pea seedlings. The ethidium bromide (EtBr)-stained total RNA is included as a loading control for each northern analysis.

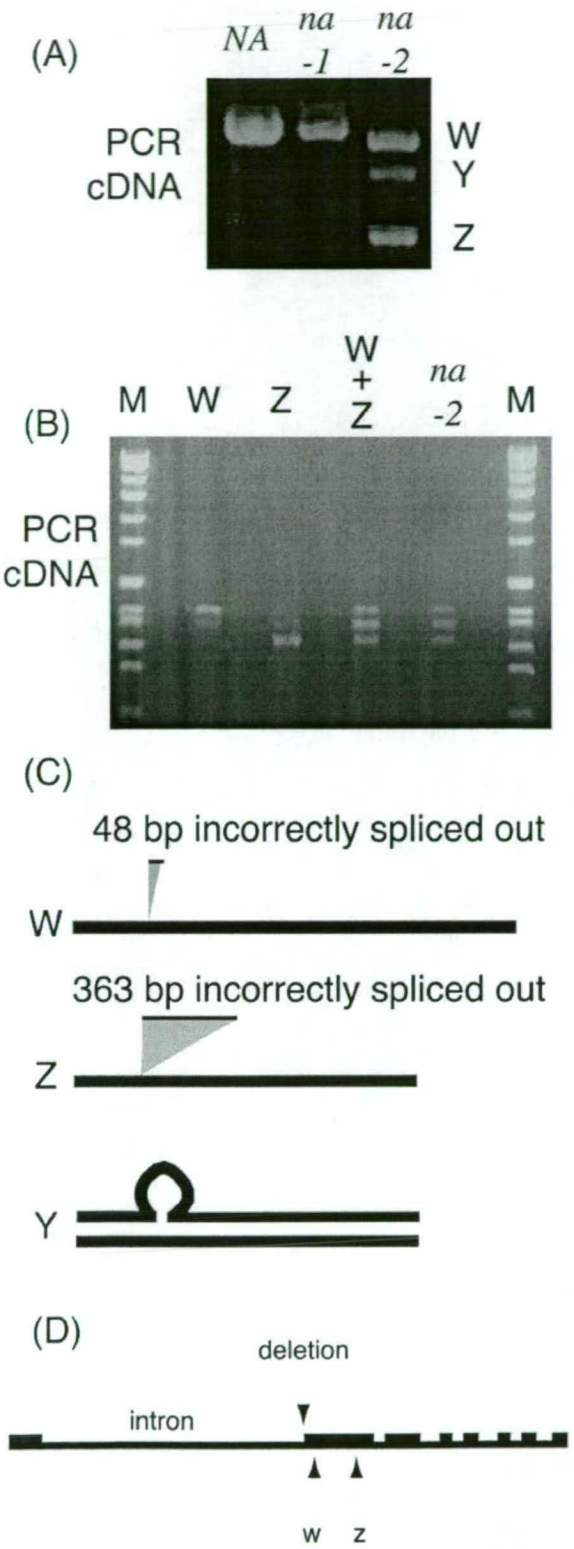


Figure 5. A, The cDNA PCR products of *PsKAO1* from wild-type *NA* (WL1769) and mutants *na-1* (WL1766) and *na-2* (L81) using the same specific primers run on 1% (w/v) agarose/Tris-acetate EDTA gel containing EtBr at 80 V for 45 min. The *na-2* bands have been labeled W, Y, and Z. B, Lanes W and Z contain previously gel-purified PCR product bands W and Z from *na-2* mutant cDNA (see A). Lane W + Z is the product formed when the gel-purified products W and Z were combined, melted, and annealed (three cycles of melting at 95°C and annealing at 55°C then a 70°C extension). Lane *na-2* is the PCR product of the *na-2* mutant cDNA as seen in A and lane M is the SPPI-*Eco*RI size marker run on 11% (w/v) agarose/Tris-acetate EDTA gel containing EtBr. C, Schematic diagram explanations of the cDNA bands W, Z, and Y of A from sequence and experimental data (B). D, Schematic diagram of genomic DNA of *PsKAO1* from sequence data. The 25-bp deletion site as well as the W and Z cryptic splice sites of the *na-2* mutation are indicated.

converted these substrates to GA_{12} although at a lower rate than expected from the KA feed data (data not shown). A substrate earlier in the GA biosynthetic pathway, *ent*-kaurene, was not metabolized by yeast expressing PsKAO1 or PsKAO2 (data not shown). Untransformed wild-type yeast did not metabolize KA to intermediates in the GA biosynthetic pathway (Table I).

The compound 7β-hydroxy-kaurenolide was detected and confirmed against authentic standard (Table II) in the samples with PsKAO1 and PsKAO2 activity after KA feeds but not when the intermediates *ent*-7α-hydroxykaurenoic acid or GA_{12}-aldehyde were used as substrates. *ent*-6α,7α-dihydroxykaurenoic acid was detected (Table I) and tentatively identified based on comparison with published spectra (Gaskin and MacMillan, 1991; Table II) in yeast with PsKAO1, PsKAO2, or AtKAO1 activity when fed with KA. However, this product was also present after feeds of *ent*-7α-hydroxykaurenoic acid (data not shown). Further conversion to fujenoic acid was not observed. Neither 7β-hydroxy-kaurenolide nor *ent*-6α,7α-dihydroxy-kaurenoic acid were detected in the wild-type untransformed yeast samples and appeared to be a result of the KAO activity. However, in the wild-type untransformed yeast and yeast expressing AtKAO1 and PsKAO1 samples, the C/D ring-rearranged compounds, stachenoic acid and trachylobanic acid, were present in significant amounts. However, neither compound was present in the more active PsKAO2-expressing samples that appeared to metabolize these compounds to the *ent*-7α hydroxy and *ent*-6α,7α-dihydroxy derivatives and also through to GA_{12}-like derivatives (Table III).

DISCUSSION

PsKAO1 and *PsKAO2* Encode CYP88A Cytochrome P450 Monooxygenases

Two genes, *PsKAO1* and *PsKAO2*, encoding cytochrome P450 monooxygenases from the subfamily CYP88A were identified in pea. They have high similarity to the recently cloned genes *AtKAO1* and *AtKAO2* of Arabidopsis (Helliwell et al., 2001) and *CmKAO1* of pumpkin (Helliwell et al., 2000). They are grouped with the GA biosynthetic KAO enzymes in the subfamily CYP88A as defined by the maize *D3* enzyme (Winkler and Helentjaris, 1995). The KAOs

Table I. *Putative products from WAT21 and WAT11 yeast strains expressing CYP88A cytochrome P450s*

The GC-MS total ion current (TIC) areas were measured for *ent*-7α-hydroxykaurenoic acid, GA_{12}-aldehyde, GA_{12}, GA_{53}, GA_{14}, 7β-hydroxy kaurenolide, and *ent*-6α,7α-dihydroxykaurenoic acid after KA feeds.

Enzyme	Yeast	KA	*ent*-7α-OH KA	GA12-Aldehyde	GA12	GA53 or GA14	7α-OH Kaurenolide	ent-6α,7α-diOH KA
					TIC (×10⁶)			
Wild-type yeast	W21	1,131	n.d.[a]	n.d.	n.d.	n.d.	n.d.	n.d.
AtKAO1	W21	1,204	49	15	≈2.5[b]	n.d.	n.d.	18
PsKAO1	W21	702	414	22	84	n.d.	≈5[b]	39
PsKAO2	W21	n.d.	520	108	265	n.d.	≈54[b]	96
PsKAO1	W11	921	437	23	95	n.d.	≈5[b]	42
PsKAO2	W11	n.d.	480	131	265	n.d.	≈49[b]	79

[a]n.d., Not detected. [b]Approximate TIC area value where baseline is too high or peak is contaminated, calculated from the uncontaminated major ion and a conversion factor based on the appropriate standard.

from the dicotelydons (pea, Arabidopsis, and pumpkin) are grouped separately from those of the monocotyledons (rice, maize, and barley; Fig. 3). In addition, the two enzymes of pea are grouped, as are those of Arabidopsis, suggesting that gene duplication occurred late in the evolutionary process. The KAO-deduced proteins contain the four catalytic domains (A–D) common to eukaryotic cytochrome P450s (Kalb and Loper, 1988). However, a critical conserved Thr of the A domain that was shown in the crystal structure of P450cam to form a hydrogen bond with Gly to produce an oxygen-binding pocket (Poulos et al., 1985), and may also be involved in oxygen transfer (Imai et al., 1989), is replaced by Ser in all the KAOs sequenced so far. In P450cam, this Thr could be replaced by Ser in site-directed mutagenesis without altering the monooxygenase activity, whereas replacement with amino acids without the hydroxyl group uncoupled the oxygen consumption of the enzyme (Imai et al., 1989).

Pea *NA* Encodes PsKAO1

The evidence shows that the pea *NA* gene encodes PsKAO1. First, *PsKAO1* from both the *na-1* and *na-2* GA-responsive dwarf mutants had altered sequences. The mutations *na-1* and *na-2* are allelic and produced by independent mutational events (Reid et al., 1983). Furthermore, the five-base deletion in *PsKAO1* associated with the *na-1* mutation cosegregated with the dwarf phenotype. *PsKAO1* is expressed in the tissues where the *na* mutant phenotype is expressed. The mutant *na* plant has dwarfed stature, reduced taproot length, and reduced leaflet area (Reid et al., 1983; Reid and Ross, 1993; Yaxley et al., 2001). The GA_1 levels are reduced in the shoot, roots, leaves, and pods (Potts and Reid, 1983; Ingram et al., 1984; Potts, 1986; Yaxley et al., 2001). *PsKAO1* was expressed in stems, roots, leaves, apical buds, pods, and seeds (Fig. 4). However, the other pea CYP88A gene, *PsKAO2*, was only expressed in developing seeds and not any of the other tissues tested (Fig. 4). Because *PsKAO2* was expressed in seeds, KAO activity could be expected in the seeds of the *na* mutant. This would explain the observation that mutant *na* plants have normal seed development and the same GA content in their seeds as wild-type pea seeds (Potts and Reid, 1983). In contrast, the two Arabidopsis genes have similar expression patterns.

Table II. *Authentication of GA biosynthetic intermediates and additional products in yeast extracts*

GC-MS relative ion abundances and Kovats' retention index (KRI) were compared with authentic standards. Samples are methyl ester (ME), trimethyl silyl (TMS), or methyl ester trimethyl silyl (METMS) derivatives.

Reference or Putative Compounds	KRI	Characteristic Ions– Relative Abundance as % of Base Peak							
		404(M+)	**389**	**314**	**299**	**255**	**254**	**239**	**223**	**199**
ent-7α-hydroxy KA METMS standard	2,459	22	8	100	32	69	59	43	19	22
ent-7α-hydroxy KA METMS	2,460	35	13	100	33	67	60	45	19	23
		330(M+)	**287**	**270**	**255**	**242**	**241**	**239**	**227**	**199**
GA_{12} aldehyde ME standard	2,437	12	14	40	14	24	100	24	14	10
GA_{12} aldehyde ME	2,436	11	14	40	15	25	100	24	15	11
		360(M+)	**328**	**300**	**285**	**241**	**240**	**225**	**185**	
GA_{12} ME standard	2,415	1	17	100	24	35	29	20	12	
GA_{12} ME	2,416	1	17	100	22	35	31	26	15	
		388(M+)	**345**	**298**	**283**	**270**	**255**	**227**	**163**	**137**
7β-Hydroxykaurenolide TMS standard	2,557	2	9	100	15	17	12	10	12	58
7β-Hydroxykaurenolide TMS	2,552	1	10	100	17	22	14	13	27	95
		492(M+)	**477**	**402**	**387**	**343**	**327**	**269**	**253**	**209**
ent-6α,7α-dihydroxy KA METMS[a]	2,593	<1	92	16	5	4	8	100	16	50

[a]Tentative identification based on comparison with published spectra (Gaskin and MacMillan, 1991).

Table III. *Putative C/D ring rearranged products in untransformed yeast and yeasts expressing KAOs fed with KA*
GC-MS relative ion abundances and KRI are indicated.

Putative Compound	Present following Feed of KA to	KRI	Characteristic Ions (Relative Abundance as % of Base Peak)
Stachenoic acid ME	Wild-type yeast, *AtKAO1*, and *PsKAO1*	2,224	316 (75), 301 (8), 273 (5), 257 (22), 256 (11), 241 (11), 194 (27), 181 (24), 159 (20), 148 (39), 135 (100)
Trachylobanic acid ME	Wild-type yeast, *AtKAO1*, and *PsKAO1*	2,278	316 (77),301 (27), 273 (5), 260 (100), 257 (58), 256 (19), 245 (45), 241 (46), 201 (30), 200 (27)
ent-7α-hydroxy stachenoic acid METMS[a]	*PsKAO1* and *PsKA02*	2,344	404 (25), 389 (7), 314 (51), 301 (18), 299 (15), 286 (76), 283 (63), 255 (54), 254 (44), 239 (32), 223 (42), 193 (24), 181 (46), 133 (97), 73 (100)
ent-7α-hydroxy trachylobanic acid METMS[a]	*PsKAO1* and *PsKA02*	2,399	404 (2), 389 (4), 314 (100), 299 (17), 255 (55), 254 (60), 239 (41), 209 (31), 185 (43), 157 (65)
ent-6α,7α-dihydroxy stachenoic acid METMS	*PsKA02*	2,471	492 (<1), 477 (57), 402 (18), 387 (4), 343 (4), 327 (11), 269 (100), 253 (15), 209 (45)
ent-6α,7α-dihydroxy Trachylobanic acid METMS	*PsKA02*	2,531	492 (<1), 477 (18), 402 (12), 387 (3), 343 (2), 327 (4), 269 (100), 253 (17), 209 (40)
Stacheno-GA_{12} ME[b]	*PsKA02*	2,299	360 (4), 328 (47), 300 (63), 285 (22), 240 (34), 225 (53), 119 (100)
Trachylo-GA_{12} ME	*PsKA02*	2,360	360 (4), 328 (21), 300 (100), 285 (84), 241 (73), 240 (29), 225 (31), 164 (68), 119 (24)

[a]Tentatively identified as *ent*-7α-hydroxy stachenoic acid METMS and *ent*-7α-hydroxy trachylobanic acid METMS based on the similarities in their mass spectra with *ent*-7α-hydroxy KA METMS, and similar relative KRI values of *ent*-7α-stachenoic (putative)/*ent*-7α-trachylobanic (putative)/*ent*-7α-kaurenoic acid METMS compared with the stachenoic/trachylobanic/kaurenoic acid ME KRI values. [b]Tentatively identified as "stacheno-GA_{12} ME" because of the similarity of its spectra to GA_{12} ME and trachylo-GA_{12} ME, and similar relative KRI values to the two groups of three above.

Probably because of this redundancy, no mutants have been found in Arabidopsis (Helliwell et al., 2001).

The *na* mutants are dwarfs with extreme reduction in internode lengths. The differences in internode lengths between WL1766 and L81 (Fig. 2) arise from differences in their genetic background (Reid et al., 1983). The putative proteins from the mutants (*na-1* and *na-2*) are severely altered from wild-type PsKAO1. However, the double recessive with other GA biosynthetic mutants (*na lh* and *na ls*) are shorter than the single *na* mutant (Reid, 1986b). This could be because of limited activity by other KAO enzymes in pea shoots because extra bands were observed on genomic southern blots at low stringency (data not shown). Alternatively, there may be movement of intermediates from other tissues where the *PsKAO2* gene is expressed. The latter is possible because the effect of the *NA* (*PsKAO1*) gene is graft transmissible (Reid et al., 1983; Proebsting et al., 1992) and mature pea seeds at sowing contain GA_{20} (Ross et al., 1993). The other *KAO* gene (*PsKAO2*) is expressed in the seed (Fig. 4, A and B) and the seeds of *na* mutant plants develop normally (Potts and Reid, 1983; Potts, 1986). This may provide a limited amount of GA precursors in the stem of the mutant *na* seedlings allowing the epicotyl to develop almost normally before the extreme dwarfism sets in (Reid et al., 1983). Furthermore, the phenotype of the double mutant seedlings appears to reflect the accumulation of GAs in the seed (*na sln*, Reid et al., 1992; Ross et al., 1995; *na le*, Reid et al., 1983; Lester et al., 1999a; and *na ls* and *na lh*, Swain et al., 1995).

PsKAO1 and PsKAO2 Catalyze KA to GA_{12}

PsKAO1 and PsKAO2, when expressed in yeast, catalyzed the three steps from KA to *ent*-7α-hydroxykaurenoic acid to GA_{12}-aldehyde to GA_{12} (Table I; Fig. 1). This was observed previously with *AtKAO1* and *AtKAO2* from Arabidopsis and *HvKAO1* of barley (Helliwell et al., 2001). Many of the enzymes in the GA biosynthetic pathway are multifunctional (Hedden, 1997). This occurs normally where the enzyme catalyzes multiple oxidations at the same carbon position (Phillips et al., 1995; Xu et al., 1995; Helliwell et al., 1999) as occurs with all three oxidations from KA to GA_{12} at the C-7 position (Hedden, 1997; MacMillan, 1997; Helliwell et al., 2001).

The *na* Mutation Blocks GA Biosynthesis before GA_{12}-Aldehyde

To see if the three-step oxidation of KA observed in the yeast expression studies is demonstrated in the plant, we can look at the corresponding mutants. The pea *na-1* and *na-2* mutants, which have a defective

PsKAO1 gene, do not metabolize [^{3}H] kaurenoic acid to substances co-eluting with GA_{20}, GA_1, or GA_8, even though *NA* plants do carry out this conversion (Ingram and Reid, 1987). This supports the yeast expression data (Table I) and the finding that the barley mutant (*grd5*) accumulates KA in its seed (Helliwell et al., 2001). However, metabolism studies show that *na* plants can convert [^{2}H] GA_{12}-aldehyde to C19-GAs such as GA_{20}, GA_{29}, GA_1, and GA_8. In addition, application studies show that although *na* plants do not respond to precursors before GA_{12}-aldehyde, including KA and *ent*-7α-hydroxykaurenoic acid, they do respond to GA_{12}-aldehyde and readily convert labeled GA_{12}-aldehyde to GA_1 (Ingram and Reid, 1987). The *na* mutation, therefore, appears to block the first two biosynthetic steps but not the final GA_{12}-aldehyde to GA_{12} step in the plant. This could occur if the nature of the *na* mutation altered the specificity of the PsKAO1 enzyme for the substrates. However, this is not likely because *na-1* (WL1766) is a null mutation.

Alternatively, there may be other specific or nonspecific enzymes present in the plant that can catalyze the last step but not the earlier steps catalyzed by PsKAO1 and PsKAO2 when expressed in yeast. A GA 7-oxidase dioxygenase has been found in pumpkin (Lange, 1997) in addition to the monooxygenase 7-oxygenase activity (Hedden et al., 1984) but has not yet been found in other species (Hedden and Phillips, 2000). Perhaps the most likely explanation is that nonspecific activity may be involved because multigene families of aldehyde oxidases have been cloned from maize and Arabidopsis (Sekimoto et al., 1997, 1998) and some of these oxidize a wide range of aldehydes (Seo et al., 1998).

PsKAO1 and PsKAO2, when expressed in yeast, did not produce GA_{53} from any of the precursors provided, although the early 13-hydroxylation GA biosynthetic pathway predominates in pea (Ingram et al., 1986; Reid and Ross, 1993; Poole et al., 1995). In immature pea and barley embryos, the formation of GA_{53} from GA_{12} was associated with the microsomal fraction and required NADPH and oxygen (Kamiya and Graebe, 1983; Groβelindemann et al., 1992), suggesting that there may be another membrane-bound cytochrome P450 monooxygenase in pea catalyzing GA_{12} to GA_{53}.

Additional Products of PsKAO Activity

In addition to *ent*-7α hydroxykaurenoic acid, GA_{12}-aldehyde, and GA_{12}, several side products of PsKAO1 and PsKAO2 activity were identified in the yeast expression studies. After either KA or *ent*-7α-hydroxykaurenoic acid feeds, the byproduct *ent*-6α,7α-dihydroxykaurenoic acid was detected (Fig. 1). We also detected this compound as a product of AtKAO1 activity. The compound, *ent*-6α,7α-dihydroxykaurenoic acid, was noted in pumpkin (Hedden, 1997; MacMillan, 1997) and related products were previously detected in pea (Ingram and Reid, 1987). The product 7β-hydroxykaurenolide was detected after KA (but not *ent*-7α-hydroxykaurenoic acid) feeds in yeast expressing PsKAOs and is presumably a side product of the formation of the double bond and epoxide from KA via *ent*-kauradienoic acid (Hedden, 1997). The P450–1 enzyme of *Gibberella fujikuroi* that catalyzes the four steps from KA to GA_{14} also produced 7β-hydroxykaurenolide and *ent*-6α,7α-dihydroxykaurenoic acid (Rojas et al., 2001). Fungi and higher plants appear to have evolved their GA biosynthetic pathway independently and P450–1 belongs to a different subfamily (CYP68) with low sequence homology to higher plant KAOs of subfamily CYP88A (Hedden et al., 2002). Because the additional products are common to both enzymes, they may be inevitable consequences of the reactions rather than specific products of the respective enzymes. In line with the expected difference between the fungi and higher plant KAO enzymes, the compound GA_{14} was not detected from PsKAO activity. It was interesting to note, however, that the C/D ring-rearranged stachenoic acid and trachylobanic acid can act as substrates for the PsKAO activity.

CONCLUSION

We have cloned two *CYP88A* genes in pea, *PsKAO1* and *PsKAO2*. Both of these genes catalyze the three steps from KA to GA_{12} when expressed in yeast. The genes have distinct expression patterns. *PsKAO1* is the pea *NA* gene and is expressed in the stem, apical bud, root, leaf, pod, and seed. Mutation in the *PsKAO1* gene results in the extreme dwarf *na* phenotype. *PsKAO2* is expressed in developing seeds, explaining the normal seed GA levels and seed development of *na* plants.

MATERIALS AND METHODS

Plant Material and Growing Conditions

Two independent mutational events in pea (*Pisum sativum*) resulted in the alleles *na-1* and *na-2* (Reid et al., 1983). The *na-1* fast neutron induced recessive mutation is in the Weibullsholm line WL1766 (genotype *na-1 LE LH LS*) and the *na-2* mutation is in the Hobart line L81 (genotype *na-2 le LH LS*). The tall *NA* WL1769 (genotype *NA LE LH LS*) was used as wild type and contains the progenitor sequence for the *na-1* mutation. The *na-1* and *NA* plants used for the cosegregation analysis were isogenic as a result of eight generations of single plant selection after a cross between lines WL1766 (*na-1*) and WL1769 (*NA*). Another wild-type Hobart line L107 (genotype *NA LE LH LS*) derived from pea cv Torsdag was used for some northern analyses.

Plants were grown two per pot in a heated greenhouse under an 18-h photoperiod (Beveridge and Murfet, 1996).

Library Screening

Both seed and shoot cDNA libraries were screened. The seed cDNA library was constructed in Lambda ZAPII (Stratagene, La Jolla, CA) with cDNA prepared from L107 pea cv Torsdag seeds at "contact point" (Ait-Ali et al., 1997). The library screening and the isolation of clones were according

to methods recommended by the manufacturer (Stratagene). The shoot cDNA library was in Lambda gt11 prepared from pea cv Alaska apical buds (CLONTECH Laboratories, Palo Alto, CA). The library screening method was similar to above; however, inserts were obtained directly by PCR with nested vector primers from original pure clones. The probe used was a 339-nucleotide fragment from a maize (*Zea mays*) *D3*-like EST from soybean (*Glycine max*). The conserved 3′ end of soybean gi9483278 (BE657386), nearly identical to gi6915567 (AW397097), was ^{32}P labeled using the Decalabel DNA labeling kit (MBI Fermentas, Burlington, ON, Canada).

Northern-Blot Analysis

Total RNA was extracted using either the Phenol/SDS Method (Ausubel et al., 1994; Fig. 4A) or the RNeasy Plant Kit (Qiagen USA, Valencia, CA; Fig. 4, B and C) consistent within the blot. The RNA (5 μg per lane) was fractionated in 1.5% (w/v) agarose gel containing formaldehyde and transferred to Genescreen Plus hybridization transfer membrane (PerkinElmer Life Sciences, Boston) using 10× SSC. The membrane was hybridized with a ^{32}P-labeled cDNA fragment of PsKAO1 or PsKAO2 at 42°C in 5× SSC, 5× Denhardts, 50% (w/v) formamide, 1% (w/v) SDS, and 200 μg mL^{-1} salmon sperm. The membrane was washed in 2× SSC and 0.1% (w/v) SDS, then 0.2× SSC and 0.1% (w/v) SDS at 65°C and exposed to Biomax x-ray film (Eastman-Kodak, Rochester, NY) at −70°C.

Cosegregation Analysis

DNA was extracted from the leaves of 50 individuals of four segregating families from the F_9 generation of cross WL1766 (*na-1*) × WL1769 (*NA*). The genomic *PsKAO1* PCR products (243 bp in the wild type) were visualized using a 5′ primer labeled with the fluorescent dye, hexachloro fluorescein. The PCR fragment encompassed the five-base deletion of the *na-1* mutant gene. The PCR products were denatured (94°C for 3 min) in loading buffer containing deionized formamide and bromphenol blue, then placed on ice before loading on a denaturing gel (5% [w/v] acrylamide gel in 0.6× Tris-borate/EDTA buffer containing 7 M urea) in the Gel-Scan 2000 (Corbett Research, Sydney).

Yeast (*Saccharomyces cerevisiae*) Expression

The constructs were prepared in the pYEDP60 plasmid vector (Pompon et al., 1996). Oligonucleotide primers with restriction sites incorporated at the 5′ end were designed and checked with the aid of the Oligo Primer Analysis Software (version 6.74, Molecular Biology Insights, Cascade, CO). The *PsKAO1* and *PsKAO2* cDNA were prepared from RNA extracted from WL1769 stems or L107 seeds, respectively. The cDNA PCR products encompassed the putative protein-coding sequence with the 5′-untranslated region as short as possible and were amplified using Pfu Turbo DNA polymerase (Stratagene). These PCR products were cloned into pGEM-T vector (Promega, Madison, WI) and sequenced to check for PCR-generated mutations. Selected clones were digested using restriction enzymes corresponding to the sites introduced in the PCR primers and ligated into pYEDP60 vector in the sense orientation with reference to the GAL10-CYC1 promoter (Pompon et al., 1996). An Arabidopsis AtKAO1 construct was used for comparison (Helliwell et al., 2001). The WAT11 and WAT21 yeast lines that are modified to express Arabidopsis NADPH-cytochrome P450 reductases, ATR1 and ATR2-1, respectively (Pompon et al., 1996; Urban et al., 1997), were transformed with the construct plasmids (Cullin and Pompon, 1988). The transformed yeasts and untransformed yeast as a control were incubated with 10 μg of the substrates (KA, *ent*-7α hydroxy kaurenoic acid, GA_{12}-aldehyde, or *ent*-kaurene) for 2 h at 28°C (Helliwell et al., 1999). In preparation for GC-MS analysis, methylation or trimethylsilylation was required. Extracts in about 2 mL of hexane/EtOAc were dried almost completely by speed vacuum, then to completion under nitrogen. Methylation was in the same test tubes by addition of 50 μL of MeOH and 400 μL of ethereal diazomethane. Samples were left for 15 min, dried as before, then transferred to reactivials using 4 × 50 μL EtOAc. These were dried and then trimethylsilylated using 5 μL of pyridine and 5 μL of bis(TMS) trifluoroacetamide + 1% (w/v) trimethylchlorosilane, which was heated at 90°C for 30 min (Helliwell et al., 1999). Injections were 1-μL samples with 0.1-μL parafilm standard. The KRIs were calculated using hydrocarbon peaks from the co-injected parafilm standard. Identities of products were confirmed by GC-MS comparison of spectra and KRI with authentic standards where possible. Alternatively, some side products were tentatively identified based on comparison with published spectra (Gaskin and MacMillan, 1991) and relative KRI values.

ACKNOWLEDGMENTS

We thank Jenny Smith and Adam J. Smolenski (University of Tasmania, Hobart, Australia) for technical assistance in the molecular laboratory, Ian Cummings and Tracey Jackson (University of Tasmania) for greenhouse assistance, Denis Pompon (Centre National de la Recherche Scientifique, Gif-sur-Yvette, France) for the WAT11 and WAT21 yeast strains, Bruce Twitchin and Professor Lewis Mander (Australian National University, Canberra) for the provision of authentic GA standards. We would also like to thank Dr. John Ross and Dr. L. Huub Kerckhoffs (University of Tasmania) for helpful discussions.

Received August 13, 2002; returned for revision September 10, 2002; accepted October 14, 2002.

LITERATURE CITED

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. Plant J **11:** 443–454

Altschul SF, Madden TL, Schaffer AA, Zhang JH, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res **25:** 3389–3402

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JG, Struhl K (1994) Current Protocols in Molecular Biology 1. Wiley Interscience, New York

Beveridge CA, Murfet IC (1996) The *gigas* mutant in pea is deficient in the floral stimulus. Physiol Plant **96:** 637–645

Bishop GJ, Nomura T, Yokota T, Harrison K, Noguchi T, Fujioka S, Takatsuto S, Jones JDG, Kamiya Y (1999) The tomato DWARF enzyme catalyses C-6 oxidation in brassinosteroid biosynthesis. Proc Natl Acad Sci USA **96:** 1761–1766

Brown JWS (1996) *Arabidopsis* intron mutations and pre-mRNA splicing. Plant J **10:** 771–780

Choe SW, Dilkes BP, Fujioka S, Takatsuto S, Sakurai A, Feldmann KA (1998) The DWF4 gene of Arabidopsis encodes a cytochrome P450 that mediates multiple 22 alpha-hydroxylation steps in brassinosteroid biosynthesis. Plant Cell **10:** 231–243

Cullin C, Pompon D (1988) Synthesis of functional mouse cytochromes P-450 P1 and chimeric P-450 P3–1 in the yeast *Saccharomyces cerevisiae*. Gene **65:** 203–217

Frohman MA, Dush MK, Martin GR (1988) Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc Natl Acad Sci USA **85:** 8998–9002

Frydman VM, Gaskin P, MacMillan J (1974) Qualitative and quantitative analyses of gibberellins throughout seed maturation in *Pisum sativum* cv. Progress no.9. Planta **118:** 123–132

Gaskin P, MacMillan J (1991) GC-MS of the gibberellins and related compounds: methodology and a library of spectra. University of Bristol, Cantock's Enterprises, Bristol, UK

Großelindemann E, Lewis MJ, Hedden P, Graebe JE (1992) Gibberellin biosynthesis from gibberellin A_{12}-aldehyde in a cell-free system from germinating barley (*Hordeum vulgare* L, Cv Himalaya) embryos. Planta **188:** 252–257

Gutierrez RA, MacIntosh GC, Green PJ (1999) Current perspectives on mRNA stability in plants: multiple levels and mechanisms of control. Trends Plant Sci **4:** 429–438

Hedden P (1997) The oxidases of gibberellin biosynthesis: their function and mechanism. Physiol Plant **101:** 709–719

Hedden P, Graebe JE, Beale MH, Gaskin P, MacMillan J (1984) The biosynthesis of 12α-hydroxylated gibberellins in a cell-free system from *Cucurbita maxima* endosperm. Phytochemistry **23:** 569–574

Hedden P, Kamiya Y (1997) Gibberellin biosynthesis: enzymes, genes and their regulation. Annu Rev Plant Physiol Plant Mol Biol **48:** 431–460

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

Hedden P, Phillips AL, Rojas MC, Carrera E, Tudzynski B (2002) Gibberellin biosynthesis in plants and fungi: a case of convergent evolution? J Plant Growth Regul **20:** 319–331

Hedden P, Proebsting WM (1999) Genetic analysis of gibberellin biosynthesis. Plant Physiol **119:** 365–370

Helliwell CA, Chandler PM, Poole A, Dennis ES, Peacock WJ (2001) The CYP88A cytochrome P450, *ent*-kaurenoic acid oxidase, catalyzes three steps of the gibberellin biosynthesis pathway. Proc Natl Acad Sci USA **98:** 2065–2070

Helliwell CA, Olive MR, Gebbie L, Forster R, Peacock WJ, Dennis ES (2000) Isolation of an *ent*-kaurene oxidase cDNA from *Cucurbita maxima*. Aust J Plant Physiol **27:** 1141–1149

Helliwell CA, Poole A, Peacock WJ, Dennis ES (1999) *Arabidopsis ent*-kaurene oxidase catalyzes three steps of gibberellin biosynthesis. Plant Physiol **119:** 507–510

Helliwell CA, Sheldon CC, Olive MR, Walker AR, Zeevaart JAD, Peacock WJ, Dennis ES (1998) Cloning of the Arabidopsis ent-kaurene oxidase gene GA3. Proc Natl Acad Sci USA **95:** 9019–9024

Hooley R (1994) Gibberellins: perception, transduction and responses. Plant Mol Biol **26:** 1529–1555

Imai M, Shimada H, Watanabe Y, Matsushima-Hibiya Y, Makino R, Koga H, Horiuchi T, Ishimura Y (1989) Uncoupling of the cytochrome P-450cam monooxygenase reaction by a single mutation, threonine-252 to alanine or valine: possible role of the hydroxy amino acid in oxygen activation. Proc Natl Acad Sci USA **86:** 7823–7827

Ingram TJ, Reid JB (1987) Internode length in *Pisum*1. Gene *na* may block gibberellin synthesis between *ent-7-alpha*-hydroxykaurenoic acid and gibberellin A12-aldehyde. Plant Physiol **83:** 1048–1053

Ingram TJ, Reid JB, MacMillian J (1986) The quantitative relationship between gibberellin A1 and internode growth in *Pisum sativum* L. Planta **168:** 414–420

Ingram TJ, Reid JB, Murfet IC, Gaskin P, Willis CL, MacMillian J (1984) Internode length in *Pisum*. Planta **160:** 455–463

Kalb VF, Loper JC (1988) Proteins from eight eukaryotic cytochrome P-450 families share a segmented region of sequence similarity. Proc Natl Acad Sci USA **85:** 7221–7225

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

Lange T (1997) Cloning gibberellin dioxygenase genes from pumpkin endosperm by heterologous expression off enzyme activities in *Escherichia coli*. Proc Natl Acad Sci USA **94:** 6553–6558

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 Regul **27:** 83–89

Lester DR, Ross JJ, Davies PJ, Reid JB (1997) Mendels stem length gene (*Le*) encodes a gibberellin 3-beta-hydroxylase. 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*. Plant J **19:** 65–73

MacMillan J (1997) Biosynthesis of the gibberellin plant hormones. Nat Prod Rep **14:** 221–243

Martin DN, Proebsting WM, Hedden P (1997) Mendel's dwarfing gene: cDNAs from the *Le* alleles and function of the expressed proteins. Proc Natl Acad Sci USA **94:** 8907–8911

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

Phillips AL, Ward DA, Uknes S, Appleford NEJ, Lange T, Huttly AK, Gaskin P, Graebe JE, Hedden P (1995) Isolation and expression of 3 gibberellin 20-oxidase cDNA clones from *Arabidopsis*. Plant Physiol **108:** 1049–1057

Pompon D, Louerat B, Bronine A, Urban P (1996) Yeast expression of animal and plant P450s in optimized redox environments. Methods Enzymol **272:** 51–64

Poole AT, Ross JJ, Lawrence NL, Reid JB (1995) Identification of gibberellin A_4 in *Pisum sativum* L. and the effects of applied gibberellins A_9, A_4, A_5 and A_3 on the *le* mutant. Plant Growth Regul **16:** 257–262

Potts WC (1986) Gibberellins in light-grown shoots of *Pisum sativum* L. and the influence of reproductive development. Plant Cell Physiol **27:** 997–1003

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. Physiol Plant **57:** 448–454

Poulos TL, Finzel BC, Gunsalus IC, Wagner GC, Kraut J (1985) The 2.6-A crystal structure of *Pseudomonas putida* cytochrome P-450. J Biol Chem **260:** 16122–16130

Proebsting WM, Hedden P, Lewis MJ, Croker SJ, Proebsting LN (1992) Gibberellin concentration and transport in genetic lines of pea: effects of grafting. Plant Physiol **100:** 1354–1360

Reid JB (1986a) Gibberellin mutants. *In* PJ King, AD Blonstein, eds, Plant Gene Research: A Genetic Approach to Plant Biochemistry, Vol 3. Springer-Verlag, New York, pp 1–34

Reid JB (1986b) Internode length in *Pisum*. Three further loci, *lh*, *ls* and *lk*. Ann Bot **57:** 577–592

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*. J Exp Bot **34:** 349–364

Reid JB, Ross JJ (1993) A mutant based approach, using *Pisum sativum*, to understand plant growth. Int J Plant Sci **154:** 22–34

Reid JB, Ross JJ, Swain SM (1992) Internode length in *Pisum*. A new, slender mutant with elevated levels of C19 gibberellins. Planta **188:** 462–467

Rojas MC, Hedden P, Gaskin P, Tudzynki B (2001) The P450-1 gene of *Gibberella fujikuroi* encodes a multifunctional enzyme in gibberellin biosynthesis. Proc Natl Acad Sci USA **98:** 5838–5843

Ross JJ, Murfet IC, Reid JB (1997) Gibberellin mutants. Physiol Plant **100:** 550–560

Ross JJ, Reid JB, Swain SM (1993) Control of stem elongation by gibberellin A1: evidence from genetic studies including the slender mutant *sln*. Aust J Plant Physiol **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*. Plant J **7:** 513–523

Sekimoto H, Seo M, Dohmae N, Takio K, Kamiya Y, Koshiba T (1997) Cloning and molecular characterization of plant aldehyde oxidase. J Biol Chem **272:** 15280–15285

Sekimoto H, Seo M, Kawakami N, Komano T, Desloire S, Liotenberg S, Marion-Poll A, Caboche M, Kamiya Y, Koshiba T (1998) Molecular cloning and characterization of aldehyde oxidases in *Arabidopsis thaliana*. Plant Cell Physiol **39:** 433–442

Seo M, Akaba S, Oritani T, Delarue M, Bellini C, Caboche M, Koshiba T (1998) Higher activity of an aldehyde oxidase in the auxin-overproducing superroot1 mutant of *Arabidopsis thaliana*. Plant Physiol **116:** 687–693

Sponsel VM, Schmidt FW, Porter SG, Nakayama M, Kohlstruk S, Estelle M (1997) Characterization of new gibberellin-responsive semidwarf mutants of *Arabidopsis*. Plant Physiol **115:** 1009–1020

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

Swain SM, Reid JB, Ross JJ (1993) Seed development in *Pisum*: The lh^i allele reduces gibberellin levels in developing seeds, and increases seed abortion. Planta **191:** 482–488

Swain SM, Ross JJ, Reid JB, Kamiya Y (1995) Gibberellins and pea seed development: expression of the lh^i, *ls* and le^{5839} mutations. Planta **195:** 426–433

Swofford DL (1999) PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland, MA

Szekeres M, Nemeth K, KonczKalman Z, Mathur J, Kauschmann A, Altmann T, Redei GP, Nagy F, Schell J, Koncz C (1996) Brassinosteroids rescue the deficiency of CYP90, a cytochrome P450, controlling cell elongation and de-etiolation in Arabidopsis. Cell **85:** 171–182

Talon M, Koornneef M, Zeevaart JAD (1990) Endogenous gibberellins in *Arabidopsis thaliana* and possible steps blocked in the biosynthetic pathways of the semidwarf *ga4* and *ga5* mutants. Proc Natl Acad Sci USA **87:** 7983–7987

Urban P, Mignotte C, Kazmaier M, Delorme F, Pompon D (1997) Cloning, yeast expression, and characterization of the coupling of two distantly related *Arabidopsis thaliana* NADPH-cytochrome P450 reductases with P450 CYP73A5. J Biol Chem **272:** 19176–19186

Winkler RG, Helentjaris T (1995) The maize *Dwarf3* gene encodes a cytochrome P450-mediated early step in gibberellin biosynthesis. Plant Cell **7:** 1307–1317

Xu YL, Li L, Wu KQ, Peeters AJM, Gage DA, Zeevaart JAD (1995) The *Ga5* locus of *Arabidopsis thaliana* encodes a multifunctional gibberellin 20-oxidase: molecular cloning and functional expression. Proc Natl Acad Sci USA **92:** 6640–6644

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

CSIRO PUBLISHING
www.publish.csiro.au/journals/fpb
Functional Plant Biology, 2003, **30**, 83–89

Developmental regulation of the gibberellin pathway in pea shoots

John J. Ross[A], *Sandra E. Davidson*[A], *Carla M. Wolbang*[A], *Emma Bayly-Stark*[A], *Jennifer J. Smith*[A] *and James B. Reid*[A,B]

[A]School of Plant Science, University of Tasmania, GPO Box 252–55, Hobart, Tas. 7001, Australia.
[B]Corresponding author; email: jim.reid@utas.edu.au

Abstract. To investigate gibberellin (GA) biosynthesis in mature tissue of pea (*Pisum sativum* L.) in the absence of potentially GA-producing meristematic tissue we grafted wild-type scions to rootstocks of the GA-deficient *ls-1* mutant and later decapitated the shoot. After 2 d, decapitated shoots contained as much GA_{19} (a precursor of the bioactive GA_1) as comparable tissue from intact plants, even though applied [^{14}C]GA_{19} was metabolised rapidly during this time. These results show that the pool size of endogenous GA_{19} was maintained, probably by *de novo* GA_{19} synthesis. We also found that the *LS* gene, which catalyses an early step in GA biosynthesis, is expressed in mature tissue, as are the shoot-expressed GA 20-oxidase and GA 3-oxidase genes. Nevertheless, mature tissue contained very low levels of GA_1 and GA_{20} compared with immature tissue. Levels of GA_{19}, GA_{29} and GA_8 were less affected by tissue age. Metabolism studies using ^{14}C-labelled GAs indicated that mature tissue rapidly converted GA_{19} to GA_{20} and GA_{20} to GA_1; the latter step was promoted by IAA. However, the 2-oxidation steps GA_1 to GA_8, GA_{20} to GA_{29} and GA_{29} to GA_{29}-catabolite appear to proceed very rapidly in mature tissue (regardless of IAA content), and we suggest this is the reason why GA_1 and GA_{20} do not accumulate. This is supported by the high level of expression of a key GA 2-oxidase gene in mature tissue.

Introduction

GAs play a critical role in stem elongation (Kende and Zeevaart 1997), and regulation of GA biosynthesis has received much attention (Hedden and Phillips 2000). Studies involving GA mutants have identified the 'feedback' phenomenon (Croker *et al.* 1990; Scott 1990) whereby bioactive GAs, such as GA_1, negatively regulate their own biosynthesis. Recent results indicate that GA biosynthesis in pea is strongly affected by the level of another growth hormone, auxin (Ross *et al.* 2000), and by the nature of the light regime (Ait-Ali *et al.* 1999; Gil and Garcia-Martinez 2000; Reid *et al.* 2002).

Gibberellin biosynthesis is also developmentally regulated (Hedden and Phillips 2000; Yamaguchi and Kamiya 2000; Chang and Sun 2002). For example, there is clear evidence that the apical portions of WT pea plants contain more GA_1 per gram fresh weight than mature tissue (Proebsting *et al.* 1992; Smith *et al.* 1992; Ross 1998; O'Neill *et al.* 2000). One explanation for this localisation is that synthesis of the GA precursor *ent*-kaurene is restricted mainly to elongating, apical tissue (Coolbaugh 1985; Chung and Coolbaugh 1986; Aach *et al.* 1997; Hedden 1999). *ent*-Kaurene is synthesised from geranylgeranyl diphosphate (GGPP) via two steps (Fig. 1). In *Arabidopsis* and pumpkin, the first of these reactions (but not the second) is thought to be developmentally regulated at the mRNA level (Smith *et al.* 1998; Hedden and Phillips 2000; Yamaguchi and Kamiya 2000). In pea, the enzyme for the first step [conversion of GGPP to copalyl diphosphate (CPP); Fig. 1] is encoded by the gene *LS* (Ait-Ali *et al.* 1997), but little is known about the *LS* expression pattern in the vegetative shoot.

The evidence that *ent*-kaurene synthesis occurs mainly in immature tissue in pea (Coolbaugh 1985; Chung and Coolbaugh 1986) might imply that the mature tissue plays little, if any, role in GA biosynthesis. On the other hand, mature tissues are not devoid of GAs (Proebsting *et al.* 1992), and mature pea internodes have been shown to contain mRNA for a later GA biosynthesis gene (a GA 20-oxidase; Garcia-Martinez *et al.* 1997). Furthermore, strong evidence that mature tissue can synthesise GA_1 precursors has been obtained from grafting studies (Reid *et al.* 1983; Proebsting *et al.* 1992). The most instructive grafts in this respect are those involving the *na* mutation, which strongly blocks GA biosynthesis, between *ent*-kaurenoic acid and GA_{12}-aldehyde (Fig. 1; Davidson *et al.* 2003). Incorporation of WT leaves and internodes ('interstocks') into grafts consisting otherwise of genotype *na* markedly stimulated elongation of the *na* scions, compared with grafts consisting entirely of *na* tissue (Reid *et al.* 1983). Grafts involving Mendel's *le-1* (dwarf) mutant provided convincing

Abbreviations used: CPP, copalyl diphosphate; GA, gibberellin; GC-MS, gas chromatography-mass spectrometry; GGPP, geranylgeranyl diphosphate; WT, wild type.

© CSIRO 2003
10.1071/FP02108
1445-4408/03/010083

evidence that it is not GA_1 itself that moves across the graft union (Reid *et al.* 1983). Proebsting *et al.* (1992) also used the *na* mutant to obtain evidence for the graft-transmissibility of GA_1 precursors. The *na*–WT grafting results (Reid *et al.* 1983; Proebsting *et al.* 1992) suggest that GA_1 precursors, such as GA_{19} and/or GA_{20} (Fig. 1), might be synthesised in mature WT tissue, exported into the *na* scion, and then converted into the growth-promoting GA_1. However, it cannot be excluded that in these experiments the mature tissue simply acted as a store for GAs that had been synthesised when mature tissue was itself immature.

Another explanation for the distribution of GA_1, which accommodates the possibility that GA_1 precursors are synthesised in mature tissue, is that only immature tissue can produce GA_1 from its immediate precursor, GA_{20} (Ingram *et al.* 1985). According to this hypothesis, mature tissue might be capable of synthesising GA_{20}, but incapable of converting GA_{20} to GA_1.

In this paper we further investigate the developmental regulation of GA biosynthesis in pea. In particular, we address the question of whether mature, fully expanded tissues can synthesise GA_1 precursors. We also compare GA metabolism in mature and immature tissue, and investigate the expression of the *LS*, GA 20-oxidase (*PsGA20ox1*), GA 3-oxidase (*PsGA3ox1*, *LE*) and GA 2-oxidase (*PsGA2ox1*) genes in a range of plant parts. Finally, we report on the role of the auxin IAA in regulating the GA pathway in mature internodes.

Materials and methods

We used two WT lines (107 and 205+) and the mutant *ls-1*, which is near-isogenic with 107 (derived from cv. Torsdag). The *ls-1* mutant is GA-deficient, with reduced elongation of both shoots and roots (Reid 1986; Yaxley *et al.* 2001). The growth medium was a 1:1 mixture of dolerite chips and vermiculite, topped with 20–30 mm of sterilised potting mix. Plants were grown in a heated glasshouse (Beveridge and Murfet 1996) with an 18-h photoperiod provided by extending the natural photoperiod at its beginning and end with a mixture of fluorescent (40 W cool white; Osram, Munich, Germany) and incandescent (100 W; Thorn, Sydney, Australia) light (25 μmol photons m^{-2} s^{-1} at pot top). Experiments were either repeated with similar results or included replicates within treatments, or both. A replicate consisted of 4–10 plants.

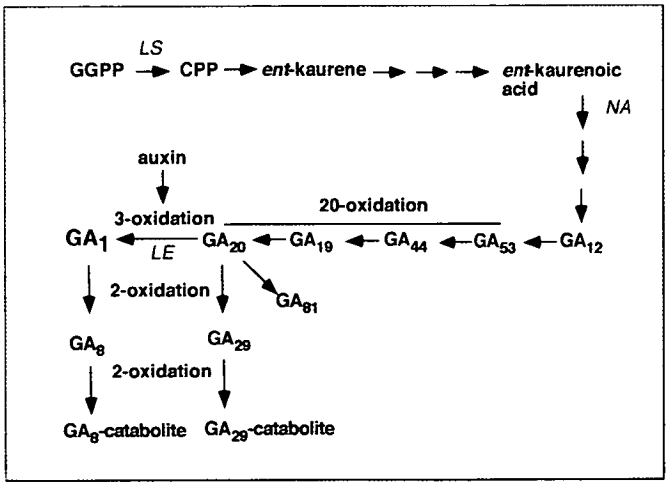


Fig. 1. GA biosynthesis pathway in pea shoots. CPP, copalyl diphosphate; GGPP, geranylgeranyl diphosphate.

To construct plants without growing tips that might act as sources of GAs, we firstly grafted WT scions to *ls-1* rootstocks, recently shown to be GA-deficient (Yaxley *et al.* 2001). Grafts were performed epicotyl-to-epicotyl, when the scions and rootstocks were 5- and 6-d-old, respectively, as described previously (Reid *et al.* 1983). When the scions possessed 10 fully-expanded leaves (counting from the cotyledons as zero, and including the two basal scale leaves), the apical portion of half of the plants was excised 15 mm above node 9, and (to prevent drying of the excision site) lanolin was applied to the stump. One-third of both decapitated and intact plants were then treated (on leaf 8) with 1500 Bq (273 ng) $plant^{-1}$ of [17-^{14}C]GA_{19} (2 GBq $mmol^{-1}$; 5.5 Bq = 1 ng; from Professor L. N. Mander, Australian National University, ACT) in 10 μL ethanol. The treated leaf was excised after 1 d. Axillary buds were removed over the course of the experiment. Two days after decapitation, the [^{14}C]GA_{19}-treated plants were harvested. Remaining plants were also harvested for determination of endogenous GA levels. Parts harvested were an apical portion (intact plants only) consisting of all material above node 10, a mature portion consisting of all material between nodes 7 and 9 (excluding the leaf at 7, but including leaves at 8 and 9), and a basal portion consisting of all remaining material down to the graft union (for measurement of endogenous GAs only). Thus, in this experiment, the apical and mature portions were separated by one internode (that between nodes 9 and 10).

We also investigated the metabolism of [^{14}C]GA_{20} (2 GBq $mmol^{-1}$; 6 Bq = 1 ng; from Professor L. N. Mander). Substrate was applied to immature or mature leaves in 10 μL ethanol. Treated leaves were harvested after 2 d and washed by brief dipping into distilled water. To investigate effects of IAA, 205+ plants that had just expanded their eleventh leaf were selected, and two-thirds were decapitated 20 mm above node 9. Substrate was then applied at a rate of 1000 Bq (166 ng) $plant^{-1}$ in 10 μL ethanol to leaf 8 of decapitated plants and half of the intact plants, and to leaf 11 of the remaining intact plants. Half of the decapitated plants were treated with lanolin containing 3000 ppm IAA (as before, Ross *et al.* 2000). The remaining decapitated plants received lanolin only. IAA and lanolin were re-applied after 8, 21, 32 and 45 h. Material was harvested 2 d after decapitation. From intact plants treated with substrate on leaf 11 we harvested the internode between nodes 11 and 12 and the internodes between nodes 12 and 14 (combined). For all other cases internode 8–9 was harvested. Leaves to which substrate was applied were also harvested.

Harvested material was immediately immersed in cold (–20°C) 80% methanol containing 250 mg L^{-1} butylated hydroxytoluene, and placed in a freezer. Material was then homogenised, extracted at 3°C for 24 h and filtered. Extracts containing ^{14}C-labelled GA metabolites were purified using Sep-Pak C18 cartridges as before (Ross *et al.* 1995), methylated and subjected to HPLC as methyl esters, as described previously (Ross *et al.* 1995). The solvent program ran from 30 to 60% methanol (exponential gradient) in distilled water over 35 min, followed by isocratic elution. The flow rate was 1.6 mL min^{-1}, and 1 min fractions were collected and assayed for radioactivity by scintillation counting. In some cases, aliquots were taken (before counting) for identification of metabolites by gas chromatography-mass spectrometry (GC-MS).

For measurement of endogenous GAs, internal standards were added, with the amount depending on previous findings. Internal standards were [$^2H_2$]GA_{19}, [$^2H_2$]GA_{20}, [$^2H_2$]GA_1, [$^2H_2$]GA_8, [$^2H_2$]GA_{29} and [$^2H_2$]GA_{29}-catabolite, provided by Professor L. N. Mander.

Extracts for endogenous GA measurements were purified by Sep-Pak (Ross *et al.* 1995) and HPLC as free acids, methylated, trimethylsilylated, and analysed by GC-MS (selected ion monitoring mode). GC-MS conditions were as described previously (Ross 1998).

Expression of the genes *LS*, *PsGA20ox1*, *PsGA3ox1* and *PsGA2ox1* was monitored by northern analysis. Total RNA was extracted using RNEasy Plant Kit (Qiagen, Hilden, Germany) and quantified by UV spectroscopy. RNA (5 μg per lane) was fractionated in 1% agarose gel (1.5% for the blot probed with the *LS* gene) containing formaldehyde and transferred to GeneScreen Plus hybridisation transfer membranes (Perkin Elmer Life Sciences, Boston, MA, USA) using 10× saline sodium citrate (SSC). Membranes were hybridised at 42°C in 5× SSC, 5× Denhardt's, 50% formamide, 1% SDS and 200 mg mL^{-1} sheared salmon sperm DNA. DNA probes were labelled with ^{32}P by random priming using a DecaLabel kit (MBI Fermentas; Progen, Heidelberg, Germany). Templates for probes were polymerase chain reaction (PCR) products generated from the cDNAs using gene-specific primers. For *LS*, nested primers were also used in a second round of PCR, and for the other genes the cDNAs were ligated into pGEM-T (Promega, Madison, WI, USA) prior to PCR. The 1.7-kb *LS* PCR product was gel-purified. The other PCR products were purified using the QIAquick PCR Purification kit (Qiagen). The *PsGA20ox1* PCR product was 700 bp, and the *PsGA3ox1* and *PsGA2ox1* PCR products were approximately 1 kb each. Blots were washed in 2× SSC, 0.1% SDS at 42°C (65°C for *LS*), then in 0.2× SSC, 0.1% SDS at 65°C, and exposed to Kodak Biomax X-ray film at −70°C.

Results and discussion

Evidence for GA biosynthesis in mature tissue

To investigate GA biosynthesis in mature tissue in the absence of potentially GA-producing meristematic tissue, we grafted WT scions to rootstocks of the GA-deficient *ls-1* mutant (Yaxley *et al.* 2001) and (after 23 d) decapitated the shoot, leaving only mature tissue in the shoot system. Decapitation and grafting to *ls-1* rootstocks precluded significant import of GAs into mature tissue from the shoot apical bud and roots, respectively. Roots of the *ls-1* mutant are grossly deficient in GAs, especially GA_{19}, previously shown to be present at less than 1% of WT level (Yaxley *et al.* 2001). Material was harvested 2 d after decapitation. The potential outgrowth of axillary buds in this time was limited (< 1 mm), but these buds were nevertheless removed. In these experiments, decapitation did not reduce GA_{19} and GA_{20} content in the remaining shoot tissue (Table 1), compared with the mature tissue of accompanying intact plants. (Similar results were obtained in an additional experiment, in which it was also shown that the level of GA_{19} in mature tissue remained constant over the 2 d of the experiment; data not shown).

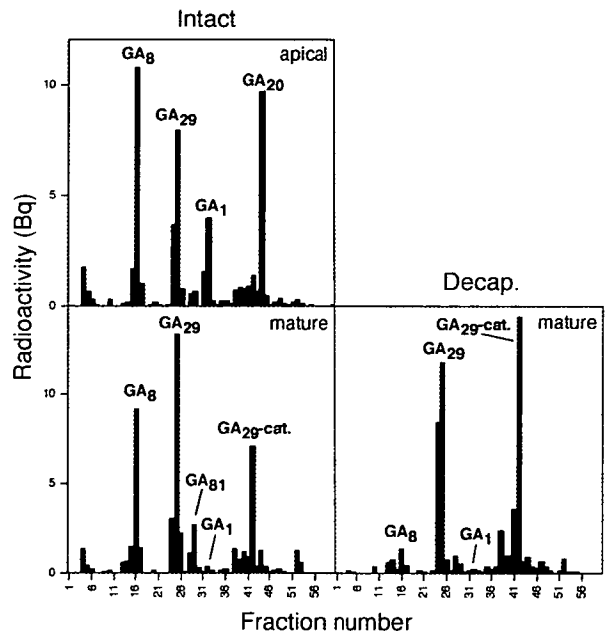


Fig. 2. $[^{14}C]GA_{19}$ metabolism in apical and mature portions (consisting of leaves and internodes) of wild-type (line 107) shoots grafted to *ls-1* rootstocks and either left intact or decapitated 2 d before harvest. Identities of peaks are indicated, based on HPLC retention times and GC-MS analyses. Amounts of radioactivity (Bq g^{-1} fresh weight) recovered were: intact apical, 155 ± 2.5; intact mature, 49 ± 0.6; decapitated mature, 104 ± 3.6. Additional data from this experiment are shown in Table 1.

In the experiment represented in Table 1, $[^{14}C]GA_{19}$ was applied to leaves of additional plants at the same time that decapitation was performed. After 2 d, the recovery of $[^{14}C]GA_{19}$ substrate from both decapitated and intact tissue (consisting of internodes and leaves other than the treated leaf) was extremely low (fractions 51–53), while metabolites of $[^{14}C]GA_{19}$ comprised large peaks (Fig. 2). These metabolites were identified by full-scan GC-MS. For all metabolites, the ^{14}C-labelled form was less abundant than the endogenous GA (Table 2). The $[^{14}C]GA_{19}$ substrate was also rapidly metabolised in leaves to which it was applied (data not shown).

Table 1. Levels of endogenous GAs (ng g^{-1} fresh weight) in apical, mature and basal portions (consisting of leaves and internodes) of WT (line 107) shoots grafted to *ls-1* rootstocks and either left intact or decapitated 2 d before harvest

Additional data from this experiment are shown in Fig. 2. Data are means ± s.e. of two replicate harvests

Portion	GA_{19}	GA_{20}	GA_1	GA_8	GA_{29}	GA_{29}-catabolite	Sum of GAs measured
Intact, apical	6.3 ± 0.2	16.0 ± 0.2	5.3 ± 0.6	21.2 ± 0.6	10.0 ± 0.7	1.1 ± 0.07	59.9
Intact, mature	3.8 ± 0.2	0.8 ± 0.06	0.18 ± 0.01	6.4 ± 0.4	4.0 ± 0.3	2.4 ± 0.13	17.6
Intact, basal	2.9 ± 0.2	0.44 ± 0.08	0.05 ± 0.01	1.5 ± 0.01	2.2 ± 0.2	6.0 ± 0.3	13.1
Decapitated, mature	4.6 ± 0.2	2.5 ± 0.1	0.23 ± 0.01	5.1 ± 0.4	5.1 ± 0.2	2.9 ± 0.2	20.4
Decapitated, basal	4.6 ± 0.5	1.5 ± 0.16	0.06 ± 0.01	1.4 ± 0.05	3.1 ± 0.1	7.8 ± 0.3	18.5

Table 2. Examples of mass spectra obtained to identify metabolites of [^{14}C]GA_{19}

Metabolites were analysed as methyl ester trimethylsilyl ethers by full scan mass spectrometry. HPLC fraction number is shown (see Fig. 2), as is the Kovats retention index (KRI), with the KRI of the corresponding authentic GA in parentheses. There was approximately 25% of unlabelled material in the [^{14}C]GA_{19} substrate. All metabolite spectra consisted mainly of endogenous (unlabelled) material

Metabolite identified	HPLC fraction	KRI	Ions (abundance)
GA_8	15	2813 (2814)	596 (60), 595 (52), 594 (100), 581 (4), 579 (9), 450 (11), 448 (22)
GA_{29}	23	2687 (2686)	508 (74), 507 (43), 506 (100), 493 (10), 491 (15), 449 (13), 447 (13), 377 (15), 375 (17), 305 (30), 303 (32), 209 (33), 207 (40)
GA_1	29, 30	2673 (2672)	508 (67), 507 (33), 506 (100), 450 (19), 449 (16), 448 (29), 447 (16), 378 (19), 376 (23)
GA_{20}	43	2494 (2497)	420 (39), 419 (36), 418 (100), 405 (8), 403 (21), 377 (35), 375 (78), 361 (9), 359 (26), 303 (10), 301 (18), 209 (13), 207 (36)

The rapid metabolism of applied [^{14}C]GA_{19} during the course of the experiment suggests that endogenous GA_{19} was also rapidly metabolised. Furthermore, there was no evidence that the maintenance of endogenous GA_{19} levels in decapitated tissue was due to a decreased rate of GA_{19} metabolism, compared with intact tissue. The observation that endogenous GA_{19} levels were maintained, even though the GA_{19} turnover rate was high, indicates a continual input into the GA_{19} pool in mature tissue.

The basis for this reasoning is that the metabolism of applied [^{14}C]GA_{19} is similar to that of endogenous GA_{19}. Evidence that this is the case comes from previous studies on GA-pathway mutants such as *le-1* and *sln* (Ingram *et al.* 1984; Proebsting *et al.* 1992; Ross *et al.* 1995). For the *le-1* mutant, both the metabolism of applied radiolabelled GA_{20} and measurements of endogenous GA levels indicate the same conclusion; that the mutation blocks GA_{20} 3-oxidation. Also, with the *sln* mutant, both approaches indicate that *sln* blocks GA_{20} 2-oxidation (Ross *et al.* 1995).

We suggest that in the present case, mature tissue maintained GA_{19} levels either by synthesising GAs *de novo* via *ent*-kaurene, or by converting a putative post-*ent*-kaurene storage product into GA_{19}. The first possibility is supported by the finding (Fig. 3) that the *LS* gene, which encodes the enzyme for the first committed step in *ent*-kaurene biosynthesis (Fig. 1), was expressed in mature internodes. Indeed, *LS* expression was greater in mature internodes than in the apical bud (Fig. 3) on the basis of band intensity observed after loading the same amount of total RNA. Thus, while there appears to be developmental regulation of *LS* expression, it is in the opposite direction to that expected if *ent*-kaurene biosynthesis is confined to the apical bud. Interestingly, the *GA1* gene of *Arabidopsis*, which also encodes a CPP synthase, is expressed in mature leaves, and it has been suggested that these leaves export GAs to other organs (Silverstone *et al.* 1997; Sun and Kamiya 1997). The gene *PsGA20ox1*, which encodes the enzyme for metabolism of GA_{53} to GA_{44}, GA_{19} and GA_{20} (Fig. 1), was also expressed in mature leaves (Fig. 4).

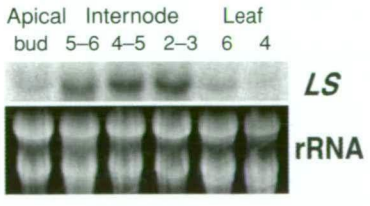


Fig. 3. Northern analysis of *LS* mRNA levels in various portions of wild-type (line 107) shoots. Fifteen-day-old plants with six fully-expanded leaves were partitioned into the following parts: apical bud, harvested above node 6; internodes between nodes 5 and 6, 4 and 5, and 2 and 3; leaf at node 6; leaf at node 4.

The evidence that mature tissue can synthesise GA_{19} is consistent with the finding that the GA_{19} content of mature tissue was not dramatically lower than in that of the apical portion (Table 1). Proebsting *et al.* (1992) also found that GA_{19} levels did not diminish in mature tissue (compared with young tissue) to the same extent as GA_1 or GA_{20}. In fact, in our experiment, the total level of all GAs and GA catabolites analysed was reduced by only about 3-fold in mature tissue, compared with the apical bud. Data in Table 1 are from shoots grafted to *ls-1* rootstocks, but similar observations have been made from intact plants (data not shown).

Evidence for the rapid 2-oxidation of GA_{20} in mature tissue

The preceding results indicate that mature tissue is capable of synthesising GA_1 precursors such as GA_{19}. However, mature tissue contained low levels of GA_{20} and GA_1 compared with the apical bud (there were reductions of 20- and 30-fold, respectively; Table 1). The low GA_{20} level in mature tissue did not appear to result from an inability to produce GA_{20} from GA_{19}, since mature tissue from both intact and decapitated shoots contained large peaks corresponding to the 2-oxidation products of [^{14}C]GA_{20} (namely [^{14}C]GA_{29} and [^{14}C]GA_{29}-catabolite) after feeds of [^{14}C]GA_{19} (Fig. 2). In the case of decapitated shoots, the [^{14}C]GA_{29} and [^{14}C]GA_{29}-catabolite peaks could not have originated in the apical bud. The low levels of GA_{20} (and GA_1) in mature tissue might be due to rapid 2-oxidation of these GAs. This idea is consistent with evidence that the

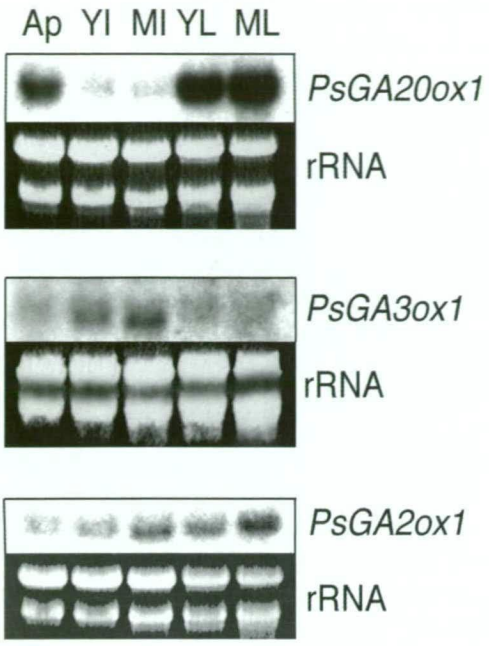


Fig. 4. Northern analysis of *PsGA20ox1*, *PsGA3ox1* and *PsGA2ox1* mRNA levels in various portions of wild-type (line 107) shoots. Forty-four-day-old plants with 12 fully-expanded leaves were partitioned into: apical bud (Ap), harvested above node 12 (including some young internode); young internode (YI), between nodes 11 and 12 (60–90% expanded); mature internode (MI), between nodes 8 and 9; young leaf (YL), at node 12; leaf (ML), at node 9. RNA (5 μg) was loaded in each lane. Exposure time was 16 h for *PsGA20ox1* and 48 h for the other genes.

gene encoding a key GA 2-oxidase, *PsGA2ox1*, was more strongly expressed in mature leaves and internodes than in immature leaves and internodes (Fig. 4).

Therefore, we directly investigated $[^{14}C]GA_{20}$ metabolism in immature and mature tissue. The $[^{14}C]GA_{20}$ substrate was applied to leaves, which were harvested after 2 d. HPLC analysis of extracts from these leaves showed that mature leaves converted a higher proportion of substrate to $[^{14}C]GA_{29}$ than immature leaves (Table 3). This was the case for two markedly different dosages of substrate. These results support the idea that GA_{20} 2-oxidation proceeds rapidly in mature tissue, resulting in low GA_{20} levels.

Interestingly, the GA 3-oxidase gene *PsGA3ox1* was also expressed slightly more in mature internodes than in immature internodes (Fig. 4). Expression of *PsGA20ox1* was consistently higher in leaves than in internodes, while *PsGA3ox1* transcript levels showed the reverse pattern (Fig. 4). These results suggest that GA_{20} production may be greater in leaves, while GA_1 production might occur mainly in internodes.

Evidence that the low level of GA_1 in mature tissue is not due to low IAA content

We also investigated the effects of IAA on $[^{14}C]GA_{20}$ metabolism in mature tissue, because our previous work revealed dramatic effects of this auxin on the GA pathway in immature tissue (Ross *et al.* 2000). We tested whether or not the inability of mature tissue to accumulate GA_1 is due to their relatively low IAA level (Ross 1998) by feeding $[^{14}C]GA_{20}$ to intact and decapitated plants.

Decapitation reduced IAA content in mature internodes (Fig. 5, caption), and this was accompanied by a reduced $[^{14}C]GA_8$ peak (compared with intact internodes; Fig. 5). Application of IAA to decapitated internodes restored the pattern of $[^{14}C]GA_{20}$ metabolites to that observed in intact mature internodes (Fig. 5). This indicates that in mature tissue, as in immature tissues (Ross *et al.* 2000), IAA is required for 3-oxidation products to accumulate to levels found in intact plants. However, even though IAA application resulted in high levels of IAA in mature internodes (see Fig. 5 caption), it did not result in chromatograms similar to those from immature internodes. IAA-treated mature internodes still contained much less $[^{14}C]GA_1$ and more $[^{14}C]GA_{29}$-catabolite than did young internodes from intact plants (Fig. 5). This indicates that the relatively low IAA content of mature internodes is not responsible for their inability to accumulate GA_1. Again, it appears that GA 2-oxidation is a dominant process in mature tissue, even in IAA-treated plants, and that as a result, GA_1 was rapidly deactivated to GA_8.

Conclusions

In conclusion, there is strong developmental regulation of GA biosynthesis and deactivation in pea shoots. The

Table 3. Effects of leaf maturity on 2-oxidation of $[^{14}C]GA_{20}$ to $[^{14}C]GA_{29}$

$[^{14}C]GA_{20}$ was applied to either leaf 12 (immature, unexpanded at time of treatment) or leaf 8 (fully mature) of line 205+ plants with 11 leaves expanded. There were two doses: 1000 Bq plant^{-1} (Experiment 1) or 67 Bq plant^{-1} (Experiment 2). Treated leaves were harvested after 2 d. Data are means ± s.e. of two replicate harvests

Experiment	Leaf to which substrate was applied	Radioactivity (Bq g^{-1} fresh weight) co-eluting with: $[^{14}C]GA_{20}$	$[^{14}C]GA_{29}$	Ratio of $[^{14}C]GA_{20}$ to $[^{14}C]GA_{29}$
1	12 (immature)	314 ± 20	22 ± 1.3	14 ± 1.5
	8 (mature)	100 ± 9	40 ± 6	2.5 ± 0.14
2	12 (immature)	9.6 ± 0.6	0.5 ± 0.1	19 ± 3.7
	8 (mature)	3.4 ± 0.4	3.8 ± 0.1	0.9 ± 0.1

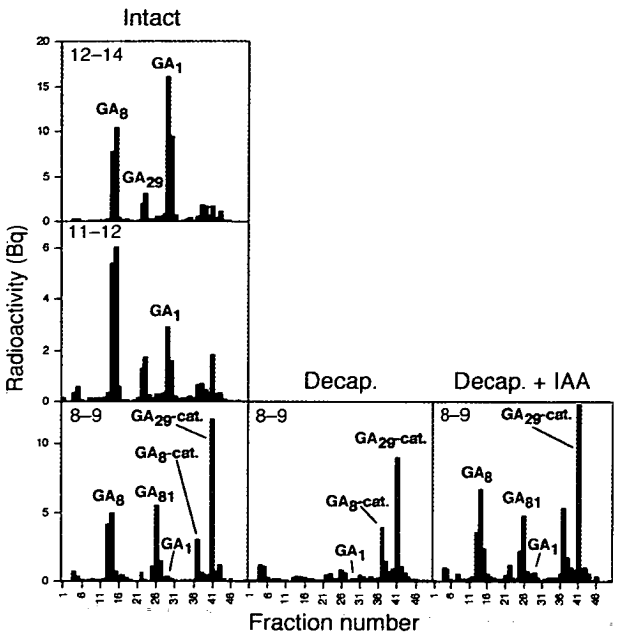


Fig. 5. [^{14}C]GA_{20} metabolism in immature internodes (internodes 12–14, combined; radioactivity recovered, 25 Bq g^{-1} fresh weight), mature internodes (8–9; 19 Bq g^{-1} fresh weight) and intermediate internodes (11–12; 10 Bq g^{-1} fresh weight) from intact plants of wild-type line 205+, and in internode 8–9 from decapitated plants (21 Bq g^{-1} fresh weight) and decapitated plants treated with IAA (40 Bq g^{-1} fresh weight), of wild-type line 205+. Plants harvested for analysis of internodes 11–12 and 12–14 were treated with substrate on leaf 11, and all other plants (intact or decapitated) on leaf 8. Identities of peaks are indicated, based on HPLC retention times and GC-MS analyses. IAA levels in internodes were (ng g^{-1} fresh weight): intact internode 12–14, 448; intact internode 11–12, 224; intact internode 8–9, 121; decapitated 8–9, 29; decapitated + IAA 8–9, 533.

bioactive GA, GA_1, and its immediate precursor, GA_{20}, are essentially restricted to young, expanding tissue in the apical portion, where GA_1 promotes growth. Levels of the other GAs monitored, however, were much less affected by tissue age. Our results suggest that mature tissue is capable of maintaining GA_{19} content without importing GA_{19} or GA_{19} precursors from other tissues. Furthermore, the *LS* gene, which encodes CPP synthase, is expressed relatively strongly in mature shoot tissue, as are genes that catalyse the later stages of GA metabolism, such as *PsGA20ox1*, *PsGA3ox1* and *PsGA2ox1*. These results contrast with the emerging idea (Hedden 1999; Davies 2002), that *ent*-kaurene biosynthesis and/or GA biosynthesis in general is restricted to immature tissues. This perception is based largely on papers by Aach *et al.* (1995, 1997), who reported that proplastids from immature pea tissues are capable of synthesising *ent*-kaurene, while mature chloroplasts cannot. However, our results do not necessarily contradict those of Aach *et al.*, who also showed that leucoplasts are capable of synthesising *ent*-kaurene (Aach *et al.* 1995). This type of plastid can occur in mature cells (Carde 1984). Interestingly, the *GA1* gene, which encodes the enzyme for *ent*-kaurene synthesis in *Arabidopsis*, is also expressed in mature tissues (Silverstone *et al.* 1997). Our present results are certainly consistent with previous grafting studies, which still provide some of the best evidence that GA_1 precursors can be transported from mature tissue to the growing tip of pea shoots (Reid *et al.* 1983; Proebsting *et al.* 1992).

In the present study, mature tissue was capable of rapidly converting [^{14}C]GA_{19} to [^{14}C]GA_{20}, [^{14}C]GA_{20} to [^{14}C]GA_1 and [^{14}C]GA_{29}, and apparently [^{14}C]GA_1 to [^{14}C]GA_8 and [^{14}C]GA_{29} to [^{14}C]GA_{29}-catabolite (Fig. 2). A major reason for the low levels of GA_{20} and GA_1 in mature pea tissue appears to be the rapid 2-oxidation (deactivation) of these GAs. It is suggested that as internodes (and leaves) complete expansion, 2-oxidation is strongly upregulated, and that this results in rapid depletion of GA_{20} and GA_1. This is supported by high expression levels of *PsGA2ox1*. While it is possible that mature tissue also synthesises slightly less GA in general than immature tissue, this does not appear to be the primary cause of the very large difference in GA_1 content between mature and immature tissue.

Acknowledgments

We thank Dr Noel Davies (Central Science Laboratory, University of Tasmania), Tracey Jackson and Ian Cummings for technical assistance, Professor Lewis Mander (Australian National University, Canberra) for labelled GAs, and the Australian Research Council for financial assistance.

References

Aach H, Bode H, Graebe JE (1995) *ent*-Kaurene biosynthesis in a cell-free system from wheat (*Triticum aestivum* L.) seedlings and the localisation of *ent*-kaurene synthetase in plastids of three species. *Planta* **197**, 333–342.

Aach H, Bode H, Robinson DG, Graebe JE (1997) *ent*-Kaurene synthase is located in proplastids of meristematic shoot tissues. *Planta* **202**, 211–219.

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

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

Beveridge CA, Murfet IC (1996) The *gigas* mutant in pea is deficient in the floral stimulus. *Physiologia Plantarum* **96**, 637–645.

Carde JP (1984) Leucoplasts: a distinct kind of organelle lacking typical 70S ribosomes and free thylakoids. *European Journal of Cell Biology* **34**, 18–26.

Chang C-w, Sun T-p (2002) Characterisation of *cis*-regulatory regions responsible for developmental regulation of the gibberellin biosynthetic gene *GA1* in *Arabidopsis thaliana*. *Plant Molecular Biology* **49**, 579–589.

Chung CH, Coolbaugh RC (1986) *ent*-Kaurene biosynthesis in cell-free extracts of excised parts of tall and dwarf pea seedlings. *Plant Physiology* **80**, 544–548.

Coolbaugh RC (1985) Sites of gibberellin biosynthesis in pea seedlings. *Plant Physiology* **78**, 655–657.

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** (in press)

Davies PJ (2002) Gibberellins: regulators of plant height. In 'Plant physiology (3rd edn)'. (Eds L Taiz and E Zeiger) pp. 461–492. (Sinauer Associates: Stamford, CT)

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.

Garcia-Martinez JL, Lopez-Diaz I, Sanchez-Beltran MJ, Phillips AL, Ward DA, Gaskin P, Hedden P (1997) Isolation and transcript analysis of gibberellin 20-oxidase genes in pea and bean in relation to fruit development. *Plant Molecular Biology* **33**, 1073–1084.

Gil J, Garcia-Martinez JL (2000) Light regulation of gibberellin A_1 content and expression of genes coding for GA 20-oxidase and GA 3β-hydroxylase in etiolated pea seedlings. *Physiologia Plantarum* **108**, 223–229.

Hedden P (1999) Regulation of gibberellin biosynthesis. In 'Biochemistry and molecular biology of plant hormones'. (Eds PJJ Hooykaas, MA Hall and KR Libbenga) pp. 161–187. (Elsevier Science: Amsterdam)

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

Ingram TJ, Reid JB, Murfet IC, Gaskin P, Willis CL, MacMillan J (1984) Internode length in *Pisum*. The *Le* gene controls the 3β-hydroxylation of gibberellin A_{20} to gibberellin A_1. *Planta* **160**, 455–463.

Ingram TJ, Reid JB, MacMillan J (1985) Internode length in *Pisum sativum* L. The kinetics of growth and [^{3}H]gibberellin A_{20} metabolism in genotype *na Le*. *Planta* **164**, 429–438.

Kende H, Zeevaart JAD (1997) The five classical plant hormones. *The Plant Cell* **9**, 1197–1210.

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

O'Neill DP, Ross JJ, Reid JB (2000) Changes in gibberellin A_1 levels and response during de-etiolation of pea seedlings. *Plant Physiology* **124**, 805–812.

Proebsting WM, Hedden P, Lewis MJ, Croker SJ, Proebsting LN (1992) Gibberellin concentration and transport in genetic lines of pea. *Plant Physiology* **100**, 1354–1360.

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

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, 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.

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

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.

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.

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

Silverstone AL, Chang C-w, Krol E, Sun T-p (1997) Developmental regulation of the gibberellin biosynthetic gene *GA1* in *Arabidopsis thaliana*. *The Plant Journal* **12**, 9–19.

Smith MW, Yamaguchi S, Ait-Ali T, Kamiya Y (1998) The first step of gibberellin biosynthesis in pumpkin is catalysed by at least two copalyl diphosphate synthases encoded by differentially regulated genes. *Plant Physiology* **118**, 1411–1419.

Smith VA, Knatt CJ, Gaskin P, Reid JB (1992) The distribution of gibberellins in vegetative tissues of *Pisum sativum* L. *Plant Physiology* **99**, 368–371.

Sun T-p, Kamiya Y (1997) Regulation and cellular localisation of *ent*-kaurene synthesis. *Physiologia Plantarum* **101**, 701–708.

Yamaguchi S, Kamiya Y (2000) Gibberellin biosynthesis: its regulation by endogenous and environmental signals. *Plant and Cell Physiology* **41**, 251–257.

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

Manuscript received 3 June 2002, received in revised form 8 October 2002, accepted 16 October 2002