Gibberellins and seed development in *Pisum*

by

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Declaration

This thesis contains no material that has been accepted for the award of any other degree or diploma in any university and contains no copy or paraphrase of material previously published or written by another person, except where due reference is made in the text.

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Abstract

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The *lh*^{i} and *ls* mutations have been used to investigate the role of the gibberellins (GAs) in seed development of the garden pea (*Pisum sativum* L.). These mutations were originally identified by their effects on internode elongation. Plants homozygous for *lh*^{i} or *ls* possess reduced levels of endogenous GA_1 in developing shoots, resulting in a dwarf phenotype compared with wild-type plants. The *Lh* locus has been shown to be linked to the *Le* locus at a distance of ca. 5cM. In conjunction with wild-type plants and other GA-deficient mutants, *lh*^{i}, *ls* and *le*^{5839} plants have been used to demonstrate a log-linear relationship between endogenous GA_1 levels and internode elongation, further supporting a role for GA_1 as the major native GA controlling internode elongation is this species. However, the *lh*^{i} mutation differs from other GA-deficient mutations, such as *lh*, since the response of *lh*^{i} plants to paclobutrazol (an inhibitor of GA-biosynthesis) is dramatically increased.

The *lh*^{i} and *ls* mutations also reduce endogenous GA levels in developing seeds. This has allowed the site of action of the *ls* mutation to be identified. Incubation of cell-free enzyme systems from developing wild-type and *ls* seeds suggests that *ls* plants possess reduced *ent*-kaurene synthetase A activity. The *lh*^{i} mutation also reduces endogenous GA_1 and GA_3 levels in young seeds (a few days after fertilization), while *ls* seeds possess similar GA_1 and GA_3 levels at this stage compared with wild-type seeds. Comparison of GA levels in *lh*^{i}*lh*^{i}, *lsls*, *Lsls* and wild-type seeds suggests that GA-biosynthesis may vary within different tissues (embryo, endosperm and testa) of developing seeds. Seeds homozygous for *lh*^{i} are more likely to abort during development, and weigh less at harvest, compared with wild-type seeds and seeds homozygous for *ls*. Altering the source/sink relations of developing *lh*^{i} plants, and ^{14}C-photoassimilate studies, both suggest that *lh*^{i} seeds possess reduced sink strength compared with wild-type seeds. Fertilizing *lh*^{i} plants with wild-type pollen produces seeds with normal GA levels and restores normal seed development. Culturing of *lh*^{i} embryos with GA_1 *in vitro* also increases embryo size. These results have been used to suggest that GA_1 and GA_3 play an important role early in pea seed development. By contrast, the high GA levels found in maturing wild-type seeds do not have a physiological role in seed development.

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Contents

Chapter 1

Hormones and seed development

Introduction

The plant hormones, gibberellins (GAs), abscisic acid (ABA), auxin and cytokinins, have all been identified in developing seeds of a range of Angiosperm species (Graebe 1987, King 1982, Karssen 1982). The presence of these hormones and observed changes in their levels during seed development has led many workers to postulate a physiological role. Although strong evidence suggests that GAs and ABA regulate seed germination and dormancy (e.g. Hilhorst and Karssen 1992), the situation is less certain for hormones in regard to other aspects of seed development. In this review some of the evidence relating to the possible role of GAs, ABA, auxin and cytokinins in Angiosperm seed development, between fertilization and desiccation, is discussed.

Seed development

Embryogenesis in Angiosperms commences with the double fertilization that gives rise to the diploid zygote and triploid endosperm tissue (Meinke 1991, Goldberg et al. 1989, Dure 1975). These events occur within the embryo sac that is embedded in maternal tissue consisting of the nucellus tissue and one or more integuments. The integument(s) is connected via the funiculus to the remainder of the ovary. Following fertilization, the integument (which develops into the testa) and endosperm tissue initially shows the most rapid growth, with the endosperm becoming the most extensive part of the seed. Meanwhile, the embryo initially grows relatively slowly giving rise to the suspensor (which may degenerate) and the embryo-proper (Meinke 1991). The embryo then grows more rapidly at the expense of the endosperm. The extent to which the embryo consumes the endosperm varies between species, particularly the monocots (endosperm retained) and dicots (endosperm absorbed). Both the embryo and endosperm initially grow by an increase in cell size and cell number. Cell division ceases when seed maturation begins, although fresh and dry weights continue to increase dramatically (Goldberg et al. 1989, Dure 1975). At some point the funiculus degenerates isolating the seed from the maternal plant. Finally

desiccation occurs as the seed looses water to the surrounding environment, the testa (seed coat) sclerifies and dies, and the seed usually enters a quiescent state prior to germination (Karssen and van Loon 1992).

Physiological roles of the plant hormones

The GAs and ABA have received the most study regarding possible physiological roles during seed development. However, excluding seed germination and dormancy, very little is known regarding the roles of the plant hormones during seed development.

Abscisic Acid (ABA).

A correlation between a peak in endogenous ABA levels and maximum seed fill has been noted in a number of species and ABA has consequently been suggested to regulate assimilate partitioning to developing seeds (Brenner 1987). However, a role for ABA in the regulation of assimilate partitioning to developing embryos *in vivo* is not supported by results obtained using genetic mutants of several species. Mutants *aba1* (*Arabidopsis*), *wil* (pea) and sit^w (tomato) are all ABA-deficient, possessing less than 10% of ABA levels found in wild-type seeds (Karssen and van Loon 1992, de Bruijn and Vreugdenhil 1992, Groot et al. 1991). By fertilizing ABA-deficient mutant plants with either wild-type or mutant (*aba1*, *wil* or sit^w) pollen, the assimilate uptake of ABA-deficient seeds can be compared with seeds possessing higher endogenous ABA levels for each species. No differences between assimilate-partitioning have been detected in *Arabidopsis* (Karssen and van Loon 1992), peas (de Bruijn and Vreugdenhil 1992) or tomatoes (Groot et al. 1991). Consequently, the majority of ABA present in developing embryos does not appear to be involved in the regulation of assimilate uptake to developing seeds. Nevertheless, ABA may be involved in phloem unloading from excised seed coats (maternal tissue) (e.g. Clifford et al. 1986), since in these genetic studies the genotype of the testa is independent of the pollen genotype (Koornneef 1991).

ABA has also been suggested to regulate the synthesis of seed storage proteins and a group of proteins that accumulate to relatively high levels during seed desiccation, at about the same time as maximal ABA levels (Quatrano 1987). This second group is termed the LEA (*L*ate *E*mbryogenesis *A*bundant, Galau et al. 1987) proteins and they have been characterised in a range of species including cotton, soybean, rapeseed and wheat (see Quatrano 1986, 1987). Several LEA proteins have a repeated

amino acid motif in common (Dure et al. 1989) that may determine the tertiary and quaternary structure of the protein (Dure 1993). LEA proteins have been suggested to play a role in desiccation protection during seed maturation, possibly by sequestering ions and preventing cell damage (e.g. Robertson and Chandler 1992, Dure 1993). Such a role is supported by the expression of similar proteins in response to water-stress or ABA application to seedlings of several species (Dure 1993). Many LEA proteins can also be induced *in vitro* by culturing partially developed embryos in a medium containing ABA or high osmotic potential (e.g. Butler and Cuming 1993, Marcotte et al. 1989). As a result, increasing ABA levels have commonly been assigned a direct role in the regulation, or at least induction, of these proteins in maturing seeds *in vivo* (Quatrano 1987, Goldberg et al. 1989).

The possible role of ABA in storage and LEA protein synthesis has been examined using ABA-deficient mutants of maize (*Zea mays*), *Arabidopsis* and tomato (*Lycopersicon esculentum*). The mutants were identified by the common phenotype of reduced seed dormancy, a process thought to be regulated by ABA (Karssen and van Loon 1992). In maize, a number of ABA-deficient mutants have been identified (e.g. Robertson 1955, Smith et al. 1989). For example, the *vp5* mutation reduces endogenous ABA levels in developing embryos to approximately 10% of the levels found in wild-type embryos (Neill et al. 1987), and was identified by the ability of homozygous recessive *vp5* seeds to germinate precociously (vivipary) on a heterozygous (*Vp5vp5*) maternal plant (Robertson 1955, Neill et al. 1986). Unlike maturing *Vp5*- (wild-type) maize embryos, which normally accumulate mRNA for a LEA protein similar to the "Em" protein found in developing wheat embryos (Morris et al. 1990, Marcotte et al. 1989), "Em" mRNA is barely detectable in ABA-deficient *vp5* embryos (Butler and Cuming 1993). Synthesis of "Em" mRNA can be restored in immature ABA-deficient *vp5* embryos cultured *in vitro* with ABA or high osmotic potential (Butler and Cuming 1993). Similar results were found when the synthesis of storage proteins was examined (Karssen and van Loon 1992). Thus, the *vp5* mutant suggests ABA plays an important role in seed development of maize by regulating, or at least inducing, storage and LEA protein accumulation in the developing embryo.

By contrast, results obtained from ABA-deficient mutants of *Arabidopsis* (*aba1*) and tomato (sit^w), and from *in vitro* culture of pea fruits, do not completely support this conclusion for these species. Seed development, including storage/LEA protein accumulation and desiccation tolerance does not appear to be altered by the *aba1* or sit^w mutations (Karssen and van Loon 1992, Koornneef et al. 1989). The levels of storage proteins were also unaffected when fluriodone was used to reduce ABA levels in pea seeds developing in pods cultured *in vitro* (Barratt et al. 1989).

Thus, it appears that the majority of ABA present in developing seeds is not required for assimilate uptake, storage and LEA protein accumulation, or desiccation tolerance in seeds of *Arabidopsis*, tomato or peas.

ABA-insensitive mutants of maize and *Arabidopsis* have also been used to investigate the role of ABA in seed development. Like the *vp5* mutant, the *vp1* mutant of maize also results in precocious germination of developing embryos (Robertson 1955, Robichaud et al. 1980, Robichaud and Sussex 1986) and reduced production of "Em" mRNA (Butler and Cuming 1993) and storege protein mRNA (Kriz et al. 1990). However, *vp1* seeds contain slightly elevated ABA levels (Neill et al. 1987) and *in vitro* incubation of *vp1* embryos with exogenous ABA or high osmoticum cannot restore "Em" mRNA synthesis to levels found in wild-type embryos (Butler and Cuming 1993). As a result, the *vp1* mutant is described as ABA-insensitive. Developing seeds of genotype *vp1* also possess reduced anthocyanin levels in the aleurone layer (outermost layer of the endosperm) compared with wild-type seeds (Robertson 1955). This may distinguish the effects of the *vp1* and *vp5* mutations, although ABA-deficient *vp5* seeds may also possess a slightly reduced anthocyanin content (Smith et al. 1989).

The *C1* gene is also expressed in wild-type maize seeds and may be a transcription regulatory factor responsible for the activation of the anthocyanin pathway in seeds (Paz-Ares et al. 1987). The accumulation of the *C1* gene-product is blocked by the *vp1* mutation (McCarty et al. 1989), suggesting that the *Vp1* protein may also be a transcription regulatory factor. The *Vp1* protein may therefore be a common regulatory component of an ABA regulated transduction chain (leading to LEA protein accumulation) and a pathway controlling anthocyanin production (that may not be regulated by ABA). The *Vp1* gene has been cloned by transposon tagging (McCarty et al. 1989), and analysis of mutations that separate control of viviparous germination and anthocyanin production suggest that these two responses are regulated by discrete regions of the *Vp1* protein (McCarty and Carson 1991), consistent with a regulatory role in several aspects of seed development.

The expression of storage and LEA mRNA's and proteins in *Arabidopsis* has also been studied using ABA-insensitive mutants identified at three loci named *ABI1*, *ABI2* and *ABI3* (Koornneef et al. 1982,1984, Nambara et al. 1992, Giraudat et al. 1992, Karssen and van Loon 1992). These mutants do not contain reduced ABA levels in developing seeds (Koornneef et al. 1984), and are all able to germinate in the presence of higher concentrations of ABA than wild-type seeds. Mutants at the *ABI1* and *ABI2* loci also have increased wilting in response to water stress but do not appear to affect aspects of seed development other than seed dormancy (Koornneef et al. 1984, 1989, Finkelstein and Somerville 1990). By

contrast, alleles at the *ABI3* locus may have a small effect on vegetative tissues (Finkelstein and Somerville 1990) and varying effects on seed development. The *abi3-3* allele has a marked effect on seed development when endogenous ABA levels are normal (genotype *ABA1*) (Nambara et al. 1992). By contrast, the *abi3-1* allele (*ABA1* genetic background) reduces storage protein accumulation by ca. 30% (Meurs et al. 1992), but otherwise only has a detectable effect on an ABA-deficient *aba1* background (Koornneef et al. 1989). The *abi3-1* allele therefore appears to be "leaky", and less severe than the *abi3-3* allele. Plants homozygous for the *aba1abi3-1* or *abi3-3* mutations produce seeds that contain reduced levels of storage and LEA proteins, remain green throughout development and are unable to survive desiccation (Koornneef et al. 1984, Nambara et al. 1992, Karssen and van Loon 1992). Viable mutant seeds can be obtained by harvesting them before desiccation and allowing them to precociously germinate or by applying exogenous ABA to the maternal plant (Koornneef et al. 1989, Nambara and Naito 1993). Viable seeds of genotype *aba1abi3-1* can also be obtained from self-pollinated *ABA1aba1abi3-1abi3-1* plants which possess normal ABA levels in maternal tissues, including the testa (Koornneef et al. 1989).

Since mutants at the *ABI3* locus affect seed dormancy without altering ABA levels, the *ABI3* protein appears to be an excellent candidate for an ABA transduction component (as is the *Vp1* protein), strongly supporting a direct regulatory role for ABA in LEA protein synthesis and desiccation tolerance. The results obtained from *in vitro* embryo culture and the analysis of ABA-deficient and ABA-insensitive mutants of *Arabidopsis* therefore suggest that ABA directly regulates certain aspects of seed maturation (e.g. LEA protein synthesis) but that only a relatively small proportion of the ABA present in wild-type seeds is essential for normal seed development. In turn, this has led to the suggestion that ABA response in developing seeds is regulated by changes in ABA sensitivity (rather than changes in ABA levels) mediated via a pathway involving the *ABI3* protein (Koornneef et al. 1989, Karssen and van Loon 1992).

The *ABI3* locus has recently been identified by positional cloning and the primary structure of the *ABI3* protein deduced from sequence analysis of a corresponding cDNA clone (Giraudat et al. 1992). The predicted protein shares discrete regions of high similarity to the maize *Vp-1* protein (McCarty et al. 1989). The *Vp1* and *ABI3* proteins may therefore have similar, or related, physiological roles in developing seeds, at least regarding ABA-induction/regulation of LEA protein accumulation.

Unfortunately, a potential problem must be mentioned at this point. Because

reduced seed dormancy was used to isolate the ABA-insensitive mutants, the effects of the *Vp1* and *ABI3* mutations must be interpreted with care when applied to other aspects of seed physiology (i.e. LEA protein synthesis). The *Arabidopsis* mutants were identified by their ability to germinate in the presence of a higher concentration of ABA than wild-type seeds (Koornneef et al. 1984) or in the presence of uniconazol (Nambara et al. 1992), an inhibitor of GA-biosynthesis (Graebe 1987). The *vp1* mutant was identified by the ability of homozygous *vp1* seeds to germinate on a heterozygous *Vp1vp1* maternal plant (Robertson 1955). Seeds of genotype *vp1* (maize), *abi1*, *abi2*, *abi3-1* and *abi3-3* (*Arabidopsis*) are also able to germinate in the presence of a higher concentration of ABA than wild-type seeds (Neill et al. 1987, Koornneef et al. 1984, Nambara et al. 1992). This fact has been used to suggest that an ABA-transduction chain involved in the inhibition of germination by ABA is impaired in these mutants, and therefore, any other phenotypic effects of the *Vp1* or *ABI3* mutants (e.g. LEA protein synthesis) must be related to ABA action. However, an alternative explanation is possible: the *Vp1* and *ABI3* mutants possess reduced dormancy because seed development is altered in some way (unrelated to ABA action) such that the mature seeds are not competent to respond to ABA. Therefore, the *Vp1* and *ABI3* proteins may not be involved in an ABA-transduction chain even though seed dormancy is affected by these proteins. Although the cloning of the *Vp1* and *Abi3* genes is an exciting step towards understanding the control of seed maturation, these studies have not yet revealed the exact mechanism by which ABA acts in developing seeds.

If the *ABI3* protein is not involved in an ABA regulated transduction chain, the markedly altered seed development of *abi3-1* seeds on an ABA-deficient (*aba1*), but the small effect on a wild-type (*ABA1*), background remains unexplained (Koornneef et al. 1984, 1989). One possibility is that ABA (in particular maternal ABA) may only have an indirect role in storage/LEA protein synthesis and desiccation tolerance by inhibiting water uptake into the developing embryo of *Arabidopsis* seeds (Koornneef et al. 1989, Galau et al. 1991). The increased water stress could then serve to induce the production of storage and LEA proteins either directly (by regulating gene expression) or indirectly (by preventing precocious germination and allowing seed maturation to continue to completion). This hypothesis is consistent with the experimental observations regarding the ABA-insensitive mutants. Firstly, the ability of exogenous or maternal ABA to overcome the effects of the *abi3-3* and *aba1abi3-1* genotypes (Koornneef et al. 1989, Nambara and Naito 1993), and secondly, the induction of storage and LEA proteins *in vitro* if ABA prevents precocious germination and allows seed development to continue (Barratt 1986a, Koornneef et al. 1989, Galau et al. 1991).

Thirdly, this hypothesis may also explain why the majority of ABA present in developing *Arabidopsis* and tomato seeds is not required for normal seed development. If maternal ABA prevents water uptake into the developing embryo, reduced maternal ABA levels may only delay embryo water stress slightly but still allow seed development (e.g. LEA protein synthesis) enough time for completion. If the *abi3-1* allele is "leaky", so that it only has a small effect on seed development (Koornneef et al. 1984), the *aba1abi3-1* double mutant may have abnormal seed development (Koornneef et al. 1989) because slightly delayed water stress (due to ABA deficiency) combined with the slightly reduced ability to induce storage/LEA protein expression and desiccation tolerance (due to the *abi3-1* allele) prevents completion of normal seed development. The results of Finkelstein and Crouch (1986) are also consistent with an indirect role for ABA late in seed development since high osmoticum can induce LEA protein expression without affecting ABA levels.

In conclusion, embryonic ABA does not appear to regulate assimilate partitioning to developing seeds, although ABA in the testa may have a physiological role. By contrast, numerous results suggest that ABA plays an important role in other aspects of seed development, particularly during seed maturation and desiccation. However, the mechanism by which ABA may regulate storage/LEA protein accumulation and desiccation tolerance has yet to be completely resolved.

Gibberellins (GAs).

Endogenous GAs have been shown to increase in abundance in parallel with increases in dry and/or fresh weights of developing seeds in a range of plants. These include peas (Wang and Sponsel 1985), Japanese morning glory (Barendse et al. 1983), barley (Mounla 1978, Mounla and Michael 1973), apricots, pears, apples, wheat, rye, maize and rice (for review of the specific GAs involved see Pharis and King 1985). These results are consistent with a physiological role for GAs in seed development, and have been used to suggest a causal relationship between GA levels and seed or grain fill. For example, GAs have been suggested to regulate assimilate partitioning to developing pea seeds based on such correlations (e.g. Stoddart 1983, Wareing and Seth 1967). However, in several species, the most abundant GAs quantified during seed development often posses little or no biological activity in bioassays involving vegetative growth (Pharis and King 1985). For example, in peas, GA_{20} and GA_{29} reach extremely high levels in developing and maturing seeds (Frydman et al. 1974, Gaskin et al. 1985), but these GAs are though to possess no biological activity *per se* in vegetative tissues (Ingram et al. 1986). Although GA_{20} can be converted to the biologically active GA_1 in pea shoots (Ingram et al. 1984), GA_1 is not present in developing pea seeds when GA_{20} levels are highest (Gaskin et

al. 1985). Thus, a role for GAs in the seeds of peas, and other species, when GAs reach their highest levels, may require biological activity of GAs that are inactive *per se* in vegetative tissues of the same plant (Pharis and King 1985).

Culture of immature seeds and embryos *in vitro*, with an exogenous GA (usually GA_3) in the incubation medium, has been reported to promote growth in several species including cotton (Beasley 1973), beans (Alpi 1990) and wheat (Kefford and Rijven 1966). Although GAs are not usually included in embryo culture media (Collins and Grosser 1984), *in vitro* culture has been used to investigate the role of GAs in the embryo/suspensor system of *Phaseolus spp.* (beans) over a number of years. The role of the suspensor in early embryogenesis is not fully understood, but some evidence suggests that the suspensor may be involved in the transfer of nutrients and GAs to the developing embryo (e.g. Alpi et al. 1975, Yeung and Sussex 1979). Endogenous GAs, including GA_1, have been reported to be present in both suspensors and embryos of very young *Phaseolus* seeds (Alpi et al. 1975,1979). Results obtained from cell-free systems suggest that suspensors are capable of synthesising these GAs *de novo* (Ceccarelli et al. 1979,1980,1981), although contamination with embryo cells during enzyme extraction is a possibility (Turnbull et al. 1986). When embryos are cultured without the suspensor attached, embryo growth and protein content are reduced compared with embryos cultured with suspensors attached. However, growth and protein content of embryos without suspensors was restored by inclusion of certain concentrations of GA_3 in the incubation medium (Brady and Walthall 1985, Cionini et al. 1976). Consequently, GAs have been suggested to have an important role in embryo/suspensor development of this species (Alpi et al. 1975, Cionini et al. 1976, Ceccarelli et al. 1981, Apli 1990). Unfortunately, these *in vitro* studies have not been extended to demonstrate a physiological role for GAs *in vivo* for beans or other species where the suspensor is less massive.

The role of the GAs in seed development has also been examined using chemical inhibitors of GA-biosynthesis. Zeevaart (1966) used chlormequat (CCC) to reduce endogenous GA levels in developing seeds of Japanese Morning Glory (*Pharbitis nil*). Seeds treated with CCC contained dramatically reduced GA levels during the later phase of seed development but obtained fresh and dry weights similar to those of untreated seeds. GA levels could not be reduced in young seeds by CCC application before anthesis, since this resulted in flower abortion (Zeevaart 1966).

Excised pea fruits developing on nutrient media with, or without AMO-1618, were used to examine the effect of reduced GA levels on developing pea seeds (Baldev et al. 1965). Fruit were removed from the maternal plant 13 days after pollination, and seed fresh weights determined after 10 days in culture. GA levels late in seed

development were reduced by all AMO-1618 concentrations, while seed weight was only reduced by the highest AMO-1618 concentration, which reduced endogenous GA levels more than 10 fold compared with seeds developing on media without the inhibitor. Similar results were also obtained with a range of GA-biosynthesis inhibitors included in the media used to culture 4 day old pea fruit explants, although GA levels were only determined approximately half-way through seed development (Garcia-Martinez et al. 1987). Together, results from the use of GA-biosynthesis inhibitors suggest that a large proportion, or possibly all, of the GAs that accumulate in developing seeds are not required for normal seed development, at least in the species studied to date.

Genetic studies have also been used to address the role of GAs in developing seeds. Groot et al. (1987) used the GA-deficient *gib1* (previously named *ga1*) dwarf mutant of tomato to investigate seed and fruit development. Vegetative tissues and intact fruits of homozygous *gib1* plants contain reduced levels of endogenous GAs, presumably due to reduced *ent*-kaurene synthetase A activity compared with wild-type plants (Bensen and Zeevaart 1990). Ultimate fresh and dry weights of mature, heterozygous *Gib1gib1* seeds (produced by fertilizing *gib1* plants with *Gib1* pollen) were higher than those of homozygous *gib1gib1* seeds. However, seed numbers and the total content and composition of seed proteins were not affected by the *gib1* mutation. Groot et al. (1987) therefore concluded that GAs are promoting, but not essential for tomato seed development.

Comparable experiments were also conducted by Barendse et al. (1986) using the *ga1* mutant of *Arabidopsis*. The *ga1* mutation is thought to reduce GA-biosynthesis in internodes and intact fruit, compared with wild-type plants, by partially blocking the production of an early GA-precursor, possibly *ent*-kaurene (Barendse et al. 1986). No detectable effect on seed development of the *ga1* mutation was observed when *ga1* plants were fertilized with either *GA1* (wild-type) or *ga1* pollen. Barendse et al. (1986) consequently found no evidence to support a role for GAs in seed development of *Arabidopsis*.

Although both the *gib1* (tomato) and *ga1* (*Arabidopsis*) mutants are thought to possess reduced GA levels in whole fruits (Barendse et al. 1986, Groot et al. 1987) GA levels have not been determined in developing isolated seeds. The small size of developing tomato and *Arabidopsis* seeds would, of course, make such quantifications difficult. However, until such measurements are made it cannot be assumed that these mutants possess lowered GA levels in developing seeds compared with wild-type plants. This statement is supported by results obtained with the *na* mutant of peas (*Pisum sativum*). Plants homozygous for the *na* mutation possess

extremely reduced GA levels in young shoots (Proebsting et al. 1992) and developing pods (Potts 1986, Potts and Reid 1983) compared with wild-type plants. By contrast, *na* seeds contain a similar complement and levels of endogenous GA-like compounds as seeds developing on wild-type plants (Potts 1986, Potts and Reid 1983). Consequently, the results of Barendse et al. (1986) and Groot et al. (1987) regarding seed development remain inconclusive until the expression of the *ga1* and *gib1* mutations in developing seeds is examined in more detail.

In conclusion, the quantification of endogenous GAs in comparison with seed growth and *in vitro* culture experiments are consistent with a role for GAs in seed development of a range of species. By contrast, results obtained from mutants of *Arabidopsis thaliana* and tomato, and GA-biosynthesis inhibitors, do not support an important role for GAs in seed development.

Auxin.

As for the GAs and ABA, peak auxin levels also correlate with maximum fresh weight increases (Jones and Stoddart 1977). In addition, auxin has long been known to influence the development of embryos cultured *in vitro* (e.g. Beasley 1973, Tilton and Russell 1984, Collins and Grosser 1984), and auxin present in the suspensor of *Tropaeolum majus* has been suggested to regulate seed development (Przybyllok and Nagl 1977). While auxin has been shown to regulate gene expression in developing fruit (e.g. Theologis 1986, Reddy and Poovaiah 1990) the exact function of these genes *in vivo* remains in question, and it is not clear whether these genes are regulated by auxin in developing seeds.

The best evidence suggesting a role for auxin in seed development appears to be the defective endosperm-B18 (de*-B18) mutant of maize. Developing de*-B18 seeds contain reduced auxin levels and accumulate substantially less dry weight in the endosperm than wild-type seeds (Torti et al. 1986). However, normal seed development can be restored by fertilizing de*-B18 plants with wild-type pollen or exogenously applying an auxin analog (naphthalene-acetic acid), suggesting that auxin is required for normal endosperm development in wild-type seeds.

Cytokinins.

The evidence suggesting a role for the cytokinins in seed development is entirely circumstantial. Cytokinins are mainly present in the early stages of seed growth, when the liquid endosperm predominates (Karssen 1982, van Staden 1983). Since cytokinins are often associated with cell division and cell enlargement, they have been suggested to affect seed size by regulating cell numbers, thereby regulating the storage capacity (and sink strength) of the developing seed (Jones et al. 1992,

Borkovec and Prochazka 1992, van Staden 1983). For example, in the embryo-suspensor system of *Phaseolus spp* (beans), the suspensor has been suggested to synthesize cytokinins that are then transported to the developing embryo (Lorenzi et al. 1978) where they may stimulate cell division.

Future directions

As can be seen from the preceding discussion, the role of GAs, auxin and cytokinins in seed development remains uncertain. For the GAs this is particularly ironic since cell-free systems from developing seeds of several species have been used to establish GA-biosynthesis pathways (e.g. Kamiya and Graebe 1983, Lange et al. 1993a,b). The situation is clearer for ABA, largely due to the use of genetic mutants and the relatively high levels of LEA mRNA and proteins in the later stages of seed development which has facilitated molecular and biochemical analysis. Since GAs, auxin and cytokinins are likely to influence aspects of seed development less easily examined than LEA mRNA/protein accumulation, a genetic approach would seem the most promising for further investigating the roles of hormones in seed development. Two techniques are commonly used to alter the genetic makeup of plant species: the insertion of genes (of known function) into transgenic plants and mutagenesis to produce plants with an altered gene (usually of unknown function). Transgenic plants containing genes that result in elevated or decreased levels of auxins and cytokinins have been produced in several species, including *Nicotiana tabacum* (tobacco) and *Arabidopsis* (e.g. Sitbon et al. 1992, Romano et al. 1993, Li et al. 1992). While this a powerful technique, problems may arise. In particular, since non-specific and/or constitutive promoters have usually been used, hormone levels may be altered at developmental stages or in organs not usually influenced by the hormone in wild-type plants. In addition, the overall plant development may be so disturbed that processes such as seed development cannot be examined. The identification of seed-specific promoters should allow transgenic plants with altered hormone levels (in developing seeds only) to be examined in the future (e.g. Schell 1987, Fujiwara et al. 1992, Karchi et al. 1993).

Alternatively, genetic mutations affecting levels of, or the response to, plant hormones can be used to investigate seed development. Such mutants can be generated in a number of ways, such as chemical mutagenesis (the most common method) or insertion of DNA sequences into the wild-type genome (e.g. transposon tagging). However, a genetic approach also entails certain difficulties. Firstly, mutations

affecting hormone levels or hormone response in developing seeds may be difficult to identify if the expected phenotype is unknown, or could result from a range of different causes, unrelated to hormone action. For example, many mutants with abnormal seed development have recently been identified in maize (e.g. Clark and Sheridan 1991) and *Arabidopsis* (Mayer et al. 1991, Robinson-Beers et al. 1992), but many (or all) of these may not result from changes in hormone mediated responses (Meinke 1991). The second difficulty may arise when mutants are identified based on their effect on another aspect of plant development thought to be controlled by a particular hormone. For example, the *gal* mutant of *Arabidopsis* was identified by its effect on seed germination and internode elongation and appears to reduce GA levels in vegetative tissues and developing fruit. However, this does not demonstrate that GA levels are altered to the same degree in developing seeds. Nevertheless, if seed hormone mutants can be correctly identified (e.g. the ABA-deficient mutants of maize and *Arabidopsis*) they are clearly of great use in investigating the roles of hormones in seed development.

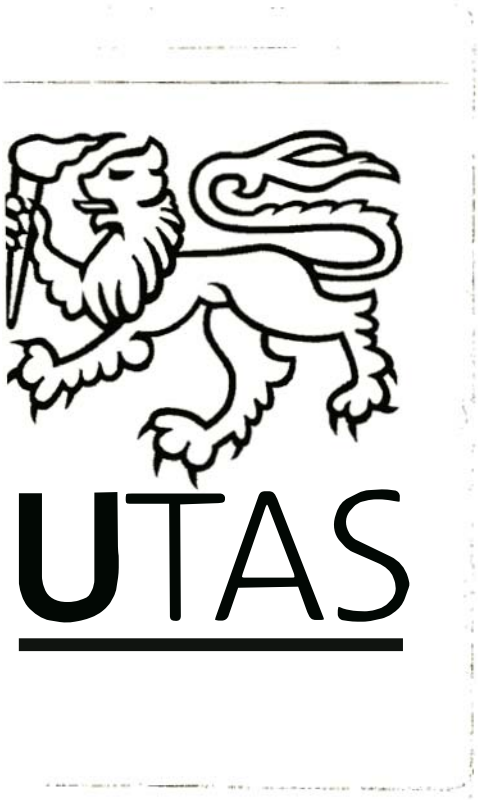
Aims of this thesis

Extensive evidence is available regarding the role of GAs in internode elongation in a range of species (Lenton et al. 1987, Fujioka et al. 1988, Reid and Ross 1993). For example, compelling evidence suggests that GA_1 regulates internode elongation in the garden pea (*Pisum sativum* L.) (Ingram et al. 1984, 1986, Reid et al. 1992, Ross et al. 1992), and a large proportion of this evidence has been obtained using mutants affecting either GA-metabolism (*le, na, ls, lh* and *sln*) or the response to GA_1 (*lk, lka, lkb, lkc, lkd, lv, lw,* and the *la* cry^S gene combination) in vegetative tissues (Fig. 1) (Reid 1990, Reid and Ross 1993, Weller and Reid 1993). The role of GAs in seed development may also be amenable to further investigation using genetic mutants. The garden pea is ideally suited for the identification and characterisation of mutants with altered GA levels in developing seeds. Potential GA-mutants can be identified based on changes in internode elongation, and the relatively large size of pea seeds, compared with *Arabidopsis* and tomato seeds, allows the expression of the mutation in developing seeds to be examined with relative ease.

GA levels have previously been determined in developing seeds of several GA-metabolism mutants (*sln, le* and *na*), originally identified by their effects on internode elongation. Dry seeds harvested from self-pollinated *sln* plants possess extremely high levels of GA_{20} due to impaired GA-catabolism late in seed development (Reid et al. 1992, Ross et al. 1993), while the *le* allele has recently been reported to reduce the level of GA_1 in young seeds (Santes et al. 1993). By contrast,

the *na* allele has been shown to drastically reduce GA levels in shoots and pods, without a substantial effect on the levels of GA-like substances in developing seeds (Potts and Reid 1983, Potts 1986). However, none of these results has defined a role for GAs in seed development.

In this thesis a new GA-metabolism mutant is fully characterised and used, in conjunction with existing GA mutants, as part of a genetic approach to examine the role of the GAs in pea seed development.



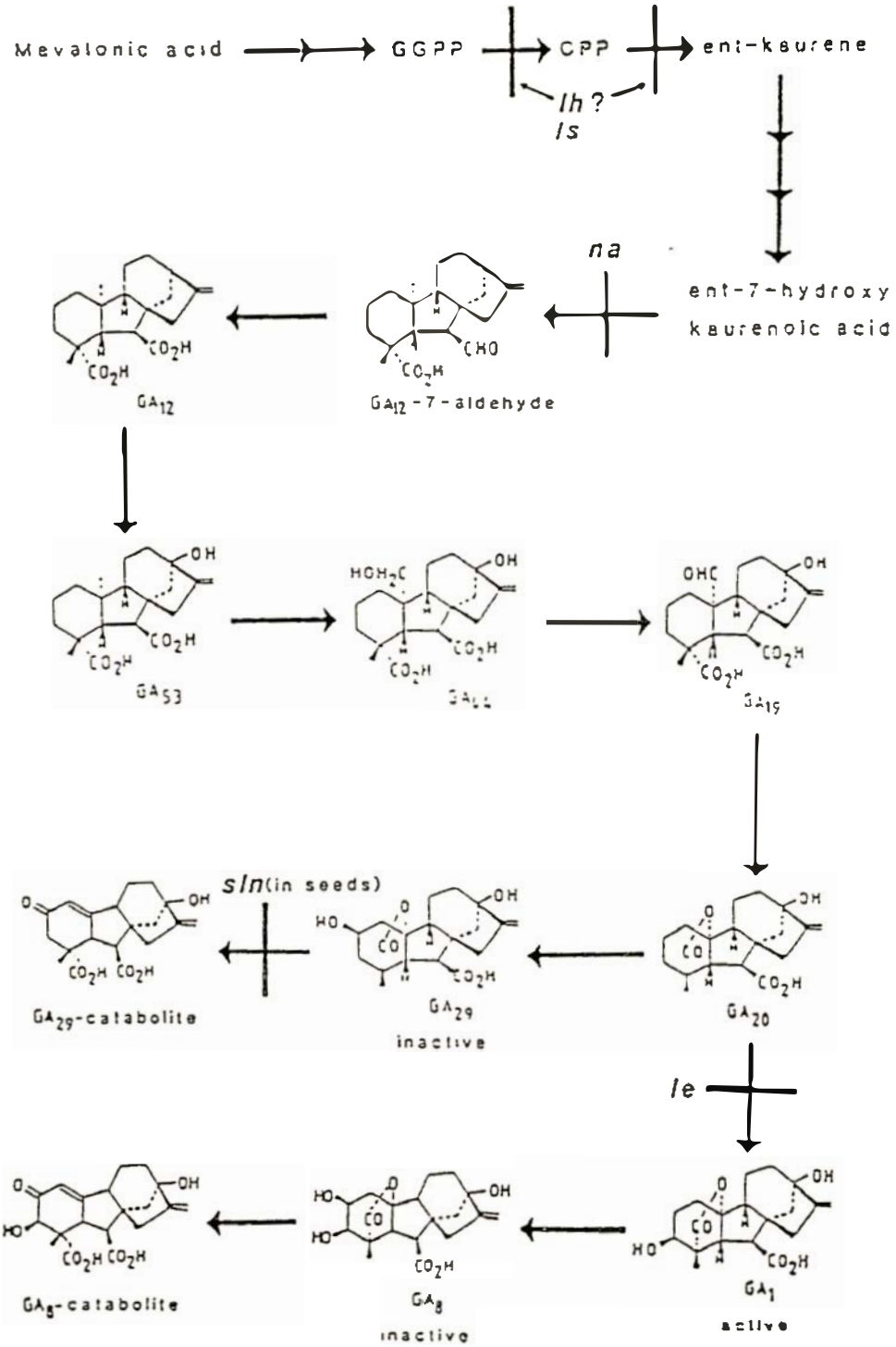


Fig. 1 The early 13-hydroxylation pathway of GA biosynthesis. This pathway predominates in garden pea (*Pisum sativum* L.) and the proposed sites of action of the GA-synthesis mutants are indicated.

Chapter 2

General materials and methods

Plant Material

The pure lines of *Pisum sativum* L. (garden pea) used during this work are held in the collection at Hobart, Australia. The previously uncharacterised dwarf mutant NGB5843 (lh^i) was derived from the wild-type tall cv. Torsdag by Dr K. K. Sidorova (Novosibirsk, Russia) using ethyleneimine (EI) (Blixt 1983). The dwarf lines K511 (*lh*) and NGB5839 (le^{5839}) were derived from the wild-type tall cv. Torsdag by Dr K. K. Sidorova using ethylmethane sulphonate (EMS) (Reid 1986a, Jolly et al. 1987). Line 181 (*ls*) was derived from a cross between cv. Torsdag and K202, which was also derived from the wild-type tall cv. Torsdag by Dr K. K. Sidorova using EMS (Reid 1986a). Line 197 ($lacry^S$) was derived from a cross between L133 and NGB1766 (Steane et al. 1989). Line NGB6074 (*sln*) was produced by J. Jaranowski (Poyon, Poland) (Reid et al. 1992). Line 23 (*r*) was derived by single plant selection from cv. Telephone (I. Murfet, unpublished data). Other genotypes used were segregating F_4 and F_6 progeny of a cross between lines 1769 (*Na*) and 1766 (*na*). All lines were homozygous dominant at the internode length loci *Le, Ls, Lh, Na, Lk, Lka, Lkb, Lkc, Lkd, Lv, Lw, La* and/or *Cry, Lm* and *Sln* unless otherwise indicated. Further details about the phenotypes and genotypes of these lines can be found in Reid and Ross (1993).

Growing conditions

Unless otherwise stated, plants were grown in a 50:50 mixture of vermiculite and dolerite chips topped with 2-3 cm of potting mix in 14 cm slim-line pots or plastic tote boxes (41 x 32 cm). A small nick was made in the testa of all seeds prior to planting to facilitate even germination. Plants were initially watered daily until they broke through the soil surface, when they were no longer watered. Watering recommenced when the seedlings had ca. 3 leaves expanded. Plants were watered either every day or 4 times weekly, depending on the size of the plant and the season. Counting of nodes started from the first scale leaf as node 1. Counting of internodes started with the internode

between nodes 1 and 2 as internode 1. Plants were provided with nutrient solution, Aquasol or Total Growth Nutrient, once a week.

All plants were initially grown in a heated glasshouse with a minimum night temperature of ca. 16°C (the temperature rarely fell below 12°C) and a maximum day temperature of ca. 25°C. The day temperature fluctuations varied with the seasons. Typical maximum/minimum day/night temperatures in summer were ca. 30°C and 19°C, respectively. Typical maximum/minimum day/night temperatures in winter were ca. 22°C and 13°C, respectively. Unless otherwise indicated, the photoperiod was extended to 18h with a mixture of fluorescent (Thorn 40W white tubes) and incandescent (Mazda 100W pearl globes) light sources (25μmol.m^{-2}.s^{-1} at pot top) (Weller and Reid 1993). Three weeks after sowing. some plants were transferred to controlled environment cabinets with an 18h photoperiod, where light was provided from a mixed fluorescent (Thorn 40W cool white tubes) and incandescent (Mazda 100W pearl globes) source (200μmol.m^{-2}.s^{-1} at pot top). Cabinets were used with day/night temperatures of 30/25, 25/20, 20/15 or 15/10°C.

For quantifications of GA levels in developing seeds, cv. Torsdag (wild-type) plants were laid down so that the height of the developing pods was approximately the same for wild-type plants and mutant (dwarf) plants. Anthesis was scored as the first day the flower fully opened and the petals were fully reflexed.

Growth regulator treatments

A range of plant growth inhibitors, AMO-1618 [(2-isopropyl-5-methyl-4-trimethylammoniumchloride)-phenyl-1-piperidiniumcarboxylate], paclobutrazol [1-(4-chlorophenyl-4,4-dimethyl-2-(1,2,4-triazol-1-yl)-1-pentan-3-ol], uniconazol [(*E*)-1-(4-chlorophenyl-4,4-dimethyl-2-(1,2,4-triazol-1-yl)-1-penten-3-ol], inabenfide [4'-chloro-2'-(α-hydroxybenzyl)-isonicotinanilide] prohexadione-calcium (calcium 3,5-dioxo-4-propionylcyclohexanecarboxylate) and ancymidol [α-cyclopropyl-α-(p-methoxyphenyl)-5-pyrimidinemethanol], were used (Graebe 1987, Miki et al. 1990, Nakayama et al. 1991). Since prohexadione-calcium (BX-112) was not soluble in ethanol it was first dissolved in an acidic solution, dried and then redissolved in ethanol. The inhibitors (except ancymidol) were applied in a few μl of ethanol to a small (ca. 4mm^2) nick in the testa of the dry seed with an autopipette or micropipette. Control plants received an identical volume of ethanol only. After the ethanol had

evaporated, the seeds were carefully sown. The seeds were not watered for 3-4 days so that the inhibitors were not washed off. After this period plants were watered as normal. Ancymidol was applied as a foliar spray to young seedlings.

Gibberellin extraction and analysis

For all extracts, harvested tissue was weighed and immersed in cold methanol (-20°C). To begin extraction, dH_2O was added to a final concentration of 20%. The tissue was homogenised and dideuterated or $^{13}C,^{3}H$-labelled internal standards were added. These were [17,17-$^{2}H_2$] GA_1, [17,17-$^{2}H_2$] GA_3, [17,17-$^{2}H_2$] GA_8, [17,17-$^{2}H_2$] GA_{19}, [17,17-$^{2}H_2$] GA_{20} and [17,17-$^{2}H_2$]GA_{29} (provided by Professor L. Mander, ANU, Canberra, Australia) or [17,17-$^{13}C^{3}H_2$]GA_{29} (provided by Prof. B.O. Phinney, UCLA, USA). The internal standards were added so that an approximate 1:1 ratio of internal standard:endogenous GA resulted (Gaskin and MacMillan 1991), such that a minimum of 5ng of internal standard was added. This minimum was chosen to ensure that the standard was recovered in cases where the endogenous GA level was below detection. In cases where the amount of GA was extremely high (i.e. extracts from several grams of mature seeds) an aliquot for extraction was taken after filtering (see below) and internal standards added at this stage. For the majority of GA quantifications, the ratio of endogenous GA to labelled internal standard was in the range 1:2 to 2:1. GAs were extracted overnight at 4°C. The extract was subsequently filtered through 1 layer of Whatman #1 filter paper. Depending on the tissue examined, one of 3 different procedures was used for the purification of GAs prior to analysis by Gas Chromatography Selected Ion Monotoring (GC-SIM).

Method 1 (vegetative tissue, pods and seeds).

The method for extraction of GAs outlined below is essentially as described in Reid et al. (1990, 1991) except that methylated GAs were not rechromatographed. After filtering the proportion of methanol was reduced to 60% by addition of dH_2O. The extract was forced through a C_{18} Sep-Pak cartridge (Millipore, Milford, USA) preconditioned with approximately 10ml of 100% methanol. The methanol, and most of the water, was removed *in vacuo*, so that approximately 15-30ml of extract remained. An equal volume of potassium phosphate buffer (pH~8) was added and the pH reduced to 2.9 by addition of 3M HCl. After partitioning against ethyl acetate

(40% of total extract volume) 5 times, the organic phases were combined and stored at -20°C.

Prior to High Performance Liquid Chromatography (HPLC), the extract was allowed to thaw, any water removed with a pasteur pipette, and toluene (5% of total volume) was added. The extract was subsequently dried *in vacuo* and redissolved in the initial conditions for HPLC. The extracts were fractionated by reverse phase C_{18}-HPLC. The equipment (all from Waters Associates, Milford, MA, USA) included two M-45 Solvent Delivery Systems, a Model U6K Universal Liquid Chromatograph Injector fitted with a 2ml sample loading loop, Model 660 Solvent Programmer, Z-Module Radial Compression Separation System and a 10ml Radial-Pak A cartridge C_{18} column (100x8mm internal diameter). Solvents were filtered regularly through 0.5µm type FH (methanol) and 0.45µm type OE 67 (dH_2O) Millipore filters.

The HPLC was programmed and approximately 3ml of initial conditions collected. The GA extract was dissolved in 3 successive volumes of 0.5, 1.0 and 1.0ml, respectively, and injected into the HPLC loading loop via a syringe and 0.45µm filter (Gelman Sciences, MI, USA). After allowing 3 minutes for the loaded material to equilibrate in the loading loop the sample was injected and run on the following program: 21-70% methanol (in 0.4% acetic acid) over 40min, gradient curve #9 (exponential), flow rate of $2ml.min^{-1}$. Appropriate fractions were collected (based on retention times of radio-labelled standards) and dried *in vacuo*. GAs were redissolved in 200µl of methanol and methylated with 750µl of ethereal diazomethane for at least 5min, and evaporated to dryness under N_2 (g).

Method 2 (young seeds).

After filtering, the methanol and most of the water were removed *in vacuo*. An equal volume of sodium phosphate buffer (pH ~8.0) was added and the pH reduced to 2.9 with 3M HCl. The extract was partitioned 5 times against 0.4 volumes ethyl acetate, and the organic fractions combined and dried. The extract was dissolved in 3ml of 0.4% acetic acid and loaded through a 0.45µm filter onto a C_{18} Sep-Pak cartridge preconditioned with 8ml of methanol and 10ml of 0.4% acetic acid. The Sep-Pak was washed with 2ml of 0.4% acetic acid and 2ml of 5% methanol in 0.4% acetic acid and the eluate discarded. GAs were eluted with 11ml of 50% methanol in 0.4% acetic acid, dried, methylated (see Method 1), and loaded in 3ml of dH_20 through a 0.45µm filter onto another Sep-Pak cartridge preconditioned with 8ml of methanol and

10ml of dH_20. The Sep-Pak was washed with 2ml of dH_20 and 10ml of 10% methanol. GAs were eluted with 10ml of 60% methanol and dried *in vacuo*. This approach could be used since GA_{81} (which can complicate analysis of GA_1) is present at very low levels in the tissues used. For example, in 7 day old *Lh* (L107) seeds GA_{81} (if present) was shown to be less than 5% of the endogenous GA_1 level by using a GC column (see Ross et al. 1990) that separates GA_1 and GA_{81} (data not shown).

Method 3 (maturing seeds).

After filtering the proportion of methanol was reduced to 60% by addition of dH_2O. The extract was forced through a C_{18} Sep-Pak cartridge preconditioned with approximately 10ml of 100% methanol. The methanol, and most of the water, was removed *in vacuo*, so that approximately 15-30ml of extract remained. An equal volume of potassium phosphate buffer (pH~8) was added and the pH reduced to 2.9 by addition of 3M HCl. After partitioning against ethyl acetate (40% of total extract volume) 5 times, the organic phases were combined and stored at -20°C. The extract was allowed to thaw, any water removed with a pasteur pipette, and toluene (5% of total volume) was added. The extract was subsequently dried *in vacuo*, redissolved in 3ml of 0.4% acetic acid, and loaded through a 0.45µm filter onto a C_{18} Sep-Pak cartridge preconditioned with 5ml of 100% methanol and 5ml of 0.4% acetic acid. The Sep-Pak was washed with 2ml of 0.4% acetic acid and the eluate discarded. GAs were eluted with 10ml of 60% methanol in 0.4% acetic acid, dried, methylated (see Method 1) and dried under $N_2(g)$.

Quantification of GAs by GC-SIM

Extracts were trimethylsilylated with 10µl bis(trimethyl-silyl)trifluoroacetamide (BSTFA) for 10min at 60°C. When necessary, up to 5µl of dry pyridine was added to enable complete dissolution.

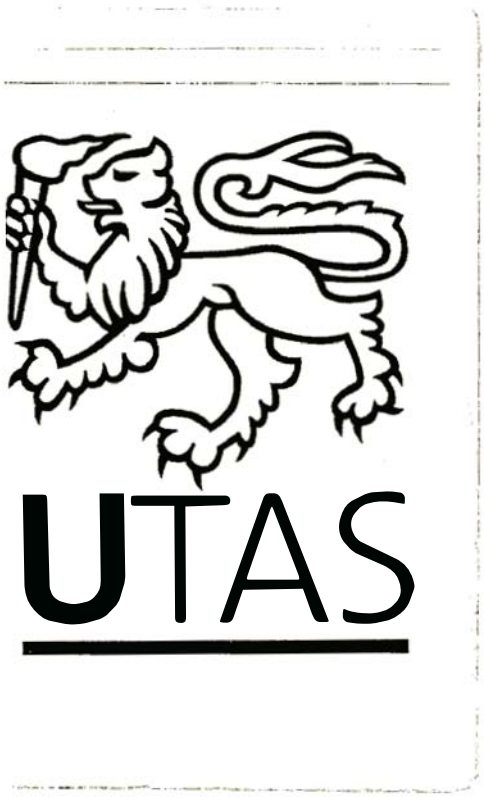
The majority of results were obtained using a Hewlett Packard 5890 GC coupled to a Hewlett Packard 5970 Mass Selective Detector. A 25m x 0.32mm internal diameter x 0.52µm film HP1 fused silica column was coupled to the detector via an open split interface. Helium was used as the carrier gas, at an initial flow rate of $2ml.min^{-1}$ at 60°C. Injections were made in the splitless mode and the oven

temperature was programmed for 60°C to 230°C at 30°C.min^{-1}, and then to 290°C at 3°C.min^{-1}. The interface temperature was 290°C and the ionization potential was 70eV. The pressure was set at between 10 and 15 PSI, depending on the level and type of GA(s) being monitored.

For certain GAs (e.g. GA_{19}) and certain tissues (e.g. young seeds), High Resolution-Selected Ion Monitoring (HR-SIM) was performed using a Hewlett-Packard 5890 Series II gas chromatograph linked via a direct inlet into a Kratos Concept ISQ mass spectrometer controlled by a MACH 3 data system. Splitless injections (1µl) were made at 60°C onto a 25m x 0.22 internal diameter SGE BP1 column with a 0.25µm film thickness (SGE, Victoria, Australia). The carrier gas was helium with the head pressure programmed to maintain a flow rate of approximately 2ml.min^{-1}. The oven temperature was programmed from 60°C to 240°C at 30°C.min^{-1}, and then to 290°C at 3°C.min^{-1}. The interface temperature was 290°C and the ionization potential was 70eV. The masses of characteristic ions (see below) were calculated to four decimal places and detection was achieved by voltage switching at a resolution of 10 000 (10% valley definition) and a cycle time of 0.6 seconds. Perfluorokerosene was used to provide lock masses. To minimse the contamination of one run with GAs from the previous run (ghosting), different GAs (with different molecular ions) were analysed in adjacent runs. Where this was not possible and ghosting was a potential problem, runs were separated by blank runs (BSTFA injected only).

The ions monitored for quantification of endogenous GAs were 506 and 508 (GA_1), 504 and 506 (GA_3), 594 and 596 (GA_8), 434 and 436 (GA_{19}), 418 and 420 (GA_{20}), and 506 and 508 (GA_{29}) (or 506 and 507 for the [$^{13}C^3H_2$]GA_{29} internal standard). Identification was confirmed on the basis of retention time and the presence of additional ions (Gaskin and MacMillan 1991). These ions were 448 and 450 (GA_1), 210 (GA_3), 375 and 448 (GA_8), 375 and 377 (GA_{19} and GA_{20}) and 491 and 493 (or 492) (GA_{29}). The dideuterated GA_1, GA_3, GA_8, GA_{20} and GA_{29} internal standards contained <1% unlabelled molecules so that the contamination of the measured endogenous GA levels by molecules present in the internal standard was negligible. The dideuterated GA_{19} and [$^{13}C^3H_2$]GA_{29} internal standards contributed 11% and 8% of the measured abundance of the internal standard ion (436 or 507) to the endogenous GA ion (434 or 506), respectively. The contribution of naturally occurring heavy isotopes (e.g. due to ^{17}O) was measured separately on the two GC-

SIM systems and used to correct the measured abundance of the internal standard ion (e.g. 508 for $^2H_2GA_1$). For GA_1, the measured abundance of naturally heavy endogenous GA_1 molecules (i.e. molecular ion of 508 instead of 506) was found to be 15.6% and 8.3% of the total endogenous GA_1 abundance on the Hewlett Packard and Kratos systems, respectively. Endogenous levels were subsequently calculated on the basis of peak areas, after corrections were made for the contribution of naturally occurring isotopes and for the presence of unlabelled GAs in the internal standards (Lawrence et al. 1992).



Chapter 3

A new allele at the *Lh* locus

Parts of this chapter are published in Swain and Reid (1992a), Physiol. Plant. **86**, 124-130.

Introduction

Mutant line NGB5843 of the garden pea (*Pisum sativum* L.) was originally identified by its dwarf phenotype and slightly darker foliage compared with the wild-type progenitor, cv. Torsdag. Application of GA_3 to NGB5843 plants also increased internode lengths compared with untreated plants. In these respects NGB5843 plants are similar to the previously isolated GA-deficient mutants of peas (e.g. le^{5839}, Jolly et al. 1987), suggesting that NGB5843 may also possess reduced endogenous GA levels in developing shoots. Initial observations of NGB5843 also suggested that seed yield was reduced compared with cv. Torsdag. In this chapter NGB5843 is shown to possess a new allele at the *Lh* locus that results in reduced GA levels in developing shoots and possible pleiotropic effects on seed development.

Materials and methods

Growing conditions.

Plants used for GA quantification were grown in plastic tote boxes containing potting mix at a density of 60 per box. The NGB5843 (lh^i) and K511 (*lh*) plants grown to measure seed abortion at different temperatures were sown 1 per pot. The cv. Torsdag (wild-type), NGB5843 (lh^i), K511 (*lh*), NGB5839 (le^{5839}) and L181 (*ls*) plants treated with paclobutrazol (PP333) or AMO-1618 were sown 3 per pot. The cv. Torsdag (wild-type), NGB5843 (lh^i), K511 (*lh*), NGB5839 (le^{5839}) and L181 (*ls*) plants treated with paclobutrazol, prohexadione (BX-112) or ancymidol were sown in tote boxes. All other plants were grown at a density of 2 per pot. The NGB5843 (lh^i) and K511 (*lh*) plants used to measure seed abortion were sown in the same glasshouse and transferred to controlled environment cabinets with day/night

temperatures of 25/20, 20/15 or 15/10°C (see Chapter 2 for more details).

Growth regulator treatments.

Growth regulators (in 2 or 5µl of ethanol per seed) were applied by micropipette or autopipette to a small nick in the testa of the dry seeds prior to sowing. The GA-biosynthesis inhibitor, paclobutrazol, was applied at a dose of 0.001, 0.01, 0.1, 1, 5 or 10µg per seed. The GA-biosynthesis inhibitors, prohexadione and AMO-1618 were applied at a dose of 10µg and 20µg per seed, respectively. GA_3 was applied at a dose of 10µg per seed. Controls were treated with an appropriate volume (2 or 5µl) of ethanol only. Since ancymidol was only available as a spray, young seedlings (4-5 fully expanded leaves) were uniformly sprayed with ca. 0.2µg of ancymidol per plant.

Gibberellin extraction and analysis.

For the quantification of GAs in cv. Torsdag, NGB5843 and K511 plants shoots were harvested at 21 days after sowing (Tab. 1). At this age all plants had approximately 5 expanded leaves. Harvested tissue (consisting of material above, and including the second highest fully expanded leaf) was weighed and immersed in cold methanol (-20°C). Extraction, purification and quantification of GAs was as described in Chapter 2 (Method 1).

Results

The nature of mutant NGB5843 (from Swain 1989).

NGB5843 exhibits a dwarf phenotype, similar to the previously characterised lines NGB5839 (*le*^{5839}, Jolly et al. 1987, Ross and Reid 1991) and K511 (*lh*, Reid 1986a), resulting from a reduction in internode length compared to its parental cultivar, Torsdag (Fig. 1). In addition, this mutant shows significant seed abortion when grown under warm (summer) temperatures (mean maximum/minimum day and night temperatures of 30.0±0.9°C and 18.7±0.3°C, respectively, Tab. 2).

A cross between NGB5843 and the slightly more severe (shorter) internode length mutant K511 (*lh*) gave dwarf F_1 plants (Fig. 2) indicating the mutation in NGB5843 is allelic with the *Lh* locus. By contrast, crosses between NGB5843 and lines homozygous for the recessive internode length alleles *le, ls, na, lka, lkb, la.*

cry^{c}, *lm*, *lw*, and *lv* all produced tall (wild-type) F_1 plants. The F_1 of cross NGB5843 by K511 more closely resembled the shorter K511 parent (Fig. 2) with a degree of dominance of 0.47 and 0.39 for lengths between nodes 1 to 4 and 4 to 6, respectively. When these F_1 plants were selfed, the F_2 resulted in a fairly continuous distribution. The genotypes of 21 of the F_2 were confirmed by growing-on at least 6 F_3 progeny from each, allowing the F_2 to be divided into two groups (Fig. 3). All the taller F_2 plants bred true while some of the shorter F_2 plants segregated in the F_3. Therefore, the F_2 appears to be segregating at 1 locus even though the ratio is slightly disturbed (χ^2_1 for 3:1 = 4.27, P<0.05).

A cross between NGB5843 (dwarf) and the tall, wild-type cv. Torsdag produced all tall F_1 plants. Dwarfism in NGB5843 consequently appears to be conferred by an allele at the *Lh* locus recessive (in regard to internode length) to both the *Lh* and *lh* alleles.

The F_2 from a cross between cv. Torsdag and NGB5843 segregated to give 73 tall and 7 dwarf plants (χ^2_1 for 3:1= 11.27, P<0.001). Progeny (6 plants) from each of the dwarf F_2 plants bred true in the F_3. Since the mutation present in NGB5843 is allelic with *lh*, a 3:1 segregation was expected in the F_2 resulting from this cross. The disturbed ratio is believed to be caused by abortion of the homozygous recessive seeds and/or gametophytic (or gametic) selection that occurs under the summer conditions (warm temperatures) under which the F_1 plants were grown (see later).

The dwarf F_2 also appeared to be more sterile than *Lh* (wild-type) plants (and similar to NGB5843) when grown under warm temperatures. A slight difference (P<0.05) was also observed between the mass of seeds harvested from the wild-type F_2 (*LhLh* and *Lhlh*^{i}) (0.236±0.002g) and seeds from the dwarf F_2 (0.220±0.006g). These results suggest that either the mutation present in NGB5843 (at the *Lh* locus) is affecting seed abortion directly or an allele at a locus closely linked to the *Lh* locus is also present in NGB5843.

It is proposed that the recessive gene affecting internode length (and possibly seed abortion) carried by NGB5843 be named *lh*^{i} and that NGB5843 should be the type line for this mutation.

Response of lh^{i} *plants to growth regulators.*

Application of GA_3 to dry seeds of genotype *lh*^{i} substantially promoted

internode elongation to lengths comparable to similarly treated *Lh* (wild-type) plants over the first few nodes (Fig. 4). In addition, the application of the gibberellin biosynthesis inhibitor, paclobutrazol, to *Lh* (wild-type) plants substantially reduced internode length, producing a dwarf phenotype similar to lh^i for early internodes (Fig. 4). When GA_3 and paclobutrazol were applied simultaneously to lh^i seeds, internode elongation was initially increased to lengths comparable to similarly treated wild-type plants and then decreased over later nodes to produce shorter internodes than control plants (Fig. 4).

The more severe effect of paclobutrazol on lh^i plants compared with *Lh* (wild-type) plants (Fig. 4) was unexpected, particularly since this did not occur for similarly treated *lh* plants (unpublished data). To further investigate the increased sensitivity of lh^i plants to paclobutrazol, near-isogenic lh/lh^i lines (descended from a common F_5 ancestor from a cross between lines NGB5843 and K511) were treated with 1μg of paclobutrazol to the dry seed before sowing. The internode lengths of lh^i plants were reduced to a far greater extent (4% of the length between nodes 2 and 5 for untreated plants, $n \geq 7$) than *lh* plants (36% of the length between nodes 2 and 5 for untreated plants, $n \geq 7$). Therefore, the response of NGB5843 (lh^i) plants to paclobutrazol appears to be a direct consequence of the lh^i mutation.

To quantify the increased sensitivity of lh^i plants to paclobutrazol, *lh* and lh^i plants, which are of similar stature (Fig. 1), were treated with a range of paclobutrazol doses to the dry seed before sowing (Fig. 5). The response of the two genotypes to paclobutrazol was clearly differentiated for the length between nodes 4 and 5. Plants of genotype lh^i required approximately 34 times less paclobutrazol than *lh* plants to reduce internode length by 50%. Similar results were obtained for other internodes (data not shown).

Like paclobutrazol, the GA-biosynthesis inhibitors uniconazol (structurally very similar to paclobutrazol), ancymidol and inabenfide are all thought to reduce the conversion of *ent*-kaurene to *ent*-kaurenoic acid (Graebe 1987, Hedden and Graebe 1985, Miki et al. 1990). Plants of genotype lh^i were also found to be more sensitive to uniconazol and inabenfide (applied to the dry seed before sowing, data not shown) and to ancymidol (sprayed onto young seedlings with 4-5 expanded leaves) than wild-type, *lh*, le^{5839} and *ls* plants (Fig. 6). The response of lh^i plants to other GA-biosynthesis inhibitors, prohexadione and AMO-1618, applied to the dry seed was also examined. The activity of *ent*-kaurene synthetase A, which converts geranyl-

geranyl-pyrophosphate to copalyl-pyrophosphate (the precursor of *ent*-kaurene), is though to be inhibited by AMO-1618 (Graebe 1987). By contrast, prohexadione is thought to act late in the GA-biosynthetic pathway (Rademacher et al. 1992), particularly reducing the 3β-hydroxylation of GA_{20} into GA_1 (Hedden 1991, Nakayama et al. 1992). Unlike paclobutrazol, and other inhibitors thought to act between *ent*-kaurene to *ent*-kaurenoic acid, AMO-1618 and prohexadione did not have an enhanced effect on lh^i plants compared with wild-type, *lh*, le^{5839} and *ls* plants (Fig. 7). The slightly increased response of *ls*, and to a lesser extent le^{5839}, plants to applied ancymidol and paclobutrazol (Figs 6,7) may be an artefact of the reduced stature of untreated plants (Reid 1986a, Jolly et al. 1987). When le^{5839} and *ls* plants were treated with GA_3 and paclobutrazol simultaneously (in the same manner as the plants in Fig. 4), paclobutrazol did not have a detectable effect after the GA_3-induced internode elongation had ceased (J.B. Reid, unpublished data), unlike the long-lasting effect of paclobutrazol on lh^i plants (Fig. 4).

The response of heterozygous $Lhlh^i$ plants to paclobutrazol was also investigated: dry seeds were treated with 1μg of paclobutrazol before sowing, and the length between nodes 1 and 4 measured. Compared with untreated plants, $Lhlh^i$ plants showed a similar response (48% reduction) to *LhLh* (wild-type) plants (50% reduction), and a far smaller response than lh^ilh^i plants (90% reduction). Thus, the *Lh* (wild-type) allele is completely dominant over the lh^i allele in terms of sensitivity to paclobutrazol.

GA levels in shoots of lh *and* lh^i *plants (from Swain 1989).*

Although GA levels have been examined in *lh* by bioassay (Reid and Potts 1986), the more accurate and precise technique of GC-SIM has not been used previously for GA quantification in K511 or NGB5843 (lh^i). Endogenous GA_1 levels decreased in the order *Lh* (wild-type), lh^i to *lh*. A clear, quantitative relationship between GA_1 levels and internode length between nodes 4 and 6 (encompassing the developmental stage at which plants were harvested) was found (Fig. 8). A comparable relationship, at a similar developmental stage, has been reported by Ross et al. (1989) for the action of the alleles at the *Le* locus. However, this result was partly based on the level of GA_1 synthesised in the apical portion from applied [^{3}H, ^{13}C]-labelled GA_{20}. Therefore, the present analysis is the first to use internal standards to quantify endogenous GA levels in a multiple allelic series.

The *lh* and lh^i alleles are thought to act very early in the GA biosynthetic pathway, reducing the level of all GAs (Ingram and Reid 1987a). The fact that the level of GA_{20} was below the level of detection in *lh* and lh^i plants (Tab. 1) is consistent with this view. By contrast, the *le* and le^d alleles are thought to act immediately prior to GA_1 formation (Ingram et al. 1984, 1986) and consequently, *le* plants possess elevated levels of GA_{20} (Ross et al. 1992). Since a quantitative relationship between internode length and endogenous GA_1 levels has been demonstrated using both loci, a clear relationship cannot be demonstrated between internode length and the levels of any GA_1 precursor. This further supports the role of GA_1 as the only biologically active native GA affecting internode length in its own right, especially since GA_3 has not been detected in pea shoots (Lawrence et al. 1992, Ross et al. 1992). A similar line of reasoning has been used for the role of GA_1 in maize (Phinney 1984).

Seed abortion on lh^i *plants.*

Compared to *Lh* (wild-type) plants, lh^i plants produce significantly ($P<0.001$) fewer seeds and fewer seeds per pod when grown under warm temperatures (Tab. 2). However, a direct comparison is complicated by the large difference in stature of the two genotypes, which may indirectly affect seed abortion. Hence, lh^i and *lh* plants, which are of similar stature (Fig. 1), were grown in controlled environment cabinets to more accurately determine seed yield (Tab. 3). In 20/15°C or 15/10°C temperature regimes lh^i plants had significantly ($P<0.001$) fewer seeds and seeds per pod, and more reproductive nodes and pods than *lh* plants. At 25/20°C lh^i plants had fewer seeds per pod ($P<0.001$) and more reproductive nodes ($P<0.01$) when compared with *lh* plants. In addition, the seed yield of both genotypes decreased with increasing day/night temperature (Tab. 3). The reduced number of seeds at the first two reproductive nodes of *lh* plants (compared to subsequent nodes, Fig. 9) is due to occasional flower abortion, the cause of which is not known.

Apical senescence was significantly delayed in lh^i plants compared with *lh* plants grown at 25/20°C ($P<0.01$), 20/15°C ($P<0.001$) and 15/10°C ($P<0.001$) (Tab. 3) due to an increase in the number of reproductive nodes (secondary growth). This is thought to result from the reduced number of seeds at early reproductive nodes

of lh^i plants (Fig. 9). A similar response has been noted for plants homozygous for *ar* or *n*. The *ar* gene acts by reducing the size of the hilum and funiculus while the *n* gene results in decreased size of the pod lumen (Murfet 1985, Reid 1989). Both genes appear to reduce seed sink strength (by reducing seed yield) leading to secondary growth and delayed apical senescence (Murfet 1985).

Discussion

A new internode length allele (lh^i), at the *Lh* locus, has been identified in the garden pea (*Pisum sativum L.*). The lh^i allele results in reduced internode length compared to its tall, wild-type progenitor cv. Torsdag (*Lh*) (Fig. 1). However, lh^i plants are slightly taller than the allelic internode length mutant, *lh*, described previously by Reid and Potts (1986). Plants of genotype lh^i also have reduced seed yield compared to cv Torsdag (*Lh*) and *lh* plants (Tabs. 2, 3).

In shoots, endogenous levels of GA_1 and GA_{20} are reduced in lh^i and *lh* plants compared to *Lh* (wild-type) plants (Tab. 1). Since lh^i is allelic with *lh*, it is also thought to partially interfere with gibberellin biosynthesis between geranylgeranyl-pyrophosphate (GGPP) and *ent*-kaurene (Ingram and Reid 1987a), hence reducing the levels of all gibberellins in young shoots. In this respect the *lh* and lh^i alleles differ from alleles at the *Le* locus. Plants homozygous for the *le* or le^d alleles possess elevated GA_{20} levels compared with wild-type plants, due to impaired ability to 3β-hydroxylate GA_{20} into GA_1 (Ingram et al. 1984, Ross et al. 1992). Both the lh^i and *lh* alleles can be described as 'leaky' since GA biosynthesis is not completely blocked. The response of lh^i plants to exogenously applied GA_3 and paclobutrazol is consistent with lh^i acting, in shoots, by reducing the level(s) of endogenous active gibberellin(s).

The reason for the increased sensitivity of lh^i plants (more than 30 times compared with *lh* plants) to the GA-biosynthesis inhibitor, paclobutrazol, is not known. However, it is not due to effects of the lh^i mutation on seed development before germination. Compared with lh^i plants treated with GA_3 only, lh^i plants treated with both GA_3 and paclobutrazol possessed early internodes of very similar length, while the later internodes were significantly ($P<0.001$) shorter (Fig. 4). By contrast, this did not occur for similarly treated *Lh* (wild-type) plants (Fig. 4), suggesting that lh^i plants are more sensitive to paclobutrazol than *Lh* (wild-type)

plants, even when early internodes are not effected. The response of lh^i plants to ancymidol sprayed onto young seedlings (Fig. 6) is also consistent with this view. The results shown in Fig. 4 also suggest that paclobutrazol is affecting internode length in both lh^i and *Lh* (wild-type) plants solely by reducing GA-biosynthesis, rather than by indirect (i.e. toxic) effects (Graebe 1987).

Plants of genotype lh^i exhibit increased sensitivity to paclobutrazol, uniconazol, inabenfide and ancymidol (which are thought to act between *ent*-kaurene and *ent*-kaurenoic acid, Graebe 1987, Miki et al. 1990), and a relatively normal response to AMO-1618 and prohexadione (which act elsewhere in the GA-biosynthetic pathway), compared with wild-type, *lh*, le^{5839} and *ls* plants. The *ls* and le^{5839} mutations are thought to reduce the conversion of geranyl-geranyl pyrophosphate to copalyl pyrophosphate (see Chapter 8) and the conversion of GA_{20} to GA_1 (Ingram et al. 1984), respectively. By contrast, although application studies suggest that *lh* (and presumably lh^i) act before *ent*-kaurene production (Ingram and Reid 1987a), the site of action of the *Lh* protein remains to be conclusively identified. Since paclobutrazol has been suggested to act as a competitive inhibitor of GA-biosynthesis (by mimicking the 3 dimensional structure of *ent*-kaurene, Sugavanam 1984), the different responses of lh^i, *ls* and le^{5839} plants to paclobutrazol suggests that the *Lh* protein may be involved in *ent*-kaurene metabolism. However, the different responses of *lh* and lh^i plants to paclobutrazol, uniconazol, inabenfide and ancymidol still remains unexplained. One possible explanation is that the *Lh* gene encodes the enzyme (or one of several enzymes) responsible for the conversion of *ent*-kaurene to *ent*-kaurenoic acid. Plants of genotype lh^i may produce an enzyme with altered 3 dimensional structure that is more severely affected by paclobutrazol, uniconazol, inabenfide and ancymidol than the enzymes produced by *Lh* (wild-type) or *lh* plants. This question cannot be resolved until the site of action of *Lh* has been identified, although preliminary results suggest that neither the *lh* nor lh^i mutations reduce *ent*-kaurene production *in vitro* in cell-free enzyme preparations from developing seeds (data not shown), consistent with the hypothesis above.

A quantitative relationship between endogenous GA_1 levels and internode length was found for plants possessing the three alleles (*lh*, lh^i and *Lh*) at the *Lh* locus (Fig. 8), further supporting a direct role for GA_1 in stem growth in peas. Although GA_1 is thought to be the major active GA in shoots *per se* (see Fujioka et al. 1988, Reid et al. 1992, Reid and Ross 1993), such a relationship has only been

shown in a limited number of species including wheat (Lenton et al. 1987) and peas (for alleles at the *Le* locus, Ross et al. 1989).

The simplest possible hypothesis explaining a 'leaky' gene is an allele that results in the formation of a partially functional enzyme, with reduced activity, compared to the enzyme produced by the wild-type allele. If both the *lh* and lh^i alleles were operating in this manner, the less severe lh^i allele might be expected to show partial or full dominance over the more severe *lh* allele in young shoots. Such a mode of action has been suggested for alleles at the *Le* locus (Phinney 1984, Ross et al. 1989) which show decreasing dominance in the order *Le*, *le*, and le^d in conjunction with decreasing GA_1 levels (Ross et al. 1989) and, presumably, increasing severity of the genetic lesion. However, the dominance relationship between lh^i and *lh* is unusual since, in young shoots, the more severe *lh* allele is partially dominant over the less severe lh^i allele (Fig. 2). Thus the above model seems unlikely to be operating for alleles at the *lh* locus. However, the wild-type allele (*Lh*) is dominant over both the *lh* (Reid 1986a) and lh^i alleles. Further work, including analysis of the *Lh* locus at the biochemical or molecular level, is required to determine the cause of the unusual dominance relationships.

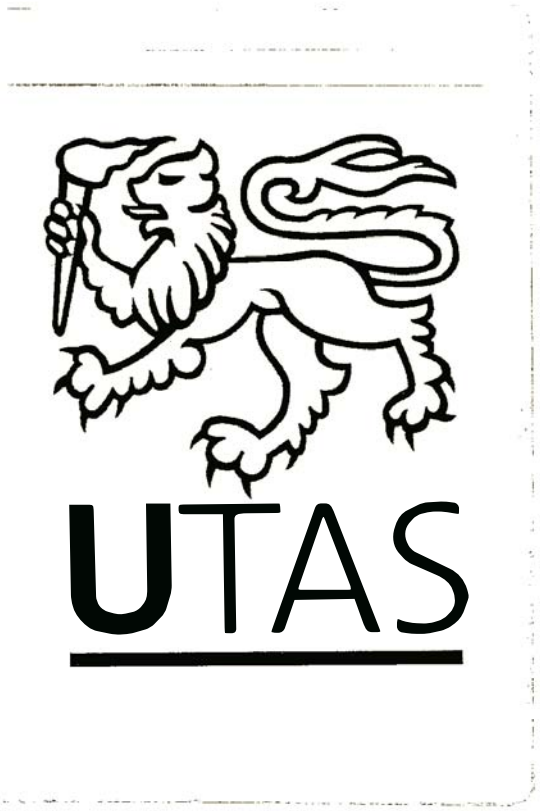
Tab. 1. Harvest details and GA levels in young seedlings of genotype *Lh* (wild-type), lh^i and *lh*. From Swain (1989).

Genotype	Number of plants	Mass (gFW)	GA_1 ($ng.(gFW)^{-1}$)	GA_{20} ($ng.(gFW)^{-1}$)
Lh	56	35.65	3.39	3.77
lh^i	58	34.85	0.50	-[a]
lh	58	30.56	0.17	-[a]

[a] no dilution of internal standard.

Tab. 2. Seed yield of self-pollinated *Lh* (wildtype) and lh^i plants grown under an 18h photoperiod during Summer (n=9).

Genotype	Flowering node	Seeds	Pods	Seeds per pod
Lh	15.5±0.2	15.3±0.5	6.3±0.2	2.4±0.1
lh^i	15.7±0.2	9.1±1.0	5.9±0.5	1.6±0.2



Tab. 3. Seed yield of *lh*^{i} and *lh* plants grown under an 18h photoperiod with day/night temperatures of 25/20, 20/25 or 15/10°C (n=15).

	Day/night temperature (°C)					
	25/20		20/15		15/10	
	lh^{i}	*lh*	*lh*^{i}	*lh*	*lh*^{i}	*lh*
Reproductive nodes	7.1 ±0.4	5.5 ±0.2	11.8 ±0.7	7.5 ±0.2	21.1 ±0.8	10.8 ±0.3
Pods[a]	7.6 ±0.4	4.7 ±0.2	14.3 ±0.7	10.2 ±0.5	26.4 ±0.9	17.9 ±0.7
Seeds	9.9 ±0.8	11.8 ±0.7	15.3 ±1.1	32.7 ±2.1	43.8 ±3.1	62.5 ±1.8
Seeds per pod	1.34 ±0.11	2.54 ±0.17	1.13 ±0.11	3.22 ±0.17	1.70 ±0.41	3.51 ±0.08

[a] maximum of 2 pods per node.

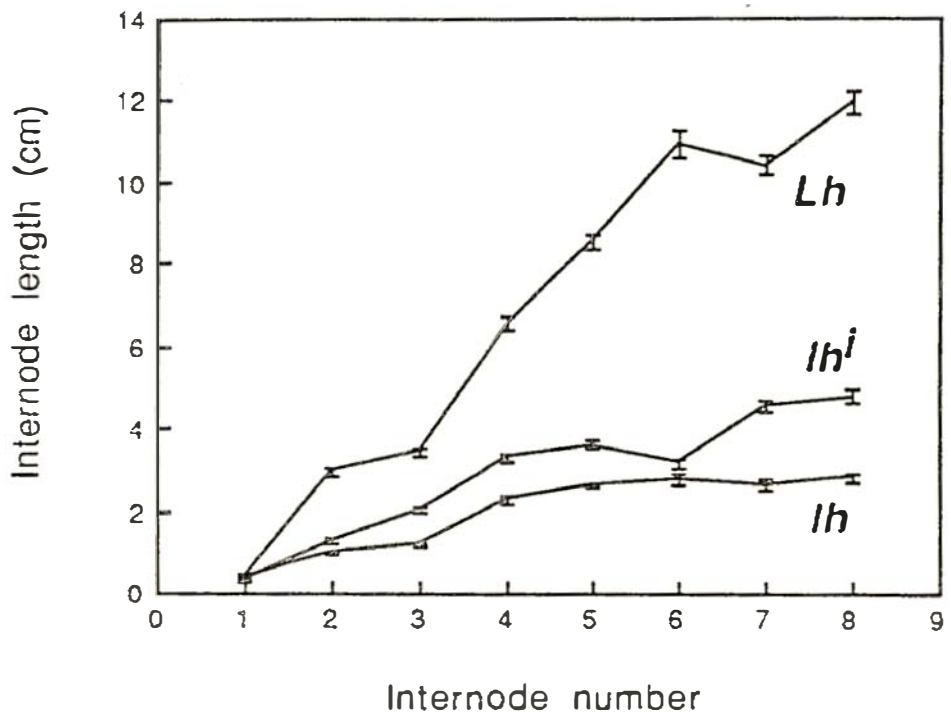


Fig. 1. Internode lengths of *Lh* (wild-type), lh^i and *lh* plants. Values are the means of 12 plants ± SE. From Swain (1989).

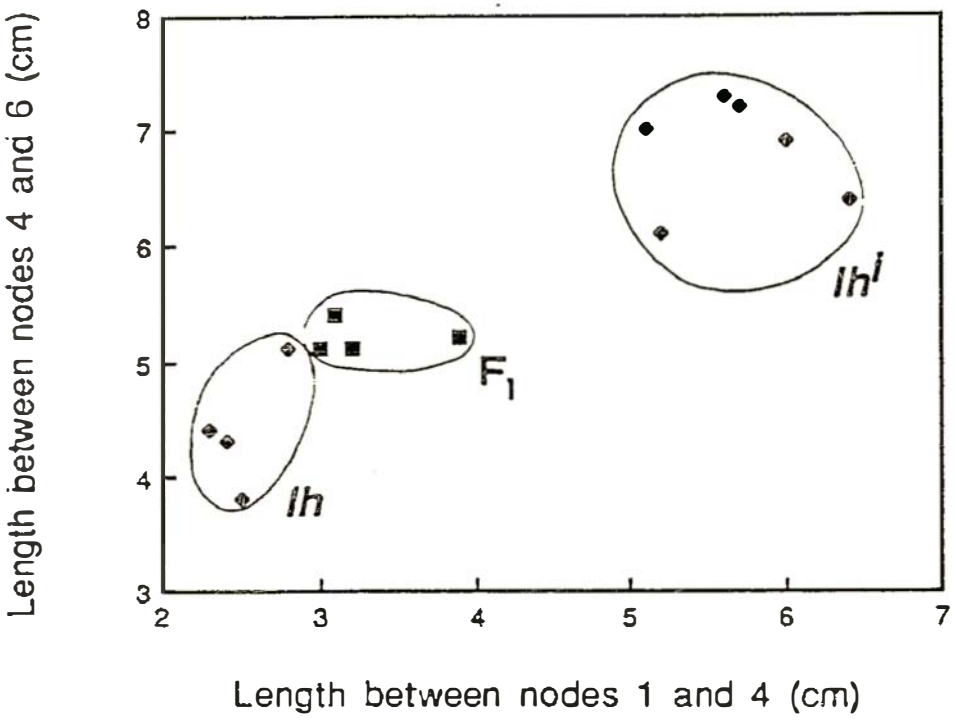


Fig. 2. Length between nodes 1 and 4 plotted against length between nodes 4 and 6 of parents and the F_1 from a cross between lines NGB5843 (lh^i) and K511 (lh). From Swain (1989).

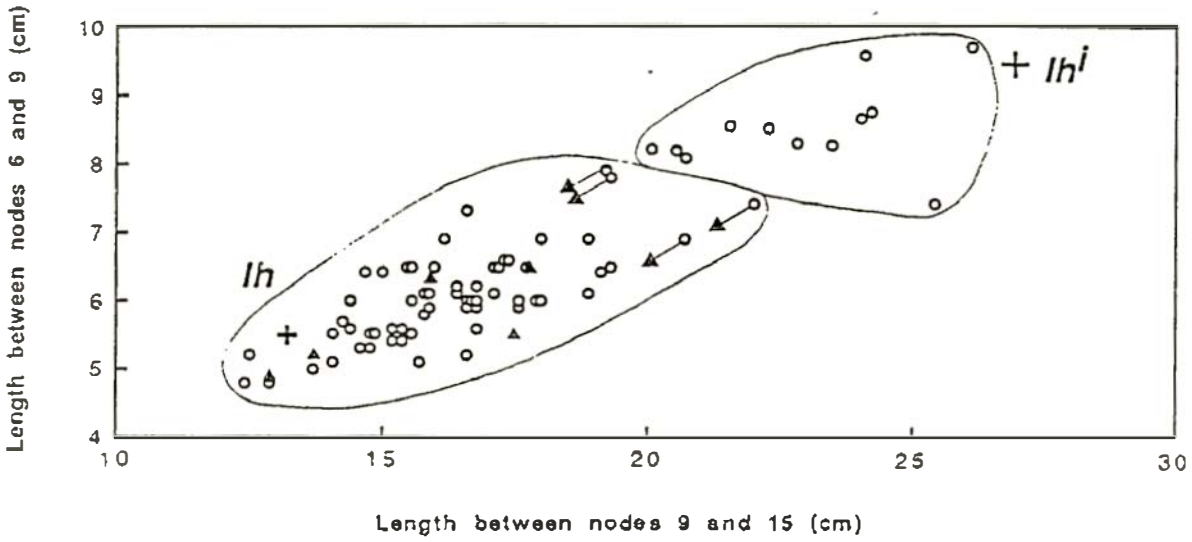


Fig. 3. Length between nodes 9 and 15 plotted against length between nodes 6 and 9 of F_2 plants from a cross between lines NGB5843 (lh^i) and K511 (lh) showing division into two classes based on the F_3. Crosses represent means and SE's of 6 NGB5843 (lh^i) and K511 (lh) parents grown at the same time. Arrows represent F_2 plants that segregated. All the plants in the taller group bred true. In addition, progeny from five F_2 plants in the shorter group (▲) were grown-on to confirm their genotype. From Swain (1989).

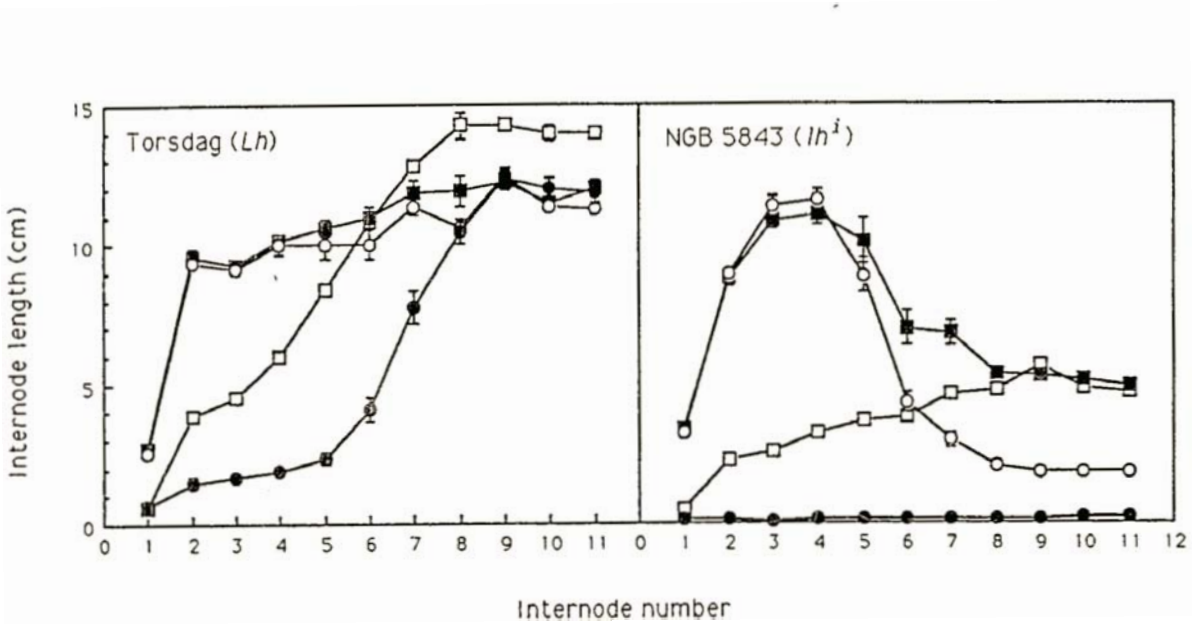


Fig. 4. Internode length plotted against internode number for *Lh* (wild-type) and lh^i (NGB5843) plants treated with ethanol only (□), 10µg GA_3 (■), 10µg GA_3+5µg paclobutrazol (○), or 5µg paclobutrazol (●). Values are the means of 8 to 12 plants ± SE.

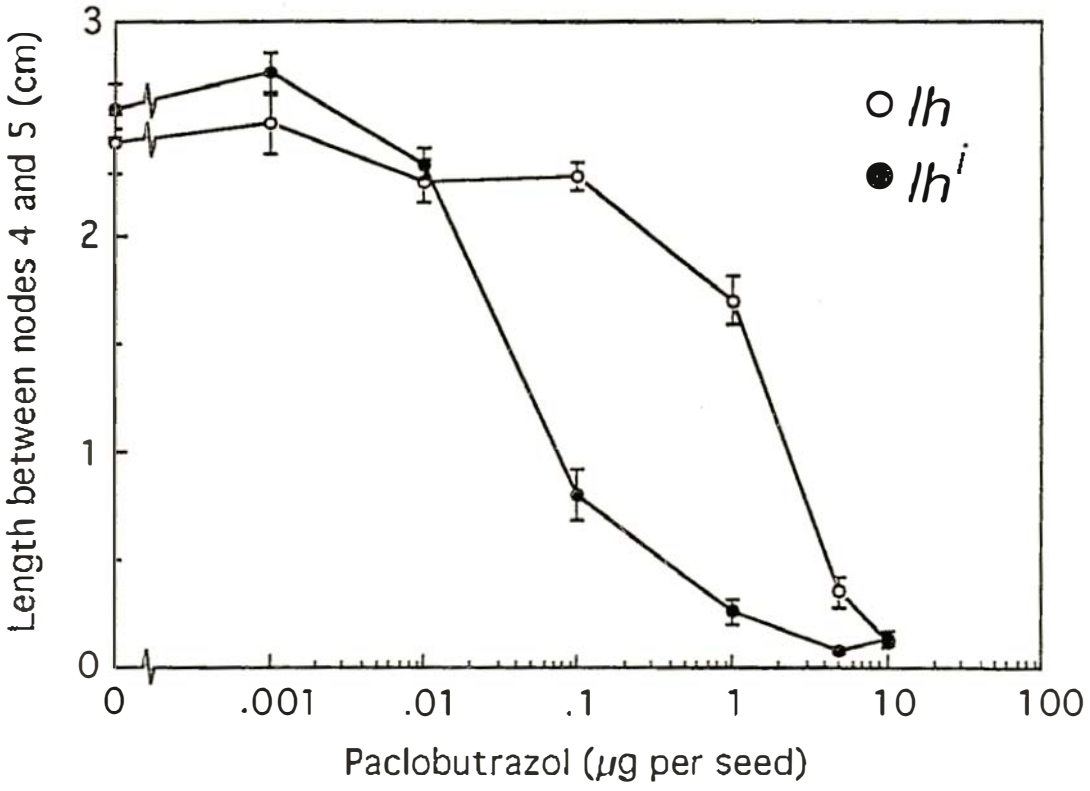


Fig. 5. Inhibition of internode elongation between nodes 4 and 5 by paclobutrazol (applied to the dry seed before germination) of *lh* and lh^i plants. Values are the means of 8 plants ± SE.

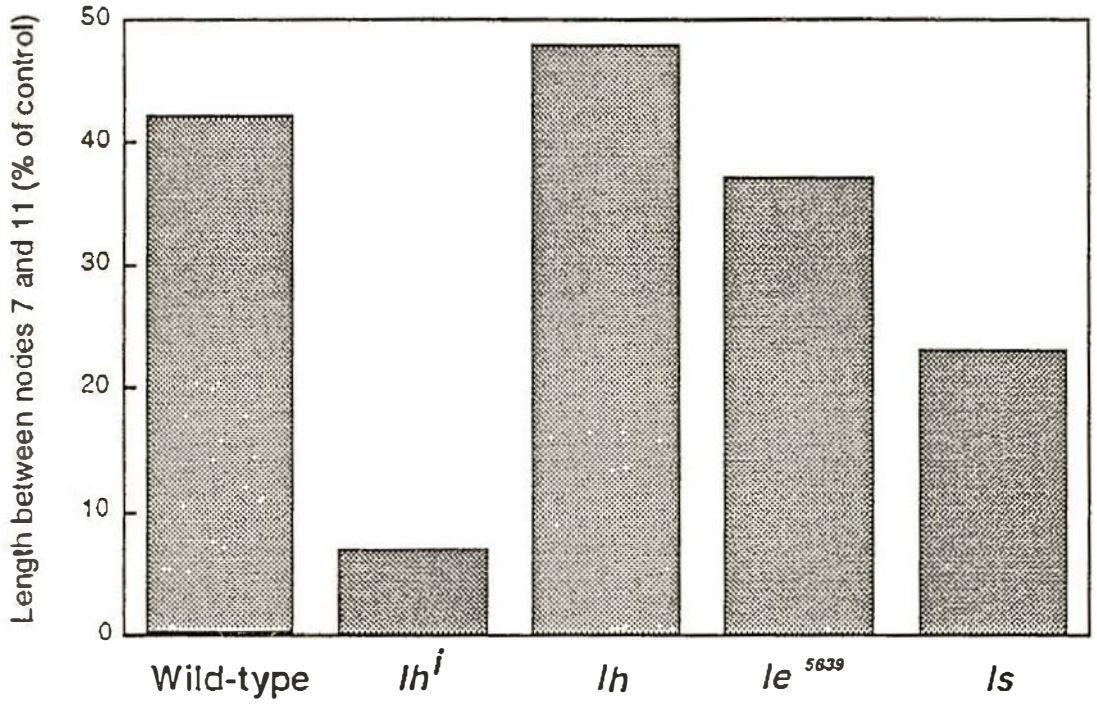


Fig. 6 Inhibition of internode elongation between nodes 7 and 11 by ancymidol (ca. 0.2µg per plant) sprayed onto young wild-type seedlings and seedlings of genotype *lh*^{i}, *lh*, *le*^{5839} and *ls*. Values are the means of 8 plants ± SE.

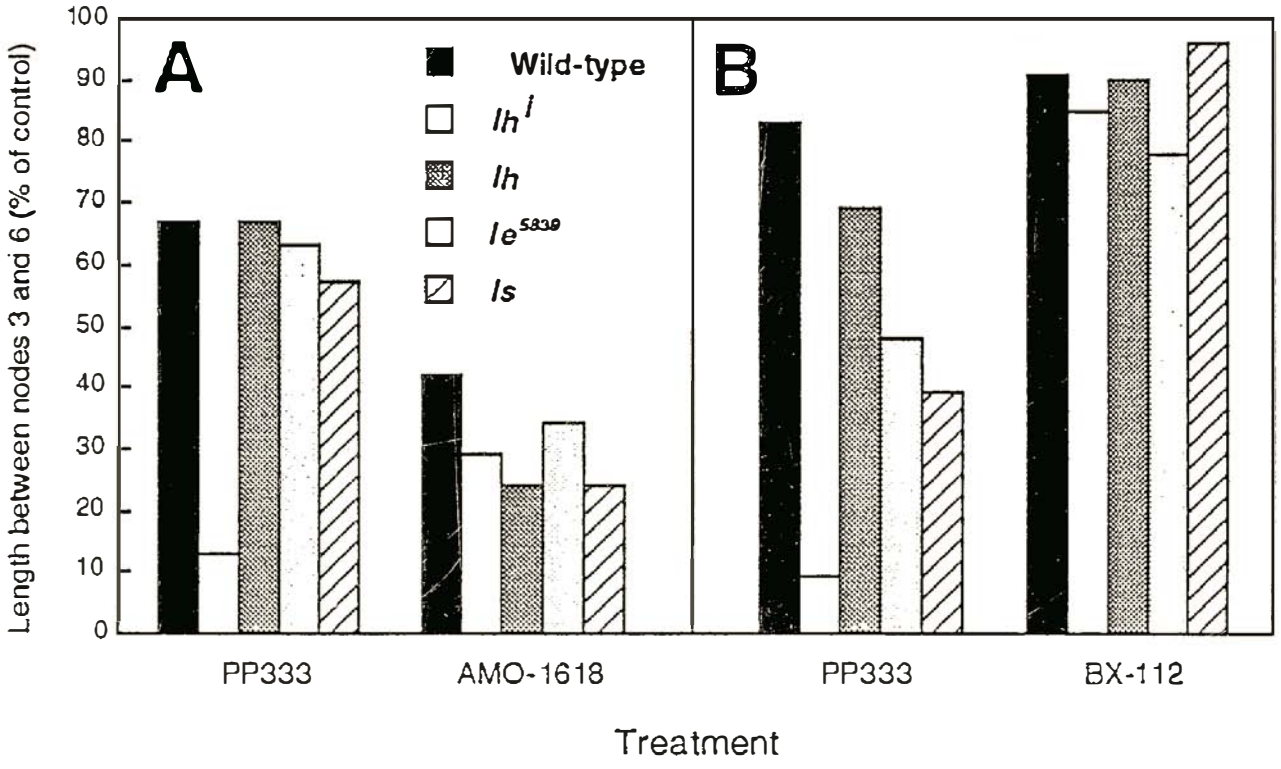


Fig. 7. Inhibition of internode elongation between nodes 3 and 6 by paclobutrazol (PP333, 1µg), AMO-1618 (20µg) and prohexadione (BX-112, 10µg) (applied to the dry seed before germination) of wild-type, lh^i, *lh*, le^{5839} and *ls* plants. Results are from two separate experiments (A and B). Values are the means of 8 plants ± SE.

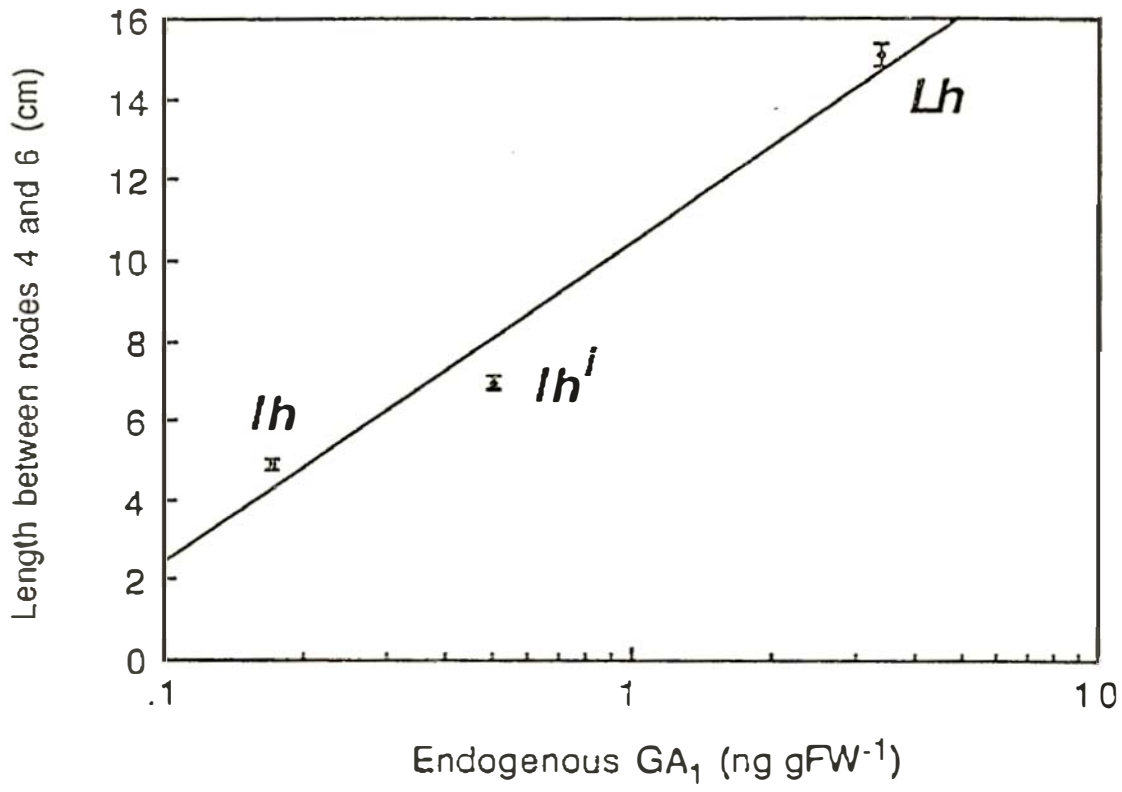


Fig. 8. Relationship between endogenous GA_1 levels in young shoots (harvested at approximately 5 nodes expanded) and internode length between nodes 4 and 6 for *Lh* (wild-type), lh^i and *lh* plants. Internode lengths are the means of 12 plants ± SE. From Swain (1989).

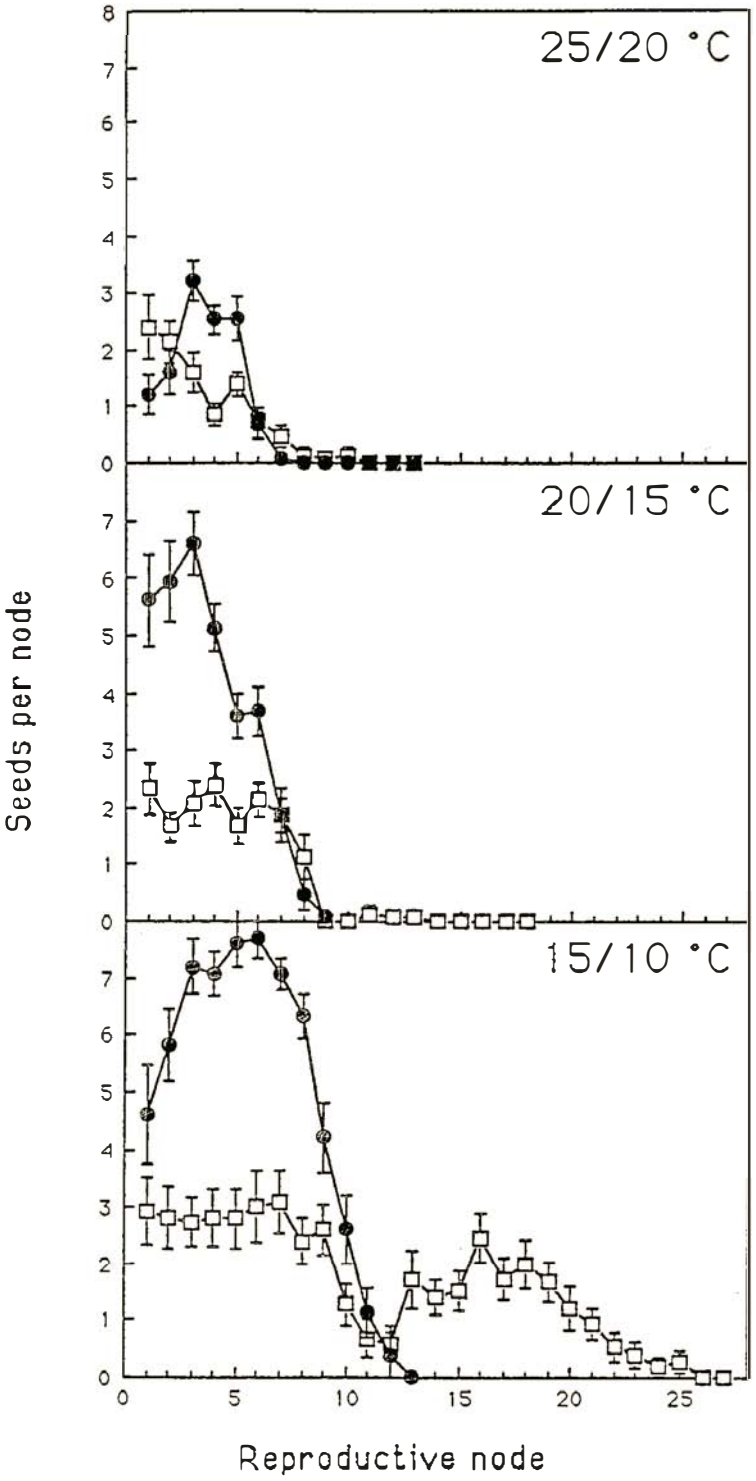


Fig. 9. Seeds harvested from each reproductive node of lh^i (□) and lh (●) plants grown at day/night temperatures of 25/20, 20/15 or 15/10°C. Values are the means of 15 plants ± SE.

Chapter 4

The lh^i allele reduces gibberellin levels in developing seeds, and increases seed abortion.

The contents of this chapter are accepted for publication in Planta (1993).

Introduction

In chapter 3 a new GA-metabolism mutant, lh^i, with pleiotropic effects on internode length and seed yield was described. Plants of genotype lh^i possess reduced seed yield compared with *Lh* plants and the allelic dwarf mutant, *lh*. In addition, when a cross between the wild-type, cv. Torsdag (*Lh*) and the mutant line NGB5843 (lh^i) was performed, the resulting F_2 did not segregate in accord with the expected ratio of 3 tall to 1 dwarf plant, due to a deficiency in lh^i (dwarf) progeny (Chapter 3). These results suggest that GAs may play an important role in seed development of *Pisum*.

In this chapter lh^ilh^i seeds are shown to possess reduced GA levels compared with *LhLh* (wild-type), $Lhlh^i$ and *lhlh* seeds. The lh^i mutant is then used to investigate the relationship between GA levels, seed weight and seed abortion.

Materials and methods

Growing conditions.

Plants were grown in pots, with the exception of F_2, F_5 and F_7 progeny from heterozygous parents selfed at various temperatures, which were grown in tote boxes and visually scored for stature (tall, dwarf or nana phenotype).

Abortion of lh^i *(NGB5843) seeds.*

To examine the developmental stage at which seed abortion was occurring seeds were allowed to develop on 6 NGB5843 (lh^i) plants grown in a controlled environment chamber at a density of 1 per pot in a day/night temperature regime of 20/15°C. At 12 days after anthesis the lower quarter (opposite the funicles) of the pods at the first two reproductive nodes (1 pod at each node) was removed to reveal the

seeds. Seeds were visually scored as being either "healthy" or "aborting" based on their size relative to other seeds (in the same and in different pods). Seeds were monitored every second day until seed development was complete (when the seeds appeared to be completely desiccated). Plastic bags (11.5cm x 7.5cm) were placed over the pods to prevent them from drying out before seed maturation; Bags were replaced every second day.

Plants used for GA extractions.

Plants from which seeds and pods were harvested for GA analysis were grown at a density of 2 per pot in a heated glasshouse. Seeds were harvested at 5,7,11,15 or 19 days after anthesis. Pods were harvested at 6 or 11 days after anthesis. Endogenous GA_3 was not identified in pod tissue, despite the use of an internal standard (endogenous level below 0.1 ng.(gFW^{-1})). In all cases seeds and pods were separated immediately after removal from the plant. Harvested seeds included the embryo, testa and liquid endosperm (if present).

Gibberellin analysis.

Harvested tissue was weighed, immersed in cold methanol and stored at -20°C. To begin extractions, dH_2O was added to a final concentration of 20%, the tissue was homogenised and di-deuterated or ^{13}C,^{3}H internal standards were added. Extraction of GAs from all pods and seeds at least 11 days of age was as described in Chapter 2 (Method 1). For seeds harvested 5 or 7 days after anthesis, GAs were extracted as described in Chapter 2 (Method 2).

Plants used to examine segregation of the Lh *and* lh^i *alleles.*

The lh^i (NGB5843) and *lh* (K511) plants used to investigate gametic/gametophytic selection were grown (2 per pot) in a glasshouse during summer (mean maximum/minimum day and night temperatures of 30.0±0.9 °C and 18.7±0.3 °C, respectively). All other plants were grown at a density of 1 per pot and transferred to controlled environment cabinets with day/night temperature regimes of 30/25, 25/20, 20/15 or 15/10°C.

Results

Abortion of lh^{t} seeds.

Under normal glasshouse conditions (Reid and Potts 1986) the majority of pods developing on *Lh* (wild-type) and *lh* (dwarf) plants contain at least 4 seeds at harvest. By contrast, pods harvested from *lh^{t}* plants typically have only one or two fully developed seeds and several partially developed but aborted seeds (Fig. 1). In order to further characterise the *lh^{t}* phenotype seeds developing on self-pollinated *lh^{t}* plants were classified as either "healthy" or "aborting" depending on their relative size and appearance at 12 days after anthesis. All the seeds classified as "aborting" (small) did not subsequently develop into normal (capable of germination) seeds. Instead, they remained smaller and eventually began to desiccate at about the same time as neighbouring seeds. Of the seeds originally labelled "healthy" (larger) some failed to grow as fast as others and eventually failed to develop into normal seeds when pod senescence occurred (data not shown). The remaining seeds continued to develop to contact point, and beyond, and finally desiccated to resemble wild-type seeds. Examination of aborted seeds revealed embryos at various stages of development (but apparently before contact point) with no common point at which development appeared to cease. In addition, the mean weight of fully developed seeds harvested from *lh^{t}* plants (0.204±0.005g, n=20) was significantly less than the mean weight of seeds from *Lh* (wild-type) plants (0.264±0.005g, n=20) grown under similar conditions ($P<0.001$).

In another experiment, heterozygous *Lhlh^{t}* seeds developing on homozygous *lh^{t}* plants were produced by fertilizing *lh^{t}* flowers with wild-type (*Lh*) pollen. The proportion of partially developed, but aborted, seeds was consequently reduced from 62% (*lh^{t}lh^{t}*) to 5% (*Lhlh^{t}*), a proportion similar to self-pollinated *Lh* (wild-type) plants (*LhLh*, 0%) (Fig. 1). In addition, the mean dry weight of *Lhlh^{t}* seeds was also greater (0.205±0.007g) than the weight of *lh^{t}lh^{t}* seeds (0.179±0.006g, $P<0.01$), and similar to the weight of *LhLh* seeds (0.202±0.005g, $P>0.70$). These results demonstrate that one copy of the *Lh* (wild-type) allele can restore normal seed development (reducing seed abortion and increasing seed weight) on a homozygous recessive *lh^{t}* plant.

Gibberellin levels in developing seeds.

Since, in developing shoots, the primary action of the *lh*^{i} mutation is to reduce internode length by a reduction of GA_1 levels (Chapter 3), GA levels were investigated in developing seeds. In two separate experiments, GA levels were determined in *Lh* (wild-type), *lh*^{i} and *lh* seeds harvested at either 5 and 7 days after anthesis, or 11,15 and 19 days after anthesis. Seeds of genotype *lh*^{i} were found to have substantially reduced levels of GA_1 compared with *Lh* (wild-type) seeds and *lh* seeds when harvested 7 days after anthesis (Tab. 1). At this stage the number of apparently healthy *lh*^{i} seeds was actually slightly greater than the number of healthy *Lh* seeds ($P<0.001$), although the average fresh weight of *lh*^{i} seeds was less than that of *Lh* seeds (Tab. 1). The level of GA_{20} was also reduced in *lh*^{i} seeds compared with *Lh* and *lh* seeds, although the effect of the *lh*^{i} allele on GA_{20} levels was much less marked than for GA_1 (Tab. 1). When compared with *Lh* seeds of a similar weight (harvested 5 days after anthesis) *lh*^{i} seeds (harvested 7 days after anthesis) had reduced levels of GA_1, GA_{20} and GA_{29} (Tab. 1). At 11,15 and 19 days after anthesis healthy *lh*^{i} seeds were again found to weigh less and possess reduced levels of endogenous GAs compared with wild-type seeds (Tab. 2). The effect of the *lh*^{i} mutation in healthy seeds, compared with *Lh*, was maximal for GA_{20} and GA_{29} levels at 15 days after anthesis (Tab. 2). At 15 and 19 days after anthesis *lh*^{i} seeds could be clearly divided into two groups based on their relative size in a similar manner to the experiment detailed above. Except in rare cases (ca. 5% of seeds) no such division could be made for seeds of any other genotype. The *lh*^{i} seeds classified as "aborting" weighed less and contained extremely low levels of GA_{20} and GA_{29} compared with healthy *lh*^{i} seeds and *Lh* (wild-type) seeds (Tab. 2).

The rate of development of *lh*^{i} seeds is also slower than that of *Lh* (wild-type) seeds. Depending on environmental conditions, contact point (the first day no liquid endosperm remains) can be delayed by up to 6 days for *lh*^{i} seeds compared with wild-type seeds. However, the large differences in GA levels between *Lh* (wild-type) and *lh*^{i} seeds (Tab. 2) are not due to different developmental ages since *lh*^{i} seeds were found to possess ca. 2% of the level of GA_{20} present in *Lh* (wild-type) seeds when both genotypes were harvested at contact point (data not shown).

Heterozygous *Lhlh*^{i} seeds developing on homozygous *lh*^{i} plants were produced by fertilizing *lh*^{i} flowers with wild-type (*Lh*) pollen. Seeds of genotype

Lhlh^{i} were of similar weight and possessed similar, or slightly higher, GA levels as seeds developing on self-pollinated wild-type plants (Tab. 2). This is consistent with the hypotheses that the GAs present in developing seeds are important for seed development and are produced *in situ* (i.e. not imported from the surrounding maternal tissue).

Seeds homozygous for the *lh* allele were also found to possess reduced levels of certain GAs, compared with *Lh* (wild-type) seeds, at some, but not all, of the seed ages examined (Tabs. 1,2). However, *lh*^{i} seeds possessed markedly lower levels of GA_1 at 7 days after anthesis (Tab. 1) and GA_{20} at 11 and 15 days after anthesis (Tab. 2) than *lh* seeds.

In agreement with results obtained by other workers (Gaskin et al. 1985), endogenous GA_1 or GA_3 were not found in seeds 15 days of age or older. In fact, endogenous GA_3 was not usually detected, even in very young seeds (Tab. 1) although GA_3 was found in Torsdag (*Lh*) seeds harvested 4 and 6 days after anthesis in another experiment (data not shown).

Segregation of the Na/na, Lh/lh, *and* Lh/lh^{i} *alleles.*

A previous cross involving the *Lh* and *lh*^{i} alleles did not segregate in agreement with expected results due a deficiency of homozygous recessive *lh*^{i} plants (Chapter 3). Since the *na* allele is not expressed in developing seeds (Potts and Reid 1983, Potts 1986) while the *lh* allele has a minor effect on seed GA levels (Tabs. 1,2), the segregation of the *Na/na* and *Lh/lh* alleles were compared with that of the *Lh/lh*^{i} alleles at various temperatures. When heterozygous *Nana* or *Lhlh* plants were allowed to self-pollinate at 30/25°C (*Nana*) or 30/25, 25/20, 20/15 and 15/10°C (*Lhlh*) the observed proportion of short (either nana or dwarf phenotype) F_2 plants was in agreement with the expected ratio of 3 tall to 1 short (Fig. 2). By contrast, when heterozygous *Lhlh*^{i} plants were allowed to self-pollinate the tall:dwarf ratio was significantly disturbed at 30/25 ($P<0.01$), 25/20, 20/15 and 15/10°C ($P<0.001$, Fig. 2). However, a tall:dwarf ratio in agreement with the expected 3:1 was obtained at 20/15°C when *Lhlh*^{i} plants were decapitated above the second reproductive node ($P>0.50$, Fig. 2).

In another experiment, when seeds obtained from *lh* plants fertilized with pollen from *Lhlh* heterozygotes were sown the observed numbers of tall and dwarf offspring did not differ significantly from the expected 1:1 ratio (Tab. 3). However, a

significant deficiency ($P<0.01$) in dwarf offspring was found when lh^i plants were fertilized with pollen from heterozygous $Lhlh^i$ plants, producing seeds of genotype $Lhlh^i$ (normal GA levels, Tab. 2) and lh^ilh^i (GA deficient, Tab. 2) on the same maternal plant (Tab. 3).

In addition, to determine whether the *lh* and lh^i alleles affected the development of healthy seeds resulting from these crosses, the dry weight of the seeds were recorded immediately before sowing. No significant difference between *Lhlh* and *lhlh* seeds was found (Fig. 3). However, seeds of genotype $Lhlh^i$ were found to be heavier than fully developed lh^ilh^i seeds developing in the same pods (Fig. 3) regardless of whether seeds from all pods ($P<0.001$), or only seeds from relatively fertile pods (at least 3 seeds) ($P<0.01$) were considered.

Since the reduced number of homozygous lh^i plants obtained in the above crosses could result from zygotic and/or gametic/gametophytic selection two further experiments were conducted. Firstly, to investigate whether zygotic selection was occurring, pollen from homozygous lh^i plants was used to fertilize heterozygous $Lhlh^i$ plants. Again, the number of tall ($Lhlh^i$) and dwarf (lh^ilh^i) offspring did not agree with the expected 1:1 ratio ($P<0.05$). When seeds from pods with marked seed abortion (i.e. no more than 3 seeds) were considered this deviation was even more marked ($P<0.01$, Tab. 3). However, when pods containing at least 4 seeds were scored the tall:dwarf ratio was not significantly disturbed ($P>0.30$, Tab. 3). These results suggest that zygotic selection favours $Lhlh^i$ seeds over lh^ilh^i seeds.

In the second experiment, the possible role of gametic or gametophytic selection was examined by crossing pollen from heterozygous $Lhlh^i$ and *Lhlh* plants onto homozygous *lh* and lh^i plants, respectively. No significant deviation from the expected ratio of 1 tall ($Lhlh^i$ or *Lhlh*) to 1 dwarf ($lhlh^i$) was found in progeny resulting from either cross (Tab. 3). Hence, no evidence for gametic or gametophytic selection was found. This result also demonstrates that seeds of genotype *Lh-* and $lhlh^i$ are equally likely to survive, at least under these conditions, suggesting that the *Lh* and *lh* alleles are completely dominant over the lh^i allele in developing seeds.

Expression of the lh^1 *allele in pods.*

Pods developing on lh^i plants were shorter ($P<0.001$), weighed less and contained lower levels of GA_1, GA_{20} and GA_{29} at 6 and 11 days after anthesis compared with pods harvested from *Lh* (wild-type) plants (Tabs. 4,5). Fertilizing lh^i

plants with *Lh* (wild-type) pollen resulted in seeds (genotype $Lhlh^i$) with increased GA levels (Tab. 2) but did not increase GA levels in the surrounding pod tissue compared with pods developing on self-pollinated lh^i plants (Tab. 5). In fact, the total amount of GA_{20} present in $Lhlh^i$ seeds is approximately 185 times higher at 11 days after anthesis (Tabs. 2,5), and 4000 times higher at 15 days after anthesis (data not shown), than the total amount of GA_{20} present in the surrounding pod tissue (genotype lh^i). These results suggest that the lh^i allele is expressed in developing pods and that, if at all, only a small proportion of the GAs present in the developing seeds are exported to, and accumulate in, the surrounding pod tissue. However, these results do not exclude the possibility that seeds influence pod GA levels during pod elongation (which occurs in the first few days after anthesis) (e.g. Ozga et al. 1992).

Discussion

The mutant allele lh^i reduces endogenous GA levels in developing seeds, decreases seed weight and increases seed abortion compared with the *Lh* (wild-type) and *lh* alleles. Although a range of GA-synthesis mutants have previously been identified in the garden pea, *Pisum sativum* L. (e.g. *na, ls, le*), this is the first mutation shown to both reduce GA levels in seeds and to show significant seed abortion (compare with results obtained for the *le* allele in Santes et al. 1993).

Seed yield of lh^i plants was markedly increased (more and heavier seeds) by fertilizing lh^i flowers with wild-type, *Lh*, pollen to produce seeds of genotype $Lhlh^i$ (Fig. 1). Heterozygous $Lhlh^i$ seeds were also found to have GA levels similar to, or slightly higher than, homozygous *Lh* seeds and substantially higher than homozygous recessive lh^i seeds (Tab. 2). This supports the hypothesis that GAs are required for normal seed development in *Pisum* and that severely reduced GA levels cause an increase in seed abortion and a decrease in final seed weight. This result also confirms that seeds can synthesize GAs *de novo* and demonstrates that the lh^i allele affects seed development after fertilization by acting in the developing embryo and/or endosperm rather than the testa or other maternal tissue.

Self-pollinated *Lhlh* and *Nana* F_1 plants produced F_2 progeny in agreement with the expected ratio of 3:1 for genotypes *Lh-:lhlh,* and *Na-:nana,* respectively. This is consistent with the *na* allele not acting in seeds at contact point (Potts and Reid 1983, Potts 1986) and with the relatively minor effect of the *lh* allele in developing

seeds (Tabs. 1,2). By contrast, a deficiency in the number of homozygous recessive *lh*^{i} dwarf plants in the F_2 progeny of crosses between *lh*^{i} (NGB5843) and its wild-type progenitor, cv. Torsdag (*Lh*) was observed (Fig. 2). This appears to result entirely from zygotic selection against the GA-deficient *lh*^{i}*lh*^{i} seeds on the parent plant rather than gametic or gametophytic selection against *lh*^{i} pollen (Tab. 3, Fig. 2).

Previous studies attempting to use either genetic (e.g. Barendse et al. 1986, Groot et al. 1987, Santes et al. 1993) or chemical (e.g. Zeevaart 1966, Garcia-Martinez et al. 1987) means to reduce GA levels in developing seeds have not demonstrated a clear role for GAs in seed development. However, this does not necessarily contradict the results presented in this paper since, in genetic studies using the *ga1* mutant of *Arabidopsis* (Barendse et al. 1986) or the *gib1* mutant of tomato (Groot et al. 1987), GA levels have not been shown to be reduced in isolated developing seeds. Chemical treatments (e.g. paclobutrazol) may have failed to affect seed development if GAs are only required early in this process (*lh*^{i} seeds weigh less than *Lh* seeds 7 days after anthesis, Tab. 1), and GA levels were not reduced by the treatment at this stage. In addition, seeds may only require a small proportion of the GAs that normally accumulate for successful development (Baldev et al. 1965, Santes et al. 1993, Tabs. 1,2).

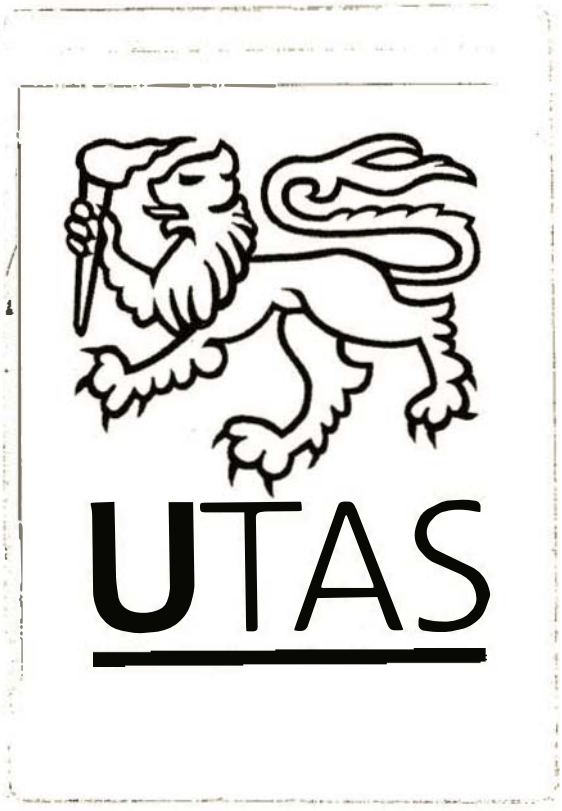
In numerous plantings involving the *Lh* (wild-type), *lh*^{i} and *lh* alleles no differences in the proportion of apparently normal seeds successfully germinating have been detected (data not shown). Therefore, the high levels of GAs that accumulate in maturing seeds of wild-type plants (Tab. 2, Gaskin et al. 1985) do not appear to be essential for germination and subsequent shoot growth under normal conditions (Sponsel 1983). However, *lh*^{i} plants often produce seeds of severely reduced size that either fail to germinate or produce unhealthy seedlings. Thus, gibberellins may have an indirect role in germination in peas by ensuring that seeds develop normally.

The *lh* and *lh*^{i} alleles demonstrate tissue-dependent regulation of GA-biosynthesis, in developing seeds and shoots of peas, since *lh*^{i} plants were found to possess more GA_1 in young shoots (Chapter 3) but less GA_1 in developing seeds (Tabs. 1,2) compared with *lh* plants.

Pods developing on self-pollinated *lh*^{i} plants are shorter ($P<0.001$), weigh less and possess reduced GA levels compared with pods from *Lh* (wild-type) plants (Tabs. 4,5). However, this may be a consequence of the reduced seed yield of pods on homozygous *lh*^{i} plants (Chapter 3) rather than a direct effect of reduced pod GA

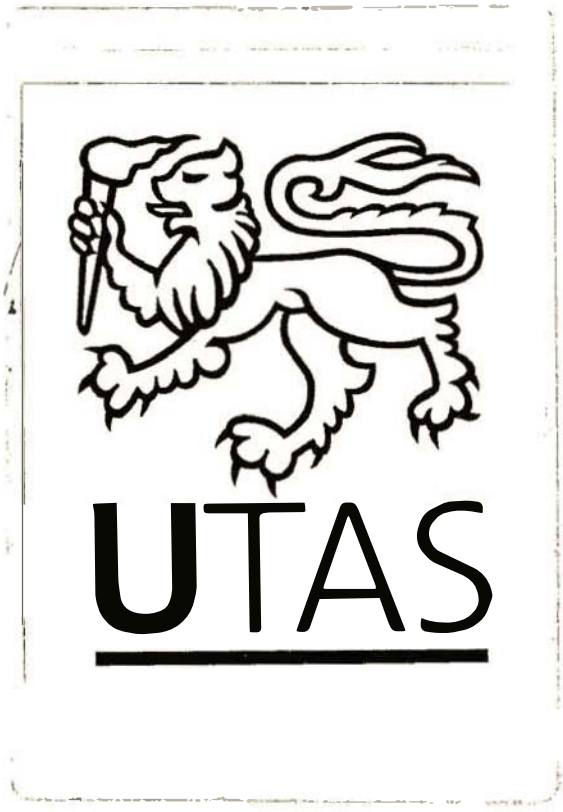
levels. The GA-deficiency of fully-elongated lh^i pods (Tab. 5) is not due to a lack of GA transport from the developing seeds. Homozygous lh^i pods containing seeds of genotype $Lhlh^i$ also have reduced GA levels compared with pods from self-pollinated *Lh* (wild-type) plants (Tab. 5) consistent with results obtained with the *na* mutant (Potts 1986).

In conclusion, the lh^i allele is expressed in developing seeds and pods, as well as young shoots (Chapter 3). Homozygous lh^i seeds have reduced GA levels, weigh less, and are less likely to develop to maturity when compared with *Lh* seeds. Fertilization of lh^i plants with *Lh* pollen increases seed GA levels, seed weight and seed survival suggesting that an increase in seed GA levels due to the *Lh* allele can restore normal seed development. The results presented in this chapter suggest that endogenous GAs may regulate seed development in *Pisum sativum*.



Tab. 1. GA levels in developing seeds from *Lh* (wild-type, cv. Torsdag), lh^i (NGB5843) and *lh* (K511) plants harvested at 5 or 7 days after anthesis. Seed weights represent the average of at least 126 seeds. Photoperiod 18h. n.d. no dilution of internal standard, endogenous level below ca. 2 ng.(gFW^{-1}).

Genotype	Age (days)	Seed weight (mgFW)	No. healthy seeds per pod±SE	Gibberellin level (ng.(gFW^{-1}))				
				GA_1	GA_3	GA_8	GA_{20}	GA_{29}
Lh	7	2.50	5.83±0.09	28.7	n.d.	12.8	19.3	23.7
lh	7	1.96	5.19±0.09	15.5	n.d.	3.2	16.5	10.5
lh^i	7	1.13	6.58±0.11	2.4	n.d.	n.d.	9.1	26.6
Lh	5	1.01	6.00±0.03	31.3	n.d.	n.d.	28.2	36.1



Tab. 2. GA levels (ng.(gFW^{-1})) and weights of developing seeds harvested from *Lh* (wild-type, cv. Torsdag), lh^i (NGB5843) and *lh* (K511) plants at 11,15 and 19 days after anthesis. Seeds of genotype $Lhlh^i$ were produced by fertilizing lh^i plants with pollen from *Lh* (wild-type) plants. Contact point (the first day no liquid endosperm remained) occurred between 11 and 15 days after anthesis for all genotypes. Seed weights represent the average of at least 26 seeds. No GA_1 was found at 15 or 19 days after anthesis. Photoperiod 18h.

Days after anthesis		*LhLh*	lh^ilh^i		*lhlh*	$Lhlh^i$
			Healthy	Aborting		
11	GA_1	1.43	0.32	-[a]	1.09	2.10
	GA_{20}	27.6	<4.0	-	33.4	110.8
	Weight (g)	0.146	0.057	-	0.114	0.131
15	GA_{20}	2450	23	2.1	518	2662
	GA_{29}	717	46	1.0	113	1119
	Weight (g)	0.402	0.250	0.135	0.378	0.358
19	GA_{20}	235	20	1.1	316	572
	GA_{29}	963	190	2.0	366	1535
	Weight (g)	0.491	0.378	0.100	0.463	0.483

[a] no seeds were identified as aborting.

Tab. 3. Observed numbers of tall (*Lh*-) and dwarf (*lhlh*, $lh^{i}lh^{i}$ or $lhlh^{i}$) progeny resulting from the crosses indicated. Photoperiod 18h.

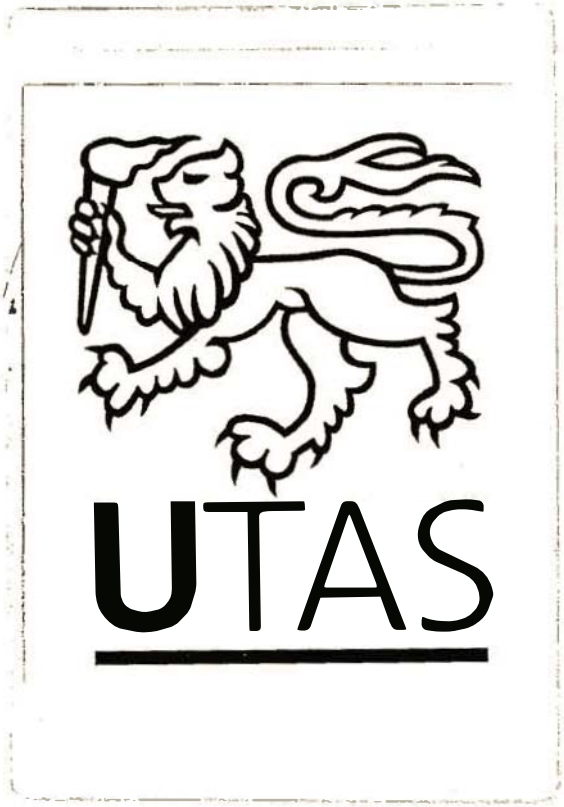
Parental genotypes		Day/night temperature	Pods	Tall	Dwarf
Female	Male	(°C)	scored	progeny	progeny
lhlh	*Lhlh*	15/10	all	24	16
$lh^{i}lh^{i}$	$Lhlh^{i}$	25/20 and 15/10	all	46	21 [a]
$Lhlh^{i}$	$lh^{i}lh^{i}$	20/15	all	51	30 [a]
			≤3 seeds	26	10 [a]
			≥4 seeds	25	20
lhlh	$Lhlh^{i}$	glasshouse [b]	all	28	25
$lh^{i}lh^{i}$	*Lhlh*			25	28

[a] significantly different from expected tall:dwarf ratio of 1:1 at $P<0.05$.

[b] mean maximum/minimum day and night temperatures of 30.0±0.9°C and 18.7±0.3°C, respectively.

Tab. 4. GA levels (ng.(gFW^{-1})), lengths and weights of developing pods harvested from *Lh* (wild-type, cv. Torsdag) and lh^i (NGB5843) plants 6 days after anthesis. Pod weights represent the average of at least 35 pods. Photoperiod 18h. n.d. no dilution of internal standard.

Genotype	Length (cm)	Weight (g)	GA_1	GA_8	GA_{19}	GA_{20}	GA_{29}
Lh	6.40±0.05	1.70	1.1	1.9	0.3	3.5	3.3
lh^i	4.23±0.06	0.69	0.2	0.8	n.d.	1.7	0.9



Tab. 5. GA levels (ng.(gFW^{-1}) and weights of developing pods harvested from *Lh* (wild-type, cv. Torsdag) and lh^i (NGB5843) plants at 11 days after anthesis. All pods were fully elongated at harvest. Pod weights represent the average of at least 11 pods. Photoperiod 18h.

	Pod genotype (seed genotype)		
	LhLh(*LhLh*)	lh^ilh^i(lh^ilh^i)	lh^ilh^i(*Lhlh*^{i})
GA_1	0.52	0.16	0.15
GA_{20}	1.10	0.67	0.17
GA_{29}	1.02	0.33	0.18
Pod weight (g)	2.50	1.75	2.59

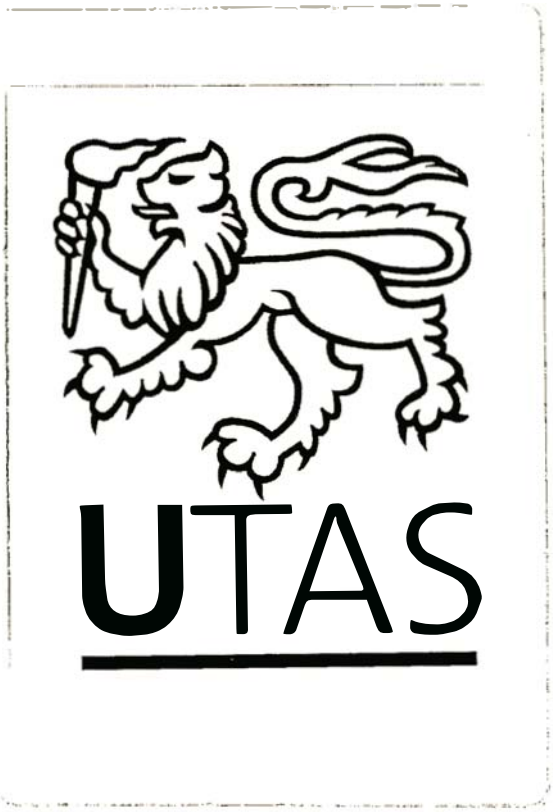


Fig.1. Seeds and pods harvested from self-pollinated *Lh* (wild-type, cv. Torsdag) plants (*left*), self-pollinated lh^i (NGB5843) plants (*right*) and lh^i (NGB5843) plants fertilized with *Lh* (wild-type) pollen (*centre*). Photoperiod 18h.

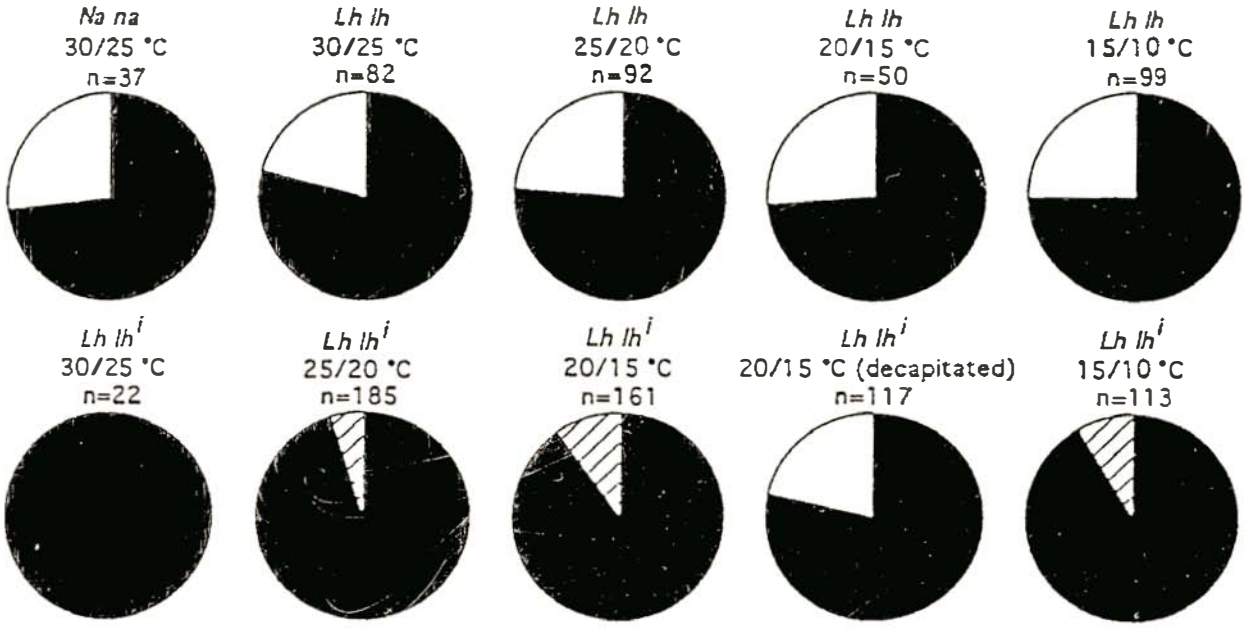


Fig. 2. Observed proportion of tall (black) and short (white) progeny resulting from the selfing of heterozygous parents grown under a range of day/night temperature regimes. Striping indicates that the observed number of short progeny was significantly ($P<0.001$) below expectation.

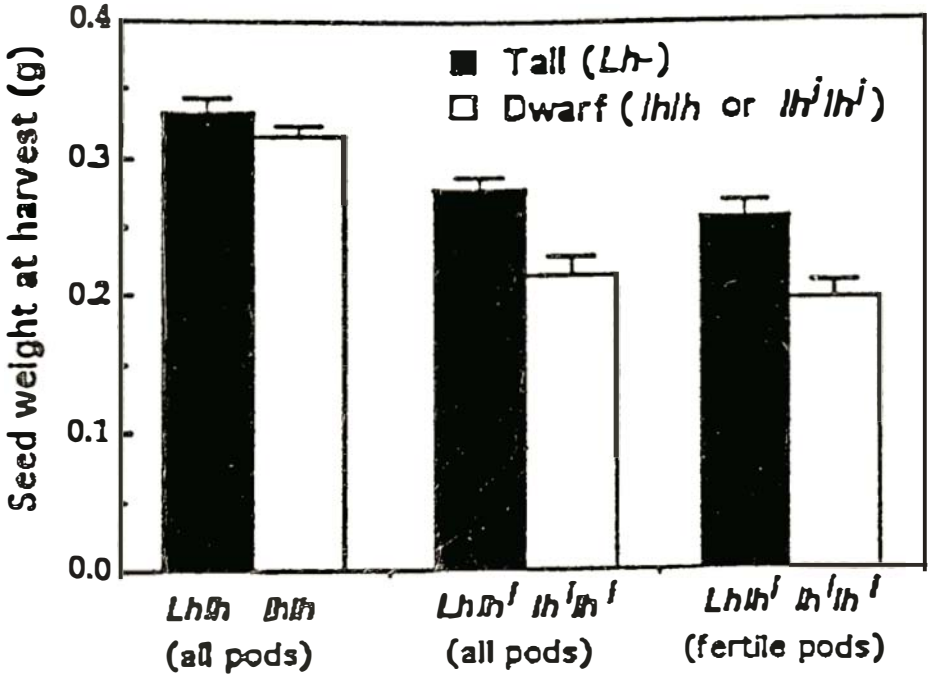


Fig. 3. Harvest weights of segregating seeds developing on either *lh* (K511) plants fertilized with pollen from *Lh/h* heterozygotes, or lh^j (NGB5843) plants fertilized with pollen from Lh/h^j heterozygotes. Pods containing at least 3 seeds are referred to as fertile. Female plants were grown in a controlled environment cabinet with day/night temperatures of 15 and 10°C, respectively. Photoperiod 18h. The genotype of segregating seeds was scored according to the phenotype of the plants obtained after sowing.

Chapter 5

Source-sink relations of gibberellin-deficient lh^i seeds

Introduction

A role for plant hormones, including gibberellins (GAs) in assimilate partitioning to developing fruits and seeds has often been suggested (e.g. Stoddart 1983, Wareing and Seth 1967, Wang and Sponsel 1985, Brenner 1987, Patrick 1988, Jahnke et al. 1989, Garcia-Martinez and Beltran 1992). A common experimental approach is to artificially manipulate the relative strengths of sinks (e.g. developing fruits, Thorne 1985, Wolswinkle and Ammerlaan 1986, Wolswinkle and Koerselman-Kooij 1992) and sources (e.g. leaf area, Clifford et al. 1987), although a mutant based approach has been used (e.g. Karssen and van Loon 1992, Groot et al. 1991, Beveridge et al. 1992, de Bruijn and Vreugdenhil 1992).

The lh^i mutant has been used to support a role for GA_1 in internode elongation (Chapter 3) and to suggest a role for GAs in pea seed development (Chapter 4). Heterozygous $Lhlh^i$ seeds can be produced by fertilizing lh^i plants with *Lh* (wild-type) pollen so that the seed coat (testa) and other maternal tissues are of the same genotype as self-pollinated lh^i plants. Seeds of genotype $Lhlh^i$ possess increased GA levels, have a higher weight and are less likely to abort during development than GA-deficient lh^ilh^i seeds (Chapter 4). Another allele, named *lh*, has also been described at this locus (Reid 1986a). Compared with lh^i seeds, developing *lh* seeds possess higher GA levels and are less likely to abort (Chapters 3,4). Since sink strength has been defined as a function of sink size and sink activity (Warren-Wilson 1972), these results suggest that GA-deficient lh^i seeds have reduced sink strength during development compared with seeds with higher GA levels. In this chapter, a genetic approach is used to investigate the relationship between source supply, sink strength and the development of GA-deficient seeds.

Materials and methods

Plant material and growing conditions.

All lines possessed flowering genotype *Sn Dne Ppd Gi Fds E Lf hr*. Further details about the flowering phenotypes and genotypes of these lines can be found in Murfet and Reid (1993).

To investigate the effects of decapitation, plants of genotype *lh* (K511) and lh^t (NGB5843) were sown at a density of 1 per pot and transferred to controlled environment cabinets with day and night temperatures of 20°C and 15°C, respectively. Flower buds were removed before anthesis to ensure that only one pod developed at the first two reproductive nodes of all plants. These flowers were allowed to self-pollinate. Plants were either allowed to develop and senesce normally (n=12 for both genotypes) or decapitated just below the third reproductive node when the flower at the second reproductive node reached anthesis (n=12 for *lh*, n=10 for lh^i). Lateral branches were removed from plants every second day.

To examine the influence of plant stature on seed yield, 5 pure-breeding tall (*LhLh*) and 6 dwarf (*lhlh*) F_3 progeny resulting from a cross between *Lh* (wild-type cv. Torsdag) and *lh* (K511) were transferred to a controlled environment cabinet with day and night temperatures of 15 and 10°C, respectively.

^{14}C-labelled assimilate partitioning measurements.

To compare assimilate partitioning between $Lhlh^t$ seeds and lh^ilh^i seeds the first flower bud developing on 14 lh^i (NGB5843) plants was hand-pollinated with either *Lh* (from cv. Torsdag flowers) or lh^i (from NGB5843 flowers) pollen. Only one pod was allowed to develop at this node. Consequently, the genotype (lh^ilh^i) of the maternal tissue (including the testa) was identical for both embryo and endosperm genotypes. To reduce the possible effects of differences in pod growth rates, measurements of ^{14}C-labelled assimilate partitioning were taken 11 days after anthesis, when pod elongation had ceased for all plants (data not shown). At 11 days after pollination the 15 lh^i (NGB5843) plants with one pod containing either $Lhlh^i$ (n=8) or lh^ilh^i (n=7) seeds were placed in an air-tight, transparent chamber (90x52x108cm). Plastic bags were placed over hand-pollinated pods, and sealed with silicone grease, to reduce direct ^{14}C uptake (Flinn 1985, Pate 1985).

Approximately 8μCi of $^{14}CO_2$ per plant was liberated within the chamber by adding 2.0mL of 1N HCl to an appropriate amount of $Na_2{}^{14}CO_3$ (55μCi.mmol^{-1}, Amersham international, Australia) placed in a small vial at one end of the chamber. Air was circulated throughout the chamber by a small electric fan. Plants were exposed to $^{14}CO_2$ for 1 hour in a controlled environment room maintained at 18°C. Light was provided by eight 40W and two 20W cool fluorescent tubes (80 μmol m^{-2} s^{-1} at plant top). After $^{14}CO_2$ exposure the chamber lid was removed and plants moved to a glasshouse where they received natural light for a further 6-7 hours (see Beveridge et al. 1992).

Plants with pods containing seeds of genotype *Lhlh*^{i} or *lh*^{i}*lh*^{i} were harvested alternatively and sectioned into the apex (apical portion above the highest partially expanded leaf), pod (excluding seeds and other floral organs) and seeds (aborted seeds excluded). All seeds had not reached contact point (i.e. some liquid endosperm remained) at harvest. The plant sections were frozen, freeze-dried, weighed and extracted in 80% ethanol at 80°C for 18h. Three 1.0mL aliquots were taken for scintillation counting (using Beckman ReadySafe scintillation liquid). Radioactivity was determined using a Beckman LS5801 scintillation counter (Beckman Instruments, USA)

In vitro *embryo culture*.

At 10 or 12 days after anthesis, *Lh* (wild-type) pods (10 days) and *lh*^{i} pods (12 days) were removed from the parent plant. The young seeds (endosperm still present) were removed (with funicle attached) and surface-sterilized for 2min. in a sodium hypochloride solution containing 1% available chlorine, with a drop of Tween 20 as a wetting agent. The seeds were rinsed twice with dH_2O. Seeds were subsequently manipulated under sterile conditions in a lamina flow cabinet with flame-sterilized forceps to prevent contamination. Seeds were kept in a small amount of dH_2O until dissection.

Embryos at the late heart or early cotyledonary stage (ca. 2.5mm total width) were removed from the seeds and blotted onto filter paper to remove any residual liquid endosperm. The embryos were then placed individually in wells of a cell culture cluster (Costar #3524) each containing 1ml of culture medium. At least 17 embryos were used per treatment, and were cultured in a controlled environment room at 25°C under constant low light intensity (ca. 20 μE.m^{-2}.s^{-1}) for 14 days. Cotyledon lengths

were measured daily using a dissecting microscope and graticule.

The method and medium used were modified from Dr T. Wang (pers. com.). The liquid medium is based on the media used by Murashige and Skoog (1962) supplemented with a full range of amino acids. After preparing the culture medium as described in Tab. 1, sucrose was added to a concentration of 14%, and the pH adjusted to 5.6 with 10M NaOH. The medium was filter-sterilized using a 0.2µm membrane filter and stored at 4°C until use. For the culture of lh^i embryos with 10^{-5}M GA_1, GA_1 was dissolved in 100µl of ethanol and added to the culture medium.

Results

Distribution of dry weights and ^{14}C-assimilates.

Seeds of genotype $Lhlh^i$ (normal GA levels) or lh^ilh^i (GA-deficient) were produced by fertilizing homozygous lh^ilh^i plants with either *Lh* (from wild-type flowers) or lh^i (from NGB5843 flowers) pollen. At 11 days after anthesis, pods containing seeds of either genotype were fully elongated (data not shown). At this time, pods containing $Lhlh^i$ seeds were longer ($P<0.001$), had a higher dry weight and a higher ratio of pod:apex dry weight ($P<0.01$) than those containing lh^ilh^i seeds (Tab. 2). The difference in pod development may be due to the increased number of $Lhlh^i$ seeds developing normally (i.e. not aborted) compared with lh^ilh^i seeds (Tab. 2, $P<0.05$), and/or a more direct effect of the elevated GA levels in $Lhlh^i$ seeds (e.g. Ozga et al. 1992). The dry weight and the ratio of seed:apex and seed:pod dry weights were greater ($P<0.001$) for $Lhlh^i$ seeds than for lh^ilh^i seeds (Tab. 2).

When lh^i plants were allowed to photosynthesise in the presence of ^{14}C-labelled CO_2 at 11 days after pollination, seeds of genotype $Lhlh^i$ (normal GA levels) were found to have received more ^{14}C-assimilates on a per seed basis than GA-deficient lh^ilh^i seeds (Tab. 2, $P<0.01$). No significant difference ($P>0.30$) in the amount of ^{14}C-assimilates found in the apex or pod of lh^i plants containing $Lhlh^i$ or lh^ilh^i seeds was observed (data not shown).

Increased assimilate flow to developing seeds.

The effect of removing sinks competing with the developing fruit on *lh* and lh^i plants was investigated. Plants of genotype *lh* and lh^i were either (i) allowed to develop and senesce normally, or (ii) decapitated so that competition between the

remaining developing seeds and other sinks was decreased. For plants allowed to develop normally (control treatment), seeds of genotype *lh* were heavier and less likely to abort than lh^i seeds (Fig. 1, $P<0.001$). Decapitation of plants of either genotype above the second reproductive node (1 flower at each node) resulted in increased pod length, leaf length, and the width of the reproductive node, compared with plants allowed to develop normally (Tab. 3, $P<0.001$). In addition, decapitation of lh^i plants increased peduncle length compared with control plants (Tab. 3, $P<0.05$). Decapitation also increased final seed weight of *lh* and lh^i seeds (Fig. 1, $P<0.001$), and the number of lh^i seeds fully developed at harvest, compared with control lh^i plants (Fig. 1, $P<0.02$), suggesting that source supply to developing seeds was increased on decapitated *lh* and lh^i plants. However, the increase in yield (change in seed number x change in seed weight) of lh^i plants after decapitation (0.570g) was less than that of *lh* seeds (1.161g), and decapitation did not fully restore the yield of lh^i plants to that of control *lh* plants (Fig. 1, $P<0.001$). Thus, the phenotypic effects on seed development of the lh^i mutation (reduced numbers and weights of seeds) were not entirely overcome by a procedure which increased the size of (and presumably assimilate supply to) the developing fruit.

Decreased assimilate flow to developing seeds.

The effect of reducing assimilate flow to lh^i seeds was also investigated. For peas, assimilate distribution between vegetative and reproductive structures has been reported to be altered by growing photoperiodic plants (genotype *Sn-Dne-Ppd-*, Murfet and Reid 1993) in long days (LD, e.g. 24h photoperiod) or short days (SD, e.g. 8h photoperiod). Compared with LD, plants grown in SD invest a reduced proportion of the available assimilates in reproductive growth (Kelly and Davies 1988). The wild-type cultivar Torsdag (*Lh*), and the mutant lines NGB5843 (lh^i) and K511 (*lh*) derived from it, are photoperiodic. When grown in SD the node of flower development was delayed and the number of reproductive nodes, pods and, as a consequence, seeds was increased compared with plants grown in LD for all three genotypes (data not shown, see Murfet 1977). In contrast to the increase in total seed yield, the number of seeds at each reproductive node was decreased for *Lh* (wild-type), lh^i and *lh* plants in SD compared with LD (Fig. 2, $P<0.001$). However, this decrease was relatively less severe for lh^i plants compared with *Lh* (wild-type) and *lh* plants. Compared with plants grown under SD conditions, the number of seeds per

node for genotypes *lh*^{i}, *lh* and *Lh* (wild-type) was increased under LD conditions by 68%, 112% and 144%, respectively (Fig. 2). In terms of fully developed seeds per node, the relative severity of the *lh*^{i} mutation (in comparison with *Lh* and *lh*) was less in SD compared with LD conditions (Fig. 2).

Influence of plant stature on seed yield.

The similar stature of *lh* and *lh*^{i} plants (Chapter 3) minimises any effects on assimilate distribution due to differences in shoot growth. However, it is possible that the dwarf stature of *lh*^{i} plants, compared with tall (wild-type) *Lh* plants, has an indirect affect on seed yield of *lh*^{i} plants. To investigate the influence of plant stature on seed yield pure-breeding tall (*LhLh*) and dwarf (*lhlh*) F_3 progeny from a cross between line K511 (*lh*, dwarf) and its wild-type progenitor, cv. Torsdag (*Lh*, tall) were allowed to self-pollinate in a day and night temperatures of 15 and 10°C, respectively. These genotypes were used (i) because of the nearly identical genetic backgrounds compared with NGB5843 (*lh*^{i}), and (ii) no differences in seed development between *Lh*- and *lhlh* seeds have been detected (Chapter 4). Self-pollinated dwarf (*lh*) plants were found to be shorter (P<0.001), have more seeds (P=0.05), fewer pods (P<0.05) and more seeds per pod (P<0.001) than tall (*Lh*) plants (Tab. 4). Thus, under these conditions, the reduced stature of *lh* (dwarf) plants slightly increased seed yield relative to *Lh* (wild-type, tall) plants. This result suggests that the dwarf stature of *lh*^{i} plants in not partially responsible for the reduced seed yield compared with *Lh* (wild-type) plants, consistent with previous results (Chapter 4). In fact, GA deficient *lh*^{i} seeds might be expected to weigh less and be more likely to abort, if developing on a tall (rather than dwarf) parent plant.

In vitro *embryo culture.*

Although *Lh* (wild-type) embryos were harvested at an earlier age (10 days after anthesis) than *lh*^{i} embryos (12 days after anthesis), the cotyledons of *Lh* embryos were initially significantly larger (Fig 3., P<0.001) than both control and GA_1-treated *lh*^{i} embryos. This is consistent with the reduced weight of *lh*^{i} seeds throughout seed development compared with *Lh* (wild-type) seeds (Chapter 4). After 14 days in culture, when embryos of both genotypes had finished elongating, this difference was no longer significant, possibly because of the variability in final cotyledon length of both genotypes (Fig. 3). However, the maximum cotyledon length

obtained by lh^i embryos cultured in the presence of 10^{-5}M GA_1 was significantly ($P<0.05$) greater than that of lh^i embryos cultured without GA_1 after 10 days in culture (Fig. 3). The relatively small size of this increase may be related to the growth of pea embryos *in vitro* or may be due to the GA_1 concentration used (10^{-5}M) not being optimal for embryo growth.

Discussion

Seeds of genotype $Lhlh^i$, developing on an lh^i plant, were produced by fertilizing lh^i flowers with pollen from *Lh* (wild-type) plants. Compared with $Lhlh^i$ seeds, lh^ilh^i seeds accumulated a lower amount of ^{14}C-labelled assimilates and possessed a lower dry weight, on an absolute basis and relative to the dry weight of apex and surrounding pod tissue (Tab. 2). GA-deficient lh^ilh^i seeds therefore appear to possess reduced sink strength compared with $Lhlh^i$ seeds (normal GA levels).

Changes in source availability, either by removing competing sinks or by growing plants in different photoperiods, affected the development of lh^i seeds (Figs 1,2). Decapitation and regular removal of lateral branches from *lh* and lh^i plants left the developing fruit as the major sink. This treatment increased yield of both *lh* and lh^i plants, compared with undecapitated controls, but did not completely overcome the effects of the lh^i mutation on seed development (Fig. 1). This result is consistent with the increased seed weight and reduced seed abortion, observed when lh^i plants are fertilized with *Lh* (wild-type) pollen (Tab. 2, Chapter 4), resulting primarily from changes confined to the developing seed (embryo/endosperm) rather than indirect effects of the increased seed GA levels on the maternal plant (e.g. increased source supply).

The growth of lh^i embryos *in vitro* was increased, relative to untreated embryos, by the addition of 10^{-5}M GA_1 to the culture medium (Fig. 3). This result supports the hypothesis that GAs play an important role in seed development and suggests that GA_1 may be biologically active in promoting embryo growth. This result is also consistent with the hypothesis that reduced endogenous GA levels cause the reduced sink strength of lh^i seeds.

For peas, SD conditions reduce the proportion of assimilates available for reproductive development compared with LD's (Kelly and Davies 1988). Relative to *Lh* (wild-type) or *lh* plants, the number of seeds per node on lh^i plants was less

severely affected by SD compared with LD conditions (Fig. 2). Thus, lh^i seeds appear to be less dependent on a high source supply than *Lh* (wild-type) or *lh* seeds, consistent with a reduction in sink strength for lh^i seeds compared with developing seeds containing higher GA levels.

The lh^i mutation is the first in peas, and may be the first in any species, where a reduction in seed (embryo/endosperm) hormone levels has been shown to reduce assimilate supply to the developing seed. Mutants deficient in ABA in the developing seed have been identified in peas (*wil*), tomatoes (sit^w) and *Arabidopsis* (*abal*), but none of these appears to influence assimilate partitioning (Karssen and van Loon 1992, Groot et al. 1991, de Bruijn and Vreugdenhil 1992, see Chapter 1). A mutant reducing auxin levels in developing seeds of maize has been reported to reduce endosperm weight (Torti et al. 1986), but assimilate supply to the developing seeds does not appear to have been examined.

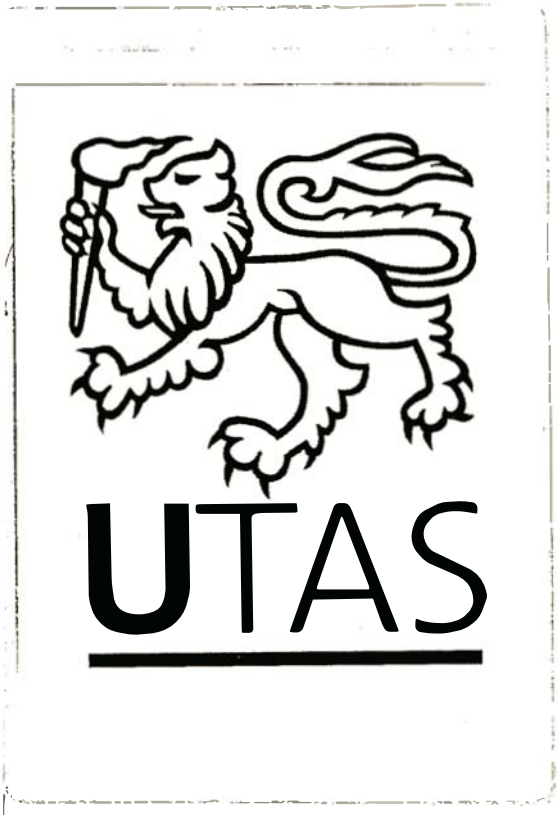
The present results do not indicate the relationship between differences in seed weight and assimilate partitioning to developing seeds containing different GA levels. The weight of lh^i seeds is less than that of *Lh* (wild-type) seeds before any obvious signs of seed abortion (Chapter 4), suggesting that the reduced GA levels in lh^i seeds directly causes the reduction in seed weight, and consequently increases seed abortion. However, whether the reduced GA levels in lh^i seeds directly results in (i) reduced assimilate partitioning to developing seeds (causing reduced seed weight) or (ii) reduced seed weight (causing reduced assimilate partitioning) remains in question. Since Clifford et al. (1986) found no significant effect of applied GA_3 and $GA_{4/7}$ on ^{14}C-unloading from excised seed coats of beans (*Phaseolus vulgaris*), the second alternative may be more likely. Although GA_1 may promote embryo growth *in vitro* (Fig. 3), GAs could still affect either embryo growth directly (e.g. cell division) or assimilate uptake from the surrounding endosperm (e.g. by regulating an active transport mechanism). This question cannot be resolved until the mechanism of GA action in developing seeds is known at the biochemical level.

Tab. 1. Components of the *in vitro* embryo culture medium.

Component	Concentration ($mg.l^{-1}$)	Component	Concentration ($mg.l^{-1}$)
KNO_3	19000	NH_4NO_3	16500
$CaCl_2.2H_2O$	4400	$MgSO_4.7H_2O$	3700
KH_2PO_4	1700		
FeNaEDTA	367	$MnSO_4$	223
$ZnSO_4$	86	H_3BO_3	62
KI	8.3	$Na_2MoO_4.2H_2O$	2.5
$CuSO_4.5H_2O$	0.25	$CoCl_2.5H_2O$	0.25
Inositol	100	Pyridoxine	5
Thiamine-HCl	1	Nicotinic acid	5
L-alanine	1000	L-methionine	1000
L-glutamic acid	1000	L-threonine	1000
L-glutamine	1000	L-serine	500
L-glycine	500	L-isoleucine	500
L-arginine	500	L-valine	300
L-histidine-HCl	200	L-asparagine	200
L-aspartic acid	200	L-leucine	200
L-lysine	100		

Tab. 2. Harvest details for, and ^{14}C-assimilate accumulation (disintegrations per minute, DPM) in, seeds harvested from self-pollinated lh^i plants (seed genotype lh^ilh^i) and lh^i plants fertilized with wild-type pollen (seed genotype $Lhlh^i$). Values are the means±SE for at least 6 seeds. Photoperiod 18h.

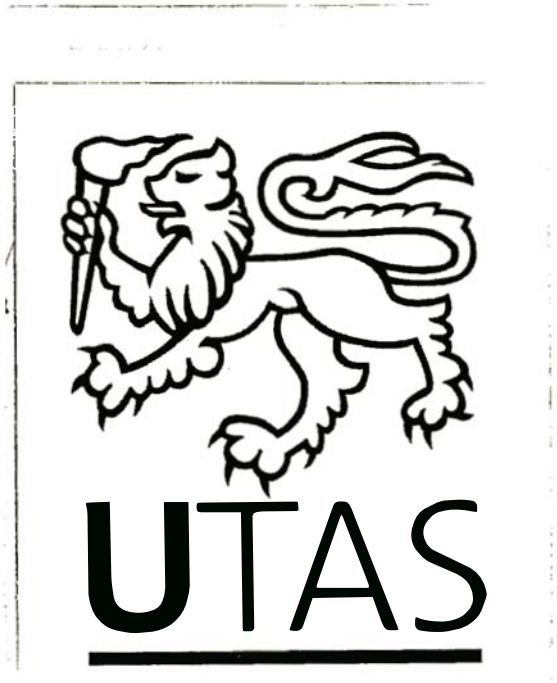
Seed genotype	Pod length (cm)	Pod dry weight (mg)	Number of healthy seeds	Seed dry weight (mg)	^{14}C-assimilate accumulation per seed (DPM)	Dry weight ratio		
						Seed: apex	Pod: apex	Seed: pod
$Lhlh^i$	6.21 ±0.20	377 ±34	4.50 ±0.96	21.4 ±2.0	18900 ±2600	0.44 ±0.06	7.76 ±1.24	0.057 ±0.002
lh^ilh^i	4.86 ±0.13	219 ±19	2.14 ±0.40	8.0 ±0.9	9600 ±1300	0.12 ±0.01	3.46 ±0.39	0.036 ±0.002



Tab. 3. Effect on the first reproductive node, and on the leaf and pod subtending that node, of decapitation of *lh* and *lh*^{i} plants above the second reproductive node. Similar results were obtained for the second reproductive node. Plants were grown in a controlled environment cabinet with day and night temperatures of 20 and 15°C, respectively. Values represent the means±SE for at least 10 plants. Photoperiod 18h.

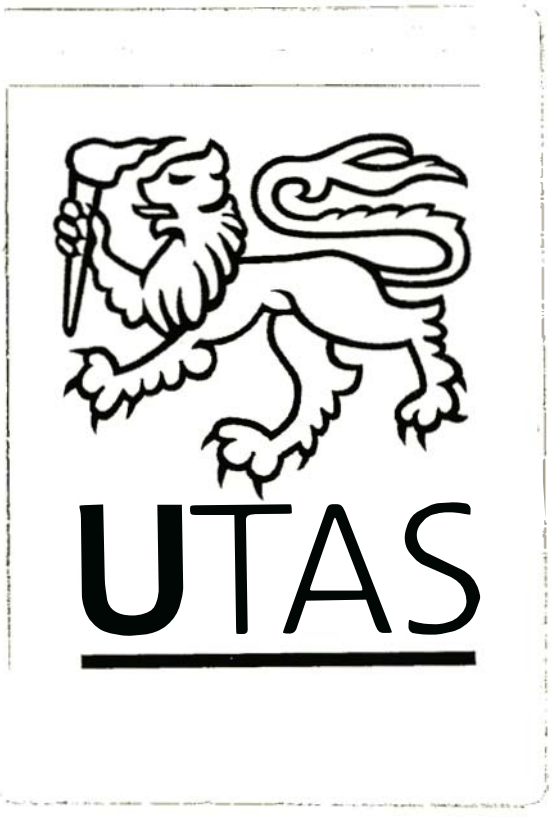
Genotype	Treatment	Node width[a] (mm)	Peduncle length (cm)	Pod length (cm)	Leaf length (cm)
lh	Control	5.0±0.1	5.6±0.2	6.6±0.1	10.6±0.2
	Decapitated	7.0±0.2	5.6±0.2	7.3±0.2	12.9±0.4
lh^{i}	Control	6.0±0.2	5.6±0.2	6.2±0.1	13.0±0.3
	Decapitated	9.8±0.3	6.5±0.3	7.3±0.1	17.5±0.6

[a] node at which the first flower subtended.



Tab. 4. Comparison of the vegetative and reproductive development of self-pollinated *LhLh* (wild-type) and *lhlh* plants grown in a controlled environment cabinet with day and night temperatures of 15 and 10°C, respectively. Values represent the means±SE for at least 5 plants. Photoperiod 18h.

Genotype	Length between nodes 6 and 9 (cm)	Flowering node	Seeds	Pods	Mean seeds per pod	Seed weight (mgFW)
LhLh (tall)	23.8 ±1.0	15.6 ±0.2	57.6 ±4.7	19.4 ±1.9	2.99 ±0.09	269 ±15
lhlh (dwarf)	5.3 ±0.2	15.2 ±0.2	71.5 ±1.7	15.0 ±0.6	4.79 ±0.17	297 ±7



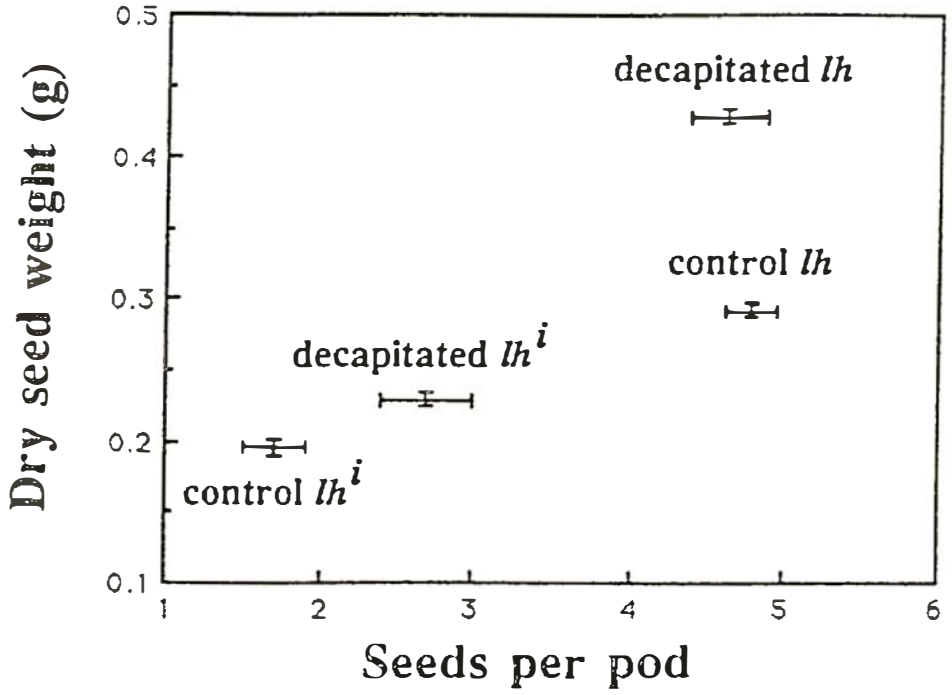


Fig. 1. Seed development on plants of genotype *lh* (K511) and *lh*^{i} (NGB5843) either decapitated above the second reproductive node (*decapitated*) or allowed to develop normally (*control*). All plants (n≥10) were grown in a day/night temperature regime of 20/15°C. Only one flower was allowed to develop at each of the first two reproductive nodes, and the data shown is for these nodes only. Photoperiod 18h.

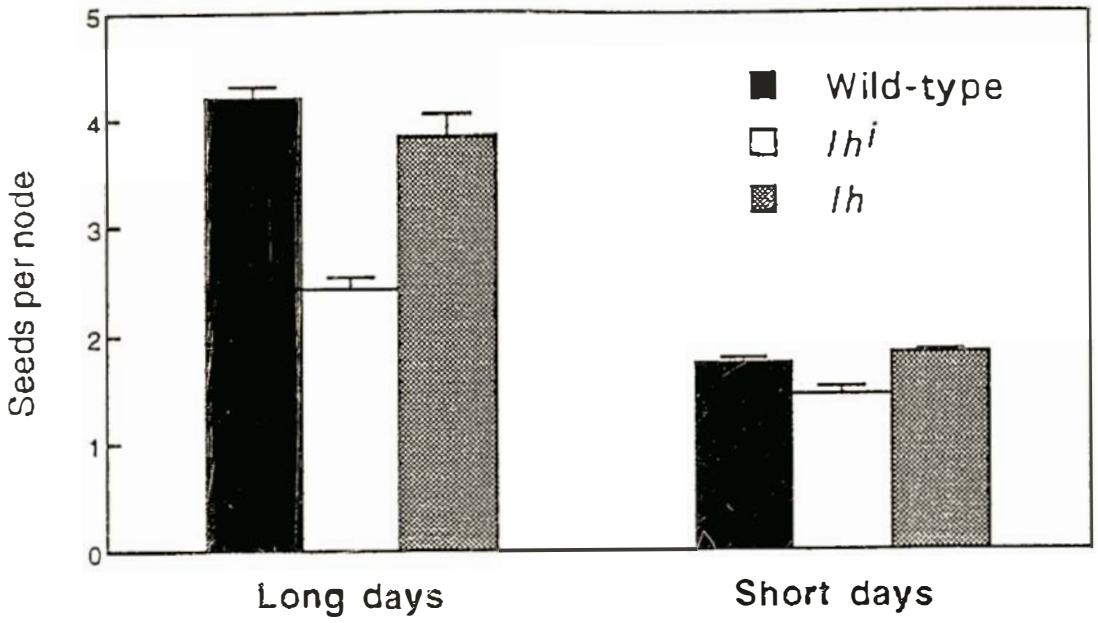


Fig. 2. Seeds per node for *Lh* (wild-type), lh^i (NGB5843) and *lh* (K511) plants grown under long days (24h: 8h natural light supplemented with 16h weak incandescent light) and short days (8h natural light).

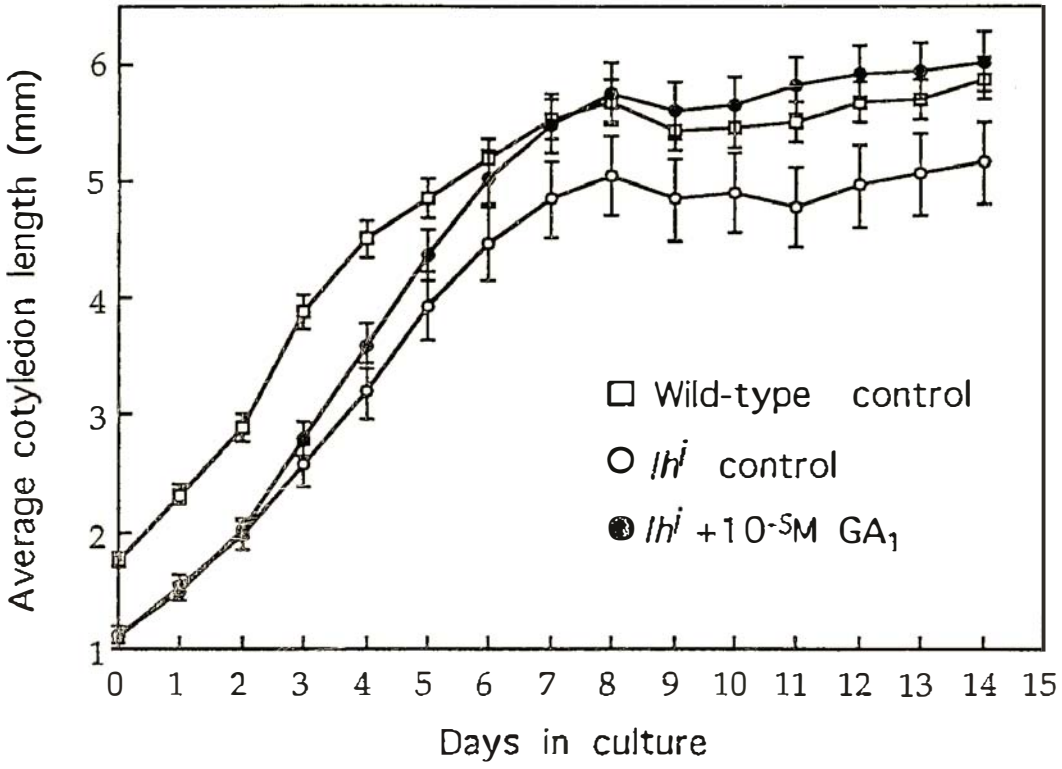


Fig. 3. Average cotyledon length of immature (liquid endosperm present) *Lh* (wild-type) and lh^i (NGB5843) embryos grown *in vitro* for 14 days. Embryos of genotype lh^i were cultured with or without 10^{-5}M GA_1.

Chapter 6

Genetic analysis of the *Lh* locus

1. Linkage of the *Lh* and *Le* loci

Introduction

One of the main advantages of a genetic approach to understanding plant development is the combination of one or more mutations in a single plant (e.g. Koornneef et al. 1989, Reid 1986b, Ingram and Reid 1987b, Weller et al. 1993). However, it may be difficult to combine two mutations if they are closely linked since they will not assort independently in the F_2 generation when crossed together in repulsion (i.e *Ab* x *aB*). Genes linked to the *Lh* locus would be particularly difficult to combine with the lh^i allele since the *Lh* (wild-type) and lh^i alleles do not usually segregate in accord with the expected 3:1 ratio (Chapter 4). Ideally, the position of the *Lh* locus should be known before the interaction of the lh^i allele with other mutations is investigated (e.g. Chapters 7,8).

Although isolating the position of a particular locus usually involves crossing to several multiple-marker (e.g. morphological or isozyme) lines, existing evidence already suggested that the *Lh* locus might be linked to *Le*. When a cross was performed between the non-isogenic lines L61a (*leLh*) and K511 (*Lelh*) no F_2 plants shorter than the shortest parent were apparent (Reid 1986a). This was unexpected since plants of genotype *lslh* possess the nana phenotype (extremely short internodes) and are considerably shorter than *Lslh* and *lsLh* plants (Reid 1986a). There are four possible explanations for the apparent absence of the nana phenotype in the L61a (*leLh*) by K511 (*Lelh*) F_2 progeny. Firstly, the *lh* gene may be epistatic over the *Le* locus so that plants of genotype *Lelh* and *lelh* are phenotypically indistinguishable. Although the *na* gene is epistatic over the *Le* locus (Reid et al. 1983), this seems unlikely to be the case for the *Lh* and *Le* loci. Treatment of *le* plants with a moderate (5µg per seed) dose of paclobutrazol (which has a similar effect on wild-type internode elongation as the *lh* allele) further reduces internode lengths (Swain and Reid 1992a) suggesting that *lelh* plants should be considerably shorter than *leLh* and

Lelh plants. Secondly, if *lelh* plants are shorter than *Lelh* plants, polygenic differences between the two parents segregating in the F_2 progeny may have prevented detection of the *lelh* double mutant. Thirdly, the *lelh* double mutant may be lethal. However, this seems unlikely since both the *le* and *lh* mutations segregate in accord with a 3 (tall):1 (dwarf) ratio (Chapter 4, Ross and Reid 1987) and the *lslh* double mutant is not lethal (Reid 1986a). The fourth explanation is that the *Le* and *Lh* loci are closely linked and, since the L61a by K511 cross was in repulsion, no recombinants (genotype *lelh*) would have been expected in the relatively small number of F_2 progeny examined (Reid 1986a).

To distinguish between these possibilities, a cross was performed between the near-isogenic lines NGB5839 ($le^{5839}Lh$, Jolly et al. 1987, Ross and Reid 1991) and K511 (*Lelh*), both of which were derived from cv. Torsdag (*LeLh*) (Chapter 2). Line K511 was chosen instead of NGB5843 (lh^i) since, unlike the *Lh* (wild-type) and lh^i alleles, the *Lh* and *lh* alleles segregate in agreement with the expected 3:1 ratio (Chapter 4, Reid 1986a). In this section the $le^{5839}lh$ double mutant is identified, and linkage between the *Le* and *Lh* loci established.

Materials and Methods

Hobart line 239 is isolated and described in this section and possesses genotype $le^{5839}lh$. All plants were grown in a glasshouse in pots or tote boxes (Chapter 2) with the exception of the F_1 plant from cross cv. Torsdag by L239 ($le^{5839}lh$), which was grown in a 21cm pot and given extra nutrient solution. In order to increase seed yield of some $le^{5839}lh$ plants, 2µg of GA_3 was applied in 10µl of ethanol when ca. 7 leaves were fully expanded.

Results

A cross between lines NGB5839 ($le^{5839}Lh$) and K511 (*Lelh*) produced tall F_1 plants confirming that the *Le* and *Lh* loci are not allelic. When these F_1 plants were allowed to self-pollinate, the F_2 progeny segregated to give 150 tall and 164 dwarf plants (data not shown). The $le^{5839}Lh$ and *Lelh* genotypes (both possess dwarf phenotypes) could not be distinguished because of the similar stature of NGB5839 and K511 plants (Fig. 1). No plants shorter than either the NGB5839 or K511 parents were

identified, consistent with the results of Reid (1986a). In a further attempt to identify the *le*^{5839}*lh* genotype, progeny from 25 dwarf F_2 plants were grown-on in the F_3. Twenty-one F_2 plants produced all dwarf F_3 progeny, while 4 F_2 plants produced both dwarf and nana (extremely short) F_3 progeny in agreement with a 3 (dwarf):1 (nana) ratio (P>0.2). The genotype of a single nana plant was subsequently shown to be *le*^{5839}*lh* by test-crossing to each parent, demonstrating that the *le*^{5839}*lh* double mutant possesses internode lengths shorter than both the NGB5839 (*le*^{5839}*Lh*) and K511 (*Lelh*) parents. Thus, there exists a significant deficiency (P<0.001) of *le*^{5839}*le*^{5839}*lhlh* (and *le*^{5839}*le*^{5839}*Lhlh* and *Lele*^{5839}*lhlh*) plants in the F_2 progeny, consistent with linkage of the *Le* and *Lh* loci, and a repulsion phase cross. Plants of genotype *le*^{5839}*lh* have been assigned Hobart line number 239 (Tab. 1, Fig. 1).

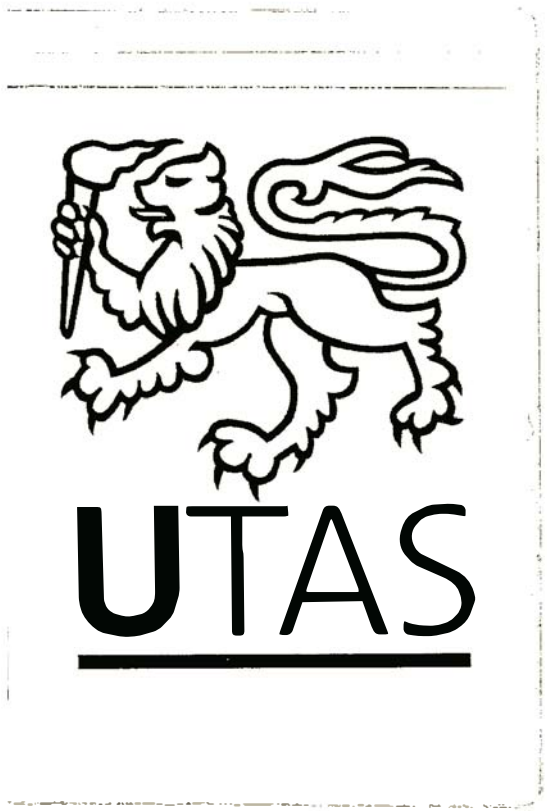
To further investigate the possible linkage of the *Le* and *Lh* loci, a cross was performed (in coupling phase) between cv. Torsdag (*LeLh*) and L239 (*le*^{5839}*lh*). A single, self-pollinated tall F_1 plant segregated to give 128 tall (*Le-Lh-*), 10 dwarf (*Le-lhlh* and *le*^{5839}*le*^{5839}*Lh-*) and 54 nana (*le*^{5839}*le*^{5839}*lhlh*) plants in the F_2 progeny. This segregation did not fit (P<0.001) the expected tall:dwarf:nana ratio of 9:6:1. In fact, analysis using a 2 by 2 contingency table shows that the *Le* and *Lh* loci are linked, regardless of the genotypes (*Le-lhlh* or *le*^{5839}*le*^{5839}*Lh-*) of the 10 dwarf plants (P<0.001). Using the maximum likelihood method of Mather (1951), which does not require a distinction between the *Le-lhlh* and *le*^{5839}*le*^{5839}*Lh-* dwarf recombinants, the linkage of the *Le* and *Lh* loci was calculated to be 4.9±1.2cM on chromosome 4 (Weeden et al. 1991). To establish the precise position of the *Lh* locus, the linkage of the *Lh* and *V* loci is being examined since *v* (sugar pods) is linked to *Le* at a distance of 12.6±0.5cM (Rasmusson 1927). A cross has been performed between plants of genotype *LeLhv* (tall, sugarpods) and *LelhV* (dwarf, normal pods), but results are not available at the time of writing.

Discussion

The *le*^{5839}*lh* double mutant has been identified and shown to possess the nana phenotype (extremely short internodes). The phenotypic interaction of the *Lh* and *Le* loci therefore differs from that of the *Na* and *Le* loci since *na* is epistatic over the *Le* locus (Reid et al. 1983). Although the biochemical basis for this epistasis is not known, it cannot result from the distance between the sites of action in the GA-

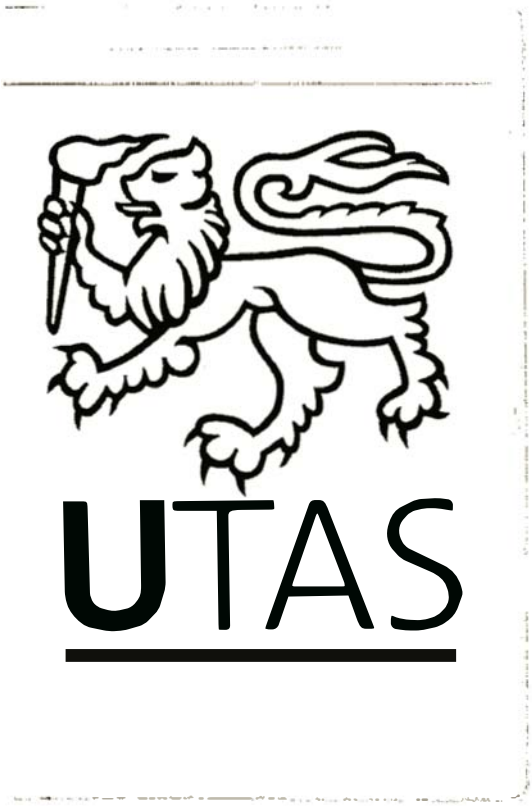
biosynthetic pathway (see Chapter 1) of the *na* and *le* mutations. While the *le* and le^{5839} alleles are thought to reduce the conversion of GA_{20} to GA_1 (Ingram et al. 1984, Jolly et al. 1987, Ross et al. 1992), both the *na* and *lh* mutations are though to reduce GA-biosynthesis prior to the formation of GA_{12}-aldehyde (Ingram and Reid 1987a). A more likely explanation for the *naLe* and *nale* phenotypes may be that the extremely low GA_{20} levels present in *na* shoots (Proebsting et al. 1992) do not require a fully-functional *Le* gene-product for efficient conversion of this small substrate pool into GA_1.

The linkage of the *Lh* and *Le* loci (4.9±1.2cM) allows the possible linkage of the *Lh* locus with genes on other chromosomes to be ignored. For example, the *La*, *Cry* and *R* loci (see Chapter 7) will not show linkage with the lh^i mutation since they are on different chromosomes (Weeden et al. 1991).



Tab. 1. Internode length phenotypes of the *lh* (K511), le^{5839} (NGB5839) and $le^{5839}lh$ (L239) mutants.

Line	Genotype	Length between nodes 3 and 6 (cm)	Phenotype
K511	*Lelh*	5.32±0.20	Dwarf
NGB5839	$le^{5839}Lh$	4.00±0.24	Dwarf
L239	$le^{5839}lh$	1.34±0.04	Nana



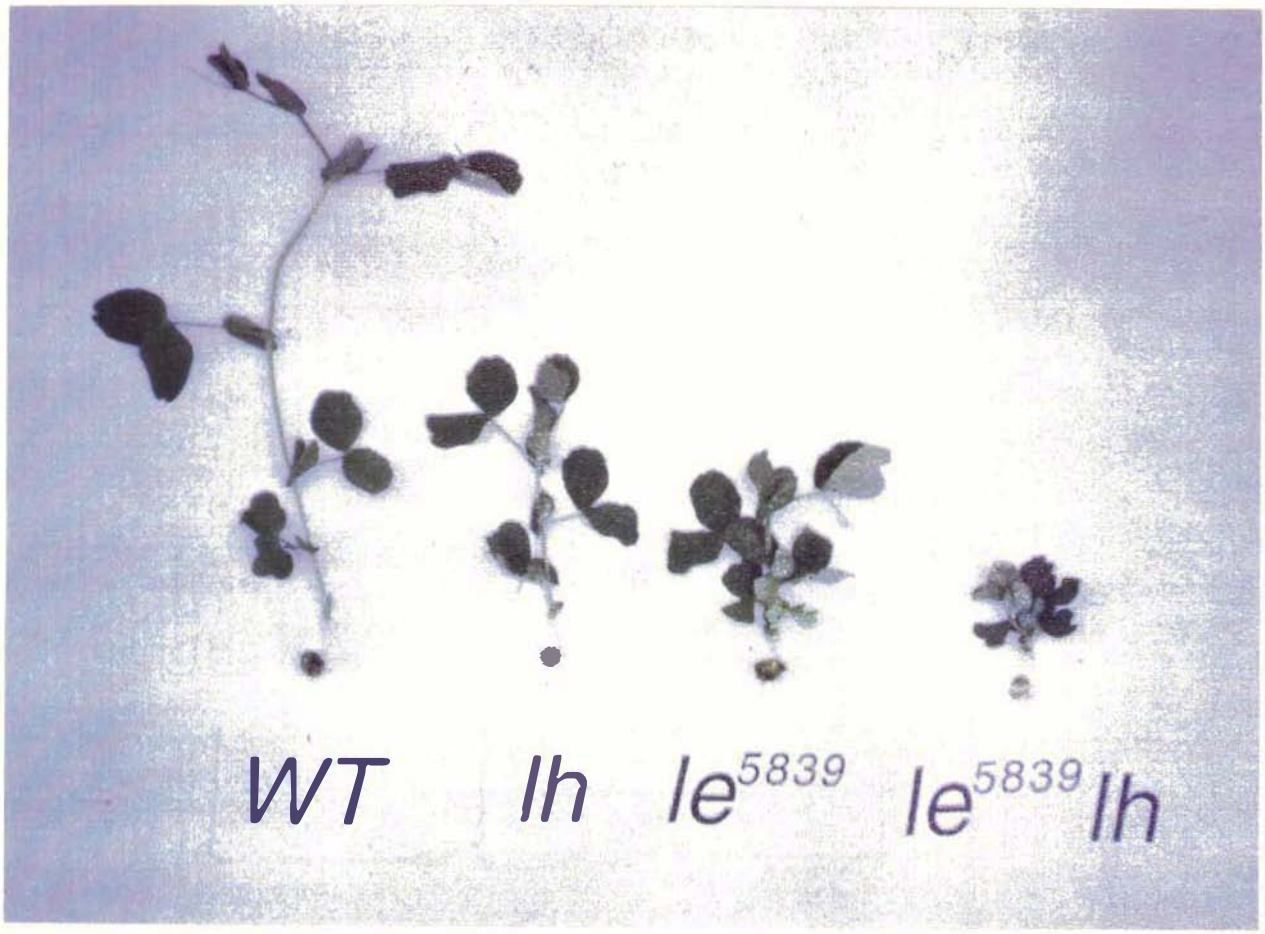


Fig. 1. Wild-type plants and plants of genotype *lh* (K511), *le*^{5839} (NGB5839) and *le*^{5839}*lh* (L239) at ca. 3 weeks after sowing.

2. Possible intragenic recombinations at the *Lh* locus

Introduction

Two alleles have been characterised at the *Lh* locus: *lh* and *lh*^{i} (Chapters 3,4, Reid 1986a). Both of these alleles reduce GA_1 levels in young shoots, thereby reducing internode elongation and causing a dwarf phenotype compared with *Lh* (wild-type) plants (Chapter 3, Reid 1986a). In shoots the *lh* allele is more severe than *lh*^{i} since *lh* plants possess lower GA_1 levels in young shoots, and are shorter, than *lh*^{i} plants (Chapter 3). The *lh* and *lh*^{i} alleles are also expressed in developing seeds (Chapter 4). However, in developing seeds the *lh*^{i} allele is more severe than the *lh* allele (*lh*^{i} seeds have lower GA levels, weigh less and are more likely to abort than *lh* seeds, Chapters 3,4). Therefore, the *lh* and *lh*^{i} alleles demonstrate tissue-dependent regulation of GA-biosynthesis in developing seeds and young shoots of peas. This suggests that discrete regions of the *Lh* locus may be involved in GA-biosynthesis in different tissues. Consequently, it may be possible to obtain additional alleles at the *Lh* locus with tissue-dependent regulation of GA-biosynthesis different from that of the *lh* and *lh*^{i} mutations. For example, a mutant resembling *lh* plants with regard to internode lengths, but resembling *lh*^{i} plants with regard to seed development, might be identified. On the other hand, the opposite combination (like *lh*^{i} in shoots but like *lh* in seeds) may also be possible. Since the molecular structure of the *Lh* locus is unknown, this hypothesis cannot be tested by constructing alleles with particular DNA sequences and examining the resulting phenotypes (GA levels in developing seeds and shoots). However, it is possible that new alleles at the *Lh* locus could result from natural recombination within this locus during meiosis in heterozygous *lhlh*^{i} plants. If, for example, a new allele with reduced GA levels in developing seeds, reduced seed weight and increased seed abortion was identified, this would lend further support for a physiological role for GAs in seed development.

The F_1 and F_2 progeny resulting from a cross between K511 (*lh*) and NGB5843 (*lh*^{i}) plants have already been described in Chapter 3. Although the majority of the F_2 progeny resembled either the *lh* or *lh*^{i} parent with regard to both internode lengths and seed development, two plants did not (data not shown). One possessed internode lengths similar to NGB5843 (*lh*^{i}) but seed development similar

to K511 (*lh*), while the other possessed internode lengths similar to K511 (*lh*) but seed development similar to NGB5843 (lh^i). These plants are therefore potential new mutants resulting from independent intragenic recombinations at the *Lh* locus. In this section, these two genotypes are examined.

Materials and Methods

All plants from which seeds were harvested were grown at a density of 2 per pot, except for the F_1 parents used to obtain the data shown in Tab. 3, which were grown at a density of 1 per pot. Plants harvested for GA determinations in young shoots were grown in tote boxes at a density of 60 per tote box (Chapter 2). Shoot tissue above, and including, the highest fully-expanded leaf was harvested from cv. Torsdag (wild-type) and lines K511 (*lh*), NGB5843 (lh^i), L237 (lh^f), L238 (lh^s), NGB5839 (le^{5839}) and L181 (*ls*) plants 20 days after sowing. Tissue of a comparable developmental stage was harvested from L236 ($lslh^i$, see Chapter 8) plants 26 days after sowing since the small size of $lslh^i$ plants (nana phenotype similar to $le^{5839}lh$ plants, Section 1) resulted in insufficient plant material for GA analysis at 20 days of age. All plants had between 4 and 7 leaves expanded at harvest. At least 6 plants were allowed to continue growth so that the length between nodes 4 and 7 could be determined after these internodes were fully expanded. GA levels in young shoots and developing seeds harvested at contact point (the first day no liquid endosperm remained) were determined as described in Chapter 2 (Method 1). GA_3 levels were not monitored since previous results (Lawrence et al. 1992, Ross et al. 1992) have shown that GA_3, if present, occurs at very low levels in pea shoots. To compare the effects of paclobutrazol on lines K511 (*lh*), NGB5843 (lh^i), L237 (lh^f) and L238 (lh^s), 1µg of paclobutrazol was applied to the dry, nicked seed ($n \geq 5$) in 2µl of ethanol before planting. Control plants ($n \geq 8$) received 2µl of ethanol only. These plants were grown in tote boxes (Chapter 2).

All plants were initially grown in a heated glasshouse (Chapter 2). At three weeks of age the F_1 plants from which the data in Tab. 3 was obtained were transferred to a controlled environment cabinet with day and night temperatures of 15°C and 10°C, respectively (Chapter 2).

Results

The two novel F_2 progeny from cross K511 (*lh*) by NGB5843 (lh^i) were allowed to self-pollinate over a further 5 generations, at which time they appeared to be pure-breeding (data not shown). The plants with internode lengths similar to NGB5843 (lh^i) but seed development similar to K511 (*lh*) were assigned Hobart line number 237 (tentatively assigned symbol lh^f, *f*ertile). The plants with internode lengths similar to K511 (*lh*) but seed development similar to NGB5843 (lh^i) were assigned Hobart line number 238 (tentatively assigned symbol lh^s, *s*terile). Details of the internode lengths and seed development of these lines are shown in Tab. 1. Internode lengths between nodes 6 and 9 decreased in the order *Lh* (wild-type), L237 (lh^f), NGB5843 (lh^i), K511 (*lh*) and L238 (lh^s) ($P<0.05$). In terms of seed development, L237 (lh^f) plants possessed more ($P<0.05$) seeds per pod than all other genotypes (Tab. 1). The number of seeds per pod developing on wild-type (*Lh*) and K511 (*lh*) plants were not significantly different ($P=1$), but both these genotypes possessed significantly more ($P<0.05$) seeds per pod than NGB5843 (lh^i) and L238 (lh^s) plants (Tab. 1). The number of seeds per pod developing on NGB5843 (lh^i) and L238 (lh^s) plants were not significantly different ($P>0.5$, Tab. 1). To compare GA levels in seeds from the various lines at the same developmental stage, seeds were harvested at contact point (the first day no liquid endosperm remained). Seeds of genotype lh^i were found to weigh less and possess dramatically reduced levels of GA_{20} compared with *Lh* (wild-type) seeds (Tab. 2), consistent with the results presented in Chapter 4. The *lh* allele was also found to reduce endogenous GA_{20} levels, compared with the *Lh* (wild-type) allele, although *lh* seeds possessed markedly more GA_{20} than lh^i seeds (Tab. 2). Fertilization of lh^i flowers with *lh* pollen (from K511 plants) produced seeds of genotype $lhlh^i$. This increased seed weight and endogenous GA_{20} and GA_{29} levels, compared with self-pollinated lh^i plants, to levels comparable to self-pollinated *lh* plants (Tab. 2). This result suggests that the *lh* allele is completely dominant over the lh^i allele in developing seeds, consistent with the results presented in Chapter 4.

Endogenous GA levels were also determined in seeds harvested at contact point (the first day no liquid endosperm remained) from L237 (lh^f) and L238 (lh^s) plants. Lines K511 (*lh*) and L237 (lh^f) possessed similar seed weights and similar

GA_{20} and GA_{29} levels in developing seeds (Tab. 2). Lines NGB5843 (lh^i) and L238 (lh^s) also possessed similar seed weights and similar GA_{20} and GA_{29} levels in developing seeds (Tab. 2).

Since, at least under certain conditions, plant stature can influence seed development (Chapter 5), the development of homozygous recessive lh^i, lh^s and lh^f seeds was compared with *Lh*- or *lh*- seeds that developed on the same heterozygous maternal plant (Tab. 3). Heterozygous $Lhlh^i$, $Lhlh^s$, $Lhlh^f$ (tall phenotype) and $lhlh^i$ (dwarf phenotype) plants were allowed to self-pollinate in day and night temperatures of 15°C and 10°C, respectively. The resulting F_2 seeds were individually weighed and subsequently sown to determine their genotype. To distinguish between genotypes *lh*- and lh^ilh^i (from $lhlh^i$) parents, 0.5µg of paclobutrazol (in 2µl of ethanol) was applied to the dry seeds before sowing since lh^i plants are more sensitive to paclobutrazol than *lh* plants (Chapter 3). Approximately three weeks later seed genotypes were determined by scoring the phenotypes of the resulting seedlings. Heterozygous $Lhlh^i$, $Lhlh^s$ and $Lhlh^f$ parents produced either tall (*Lh*-) or dwarf (lh^ilh^i, lh^slh^s or lh^flh^f) seedlings. Heterozygous $lhlh^i$ parents produced either dwarf (*lh*-) or nana (lh^ilh^i) plants because of the different response to paclobutrazol (Chapter 3). Having established the genotypes of individual seeds, mean seed weights were calculated for the different seed genotypes.

Consistent with previous comparisons between seeds of genotype *Lh*- and lh^ilh^i (Chapters 3,4), the observed proportion of tall (*Lh*-) and dwarf (lh^ilh^i) F_2 progeny did not agree with the expected 3:1 ratio ($P<0.001$, Tab. 3). In addition, the Lh/lh^s and lh/lh^i alleles did not segregate in agreement with the expected 3:1 ratio due to a deficiency of lh^ilh^i and lh^slh^s F_2 progeny, respectively ($P<0.001$, Tab. 3). The mean seed weight of lh^ilh^i and lh^slh^s seeds was also less than that of *Lh*- and *lh*- seeds ($P<0.01$, Tab. 3). The deficiency and reduced weight of lh^ilh^i and lh^slh^s seeds is consistent with the reduced GA levels of these seeds (Tab. 2, Chapter 4) directly causing reduced seed weight and increased seed abortion compared with *Lh*- (wild-type) seeds (Tabs. 1,3). By contrast, the *Lh* (wild-type) and lh^f alleles segregated in agreement with expected results (Tab. 3, $P>0.10$). Furthermore, homozygous lh^f seeds did not weight less than *Lh*- seeds, and in fact were slightly heavier ($P<0.05$, Tab. 3).

Therefore, the levels of GA_{20} and GA_{29} in developing seeds correspond to the seed yield observed for the four dwarf genotypes (Tab. 1) and to the development

of homozygous recessive seeds on heterozygous parent plants (Tab. 3). GA-deficient seeds (genotypes $lh^{i}lh^{i}$ and $lh^{s}lh^{s}$) are lighter and more likely to abort than seeds with higher GA levels (genotypes *Lh*-, *lh*- and $lh^{f}lh^{f}$), consistent with a role for the GAs in seed development.

The levels of endogenous GAs were also determined in developing shoots of mutant lines K511 (*lh*), NGB5843 (lh^{i}), L237 (lh^{f}), L238 (lh^{s}), NGB5839 (le^{5839}), L181 (*ls*), L236 ($lslh^{i}$) and cv. Torsdag (wild-type) (Tab. 4). A quantitative relationship between endogenous GA_1 levels and internode lengths between nodes 4 and 7 (encompassing the developmental stage at which plants were harvested) was found (Fig. 1). Plants of genotype le^{5839} did not fit this relationship as well as other genotypes (Fig. 1), possibly because several tissues were harvested together for GA analysis (e.g. leaf, petiole, internode and apical bud tissues). Since 3β-hydroxylation may vary in these tissues (Smith et al. 1992), the severity of the le^{5839} mutation may differ between different tissues. Hence, the expression of the le^{5839} mutation in expanding internodes may be more severe than appears when the whole apical region is examined, explaining the stature of le^{5839} plants (Fig. 1). Such problems are unlikely to occur for the other mutants used, since they all block prior to GA_{12}-aldehyde (Ingram and Reid 1987a, see Chapter 8) The relationship between GA_1 levels and internode lengths reported here is consistent with the results presented in Chapter 3 (for alleles at the *Lh* locus), and with results from maize (e.g. Phinney and Spray 1982, Spray et al. 1984, Fujioka et al. 1988), wheat (Lenton et al. 1987) and peas (for alleles at the *Le* locus, Ross et al. 1989). This relationship does not hold for GA_{20} levels since le^{5839} plants possess more GA_{20}, but are considerably shorter, than wild-type plants (Tab. 4, Fig. 1) due to impaired 3β-hydroxylation of GA_{20} to GA_1 (Ingram et al. 1984). These results lend further support to the hypothesis that GA_1 is the major native GA controlling internode elongation in peas (Reid and Ross 1993). Furthermore, the GA_1 levels in shoots of lines K511 (*lh*), NGB5843 (lh^{i}), L237 (lh^{f}) and L238 (lh^{s}) are consistent with the internode lengths of these lines (Tabs. 1,4, Fig. 1). Comparison of GA levels in shoots (Tab. 4) and developing seeds (Tab. 2) also confirms that the *lh* and lh^{i} alleles demonstrate tissue-dependent regulation of GA biosynthesis in these tissues (Chapter 4).

Since lh^{i} plants are more sensitive to exogenously applied paclobutrazol than *lh* plants (Chapter 3), the response of L237 (lh^{f}) and L238 (lh^{s}) plants to paclobutrazol applied to the dry seed before sowing was examined (Fig. 2). The

degree of sensitivity to paclobutrazol was associated with seed GA levels and seed development rather than shoot GA_1 levels and internode lengths of untreated plants. Compared with K511 (*lh*) and L237 (*lh*^{f}) plants (relatively high yield), NGB5843 (*lh*^{i}) and L238 (*lh*^{s}) plants (relatively low yield) are extremely sensitive to applied paclobutrazol (Fig. 2).

Discussion

The endogenous levels of GAs in both developing seeds and young shoots of L237 (*lh*^{f}) and L238 (*lh*^{s}) plants are consistent with the observed seed development and internode length phenotypes of these lines. Furthermore, these results are consistent with these lines having arisen from independent intragenic crossovers at the *Lh* locus.

On the basis of these results, a hypothetical model to explain the phenotypes of the *Lh* (wild-type), *lh*^{i},*lh*, *lh*^{f} and *lh*^{s} genotypes can be constructed. In this model, part of the *Lh* locus encoding the structural gene (i.e. directly encoding the *Lh* protein) is comprised of three regions (Fig. 3). Mutations within these regions alter the three-dimensional structure of the *Lh* protein so that (i) protein function in young shoots is impaired, (ii) protein function in developing seeds is impaired, and/or (iii) inhibition of GA-biosynthesis by paclobutrazol in increased. A further assumption is that the *lh*^{i} allele contains two base-pairs different from the *Lh* allele, while the *lh* allele contains one base-pair different for the *Lh* allele. This is a reasonable assumption since both the *lh* and *lh*^{i} mutations were produced using mutagens that tend to cause numerous mutations to single nucleotide bases throughout the genome (Chapter 2). If these lesions occurred in the regions indicated in Fig. 3, the phenotypes of the *lh* and *lh*^{i} mutants can be explained. Both the *lh* and *lh*^{i} alleles differ from the *Lh* allele in the region responsible for altering protein activity in both young shoots and developing seeds. The positions of these lesions is such that the *lh* protein (lesion at position "c") results in lower GA_1 levels in young shoots (Tab. 4) and slightly shorter internode lengths (Fig. 1), compared with the *lh*^{i} allele (lesion at position "b"). The second lesion (position "a") in the *lh*^{i} allele occurs in the region affecting both protein activity in the developing seed and sensitivity to paclobutrazol. Hence GA_{20} levels in developing seeds decrease in the order *Lh* (wild-type), *lh* to *lh*^{i} (Tab. 2), and *lh*^{i} plants are more sensitive to applied paclobutrazol than *Lh* (wild-type) or *lh* plants (Chapter 3).

The phenotypes of L237 (lh^f) and L238 (lh^s) plants can then be explained by independent intragenic crossovers between positions "a" and "b" (Fig. 3) when heterozygous $lh^s lh^i$ plants were allowed to self-pollinate (Chapter 3). The lh^s allele possesses lesions at positions "a" and "c", resulting in increased sensitivity to paclobutrazol (similar to lh^i plants, Fig. 2), dramatically reduced GA levels in developing seeds (similar to lh^i plants, Tab. 2) and reduced GA_1 levels in young shoots (similar to *lh* plants, Tab. 4). The lh^f allele possesses a lesion at position "b" only, resulting in normal sensitivity to paclobutrazol (similar to *lh* plants, Fig. 2), reduced GA levels in developing seeds (similar to *lh* plants, Tab. 2) and reduced GA_1 levels in young shoots (similar to lh^i plants, Tab. 4).

This model can also explain the phenotypes of the original F_2 plants, from which lines L237 (lh^f) and L238 (lh^s) are descended (Chapter 3), if they possessed genotypes $lh^f lh^i$ and $lh^s lh^i$, respectively. Plants (F_2 progeny) of genotype $lh^f lh^i$ would be expected to resemble $lh^i lh^i$ plants in terms of internode elongation, but show increased seed yield compared with $lh^i lh^i$ plants since approximately three quarters of the F_3 seeds would possess genotype lh^f- (seed GA levels and seed development similar to *lh* seeds since *lh* is dominant over lh^i in seeds, Tab. 2). Since the lh^f phenotype (Tab. 1) was selected over 5 generations and L237 (lh^f) appears to breed true, the lh^f allele is likely to be homozygous in L237 (lh^f).

Plants (F_2 progeny) of genotype $lh^s lh^i$ would be expected to resemble *lhlh* plants in terms of internode elongation since the *lh* allele (and presumably the lh^s allele) is partially dominant over the lh^i allele in young shoots (Chapter 3). However, F_3 seeds developing on self-pollinated $lh^s lh^i$ plants (genotype $lh^s lh^s$, $lh^s lh^i$ or $lh^i lh^i$) would all be GA-deficient leading to significant seed abortion, similar to that on self-pollinated $lh^i lh^i$ plants. Again, since the lh^s phenotype (Tab. 1) was selected over 5 generations and L238 (lh^s) appears to breed true, the lh^s allele is likely to be homozygous in L238 (lh^s).

The reason for the differences in internode elongation between K511 (*lh*) and L238 (lh^s) plants, and between NGB5843 (lh^i) and L237 (lh^f) plants (Tab. 1, Fig. 4), can be explained if small amounts of GA_1-precursors (e.g. GA_{19} and GA_{20}) are carried over in the dry seed. Such a theory is supported by the presence of GA_{19} and GA_{20} in dry *Lh* (wild-type) seeds (J. Ross, unpublished data). Seedlings of lines K511 (*lh*) and L237 (lh^f) may receive more GA precursors at germination than lines NGB5843 (lh^i) and L238 (lh^s) since developing *lh* and lh^f seeds contain higher

GA_{20} levels than lh^i and lh^s seeds (Tab. 2). Such GA carry-over may explain the increased stature of K511 (*lh*) and L237 (lh^f) plants compared with L238 (lh^s) and NGB5843 (lh^i) plants, respectively.

Intragenic crossovers have been reported for the *VP1* (McCarty and Carson 1991) and *R* (Robbins et al. 1991) loci of maize, and the *GA1* locus of *Arabidopsis* (Koornneef 1979). Although the results presented in this section are consistent with independent intragenic crossovers at the *Lh* locus, the relatively similar stature of the dwarf lines K511 (*lh*), NGB5843 (lh^i), L237 (lh^f) and L238 (lh^s) does not allow polygenic influences to be completely discounted. For example, if small polygenic differences occur between lines K511 (*lh*) and NGB5843 (lh^i), these may have segregated in the F_2 progeny of the cross between these lines so that the L237 and L238 phenotypes (Tab. 1) do not result from changes at the *Lh* locus. The segregation of internode length phenotypes was examined in the F_2 progeny resulting from crosses between L237 (lh^f) and cv. Torsdag (*Lh*), and between L238 (lh^s) and cv. Torsdag (*Lh*), but the similar phenotypes of lines K511 (*lh*), NGB5843 (lh^i), L237 (lh^f) and L238 (lh^s) prevented a clear interpretation of the results obtained (data not shown). Characterization of the biochemical and/or molecular nature of the *Lh* locus is required before the model outlined above can be tested in more detail, and the influence of possible polygenic differences examined.

Tab. 1. Phenotypes of cv. Torsdag (wild-type), K511 (*lh*), NGB5843 (lh^i), L237 (lh^f) and L238 (lh^s) plants. Values represent the means of at least 11 plants±SE. Photoperiod 18h.

Line	Genotype[a]	Length between nodes 6 and 12 (cm)	Fully developed seeds per pod
Torsdag	*Lh*	45.9±0.9	3.00±0.20
L237	lh^f	18.0±0.3	3.71±0.19
K511	*lh*	11.9±0.1	3.00±0.26
NGB5843	lh^i	16.9±0.3	2.02±0.26
L238	lh^s	11.0±0.1	1.88±0.13

a lh^f and lh^s are tentative alleles.

Tab. 2. Fresh weights and GA levels in seeds (including testa) harvested at contact point (the first day no liquid endosperm remained) for various genotypes at the *Lh* locus. Heterozygous $lhlh^i$ seeds were produced by fertilizing lh^i (NGB5843) plants with *lh* (K511) pollen. GA_1 and GA_3 are not present in seeds at this developmental stage (Gaskin et al. 1985).

Experiment	Line	Seed genotype[a]	Average seed weight at harvest (mg)	GA level (ng.(gFW^{-1}))	
				GA_{20}	GA_{29}
	Torsdag (wild-type)	*LhLh*	227	775	-[c]
1[b]	NGB5843	lh^ilh^i	195	14	-
	K511	*lhlh*	255	165	-
	K511	*lhlh*	280	248	37
2	NGB5843	lh^ilh^i	180	7	1
	5843xK511 pollen	$lhlh^i$	260	218	31
	L237	lh^flh^f	235	298	44
3	K511	*lhlh*	229	278	33
	NGB5843	lh^ilh^i	207	7	2
	L238	lh^slh^s	201	7	3

a lh^f and lh^s are tentative alleles.

b from Swain (1989).

c not measured.

Tab. 3. Observed segregation of the numbers and dry seed weights of the F_2 progeny (seeds) resulting from self-pollination of $Lhlh^i$, $Lhlh^s$, $Lhlh^f$ and $lhlh^i$ F_1 plants in day (18h) and night (6h) temperatures of 15°C and 10°C, respectively.

Parent genotype (F_1)	Observed F_2 segregation					
	Dominant			Recessive		
	Genotype	No.	Weight (mg)	Genotype	No.	Weight (mg)
$Lhlh^i$	*Lh-*	161	226±3	lh^ilh^i	9[a]	187±14[b]
$Lhlh^s$	*Lh-*	105	297±3	lh^slh^s	12[a]	245±9[b]
$Lhlh^f$	*Lh-*	69	268±3	lh^flh^f	15	282±5[c]
$lhlh^i$	*lh-*	92	293±3	lh^ilh^i	7[a]	236±7[b]

a significant deficiency ($P<0.001$) of homozygous recessive progeny.

b homozygous recessive seeds significantly lighter ($P<0.01$) than seeds with at least one dominant allele.

c homozygous lh^f seeds significantly heavier ($P<0.05$) than *Lh-* seeds.

Tab. 4. GA levels in apical tissue (above and including the highest fully-expanded leaf) harvested from cv. Torsdag (wild-type), K511 (*lh*), NGB5843 (lh^i), L237 (lh^f), L238 (lh^s), NGB5839 (le^{5839}), L181 (*ls*) and L236 ($lslh^i$) plants. All plants had between 4 and 7 nodes expanded at harvest.

Line	Genotype[a]	Age (days)	GA level (ng.(gFW^{-1}))			
			GA_1	GA_8	GA_{20}	GA_{29}
Torsdag	wild-type	20	8.35	25.16	5.82	10.63
L237	lh^f	20	0.46	6 02	0.55	3.51
NGB5843	lh^i	20	0.43	1.94	0.37	1.56
K511	*lh*	20	0.40	2.45	0.38	1.54
L238	lh^s	20	0.28	1.29	0.18	1.02
NGB5839	le^{5839}	20	0.50	4.24	14.08	41.76
L181	*ls*	20	0.14	0.61	0.16	0.48
L236	$lslh^i$	26	0.06	0.08	0.05	0.06

[a] lh^f and lh^s are tentative alleles.

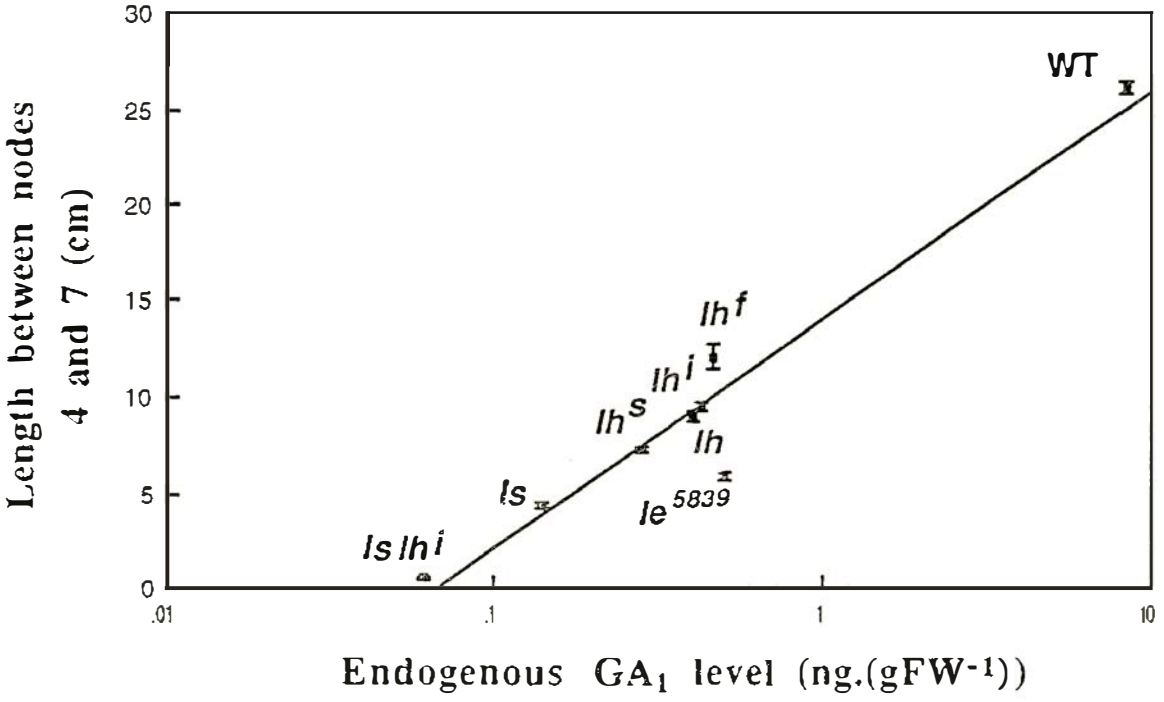


Fig. 1. Relationship between endogenous GA_1 levels (ng.(gFW^{-1})) and internode lengths between nodes 4 and 7 for wild-type (WT) plants and the mutant genotypes indicated. All genotypes had between 4 and 7 leaves fully expanded at harvest.

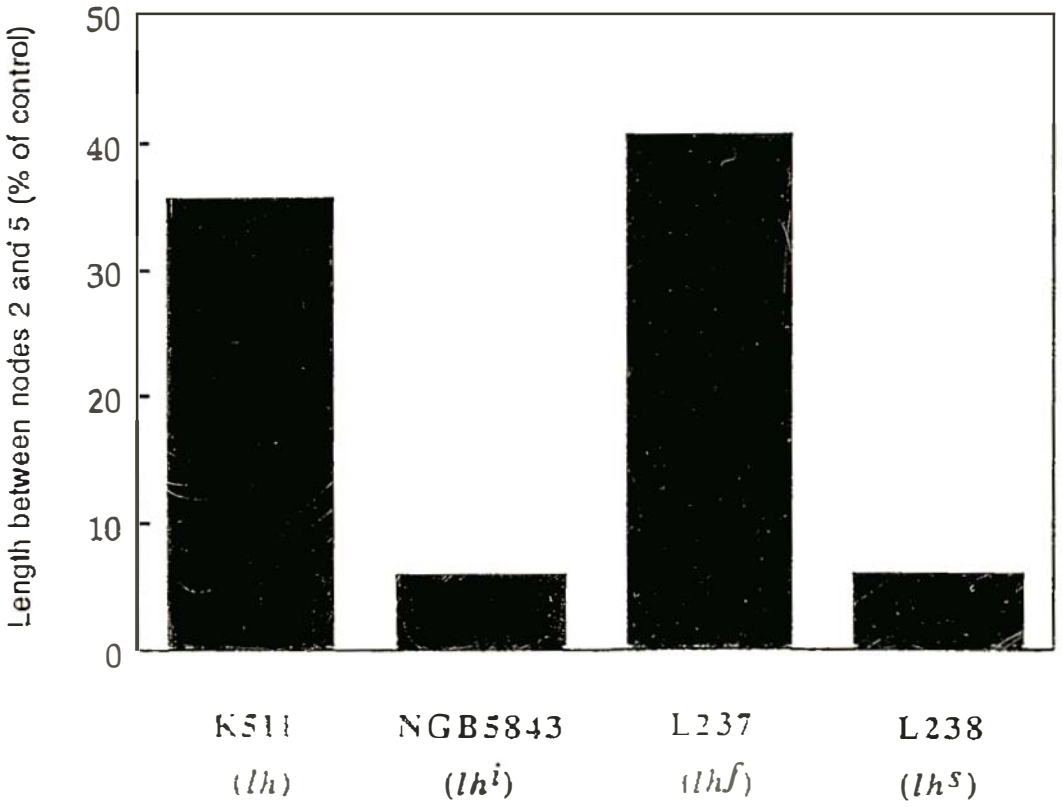


Fig. 2. Response of K511 (*lh*), NGB5843 (lh^i), L237 (*lhf*) and L238 (lh^s) plants (n≥5) to 1μg of paclobutrazol applied to the dry seed before germination.

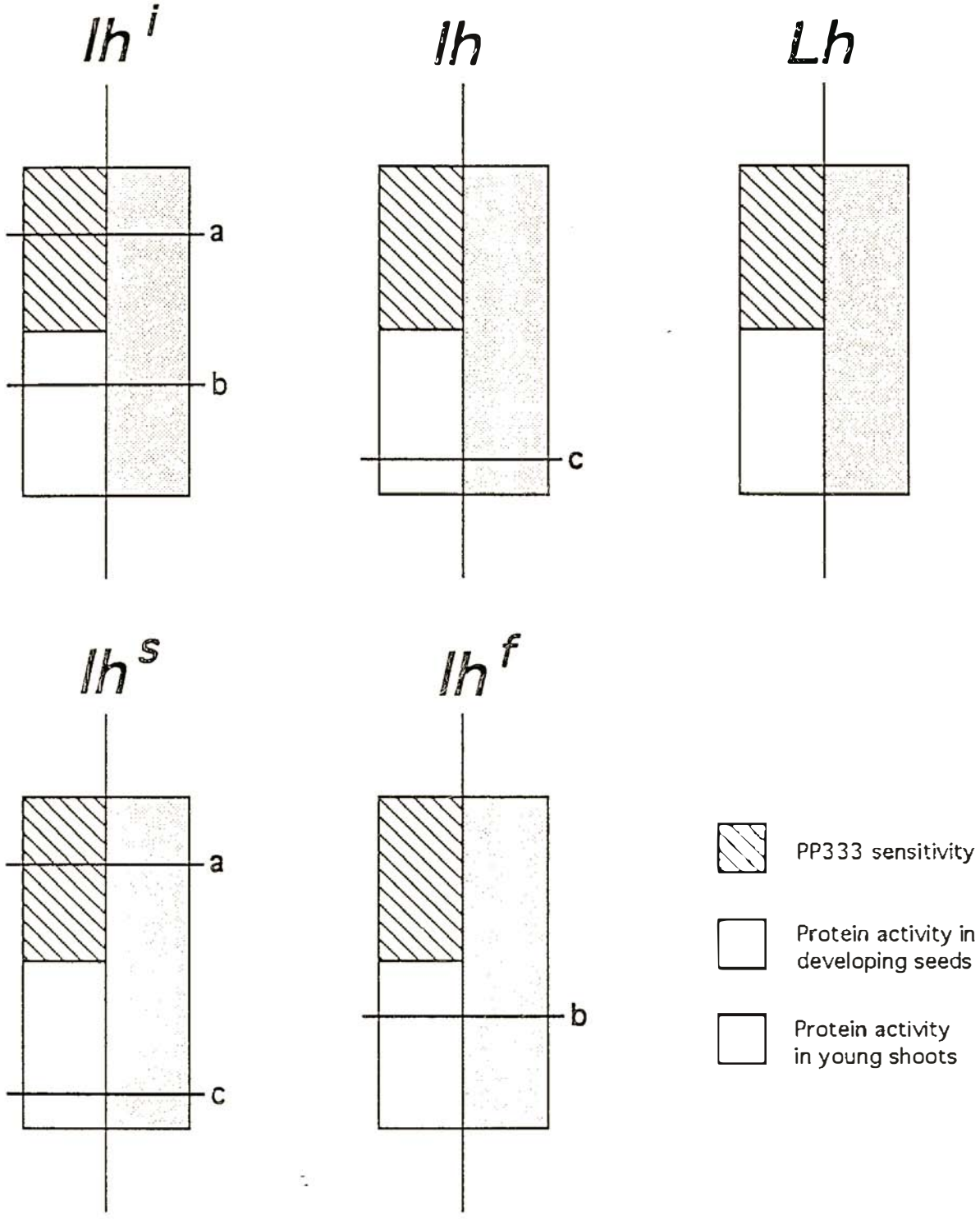


Fig. 3. Hypothetical model to explain the phenotypes of mutant lines NGB5843 (lh^i), K511 (lh), L238 (lh^s) and L237 (lh^f). The model represents the portion of the *Lh* locus encoding the structural protein, and "a", "b" and "c" indicate the positions of base-pairs differing from those of the *Lh* (wild-type) allele.

Chapter 7

Genetic interactions with the *lh*^{i} mutation

Parts of this chapter are published in:

Swain and Reid (1992b), Pisum Genetics **24**, 83-85

Reid, Ross and Swain (1992), Planta **188**, 462-467

Ross, Reid and Swain (1993), Aust. J. Plant Physiol., in press.

Introduction

Homozygous *lh*^{i} seeds possess reduced GA levels, are more likely to abort during development and weigh less, compared with *Lh* (wild-type) seeds. Fertilizing *lh*^{i} flowers with *Lh* (wild-type) pollen increases seed GA levels and restores normal seed development (Chapter 4), suggesting that the reduced GA levels of *lh*^{i} seeds directly causes the alterations in seed development compared with wild-type seeds. Consequently, other factors that increase GA levels or GA-response in developing *lh*^{i} seeds would be expected to overcome the effects of the *lh*^{i} mutation on seed development. Application of exogenous GA_3 has already been used successfully to restore normal internode elongation in *lh*^{i} plants (Chapter 3). Unfortunately, the small size of young developing pea seeds, and their location within rapidly growing pod tissue, prevents GA application *in vivo* without dramatic alterations in shoot and pod growth, which may adversely affect seed development (Barratt 1986b). Although incubation of *lh*^{i} embryos with GA_1 *in vitro* appears to increase embryo growth (Chapter 5), this may not mimic the situation *in vivo* (e.g. Ambrose et al. 1987).

In this chapter a genetic approach is used to further explore the role of GAs in seed development by examining the interaction of the *lh*^{i} gene with the *sln*, *la*, *cry*^{s} and *r* genes. In particular, since *lh*^{i} seeds weigh less and are more likely to abort than *Lh*- seeds, mutations partially or fully epistatic over *lh*^{i} would be expected to cause increased seed weight and survival of the double (*lh*^{i}*sln* or *lh*^{i}*r*) or triple mutants (*lh*^{i}*lacry*^{s}) compared with non-recombinant seeds.

1. Interaction of genes *lh*^{i} and *sln*

Introduction

The *lh*^{i} mutation is thought to partially block GA-biosynthesis prior to the formation of GA_{12}-aldehyde (Chapter 3, Ingram and Reid 1987a). Consequently, an additional mutation that reduced GA-catabolism might increase GA levels on a *lh*^{i} background. One such mutant, named *sln*, that may act in this manner has already been identified. Seeds maturing on a homozygous *sln* plant have a reduced ability to catabolise GA_{20} to GA_{29}-catabolite (Ross et al. 1993, unpublished results), and this results in significant GA_{20} carry-over in the dry seed (Reid et al. 1992). Upon germination, the GA_{20} is thought to be converted into GA_1 resulting in increased internode elongation and the slender phenotype. This hypothesis is consistent with the inability of the GA-biosynthesis inhibitors paclobutrazol and AMO-1618 (which act before GA_{12}-aldehyde production, Graebe 1987) to reduce internode elongation of *sln* plants when applied to germinating seeds (Reid et al. 1992). In contrast to the effects of the *sln* mutation in maturing seeds, *sln* shoots do not appear to possess significantly impaired GA-catabolism (J. Ross, pers. com.).

Seeds of genotype *lh*^{i}*sln* may possess increased GA levels compared with *lh*^{i}*Sln* seeds, and this would be expected to cause increased seed weight and reduced seed abortion. Such a result would lend considerable support to the hypothesis that GAs are required for normal seed development in the garden pea. However, the *sln* mutation cannot be combined with other mutations, such as *lh*^{i}, since the inheritance of the *sln* (slender) phenotype is unclear. In particular, when *Sln* (wild-type) and slender (*sln*) plants are crossed, no phenotypically slender plants appear in the F_2 generation (Reid et al. 1992). In this chapter a model is developed to explain the inheritance of the *sln* phenotype, the *lh*^{i}*sln* double mutant is isolated and the mode of action of the *sln* mutation is confirmed.

Materials and Methods

Plant Material.

The *Lhsln* parent used in the cross with *lh*^{i}*Sln* (NGB5843) was derived

form a cross between cv. Torsdag (*LhSln*) and NGB6074 (*Lhsln*). This parent was chosen in an attempt to minimise any polygenic differences that may exist between lines NGB6074 and NGB5843, since only the latter is derived from cv. Torsdag.

Growth regulator treatments.

To identify plants of genotype *Lh-* or $lh^{i}lh^{i}$ resulting from a cross between plants of genotype *Lhsln* and $lh^{i}Sln$ (NGB5843), four F_3 seeds from 32 F_2 plants were treated with 1µg of paclobutrazol in 2µl of ethanol to the dry seed before sowing.

Prohexadione-calcium (BX-112) (Kumiai Chemical Industry Company, Tokyo) was applied to the dry nicked seed of lines NGB6074, 197 and Torsdag, at a rate of 100µg per seed in 4µl of ethanol. Control plants received 4µl of an identical solution without prohexadione (see Chapter 2).

Results

Inheritance of the sln *phenotype.*

The absence of the slender phenotype in the F_2 progeny resulting from crosses between *Sln* (wild-type) and *sln* (slender) parents (both homozygous for the *Lh* allele) may be explained if both the seed coat (testa) and the cotyledons are able to catabolise GA_{20} provided at least one wild-type allele (*Sln*) is present. According to this hypothesis, since the genotype of the testa is the same as that of the maternal plant, all seeds produced by F_1 (wild-type phenotype) plants (genotype *Slnsln*) would be capable of GA catabolism. Approximately 75% of these F_2 seeds (genotype *Sln-*) would be able to catabolise GA_{20} in both the cotyledons (genotype *Sln-*) and testa (genotype *Slnsln*), while in the remainder (cotyledonary genotype *slnsln*) catabolism could proceed only in the testa (genotype *Slnsln*). However, the end result is the same: GA_{20} would not accumulate in any of the F_2 seeds. Thus, seeds of genotype *slnsln* which developed on a F_1 plant (genotype *Slnsln*) will germinate to produce phenotypically tall seedlings. Therefore, it is suggested that the genotype of the parent plant influences the expression of the *slnsln* genotype. These genetically slender, phenotypically tall F_2 seedlings would, on the above hypothesis, be expected to produce all slender F_3 offspring since the testa around the F_3 seeds would be of genotype *slnsln*.

The above model was confirmed when six F_3 seeds from each of 49 F_2 plants from a cross between cv. Torsdag (*Sln*) and NGB6074 (*sln*) were sown (Reid et al. 1992). Approximately one quarter (16 plants) of the F_2 produced all slender progeny in the F_3 demonstrating that the *Sln/sln* alleles segregated in agreement with the expected 3:1 *Sln-:slnsln* ratio in the F_2 progeny even though the *sln* mutation was not expressed phenotypically (Reid et al. 1992).

Mode of action of the sln *gene.*

The new class of GA-biosynthesis inhibitors, acylcyclohexanediones (including prohexadione), interfere with the late steps of GA-biosynthesis (Rademacher et al. 1992). They are particularly effective at blocking the 3β-hydroxylation of GA_{20} to GA_1 (Hedden 1991, Nakayama et al. 1992) and consequently result in pronounced dwarfing of wild-type peas (Fig 1, Sponsel and Reid 1992). The slender phenotype of *sln* plants (from *slnsln* parents) is believed to be caused by the conversion of GA_{20} (carried over in the seed) into GA_1 following germination (Reid et al. 1992). If this hypothesis is correct, prohexadione would be expected to reduce internode elongation of seedlings if applied to seeds at the time of planting, unlike the effects of paclobutrazol and AMO-1618 treatment at this time (Reid et al. 1992). This indeed occurs since prohexadione reduced the length between nodes 1 and 4 by 73% (P<0.001, Fig. 1). As would be expected, phenotypically slender plants caused by the duplicate gene combination *lacry*^{S} were largely unaffected by a similar prohexadione treatment (Fig. 1) although a significant (P<0.001) reduction in height was observed and may reflect a toxic effect of the high dose used. This differential response clearly separates the two slender mutants and is consistent with previous suggestions that the *lacry*^{S} combination acts at or after perception of the GA_1 signal, rather than by an alteration in GA levels or metabolism. It also suggests that the large effect of prohexadione on *sln* plants is not due, at least in any substantial way, to a general toxicity of this compound.

Isolation of the lh^{i}sln *genotype.*

Having established the genetic inheritance of the *sln* gene, a cross was performed between plants of genotype *Lhsln* (slender) and *lh*^{i}*Sln* (dwarf, NGB5843). The resulting F_1 plants possessed a tall phenotype, consistent with genotype *Lhlh*^{i}*Slnsln*. When these F_1 plants were allowed to self-pollinate the F_2

progeny produced a range of internode length phenotypes (Fig. 2). No phenotypically slender F_2 plants were identified, consistent with the previously reported inheritance of the *sln* phenotype (Reid et al. 1992) and the model described above. The F_2 progeny was divided into *Lh-* (148 plants) and lh^ilh^i (33 plants) genotypes (Fig. 2) by sowing 4 paclobutrazol-treated F_3 seeds from 32 F_2 plants (data not shown). This method was used since lh^ilh^i plants are more sensitive to exogenously applied paclobutrazol than *Lh-* plants (Chapter 3). The number of tall (*Lh-*) and dwarf (lh^ilh^i) F_2 progeny did not agree with the expected tall:dwarf ratio of 3:1 ($P<0.05$) due to a deficiency of dwarf plants caused by zygotic selection against lh^ilh^i seeds on the parent F_1 plant (Chapter 4). Untreated F_3 progeny were also grown-on from a range of F_2 parents. When 4 F_3 progeny were grown-on from 32 phenotypically tall (*Lh-*) F_2 plants, the segregation of *Sln-* (24 plants produced all tall F_3 progeny) and *slnsln* (8 plants produced some or all slender F_3 progeny) F_2 plants was found to agree with the expected 3:1 ratio ($P=1$). Significantly, 2 of the phenotypically tall (but genetically slender, *slnsln*) F_2 plants produced 3 slender and 1 dwarf offspring in the F_3 (Fig. 3). The genotype of these F_2 parents was therefore $Lhlh^iSlnsln$, and the dwarf F_3 offspring possessed genotype $lh^ilh^islnsln$ (the "double mutant"). As genotype lh^isln has a dwarf rather than slender phenotype (even when developing on a homozygous *sln* F_2 parent), the lh^i mutation is epistatic over the *sln* mutation, at least in terms of the GA_{20} carry-over in the dry seed and the slender phenotype of the resulting seedling. This explains the absence of slender plants in the F_3 progeny obtained when 4 seeds from 32 of the dwarf (lh^ilh^i) F_2 plants (Fig. 2) were grown-on (data not shown, see Fig. 3).

To investigate the seed yield of lh^isln plants, F_4 progeny from a single lh^isln F_3 (dwarf) plant from the cross described above were compared with lh^iSln (NGB5843) plants. Plants of genotype lh^isln did not exhibit the slender phenotype (Fig. 3) and possessed more seeds ($P<0.01$), fewer pods ($P<0.01$) and more seeds per pod ($P<0.001$) compared with lh^iSln plants (J. Ross, pers. com.). However, this difference in seed yield is not necessarily caused by the *sln* mutation. When the lh^isln F_3 parent was back-crossed to lh^iSln (NGB5843), the majority of the F_2 progeny possessed a seed yield considerably higher than that of the lh^iSln (NGB5843) parent (data not shown). This suggests that the increased yield of lh^isln plants compared with lh^iSln (NGB5843) plants is due, at least partially, to differences between the two genotypes at loci other than *Sln*.

Discussion

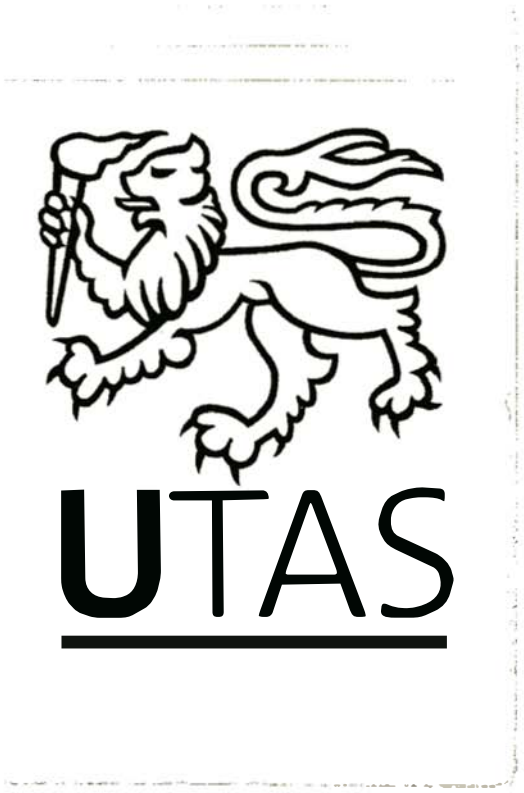
A model that successfully explains the inheritance of the *sln* phenotype has been developed (see Fig. 3). This has allowed the interaction between the lh^i and *sln* mutations to be explored, and in future will allow the *sln* mutation to be combined with a range of GA-synthesis and GA-response mutants (e.g. *na, le* and *lv*).

Two lines of evidence support the hypothesis that the high levels of GA_{20} carried-over into germinating *sln* seeds is responsible for the elevated GA_1 levels and increased internode elongation (slender phenotype) of *sln* plants compared with *Sln* (wild-type) plants. Firstly, treatment of dry *sln* seeds with prohexadione, an inhibitor of the 3β-hydroxylation of GA_{20} to GA_1 (Hedden 1991, Nakayama et al. 1992), prevents expression of the *sln* phenotype. Secondly, the lh^i mutation, which dramatically reduces GA_{20} levels in maturing seeds (Chapter 4), also prevents expression of the *sln* mutation (lh^isln plants do not possess the slender phenotype). It appears that lh^i seeds are so severely GA-deficient that even in the presence of the *sln* allele sufficient GA_{20}-catabolism occurs to prevent GA_{20} accumulation in the dry seed, and the slender phenotype upon germination.

The inheritance of the *sln* phenotype appears to be unique amongst internode length mutations since the *sln* (slender) phenotype is absent in the F_2 generation, but reappears in the F_3. A similar situation exists for the *aba1abi3-1* double mutant of *Arabidopsis* (see Chapter 1). Homozygous *aba1abi3-1* (ABA-deficient) seeds developing on a heterozygous *ABA1aba1abi3-1abi3-1* (normal ABA levels in maternal tissues) parent plant exhibit normal seed development in terms of LEA protein accumulation and desiccation tolerance. However, when homozygous *aba1abi3-1* (ABA-deficient) plants are self-pollinated the resulting seeds (genotype *aba1abi3-1*) have reduced levels of LEA proteins and are not desiccation tolerant (Koornneef et al. 1989). Thus, the genotype of the maternal plant influences the phenotype of the next generation for both the *sln* and *aba1abi3-1* mutations.

The increased seed yield of lh^isln plants compared with lh^iSln (NGB5843) plants is consistent with a physiological role for the GAs in seed development. However, the existence of polygenic differences between lines NGB6074 and NGB5843 prevent a valid interpretation of this result. To overcome this problem near-isogenic lh^isln/lh^iSln lines are being generated by growing-on from self-pollinated $lh^ilh^iSlnsln$ heterozygotes obtained from the F_2 progeny of cross lh^isln by lh^iSln

(NGB5843). These heterozygotes can be identified by test-crossing onto *Lhsln* female plants (e.g. NGB6074) since an approximately 1:1 ratio of tall (*Lhlh^{i}Slnsln*) to slender (*Lhlh^{i}slnsln*) offspring will be produced (Reid et al. 1992). Although *lh^i* appears to be epistatic over *sln* in terms of the GA_{20} carry-over in the dry seed, this may not be the case in developing seeds, when the abortion of *lh^i* seeds occurs (Chapter 4).



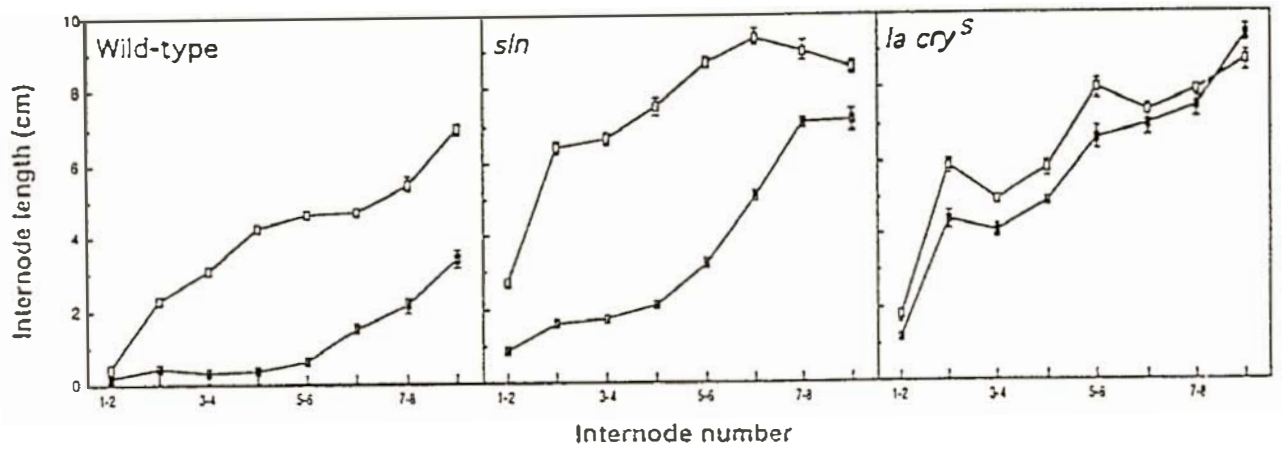


Fig. 1. Internode length plotted against internode number for cv. Torsdag (wild-type, tall, *SlnLaCry*), and lines NGB6074 (slender, *slnLaCry*) and 197 (slender, *Slnlacry*^{S}), treated on the dry seed with either 100µg BX-112 in ethanol (■) or ethanol only (□), prior to sowing (n≥8).

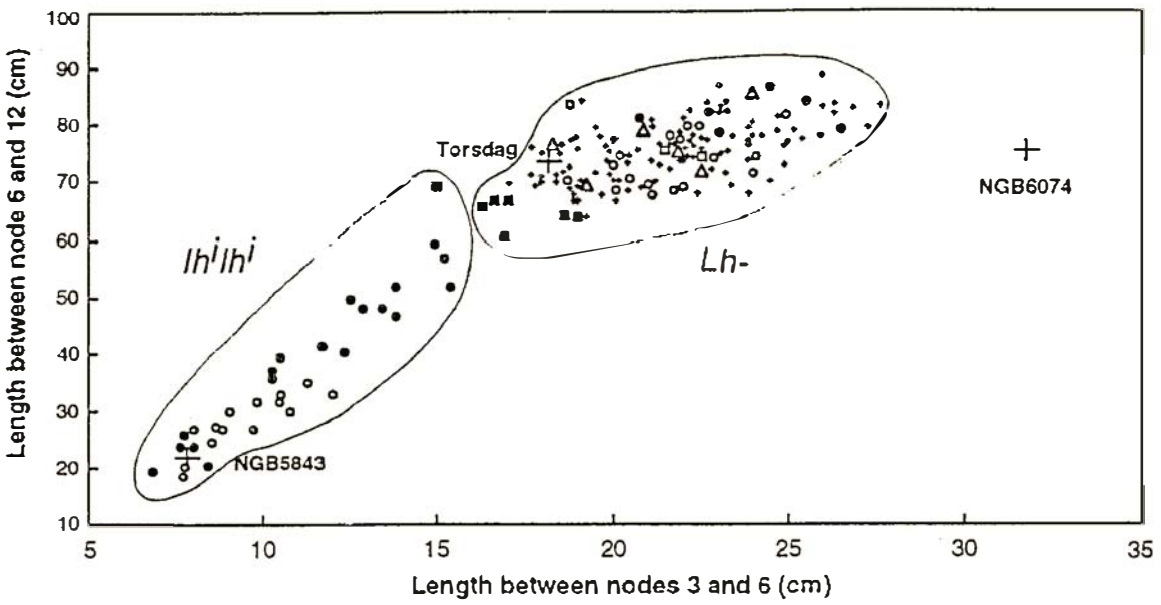


Fig. 2. Lengths between nodes 3 and 6 plotted against lengths between nodes 6 and 12 for the F_2 progeny resulting from a cross between plants of genotype *lh^{i}Sln* (dwarf, NGB5843) and *Lhsln* (slender). Two separate F_3 progeny were grown-on from various F_2 parents. In the first planting, 4 F_3 seeds from 32 F_2 parents (■,●) were treated with 1µg of paclobutrazol before sowing to divide the F_2 progeny into genotypes *Lh-* and *lh^{i}lh^i*. In the second planting, 4 seeds from 32 tall (*Lh-*) and 32 dwarf (*lh^{i}lh^i*) F_2 parents were grown-on without paclobutrazol treatment (○,●,△,□). Twenty-four tall F_2 parents (genotype *Lh-Sln-*) produced F_3 plants with either tall or dwarf phenotypes (○,●). Six tall F_2 parents (genotype *Lh-slnsln*) produced all slender F_3 plants (△). Two tall F_2 parents (genotype *Lhlh^{i}slnsln*) produced 3 slender and 1 dwarf F_3 offspring (□). No dwarf (*lh^{i}lh^i*) F_2 parents produced any slender F_3 offspring. Means for cv. Torsdag (wild-type, *LhSln*) and lines NGB6074 (*Lhsln*) and NGB5843 (*lh^{i}Sln*) are indicated by large crosses (n≥5).

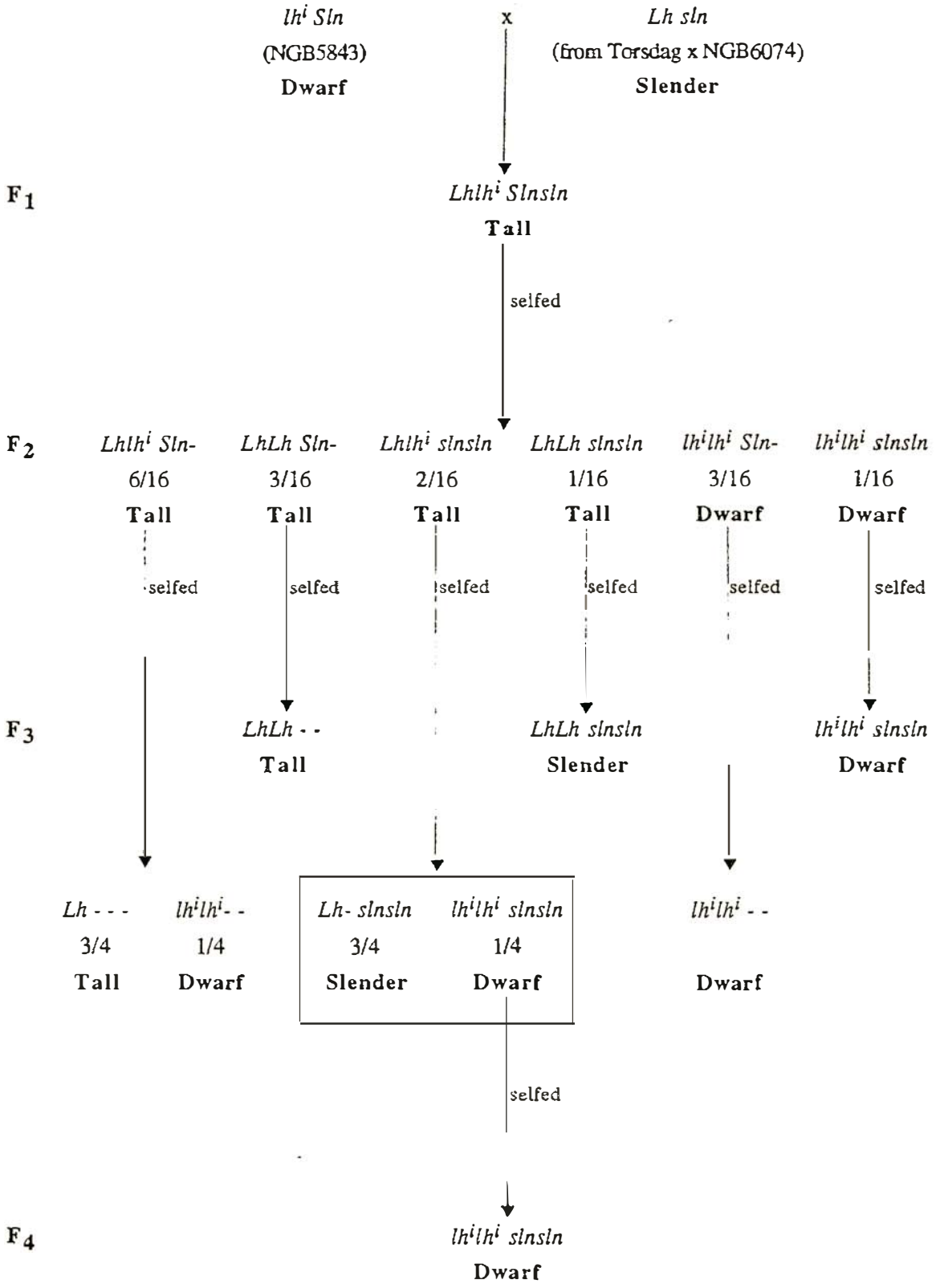


Fig. 3. Segregation of the genotypes and phenotypes of the F_1, F_2, F_3 and F_4 progeny resulting from a cross between plants of genotype *lh^{i}Sln* (dwarf, NGB5843) and *Lhsln* (slender).

2. Interaction of genes lh^i, *la* and cry^s

Introduction

Plants homozygous for both the *la* and cry^s alleles exhibit the slender phenotype (long, thin internodes, de Haan 1927, de Haan 1930, Reid et al. 1983) and resemble wild-type plants treated with a saturating dose of GA_1, regardless of endogenous GA levels (Ingram and Reid 1987b, Potts et al. 1985). Hence, the *La* and *Cry* gene-products are thought to act at or after GA perception in the transduction chain linking GA_1 levels to changes in internode length (Potts et al. 1985).

A GA response mutant that is epistatic to the lh^i allele in developing seeds would lend support to the hypothesis that GAs play an important role in seed development. Therefore, the interaction between the $lacry^s$ gene combination and the lh^i allele was investigated.

Materials and Methods

All F_1, F_2, F_3, F_4 and F_5 plants resulting from the cross between lines 197 (*Lh* $lacry^s$) and NGB5843 (lh^i *LaCry*) were initially grown in a heated glasshouse (Chapter 2). Some of the F_3 plants were transferred 3 weeks after planting to a controlled environment cabinet with a day/night temperature regime of 25/20°C (Chapter 2).

Results and Discussion

The F_1 of the cross between lines NGB5843 (dwarf; lh^iLaCry) and 197 (slender; $Lhlacry^s$) had a tall phenotype and the F_2 (Fig. 1) segregated into three classes: tall, dwarf and slender. Seeds from some of the tall F_2 plants (●, Fig. 1) and some of the slender F_2 plants (■, Fig. 1) were grown-on to confirm their genotypes. One phenotypically slender F_2 plant was found to be genetically tall since its progeny segregated to give 5 tall and 1 slender F_3 plant (data not shown). Three phenotypically slender F_2 plants bred true in F_3. The remaining four slender F_2 plants could not be progeny tested because they produced only parthenocarpic pods and no seeds. This is

a pleiotropic effect of the slender gene combination (Potts et al. 1985) and is consistent with these plants having genotype *lacry*^{S}. All seeds from the four dwarf F_2 plants were grown on in the F_3. One dwarf F_2 plant produced in F_3 13 dwarf and 1 slender offspring. Hence, the genotype of this slender F_3 plant was *lh*^{i}*lacry*^{S}, demonstrating that the slender phenotype is expressed on a *lh*^{i} background. This result is similar to that obtained for the *nalacry*^{S} and *lelacry*^{S} genotypes (Ingram and Reid 1987b, Lamm 1937, Potts et al. 1985) and demonstrates that the *lacry*^{S} gene combination is epistatic to the *na, le* and *lh*^{i} genes in young shoots, and is expressed regardless of GA levels in this tissue. Plants of genotype *lh*^{i}*lacry*^{S} also developed parthenocarpic pods in subsequent generations.

Having established that plants homozygous for *lh*^{i}, *la* and *cry*^{S} have a slender phenotype, the expected F_2 ratio is 45 tall: 15 dwarf: 4 slender. The observed F_2 numbers of 74 tall, 4 dwarf and 7 slender plants (Fig. 1) are not in agreement with the expected ratio ($P<0.001$). However, the number of slender plants agreed with expected results when compared with the total number of F_2 plants ($P>0.3$). By contrast, there was a significant deficiency in the observed number of dwarf compared with tall F_2 plants ($P<0.001$) as observed in other crosses segregating for the *Lh* and *lh*^{i} alleles (Chapters 3,4).

Eleven of the dwarf F_3 plants, from the family containing 1 slender plant, were allowed to self-pollinate in a day/night temperature regime of 25/20°C. This temperature regime was used since the segregation of the *Lh* (wild-type) and *lh*^{i} alleles is strongly disturbed under these conditions (chapter 4). However, no significant deviation from expected results was observed in any of the resulting progeny. Four F_4 families segregated in accordance with a ratio of 3 dwarf to 1 slender plant (totals: 83 dwarf, 26 slender, $P>0.7$). Three F_4 families segregated in accordance with a ratio of 15 dwarf to 1 slender plant (totals: 73 dwarf, 4 slender, $P>0.7$). One F_4 family segregated in accordance with both a 3:1 and a 15:1 ratio (16 dwarf, 2 slender). Three F_4 families bred true.

Seeds possessing genotype *lh*^{i}*lh*^{i} have previously been shown to weigh less than *Lh*- seeds (Chapter 4). Since the *lacry*^{S} gene combination is epistatic over the *lh*^{i} allele in shoots, the possible epistasis over final seed weight of *lh*^{i} seeds was examined. Six dwarf plants from F_4 families containing approximately 1/4 slender segregants were allowed to self-pollinate in a glasshouse during winter (day and night temperatures of between 20-25 and 10-15 °C, respectively). The resulting seeds were

weighed immediately prior to sowing to determine if the slender F_5 plants (segregating in four families) developed from heavier seeds than the dwarf F_5 plants. However, no significant difference ($P>0.5$) was found between the weight of seeds which gave rise to slender plants (0.298 ± 0.010g.seed^{-1}, n=13) and those which gave rise to dwarf plants (0.293 ± 0.007g.seed^{-1}, n=48). Hence, no evidence was found for epistasis of the *lacry^S* gene combination over the *lh^i* allele in developing seeds. Possible explanations for this result include: (i) the *la* and *cry^S* mutations are not expressed in developing seeds, and (ii) the *la* or *cry^S* mutation is not segregating in this cross and that this mutation is fully epistatic over *lh^i*. For example, if the *la*, but not the *cry^S*, mutation is epistatic over the *lh^i* mutation, and the F_4 plants examined possessed genotype *lh^{i}lh^{i}lalaCrycry^S*, all F_5 seeds would be of a similar weight at harvest.

In conclusion, the *lacry^S* gene combination is epistatic over the *lh^i* allele in young shoots. The results support the hypothesis that the slender phenotype caused by *lacry^S* (long, thin basal internodes) is independent of endogenous GA levels since the *lh^i* allele is thought to block GA production before GA_{12}-aldehyde, the precursor to all GAs in peas (Chapter 3, Ingram and Reid 1987a). By contrast, the *lacry^S* gene combination does not provide further support for a physiological role for the GAs in seed development.

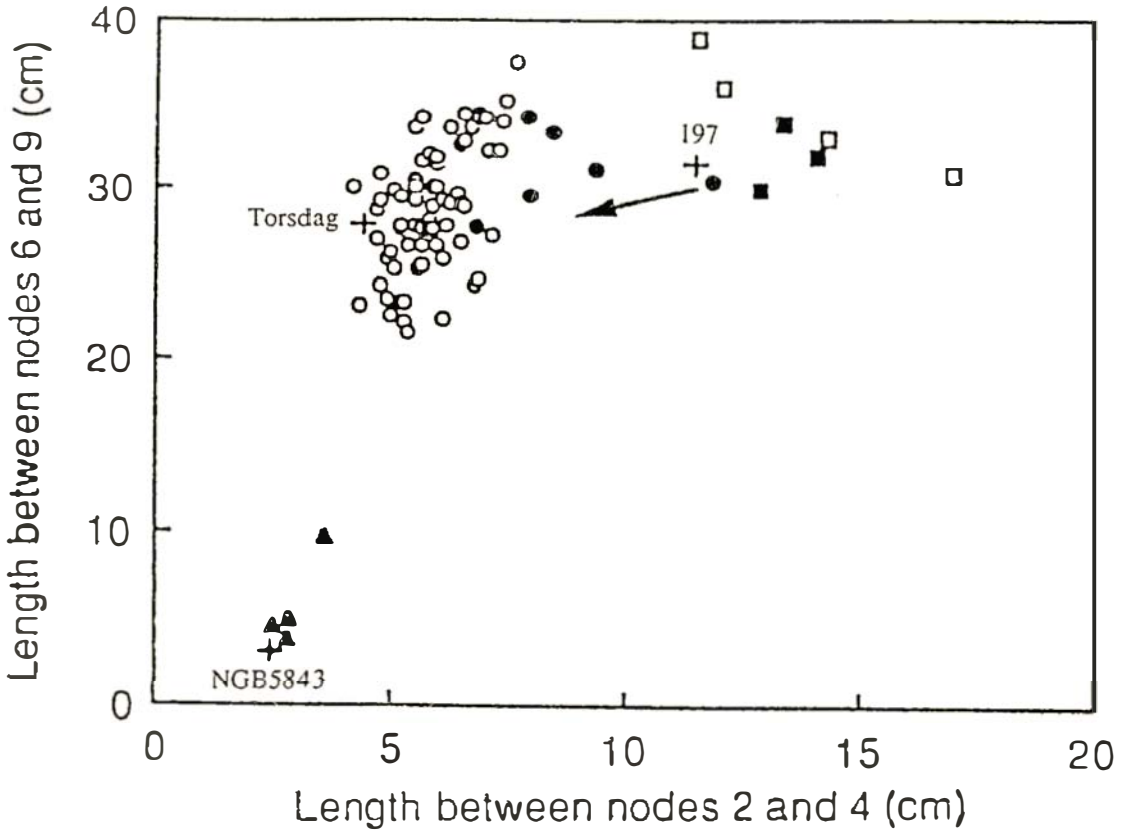


Fig. 1. Internode lengths for the F_2 resulting from a cross between line 197 (*Lhlacry*^{S}) and NGB5843 (*lh*^{i}*LaCry*), showing segregation into slender (□, ■), tall (○,●) and dwarf (▲) phenotypic classes. Solid symbols represent plants grown-on to confirm their genotype. Means for parental lines 197 and NGB5843, and for the wild-type cv. Torsdag (*LhLaCry*), are indicated by crosses (n≥5). Photoperiod 18 h.

3. Interaction of genes *lh*^{i} and *r*

Introduction

Seeds homozygous for the recessive mutation, *r*, possess the wrinkled phenotype (numerous small indentations in the testa) rather than the smooth, nearly spherical shape of *R* seeds (Mendel 1865). The *R* locus has been shown to encode one isoform of the starch-branching enzyme (SBEI), and an aberrant transcript for SBEI is produced by *rr* embryos (Bhattacharyya et al. 1990). Embryos of genotype *r* consequently produce lower levels of starch than *R* embryos and this leads to a range of pleiotropic effects, particularly once the liquid endosperm has been consumed (for review see Wang and Hedley 1991). Firstly, in *r* embryos starch synthesis is reduced so that sucrose accumulates to higher levels than in *R* embryos. In turn, elevated sucrose levels lead to increased osmotic potential in developing *r* seeds, causing greater water uptake, larger embryo cells and a higher fresh weight compared with *R* seeds. When the seed dehydrates the embryo (largely composed of the cotyledons) shrinks, but the testa does not, resulting in the characteristic wrinkled phenotype of *r* seeds. The *r* mutation also has other effects in developing embryos, such as increasing the levels of lipids and decreasing legumin content (Wang and Hedley 1991). Seeds of genotype *rr* also possess reduced dry weight compared with *RR* seeds, at least when developing on self-pollinated homozygous parents (Hedley et al. 1986).

Since the *lh*^{i} mutation reduces the sink-strength of, and ^{14}C-labelled assimilate supply to, developing seeds (Chapter 5), while the *r* mutation increases sucrose levels and embryo cell-size, the interaction of the *lh*^{i} and *r* genes was investigated. Although no evidence suggests that the *r* mutation affects any aspects of plant growth besides seed development (Hedley et al. 1986), *lh*^{i}*lh*^{i}*R*- and *lh*^{i}*lh*^{i}*rr* seeds were compared on the same heterozygous maternal plant (genotype *lh*^{i}*lh*^{i}*Rr*) so that any effects of the *r* mutation on the maternal plant were excluded.

Materials and Methods

All F_1, F_2, F_3, F_4, F_5 and F_6 plants resulting from the cross between L23 (*Lhr*) and

NGB5843 (*lh*^{i}*R*) were initially grown in a heated glasshouse (Chapter 2). All of the dwarf F_2, F_3 and F_5 plants that germinated from round seeds (genotype *lh*^{i}*lh*^{i}*R*-) were transferred 3 weeks after planting to controlled environment cabinets with day/night temperature regimes of 25/20°C, 20/15°C and 15/10°C, respectively (Chapter 2). All other plants were allowed to complete development in the glasshouse (Chapter 2).

The genotype (*R*- or *rr*) of all seeds not clearly identified by their appearance was determined after sowing, allowing the plants to self-pollinate, and scoring the resulting seeds (the next generation) for round or wrinkled phenotypes. All wrinkled seeds identified in this manner produced at least 7 wrinkled seeds in the following generation ($P<0.13$ for genotype *Rr*). A total of 6 seeds from the 6 generations scored could not be identified in this manner, since they grew into unhealthy plants that produced fewer than 7 seeds, and were excluded from the analysis.

Results and Discussion

To investigate the interaction between the *lh*^{i} and *r* genes, a cross was performed between NGB5843 (*lh*^{i}*R*) and L23 (*Lhr*). The resulting F_1 seeds had a round (unwrinkled) appearance and grew into tall plants (genotype *Lhlh*^{i}*Rr*). Six F_1 plants were allowed to self-pollinate and 81 F_2 progeny selected from pods with relatively few aborted seeds. The F_2 progeny segregated to give 53 round seeds that grew into tall plants (*Lh-R*-), 18 wrinkled seeds that grew into tall plants (*Lh-rr*), 9 round seeds that grew into dwarf plants (*lh*^{i}*lh*^{i}*R*-) and 1 wrinkled seed that grew into a dwarf plant (*lh*^{i}*lh*^{i}*rr*), demonstrating that the wrinkled phenotype is expressed on a *lh*^{i} background. The deficiency in the number of observed dwarf F_2 progeny ($P<0.001$) is due to zygotic selection against the homozygous *lh*^{i} seeds on the parent F_1 plant (Chapter 4), which occurred in spite of the selection of F_2 progeny from pods with relatively few aborted seeds. The observed number of *R*- and *rr* seeds agreed with expected results ($P>0.70$). No evidence for linkage between the *Lh* and *R* loci was found ($P>0.20$), consistent with the results presented in Chapter 6. The F_2 progeny also exhibited wide variation in characters such as internode elongation, pod length and seeds per pod (data not shown), suggesting that NGB5843 and L23 differ at many loci in addition to *Lh* and *R*. To allow more detailed comparisons of the *lh*^{i}*R* and *lh*^{i}*r* genotypes near isogenic lines were generated by allowing plants of

genotype lh^ilh^iRr to self-pollinate over several generations (Tab. 1). With the exception of a small deficiency of lh^ilh^iR- (round) seeds in the F_5 progeny, the observed segregation of lh^ilh^iR- (round) and lh^ilh^irr (wrinkled) seeds agreed with the expected round:wrinkled ratio of 3:1 (Tab. 1, P>0.10). The observed proportion of lh^ilh^iRr:lh^ilh^iRR seeds also agreed with the expected ratio of 2:1 (Tab. 1, P>0.80). Therefore, there is no evidence to suggest that the abortion of GA-deficient lh^i seeds is influenced by the R or r alleles on plants of genotype lh^ilh^iRr.

Three round F_5 seeds (genotype lh^ilh^iRr) were allowed to self-pollinate in a day/night temperature regime of 15/10°C (Tab. 1) since these conditions allow differences between the final weight of Lh- and lh^ilh^i seeds to be detected (Chapters 4,6). The resulting seeds (F_6 progeny) were weighed to determine the weights of lh^ilh^iR- and lh^ilh^irr seeds developing on the same maternal plants. No significant difference (P>0.90) in the final weight of lh^ilh^iR- (0.224±0.005g.seed^{-1}) and lh^ilh^irr (0.225±0.007g.seed^{-1}) seeds was found. This result contrasts with the reduced weight of fully developed Lhr seeds (on self-pollinated Lhr plants) compared with LhR seeds (on self-pollinated LhR plants) (Hedley et al. 1986). However, when fully developed seeds from self-pollinated homozygous lh^iR and lh^ir F_6 plants (n≥8) grown in a heated glasshouse (Chapter 2) were compared the mean weight of lh^ir seeds (0.237±0.009g.seed^{-1}) was found to be less (P<0.01) than that of lh^iR seeds (0.261±0.007g.seed^{-1}). Therefore, the simplest explanation for the difference in final weight of lh^ilh^iR- and lh^ilh^irr seeds on homozygous plants, but not on heterozygous lh^ilh^iR- plants, is that the r mutation has a small effect on whole plant development, slightly reducing the weight of fully developed seeds. If this hypothesis is correct, then no evidence exists to suggest that the lh^i and r mutations interact to modify the expression of the other gene.

Tab. 1. Observed segregation of the *R* and *r* alleles on a lh^i background. Dwarf plants germinating from round seeds (genotype lh^ilh^iR-) from the F_2, F_3, F_4 and F_5 generations of a cross between L23 (*Lhr*) and NGB5843 (lh^iR) were allowed to self-pollinate. The resulting seeds were genotyped by their external morphology (round or wrinkled phenotype) or by growing-on and scoring the next generation.

Parent plant generation	Total plants grown	Plants segregating for *R*/*r*	No. of progeny		χ^2 (3:1)
			lh^ilh^iR-	lh^ilh^irr	
F_2	9	7	51	25	2.53
F_3	29	17	407	143	0.29
F_4	6	5	33	20	4.58[a]
F_5	5	3	105	35	0.00
Total	49	32	596	223	2.17

[a] significantly different from expected results at $P<0.05$.

Chapter 8

Gibberellins, possibly GA_1 and GA_3, act early in seed development

Introduction

The new allele at the *Lh* locus, named lh^i, which dramatically reduces GA levels in developing seeds and increases seed abortion compared with *Lh* (wild-type) plants, has shown that GAs may play an important role in seed development. However, the stage during pea seed development at which GAs are required, or which GAs have biological activity *per se*, has not yet been examined.

Mutations affecting GA levels in developing shoots have been identified at the *Le*, *Na*, *Lh*, *Ls* and *Sln* loci (Reid and Ross 1993). The *na* and *lh* loci demonstrate tissue-specific differences in GA metabolism in shoots and developing seeds. The *na* mutation reduces GA levels in shoots (Ingram et al 1985, Potts and Reid 1983, Proebsting et al. 1992), but not in developing seeds (Potts and Reid 1983, Potts 1986). Comparison of the *lh* and lh^i alleles, which both reduce GA levels in shoots and developing seeds, demonstrates that different alleles can result in reductions of GA levels of differing severity, depending on the tissue examined (Chapters 3,4,6). However, tissue-dependent GA metabolism has not been demonstrated for the *le* or *ls* loci. GA levels have been determined in vegetative tissues (Ross et al. 1992, Smith et al. 1992), seeds and pods (Santes et al. 1993) of isogenic *Le* (205^+, wild-type) and *le* (205^-) plants. These results suggest that the *le* allele reduces GA_1 levels in all tissues so far examined. However, the expression in developing seeds has not been examined for another mutation at the *le* locus, le^{5839} (Jolly et al. 1987, Ross and Reid 1991), or for the *ls* mutant.

The steps in the GA-biosynthetic pathway controlled by the *Le*, *Sln* and *Na* genes have been indicated by metabolism studies. The *le* allele reduces 3β-hydroxylation of GA_{20} to GA_1 in shoots (Ingram et al. 1984), while the *sln* allele reduces the conversion of GA_{29} to GA_{29}-catabolite in seeds (Reid et al. 1992, Ross et al. 1993). The *na* allele acts prior to GA_{12}-aldehyde, possibly by reducing the conversion of *ent*-7α-hydroxykaurenoic acid to GA_{12}-aldehyde (Ingram and Reid 1987a). The situation is less clear for the *ls* and *lh* genes, although application studies suggest that both the *ls* and *lh* alleles act early in the GA-biosynthetic

pathway, possibly prior to the formation of *ent*-kaurene (Ingram and Reid 1987a).

In this chapter, the expression of the *ls* and le^{5839} alleles in developing seeds is investigated and the site of action in the GA biosynthetic pathway of the *ls* gene determined. The *ls* and lh^i alleles are subsequently used to examine the timing of GA action, tissue-dependent GA-biosynthesis within developing seeds, and the role of GA_1 and GA_3 in the regulation of seed development.

Materials and Methods

Growing conditions.

For quantification of GA levels in 21 day old *Ls* (wild-type) and *ls* shoots, 48 seeds of each genotype were sown in two separate tote boxes (Chapter 2). All plants from which seeds were harvested were grown at a density of 2 per pot, except for the plants used to examine seed development on heterozygous parents (Tab. 2), which were grown at a density of 1 per pot (Chapter 2). Plants grown under day/night temperature regimes of 20/15°C or 15/10°C were transferred to controlled environment cabinets 3 weeks after sowing. A single planting was used to provide (i) seeds at 6 days of age for GA analysis, and (ii) embryos at contact point for GA analysis and cell-free enzyme preparations. Since the extremely reduced stature of $ls lh^i$ plants (Fig. 1) adversely affects reproductive development 2µg of GA_3 (in 5µL of ethanol) was applied to the dry seeds before sowing. This treatment increased the length of early internodes, resulting in $ls lh^i$ plants of similar total height as lh^i (NGB5843) plants, and improved fruit development. Approximately 6 nodes before flowering commenced for $ls lh^i$ plants, internode lengths decreased dramatically, suggesting that no exogenous GA_3 remained when seeds were harvested (data not shown). Anthesis was scored as the first day the flower fully opened. Progeny resulting from segregating plants were sown in pots or tote boxes (Chapter 2), and visually scored for stature (tall, dwarf or nana phenotypes).

Gibberellin extraction and analysis.

For shoots and intact seeds at contact point, extraction and purification of GAs was achieved using Method 1 (Chapter 2). GAs from young seeds (5,6,7,9 or 11 days old) were quantified using Method 2 (Chapter 2), except for GAs from 11 day old wild-type seeds. These were quantified using Method 1 (Chapter 2), except that

the three GA fractions from HPLC were combined prior to GC-MS. For embryos harvested at contact point (the first day no liquid endosperm remained) the surrounding testa was removed immediately after harvesting and GAs quantified using Method 3 (Chapter 2).

Cell free enzyme systems.

Seeds were harvested from *Ls* (wild-type) and *ls* plants at contact point (the first day no liquid endosperm remained). The embryos (including cotyledons) were removed and cell-free enzyme systems prepared at 4°C (Coolbaugh and Moore 1971a,b) using the following method. After rinsing with extraction buffer (0.1M K_2HPO_4-KH_2PO_4, 100μM chloramphenicol, pH 7.1) the embryos were homogenised in the same buffer (ca. 1ml per g.FW embryos) using a mortar and pestle. The resulting slurry was centrifuged at 40 000g for 15min. and the supernatant (enzyme preparation) decanted. Extracts were frozen at -80°C until use. Subsequent work was performed at RIKEN (Tokyo, Japan) with the assistance of Dr Y. Kamiya, Dr T. Saito and Mr K.Furukawa. After defrosting at 4°C, enzyme preparations from *Ls* (wild-type) and *ls* embryos were concentrated using a Centricon 30 microconcentrator (Amicon, Beverly, MA, USA) so that incubations from both genotypes represented equivalent embryo fresh weights.

The radiolabelled substrates, ^{14}C-labelled mevalonic acid (^{14}C-MVA), ^{3}H-labelled geranyl-geranyl pyrophosphate (^{3}H-GGPP) and ^{3}H-labelled copalyl pyrophosphate (^{3}H-CPP) were prepared by Drs Y. Kamiya and T. Saito (RIKEN, Tokyo, Japan). For incubations with ^{14}C-MVA (free acid form), 20μl of ^{14}C-MVA (500nCi), 860μl of enzyme preparation (corresponding to 0.6g.FW embryo tissue for both genotypes) and 120μl of cofactors were incubated for 2h in a shaking waterbath at 30°C. The cofactor solution comprised 0.5M $MgCl_2$ (10μl), 0.1M $MnCl_2$ (40μl), 0.5M phosphoenolpyruvate (10μl), 0.5M ATP (20μl) and 0.05M NADPH (40μl). For incubations with ^{3}H-GGPP and ^{3}H-CPP, 188μl of enzyme preparation (corresponding to 1.3g.FW embryo tissue for both genotypes), 4μl of uniconazol (final concentration 10μM), 4 μl of $MgCl_2$ (final concentration 5mM) and 4μl of either ^{3}H-GGPP (120 000DPM) or ^{3}H-CPP (180 000DPM) were incubated for 2h in a shaking waterbath at 30°C.

To terminate incubations, the volume was made up to 1ml with dH_2O, and ca. 15μl of 3M HCl was added. To extract radiolabelled *ent*-kaurene, acetone (1ml)

and ethyl acetate (1ml) were added and mixed thoroughly. The extract was centrifuged for 1 min. at 4000rpm to separate the solvents, and the ethyl acetate (upper layer) removed. The ethyl acetate fraction was partitioned against a further 0.5ml of dH_2O and the ethyl acetate separated as before. The extract was gently evaporated to dryness under $N_2(g)$, ensuring that the $N_2(g)$ was removed as soon as the solvent disappeared. Such care was taken since *ent*-kaurene is volatile at room temperatures (Graebe 1987). After redissolving in 100µl of methanol, the extract was further purified by Thin Layer Chromatography (TLC) in *n*-hexane. The co-elution of radiolabelled *ent*-kaurene and another non-polar radiolabelled compound prevented analysis of *ent*-kaurene accumulation by TLC. A HPLC system (Beckman, System Gold) with an ODS (nucleosil) C_{18} column (4.6mm internal diameter, 5cm length) was used for further purification and quantification of radiolabelled *ent*-kaurene. Two HPLC solvents were used. Solvents A (80% methanol in dH_2O) and B (100% methanol) both contained 0.05% acetic acid.

The portion of the TLC plate corresponding to non-polar compounds (0.7 to 1.0 R_f) was removed and radiolabelled *ent*-kaurene extracted with 3 1ml washes of a 1:1 mixture of *n*-hexane and ethyl acetate. The solvent was evaporated under $N_2(g)$ and the extract redissolved in initial conditions. A HPLC gradient running from 80% solvent B to 100% solvent B over 10 min. (linear), and then isocratic at 100% solvent B for a further 10 min. was used for further *ent*-kaurene purification. The flow rate was 1ml per minute and 1ml fractions were collected. Radioactivity was determined using a Beckman scintillation counter. Radiolabelled *ent*-kaurene was identified on the basis of retention time compared to ^{14}C-labelled *ent*-kaurene standard.

Results

Seed yield of genotypes lh^i, ls *and* le^{5839}

Since the lh^i mutation has been shown to reduce seed yield compared with wild-type plants (Chapter 3), the yield of other GA-synthesis (dwarf) mutants was examined. Wild-type plants and plants of genotype le^{5839}, lh^i, *lh* and *ls* were allowed to flower in controlled environment cabinets with day (18h) and night (6h) temperatures of 20°C and 15°C, respectively. In agreement with previous results (Chapter 3), lh^i plants possessed more reproductive nodes ($P<0.05$), fewer seeds, on a per plant and per pod basis ($P<0.001$) and reduced mean seed weight at harvest

(P<0.001) compared with wild-type plants (Tab. 1). By contrast, the other three dwarf lines examined, *le*^{5839}, *lh* and *ls*, did not have reduced seed yield compared with wild-type plants, although the final weight of *ls* seeds was slightly less (P<0.05) than that of wild-type plants (Tab. 1). However, this comparison of seed development is complicated by the differences in stature between the different dwarf mutants and wild-type plants (Fig. 1), that may indirectly affect seed yield (e.g. Chapter 5). To overcome this problem, the development of homozygous recessive *lh*^{i}, *ls* and *le*^{5839} seeds was compared with that of *Lh-Ls-Le-* seeds on the same maternal plant. Heterozygous *Lhlh*^{i}, *Lsls* and *Lele*^{5839} F_1 plants (all possessing a tall phenotype) were allowed to self-pollinate in controlled environment cabinets with day (18h) and night (6h) temperatures of 15°C and 10°C, respectively. The resulting F_2 seeds were individually weighed and subsequently sown to determine their genotype. Approximately 3 weeks later, seedlings were classified as either tall (genotype *Lh-*, *Ls-* or *Le-*) or dwarf (genotype *lh*^{i}*lh*^{i}, *lsls* or *le*^{5839}*le*^{5839}) so that mean seed weights could be calculated for the F_2 seeds of different genotypes from the parent F_1 plants. Consistent with previous comparisons between seeds of genotype *Lh-* and *lh*^{i}*lh*^{i} (Chapters 4,6), the observed proportion of tall (*Lh-*) and dwarf (*lh*^{i}*lh*^{i}) F_2 progeny did not agree with the expected 3:1 ratio (P<0.001, Tab. 2), due to zygotic selection against the *lh*^{i}*lh*^{i} seeds (Chapter 4). For crosses involving both the *Ls*/*ls* and *Le*/*le*^{5839} alleles, no significant difference in seed weights were detected (P>0.70, Tab. 2). The observed proportion of tall (*Ls-* or *Le-*) and dwarf (*lsls* or *le*^{5839}*le*^{5839}) F_2 progeny was also in agreement with the expected 3:1 ratio for both loci (P>0.30, Tab. 2). The observed proportion of *Ls-* and *lsls* F_2 progeny was also in agreement with the expected 3:1 ratio when *Lsls* plants, on a *lkc* background (Reid et al. 1991), were allowed to self-pollinate under day (18h) and night (6h) temperatures of 25°C and 20°C, respectively (data not shown). These results suggest that the development of *ls*, *lh* and *le*^{5839} seeds does not differ from that of wild-type seeds.

Isolation of genotype lslh^{i}.

The expression of the *ls* mutation in shoots and developing seeds was also examined on a *lh*^{i} genetic background by isolating the *lslh*^{i} double-mutant. A cross between lines NGB5843 (*Lslh*^{i}) and 181 (*lsLh*) produced all tall F_1 plants. The F_2 progeny segregated to give 103 tall (*Ls-Lh-*), 41 dwarf (*lslsLh-* and *Ls-lh*^{i}*lh*^{i}) and 1

extremely short plant, possessing the "nana" phenotype. The genotype of the extremely short plant was subsequently shown to be $ls ls lh^i lh^i$ by test-crossing to each parent. The observed ratio of tall:dwarf:nana in the F_2 progeny did not fit the expected ratio of 9:6:1 ($P<0.01$). Since there is no evidence to suggest that the *ls* and *lh* loci are linked (Chapter 6, Reid 1986a), the observed deficiency in the dwarf and nana phenotypic classes probably results from zygotic selection against homozygous lh^i seeds (Tab. 4, Chapter 4).

Plants of genotype $ls lh^i$ resemble *ls lh* (Reid 1986a) and $le^{5839} lh$ (Chapter 6) plants previously isolated. This result confirms GA quantifications which suggest that both the *ls* and lh^i alleles are "leaky" (Chapters 3,6) in regard to GA-biosynthesis in shoots. Plants of genotype $ls lh^i$ have been assigned Hobart line number 236.

A valid comparison between seed yield from self-pollinated $Ls lh^i$ and $ls lh^i$ plants cannot be made because of the large difference in plant stature (Fig. 1). Consequently, the development of $Ls\text{-}lh^i lh^i$ and $ls ls lh^i lh^i$ seeds was compared by allowing heterozygous $Ls ls lh^i lh^i$ plants to self-pollinate in day (18h) and night (6h) temperatures of 15°C and 10°C, respectively. After recording seed weights, the genotypes of the developing seeds were determined by sowing and scoring for plant stature (dwarf: genotype $Ls\text{-}lh^i lh^i$ or nana: genotype $ls ls lh^i lh^i$). No significant difference in final seed weight was found between seeds of either genotype ($P>0.90$, Tab. 2). In addition, the observed proportion of dwarf and nana F_2 progeny did not differ significantly from the expected ratio of 3:1 ($P>0.70$, Tab. 2). Thus, the development of $ls lh^i$ seeds did not differ from that of $Ls lh^i$ seeds on the same maternal plant.

Gibberellin levels in young seeds.

GA levels were determined in young *ls* and *Ls* (wild-type) seeds harvested 6 days after anthesis. In addition, the expression of the le^{5839} allele (Jolly et al. 1987) in 6 day old seeds was examined since another allele at this locus, *le* (Ross and Reid 1991), has been reported to reduce GA levels in young seeds (Santes et al. 1993). Wild-type seeds and seeds of genotype *ls* and le^{5839} all contained similar levels of GA_1 at 6 days of age (Tab. 3). Seeds of genotype *ls* contained reduced levels of GA_{20} and GA_{29} compared with *Ls* (wild-type) seeds at 6 days of age (Tab. 3). However, le^{5839} seeds contained higher levels of GA_{20} and GA_{29} than *Le* (wild-

type) seeds (Tab. 3). Since GA_1 levels were not decreased, this may result from expression of the *le*^{5839} mutation in the developing pod (see Santes et al. 1993), and transport of the elevated GA_{20} and GA_{29} levels to the developing seed.

To compare the relative effects of the *ls* and *lh*^{i} mutations in young seeds (before contact point) grown under the same conditions, *ls*, *lh*^{i} and wild-type seeds were harvested at ages ranging between 5 and 11 days after anthesis. In agreement with the results of Garcia-Martinez et al (1991) and Santes et al. (1993), GA_1 and GA_3 were detected in young wild-type seeds (Tab. 4). The levels of both these GAs peaked at 7 days after anthesis (seed weight ca. 2mg) and then declined, with GA_3 levels decreasing faster than GA_1 levels (Tab. 4). This may explain the presence of GA_1, but the absence of detectable levels of GA_3, in larger seeds (Tab. 3) since at 9 days after anthesis (seed weight ca. 7mg) GA_1 levels were approximately 9 times higher than GA_3 levels in wild-type seeds (Tab. 4). The levels of GA_8, GA_{20} and GA_{29} also fell during the period examined (5 to 11 days after anthesis). Consequently, during the development of wild-type pea seeds, GA_{20} and GA_{29} levels peak at least twice (Ingram and Browning 1979). The first peak is at, or before, ca. 5 days of age, and the second peak (to much higher levels) is after contact point (Chapter 4, Frydman et al. 1974, Gaskin et al. 1985).

The average seed weight at harvest was similar for wild-type and *ls* seeds at all ages examined (Tab. 4). By contrast, *lh*^{i} seeds weighed less than wild-type seeds at 7,9 and 11 days after anthesis. Furthermore, this difference increased with seed age (Tab. 4), consistent with the reduced weight of *lh*^{i} seeds (compared with wild-type seeds) throughout seed development up to, and including, seed desiccation (Chapter 4). Since GA levels change rapidly in young wild-type seeds (Tab. 4, Garcia-Martinez et al. 1991), a comparison between GA levels in wild-type and *lh*^{i} seeds of a similar developmental age may be more valid than a comparison between seeds of the same chronological age. For example, 9 day old *lh*^{i} seeds (2.48mg) should be compared with 7 day old wild-type seeds (2.27mg), rather than 9 day old wild-type seeds (7.30mg) (Tab. 4).

Wild-type pods contained fewer apparently healthy seeds than *lh*^{i} pods (P<0.001) at 7 days after anthesis (Tab. 4, Chapter 4). However, between 7 and 11 days after anthesis, significant (P<0.001) abortion of *lh*^{i} seeds occurred (Tab. 4), consistent with the reduced yield of *lh*^{i} plants compared with wild-type plants (Tab. 1, Chapter 4).

In agreement with the results in Chapter 4 and Tab. 3, the *ls* and *lh*^{i} mutations were found to have different effects on GA-biosynthesis in developing seeds (Tab. 4). Compared with wild-type seeds of a similar fresh weight at harvest, *lh*^{i} seeds possessed reduced levels of GA_1,GA_3, GA_8, and to a lesser extent, GA_{20} and GA_{29} (Tab. 4). By contrast, the *ls* mutation did not have a marked effect on GA_1 and GA_3 levels, but did reduce the levels of GA_8, GA_{20} and GA_{29} compared with wild-type seeds (Tab. 4).

These results are consistent with a biological role for GA_1 and/or GA_3, but not for GA_8, GA_{20} or GA_{29}, early in seed development. However, since both the *ls* and *lh*^{i} mutations are thought to act early in the GA-biosynthetic pathway (Ingram and Reid 1987a, see later), the differential effects of these mutations on GA levels in young seeds was unexpected. Young pea seeds are composed of three different tissues: testa, embryo and liquid endosperm. The genotypes of the embryo (2N) and endosperm (3N) are determined by both the maternal and pollen genotypes. By contrast, the testa is solely of maternal origin and its genotype is therefore independent of the pollen genotype. In order to further investigate the expression of the *ls* mutation in the embryo, endosperm and testa of young seeds, GA levels in seeds developing on homozygous *ls* plants fertilized with either *Ls* (wild-type) or *ls* pollen were examined. If the reduced GA_8, GA_{20} and GA_{29} levels in young *ls* seeds (Tab. 4) results from expression of the *ls* mutation in the embryo/endosperm, fertilization with *Ls* (wild-type) pollen should increase the levels of these GAs in heterozygous *Lsls* seeds. However, regardless of the pollen genotype, the levels of GA_1, GA_3, GA_8, GA_{20} and GA_{29} in seeds developing on *ls* plants were very similar (Tab. 5). This result suggests that the reduced GA_{20} and GA_{29} levels in young *ls* seeds are due to reduced levels of the these GAs in *ls* testae compared with *Ls* (wild-type) testae (Tabs. 4,5). The different effects of the *ls* and *lh*^{i} mutations on GA_{20} and GA_{29} levels in young seeds can then be explained if the *lh*^{i} mutation does not have a strong effect in developing testae, and a significant proportion of the GA_{20} and GA_{29} present in young seeds is produced in this tissue. This hypothesis is consistent with the presence of GA_{20} in the embryo, endosperm and testa of young pea seeds (Ingram and Browning 1979) and GA-biosynthesis from GA_{12}-aldehyde in the testae of maturing seeds (Zhu et al. 1991). This hypothesis is also consistent with the expression of the *lh*^{i} mutation in the embryo/endosperm of developing seeds and the restoration of normal GA_1 and GA_{20} levels in *Lhlh*^{i} seeds before contact point (when

the liquid endosperm is still present) by fertilizing *lh*^{i} plants with *Lh* (wild-type pollen) (Chapter 4).

The very similar GA_1 levels in seeds developing on homozygous *ls* plants fertilized with either *Ls* (wild-type) or *ls* pollen also suggests that the slight decrease in GA_1 levels in homozygous *ls* seeds compared with *Ls* (wild-type) seeds (Tab. 4) may result from reduced GA_1 levels in *ls* testae. This may also explain the reduced GA_8 levels in *lsls* seeds compared with *LsLs* (wild-type) seeds (Tab. 4), but not compared to *Lsls* seeds (Tab. 5), if GA_8 is also produced from GA_1 in young testae.

Gibberellin levels in shoots and seeds at contact point.

Since GA_{20} and GA_{29} levels peak relatively late in seed development (Chapter 4, Frydman et al. 1974, Gaskin et al. 1985), the expression of the *ls* mutation in older seeds was investigated. Intact seeds developing on self-pollinated *ls* plants were found to possess dramatically reduced levels of GA_{19}, GA_{20} and GA_{29} compared with seeds developing on *Ls* (wild-type) plants when harvested at contact point (the first day no liquid endosperm remained) (Tab. 6). No evidence was found for the presence of GA_1 in seeds at contact point, in agreement with the results of other workers (Gaskin et al. 1985). Young shoots of genotype *ls* were also found to possess reduced levels of GA_1, GA_{19}, GA_{20} and GA_{29} compared with *Ls* (wild-type) shoots (Tab. 6). These results are consistent with previous bioassay results (Reid and Potts 1986), and the results in Chapter 6, and support the hypothesis that *ls* reduces GA-biosynthesis in shoots and developing seeds prior to the formation of GA_{19}.

Since intact pea seeds at contact point are composed of both testa and embryo tissue, the expression and interaction of the *ls* and *lh*^{i} alleles in isolated developing embryos was examined. Embryos of genotype *lh*^{i}, *ls* and *lslh*^{i} all contained reduced levels of GA_{20} and GA_{29} compared with wild-type embryos at contact point (Tab. 7). The 97% reduction in GA_{20} and GA_{29} levels in maturing *ls* embryos (at contact point) is consistent with the reduction in the levels of these GAs found in maturing intact *ls* seeds compared with *Ls* seeds (Tab. 6). Embryos of genotype *lslh*^{i} were found to possess even less GA_{20} and GA_{29} than *lh*^{i} and *ls* embryos at contact point (Tab. 7). Thus, the effects of the *ls* and *lh*^{i} alleles (reducing GA levels) were found to be cumulative in embryos at this developmental stage.

Site of action of the ls *gene.*

Cell-free enzyme preparations from developing seeds of a range of species have been used to investigate GA-biosynthesis (e.g. Albone et al. 1990, Coolbaugh and Moore 1971a,b, Graebe et al. 1972, Hedden and Phinney 1979). In particular, cell-free systems from developing pea seeds have proved extremely useful in defining the GA-biosynthetic pathway in peas (e.g. Kamiya and Graebe 1983). By contrast, the preparation of cell-free systems from pea shoots is much more difficult (Coolbaugh et al. 1973, Groβelindemann et al. 1992). Since the *ls* mutation reduces GA levels in developing embryos (Tab. 7), cell-free systems from seeds at contact point were used to investigate the site of action of the *ls* gene. Exogenous application of various GA-precursors have suggested that *ls* may reduce GA-biosynthesis prior to the formation of *ent*-kaurene (Ingram and Reid 1987a). In addition, since there is no evidence of reduced carotenoid levels in *ls* plants compared to *Ls* (wild-type) plants, the *ls* gene may act after GGPP. Preliminary results therefore suggested that the *ls* mutation may reduce *ent*-kaurene synthetase A or B activity (Ingram and Reid 1987a).

Inclusion of ^{14}C labelled MVA in the incubation medium prepared from *Ls* (wild-type) and *ls* seeds revealed that *ls* extracts produced markedly less ^{14}C-*ent*-kaurene compared with *Ls* (wild-type) extracts, confirming that *ls* acts prior to *ent*--kaurene (Tab. 8). The metabolism of ^{3}H-labelled GGPP (substrate for *ent*-kaurene synthetase A) and of CPP (substrate for *ent*-kaurene synthetase B) (see Duncan and West 1981, Coolbaugh 1983, Graebe 1987) was then compared in extracts from *Ls* (wild-type) and *ls* seeds. When ^{3}H-CPP was used as a substrate extracts from *Ls* (wild-type) and *ls* seeds produced similar amounts of ^{3}H-*ent*-kaurene, and there may even be a small increase in *ls* extracts (Tab. 8). By contrast, the conversion of ^{3}H-GGPP into ^{3}H-*ent*-kaurene was greater in extracts from *Ls* (wild-type) seeds compared with extracts from *ls* seeds (Tab. 8). For feeds of all three substrates (^{14}C-MVA, ^{3}H-CPP and ^{3}H-GGPP) similar results were obtained with incubations of several different enzyme preparations from two separate plantings (data not shown). These results suggest that the *ls* gene acts prior to the formation of *ent*-kaurene and reduces *ent*-kaurene synthetase A activity (Fig. 2). This is consistent with the dramatically reduced GA_{20} and GA_{29} levels of *ls* embryos compared with *Ls* (wild-type) embryos (Tab. 7) harvested in the same experiment as the embryos used to prepare cell-free systems.

Discussion

The *lh*^{i} mutation decreases seed weight and increases seed abortion compared with wild-type seeds by affecting the development of the embryo and/or endosperm (Tabs. 1,2, Chapters 3,4). By contrast, the *ls* mutation does not have a detectable effect on seed development (Tabs. 1,2). Unlike the *lh*^{i} mutation, which markedly reduces endogenous GA_1 and GA_3 levels in young seeds a few days after anthesis (Tab. 4, Chapter 4), the *ls* mutation is not expressed in the embryo/endosperm of young seeds (Tabs. 3,4,5). However, both the *lh*^{i} and *ls* mutations reduce GA_{20} and GA_{29} levels in seeds and embryos at contact point (Tabs. 6,7).

Taken together, these results suggest that GA_1 and/or GA_3 may have an important role in seed development in the first few days after fertilization, and that GA_{20} and GA_{29} have no physiological role in young seeds or seeds at contact point. This hypothesis is consistent with several observations. (i) The *lh*^{i} mutation reduced both GA_1 and GA_3 levels, and seed weights, compared with *Lh* (wild-type) seeds as early as 7 days after anthesis (Tab. 4). (ii) Some *lh*^{i} seeds abort in the first 11 days after anthesis (Tab. 4) and *lh*^{i} seeds have been correctly identified as aborting as early as 12 days after anthesis (Chapter 4). (iii) When aborted seeds are examined at harvest, none appear to have reached contact point (Chapter 4). (iv) Since GA_1 and GA_3 are both active *per se* in increasing internode elongation in peas (Chapters 3,6, Ingram et al. 1984, Ross et al. 1989) and other species (Fujioka et al. 1988, Lenton et al. 1987, Phinney and Spray 1982, Spray et al. 1984), these GAs are perhaps the most likely candidates for biologically active GAs in seeds. However, GA_1 and GA_3 have only been detected in young seeds before contact point (Tab. 4, Chapter 4, Gaskin et al. 1985, Garcia-Martinez et al. 1991, Santes et al. 1993). (v) A role for GAs early in seed development, but not at contact point, may explain why application of GA-biosynthesis inhibitors, which markedly reduced GA levels late in seed development, did not have a detectable effect on seed weight (e.g. Garcia-Martinez et al. 1987). (vi) The *in vitro* growth of young *lh*^{i} embryos is increased by the inclusion of GA_1 in the incubation medium (Chapter 5). (vii) Finally, *le*^{5839} plants do not have either reduced GA_1 levels in young seeds (6 days after anthesis) or reduced seed yield compared with *Le* (wild-type) plants (Tabs. 1,2,3). This last finding contrasts with the report that the *le* allele reduces GA_1 and GA_3 levels in young seeds without affecting final seed yield (Santes et al. 1993). Although *Le* (wild-type) and *le* seeds

were harvested at the same chronological age, the weights of *le* seeds was less than that of *Le* (wild-type) seeds (Santes et al. 1993), suggesting that the seeds may have been of different developmental ages. Since GA levels have been shown to change rapidly during development (e.g. Tab. 4, Chapter 4, Frydman et al. 1974, Garcia-Martinez et al. 1991, Sponsel 1985) it may not be valid to directly compare the GA levels in these seeds.

Plants of genotype $ls lh^i$ were shown to possess the nana phenotype (extremely short internodes) and contain reduced GA levels in developing seeds compared with wild-type, *ls* and lh^i plants (Tab. 7). This result is consistent with a role for GAs early in seed development, but not at contact point, since embryos of genotype $ls lh^i$ contained even lower GA levels than lh^i embryos at contact point (Tab. 7), but did not differ in their development from lh^i seeds developing on the same parent plant (Tab. 2).

Since the *ls* mutation reduces GA levels in embryos at contact point, cell-free enzyme systems were used to investigate the site of action of the *ls* mutation. Incubations with radiolabelled MVA, GGPP and CPP demonstrated that the *ls* mutation reduces the conversion of GGPP to CPP (i.e. reduced *ent*-kaurene synthetase A activity) compared with *Ls* (wild-type) seeds (Fig. 2). This is consistent with results from application studies that suggested *ls* acts early in the GA-biosynthetic pathway, between GGPP and *ent*-kaurene (Ingram and Reid 1987a). In addition, the *gib1* mutant of tomato has been reported to reduce the conversion of GGPP to CPP (Bensen and Zeevaart 1990), while *ga1* in *Arabidopsis* (Zeevaart and Talon 1992) and an_1 in maize (Reid 1993) may also possibly block this step.

Comparison of GA levels in le^{5839} shoots (Chapter 6, Jolly et al. 1987) and young seeds (Tab. 3) demonstrates that the expression of the le^{5839} mutation is tissue-dependent since this mutation reduces GA_1 levels in shoots but not seeds, compared with *Le* (wild-type) plants. This result contrasts with the expression of the *le* allele, which appears to reduce GA_1 levels in vegetative tissues, pods and developing seeds (Ross et al. 1992, Smith et al. 1992, Santes et al. 1993). Comparison of GA levels in *ls* shoots (Tab. 6) and *Lsls* and *lsls* seeds (Tabs. 4,5,6,7) suggests that the *ls* mutation is expressed in shoots, young testae, and embryos and whole seeds at contact point. By contrast, the *ls* mutation does not appear to be expressed in the embryo/endosperm of young seeds (Tab. 5), suggesting that the expression of the *ls* mutation is also tissue-dependent. Tissue-dependent GA-

biosynthesis has now been identified at the *Na*, *Lh*, *Le* and *Ls* loci, suggesting that this phenomenon may be common, at least in peas. Two explanations are possible. Firstly, the genes controlling GA-biosynthesis may be duplicated throughout the genome, with different members of these gene families controlling GA-biosynthesis in different tissues. Hence, a mutation at a single locus would not affect GA-biosynthesis in all tissues. Secondly, discrete regions of the *Na*, *Lh*, *Le* and *Ls* loci may be involved in tissue-dependent expression of the encoded proteins, and this may determine GA-biosynthesis in different tissues. Therefore a change (e.g. base-pair substitution) to one region of the gene might not affect GA-biosynthesis in all tissues. This question cannot be resolved until molecular probes for these genes become available.

In conclusion, the *ls* mutation reduces the conversion of GGPP to CPP and consequently reduces GA_1 levels in shoots and GA_{20} and GA_{29} levels in seeds (5,6,7 and 9 days of age and at contact point) compared with wild-type plants. However, the *ls* mutation does not markedly reduce GA_1 or GA_3 levels in the embryo/endosperm of young seeds or seed yield compared with wild-type plants. By contrast, the lh^i mutation reduces GA_1 and GA_3 levels in young seeds and increases seed abortion compared with wild-type plants. These results suggest GA_1 and/or GA_3 have an important physiological role in young seeds (before contact point). By contrast, GA_{20} and GA_{29} appear to be biologically inactive in developing seeds, despite their relatively high levels in seeds at contact point.

Tab. 1. Seed yield from *LsLhLe* (wild-type, cv. Torsdag), *le*^{5839} (NGB5839), *lh*^{i} (NGB5843), *lh* (K511) and *ls* (L181) plants grown in a day/night temperature regime of 20°C and 15°C, respectively. Maximum of one pod allowed to develop at each reproductive node. Photoperiod 18h. Values are shown ±SE. n≥8 for all genotypes.

	Wild-type	*lh*^{i}	*lh*	*le*^{5839}	*ls*
Seeds	33.9±2.0	20.3±2.1	30.6±1.8	31.3±1.3	29.0±1.2
Pods	9.5±0.5	10.8±0.6	7.8±0.4	7.3±0.2	6.9±0.3
Reproductive nodes	12.8±1.2	16.6±0.6	8.5±0.3	7.9±0.2	7.1±0.2
Seeds per pod	3.60±0.19	1.88±0.18	3.96±0.17	4.30±0.16	4.24±0.17
Mean seed weight (mg)	287±4	201±13	300±10	296±5	274±3

Tab. 2. Observed segregation of the numbers and dry seed weights of the F_2 progeny (seeds) resulting from self-pollination of *Lhlh*^{i}, *Lsls*, *Lele*^{5839} and *Lslslh*^{i}*lh*^{i} F_1 plants in day (18h) and night (6h) temperatures of 15°C and 10°C, respectively.

Parent genotype (F_1)	Observed F_2 segregation					
	Dominant			Recessive		
	Genotype	No.	Weight (mg)	Genotype	No.	Weight (mg)
Lhlh^i	*Lh-*	63	242±5	*lh*^{i}*lh*^{i}	3[a]	192±37
Lsls	*Ls-*	56	251±4	*lsls*	23	247±4
Lele^{5839}	*Le-*	52	239±6	*le*^{5839}*le*^{5839}	19	237±7
Lsls lh^i*lh*^{i}	*Ls-lh*^{i}*lh*^{i}	118	201±6	*lslslh*^{i}*lh*^{i}	37	200±9

[a] not in agreement (P<0.001) with the expected *Lh-*:*lh*^{i}*lh*^{i} ratio of 3:1.

Tab. 3. Seed weights and endogenous GA levels in *lsLe* (L181), *Lsle*^{5839} (NGB5839) and *LsLe* (wild-type, cv. Torsdag) seeds harvested 6 days after anthesis. Seed weights represent the average of at least 165 seeds. No endogenous GA_3 was detected. Photoperiod 18h.

Genotype	Seed weight (mg.FW)	GA level (ng.(gFW^{-1}))		
		GA_1	GA_{20}	GA_{29}
Wild-type	7.8	18.2	17.9	12.2
ls	5.8	23.5	2.8	1.7
le^{5839}	5.9	26.2	42.9	63.4

Tab. 4. Harvest details for, and endogenous GA levels in, *LsLh* (wild-type, cv. Torsdag), *lh^{i}Ls* (NGB5843) and *Lhls* (L181) seeds harvested at 5,7,9 and 11 days after anthesis. Seed weights represent the average of at least 136 seeds. Photoperiod 18h.

Genotype	Age (days)	Healthy seeds per pod	Seed weight (mg.FW)	GA level (ng.(gFW^{-1}))				
				GA_1	GA_3	GA_8	GA_{20}	GA_{29}
Wild-type	5	5.78±0.10	0.75	15.9	15.3	18.8	29.7	44.3
	7	5.74±0.09	2.27	26.0	33.2	18.1	25.1	24.2
	9	5.69±0.10	7.30	11.0	1.3	12.8	12.6	14.3
	11	5.20±0.12	22.79	6.5	<1.8	11.3	4.6	15.4
lh^i	7	6.29±0.08	1.07	2.4	3.9	6.2	11.8	28.6
	9	5.40±0.11	2.48	2.5	<8.0	7.3	8.6	19.9
	11	4.95±0.11	6.54	1.3	1.0	4.6	5.8	15.1
ls	5	5.67±0.12	0.85	6.1	18.5	2.2	4.2	2.6
	7	5.38±0.13	2.19	13.1	29.0	1.9	4.6	1.4
	9	5.23±0.11	5.91	8.9	<2.3	3.9	-[a]	0.6

[a] no result obtained.

Tab. 5. Harvest details for, and endogenous GA levels in, 5 day old seeds from *ls* (L181) plants fertilized with pollen from either *Ls* (wild-type) or *ls* plants. Seed weights represent the average of at least 120 seeds. Photoperiod 18h.

Pollen genotype	Healthy seeds per pod	Seed weight (mg.FW)	GA level (ng.(gFW^{-1}))				
			GA_1	GA_3	GA_8	GA_{20}	GA_{29}
ls	5.48±0.11	1.04	7.0	36.9	1.7	3.6	1.5
Ls	5.22±0.19	1.40	10.3	34.1	1.9	3.8	2.1

Tab. 6. Gibberellin levels (ng.(gFW^{-1})) in shoots harvested 21 days after sowing and seeds (including testa) harvested at contact point (the first day no liquid endosperm remained) from *Ls* (wild-type, cv. Torsdag) and *ls* (L181) plants. Seed weights represent the average of at least 13 seeds. Photoperiod 18h.

	Shoots				Seeds			
	GA_1	GA_{19}	GA_{20}	GA_{29}	GA_{19}	GA_{20}	GA_{29}	Weight (g)
Ls	2.6	3.3	2.1	1.8	97	2925	467	0.235
ls	0.2	<1.0	0.2	<0.5	<14	45	-[a]	0.258

[a] no result obtained.

Tab. 7. Gibberellin levels (ng.(gFW^{-1})) in embryos (testa removed) harvested at contact point from *LsLh* (wild-type, cv. Torsdag), *lsLh* (L181), *Lslh^{i}* (NGB5843) and *lslh^{i}* (L236) plants. Photoperiod 18h.

Embryo genotype	GA level (ng.(gFW^{-1}))	
	GA_{20}	GA_{29}
Wild-type	5603	4624
ls	72	145
lh^{i}	29	93
lslh^{i}	13	62

Tab. 8. Accumulation of radiolabelled *ent*-kaurene produced when cell-free enzyme preparations from *Ls* (wild-type) and *ls* embryos were incubated with ^{14}C-MVA, ^{3}H-GGPP or ^{3}H-CPP. Similar results were obtained with incubations of several different enzyme preparations from two separate plantings (data not shown).

Substrate	Radio-labelled *ent*-kaurene (DPM)	
	Ls (wild-type)	*ls*
^{14}C-MVA	9141	660
^{3}H-GGPP	333	86
^{3}H-CPP	1459	1989

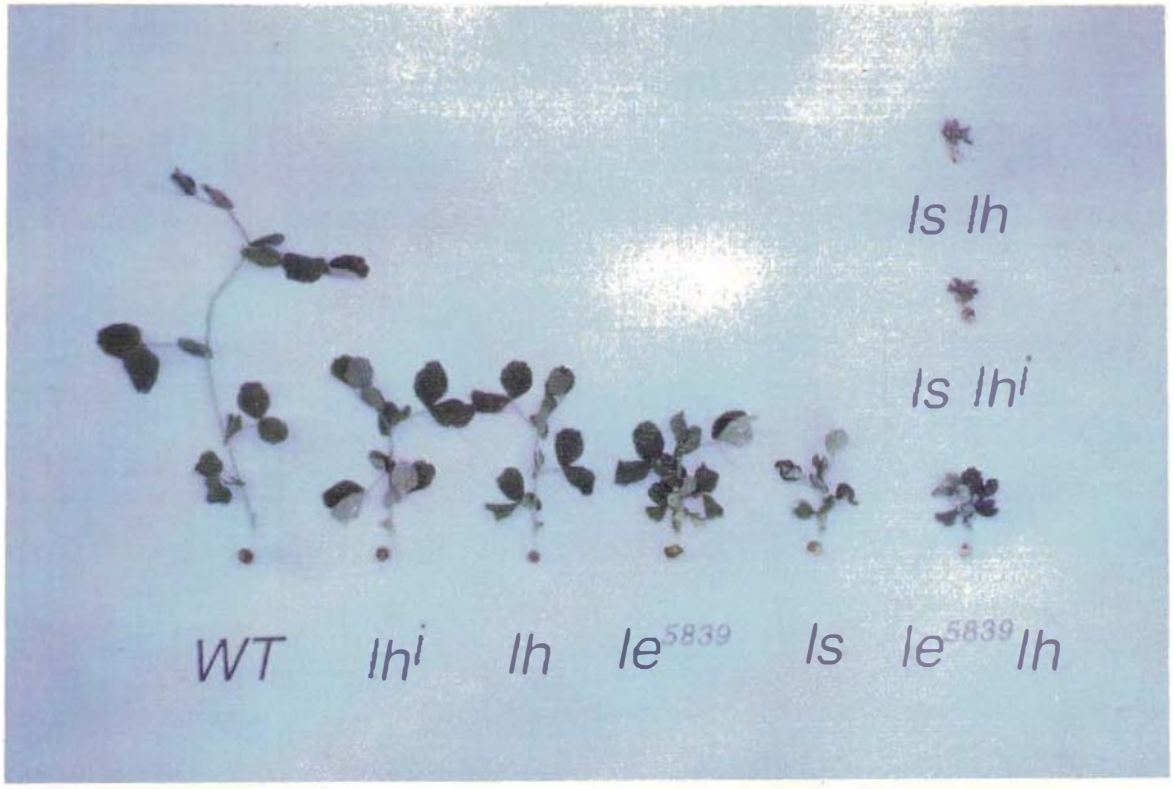


Fig. 1. Wild-type plants (WT) and plants homozygous for the mutations indicated at ca. 3 weeks after sowing.

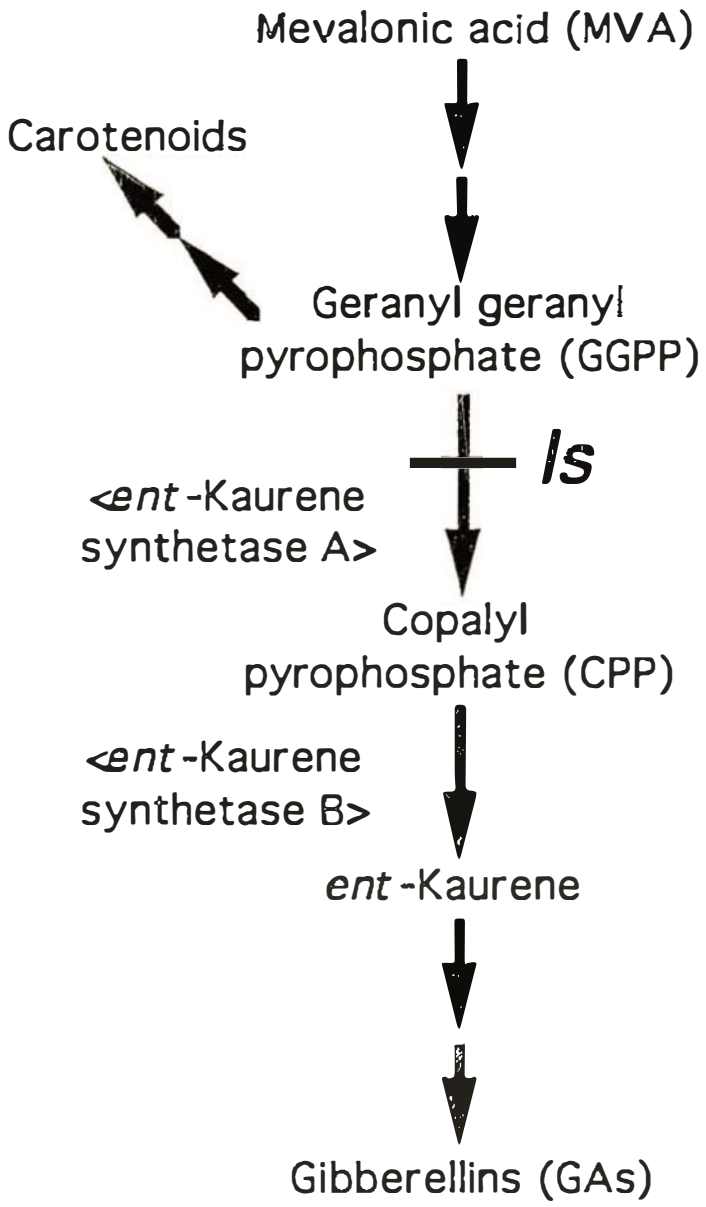


Fig. 2. The GA-biosynthetic pathway from mevalonic acid (MVA). The *ls* mutation reduces the conversion of geranyl geranyl pyrophosphate (GGPP) to copalyl pyrophosphate (CPP).

Chapter 9

General discussion

A new GA-metabolism mutant, lh^i, has been fully characterised and used to investigate the role of the GAs in seed development of the garden pea (*Pisum sativum* L.). The lh^i mutation is allelic with the previously identified *lh* mutation, and reduces endogenous GA_1 levels in developing shoots resulting in a dwarf phenotype compared with wild-type plants (Chapter 3). The lh^i mutation is also expressed in the developing embryo and endosperm of developing seeds and reduces endogenous GA levels, in both young and maturing seeds, leading to a number of phenotypic effects (Chapters 3,4,5). Homozygous lh^i seeds weigh less, develop more slowly, possess reduced sink strength and are more likely to abort during development, compared with seeds possessing higher endogenous GA levels. Consequently self-pollinated lh^i plants have reduced seed yield and delayed senescence compared with self-pollinated wild-type plants. Fertilizing lh^i plants with *Lh* (wild-type) pollen results in increased seed GA levels and normal seed development. This result suggests that one copy of the *Lh* (wild-type) allele in developing embryo/endosperm tissue can overcome the effects of the lh^i mutation on seed development (Chapter 4). When homozygous lh^i seeds (GA-deficient) develop alongside *Lh-* seeds (normal GA levels) zygotic selection favours the development of *Lh-* seeds over lh^ilh^i seeds. By contrast, the lh^i mutation does not appear to affect pollen production or pollen-tube growth on heterozygous $Lhlh^i$ plants (Chapter 4). These results, and the response of lh^i seeds to altered source supply (Chapter 5), demonstrate that the lh^i mutation increases seed abortion and reduces seed weight solely by altering the development of the embryo and/or endosperm.

The expression of another GA-metabolism dwarf mutant, *ls*, has also been examined in developing seeds. Homozygous *ls* seeds do not differ in their development compared with wild-type seeds. However, the *ls* mutation is expressed in young testae of developing seeds (a few days after anthesis) and embryos (including the cotyledons) at contact point (the first day the liquid endosperm disappears). By contrast, the *ls* mutation does not appear to have a marked effect on endogenous GA levels in the embryo or endosperm tissue of young seeds prior to

contact point. Comparison of the expression and effects on seed development of the *ls* and *lh*^{i} mutations therefore suggests that GAs, possibly GA_1 and/or GA_3, are required early in pea seed development. Furthermore, GA_1 appears to have a physiological role in young embryos (harvested before contact point) since *in vitro* culture with GA_1 increases the growth of *lh*^{i} embryos. By contrast, the relatively high GA levels that accumulate in maturing seeds do not appear to have a physiological role. The expression of the *ls* mutation in seeds at contact point has also allowed the site of action in the GA-biosynthetic pathway of this mutant to be determined. The *Ls* gene appears to encode a protein (possibly the *ent*-kaurene synthetase A enzyme) involved in the conversion of geranyl geranyl pyrophosphate to copalyl pyrophosphate (CPP).

The interaction of the *lh*^{l} mutation with other genes, that affect either seed development or internode elongation, has also been investigated. However, none of these studies have confirmed a role for GAs in seed development, although the *sln* mutation may do so in the future (Chapter 7). The interaction of the *lh* and *le*^{5839} mutations has been used to establish that the *Lh* locus is linked to the *Le* locus at a distance of 4.9±1.2 cM on chromosome 4 (Chapter 6). Two new mutant phenotypes that may result from intragenic crossovers within the *Lh* locus have also been characterised (Chapter 6). The results obtained from these mutants support a role for the GAs in seed development, but the origin of these genotypes has not yet been confirmed.

Although a role for the GAs in internode elongation in peas and other species is well established (Fujioka et al. 1988, Lenton et al. 1987, Reid and Ross 1993), and GAs may also regulate leaf growth (e.g. sweet peas, Ross et al. 1993) and pod elongation (e.g. peas, Garcia-Martinez et al. 1991), a role for the GAs in seed development of any species has not been established. In fact, with the exception of ABA and seed dormancy (Hilhorst and Karssen 1992), and possibly desiccation tolerance, none of the plant hormones have been shown to regulate seed development (Chapter 1). The results presented in this thesis therefore represent a significant advance in the understanding of seed development, particularly in regard to the possible role of plant hormones in this process. A considerable amount of research has focused on the later stages of seed development when seeds accumulate storage and LEA proteins, become desiccation tolerant and develop primary dormancy (e.g. Hilhorst and Karssen 1992, Quatrano 1987, Galau et al. 1991, Dure 1993). While the

biochemical and molecular basis of storage/LEA protein accumulation and desiccation tolerance has been studied in detail, the possible hormonal (i.e. ABA) control of this process is less well understood (Chapter 1). By contrast, this thesis presents results at the whole plant (i.e. seed development) and biochemical (i.e. GA quantifications) levels that provide a foundation for detailed analysis of the role of the GAs in the early cellular and molecular events of seed development. Such a foundation is of value if the results obtained from molecular studies, for example, are to be put in context of the development of the whole plant *in vivo*.

Previous studies, using chemical or genetic means to attempt to reduce GA levels in developing seeds, have failed to demonstrate a clear and important role for the GAs in seed development (Chapter 1). However, experiments using chemical inhibitors of GA-biosynthesis (e.g. paclobutrazol) have concentrated on reducing GA levels late in seed development when, at least in peas, GAs do not appear to have a physiological role. The *ga1* mutation of *Arabidopsis* has not been shown to act in developing seeds so it is difficult to interpret results obtained from this mutant regarding seed development. Similarly, the *gib1* mutation of tomato has not been shown to reduce GA levels in developing seeds, although *gib1gib1* seeds are lighter than *Gib1gib1* seeds developing on *gib1gib1* maternal plants (Groot et al. 1987). This result is consistent with a physiological role for the GAs in tomato seed development, but until the severity of the *gib1* mutation in isolated developing seeds is determined, the extent of this role cannot be established.

One question arising from the results presented in this thesis is why do pea seeds accumulate such high levels of GAs (e.g. GA_{20} and GA_{29}) if these GAs do not have a physiological role in seed development? A significant role during subsequent germination seems unlikely for two reasons. Firstly, wild-type pea seeds only possess relatively low levels of GA_{20}, the precursor of the biologically active GA_1 (Ingram et al. 1984), at harvest (Sponsel 1983, Ross et al. 1993). Secondly, if GA-biosynthesis inhibitors or genetic means (e.g. *ls*) are used to reduce GA levels during the later stages of pea seed development, germination does not appear to be affected (Chapter 4, Garcia-Martinez et al. 1987). The simplest explanation may be that the GAs present in maturing seeds of the garden pea (and possibly other species) are just secondary metabolites with no physiological function (Reid 1986b). If this is indeed the case, the high ABA, auxin and cytokinin levels that occur during the development of pea seeds, and seeds of other species (Chapter 1), do not necessarily imply a physiological role

for these hormones in seed development. However, such correlations between peaks in hormone levels and rapid or maximal seed growth rates have often been used to support a biological role for the plant hormones (see Chapter 1), demonstrating that further research is essential.

Although the results presented in this thesis support an important role for the GAs early in seed development, the nature of this role has not been established. GAs appear to regulate both seed weight and seed sink strength, but the cellular and molecular basis of this regulation is not known. Since the lh^i mutation affects the development of seeds that do not abort, GAs do not act merely as a crude switch determining seed survival. Instead, GAs appear to regulate the early stages of seed development so that final seed size is decreased and the probability of seed abortion is increased for seeds with reduced GA levels in the first few days after anthesis. The reason why some lh^i seeds survive while others abort is not known at present. However, reduced availability of assimilates does not appear to be the limiting factor since marked seed abortion still occurs when sinks competing with the developing fruit are removed (Chapter 5). One possibility is that the GA levels in young lh^i seeds are close to the threshold between seed survival and seed abortion, so that small environmental influences may determine the fate of individual seeds.

The two best understood physiological processes known to be controlled by the GAs are reserve mobilization during seed germination and internode elongation. Extensive research has focused on the action at the molecular level of the GAs in release of hydrolytic and proteolytic enzymes in germinating seeds (Jacobson and Chandler 1987, Lenton and Appleford 1991, Akazawa et al. 1991). The transduction chain between GA production and the regulation during germination of genes encoding various proteins, such as α-amylase, has been partially elucidated (e.g. Bush et al. 1991, Gubler and Jacobson 1992). In vegetative tissues, GAs gave been shown to regulate internode elongation in a range of species including maize (Fujioka et al. 1988), wheat (Lenton et al. 1987) and peas (Reid and Ross 1993). In elongating pea internodes GAs promote both the division and elongation of epidermal and cortical cells (Reid et al. 1983). However, very little is known regarding GA action at the molecular level, although a GA-induced gene from vegetative tissues of tomato has been identified (Shi et al. 1992). It is not known how many, if any, components of the transduction pathways of these two physiological processes (reserve mobilization and internode elongation) may have in common. Although GA action in developing

pea seeds presumably also involves a transduction pathway (and a GA receptor), this pathway may be partially, or completely, different from the pathway involved in GA action in germinating seeds or vegetative tissues.

Although GAs act in germinating seeds and elongating internodes by modifying the expression of a particular developmental program (e.g. α-amylase synthesis or cell elongation), they do not regulate the the cellular differentiation of the organs themselves. This may not be the case in developing seeds. Since GAs appear to act early in seed development, but after fertilization, they may influence either the cellular differentiation of the developing zygote, or the growth of the resulting embryo. Young developing seeds grow by increases in both cell numbers and cell sizes (Dure 1975). Since GAs act in pea internodes by stimulating cell division and elongation (Reid et al. 1983), GAs may may act by regulating one, or both, of these processes in developing seeds. However, if GAs do regulate cell division and expansion, many possible sites of action still exist. For example, GAs may regulate the expression of genes required for normal seed development. One possibility is that GAs down-regulate protease expression in young fertilized seeds (Granell et al. 1992). Alternatively, GAs may be involved in an active transport mechanism responsible for nutrient uptake to the developing seed (Stoddart 1983, Wareing and Seth 1967).

The exact site of action and synthesis of the GAs has also not been determined, although the results presented in this thesis suggest that GAs act, and are produced, in young embryo and/or endosperm tissue. In particular, the results from *in vitro* culture of *lh*^{1} embryos (Chapter 5) suggest that GA_1 may act in the young embryo. However, this result does not in itself demonstrate that GA_1 and/or GA_3 are produced in this tissue. If GA_1 and GA_3 are only produced in the liquid endosperm of pea seeds, and then move into the embryo, this would explain the absence of these GAs when the endosperm has disappeared (Chapter 4, Gaskin et al. 1985). Alternatively, GA_1 and GA_3 production may occur in the embryo and be developmentally regulated so that these GAs are not produced in older seeds. This question is difficult to answer at present since although the GA-biosynthetic pathway has been established in older pea seeds (Kamiya and Graebe 1983), the pathway has not been examined in young pea embryo or endosperm tissue.

The tissue-dependent biosynthesis of GAs and the biological activity of different GAs in young embryos could be investigated using *in vitro* embryo culture.

For example, incubation of embryos with GA_{20} with or without BX-112 may allow the possible conversion of GA_{20} to GA_1 to be examined, and the biological activity of GA_{20} to be determined (e.g. Sponsel and Reid 1992). The effects of GA_1 and GA_3 on increasing embryo growth could also be compared to determine the relative activity of these two GAs in this tissue. Experiments of this nature would be particularly useful since the preliminary results presented in Chapter 5 were obtained using a single concentration of GA_1, which may not be optimal for embryo growth.

The *lh*^{i} mutant, in conjunction with other GA-metabolism mutants, has been used to further investigate the relationship between internode elongation and endogenous GA levels. A clear quantitative relationship between the logarithm of the GA_1 concentration and internode lengths has been demonstrated for wild-type plants, 6 dwarf mutants and the extreme dwarf double mutant (genotype *ls*/*h*^{i}). These results are consistent with a regulatory role for GA_1 and confirm that GA_{20} does not possess biological activity in its own right (Chapters 3,6). In addition to its effects on seed development, the *lh*^{i} mutant also differs from other GA-metabolism mutants in its response to certain GA-biosynthetic inhibitors. Plants of genotype *lh*^{i}*lh*^{i} are more sensitive to paclobutrazol, uniconazol (structurally very similar to paclobutrazol), ancymidol and inabenfide than wild-type plants and *lh*, *ls* and *le*^{5839} plants (Chapter 3). All these inhibitors reduce the conversion of *ent*-kaurene to *ent*-kaurenoic acid (Graebe 1987, Hedden and Graebe 1985, Miki et al. 1990), suggesting that the *Lh* locus may directly effect this region of the GA-biosynthetic pathway. However, the site of action of the *lh* mutation has been suggested to be before GA_{12}-aldehyde formation (Ingram and Reid 1987a). Since the *lh*^{i} mutation is expressed in developing seeds, analysis of the metabolism of radiolabelled (e.g. ^{13}C) *ent*-kaurene and CPP in *lh*^{i} seeds *in vivo*, or in cell free extracts from *lh*^{i} seeds *in vitro*, may allow the site of action of *lh*^{i} to be determined.

Analysis of the *lh*^{i} mutation has opened up several potential avenues of future research. Firstly, isogenic lines segregating for the *Sln* and *sln* alleles on a *lh*^{i} genetic background are presently being generated (Chapter 7). In combination with GA quantifications, this will allow the interaction of the *lh*^{i} and *sln* mutations to be investigated in young seeds. Seeds of genotype *lh*^{i}*sln* may possess higher GA levels in the first few days after anthesis, increased seed weight and an increased chance of survival compared with *lh*^{i}*Sln* seeds (Chapter 7). If the GA levels in young *lh*^{i}*sln* seeds are intermediate between the levels in *lh*^{i}*Sln* and wild-type seeds, this may

allow a direct relationship between endogenous GA levels and seed development to be established. Such a relationship has already been shown for GA_1 levels and internode lengths in the GA-metabolism mutants and has been used to support a biological role for GA_1 in internode elongation (Chapters 3,6, Ross et al. 1989).

Another area of great potential is the analysis of the *ls* and lh^i mutations at the molecular level. Since a cDNA clone for the *Lh* gene or antibody to the *Lh* protein are not available, the prospects for molecular analysis of the *Lh* locus are limited at present. However, if the site of action of the lh^i mutation can be determined this deficiency may be rectified in one of several ways. Firstly, the *Lh* locus may be found to affect a step in the GA-biosynthetic pathway for which a cDNA probe or antibody is already available. The most likely possibility may be the *ga3* mutation of *Arabidopsis* since this mutation may also affect *ent*-kaurene metabolism (Zeevaart and Talon 1992). However, the site of action of the *ga3* mutation, or its sequence, has not yet been determined. A second alternative is that the protein encoded by the *Lh* locus may be isolated from wild-type plants. For example, if the *Lh* locus encodes an enzyme responsible for one or more steps in the GA-biosynthetic pathway an antibody to the wild-type enzyme could be used to analyse the expression of this protein in different tissues of wild-type, *lh* and lh^i plants. This antibody could also be used to isolate a cDNA clone from an expression system (e.g. *E. coli*) synthesizing the wild-type protein. The third possibility is that the linkage of the *Le* and *Lh* loci (Chapter 6) is used to isolate the *Lh* locus once the *Le* locus has been cloned. The enzyme responsible for the 3β-hydroxylation of GA_{20} to GA_1 may be isolated in the near future (Y. Kamiya, pers. com.), and this may allow a cDNA clone to be developed based on the amino-acid sequence of part, or all, of this protein. Chromosome walking or positional cloning (e.g. Giraudat et al. 1992) could then be used to isolate the *Lh* locus. However, these techniques may not be practical in *Pisum*, since they have proved difficult in *Arabidopsis*, and this species is easier to analyse at the molecular level (Koornneef 1991).

Analysis of the *Lh* locus would be of interest since the *lh* and lh^i alleles demonstrate tissue-dependent expression of GA-biosynthesis in shoots and developing seeds (Chapters 3,4,6). Consequently, comparison of the sequences of the *Lh*, lh^i and *lh* genes may reveal how GA-biosynthesis is regulated in different plant organs. Furthermore, the sequencing of the *Lh* locus in lines L237 (lh^f) and L238 (lh^s) may reveal whether these lines result from intragenic crossovers at the *Lh* locus

(Chapter 6).

The immediate prospects for molecular analysis of the *Ls* locus are considerably more promising. In *Arabidopsis*, the *GA1* protein is believed to act in the same region of the GA-biosynthetic pathway as the *Ls* protein of pea (Chapter 8, Zeevaart and Talon 1992). A cDNA clone for the *GA1* gene has been isolated using a *ga1* allele containing a large deletion and genomic subtraction (Sun et al. 1992). If *GA1* and *Ls* are homologous genes, it may be possible to analyse the expression of the *Ls* and *ls* genes using the *Arabidopsis GA1* cDNA clone. Alternatively, if the *GA1* and *Ls* genes are related, but not similar enough to use the *GA1* cDNA clone directly, the *GA1* clone could be used to screen a mRNA library from pea tissue and identify the pea *Ls* gene. The sequence of the *Ls* and *ls* genes could then be determined and compared, and this may allow the reduced GA levels in *ls* plants and control of the tissue-dependent expression of the *ls* mutation to be investigated at the molecular level. The existence of a clone for the *GA1* gene may also allow the *GA1* protein to be expressed in a bacterial culture, and this may allow the protein to be easily purified and a monoclonal antibody developed. An antibody to the *GA1* protein might then be used to examine the expression of the *Ls* and *ls* proteins in pea. This type of analysis would be a great use in examining the tissue and developmental regulation of GA-biosynthesis, a subject that has received relatively little attention in peas (e.g. Potts 1986) and other species (e.g. rice, Takahashi and Kobayashi 1991)

Finally, the lh^i mutation may allow deliberate screening for GA-metabolism and GA-response mutations in mutagenesis programs. Mutants have already proved extremely useful tools for examining plant development (e.g. Klee and Estelle 1991, Reid 1990), and additional GA-metabolism and GA-response mutants may allow the roles of the GAs in various aspects of plant development (e.g. seed development) to be further investigated. The lh^i mutation reduces GA levels in developing seeds and increases seed abortion. Consequently, an additional mutation that increased either GA response or GA levels, on a lh^i genetic background, would be expected to decrease seed abortion. Germinating lh^i seeds could be exposed to a mutagenic agent and the resulting M_1 plants sown and allowed to self-pollinate. The seeds developing on these M_1 plants would be the M_2 generation and approximately one quarter would be homozygous for any new mutations in addition to the lh^i allele. If any of the M_1 plants contained a single recessive mutation, henceforth named *a*, that increased either GA response (e.g. by increasing GA-reception) or GA levels (e.g. by reducing GA-catabolism) in developing seeds then the abortion of approximately one quarter of the

M_2 seeds (genotype lh^ilh^iaa) would be decreased relative to the remaining M_2 seeds (genotype lh^ilh^iA-). This would have two consequences. Firstly, if these M_1 plants (genotype lh^ilh^iAa) were grown under conditions that maximised the difference in seed yield between wild-type and lh^i plants (see Chapter 3), the yield of these M_1 plants would be increased relative to M_1 plants that did not contain the *a* mutation, due to reduced abortion of the lh^ilh^iaa seeds compared with lh^ilh^iA- seeds. Secondly, the ratio of lh^ilh^iA- to lh^ilh^iaa plants in the M_2 progeny form a single M_1 plant (genotype lh^ilh^iAa) would be disturbed since lh^ilh^iaa seeds would be less likely to abort than lh^ilh^iA- seeds. In addition, if the *a* mutation affected GA levels or GA-response in developing seeds, it might also be expressed in developing shoots and result in increased internode lengths of lh^ilh^iaa plants compared with lh^ilh^iA- plants in the M_2 generation. Consequently, the M_1 plants containing a mutation affecting seed development would be relatively easy to identify (before sowing the M_2 seeds) and fewer M_2 progeny would need to be planted in order to ensure the double recessive genotype (e.g. lh^ilh^iaa) was recovered, particularly if the largest M_2 seeds were sown. Plants of genotype lh^ilh^iaa would be expected to have increased yield, and might also be taller, compared with lh^ilh^iA- plants. If the *Lh* and *A* loci were not linked the *a* mutation could easily be transferred to a *Lh* (wild-type) genetic background by crossing to a wild-type plant (e.g. cv. Torsdag) and selecting the *LhLhaa* progeny in the F_2 or F_3 generations. Any fully, or partially, dominant mutations could also be identified in this manner.

Although the above discussion assumes that any new mutations do not occur at the *Lh* locus, a less likely possibility is that the *Lh* locus itself will be mutated again so that the new allele results in increased GA levels in developing seeds and decreased seed abortion. If this new allele was less severe than the lh^i allele in elongating internodes, it would be expected be at least partially dominant over the lh^i allele so that the M_1 plant would possess a taller phenotype than M_1 plants that were homozygous for lh^i. If a new *Lh* allele was not expressed in vegetative tissues it could still be identified by back-crossing to homozygous lh^i plants and examining the segregation for increased seed yield in the B_2 and B_3 generations. In any case, the chances of obtaining new mutations that affect GA levels or GA-response in developing seeds, and possibly shoots, should be considerably higher than if mutants from mutagenesis programs using wild-type (*Lh*) plants are examined, particularly since seed development may be affected by a wide range of mutations unrelated to GA action (Chapter 1).

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Appendix

Published papers containing results from this thesis

Swain, S.M. and Reid, J.B. (1992a) Internode length in *Pisum*. A new allele at the *Lh* locus. Physiol. Plant. **86**, 124-130.

Swain, S.M. and Reid, J.B. (1992b) Internode length in *Pisum*. Interaction of genes lh^i, *la* and cry^s. Pisum Gen. **24**, 83-85.

Swain, S.M., Reid, J.B., Ross, J.J. (1993) Seed development in *Pisum*. The lh^i allele reduces gibberellin levels in developing seeds, and increases seed abortion. Planta (in press).

Reid, J.B., Ross, J.J. and Swain, S.M. (1992) Internode length in *Pisum*. A new, slender mutant with elevated levels of C_{19} gibberellins. Planta **188**, 462-467.

Ross, J.J., Reid, J.B. and Swain S.M. (1993) Control of stem elongation by gibberellin A_1: evidence from genetic studies including the slender mutant, *sln*. Aust. J. Plant Physiol. (in press).