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Genetic and Hormonal Regulation of Stem Vascular Tissue Development In Flax (*Linum usitatissimum* L.)

by

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The long lasting rewards [of Science] come from the total involvement the work demands, the excitement of discovery and the camaraderie of those who share in the search ... [Science] involves going where your curiosity leads you, taking you into blind alleys and down side streets, often landing you far away from where you intended.

- David Suzuki

Abstract

Flax (*Linum usitatissimum*) has been grown as a source of oil and fibre for several millennia. Linseed flax varieties are cultivated for their seed (flaxseed) and for their seed oils (linseed oil), which are used for many industrial applications. The fibres that form in linseed straws are generally considered too poor to make these industrial uses economical. In order to better understand how the flax stem and its component fibres develop, the effects of two plant hormones, gibberellin (GA) and auxin (indole-3-acetic acid, IAA), on stem tissue properties were examined. GA levels were determined to be a particularly important factor in many aspects of linseed stem development, including bast fibre cellular elongation and expansion. The spatial, temporal and hormonal-responsive expression patterns of five genes putatively involved in GA response (*LuGAST1*), GA biosynthesis (*LuGA2ox1*, *LuGA3ox1*), IAA response (*LuIAA1*) and IAA transport (*LuPIN1*) were also examined. A potential association of increases in *LuGAST1* transcription with the cessation of bast fibre elongation and onset of secondary cell wall biosynthesis suggested a potential involvement of *LuGAST1* in these processes. Through a mutant screen of an elite linseed cultivar, a novel mutant was identified, *reduced fibre1* (*rdf*), which lacks a normal complement of fully differentiated fibres in its stem. The preliminary characterization of *rdf* shows that *RDF* function may be required for bast fibre elongation. Polymorphic simple sequence repeat (SSR) markers were also identified which will be useful in future for facilitating the cloning and sequencing of *RDF* through map-based cloning.

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List of Abbreviations

ΔΔCT	delta delta threshold cycle method
ABA	abscisic acid
ADP	adenosine diphosphate
AFLP	amplified fragment length polymorphism
AGP	arabinogalactan protein
ANOVA	analysis of variance
ARF	auxin response factor
ARF GAP	ADP ribosylation factor guanosine triphosphatase activating protein
ARF GEF	ADP ribosylation factor guanine exchange factor
bdl	*bodenlos*
BLAST	basic local alignment search tool
bp	base pair
cDNA	complimentary DNA
cM	centimorgan
CMF	cellulose microfibril
CPS	*ent*-copalyl diphosphate synthase
CRP	Cysteine Rich Protein
C_T	threshold cycle
DAP	days after planting
dlpf	*deficient lignified phloem fibre*
DNA	deoxyribonucleic acid

EST	expressed sequence tag
EMS	ethyl methyl sulfonate
EF1α	elongation factor 1-α
FT-IR	Fourier transform infrared spectroscopy
fra2	*fragile fiber 2*
GA	gibberellic acid
GA 2 ox	gibberellin 2 oxidase
GA 3 ox	gibberellin 3 oxidase
GAI	GA-INSENSITIVE
GGPP	geranyl geranyl diphosphate
goe	*gasa1 overexpressed*
ha	hectare
HD-ZIP	homeodomain-leucine zipper
IAA	indole-3-acetic acid
ifl	*interfascicular fiberless*
IPP	isopentyl diphosphate
irx	*irregular xylem*
KAO	kaurenoic acid oxidase
KO	*ent*-kaurene oxidase
KS	*ent*-kaurene synthase
Mbp	million base pairs
mp	*monopteros*
mRNA	messenger RNA

MT	microtubule
PBZ	paclobutrazol
PCR	polymerase chain reaction
qRTPCR	quantitative real time PCR
QTL	quantitative trait loci
RAPD	random amplification of polymorphic DNA
rdf	*reduced fibre1*
rev	*revolute*
RFLP	restriction fragment length polymorphism
RGA	REPRESSOR OF GA1-3
RNA	ribonucleic acid
RNAi	RNA interference
SEM	scanning electron microscopy
sfc	*scarface*
SSR	simple sequence repeat
TE	tracheary element
TriET	trinexapac-ethyl
Ubq	ubiquitin

1. Literature Review

Flax (*Linum usitatissimum* L.) has been grown for centuries as an important source of seed and fibre, with its use as a crop originating in the "Fertile Cresent" region of the Neolithic Middle East, geographically near the upper reaches of the Tigris and Euphrates rivers in present-day Turkey and Syria (Lev-Yadun et al., 2000). Distinct oilseed (linseed) and bast (phloem) fibre types of flax have been cultivated since flax was first domesticated as a crop, approximately 10,000 years ago (Zohary and Hopf, 2000; Allaby et al., 2005). Largely due to the increasing availability of alternative textile fibre sources - such as cotton - the cultivation of fibre flax has declined in the modern era (Vaisey-Genser and Morris, 2003). However, linseed flax continues to be an important crop, as linseed is valued for nutritional reasons (Agriculture and Agri-food Canada, 2007), while linseed oil can be used both as a nutritional supplement as well as for several industrial applications (Vaisey-Genser and Morris, 2003). Canada is the dominant international exporter of linseed, accounting for approximately 80% of the world market (Agriculture and Agri-food Canada, 2007).

The bast fibres that form in linseed flax stems are typically of an overall poorer quality than in fibre flax (Deyholos, 2006). Bast fibres are an impediment for linseed farmers, as they tend to bind to harvesting and processing equipment and are slow to decompose in the soil (Deyholos, 2006). Given that the straw is slow to biodegrade and costly to bale and store, in the past, farmers have tended to burn their straw in order to destroy it (Melitz, 2005; Deyholos, 2006). While flax

straw can be used for industrial applications, the market for waste straw is relatively low; at present, only 15-20% of waste straw is re-used in Canada (Ulrich, 2008). Increasing the marketability of linseed straw will require numerous improvements to the business relationships between all stakeholders in the flax fibre supply chain: producers, fibre processors and manufacturers of goods that make use of the fibre (for review see Melitz, 2005). A shortage of knowledge on the properties of flax fibres and how these properties could be modified has been cited as a limiting factor in industrial growth (Melitz, 2005). Several targets for the biotechnological manipulation of fibres have been identified: in particular, increasing the strength and hydrophobicity of fibres would make them useful for the manufacturing of composites; increasing the softness and pliability of fibres would make them more useful for linen production; and, increasing fibre yield and processing efficiency would be useful for all applications (Ebskamp, 2002).

This thesis describes work that was aimed towards furthering the current level of understanding of the hormonal and genetic basis for flax bast fibre differentiation. Before outlining the work completed in this thesis, background material will be presented here describing the properties of the tissues that form in the stem, and the basic patterns of stem growth. Following this, the specific properties of flax bast fibres will be outlined. As a significant portion of this thesis concerns the roles of two hormones, gibberellin (GA) and auxin (indole-3-acetic acid; IAA) as regulators of stem growth and of bast fibre properties in flax, the

biosynthesis and response pathways and the general roles played by these hormones in the differentiation of stem tissues will also be described.

1.1 The Cells and Tissues of the Plant Stem

The following outline of the different types of cells and tissues that form in plants is synthesized from more detailed overviews presented in plant anatomy textbooks (e.g. Esau, 1977; Raven et al., 1999; Evert, 2006).

The initial, primary growth of plants is derived from cellular divisions within the shoot and root apical meristems. Derivatives of the apical meristems give rise to three primary meristems: the protoderm, ground meristem and procambium. Derivatives of these primary meristems ultimately differentiate to form the epidermis, ground tissues and primary vascular tissues, respectively.

The ground tissue system consists of three basic types of tissues: parenchyma, collenchyma and schlerenchyma. Parenchyma cells are characteristically living at maturity, maintain a capability for cell division, and predominantly have been found to only form primary cell walls. Like parenchyma, collenchyma cells also remain living at maturity. In addition, collenchyma cells are typically quite elongated, and characteristically possess an unevenly thickened, non-lignified primary cell wall. Collenchyma tissues have sometimes been observed to form adjacent to the epidermis in stems and petioles of some plant species (e.g. the “strings” in celery stalks), and collenchyma is generally thought to provide a structural support for growing organs. Schlerenchyma cells are generally sub-divided into two basic types: fibres and sclereids. Both schlerenchyma cell types have thick, often lignified secondary

cell walls, and play a strengthening and supportive role in the plant body. Fibres are generally long and slender cells, commonly occurring in strands or bundles. Sclereids are more variable in shape, often are branched and, compared with fibres, are shorter in length.

The vascular tissues of plants are sub-divided into two basic tissues: xylem and phloem. Xylem is principally responsible for conducting water through plants, through tracheary elements (TEs). Two types of TEs have been identified in plants: tracheids and vessel elements. Both types of TEs are characteristically elongated cells that have secondary cell walls but lack protoplasts at maturity. Both types of cells also show pits in their secondary cell walls, but mature vessel elements, unlike tracheids, also show perforations at their apical and basal ends in which both the primary and secondary cell walls have been lost. The tracheid is the only type of TE found in most seedless vascular plants and gymnosperms, while both tracheids and vessel elements are found in most angiosperms. Phloem is principally responsible for conducting photosynthate through the plant, through sieve elements. Two types of sieve elements may be observed in plants - sieve cells and sieve tube members - which differ based on the types of plants that they are found in, and morphologically differ based on the structure of the sieve area at which the adjacent sieve elements are connected. Sieve cells, which form in gymnosperms, show narrow pores and relatively uniform sieve areas. Sieve tube elements, which form in angiosperms, show characteristic sieve plate regions where larger pores are concentrated. Unlike TEs, sieve elements have living protoplasts at maturity. However, mature sieve elements lose many of their

cellular organelles, including the nucleus. Thus, sieve elements are characteristically associated with specialized parenchyma cells, termed companion cells (for sieve tube elements) or albuminous cells (for sieve cells). Parenchyma is also associated with the xylem and is thought to serve as a sink for the storage of various substances. Fibres and sclereids can also be associated with both the xylem and phloem, providing support to the plant and sometimes allowing additional storage.

1.2 Secondary Growth of the Stem

While primary growth establishes the basic structure of the plant, further increases in stem and root thickness occur in some plants through secondary growth, which results from the activity of the lateral meristems: the vascular cambium and the cork cambium. Secondary growth is commonly observed in gymnosperms and most dicots, but is generally absent in monocots. In plants that undergo secondary growth, a cork cambium may form within the layers of ground tissues that are positioned towards the periphery of the stem or root – the cortex. The cork cambium gives rise to two tissues: cork, which forms a protective layer on the outside of the cork cambium, and phelloderm, a living parenchyma that forms to the inside of the cork cambium. Collectively, the cork, cork cambium and phelloderm are referred to as the periderm, and the periderm replaces the epidermis as the outer protective tissues of woody plants. The vascular cambium forms between the primary xylem and primary phloem and consists of two cell types: the fusiform initials and the ray initials. Periclinal divisions of the cambial initials give rise to secondary xylem and secondary phloem, with divisions of the

fusiform initials giving rise to axially-oriented TEs and sieve elements, while divisions of the ray initials give rise to vascular rays. In a typical stem that has completed its primary growth, the primary vascular tissues are arranged within discrete vascular bundles. Secondary growth, however, occurs in a continuous ring around the entire plant, giving rise to an extensive proliferation of vascular tissue. Primary phloem sieve elements are often crushed by the expanding secondary growth, while the primary xylem remains recognizable within xylem poles, located nearer the interior of the stem.

1.3 Properties of Flax Bast Fibres

Flax bast fibres form as a characteristic layer of schlerenchyma fibres, several cell layers thick, located on the outer periphery of the vascular bundles. Esau (1943) examined the ontogeny of bast fibre development in *Linum perenne*, a related species from the *Linum* genus that Esau considered to be an appropriate substitute for *L. usitatissimum*. Esau (1943) characterizes *Linum* bast fibres as a component of the protophloem, and traces their origin back to larger cells that form in a heterogeneous association with smaller sieve tube elements and companion cells. As the fibres continue to expand and undergo longitudinal growth, primary phloem sieve tubes and companion cells collapse and are destroyed, causing the fibre region to appear more homogenous (Esau, 1943; Figure 4). The outermost fibre cells are the first to expand and the first to develop secondary cell walls (Esau, 1943).

Flax bast fibres elongate through a combination of both intrusive growth – the slipping of cells between each other - and coordinate growth – the elongation

of the fibre in coordination with surrounding tissues (Anderson, 1927; Esau, 1943; Gorshkova et al., 2003; Ageeva et al., 2005). Recent investigations have demonstrated that the coordinate growth of flax bast fibres, which is observed in the first few millimetres below the stem apex, occurs through an intercalary (diffuse) growth, rather than tip growth (Ageeva et al., 2005). Fibre elongation is restricted to near the apex of the stem and is completed in a few days, whereas secondary cell wall biosynthesis occurs in lower portions of the stem (Gorshkova et al., 1996). A region termed the 'snap-point' can be identified in the growing stem. Above the snap-point the stem is somewhat flexible, whereas below the tensile strength of the stem increases. The snap-point marks the location at which bast fibre elongation ceases and fibre cell wall thickening begins (Gorshkova et al., 2003).

1.4 GA Biosynthesis and Response Pathways

The structure of the GA biosynthetic pathway is outlined in Figure 1-1. GA biosynthesis can be traced back to an isoprenoid, geranyl geranyl diphosphate (GGPP) (Hedden and Phillips, 2000). In addition to its role in GA biosynthesis, GGPP serves as a precursor for several other metabolic pathways, including carotenoid biosynthesis (reviewed in Hedden and Phillips, 2000; Yamaguchi, 2008). GGPP is formed from the linkage of two isopentyl diphosphate (IPP) units (Croteau et al., 2000). Two pathways have been identified in plastids that allow IPP to be synthesized from pyruvate: one pathway involves glyceraldehyde-3-phosphate, while the other involves the condensation of three molecules of acetyl-

CoA, the subsequent reduction of the product to form mevalonic acid, followed by the conversion of mevalonic acid to IPP (Croteau et al., 2000).

The plastid-localized enzyme *ent*-copalyl diphosphate synthase converts GGPP into, *ent*-copalyl diphosphate (CPS; Hedden and Phillips, 2000). Following this, e*nt*-kaurene synthase (KS) converts *ent*-copalyl diphosphate into *ent*-kaurene. *ent*-kaurene then undergoes a series of conversions, catalyzed by cytochrome P450 monoxygenases. The plant growth regulator paclobutrazol, which was used as a GA biosynthesis inhibitor in the work described in Chapters 2 and 3 of this dissertation, is similar in structure to *ent*-kaurene and inhibits the function of one of these enzymes, *ent*-kaurene oxidase (KO) (Rademacher, 2000). The effects of paclobutrazol treatment on stem development that have been described in other work will also be discussed in more detail later in this review. KO has been observed to be localized to the outer membrane of the plastid, while the next enzyme in the series, kaurenoic acid oxidase (KAO) is localized to the endoplasmic reticulum, where the rest of the cytochrome-P450-catalyzed conversions take place (reviewed in Yamaguchi, 2008).

Following several cytochrome P450-catalyzed steps, the GA precursor GA_{12} is synthesized (Hedden and Phillips, 2000). In its late stages, the GA pathway shows some variation in different plant and fungal species (Hedden and Phillips, 2000). A particular source of variation is imposed by the GA13-hydroxylase enzyme, which catalyzes a hydroxylation at the C-13 position of GA_{12}, forming GA_{53}. Both GA_{12} and GA_{53} can be converted into bioactive GAs, and thus two parallel pathways exist, depending on whether the 13-hydoxylated

(i.e. GA_{53}) or the non-13-hydroxylated (i.e. GA_{12}) precursor is used. Both pathways have been found in vegetative tissue, and the relative predominance of one over the other simply depends on the strength of the GA 13-hydroxylase activity in each plant species (Hedden and Phillips, 2000).

GA 20-oxidase catalyzes several further oxidations, ultimately converting GA_{12}/ GA_{53} into GA_9/ GA_{20}. GA 3-oxidase encodes as a 3β-hydroxylase which further converts GA_9/ GA_{20} into the bioactive GA_4/ GA_1 forms. In some plant and fungal species, alternative bioactive GAs, including GA_7 and GA_3, may also be produced (Hedden and Phillips, 2000)**.** How GA_7 and GA_3 are synthesized is not fully known, although they are thought to probably originate from GA_9/ GA_{20} and to be produced by side-reactions catalyzed by GA 3 oxidase (Hedden and Phillips, 2000). The final member of the GA biosynthetic pathway, GA 2-oxidase, encodes a 2β-hydroxylase that catalyzes that attachment of a hydroxyl at the C-2 position (Hedden and Phillips, 2000). 2β-hydroxylation converts the bioactive GA_4/ GA_1 forms of GA into the biologically-inactive GA_{34}/ GA_8 catabolites, and also converts GA_9/ GA_{20} into the biologically-inactive GA_{51}/GA_{29} catabolites (Hedden and Phillips, 2000).

The developmental and physiological responses to GA occur through a tightly regulated pathway. The rice *OsGID1* gene was demonstrated to encode a GA receptor, working as a component of the $SCF^{SLY1/GID2}$ E3 ubiquitin-ligase (Ueguchi-Tanaka et al., 2005; Sun, 2010). Putative *GID* orthologues have been identified in other plant species, including *Arabidopsis* (Nakajima et al., 2006). The binding of GA to its receptor is hypothesized to trigger the degradation of

repressor proteins – the DELLA proteins – through the ubiquitin-proteasome pathway (Figure 2; reviewed in Fleet and Sun, 2005; Sun, 2010). In *Arabidopsis*, five DELLA genes have been identified, with the *GA-INSENSITIVE* (*AtGAI*) and *REPRESSOR OF GA1-3* (*AtRGA*) DELLA genes acting as the major GA repressors during vegetative growth and flower production (Fleet and Sun, 2005).

1.5 IAA Response Pathways

Unlike GA, IAA biosynthesis does not follow a single pathway. Instead, five different biosynthetic pathways have been proposed to date, four of which depend on the amino acid tryptophan as a precursor (reviewed in Vanneste and Friml, 2009). Due to extensive functional redundancy, the structure and relative importance of each biosynthesis pathway have not been clearly defined (Vanneste and Friml, 2009). However, the mechanisms underlying auxin response are well-known (Figure 1-3). At low IAA concentrations, Aux/IAA proteins are proposed to dimerize with auxin response transcription factors (ARFs), blocking IAA-mediated gene expression (reviewed in Berleth et al., 2004; Vanneste and Friml, 2009). When IAA levels increase, IAA facilitates the binding of the $AtSCF^{AtTIR}$ E3 ubiquitin-ligase complex to Aux/IAA proteins, specifically between the Aux/IAA and the TIR1/AFB F-box protein subunit of SCF^{TIR} (Berleth et al., 2004; Vanneste and Friml, 2009). This triggers the ubiquitination and subsequent proteolysis of Aux/IAA proteins, alleviating the repression of ARFs by Aux/IAAs and thus triggering auxin responsive gene expression (Berleth et al., 2004). The GA and IAA response pathways are very similar, although one difference that has been noted between them is that IAA facilitates the binding of its receptor, TIR1,

to an Aux/IAA protein substrate without inducing any change in the SCF^{TIR} conformation, whereas the binding of GA to GID1 induces a conformational change that thereby facilitates the binding of the DELLA protein substrate to the $SCF^{SLY1/GID2}$ E3 ubiquitin-ligase (reviewed in Sun, 2010).

1.6 IAA Response Pathway Disruption and its Effects on Vascular Patterning

Mayer et al. (1991) conducted a genetic screen for *Arabidopsis* embryonic patterning mutants. Among the mutants that they identified was *monopteros* (*mp*), which fails to form normal basal seedling structures, such as a hypocotyl or root (Mayer et al., 1991; Berleth and Jürgens, 1993). It was further determined that vascular patterning was disrupted in *mp* seedlings, as the vasculature of its cotyledons and leaves is substantially reduced, while the tracheids within the vascular tissues that do form were improperly connected (Berleth and Jürgens, 1993; Przemeck et al., 1996). *MP* encodes an auxin response factor, *AtARF5* (Hardtke and Berleth, 1998), thus demonstrating a connection between the auxin response pathway and the regulation of vascular patterning. Seedlings and embryos of the *bodenlos* (*bdl*) mutant resemble *mp* mutants and likewise failed to form a root or hypocotyl (Hamann et al., 1999). *AtBODENLOS* encodes an Aux/IAA protein (AtIAA12) that specifically heterodimerizes with MP (Hamann et al., 2002). Downstream auxin-responsive targets whose expression is induced by MP have been shown to include a gene encoding a class III homeodomain leucine zipper, *AtHB8*, which has been implicated as a marker for and regulator of vascular tissue differentiation (Kang et al., 2003; Mattsson et al., 2003; Kang and Dengler, 2004; Scarpella et al., 2004).

Unlike *mp* and *bdl*, single mutations in other ARFs and Aux/IAAs tend to result in relatively weak or completely indistinct phenotypes, at least in *Arabidopsis* (Liscum and Reed, 2002; Guilfoyle and Hagen, 2007). *nph4*, which is mutated at the *AtARF7* gene and impaired in phototropic responses, is one of the few *Arabidopsis* ARF mutants that has been found to show a discernable phenotype (Liscum and Briggs, 1995; Watahiki and Yamamoto, 1997; Stowe-Evans et al., 1998; Harper et al., 2000). While *nph4* single mutants lack discernible vascular patterning abnormalities, the observation that the *mp* phenotype is enhanced in the *mp;nph4* double mutant background suggests that *NPH4* also has a role in vascular patterning (Hardtke et al., 2004).

1.7 IAA Distribution and its Role in Vascular Tissue Differentiation

Here, general models will be described for the regulation of IAA distribution and its effects on vascular tissue differentiation. While these studies primarily explain patterns in the leaves and cotyledons, they have possible implications for understanding how patterns form in the stem.

Two theories have generally been presented to explain how IAA cues might regulate vascular tissue patterning: the 'auxin canalization hypothesis' and the 'diffusion-reaction hypothesis' (Figure 5, reviewed in Nelson and Dengler, 1997; Berleth et al., 2000). The auxin canalization hypothesis was initially proposed by Tsvi Sachs (Sachs, 1981) and further supported by studies of the effects of applying auxin transport inhibiting chemicals to plant tissues (e.g. Mattsson et al., 1999; Sieburth, 1999), which show that vascular differentiation proliferates near the source of IAA when polar auxin transport has been blocked.

Mutants impaired in polar auxin transport, such as *pin1* (Okada et al., 1991; Galwëiler et al., 1998), also show vascular tissue patterns that are consistent with the effects of applying polar auxin transport inhibitors and thus with the auxin canalization hypothesis (reviewed in Scarpella and Meijer, 2004).

The auxin canalization hypothesis posits that auxin flow through a preferred channel is a cue for the formation of a complex vascular pattern. Initially all of the cells surrounding an auxin source show equivalent capabilities as auxin transporters. Eventually, some cells become better transporters, establishing a preferred channel for auxin transport. Once the auxin concentration reaches a threshold, the ground tissue cells serving as the auxin channel are then induced to differentiate as provascular tissue, while surrounding cells are drained of auxin and inhibited from forming into vascular tissues (Nelson and Dengler, 1997; Berleth et al., 2000).

In contrast to the auxin canalization hypothesis, the 'diffusion-reaction' hypothesis, proposed by Hans Meinhardt and colleagues based on computer-modelling studies, posits that complex vascular patterns form through interactions between locally activating substances and long-distance inhibitory substances (for a review, see Nelson and Dengler, 1997). Initially, a local activator would be uniformly distributed throughout the tissue. However, small, random, fluctuations might occur in the activator concentration. The hypothesis theorizes that a local autocatalysis might cause these fluctuations to be amplified, triggering the formation of specialized tissues, such as provascular tissues. Meanwhile, a

hypothetical fast-diffusing antagonist prevents the spread of the self-enhancing reaction into neighbouring tissues (Nelson and Dengler, 1997).

The *AtPIN1* gene was identified through the characterization of the *pin-formed1* (*pin1*) mutant, which forms a naked, 'pin-like' inflorescence stem possessing few, or no, flowers (Galwëiler et al., 1998). Cross-sections through stems of *pin1* mutants reveal increased xylogenesis, which, as noted above, is consistent with the effects of applying a polar auxin transport inhibitor (Galwëiler et al., 1998). Immunolocalization of the AtPIN1 protein demonstrated that it was localized to the basal end of the plasma membrane of xylem parenchyma, consistent with a potential function for PIN1 as an auxin efflux carrier (Galwëiler et al., 1998). This putative function has since been experimentally confirmed (Petrásek et al., 2006).

One of the key distinctions between the auxin canalization hypothesis and the diffusion-reaction hypothesis is that the auxin canalization hypothesis assumes a continuous flow of signal along a preferred channel, thus requiring that vascular strands remain connected at some stage of development. The *Arabidopsis vascular network* (*van*) mutants and the *scarface* (*sfc*) mutant show fragmented vein patterns (Deyholos et al., 2000; Koizumi et al., 2000). Such observations would appear to conflict with the canalization hypothesis, as the fragmentation would imply a break in auxin flow (Deyholos et al., 2000; Koizumi et al., 2000). By observing the expression of AtHB8:GUS and AtPIN1:GFP reporter proteins in *van3*, Scarpella et al. (2006) determined that *van3/sfc* demonstrates a normal ability to establish provascular strands during early stages of leaf development,

but it is the maintenance of these strands is compromised. That a provascular strand is indeed able to form at some stage of development thus reconciles the *van3/sfc* phenotype with the auxin canalization hypothesis.

The *GNOM/EMB30* gene encodes an adenosine diphosphate (ADP) ribosylation factor guanine exchange factor (ARF GEF), which regulates the ADP ribosylation factor, a GTPase involved in membrane trafficking (Steinmann et al., 1999). *SFC/VAN3* encodes an ADP ribosylation factor guanosine triphosphatase activating protein (ARF GAP), which also regulates the ADP ribosylation factor (Koizumi et al., 2005; Sieburth et al., 2006). ARF GEFs mediate the conversion of the inactive ADP-ribosylation factor guanosine diphosphate (ARF GDP) form into the active ADP-ribosylation factor guanosine triphosphate (ARF GTP) form. Conversely, ARF GAPs mediate the conversion of ARF GTP back to ARF GDP (reviewed in Schmidt and Hall, 2002; Sieburth et al., 2006). In *gnom/emb30*, too many, overconnected cotyledon veins are formed, as compared with too few, disconnected veins in *sfc/van3* (Steinmann et al., 1999; Sieburth et al., 2006). Impaired GNOM/EMB30 function was shown to disrupt vesicle trafficking from the endosome to the plasma membrane (Steinmann et al., 1999), whereas vesicle trafficking was disrupted in the opposing direction in *sfc/van3* - from the plasma membrane to the endosome (Sieburth et al., 2006). *gnom/emb30* mutants fail to show polar PIN1 localization, suggesting an absence of polar auxin transport which thus disrupts any sense of polarity in the developing seedling (Steinmann et al., 1999). Normal polarity is established in *sfc/van3*, and the formation of lower order veins occurs as normal, but a redirection of PIN1 localization in order to

adjust auxin flow – a prerequisite to the formation of higher order veins – was found to be compromised in the mutant (Sieburth et al., 2006).

1.8 Regulation of Xylem Tracheary Element Differentiation

The molecular cues underlying the acquisition of tracheary element (TE) cell fate have been dissected by Fukuda and colleagues, using an artificial cell culture system based on *Zinnia elegans* (Fukuda and Komamine, 1980). In the *Zinnia* model, through wounding and the application of auxin and cytokinin, leaf mesophyll cells are stimulated to transdifferentiate from a mesophyll cell fate into the TE fate (for review, see Fukuda, 1997, 2004). Further investigations with this model system have identified hormonal and genetic cues that underlie the acquisition of TE fate (Fukuda, 1997, 2000, 2004). Due to its artificial nature, legitimate questions have been raised about how well the *Zinnia elegans* model system can be used to generally explain xylem differentiation in living plants (Chaffey, 1999). However, the *Zinnia* model is generally thought to be at least partially useful, particularly as a similar series of cytological and regulatory events have been shown to underlie both the acquisition of TE fate in the *Zinnia elegans* model and secondary xylem differentiation in wood (Samuels et al., 2006).

Auxin, cytokinin and wounding are involved in stimulating the first stage of the TE differentiation process – the dedifferentiation of mesophyll cells into a pluripotent cell (Fukuda, 1997). Further differentiation of a TE precursor into a mature TE involves a number of cellular changes, including the formation of a secondary cell wall and, in late stages of the process, the induction of

programmed cell death (Fukuda, 1997). Calcium, cysteine proteases and endogenous brassinosteroids appear to be involved in the early stages of the differentiation of a TE precursor into a maturing TE (Fukuda, 1997; Yamamoto et al., 2001). Brassinosteroids have been shown to be implicated in the induction of Class III HD-ZIP genes, which normally accumulate as the cell culture begins to differentiate into xylem cells (Ohashi-Ito et al., 2002). Calcium and cysteine protease functions have been found to be involved in inducing programmed cell death during the later stages of TE differentiation (Fukuda, 2000).

1.9 Effects of GA and IAA Treatment on Secondary Growth of the Stem

Snow (1935) demonstrated that applications of IAA to the apical surfaces of decapitated sunflower (*Helianthus annus*) stems and hypocotyls stimulated secondary growth. Likewise, Bradley and Crane (1957) presented the first evidence that gibberellic acid (GA_3) treatments, applied as a spray, stimulated cambial activity in a tree species, using apricot (*Prunus armeniaca*) as their experimental model. Wareing (1958) tested the relative effects of applying lanolin paste mixed with IAA alone, GA_3 alone, or both IAA and GA_3 together, to several tree species. By decapitating and defoliating the stems, Wareing (1958) ensured that normal (endogenous) sources of GA and IAA were removed, thus allowing the effects of each hormone to be independently tested (reviewed by Fukuda, 2004). When GA_3 alone was applied, Wareing (1958) observed that secondary growth occurred, but the secondary xylem that formed was undifferentiated, lacking vessels and consisting of small, unlignified parenchyma. If IAA was applied on its own, secondary growth likewise occurred, but the secondary xylem

that formed was more lignified and contained vessels (Wareing, 1958). If both GA_3 and IAA were applied together, increased secondary growth was observed as compared with the amount that occurred when either hormone was applied on its own (Wareing, 1958). From these results, Wareing (1958) proposed that IAA is primarily responsible for regulating the differentiation of xylem, while GA has a role as a stimulator of cambial activity.

Digby and Wareing (1966) followed up on the study by Wareing (1958), further testing the effect of manipulating the ratio between the IAA and GA_3 concentrations that were applied. Digby and Wareing (1966) found that the greatest degree of xylem differentiation occurred when the level of applied GA_3 was relatively low and level of applied IAA relatively high. It was also observed that phloem differentiation was stimulated by high GA levels and inhibited by high IAA levels (Digby and Wareing, 1966). Much more recently, Wang and colleagues (Wang et al., 1992; Wang et al., 1995; Wang et al., 1997) investigated the effects of GA_3 application on cambial development and longitudinal growth in pine, and documented a positive effect of GA on the stimulation of secondary growth. However, the stimulatory effects of GA_3 on secondary growth were only observed when a source of IAA was available (Wang et al., 1995; Wang et al., 1997).

1.10 Effects of Applying Inhibitors of GA Biosynthesis on Stem Growth

A wide variety of compounds can be applied to crops to suppress longitudinal growth, and many of these plant growth regulators act by inhibiting GA biosynthesis (for review, see Rademacher, 2000). One such compound is

paclobutrazol (PBZ), which inhibits a cytochrome-dependent P450 monooxygenase that converts the GA precursor *ent*-kaurene into *ent*-kaurenoic acid (Figure 1; Rademacher, 2000). McDaniel et al. (1990) observed that PBZ application suppressed cell wall thickening in phloem fibres in poinsettia (*Euphorbia pulcherrima*) stems, while PBZ-treated plants also had a decreased xylem radius and lacked interfascicular xylem fibres. Burrows et al. (1992) observed that PBZ-treated *Chrysanthemum* stems showed decreased phloem fibre content, but determined that the PBZ treatment did not affect phloem fibre cell wall thickening. Burrows et al. (1992) also reported that the stem diameter of PBZ-treated *Chrysanthemum* stems was reduced, while the xylem thickness in the treated plants remained unaltered. A dose-responsive suppression of stem diameter expansion was observed in apricot trees that had been treated with PBZ (Jacyna and Dodds, 1995). The suppression of stem expansion and cambial growth following PBZ treatment was also observed in a comparative study of several tree species (Bai et al., 2004).

One of the most comprehensive studies that investigated the effects of applying a GA biosynthesis inhibitor on vascular tissue development in the stem of a woody plant was conducted by Ridoutt et al. (1996). Acylcyclohexanediones, such as trinexapac-ethyl (TriEt), block late stages in GA biosynthesis, particularly the 3β-hydroxylation that is catalyzed by GA 3 oxidase (Rademacher, 2000). Increasing TriEt concentrations were shown by Ridoutt et al. (1996) to suppress xylem fibre cellular elongation. Levels of GA_1 and GA_{20} in the

stem were also observed to be suppressed by the TriEt treatments, whereas IAA concentrations were unaffected (Ridoutt et al., 1996).

1.11 Effects of the Transgenic Manipulation of GA Biosynthesis and Response on Stem Growth

Eriksson et al. (2000) investigated the effects of overexpressing a GA 20 oxidase gene in hybrid aspen (*Populus tremula x treumuloides*); as an endogenous GA 20 oxidase sequence was unavailable at the time that that the study was initiated, Eriksson et al. (2000) heterologously expressed the *Arabidopsis AtGA20ox1* sequence in these lines. *35S::AtGA20ox1* transgenic trees were found to show increased cambial activity and increased xylem fibre cellular lengths (Eriksson et al., 2000). The growth rate, plant biomass and GA biosynthesis rate were also found to have increased in *35S::AtGA20ox1* trees relative to control plants (Eriksson et al., 2000). Follow-up investigations showed that *35S::AtGA20ox1* trees also demonstrate increased xylem cell expansion, and that the compound middle lamella in xylem cell walls possessed an increased lignin content (Dünisch et al., 2006). Israelsson et al. (2003) proposed that increases in secondary growth in the *35S::AtGA20ox1* trees might be due to a combined effect of the elevated IAA and GA levels in the trees. In contrast to the effects of overexpressing a GA 20 oxidase gene, overexpressing a GA 3 oxidase gene was not shown to significantly affect the stem morphology or bioactive GA content in hybrid aspen (Israelsson et al., 2004). Based on this, it was proposed that GA 20 oxidase serves as the rate-limiting enzyme in the formation of bioactive GAs, at least in hybrid aspen (Israelsson et al., 2004).

Biemelt et al. (2004) also investigated the effects of heterologously expressing the *Arabidopsis* GA 20 oxidase gene in tobacco. Consistent with what was observed in poplar, *35S::AtGA20ox1* tobacco plants demonstrated stimulated growth rates and an increased bioactive GA content relative to their controls (Biemelt et al., 2004). The intensity of xylem cell wall lignification and degree of secondary growth in the stem were also found to increase in the transgenic lines (Biemelt et al., 2004). In contrast, the effects of heterologously overexpressing an *Arabidopsis* GA 2 oxidase gene, which reduced the availability of bioactive GAs, were, as expected, opposite to the effects of overexpressing GA 20 oxidase (Biemelt et al., 2004).

Mauriat and Moritz (2009) tested the effect of overexpressing a putative hybrid aspen orthologue of the GA receptor *GID1*. *35S::PttGID* overexpressers shared many features of *35S::AtGA20ox1* overexpressers, including increased stem elongation and increased secondary growth (Mauriat and Moritz, 2009). However, the enhancement of xylem fibre elongation that was observed for the *35S::AtGA20ox1* trees was absent in the *35S::PttGID* trees. Mauriat and Moritz (2009) also investigated the effects of expressing both *AtGA20ox1* and *PttGID1* using a xylem-specific *LMX5* promoter, finding that *LMX5:AtGA20ox1* and *LMX5:PttGID1* trees were not substantially different from the controls, with the exception that xylem fibre lengths had increased in the *LMX5::AtGA20ox1* line. As they were able to separate the effects of GA on cambial activity and xylem fibre elongation, Mauriat and Moritz (2009) propose that the roles of GA as a stimulator of xylem fibre elongation in the developing xylem and as a stimulator

of cambial activity are distinct, and are likely regulated through genetic pathways that are only partially overlapping.

1.12 Radial Distribution of GA and IAA in the Stem

A gas chromatography-mass spectroscopy (GC/MS) technique has been successfully used to quantify IAA levels within specific tissues in tree stems (Uggla et al., 1996; Tuominen et al., 1997; Uggla et al., 1998). These studies of IAA distribution have generally revealed that IAA levels are expressed in a wave-like pattern across the stem, with the IAA levels highest within the vascular cambium and actively differentiating xylem (Figure 1-6). From these observations, a long-standing hypothesis has been that auxin acts as a morphogen, with the gradient of IAA levels providing positional cues that induce different stages of the xylem differentiation process (Sundberg et al., 2000).

Moyle et al. (2002) measured the transcript abundance of several putative *Aux/IAA* genes (*PttIAA*s) in samples that correspond to the different vascular cell types across hybrid aspen stems. In most cases, the transcript abundance of the *PttIAA* genes was highest in cambial and dividing xylem tissues, where IAA levels had been shown to be highest (Moyle et al., 2002). However, for some of the genes, their transcript abundance peaked in tissues that were not associated with the highest IAA levels (Moyle et al., 2002). Moyle et al. (2002) proposed that to indicate Aux/IAA proteins may have specific functional roles in wood formation, regulating specific stages of xylem differentiation in response to the positional cues inferred by the IAA concentration gradient (Moyle et al., 2002). Schrader et al. (2003) further determined that the putative hybrid aspen

orthologues of the *Arabidopsis AUX1* (*PttLAX*) and *PIN* (*PttPIN*) auxin influx and efflux carrier gene families also show expression patterns in different vascular cell types that are predominantly, but not always, consistent with the IAA concentration gradient.

The hypothesis that the radial IAA concentration gradient might be indicative of IAA functioning as a morphogen in its regulation of xylem differentiation has had its detractors. Berleth and Sachs (2001), in particular, note that commonly-studied auxin responses are generally not concentration-specific. An experimental attempt to change the distribution of IAA through the overexpression of IAA biosynthetic genes in hybrid aspen was demonstrated to affect IAA distribution, but xylogenesis was unaffected in the transgenic lines (Tuominen et al., 2000). Nilsson et al. (2008) determined that relatively few IAA-responsive genes show an expression pattern that matches the IAA gradient in hybrid aspen (Nilsson et al., 2008). Moreover, many of the genes whose expression patterns mirrored the IAA gradient responded dynamically to IAA treatments (Nilsson et al., 2008). A transgenic line expressing a mutated form of an *Aux/IAA* gene, *PttIAA3m*, demonstrated reduced auxin responsiveness accompanied by reduced secondary growth (Nilsson et al., 2008). Nilsson et al. (2008) propose that IAA levels might directly regulate the transcription of a few key genes, which they determined to include *Aux/IAA* genes and a putative hybrid aspen orthologue of the *AtHB8* gene (*PttHB8*). Nilsson et al. (2008) also suggest that IAA might also regulate secondary xylem differentiation post-

transcriptionally, perhaps by influencing the stability of that transcription factors that regulate xylogenesis (Nilsson et al., 2008).

Israelsson et al. (2005) used GC/MS to quantify levels of GAs across poplar stems. Like IAA, Israelsson et al. (2005) observed that bioactive GA levels form a gradient across the stem, although whereas IAA levels peak near the vascular cambium, the bioactive GA concentration was relatively high in xylem fibres undergoing elongation, but relatively low in cambial and phloem tissues (Figure 1-6). However, GA precursors (GA_9 and GA_{20}) were found to be highly abundant in the phloem and, to a lesser extent, in expanding xylem cells (Israelsson et al., 2005). Israelsson et al. (2005) also observed that the expression of a gene encoding ent-copalyl diphosphate synthase, a regulator of an earlier step in the GA biosynthetic pathway, also was highest in the phloem, while the expression of GA 20 oxidase was co-localized to regions of the stem that are associated with the highest bioactive GA levels. From this data, Israelsson et al. (2005) propose that GA mainly acts as a regulator of xylem fibre elongation, while its effects on the vascular cambium appear to be more indirect .

Björklund et al. (2007) repeated and extend the earlier by Wareing et al. (Wareing, 1958; Digby and Wareing, 1966), confirming that the application of IAA but not GA_3 to defoliated and decapitated hybrid aspen stems stimulated vessel differentiation, and confirming that the application of both hormones in tandem led to an enhancement of the effects of applying either hormone alone. The IAA content of the stem was also found to increase when both IAA and GA_3 were applied, as compared with when IAA was applied on its own (Björklund et

al., 2007). By following isotope-labelled IAA, Björklund et al. (2007) demonstrated that the increased IAA content was due to a stimulation of polar auxin transport through the stem. Through gene expression profiling it was found that both GA and IAA stimulate the expression of the poplar orthologues of a polar auxin transporter, *PttPIN1*, and that the expression of this gene was most enhanced when both IAA and GA_3 were applied together (Björklund et al., 2007). Global gene expression profiling revealed that IAA stimulates many GA biosynthesis genes, indicating that the GA/auxin balance, and the effects of these hormones on vascular tissue development, are regulated by an intricate cross-talk (Björklund et al., 2007). Björklund et al. (2007) agree with the observation by Israelsson et al. (2005) that auxin is the primary regulator of cambial divisions, hypothesizing that GAs might indirectly regulate secondary growth by stimulating polar auxin transport in the stem.

1.13 GAST/GASA *Gene Family*

The GAST/GASA proteins comprise a subset of the larger Cysteine-Rich Peptide (CRP) family (Silverstein et al., 2008). CRPs have been identified in a wide variety of plant species and grouped into different classes based on the numbers and arrangements of cysteine residues in their primary sequences (Silverstein et al., 2008). Most classes of CRPs that have been characterized mainly are involved in plant defense responses, although other classes, including most of the members of the GAST/GASA family described in Table 1-1, have been reported to have roles as regulators of plant growth and development (Silverstein et al., 2008). The *GA Stimulated Transcript 1* (*SlGAST*) gene, isolated

from the tomato (*Solanum lycopersicum*) *gib1* mutant, was the first gene from the GAST/GASA family that was characterized (Shi et al., 1992; Shi and Olszewski, 1998). The reader should note that although tomato was identified by its historic species name by Shi and colleagues (*Lycopersicon esculentum*), recent phylogenetic analyses have determined that the *Lycopersicon* genus belongs within *Solanum* (Olmstead et al., 2008), supporting a renaming. Members of the GASA/GAST gene family have also been identified in petunia (*PhGIP* family from *Petunia x hybrida*; Ben-Nissan and Weiss, 1996; Ben-Nissan et al., 2004), Gerbera (*GhGEG* from *Gerbera hybrida*; Kotilainen et al., 1999), potato (*StSN1* and *StSN2* from *Solanum tuberosum*; Segura et al., 1999; Berrocal-Lobo et al., 2002), strawberry (*FaGAST* from *Frageria x ananassa*; de la Fuente et al., 2006); rice (*OsGASR1* and *OsGASR2* from *Oryza sativa*; Furukawa et al., 2006) and *Arabidopsis* (*AtGASA* family; Herzog et al., 1995; Aubert et al., 1998; Roxrud et al., 2007; Zhang et al., 2009).

Among the above GAST/GASA genes, the two potato genes, *StSN1* and *StSN2*, are exceptional in terms of their proposed function, as they have been mainly found to have a role in antimicrobial defense responses (Segura et al., 1999; Berrocal-Lobo et al., 2002). Most of the GAST/GASA genes have been reported to be positively regulated by GA, although exceptions exist: the *Arabidopsis AtGASA4* gene was determined to be positively regulated by GA in flowers and rosette leaves, but negatively regulated by GA in cotyledons and cauline leaves (Aubert et al., 1998); *AtGASA5* was reported to be negatively regulated by GA (Zhang et al., 2009). Temporal and spatial expression patterns

for the reported GAST/GASA genes have also been variable. Some of the sequences, e.g. *PhGIP4* and *PhGIP5*, *OsGASR1* and *OsGASR2*, and *AtGASA4* have been hypothesized to be involved in cell division (Ben-Nissan et al., 2004; Furukawa et al., 2006; Roxrud et al., 2007), while others, e.g. *PhGIP1* and *PhGIP2, GhGEG* and *AtGASA5*, have been reported to have an involvement in cell elongation (Kotilainen et al., 1999; Ben-Nissan et al., 2004; Zhang et al., 2009).

Three *Arabidopsis GASA overexpressed* (*goe*) mutants have also been described, all of which exhibited increased expression of a reporter gene placed under the control of the *AtGASA1* promoter (Raventos et al., 2000). The biochemical functions of the *AtGOE* genes remain unknown, as the genetic mapping of these lines has not been reported to date. All three *goe* mutants exhibit some degree of alteration in their sensitivities to GA and paclobutrazol (Raventos et al., 2000). However, the effects of each *goe* mutation on GA responsiveness and the expression of GA biosynthesis and response genes were found to be inconsistent; for example, *goe1* was observed to be more sensitive to paclobutrazol than the wild-type, *goe2* demonstrated normal sensitivity and *goe3* was resistant to paclobutrazol (Raventos et al., 2000).

1.14 Effects of Gibberellin and Auxin Treatment on Fibre Cell Differentiation

In addition to exploring the effects of GAs and IAA on cambial activity, Digby and Wareing (1966) characterized the effects of these hormones on xylem fibre length. They found that applications of GA_3 alone did not significantly affect xylem fibre length. IAA on its own, however, stimulated an increase in fibre

length. When IAA and GA_3 were applied together, xylem fibre lengths were found to increase to a more substantial length compared with if IAA was applied on its own, suggesting that, as with the regulation of secondary growth, the two hormones have a synergistic relationship (Digby and Wareing, 1966).

Atal (1961) and Stant (1961, 1963) tested the effects of GA_3 treatments on phloem (bast) fibre properties in several species, including hemp (*Cannibis sativa*) (Atal, 1961; Stant, 1961, 1963) jute (*Corchorus olitorius*) and kenaf (*Hibiscus cannabinus*) (Stant, 1961, 1963). In these studies, it was generally shown that GA_3 treatments stimulated increases in bast fibre content, length, cell diameter and cell wall thickness. Aloni (1979) investigated the relative effects of IAA and GA_3 on phloem fibre differentiation in *Coleus blumei*, finding that high concentrations of IAA stimulated the differentiation of shorter fibres with relatively thick secondary cell walls, while high concentrations of GA_3 stimulated the differentiation of longer phloem fibres with thinner cell walls.

El-Shourbagy et al. (1995) and Ayala-Silva et al. (2005) tested the effects of IAA and GA_3 treatments on flax bast fibre properties (Table 1-2). In the study by El-Shourbagy et al. (1995), greenhouse-grown plants from a fibre flax variety were sprayed early (two weeks after planting, during seedling growth) and late (19 weeks after planting) in their growth with either GA_3 or IAA. Both hormone treatments were reported to stimulate secondary growth of the stem. The fibre yield was calculated by measuring the weight of retted fibres relative to the weight the air-dried stems. Both hormone treatments were also reported to stimulate increases in the bast fibre yield from the plant. Both treatments likewise

stimulated increases in industrially-desirable bast fibre properties, including the tensile strength and fibre fineness (length per unit weight) (El-Shourbagy et al., 1995). In the study by Ayala-Silva et al. (2005), field-grown plants were sprayed with hormonal treatments once floral buds were visible on the plants and repeating 15 days later. As with the study by El-Shourbagy et al. (1995), both hormone treatments were reported to stimulate increases in fibre yield and fineness (Ayala-Silva et al., 2005). However, the observations in the latter study conflicted in some respects with the previous study: while the IAA-treated plants were reported to show an increased stem diameter in both studies, GA_3-treated plants were reported to have a decreased diameter in the Ayala-Silva et al. study (2005). Furthermore, while fibres extracted from the GA_3-treated plants demonstrated an increase in tensile strength relative to controls in the study by El-Shourbagy et al. (1995), Ayala-Silva et al. (2005) found that IAA treatments were more effective in stimulating the tensile strength of the fibres. Methodological approaches between the two studies differed slightly, and the possibility cannot be excluded that differences in the accuracy of these approaches might have contributed to the differences that were observed. El-Shourbagy et al. (1995) cross-sectioned the plants that they examined at the second internode and measured the width of stem tissues while observing the cross-sections under the microscope. Ayala-Silva et al. (2005) measured the stem diameter more crudely, using a digital calliper to measure the girth of the stem at its midpoint.

1.15 Present Study

The objectives of the present study were to investigate how GA and IAA affect the properties of the flax stem and its component fibres (Chapter 2), and to initiate investigations into how these responses are controlled at the transcriptional level by characterizing the expression of putative orthologues of GA 2 oxidase and GA 3 oxidase genes (*LuGA2ox1*, *LuGA3ox1*), a gene putatively encoding a member of the Aux/IAA transcription factor family (*LuIAA1*), and a gene putatively encoding an orthologue of the AtPIN1 auxin efflux carrier protein. A fifth gene, *LuGAST1*, was also characterized in this study. *LuGAST1* putatively encodes a member of the GASA/GAST protein family, and was selected for further study based on that its closest putative orthologues in other plant species have been generally observed to be GA-responsive and to have potential roles in the regulation of flowering and the elongation of cells in the stem (Zhang et al., 2009). A potential correlation between changes in the transcriptional abundance of *LuGAST1* in flax hypocotyls with the onset of different stages of bast fibre differentiation has also been previously documented (Roach and Deyholos, 2008).

In parallel with this work, a screen for bast fibre mutants of flax was initiated. A general outline of the mutant screen is presented in Chapter 4, while a detailed characterization of a new mutant isolated in this screen, *reduced fibre1*, is presented in Chapter 5. Finally, primer pairs for 331 flax simple sequence repeat (SSR) markers, including 299 primer pairs identified at the University of Alberta, were tested for polymorphisms between the CDC Bethune linseed variety – the parental line for the mutant screen - and the Bolley Golden linseed variety. 36

polymorphic markers were identified which will be useful in future for the genetic mapping of *rdf* and other mutants that were identified in the screen, as well as having other potential uses for breeding and phylogenetic diversity studies.

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1.17 Tables and Figures

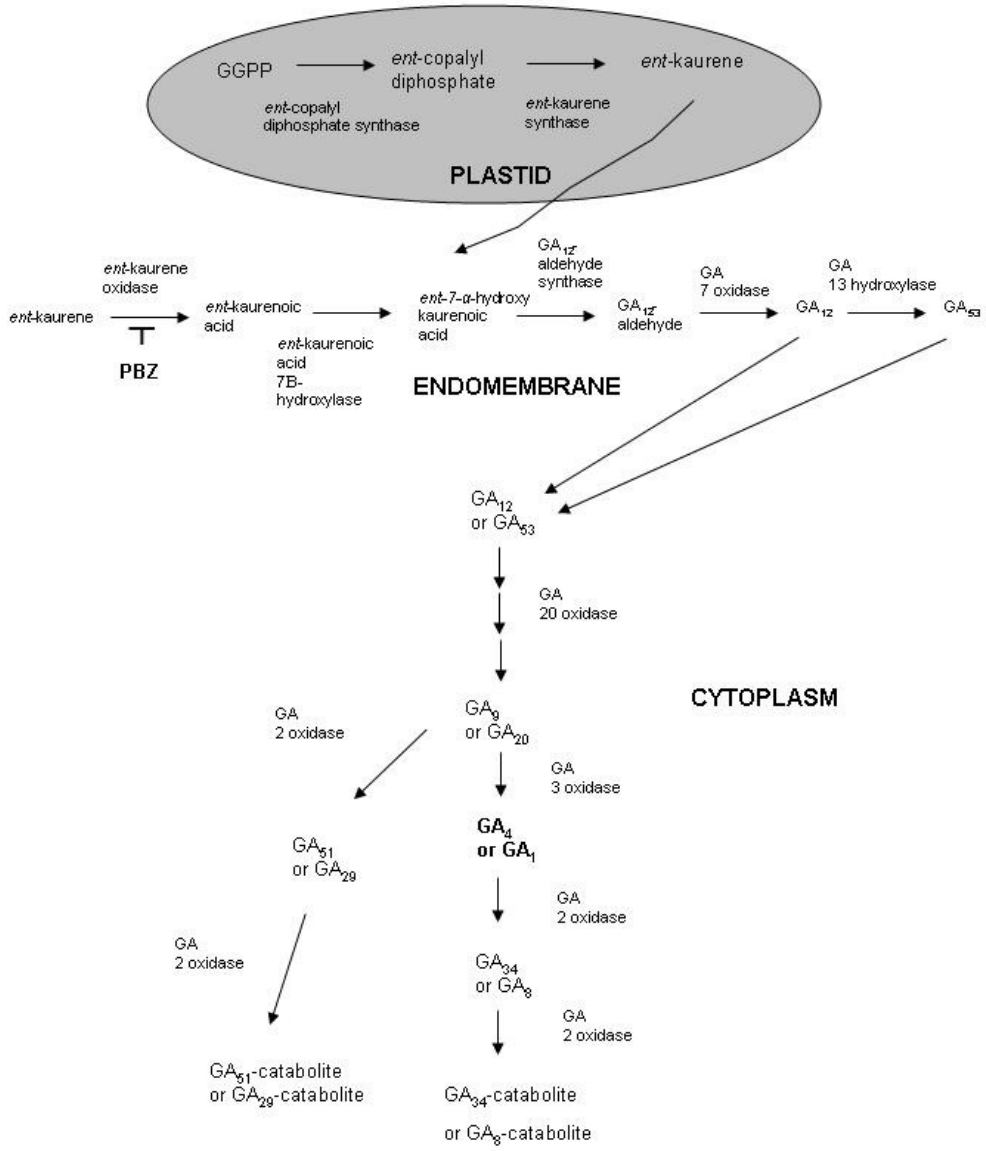

Figure 1-1. General outline of the gibberellin biosynthetic pathway in plants, modified from Hedden and Phillips (2000). In the plastid, geranyl geranyl diphosphate is sequentially converted in plastids to *ent*-copalyl diphosphate and *ent*-kaurene, catalyzed by *ent*-copalyl diphosphate synthase and *ent*-kaurene synthase, respectively. Through a series of oxidations in the endomembrane system that are catalyzed by cytochrome P450 monoxygenases, *ent*-kaurene is sequentially converted to GA_{12}. Both GA_{53}, which is formed from GA_{12}, and GA_{12} are exported to the cytoplasm and converted in parallel pathways to either GA_9 or GA_{20}, catalyzed by GA 20 oxidase. Through a 3β-hydroxylation reaction catalyzed by GA 3 oxidase, GA_9 or GA_{20} are converted to the bioactive GA_1 or GA_4 forms. 2β -hydroxylation, catalyzed by GA 2 oxidase, converts GA_1 or GA_4 into the biologically-inactive GA_{34} or GA_8 forms, which are finally converted to GA_{34}- or GA_8-catabolites. 2β -hydroxylation, catalyzed by GA 2 oxidase, also converts GA_9 or GA_{20} into the biologically-inactive GA_{34} or GA_{51} forms, which are finally converted to GA_{34}- or GA_{51}-catabolites. The plant growth regulator paclobutrazol (PBZ) partially impairs the function of *ent*-kaurene oxidase, blocking the GA biosynthetic pathway.

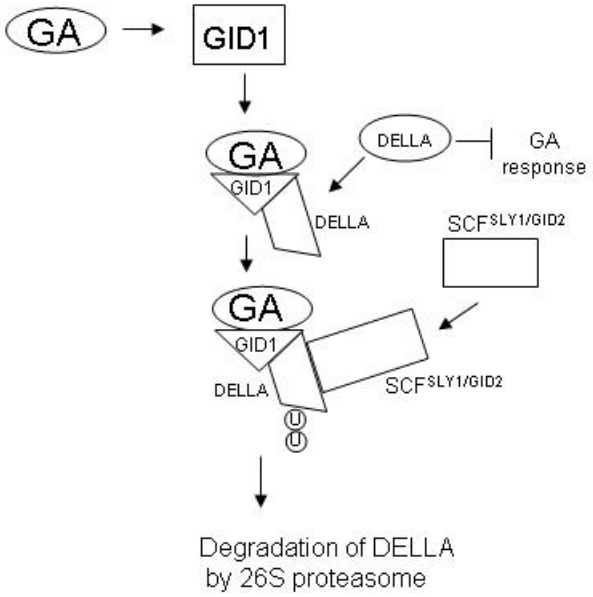

Figure 1-2. General outline of the gibberellin response pathway in plants, modified from Sun (2010). Under lower GA levels, DELLA proteins block the expression of GA-responsive genes. The binding of GA to its receptor, GID1, triggers a conformational change in the GID1 protein structure. The conformational change facilitates the binding of DELLA to GID1, which in turn induces a conformational change in the DELLA structure which facilitates recognition by the $SCF^{SLY1/GID2}$ E3 ubiquitin ligase. $SCF^{SLY1/GID2}$ polyubiquitinates the DELLA protein, facilitating its degradation by the 26S proteasome.

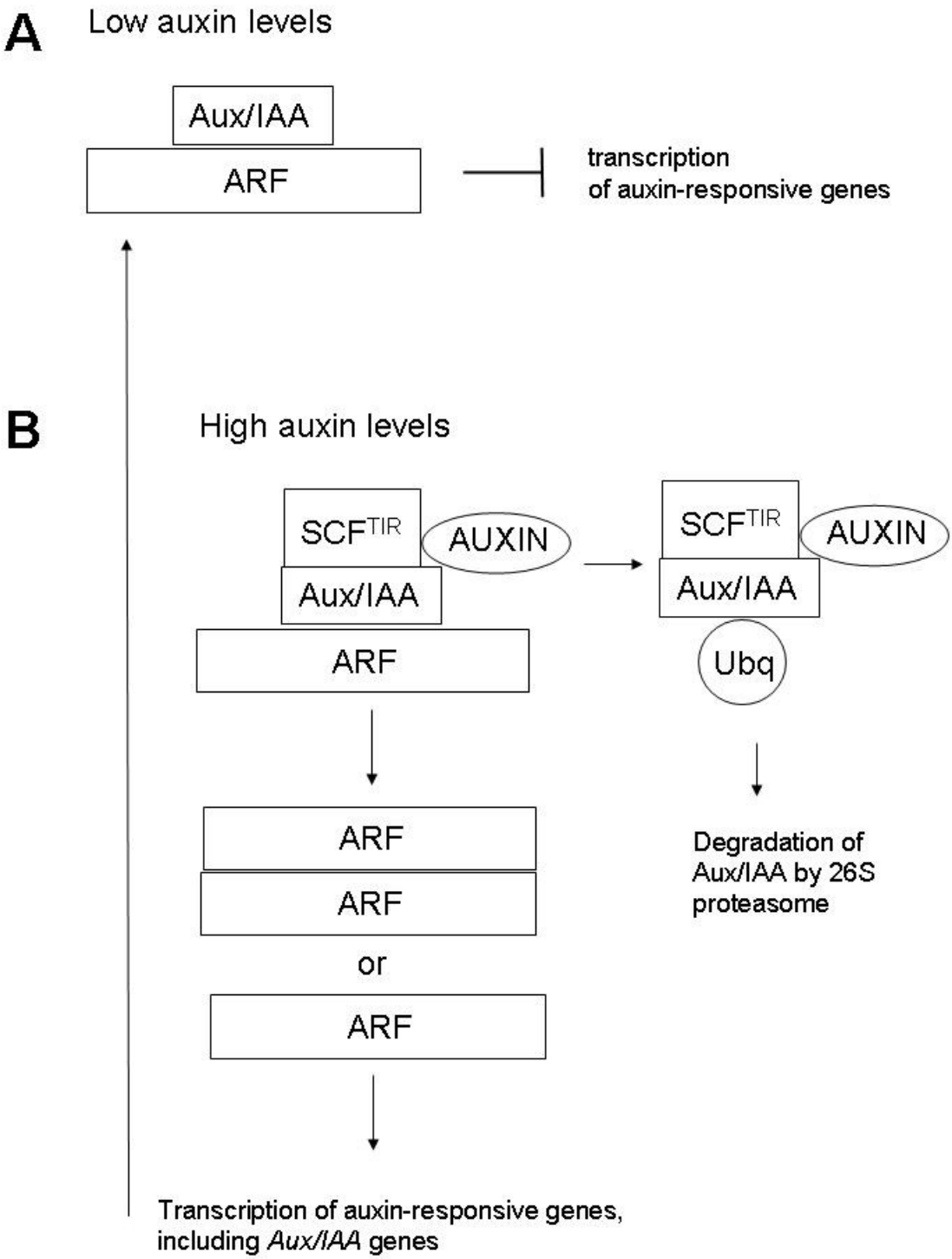
A
Low auxin levels
Aux/IAA
ARF
transcription
of auxin-responsive genes
B
High auxin levels
SCF^TIR
AUXIN
Aux/IAA
ARF
SCF^TIR
AUXIN
Aux/IAA
Ubq
ARF
ARF
or
ARF
Degradation of
Aux/IAA by 26S
proteasome
Transcription of auxin-responsive genes,
including Aux/IAA genes

Figure 1-3. General outline of the auxin signalling pathway, modified from Berleth et al. (2004). Under low auxin levels (A), Aux/IAA proteins dimerize with auxin response transcription factors (ARFs), repressing the transcription of auxin-responsive genes. When auxin levels increase (B), auxin mediates the binding of Aux/IAA proteins to the SCF^{TIR} E3 ubiquitin ligase complex, facilitating the ubiquitination of the Aux/IAA protein, thereby targeting it for degradation via the 26S proteasome. It is unclear whether the free ARFs homodimerize or remain as monomers (Vanneste and Friml, 2009). Nonethless, they become free to induce the transcription of their target genes, including *Aux/IAA* genes. The newly transcribed Aux/IAAs bind to the ARFs, allowing for stable Aux/IAA protein levels to rapidly be restored to abundance when auxin levels decrease.

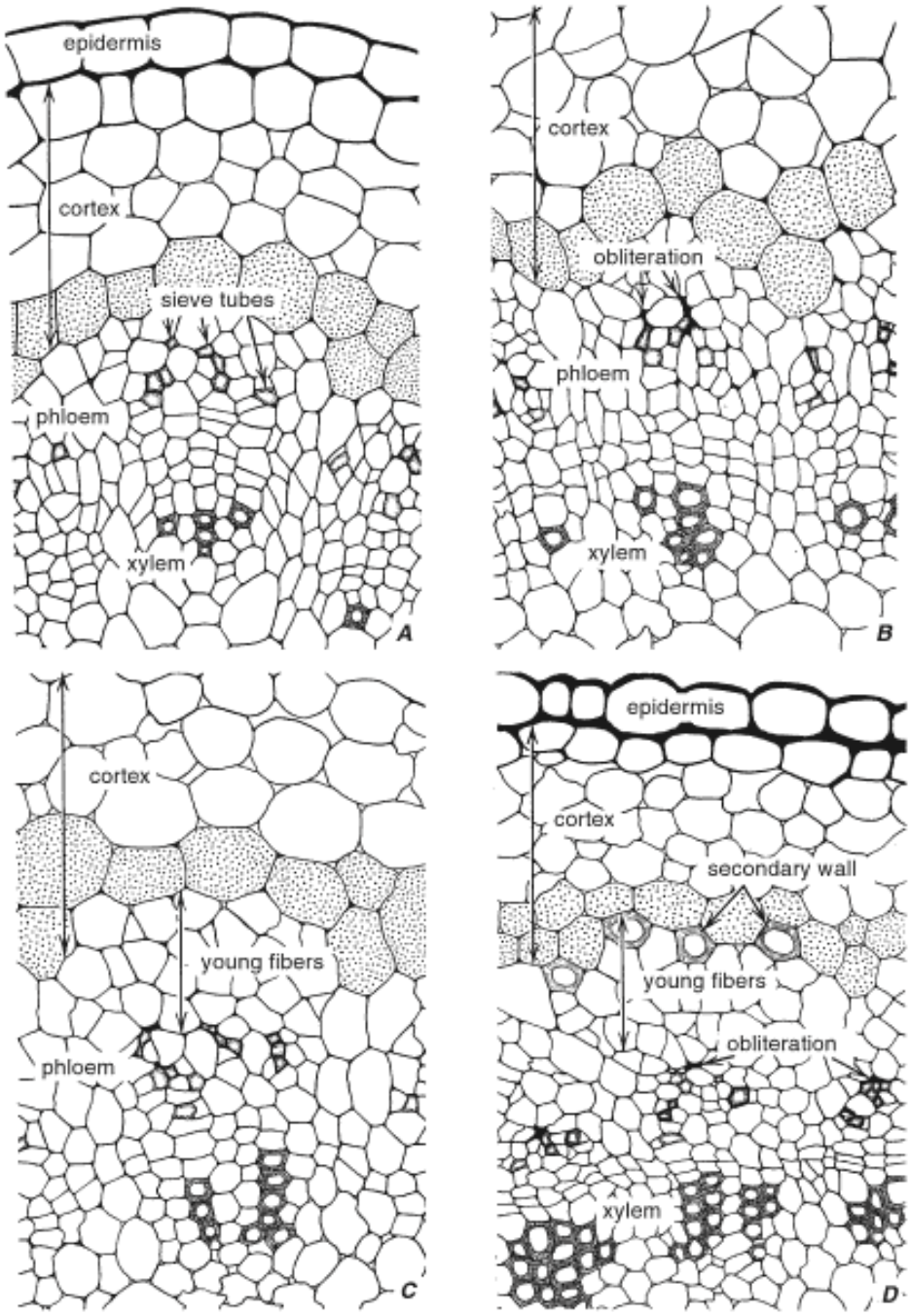
epidermis
cortex
sieve tubes
phloem
xylem
A
cortex
obliteration
phloem
xylem
B
cortex
young fibers
phloem
C
epidermis
cortex
secondary wall
young fibers
obliteration
xylem
D

Figure 1-4. Early stages of bast fibre formation in the *Linum perenne* stem (adapted from Evert, 2006). (A) First primary sieve tube elements mature. (B) and (C) New sieve tube elements differentiate while older sieve tubes are obliterated by expanding fibre initials. (D) Fibre cells positioned nearest the cortex begin forming secondary cell walls.

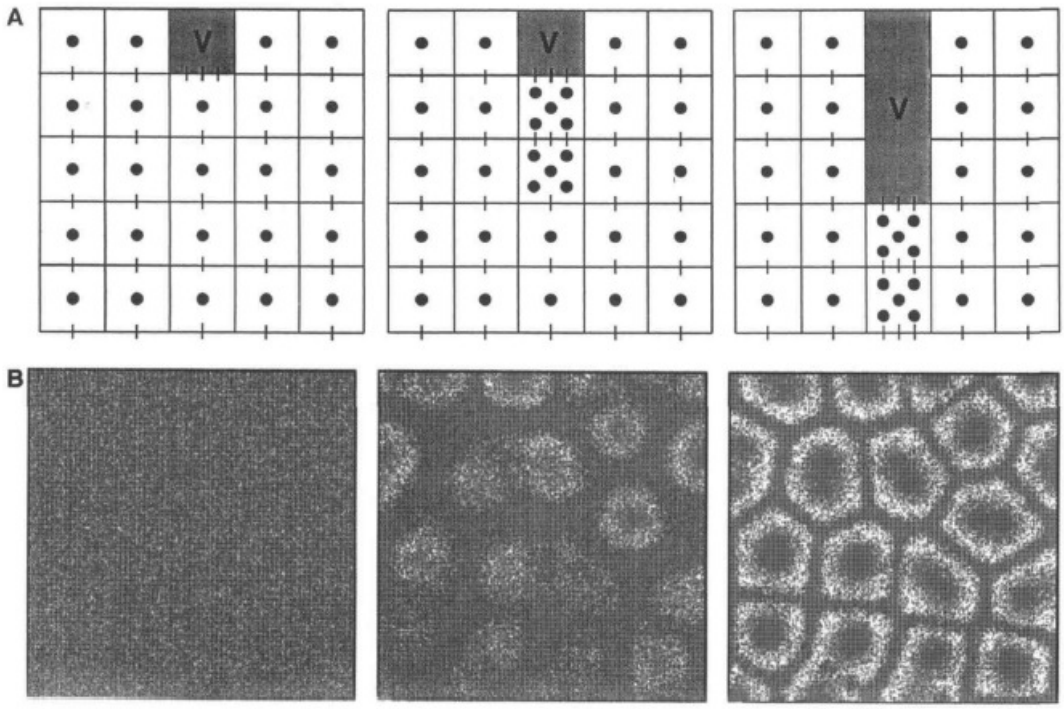

Figure 1-5. Hypotheses for vascular pattern formation (adapted from Nelson and Dengler, 1997). (A) Auxin canalization hypothesis. Initially, all cells adjacent to a severed vein (V) are equivalent transporters of auxin (dots). Cells at the terminus of the severed vein are induced to become better transporters, and the transporting cells eventually become vascular tissue. (B) Diffusion-reaction hypothesis. Computer simulation of a system forming patches combined with a system forming stripes. The patches specify where no stripes are allowed, and thus the stripes form at the largest possible distance away from other stripes, forming a distinct pattern.

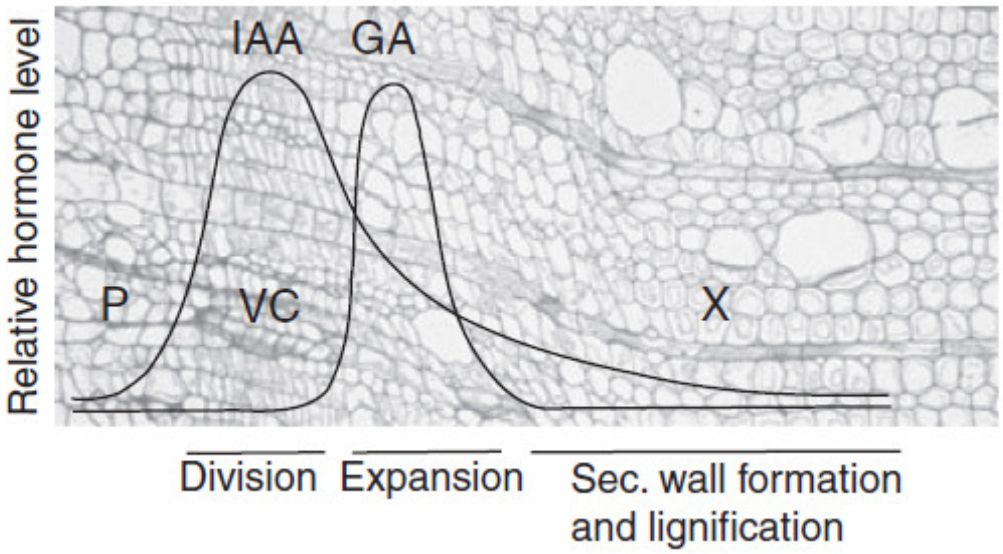

Figure 1-6. Relative spatial distribution of IAA and GA across the cambial zone of the poplar stem (adapted from Björklund et al., 2007). IAA concentrations are highest in the vascular cambium, sharply decreasing across the phloem and gradually decreasing across the xylem. Bioactive GA concentrations increase in the expanding xylem, and are relatively low in the vascular cambium and in tissues associated with secondary cell wall formation and lignification.

Table 1-1. Expression patterns for previously characterized members of the GAST/GASA family from different plant species.

Species	Gene(s) Characterized	Plant Growth Regulator Responses	Spatial Expression Pattern	Temporal Expression Pattern	Other Observations	Reference
Tomato (*Solanum lycopersicum*)	*SlGAST*; *SlRSI-1*	*SlGAST*: Positively regulated by GA, negatively regulated by ABA *SlRSI-1*: Positively regulated by auxin	*SlGAST*: Expressed in stems, leaves, petioles and flowers. Not detected in roots. *SlRSI-1*: Expressed in roots, especially in lateral root initials. Also detected at root cap and in vascular tissue. Low expression in shoot.	Not examined	*35S::GAST1* and antisense lines expressing reduced *GAST1* mRNA levels did not show detectable phenotypic differences.	Shi et al. (1992); Taylor and Scheuring (1994); Shi and Olszewski (1998)
Strawberry (*Fragaria x ananassa*)	*FaGAST*	Positively regulated by GA	Expressed in fruit. Also expressed in root, confined to end of elongation zone.	Expression in fruit concurrent with arrest in growth.	Overexpression of *FaGAST*, as well as heterologous expression in *Arabidopsis*, inhibit plant growth and reduce sensitivity to GA_3.	de la Fuente et al. (2006)
Gerbera (*Gerbera hybrida*)	*GhGEG*	Positively regulated by GA	Predominantly expressed in flowers, especially	*GhGEG* expression is associated with	Overexpression of *GhGEG* causes shortening of the	Kotilainen et al. (1999)

Species	Gene(s) Characterized	Plant Growth Regulator Responses	Spatial Expression Pattern	Temporal Expression Pattern	Other Observations	Reference
			in corollas and carpels.	cessation of cellular elongation.	corolla and carpel and an increase in radial cellular expansion.	
Petunia (*Petunia x hybrida*)	*PhGIP* family (*PhGIP1*, *PhGIP2*, *PhGIP4*, *PhGIP5*)	All four genes positively regulated by GA	Stems, floral organs. PhGIP1 and PhGIP4 show endoplasmic reticulum subcellular localization; PhGIP2 and PhGIP5 localized to cell wall.	*PhGIP1* and *PhGIP2* expression is associated with cell elongation stage; *PhGIP4* and *PhGIP5* expression is associated with cell division stage.	*PhGIP2 RNAi* lines show decreased stem elongation and flowering delay, but only observed if plants grown at an abnormally low temperature.	Ben-Nissan and Weiss (1996); Ben-Nissan et al. (2004)
Rice (*Oryza sativa*)	*OsGASR1* and *OsGASR2*	Both genes positively regulated by GA	High expression in shoot apical meristem and suspension cultured cells. Moderate expression in young leaves and roots. No expression detected in mature leaves. *OsGASR1* highly expressed in panicles; *OsGASR2* weakly expressed.	Increased expression when cell proliferation increases; potential role in cell division.		Furukawa et al. (2006)

Species	Gene(s) Characterized	Plant Growth Regulator Responses	Spatial Expression Pattern	Temporal Expression Pattern	Other Observations	Reference
			Both proteins localized to cell wall/apoplast.			
Potato (*Solanum tuberosum*)	*Snakin-1* and *Snakin-2* (*StSN1* and *StSN2*)	*StSN1* expression is unaffected by a wide variety of abiotic and biotic stimuli, including GA and IAA treatment. *StSN2* is locally up-regulated by ABA.	Both genes expressed in tubers, stems, axillary buds and young floral buds.	Not examined.	Both proteins have antimicrobial properties against bacterial and fungal pathogens.	Segura et al. (1999); Berrocal-Lobo et al. (2002)
Arabidopsis (*Arabidopsis thaliana*)	*AtGASA* family	*AtGASA1* and *AtGASA4* positively regulated by GA in rosette leaves and flower buds; *AtGASA4* negatively regulated by GA in cotyledons and cauline leaves. *AtGASA1* is negatively regulated by brassinosteroids. *AtGASA5* was found to be	*AtGASA1*: flower buds/immature siliques *AtGASA2, AtGASA3*: siliques/dry seeds *AtGASA4*: growing roots, flower buds, meristematic tissues *AtGASA5*: detected in all tissues & organs; high expression in meristematic tissues. AtGASA5	*AtGASA4*: expressed in actively dividing cells. *AtGASA5:* positively regulates *GAI* (*GA INSENSITIVE*) and *FLOWERING LOCUS C*, negatively regulates *FLOWERING LOCUS T* and *LEAFY*.	Analysis of overexpresser and null mutant lines: AtGASA4 regulates floral meristem identity and also positively affects both seed size and total seed yield. AtGASA5 negatively regulates flowering and stem elongation.	Herzog et al. (1995); Aubert et al. (1998); Raventos et al. (2000); Roxrud et al. (2007); Zhang et al. (2009)

Species	Gene(s) Characterized	Plant Growth Regulator Responses	Spatial Expression Pattern	Temporal Expression Pattern	Other Observations	Reference
		negatively regulated by GA.	protein is localized to cell wall/extracellular matrix. *AtGASA8*: roots, seeds *AtGASA10*: vasculature, meristems, seeds *AtGASA14*: hypocotyl, flowers, roots.			

Table 1-2. Comparison of experimental approaches and observational trends for previous studies on the effects of applying GA and IAA to flax. Trends stated are relative to data for their respective controls.

		El-Shourbagy et al. (1995)	Ayala-Silva et al. (2005)
Types of Flax Varieties Tested		Fibre & Dual-Use	Fibre
Where Varieties Grown		Greenhouse	Field
When Treatments Applied		2 & 19 weeks after planting	After flowering, repeating 15 days later
Stem Diameter	GA_3	Increased	**Decreased**
	IAA	Increased	Increased
Fibre Yield (fibre mass per unit weight straw)	GA_3	Increased	Increased
	IAA	Increased	Increased
Fibre Fineness (fibre mass per unit length)	GA_3	Increased	Increased
	IAA	**Decreased**	Increased
Fibre Tensile Strength	GA_3	Increased	Generally unchanged
	IAA	**Decreased**	**Increased**

2. Morphological and Anatomical Effects of Plant Growth Regulator Treatments on Stem Vascular Tissue Development in Linseed (*Linum usitatissimum* L.)[1]

2.1. Introduction

Linum usitatissimum L. has been cultivated as a source of both oil and fibre for thousands of years. The long and strong bast (phloem) fibres of flax varieties of *L. usitatissimum*, are woven into textiles. Linseed varieties are cultivated for their seed oil, which is rich in α-linolenic acid, an omega-3 fatty acid (Foster et al., 2009). Linseed oil is used as a nutritional supplement with reported healthful properties and is added as a base for paints, applied as a wood finish, and used to bind wood particles to produce linoleum flooring (Vaisey-Genser and Morris, 2003). In 2009, the top five producers of linseed were Canada, China, the USA, India and Russia (FAO, 2009). 45% of world linseed production occurred in Canada in 2009, where linseed flax was harvested from 623,300 ha of land (FAO, 2009).

Compared to flax varieties cultivated for fibre, linseed varieties are typically shorter, have more branches, and produce more seeds (Deyholos, 2006). The bast fibres that form in linseed stems are typically shorter, less numerous and of an overall poorer quality than in flax stems (Deyholos, 2006). Nevertheless, there is growing interest in utilizing bast fibres of linseed in applications such as composite manufacturing. The development of dual-purpose flax from existing

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linseed germplasm requires a more complete understanding of the factors that influence bast fibre properties in linseed.

Bast fibres of *L. usitatissimum* are part of the primary vascular tissues of the stem; unlike hemp, kenaf, and other bast fibre crops, there are no secondary bast fibres in flax or linseed (Esau, 1943). Phytohormones including auxins and gibberellins (GAs) influence the development of vascular tissues (Fukuda, 2004; Scarpella and Meijer, 2004). Spray treatments with gibberellic acid (GA_3) under both field and greenhouse conditions have been reported to induce increased fibre yield in two flax varieties and one dual-purpose variety (El-Shourbagy et al., 1995; Ayala-Silva et al., 2005). GA_3 treatments have also been shown to stimulate cambial divisions in several plant species, promoting xylem differentiation and stimulating increased secondary growth (Bradley and Crane, 1957; Wareing, 1958; Björklund et al., 2007). El-Shourbagy et al. (1995) and Ayala-Silva et al. (2005) report opposing observations as to the effects of GA_3 treatment on flax stem expansion, with El-Shourbagy et al. (1995) reporting increased stem expansion, while Ayala-Silva et al. (2005) reported that GA_3-treated stems were thinner than control plants. IAA treatments, however, reportedly stimulate secondary growth in the flax stem in both studies. El-Shourbagy et al. (1995) and Ayala-Silva et al. (2005) have also documented the effects of GA_3 and IAA treatments on industrially important bast fibre properties, such as tensile strength, fibre abundance and fibre fineness (mass per unit area). However, these properties were largely determined indirectly, and neither study has shown whether these

properties can be directly connected to the cellular morphology or development of the bast fibres in treated plants.

In this study, the effects of applying GA_3, IAA and a GA biosynthesis inhibitor (paclobutrazol) via spray treatments were tested in an elite linseed variety. Given the inconsistencies in previous reports of hormone treatments in flax, the objective was to definitively establish the relationship between growth regulators and development with industrially relevant properties of linseed stems and their constituent fibres. This will provide a much-needed physiological framework for further studies of the cellular processes that determine fibre properties, and facilitate manipulation of linseed fibres for the development of dual-purpose crops.

2.2. Materials and Methods

2.2.1 Plant Material

Experiments were conducted in the *L. usitatissimum* L. linseed variety CDC Bethune (Rowland et al., 2002). Plants were grown in Metromix 360 (Scotts, Maryland, OH), planted in round pots (7 cm height, 9.5 cm diameter at the top) to a depth of approximately 1 cm, at a density of 4 - 6 seeds per pot. The plants were grown in controlled environment chambers at 24°C with 50% humidity, and a light intensity of 200 µE supplied by high output fluorescent bulbs (CRI of 85, colour temperature of 3,500 K) on a 16 h light/8 h dark cycle.

2.2.2 Plant Growth Regulator Treatments

Growth regulator treatments were initiated 2 weeks after the seeds were planted, and continued on a weekly basis. Treatments were applied to foliage as a spray, commencing 2 weeks after seeds were planted.

All growth regulator treatments were freshly prepared prior to each treatment. Gibberellic acid (GA_3; Sigma, St. Louis, MO) and indole-3-acetic acid (IAA; Sigma, St. Louis, MO) were dissolved in 95 - 100% ethanol to produce a 100 mM stock solution; the stock solutions were then diluted in Milli-Q water to a desired final concentration (typically 250 µM). Paclobutrazol (Bonzi®; Syngenta Professional Products, Greensboro, NC) was obtained as a 4 g/L (13.8 mM) solution, and was likewise diluted in water to a concentration of 250 µM. In order to ensure that the ethanol concentration was kept constant, both the mock treatment and treatments containing paclobutrazol were supplemented with ethanol (typically 0.25% (v/v) for a 250 µM solution). Finally, 0.05% Tween-20 was added to all of the treatment solutions as a surfactant.

2.2.3 Sample Preparation for Light Microscopy

Tissues were cross-sectioned by hand and stained with phloroglucinol - HCl (2% (w/v) phloroglucinol (Sigma, St. Louis, MO) in 16% (v/v) ethanol and 20% (v/v) hydrochloric acid). Sections were rinsed in water, mounted in water on a microscope slide, and photographed using an Olympus BX51 microscope (Olympus Corporation, Tokyo, Japan).

2.2.4 Sample Preparation for Scanning Electron Microscopy

Samples approximately 1 mm in length were cut from the mid-point of the stem and fixed in formalin - acetic acid - alcohol (10% (v/v) formalin; 5% (v/v) glacial acetic acid; 50% (v/v) ethanol). After 24 hours of fixation, the samples were dehydrated in a graded ethanol solution series. Hexamethyldisilazene (HMDS; Electron Microscopy Sciences, Fort Washington, PA) was introduced through a graded ethanol-HMDS series (25% (v/v) HMDS/75% (v/v) ethanol; 50% HMDS/50% ethanol; 75% HMDS/25% ethanol; two changes in 100% HMDS). After HMDS removal, the samples were left to air dry overnight, then mounted on SEM stubs and sputter-coated with gold/palladium using a Ladd/Hummer 6.2 Sputter Coater (Ladd Research, Williston, VT). The samples were then viewed using a Philips/FEI LaB6 Environmental Scanning Electron Microscope (FEI, Hillsboro, OR).

2.2.5 Tissue Measurements

Measurements of the xylem and outer tissue radii and stem diameter were determined from photographs of stem cross-sections, taken from within the first internode at the base of the primary stem. Measurements were made using ImageJ (Abramoff et al., 2004). Analysis of the measurements was conducted within R (http://www.r-project.org; R Development Core Team, 2009). Average measurements were compared to each other for each measured parameter using a one-way analysis of variance. Variances were checked for relative equality using Bartlett's test and, where necessary, data was transformed to achieve equal variances prior to further statistical analysis. Treatments showing significant

(p<0.05) differences compared with the mock treatment were identified using Dunnett's test.

2.2.6 Stem Height Measurements

To assess whether any relationship exists between increases in the stem height and expansion of the stem girth, pots selected to receive each growth regulator treatment were randomly distributed in flats. A pot for each treatment was removed periodically, and the height of each plant, from the soil level to the apex of the primary stem, was measured. The length of the first internode at the base of the stem was also measured.

2.2.7 Fibre Length Measurements

Stem tissue was macerated using Franklin's maceration method (Chaffey, 2002). The macerate was viewed under the microscope, and xylem and bast fibres were manually measured.

2.3. Results

2.3.1 Effects of Growth Regulator Treatments on Plant Height

To evaluate the effects of giberellic acid (GA_3) and auxin (IAA) on the development of stems and bast fibres in a linseed variety of *L. usitatissimum*, growth regulators were sprayed on plants, starting 14 days after planting (DAP). An inhibitor of GA_3 biosynthesis, paclobutrazol (PBZ), was also used in some treatments. After four weeks of treatments (42 DAP), GA_3 treated plants were taller, and PBZ treated plants were shorter than mock-treated plants (Figure 2-1

A). Quantitative data, measured at 49 DAP, showed that plants treated with GA_3 or GA_3 + IAA were significantly taller than the mock-treated plants, with stem heights increasing by 20-40%; plants treated with PBZ were 65% shorter than mock-treated plants; and plants treated with IAA showed a more moderate reduction in height (Figure 2-1 B).

2.3.2 Effects of Plant Growth Regulator Treatments on Stem Tissue Development

To determine whether stem girth was affected by growth regulators, stem diameter was measured in transverse sections. The radii of the xylem and outer tissues (i.e. phloem and all other tissues between the cambium and epidermis, inclusively) were also measured. The xylem radius and the ratio of xylem radius/stem diameter decreased in plants treated with PBZ and significantly increased following any treatment that included GA_3, namely: GA_3 alone, PBZ + GA_3, and IAA + GA_3 (Table 2-1). Pair-wise comparisons using Tukey's test demonstrated that the measurements from the GA_3, IAA (25 µM) + GA_3 , and IAA (250 µM) + GA_3 treatments were not statistically significant from each other, but were significantly greater than measurements from plants treated with PBZ + GA_3. Plants treated with IAA (250 µM) also demonstrated a modest but significant reduction in the xylem/stem diameter ratio.

The observed increases in stem and xylem diameter could result from increases in either cell number, cell diameter, or both. To distinguish between these possibilities, the number of cells present along the xylem radius were counted, and the number of cells per unit area within randomly selected regions of

the xylem were also counted. The number of cells that comprised the xylem radius was decreased in PBZ-treated stems and significantly increased in plants treated with GA_3 or IAA + GA_3 (Table 2-1). The density of cells within the xylem was increased in plants treated with PBZ and decreased in plants treated with both PBZ + GA_3, IAA + GA_3, and GA_3 alone (Table 2-1). Thus, both cell number and cell diameter increased in plants treated with GA_3 and IAA + GA_3. Conversely, both cell number and cell diameter decreased in plants treated with PBZ.

Because some treatments affected both stem height and stem diameter, the correlation between these measurements was tested using mock, GA_3 and PBZ treated plants. To stabilize the variance, all measurements were log-transformed. The stem diameter was poorly correlated with the length of the first internode at the base of the stem (Figure 2-3A; for mock-treated plants, $r(19) = 0.20$, $p = 0.37$; for GA_3-treated plants, $r(25) = 0.12$, $p = 0.54$; for PBZ-treated plants, $r(22)=-0.18$, $p=0.42$). The stem diameter was correlated with the height of the stem, and this relationship was also observed in plants that had been treated with both plant growth regulators (Figure 2-3B; for mock-treated plants, $r(19) = 0.79$, $p <0.001$; for GA_3-treated plants, $r(25) = 0.74$, $p <0.001$; for PBZ-treated plants, $r(22) = 0.66$, $p <0.001$). The radius of the xylem was likewise correlated with the height of the stem (Figure 2-3C; for mock-treated plants, $r(19) = 0.81$, $p <0.001$; for GA_3-treated plants, $r(25) = 0.78$, $p <0.001$; for PBZ-treated plants, $r(22) = 0.40$, $p = 0.06$).The outer tissue content was only weakly correlated with stem height for the mock- and GA_3-treated plants (Figure 2-3D; for mock-treated plants, $r(19) =$

0.52, $p = 0.02$; for GA_3-treated plants, $r(25) = 0.45$, $p = 0.02$), but not correlated with height for the PBZ-treated plants ($r(22) = -0.04$, $p = 0.83$).

2.3.3 Effects of Plant Growth Regulator Treatments on Fibre Length

To determine whether plant growth regulators affected the length of either xylem or bast fibres, stem tissues were macerated and the lengths of samples of xylem fibres and bast fibres were determined. Neither the xylem fibre length nor the bast fibre length was affected in plants treated with GA_3 or IAA. However, both fibre types were significantly shorter in the PBZ-treated plants relative to the mock-treated controls, with xylem fibres reduced in length by 30% and bast fibre length reduced by 55% (Table 2-2).

To test whether the length of either bast fibres or xylem fibres was correlated with stem height, a correlation analysis was conducted (Figure 2-4). Because the stem height and xylem fibre length data were found to be heteroscedastic, and as this heteroscedasticity could not be corrected through a data transformation, the non-parametric Spearman rank correlation test was used. Xylem fibre length was correlated with the stem height ($r_s=0.57$, $p<0.001$). The stem height and bast fibre length data were log-transformed to introduce homoscedasticity; as with xylem fibres, the length of bast fibres was correlated with stem height ($r(30)=0.81$, $p<0.001$).

2.3.4 Effects of Plant Growth Regulator Treatments on Stem Bast Fibre Quantity

The total number of bast fibres observed in a stem cross-section was estimated from counts of the number of fibre bundles and the number of fibres within each bundle (Table 2-2). The number of fibres per bundle observed in the cross-section was significantly decreased in the PBZ-treated plants ($p<0.05$). The number of fibre bundles observed was not found to differ in any of the treatments relative to the mock-treated controls. Overall, the estimates of the number of fibres in stem cross-sections were significantly increased in plants treated with GA_3 and significantly decreased in plants treated with either PBZ or IAA (250 μM).

2.3.5 Effects of Plant Growth Regulator Treatments on Bast and Xylem Fibre Expansion

A scanning electron microscope (SEM) was used to obtain higher resolution images of bast and xylem fibres in cross-section. The bast fibres in plants treated with GA_3 or with both IAA + GA_3 appeared noticeably larger in diameter and had thicker cell walls than fibres in the mock-treated plants, while the bast fibres in the PBZ-treated plants appeared smaller with thinner cell walls (Figure 2-5). Quantitative analysis showed that bast fibre cell walls were significantly thicker in plants treated with both IAA + GA_3 (Table 2-3). Bast fibres from plants treated with either IAA or both PBZ + GA_3 appeared similar in appearance to fibres in the mock-treated plants. Measurements of xylem fibres showed that cell wall width was significantly increased ($p<0.05$) in plants treated with GA_3, IAA and both IAA + GA_3 (Table 2-3). Differences in bast fibre area were not statistically significant for any treatment.

2.4 Discussion

Application of GA_3 to linseed stimulated stem growth and xylogenesis (Table 2-1) and increased the number of bast fibres observed in stem sections (Table 2-2). Conversely, depletion of endogenous GA by application of PBZ, inhibited stem growth and xylogenesis and decreased the number of bast fibres observed in stem sections (Table 2-2). As detailed below, these observations confirm that GA_3 is an important positive regulator of stem elongation and xylogenesis in linseed, and that GA also positively regulates some industrially relevant properties of primary phloem fibres, as detailed below.

PBZ inhibits a cytochrome P450 monoxygenase responsible for regulating several steps of the GA biosynthetic pathway (Rademacher, 2000). Cytochrome P450 monoxygenases are also involved in the biosynthesis of brassinosteroids, a class of plant hormone that has also been shown to affect stem elongation and vascular tissue development (Rademacher, 2000; Clouse and Sasse, 2003). Although PBZ can also affect sterol biosynthesis, it generally has been considered to primarily inhibit the GA pathway (Asami et al., 2003). As a simple control to confirm that the effects of PBZ observed in this study were the result of GA depletion, PBZ was also applied in combination with GA_3 (PBZ + GA_3). In each experiment, the additional GA_3 in PBZ + GA_3 treatments appeared to overcome the inhibitory effects of PBZ alone (Figure 2-1B, Figure 2-2, Table 2-1, Table 2-2, Table 2-3). Thus, the observed effects of PBZ on linseed stem and fibre development can be considered to be related to the inhibition of GA biosynthesis, specifically.

GA_3 treatment is known to increase xylem but not phloem expansion in apricot (Bradley and Crane, 1957). Likewise, PBZ has been shown to reduce the xylem radius in several tree species (Bai et al., 2004). The results reported here for linseed are consistent with these studies from other species. The increased proportion of xylem within GA_3-treated stems, and the decreased xylem production in PBZ-treated stems (Table 2-1) could be attributed to one or both of the following factors: a GA-dependent change in the dimensions of the individual xylem cells, and a GA-dependent change in the number of xylem cells. Observations of transverse sections of linseed stems (Table 2-1) showed that both the number of xylem cells, and the diameter of individual xylem cells increased in the presence of exogenous GA_3 and decreased in the presence of PBZ. Thus, both the division and expansion of xylem precursors in linseed are stimulated by GA.

The GA_3 and PBZ treatments affected both stem elongation and xylem differentiation. The correlation between these parameters was measured in order to investigate whether these processes are physiologically dependent. The length of the first internode, where the sections were made, was poorly correlated with the stem diameter (Figure 2-4A). However, the stem diameter and xylem radius were both significantly correlated with the stem height (Figure 2-4 B, C). Likewise, the outer tissue radius for the mock- and GA_3-treated plants was also correlated with the stem height (Figure 2-4 D). The correlation between stem diameter and xylem radius with the stem height raises the possibility that increased xylogenesis is a consequence of stem elongation, rather than a direct result of stimulation by GA. However, the poor correlation between the stem

diameter and the length of the first internode indicates that cambial activity can be stimulated independently of elongation, since this internode was already fully elongated at the start of hormone treatments. Thus, the stimulatory effects of GA on xylogenesis in linseed are likely independent of its effects on stem elongation.

The growth regulatory effects of indole acetic acid (IAA) were examined alone and in combination with GA, because GA and IAA are reported to act synergistically in some circumstances (Björklund et al., 2007). In the results presented here, IAA treatments at two different concentrations inhibited stem elongation (Figure 2-1B). A reduction in stem height following IAA treatment has been reported in a number of plant species, including flax, and might be at least partly due to cross-talk with the ethylene biosynthetic pathway (Romano et al., 1993; Ayala-Silva et al., 2005). IAA treatments did not significantly affect the xylem or outer tissue radii (Table 2-1). These results conflict with several studies that have generally reported that exogenous IAA treatments stimulate xylem expansion, including one study in flax (Snow, 1935; Samantarai and Nanda, 1979; El-Shourbagy et al., 1995). Applying both GA_3 and IAA together has been reported to have an additive effect on xylem differentiation (Wareing, 1958; Björklund et al., 2007). Differences between these results and those of the current study may be due to the treatment conditions: in previous studies, stems were decapitated and defoliated to impair endogenous hormone biosynthesis.

The lack of stimulatory effect of IAA (alone or with GA_3) on the development of stem vascular tissues in linseed was somewhat surprising in light of results reported in flax and other species (Snow, 1935; Digby and Wareing,

1966; Samantarai and Nanda, 1979; El-Shourbagy et al., 1995; Little et al., 2002). Several lines of evidence show that the lack of response to IAA in linseed stems could not be attributed to trivial explanations, such as a failure to apply sufficiently active IAA: both 25 μM and 250 μM IAA treatments had equivalent inhibitory effects on stem elongation (Figure 2-1B), IAA increased xylem fibre cell wall thickness (Table 2-3) and IAA in combination with GA_3 increased bast fibre cell wall thickness (Table 2-3). Furthermore, the transcript abundance of a linseed *Aux/IAA* gene was increased significantly after 1 h of IAA treatment (data not shown). Therefore, the IAA treatments used in this study were sufficiently active to induce various biological responses, and the observed lack of effects within the stem vascular tissue suggest that the role of IAA in linseed stem development may be somewhat different from other species. Furthermore, these results indicate that the responses to GA reported here do not generally involve crosstalk with IAA signalling pathways.

It has been proposed that the main role of GA in secondary xylem differentiation is to stimulate cell elongation (Israelsson et al., 2005), while the effects of GA on cambial activity may be indirect, caused by modulated polar auxin transport through the stem (Björklund et al., 2007). In the current study, xylem fibres from the PBZ treated plants were approximately 30% shorter than the fibres from the mock-treated plants (Table 2-2), consistent with the proposed requirement of GA for xylem fibre elongation. However, exogenous application of GA_3 did not increase length of xylem fibres in the current study (Table 2-2),

indicating that GA levels are not a limiting factor during xylem fibre elongation in linseed.

Spray treatments with GA_3 have been shown to stimulate bast fibre elongation in hemp, jute and kenaf (Stant, 1961, 1963) and to increase bast fibre number in stem cross-sections of hemp (Atal, 1961). However, these three species all produce secondary phloem fibres (McDougall et al., 1993), whereas the bast fibres of flax and linseed develop exclusively from primary tissue (Esau, 1943). In the current study, bast fibre length increased an average of 17% following GA_3 treatment, although this increase was not statistically significant at α=0.05 due to the large variance in fibre lengths (Table 2-2).

PBZ-treated plants, however, produced bast fibres that were approximately 55% shorter than in the mock-treated plants. As it was also found that fibre length and stem height were strongly correlated (Figure 2-4) the reduced bast fibre length in the PBZ-treated plants may simply be a consequence of the reduced stem elongation. No evidence could be found to support previous reports that treatment with exogenous IAA stimulated bast fibre elongation.

Potential differences in bast fibre quantity were assessed in this study by counting the number of fibre bundles, and the number of fibres per bundle, observed in transverse stem sections (Table 2-2). The total number of fibres represented in the sections was calculated by multiplying these values together. The total fibre number was significantly increased following GA_3 treatment, but not IAA treatment (Table 2-2). A corresponding decrease in total fibre number was observed following PBZ treatment. Because flax and linseed bast fibres

elongate through a process of intrusive growth (Esau, 1943; Ageeva et al., 2005), the number of fibres observed in transverse sections is highly correlated with fibre length (Gorshkova et al., 2003). The observed effects of GA_3 and PBZ on total bast fibre number in transverse section may therefore be related to changes in either fibre length, or proliferation of fibre precursors.

Treatments with either IAA or GA_3 have been reported to increase bast fibre yield in flax (El-Shourbagy et al., 1995; Ayala-Silva et al., 2005); this effect was reportedly more pronounced for GA_3 treatments than for IAA treatments (El-Shourbagy et al., 1995). The slight increase in the fibre content in cross-sections of the GA_3-treated plants would be consistent with these observations, whereas the decrease in the IAA-treated plants is not. However, it is worth emphasizing that in the study by El-Shourbagy et al. (1995) the bast fibre quantity was determined by mechanically separating fibres from the straw of retted flax plants and then determining the fibre weight relative to the weight of the straw. Hence, the increased fibre yield that was previously reported in IAA treated plants might demonstrate that the fibre fraction was heavier but does not necessarily indicate that more fibres differentiate in the stem.

Bast fibres from plants treated with GA_3 alone, both IAA and GA_3 and both PBZ and GA_3 typically appeared larger in area, with thicker cell walls compared with the fibres from mock-treated plants (Figure 2-5). Only the thickening of the cell walls from the plants treated with IAA + GA_3 was statistically significant, suggesting that IAA + GA_3 may act synergistically in thickening of bast fibre secondary walls in linseed (Table 2-3). A trend towards an

increase in bast fibre diameter was also evident in any of the plants that had received GA_3 treatments, with and without other plant growth regulators. Likewise, bast fibres from PBZ-treated plants had significantly thinner cell walls than the mock-treated controls (Figure 2-5, Table 2-3), and the overall size of the fibres also decreased, suggesting that GA may be required for fibre cell wall thickening and fibre expansion. In kenaf, the degree of cell wall thickening has been shown to be a major determinant of fibre viscoelasticity (Ayre et al., 2009). Manipulation of GA (in combination with IAA) within developing bast fibres of linseed may therefore be useful in production of fibres with specific physical and mechanical properties.

The GA-dependent responses of linseed stems reported here are generally consistent with reports from other species, i.e. GA stimulates stem elongation and thickening of the stem through xylogenesis, and promotes cell wall expansion (Bradley and Crane, 1957; Digby and Wareing, 1966; Eriksson et al., 2000; Dünisch et al., 2006; Mauriat and Moritz, 2009). However, the absence of a clear IAA response during secondary growth of the linseed stem (Table 2-2) was surprising, especially considering that the treatment conditions used here were at more frequent and covered a broader range of concentrations that reported by El-Shourbagy et al. (1995), who applied IAA (10 µM) twice during development, (2 and 19 weeks after planting). In comparing hormone treatment studies, it must always be considered that the timing of the treatment and location of tissue sections along the stem can have a significant effect on the observations. Indeed, the observations presented by El-Shourbagy et al. (1995) and Ayala-Silva et al.

(2005) conflict in some aspects. For example, while El-Shourbagy et al. (1995) determined that GA_3 treatment increased the stem diameter, Ayala-Silva et al. (2005) conversely report that GA_3 treatment reduces the stem thickness. However, while El-Shourbagy et al. (1995) applied the hormone treatments during vegetative stages of flax stem growth, Ayala-Silva et al. (2005) did not begin applications until after the plants began to show flower buds. The decreased stem diameter – which was measured at the midstem – might simply reflect an increase in cellular elongation in response to GA_3 that occurs at a stage of growth when the vascular cambial activity has decreased, thereby causing the treated plants to appear thinner than the control plants.

A major goal of this study was to document the effects of hormonal treatments on the morphology and development of linseed bast fibres, and the data presented indeed shows that GA levels do influence fibre elongation and cell wall thickening. Hence, in order to develop dual-use flax varieties, an improvement in useful bast fibre properties, such as length and tensile strength, might be potentially introduced through the mis-expression of gibberellin biosynthetic genes. As transgenic linseed is not currently accepted by the market (Breckling, 2010), changes in GA biosynthesis could be introduced through non-transgenic approaches such as TILLING (Colbert et al., 2001). A substantial portion of the CDC Bethune genomic sequence was recently assembled (M. Deyholos, manuscript in preparation), and reverse genetics platform similar to TILLING is being developed in the authors' laboratory.

The mechanism by which GA regulates bast fibre secondary wall properties is intriguing, and bears further investigation because of its industrial relevance. Several studies have shown that GAs regulate microtubule dynamics, thus controlling cellulose deposition into cell walls (Shibaoka, 1993). Arabinogalactan (AGP) proteins, which are enriched in flax bast fibres, have been hypothesized to aid in establishing cellulose microfibril axial orientations (Hobson et al., 2010). There may be connections between gibberellin signalling and the expression of AGP proteins (Suzuki et al., 2002; Masiguchi et al., 2008). Flax bast fibres are also enriched in β-galactosidases, a class of proteins responsible for the hydrolysis of galactose (Roach and Deyholos, 2007; Hotte and Deyholos, 2008; Roach and Deyholos, 2008). The phenotype of transgenic UDP-galactose transporter overexpressers in tobacco was reported to be similar to plants that had been treated repeatedly with GA_3, i.e. the plants became taller and demonstrated increased secondary growth (Khalil et al., 2010). These transgenic tobacco also showed increased galactose residues associated with AGPs. However, GA biosynthesis gene expression was unaltered. The authors speculated that the phenocopying of GA responses in the transgenic tobacco indicated that increased glycosylation of AGPs may be a mechanism for GA to regulate fibre growth in stems. The potential relevance of these results to GA signalling in linseed fibre development is currently being investigated.

2.5 Conclusion

Using exogenous application of GA_3 and an inhibitor of GA biosynthesis (PBZ), GA was demonstrated to positively regulate stem elongation, xylogenesis

and xylem fibre expansion in linseed (Table 2-1). Effects of GA on bast fibres of linseed were less obvious, but an increase in the number of bast fibres was observed in transverse sections following GA_3 treatment (Table 2-2), presumably due in part to a moderate increase in bast fibre elongation. GA, in combination with IAA, also increased the thickness of cell walls in bast fibres by almost two fold (Table 2-3). Except for the synergistic thickening of the bast fibre cells walls, the stimulatory effects of GA in linseed development were largely independent of IAA, unlike other species in which greater crosstalk between these hormones has been reported. These results provide a framework for targeting GA signalling pathways to manipulate industrially relevant properties of bast fibres of linseed, including fibre length or quantity, and their mechanical and physical properties. This manipulation may be feasible through a variety of non-transgenic or transgenic approaches, or possibly through direct application of growth regulators to linseed in the field. This research therefore facilitates a long-term goal of developing dual-purpose flax varieties based on existing elite linseed germplasm.

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2.7 Tables and Figures

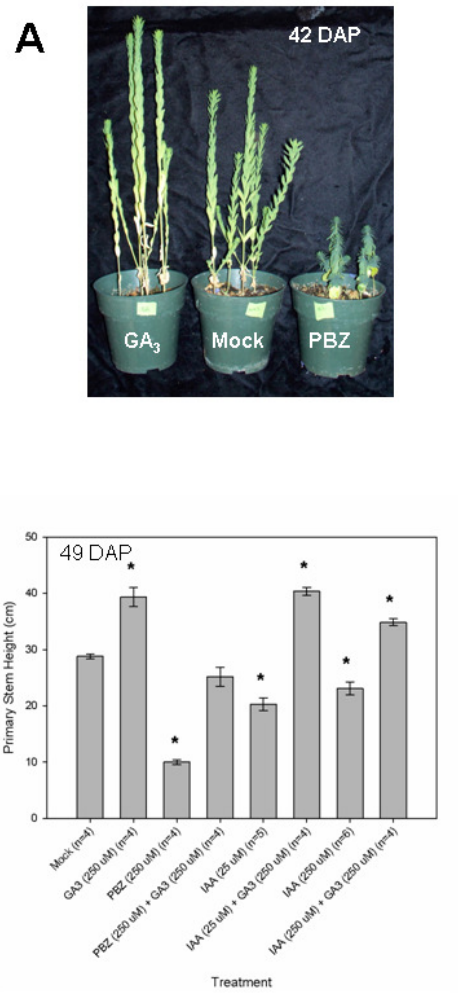

Figure 2-1. Appearance and primary stem length of treated plants. (A) Appearance of plants treated with the mock, GA_3 and PBZ treatments, 42 days after planting (DAP). (B) Primary stem length of plants sprayed on a weekly basis with plant growth regulators, measured 49 DAP. The symbol * indicate groups of treated plants that significantly differed ($p<0.05$) from the mock-treated plants. Error bars denote the standard error for each set of measurements.

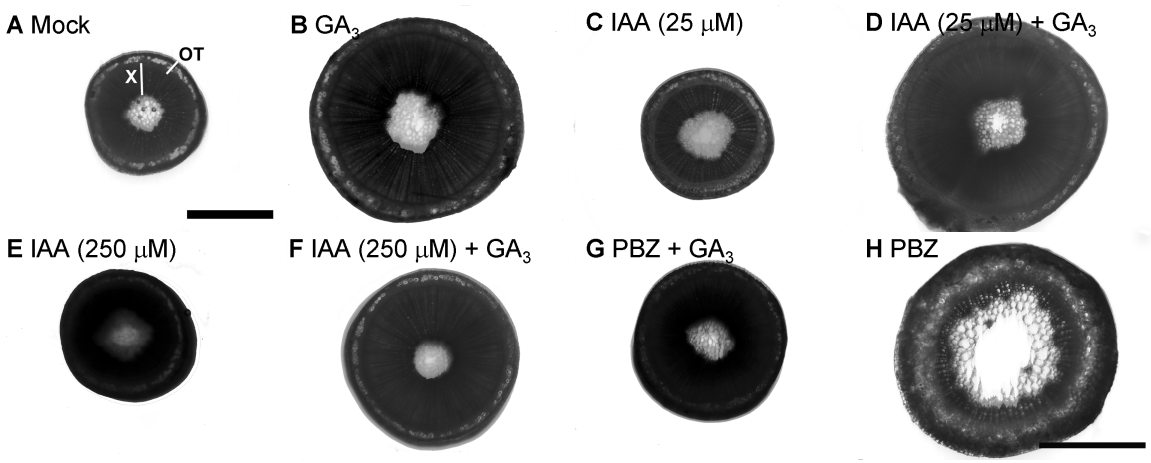

Figure 2-2. Representative transverse-sections of treated plants, sampled from the first internode at the base of the primary stem. The plants were sectioned 49 days after planting. Bar in A = 1000 μm; this bar applies to all images except H. Bar in H = 500 μm. Symbols: X = xylem, OT = outer tissue.

Table 2-1. Stem vascular tissue properties for treated plants. Measurements are shown of the xylem and outer tissue radii, stem diameter, xylem and outer tissue radii divided against the stem diameter, number of xylem cells across the xylem radius and density of xylem cells. Averages of all measurements are shown along with their standard error values. The symbol * denotes measurements that were determined to significantly differ (p<0.05) from the mock-treatment.

Measurement	**Treatment**							
	Mock (n=4)	GA_3 (250 µM) (n=4)	PBZ (250 µM) (n-4)	PBZ (250 µM) + GA_3 (250 µM) (n=4)	IAA (25 µM) (n=5)	IAA (250 µM) (n=6)	IAA (25 µM) + GA_3 (250 µM) (n=4)	IAA (250 µM) + GA_3 (250 µM) (n=4)
Xylem Radius (µm)	312.5 +/- 10.9	669.2 +/- 11.4*	109.8 +/- 9.9*	489.3 +/- 11.6*	295.1 +/- 7.0	253.1 +/- 22.6	804.5 +/- 21.6*	635.1 +/- 17.4*
Outer Tissue Radius (µm)	204.6 +/- 4.9	214.5 +/- 2.3	226.1 +/- 12.5	195.6 +/- 5.8	180.9 +/- 4.9	207.4 +/- 10.4	236.2 +/- 4.7	198.1 +/- 4.6
Stem Diameter (µm)	1590.0 +/- 51.9	2410.4 +/- 82.6*	1244.1 +/- 53.8	2013.9 +/- 127.6*	1553.3 +/- 108.0	1702.5 +/- 116.6	2829.0 +/- 134.3*	2174.2 +/- 53.9*
Xylem Radius/Stem Diameter	0.20 +/- 0.01	0.28 +/- 0.01*	0.09 +/- 0.004*	0.24 +/- 0.004*	0.19 +/- 0.01	0.15 +/- 0.01*	0.28 +/- 0.01*	0.29 +/- 0.01*
Outer Tissue Radius/Stem Diameter	0.13 +/- 0.003	0.09 +/- 0.002*	0.18 +/- 0.01*	0.10 +/- 0.01*	0.12 +/- 0.01	0.12 +/- 0.01	0.08 +/- 0.004*	0.09 +/- 0.01*
Number of Xylem Cells Across Xylem Radius	17.5 +/- 0.5	25.75 +/- 1.1*	7.0 +/- 0.4*	20.8 +/- 1.8	14.8 +/- 0.8	14.3 +/- 0.9	29.3 +/- 2.3*	27.0 +/- 2.3*
Xylem Cellular Density (Cells/mm Xylem Radius^2)	4.99 +/- 0.12	2.77 +/- 0.06*	6.09 +/- 0.17*	3.58 +/- 0.04*	5.18 +/- 0.12	5.05 +/- 0.16	3.16 +/- 0.18*	3.17 +/- 0.19*

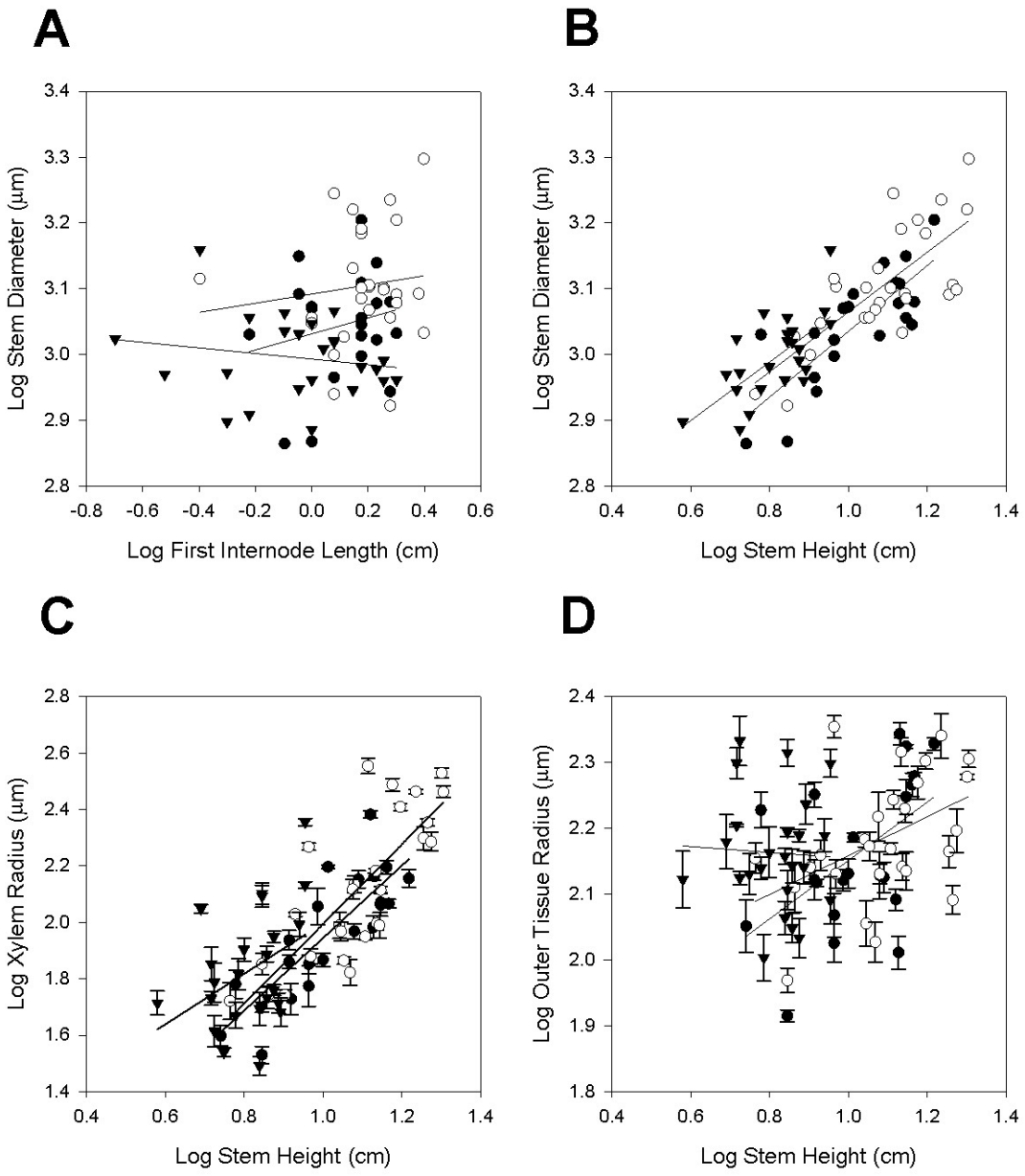
A
Log Stem Diameter (µm)
Log First Internode Length (cm)
B
Log Stem Diameter (µm)
Log Stem Height (cm)
C
Log Xylem Radius (µm)
Log Stem Height (cm)
D
Log Outer Tissue Radius (µm)
Log Stem Height (cm)

Figure 2-3. Analysis of allometric relationships. (A) Length of the first internode at the base of the primary stem vs the stem diameter. (B) Height of the primary stem vs the stem diameter. (C) Xylem radius vs the stem diameter. (D) Outer tissue radius vs the stem diameter. All regression plots are expressed on a $\log_{10}$ scale. Symbols: ● = measurement from a mock-treated plant; ○ = measurement from a GA_3-treated plant; ▼= measurement from a PBZ-treated plant.

Table 2-2. Average xylem and bast fibre lengths, sampled from stem macerates. Average bast and xylem fibre lengths for each plant were determined from 20 xylem and 10 bast fibres; n = 4 plants per treatment. Counts are also shown for the number of bast fibres per fibre bundle, and number of fibre bundles observed per stem cross-section. These values were determined from cross-sections taken within approximately the seventh internode above the base of the primary stem. The total number of fibres per stem cross-section was estimated by multiplying the measurements of the number of bast fibres per fibre bundle and the number of fibre bundles per cross-section together. All measurements are presented along with their standard error values. The symbol * denotes measurements that significantly differed (p<0.05) from the mock-treatment.

Measurement	**Treatment**							
	Mock	GA_3 (250 µM)	PBZ (250 µM)	PBZ (250 µM) + GA_3 (250 µM)	IAA (25 µM)	IAA (250 µM)	IAA (25 µM) + GA_3 (250 µM)	IAA (250 µM) + GA_3 (250 µM)
Xylem Fibre Length (mm)	0.57 +/- 0.05	0.53 +/- 0.03	0.41 +/- 0.06*	0.52 +/- 0.04	0.50 +/- 0.05	0.50 +/- 0.04	0.52 +/- 0.04	0.53 +/- 0.05
Bast Arithmetic Fibre Length (mm)	7.8 +/- 2.0	9.5 +/- 2.6	3.6 +/- 0.7*	8.9 +/- 2.8	6.9 +/- 1.4	7.5 +/- 2.3	9.2 +/- 2.9	10.1 +/- 2.6
Bast Fibres per Fibre Bundle	15.2 +/- 0.7	17.4 +/- 0.6	11.2 +/- 2.0*	11.7 +/- 1.5	16.4 +/- 0.2	12.6 +/- 0.8	15.5 +/- 1.3	17.4 +/- 0.4
Fibre Bundles per Stem Cross Section	31.4 +/- 0.6	34.7 +/- 1.1	30.4 +/- 0.7	31.7 +/- 0.3	29.5 +/- 2.5	27.3 +/- 2.1	32.3 +/- 1.3	34.3 +/- 2.0
Fibres per Stem Cross Section	474.6 +/- 23.8	597.0 +/- 29.3*	335.0 +/- 52.8*	369.4 +/- 50.5	485.4 +/- 48.0	344.6 +/- 41.9	501.9 +/- 30.5	599.0 +/- 40.9

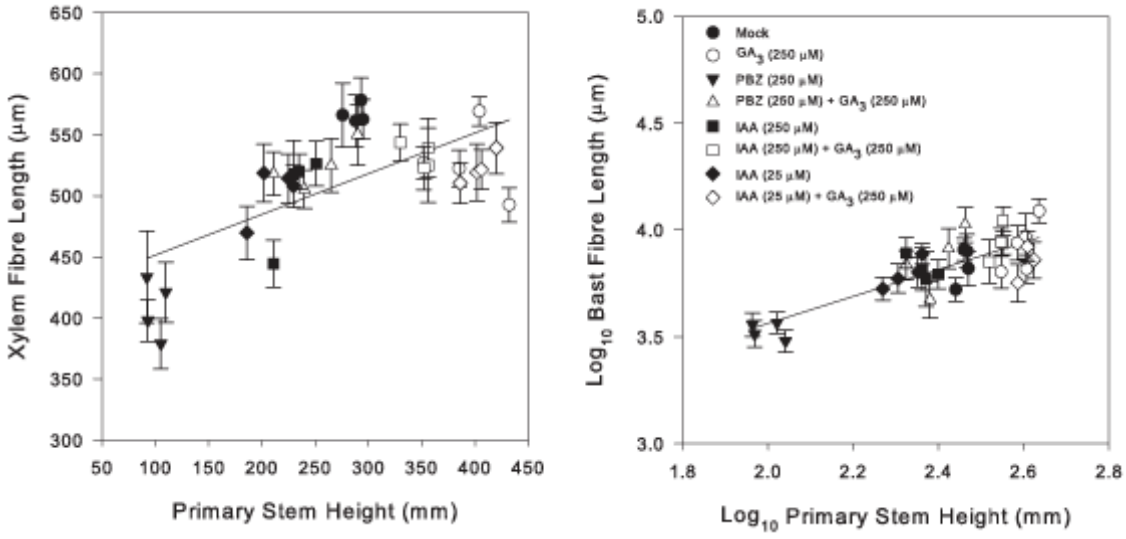

Figure 2-4. Correlation analysis. Correlation between the average arithmetic (A) xylem and (B) bast fibre lengths and the height of the primary stem.

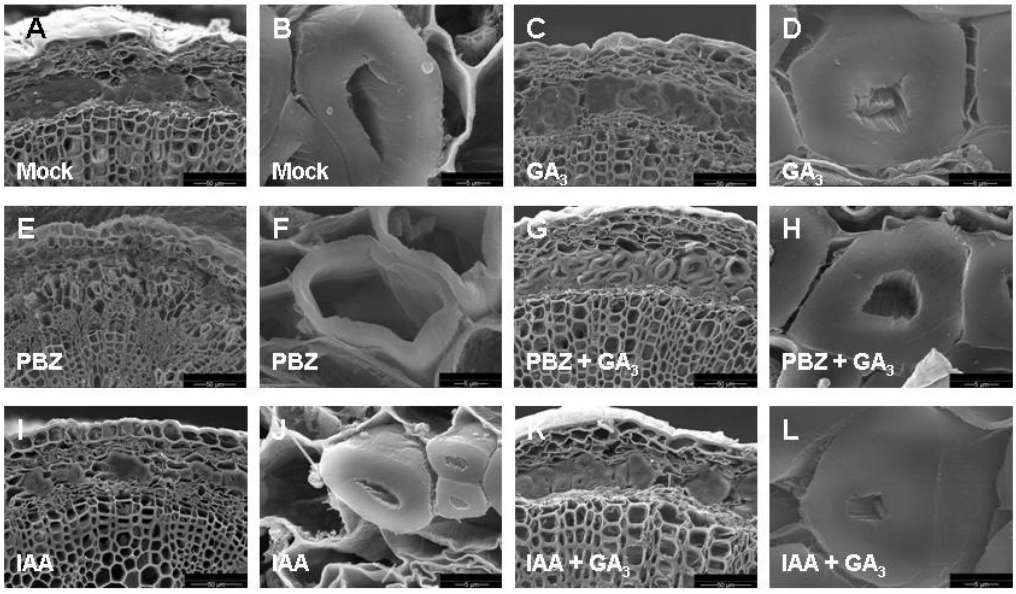

Figure 2-5. Representative examples of fibre bundles and individual bast fibres, viewed within cross-sections taken at the midstem using a scanning electron microscope. (A-B) plants treated with the mock-treatment; (C-D) plants treated with 250 µM GA_3; (E-F) plants treated with 250 µM PBZ; (G-H) plants treated with both 250 µM PBZ and 250 µM GA_3; (I-J) plants treated with 250 µM IAA; (K-L) plants treated with both 250 µM IAA and 250 µM GA_3. Scale bars for each image are displayed on the bottom right. n=3 plants per treatment.

Table 2-3. Bast and xylem fibre cell wall width and bast fibre cellular area. Measurements were made of fibres that were viewed using scanning electron microscopy. Measurements of all treated plants were compared to measurements of the mock-treated plants using a one-way analysis of variance test, followed by Dunnett's *post-hoc* test. The symbol * denotes measurements that were determined to be significantly different ($p<0.05$) compared with the mock-treatment.

Measurement	**Treatment**					
	Mock	GA_3 (250 µM)	PBZ (250 µM)	PBZ (250 µM) + GA_3 (250 µM)	IAA (250 µM)	IAA (250 µM) + GA_3 (250 µM)
Xylem Fibre Cell Wall Width (µm)	0.9 +/- 0.1	1.3 +/- 0.2*	0.8 +/- 0.1	1.3 +/- 0.2	1.4 +/- 0.3*	1.5 +/- 0.3*
Bast Fibre Cell Wall Width (µm)	3.8 +/- 0.6	5.2 +/- 0.8	1.5 +/- 0.2	5.0 +/- 0.8	3.9 +/- 0.6	7.3 +/- 1.1*
Bast Fibre Area (μm^2)	207.1 +/- 53.0	267.8 +/- 67.2	86.4 +/- 27.1	274.1 +/- 73.0	128.8 +/- 33.0	366.8 +/- 100.4

3. Expression Patterns of GA- and Auxin-Responsive Genes in Flax Stems (*Linum usitatissimum* L.)

3.1 Introduction

Linum usitatissimum L. has been cultivated as a source of both oil and fibre for several millennia. The long and strong bast (phloem) fibres of fibre flax varieties of *L. usitatissimum* are woven into textiles. Linseed flax varieties are cultivated for their seed oil, which is rich in α-linolenic acid, an omega-3 fatty acid (Foster et al., 2009). In 2009, the top five producers of linseed were Canada, China, the USA, India and Russia (FAO, 2009). 45% of world linseed production occurred in Canada in 2009, where linseed flax was harvested from 623,300 ha of land (FAO, 2009). Bast fibres of *L. usitatissimum* are part of the primary vascular tissues of the stem; unlike hemp, kenaf, and other bast fibre crops, there are no secondary bast fibres in flax or linseed (Esau, 1943).

Experiments involving the application of gibberellic acid (GA_3) (Bradley and Crane, 1957; Wareing, 1958; Stant, 1961; El-Shourbagy et al., 1995; Björklund et al., 2007), applications of inhibitors of gibberellin (GA) biosynthesis (Jacyna and Dodds, 1995; Ridoutt et al., 1996; Bai et al., 2004), and the overexpression of components of gibberellin biosynthetic and signalling pathways (Eriksson et al., 2000; Biemelt et al., 2004; Mauriat and Moritz, 2009) have supported roles for GA as a regulator of cambial activity and the elongation of xylem and phloem fibre cells. Concentration of the bioactive GAs GA_4 and GA_1 peak in the elongating/expanding xylem cells of aspen (*Populus tremula*) stems, while only trace concentrations of bioactive GAs were detectable either in the

dividing cambium or in xylem tissue that is spatially associated with secondary cell wall formation and lignification (Israelsson et al., 2005; Björklund et al., 2007). Correspondingly, the putative orthologues of several genes coding for GA biosynthetic enzymes and putative GA signalling genes show radial expression patterns that are consistent with the bioactive GA concentration profile (Israelsson et al., 2005).

Like GA, auxin (indole-3-acetic acid; IAA) has also been proposed to regulate vascular tissue differentiation. It has been proposed that IAA is transported in an apical-basal direction through the stem from the tissues where it is biosynthesized – typically apical meristems and lateral organs – downward through the cambium, and that the lateral transport of IAA away from the cambium forms a concentration gradient (Schrader et al., 2003). IAA concentrations are highest in the cambium, while concentrations gradually decrease through the surrounding xylem and sharply decrease through the surrounding phloem as the distance from the cambium increases (Sundberg et al., 2000). Given that the cambium has a low concentration of bioactive GA and high concentration of IAA, it has been proposed that IAA acts as the primary regulator of cambial activity, while GA primarily acts as a regulator of fibre cell elongation (2005; Björklund et al., 2007; Mauriat and Moritz, 2009). However, GA is thought to indirectly stimulate cambial divisions through its cross-talk with IAA signalling genes; this cross-talk been proposed to cause increased polar auxin transport through the cambium, consequentially stimulating cambial divisions (Björklund et al., 2007).

Spray treatments with GA_3 and IAA under both field and greenhouse conditions have been reported to lead to increased fibre yield and improved fibre properties in two fibre flax varieties and one dual-purpose variety (El-Shourbagy et al., 1995; Ayala-Silva et al., 2005). However, while most observations reported in these previous studies were congruent, some observations conflict. In an attempt to further clarify the relationship between plant growth regulator treatment and fibre properties, we investigated the effects of applying GA_3, IAA and a GA biosynthesis inhibitor (paclobutrazol) via spray treatments to an elite linseed variety (Chapter 2; McKenzie and Deyholos, 2011). GA was demonstrated to positively regulate stem elongation, xylogenesis and xylem fibre expansion in linseed (Chapter 2; McKenzie and Deyholos, 2011). GA was also demonstrated to stimulate a modest increase in the number of bast fibres in transverse sections of the stem, presumably due to a moderate increase in bast fibre elongation (Chapter 2; McKenzie and Deyholos, 2011). Unlike GA, IAA treatments were not determined to significantly affect most stem properties examined (Chapter 2; McKenzie and Deyholos, 2011). However, a synergistic positive effect of GA and IAA was observed on the expansion of the bast fibre cell wall (Chapter 2; McKenzie and Deyholos, 2011).

To further clarify the role that IAA and GA play in the regulation of flax stem growth and development, expression profiles are reported here for five genes. The first of these five genes characterized, *LuGAST1*, is a putative member of the GAST/GASA gene family. Members of the GAST/GASA family have been characterized from several plant species, including *Arabidopsis*, tomato (*Solanum*

lycopersicum), potato (*Solanum tuberosum*), strawberry (*Frageria x ananassa*), Gerbera (*Gerbera hybrida*) and Petunia (*Petunia x hybrida*) (Shi et al., 1992; Herzog et al., 1995; Ben-Nissan and Weiss, 1996; Aubert et al., 1998; Shi and Olszewski, 1998; Kotilainen et al., 1999; Segura et al., 1999; Berrocal-Lobo et al., 2002; Ben-Nissan et al., 2004; de la Fuente et al., 2006; Roxrud et al., 2007; Zhang et al., 2009). Most members of this family have been determined to be GA responsive, while expression and mutant analyses have suggested a possible association of GAST/GASA genes in cell division or cell elongation, flower, root and fruit development, defense and/or stress-signalling responses (Zhang et al., 2009). *LuGAST1* was initially isolated from an EST library from flax outer stem tissues (Roach and Deyholos, 2007). The transcript abundance of *LuGAST1* (referred to by Roach and Deyholos as a 'GASA5-like protein') was enriched in hypocotyls sampled 15 days after planting (DAP) relative to 7 DAP and 9 DAP hypocotyls (Roach and Deyholos, 2008). The three hypocotyl developmental timepoints examined by Roach and Deyholos (2008) represent different stages in bast fibre development (7 DAP, fibre elongation; 9 DAP completion of fibre elongation and onset of cell wall expansion; 15 DAP late cell wall expansion). Given the possible relationship of *LuGAST1* with the GA response pathway and its possible involvement in bast fibre development, further profiling of the *LuGAST1* gene expression pattern was pursued.

The second and third genes analyzed in this study, *LuGA3ox1* and *LuGA2ox1*, putatively encode GA 3 oxidase (GA 3 β-hydroxylase) and GA 2 oxidase (GA 2 β-hydroxylase) enzymes that respectively catalyze the final step in

the conversion of biologically-inactive GA precursors into bioactive forms of GA and the conversion of bioactive GAs into biologically-inactive catabolites (Hedden and Phillips, 2000). Feedback of GA on the transcription of GA 2 oxidase and GA 3 oxidase genes has been documented in other plant species (Hedden and Phillips, 2000; Olszewski et al., 2002). As both genes are potential markers for GA responsiveness, the expression profiling of *LuGA2ox1* and *LuGA3ox1* was initially pursued in an attempt to identify experimental conditions under which the GA responsiveness of other genes could be suitably tested.

LuIAA1 putatively encodes a member of the Aux/IAA family of transcription factors, which negatively regulate auxin responses (Vanneste and Friml, 2009). Aux/IAA genes have been reported to be stimulated very shortly following IAA treatment (Theologis et al., 1985; Abel et al., 1995). In our hands, exogenous IAA had been previously observed to have relatively weak effects on flax stem properties (Chapter 2; McKenzie and Deyholos, 2011). Hence, the expression profiling of *LuIAA1* was pursued because the gene is a potential marker for IAA-responsiveness, and a response would confirm that the IAA treatments were bioactive.

Finally, *LuPIN1* putatively encodes a member of the PIN family of functional auxin efflux transporters (Petrásek et al., 2006). Björklund et al. (2007) determined that local GA_3 application to hybrid aspen (*Populus tremula x tremuloides*) stems weakly stimulated *PttPIN1* expression in the stem, which they proposed as evidence for an increase in polar auxin transport. *LuPIN1* was

characterized primarily to determine whether a similar effect might occur in the flax stem following GA_3 treatment.

Experimental conditions were chosen for this study that would enable the relationship to be examined between transcript expression, tissue type, developmental stage, and exogenous plant growth regulator treatments. The gene expression responses of the five genes were generally consistent with expectations. Novel insight has also been gained relating to the development of the flax stem, as the expression profiling revealed a potential role for *LuGAST1* in regulating the differentiation of flax bast (phloem) fibre cells. Overall, the data reported here provides a basis for understanding how responses to gibberellins and auxins are regulated at the transcriptional level in linseed flax, and provides valuable information that will aid future attempts to understand the development of vascular tissues, and particularly phloem fibres.

3.2 Materials and Methods

3.2.1 Plant Material

Experiments were conducted in the *Linum usitatissimum* L. flax variety CDC Bethune (Rowland et al., 2002). CDC Bethune is a linseed flax variety that is grown primarily for its seed oil and is currently the most popular flax variety grown in western Canada. Plants were grown in Metromix 360 (Scotts, Maryland, OH), planted in round pots (7 cm height, 9.5 cm diameter at the top) to a depth of approximately 1 cm, at a density of 4-6 seeds per pot. The plants were grown in controlled environment chambers at 24°C with 50% humidity, and a light intensity

of 200 µE supplied by high output fluorescent bulbs (CRI of 85, colour temperature of 3,500 K) on a 16 h light/8 h dark cycle.

3.2.2 Gene Identification and Multiple Sequence Alignments

The sequence of *LuGAST1* corresponds to probeset 138 from a previously described cDNA library (Roach and Deyholos, 2007, 2008). Nucleotide sequences for the open reading frames (ORFs) of the hybrid aspen *PttGA2ox1, PttGA3ox1, PttPIN1* and *Arabidopsis AtIAA1* genes were aligned against flax genomic sequences from a whole-genome shotgun assembly (http://www.linum.ca; M. Deyholos, manuscript in preparation) using the BLASTX tool (Altschul et al., 1990) in order to identify putative flax orthologues. ORF predictions for the flax sequences were determined using the Augustus Gene Prediction tool (Stanke and Morgenstern, 2005). ORFs were aligned against Genbank using BLASTX in order to confirm their identities.

Multiple sequence alignments were completed using ClustalX, version 2.1 (Larkin et al., 2007). Phylograms were prepared in ClustalX using the Phylip package (Felsenstein, 1981). The neighbour-joining method was used for phylogenetic tree construction, using random number generator seed of 111 and 1000 bootstrap trials. Phylogenetic trees were drawn using TreeView version 1.6.6 (Page, 1996).

3.2.3 Plant Growth Regulator Treatments

Growth regulator treatments were applied to foliage as a spray. Treatments were freshly prepared prior to each treatment. Gibberellic acid (GA_3; Sigma, St.

Louis, MO) and Indole-3-Acetic Acid (IAA; Sigma, St. Louis, MO) were dissolved in 95-100% ethanol to produce a 100 mM stock solution; stock solutions were diluted in Milli-Q water to a 250 µM final concentration. Paclobutrazol (trade name Bonzi®; Syngenta Professional Products, Greensboro, NC) was obtained as a 4 g/L (13.8 mM) solution, and diluted in water to 250 µM. 0.05% Tween-20 was added to all of the treatment solutions as a surfactant.

3.2.4 Quantitative Real-Time PCR (qRT-PCR)

RNA was extracted using the Qiagen RNeasy Plant Mini Kit (Qiagen, Germantown, MD). cDNA was synthesized using Revertaid® H Minus M-MuLV reverse transcriptase (Fermentas, Glen Burie, MD) and oligo-dT_{18} primers (Fermentas), following the manufacturer's protocol. The Primer Express software (Applied Biosystems) was used to design gene-specific primers (Table 1). Real-time PCR was conducted using the Applied Biosystems 7500 Fast Real-Time PCR System. 2.5 µL of a 1/400 dilution of the reverse transcription reaction was used in a total volume of 10 µL, with 0.4 µM of each forward and reverse gene-specific primer, 0.2 µM dNTPs, 0.25X SYBR Green, 1X ROX and 0.075U Platinum Taq (Invitrogen). Threshold values (C_T) were determined using 7500 Fast software.

qRT-PCR was employed for a number of separate experiments, with the tissue samples obtained as follows: (i) for short term experiments, tissue samples were obtained from the mid-stem or stem apex of previously untreated plants sampled 28 days after planting (DAP), at varying timepoints following treatment application; (ii) for long term experiments, tissue samples were obtained from the

mid-stem at 28 DAP from plants that had been treated 14 and 21 DAP; (iii) stem tissues from plants grown and treated as in (ii) were separated into "inner" (inside the cambium) and "outer" (outside the cambium) tissue fractions; (iv) mid-stem and stem apex tissues from untreated plants were sampled 28 DAP; (v) mid-stem tissues were sampled from untreated plants 14, 28 and 42 DAP.

Gene expression levels determined by qRT-PCR were measured using the relative quantification method (Livak and Schmittgen, 2001). C_T values were normalized against the C_T values for the elongation factor 1-α (*EF1α*) reference gene (see Table 1 for primer sequences). In order to validate the suitability of *EF1α* as a reference, an experiment was conducted in which the raw C_T values for *EF1α* were also measured simultaneously on cDNA samples from all biological replicates that were obtained for each expression profiling experiment. Where appropriate, either a one-way ANOVA or two-way *t*-test was used to determine whether these raw C_T values significantly varied among treatments; the absence of significant variation ($p > 0.05$) supported the suitability of *EF1α* as a reference gene. The primers for *LuGAST1*, *LuGA2ox1*, *LuGA3ox1*, *LuIAA1* and *LuPIN1* were also quantified over a range of cDNA dilutions to confirm that their primer efficiencies amplified at an equivalent efficiency to primers for *EF1α*.

For each analysis reported, statistical significance was tested against delta-C_T values using either a one-way ANOVA followed by Dunnett's or Tukey's *post-hoc* test or a two-way *t*-test, as appropriate. For ANOVAs, variances were tested for homogeneity using Bartlett's test and data was tested for normality using the Anderson-Darling test. F-tests for variance were conducted prior to each

t-test to determine whether a *t*-test assuming equal or unequal variance would be appropriate. ANOVA and follow-up tests were conducted in R (R Development Core Team, 2009), while *t*-tests were conducted in Microsoft Excel.

3.3 Results

3.3.1 Multiple Sequence Alignments

The predicted amino acid sequence for LuGAST1 aligns closely to AtGASA5 (64% identity), the tomato (*Solanum lycopersicum*) SlRSI-1 (67% identity) and SlGAST1 sequences (56% identity) and petunia (*Petunia hybrida*) PhGIP1 and PhGPI2 sequences (58% identity to both) (Figure 3-1; see Appendix for relevant multiple sequence alignments). The predicted amino acid sequence for *LuGA2ox1* is most closely aligned among hybrid aspen sequences with PtGA2ox3 (68% identity) and PtGA2ox1 (62% identity), while among *Arabidopsis* sequences it aligns well to AtGA2ox1 (58% identity), AtGA2ox2 (59% identity) and AtGA3ox3 (56% identity) (Figure 3-2). The predicted amino acid sequence for *LuGA3ox1* aligns to the hybrid aspen PttGA3ox1 sequence (69% identity), and more distantly related to the *Arabidopsis* AtGA3ox1 (58% identity) and AtGA3ox2 (57% identity) sequences (Figure 3-3). The predicted *LuIAA1* sequence most closely aligns to PoptrIAA3.6 (67% identity) and PoptrIAA3.5 (64% identity), and among *Arabidopsis* sequences is closest to the AtIAA1-4 sequences (58-64% identity) (Figure 3-4). Among *Arabidopsis* and *Populus trichocarpa* PIN protein sequences, the predicted LuPIN1 sequence most closely aligns with the PtPIN1 and PtPIN7 sequences (78% identity) and

Arabidopsis AtPIN1 sequence (74% identity) (Figure 3-5, see Appendix for multiple sequence alignments).

3.3.2 Short Term Responses to Plant Growth Regulators

For the GA_3 treatment, the transcript abundance of *LuGA3ox1* was decreased relative to the mock treatment in all short-term responses tested (Figure 3-6 A, C). These differences were determined to be significant ($p<0.05$) for stem apex samples tested 4 and 8 hours after treatment. For the PBZ treatment, the expression of most genes did not significantly differ in PBZ-treated plants relative to mock-treated plants (Figure 3-6 B, D). An exception to this was a significant increase in *LuGAST1* transcript abundance in stem apex tissue from PBZ-treated plants that were sampled 24 hours after treatment. For the IAA treatment, the transcript abundance significantly increased in stem apex tissues from IAA-treated plants for *LuGAST1*, *LuIAA1* and *LuPIN1* and in the mid-stem for *LuIAA1* (Figure 3-6 E).

3.3.3 Long Term Responses to Plant Growth Regulators

The expression of *LuGAST1* and *LuGA2ox1* was not significantly altered in any of the treated plants (Figure 3-7 A). For *LuGA3ox1*, a significant decrease in transcript abundance was observed in plants treated with both PBZ and GA_3. In comparisons of the same tissue type from the GA_3-treated relative to the mock-treated plants, the transcript abundance of *LuGA3ox1* was also determined to be significantly decreased in the inner and outer stem tissues (Figure 3-7 B). The transcript abundance of *LuIAA1* significantly decreased in IAA-treated plants,

while the transcript abundance of *LuPIN1* significantly decreased in plants treated with GA_3, PBZ, and IAA + GA_3.

3.3.4 Spatial Variation in Transcript Abundance

The transcript abundance of *LuGA2ox1* significantly increased in the inner stem tissue of mock-treated plants relative to the outer stem tissues (Figure 3-7 C). The transcript abundance of *LuGAST1*, *LuIAA1* and *LuPIN1* was determined to be significantly increased in the midstem relative to the stem apex (Figure 3-8 A), while the transcript abundance of *LuGA2ox1* was determined to be significantly decreased in the midstem relative to the apex.

3.3.5 Temporal Variation in Transcript Abundance

Relative to 14 DAP tissues, the transcript abundance of *LuGAST1* was determined to be significantly increased by 91-fold in 28 DAP tissues and by 25-fold in 42 DAP tissues (Figure 3-8 B). The transcript abundance of *LuGA2ox1 was* significantly increased by 8-fold in 42 DAP tissues relative to 14 DAP tissues (Figure 3-8 C). The transcript abundance of *LuPIN1* did not significantly vary relative to 14 DAP in the 28 DAP and 42 DAP samples; however, the transcript abundance significantly varied, by 4.5-fold, between the 42 and 28 DAP samples.

3.4 Discussion

3.4.1 Short Term Responses to Plant Growth Regulators

A statistically significant positive response by any gene to GA_3 treatment was not documented in any of the tissues examined at any timepoint (Figure 3-1). If the short-term responsiveness to GA_3 is tested in a GA-deficient background, it

may be possible to obtain more conclusive data regarding its GA responsiveness; however, this experiment was not pursued because a flax GA-deficient genotype has not been identified. Substantial transcriptional up-regulation following GA_3 application typically has only been documented in GA biosynthetic mutant backgrounds, as transcriptional responses to GA are generally thought to be near saturation under normal backgrounds (Nemhauser et al., 2006). An illustration of this effect is that the transcript abundance of *SlGAST1* was induced by more than 20-fold in vegetative shoot apices of a tomato GA biosynthetic mutant, but induced only by 1.7-fold in a wild-type background (Shi et al., 1992).

In general, a large number of *GAST/GASA* genes have been documented to exhibit GA-responsiveness, including the tomato *SlGAST* gene, strawberry *FaGAST* gene, *Gerbera hybrida GhGEG* gene, all petunia *PhGIP* genes that have been examined, and the *Arabidopsis AtGASA1* gene (Shi et al., 1992; Herzog et al., 1995; Ben-Nissan and Weiss, 1996; Aubert et al., 1998; Kotilainen et al., 1999; Ben-Nissan et al., 2004; de la Fuente et al., 2006). However, *AtGASA4* expression was determined to be induced by GA_3 in floral buds (Herzog et al., 1995) but repressed in *Arabidopsis* cotyledons and leaves (Aubert et al., 1998), suggesting that responses may also depend on the tissue context that is examined.

If *LuGAST1* is positively regulated by GA levels, it would be expected that the *LuGAST1* transcript abundance should decrease following PBZ treatment. However, the *LuGAST1* transcript abundance significantly increased in PBZ-treated plants 24 hours after treatment. Among the 13 *Arabidopsis* AtGASA protein sequences that the LuGAST1 predicted amino acid sequence was aligned

to, the closest match was to the AtGASA5 sequence (Figure 3-1). Interestingly, the transcript abundance of *AtGASA5* was also shown to weakly decline following GA_3 treatment and to weakly increase following PBZ treatment in *Arabidopsis* seedling tissue, suggesting that it is negatively regulated by GA (Zhang et al., 2009). If *LuGAST1* is also negatively regulated by GA levels, as PBZ-response data suggests, it is not surprising that this trend was not evident 4- and 8-hours after treatment, as a delayed transcriptional response to PBZ would not be unprecedented. Likewise, the protein levels of RGA, an *Arabidopsis* DELLA protein that positively responded to PBZ, did not clearly increase until at least 24 hours after PBZ treatment (Silverstone et al., 2001). It was suggested by Silverstone et al. (2001) that the delay might reflect the time required for PBZ to inhibit GA biosynthesis, as well as the time required for the GA catabolism to reduce the availability of bioactive GAs.

The *LuGA3ox1* transcript abundance significantly decreased in all of the tissues and timepoints following GA_3 treatment, and the differences were determined to be significant for stem apex samples obtained 4 and 8 hours after treatment. These observations are consistent with what has typically been observed for GA 3 oxidase genes in *Arabidopsis* and other plants, and has been explained by negative feedback on GA biosynthesis genes by changes in GA availability (Hedden and Phillips, 2000; Olszewski et al., 2002; Figure 9 A). Conversely, responses by *LuGA3ox1* to PBZ were less apparent. Due to the feedback of GA on its transcription, PBZ treatment might reduce the bioavailability of GA, thus stimulating *LuGA3ox1* transcription in order to

stimulate increased GA biosynthesis. Accordingly, PBZ treatments have been shown to induce *AtGA3ox1* by more than 60-fold in *Arabidopsis* seedlings (Cowling et al., 1998). As described above for *LuGAST1*, the PBZ treatment may require a longer time-period to have an effect on transcription as compared with the GA_3 treatment. Thus, tissue sampling undertaken 24 to 72 hours after PBZ treatment may be required in order to conclusively determine whether PBZ affects the transcription of *LuGA3ox1*.

The transcript abundance of *Aux/IAA* genes tends to increase immediately following IAA treatment (Theologis et al., 1985; Abel et al., 1995). Thus, *LuIAA1* was initially tested in order to determine whether IAA treatments were bioactive. Indeed, the relative expression of *LuIAA1* was significantly higher in both the stem apex and midstem of IAA-treated plants relative to mock-treated plants, confirming that *LuIAA1* is IAA-responsive.

LuPIN1 also demonstrated significantly higher transcript abundance at the stem apex of IAA-treated plants relative to mock-treated plants, in accordance with similar observations for the *Arabidopsis AtPIN1* and hybrid aspen *PttPIN1* genes (Vieten et al., 2005; Nilsson et al., 2008). It was very interesting that the *LuGAST1* transcript abundance was significantly higher at the stem apex in IAA-treated plants. Although the effects of IAA treatment on the expression of *GAST/GASA* genes have not been widely documented, a tomato gene belonging to this family, *RSI-1*, has also been shown to be auxin-inducible (Taylor and Scheuring, 1994).

3.4.2 Long Term Responses to Plant Growth Regulators

Unlike the short term responses, the long term responses were determined from plants that had been repeatedly treated with the growth regulators. As the most recent treatments were applied one week prior to tissue sampling, it might be presumed that the responses would be influenced by changes to the plant morphology and physiology caused by these treatments, and not just a direct reflection of responses to the treatments. Preliminary data for hormonal profiles of the long term samples (National Research Council of Canada – Plant Biotechnology Institute, Saskatoon) shows that the GA_3 contents of plants that had been treated with GA_3, PBZ+GA_3 and IAA+GA_3 remain substantially elevated (data not shown), suggesting that feedback on the GA pathway remains a substantial factor that must be taken into account when explaining the responses.

As with the short term responses, the *LuGA3ox1* transcript abundance tended to be decreased in plants that had been treated with GA_3, with or without other growth regulators (Figure 3-7 A). Likewise, *LuGA3ox1* transcript abundance was determined to be decreased in the inner and outer tissues of GA_3-treated plants relative to mock-treated plants (Figure 3-7 B). These responses likely reflect persistent negative feedback on the GA biosynthesis pathway due to the elevated GA_3 content of the treated plants.

Like *LuGA3ox1*, the transcript abundance of *LuPIN1* was decreased in GA_3- and IAA+GA_3-treated plants (Figure 3-7 A). Levels of *LuPIN1* were also decreased in PBZ-treated plants and trended towards being decreased in plants treated with PBZ+GA_3. Björklund et al. (2007) determined that GA_3 application

could weakly stimulate *PttPIN1* expression. In turn, they propose that increased *PttPIN1* transcript abundance stimulates increased polar auxin transport, which further stimulates *PttPIN1* transcription in the stem (Björklund et al., 2007). The decreased *LuPIN1* transcript abundance in PBZ-treated plants suggests that a slight decrease in GA levels might have a similar, but opposing effect on *LuPIN1* expression in the stem. However, this model would also predict that the GA_3- and IAA+ GA_3-treated plants would show increased *LuPIN1* transcript abundance, whereas the opposite was observed. The discrepancy may be related to the context in which the measurements were made. While stem segments were defoliated during sampling, remaining axillary buds may be significant sources of the *LuPIN1* mRNA. GA_3 treatments stimulate internode elongation, and this may have led to a proportionate reduction in the number of axillary buds in the sample relative to samples from the mock-treated plants, potentially causing a net decrease in the *LuPIN1* transcript abundance. Björklund et al. (2007) applied treatments more locally to the stem, via a lanolin paste, which may have reduced this effect.

The transcript abundance of *LuIAA1* decreased, by nearly 4-fold, in IAA- relative to mock-treated plants (Figure 3-7 A). This response strongly contrasts with the significant increase in the transcript abundance of *LuIAA1* that was observed shortly after treatment (Figure 3-6 E). Models for the auxin response pathway suggest that auxin facilitates the proteolysis of Aux/IAA proteins, relieving their ability to restrain auxin response transcription factors, or ARFs, thus stimulating the transcription of auxin responsive genes (Vanneste and Friml,

2009). Targets of ARFs have been shown to include Aux/IAA genes, explaining why Aux/IAA transcript abundance typically increases shortly following IAA treatment (Theologis et al., 1985; Abel et al., 1995; Berleth et al., 2004). Guilfoyle and Hagen (2001) propose a model in which the induction of Aux/IAA transcription by IAA treatment would cause Aux/IAA protein levels to increase (Figure 3-9 C). As long as the IAA concentration in a tissue remains high, Aux/IAA proteins would be rapidly degraded, causing the induction of Aux/IAA genes to persist. However, when IAA concentrations decrease, Aux/IAA proteins would be degraded less rapidly, thus increasing the likelihood that the transcription of auxin-responsive gene targets, including Aux/IAA genes, would be repressed. The decreased transcript abundance of *LuIAA1* may simply be a reflection of the negative feedback on *LuIAA1* transcription. Indeed, whereas GA_3 levels remain elevated, preliminary data from hormonal profiling revealed that IAA levels do not dramatically differ among IAA-treated plants compared with the mock-treated plants (data not shown), suggesting that IAA levels may have returned to near normal levels by 1 week after treatment.

3.4.3 Spatial Variation in Transcript Abundance

The expression of putative *Populus* orthologues for all of the genes tested in this study have been spatially profiled across *Populus* stems (Moyle et al., 2002; Schrader et al., 2003; Israelsson et al., 2005). The expression profiles reported here for flax are at a much lower spatial resolution, but nonetheless some degree of comparison is possible. A variation in gene expression along the vertical axis of the flax stem is also particularly relevant to understanding how the primary

bast (phloem) fibres form in the stem (Roach and Deyholos, 2007; Figure 9 D), and thus expression levels from the mid-stem and stem apex were compared. Samples examined in this study for the stem apex correspond to the 'TOP' samples analyzed by Roach and Deyholos (2007), while mid-stem material was sampled midway between the locations of the 'MIDDLE' and 'BOTTOM' samples (Figure 3-8 A).

The transcript abundance of *LuGAST1* did not differ between either the inner or the outer stem tissues (Figure 3-7 C). Conversely, the expression of a hybrid aspen GAST gene, *PttGIP-like1,* increased in the expanding xylem (Israelsson et al., 2005). The similarity of *LuGAST1* across stem tissues suggests that it may have a role both inside the cambium, perhaps in the expanding xylem, as well as outside the cambium. The observation that *LuGAST1* transcripts were more abundant in the midstem than in the apex (Figure 3-8 A) may then be consistent with it having a role in late stages of bast fibre differentiation.

LuGA2ox1 was found to show significantly higher expression in the inner tissues relative to the outer tissues in mock-treated plants (Figure 3-7 C). A similar trend was also potentially apparent for GA_3-treated plants. Israelsson et al. (2005) reported that *PttGA2ox2* transcript abundance increased inside the cambium, particularly in expanding xylem cells. Israelsson et al. (2005) also determined that levels of bioactive GAs and their catabolites are spatially expressed across the stem in accordance with the *PttGA2ox2* profile. Thus, the increase in *LuGA2ox1* abundance inside the cambium suggests that the deactivation of endogenous GAs through 2β-hydroxylation may occur to a high

degree within these tissues. The *LuGA2ox1* transcript abundance was lower in the midstem than in the stem apex (Figure 3-8 A). However, the functional significance, if any, of variation in the relative levels of *LuGA2ox1* and *LuGA3ox1* above and below the snap-point is unclear.

LuGA3ox1 transcript abundance did not significantly differ between the inner and outer tissues (Figure 3-7 C). In hybrid aspen, the transcript abundance of *PttGA3ox1* tended to be high in the phloem, although in one of three samples tested a secondary peak in expression was also observed in the xylem (Israelsson et al., 2005). The substrates for GA 3 oxidase, GA_9 and GA_{20}, were determined to be highly prevalent in the phloem, while the products of GA 3 oxidase, GA_4 and GA_1, were prevalent in the xylem (Israelsson et al., 2005). Israelsson et al. (2005) propose that the GA precursors might be synthesized in the phloem and then transported through the symplast to the expanding xylem, where they are converted into bioactive GAs (Figure 3-9 B). In *Arabidopsis* embryos, *AtGA3ox1* and *AtGA3ox2* have also been found to be highly expressed in the cortex and endodermis (Yamaguchi, 2008). Thus, the relatively equivalent transcript abundance of *LuGA3ox1* in the inner compared with the outer tissues might be explained by expression both inside the cambium, perhaps in the expanding xylem, and outside the cambium, perhaps in the cortex.

Moyle et al. (2002) measured the transcript abundance in different tissues across hybrid aspen stems of eight putative Aux/IAA orthologues. The most closely-related of these sequences to *LuIAA1*, *PttIAA8* (*PoptrIAA3.2*), was determined to be most strongly expressed in the expanding xylem (Moyle et al.,

2002). Unlike the analysis by Israelsson et al. (2005), Moyle et al. (2002) tested expression levels in the cortex and phloem fibres, and thus the skewed expression profile that they observed likely should correspond to higher transcript abundance in the inner relative to the outer tissues assuming that the same pattern held true for *LuIAA1*. Kalluri et al. (2007) completed a genome-wide analysis of the Aux/IAA family in black cottonwood (*Populus trichocarpa*), and determined that a *P. trichocarpa* homologue of *PttIAA8* (*PoptrIAA3.2*) is one of six putative *Populus* orthologues of the *Arabidopsis AtIAA3* gene. Kalluri et al. (2007) found that *PttIAA8/PoptrIAA3.2* ESTs were prevalent in the cambial zone. ESTs for the genes that most closely align with *LuIAA1*, *PoptrIAA3.5* or *PoptrIAA3.6* (Figure 3-4), were detected in the active cambium, petioles and stressed leaves, in the case of *PoptrIAA3.5*, and in senescent leaves and dormant buds, in the case of *PoptrIAA3.6* (Kalluri et al., 2007), suggesting a possible association of *PoptrIAA3.5* with stress responses and *PoptrIAA3.6* with the onset of dormancy.

Finally, *PttPIN1* showed higher expression in inner stem tissue and lower expression in outer stem tissue (Schrader et al., 2003). These observations are consistent with observations that AtPIN1 transcript and protein levels are most prevalent in xylem and cambial cells of the *Arabidopsis* inflorescence stem (Galwëiler et al., 1998). Trends in *LuPIN1* expression also slightly favoured the inner tissue in the mock-treated plants (Figure 3-7 C), suggesting possible concordance with the *PttPIN1* and *AtPIN1* patterns. The *LuPIN1* transcript abundance was also observed to be higher in the midstem relative to the stem

apex (Figure 3-8 A). This pattern is likewise in accordance with expression data for *AtPIN1* (Galwëiler et al., 1998).

3.4.4 Temporal Variation in Transcript Abundance

The transcript abundance of *LuGAST1* was substantially higher, by 91-fold in the 28 DAP stems and 25-fold in 42 DAP stems, compared to the 14 DAP stems. Flax plants have been typically observed to form a snap point – a region on the vertical axis of the stem marking a change in tensile strength – approximately 3 weeks after germination, or 4 weeks after planting (Gorshkova et al., 2003). The stem enters a fast growth stage once the snap point becomes evident, with rapid growth and the presence of the snap point persisting until the onset of flowering, approximately 8 weeks after planting (Gorshkova et al., 2003; Figure 9 E). *LuGAST1* is a potential marker for this transition, as its transcript abundance dramatically increases approximately as the plant enters the fast growth stage. Roach and Deyholos (2008) investigated differences in gene expression between 7 DAP hypocotyls, 9 DAP hypocotyls, which were sampled as the onset of fibre secondary cell wall expansion became apparent, and 15 DAP hypocotyls, which were sampled after a rapid increase in secondary cell wall expansion was apparent. The transcript abundance of *LuGAST1* (described as a GASA5-like protein) was enriched in 15 DAP hypocotyls relative to the other two time-points (Roach and Deyholos, 2008), suggesting that *LuGAST1* expression possibly increases with the onset of secondary cell wall expansion. Gorshkova et al. (2003) determined that bast fibre elongation terminates below the snap point. Taken together, the spatial and temporal expression profiles imply a potential role for

LuGAST1 either in the stimulation of bast fibre secondary cell wall expansion or in the termination of fibre elongation. *LuGAST1* transcript abundance was also determined to increase in the *reduced fibre1* mutant, which possesses shortened bast fibres and a decreased number of bast fibres in stem transverse sections (Chapter 5). This observation further supports the possible involvement of *LuGAST1* in bast fibre development.

Like *LuGAST1*, a significant increase in transcript abundance was also detected for *LuGA2ox1* in 42 DAP relative to the 14 DAP stems (Figure 3-8 C). The increased *LuGA2ox1* abundance would be potentially in accordance with the model proposed by Zhang et al. (2009) proposing that *LuGAST1* is negatively regulated by GA levels. However, the likelihood that the increased *LuGAST1* expression might be related to a modulation in GA biosynthesis is questionable, particularly given that *LuGAST1* is not strongly GA_3-responsive (Figure 3-6 A). Furthermore, the other three genes examined in this study tended to show increased expression in either 28 DAP or 42 DAP stems relative to the 14 DAP stems, including *LuGA3ox1*, whose function opposes that of *LuGA2ox1*. Thus, the functional significance, if any, of any temporal variation in the expression of the other genes remains unclear.

3.5 Conclusions

Short term responses to GA_3 treatment by *LuGA2ox1* and *LuGA3ox1* appear to be consistent with a well-established feedback pathway by GA on its own biosynthesis (Figure 3-9 A). Negative feedback on *LuGA3ox1* transcription appeared to persist in plants treated over a longer term with GA_3. Preliminary data

demonstrating that the GA_3 content is elevated in these tissues suggests that the persistent negative feedback might be explained by the continued high availability of bioactive GAs in the treated stems. The spatial profiling showed that *LuGA2ox1* transcript abundance is increased inside the cambium, in accordance with observations for its putative hybrid aspen orthologue and a proposed model for the spatial distribution of GA biosynthesis in the stem (Figure 3-9 B).

LuIAA1 was determined to be closest to the *Arabidopsis* AtIAA1, AtIAA2, AtIAA3 and AtIAA4 sequences and to the *P. trichocarpa* PoptrIAA3.5 and PoptrIAA3.6 sequences. The transcript abundance of *LuIAA1* was significantly higher in IAA- relative to mock-treated plants in the short term experiment, whereas the transcript abundance of *LuIAA1* was significantly decreased in IAA- relative to mock-treated plants for the long term experiment. The short term induction of *LuIAA1* is consistent with typically-described responses by Aux/IAA genes. Long term responses by Aux/IAAs have not been reported, but the decreased transcript abundance of *LuIAA1* is consistent with models predicting negative feedback on Aux/IAA expression as IAA levels decrease (Figure 3-9 C).

In accordance with observations for its putative orthologues, *AtPIN1* and *PtPIN1*, *LuPIN1* demonstrated short term IAA-responsiveness. Long term responses by *LuPIN1* by plant growth regulators trended towards decreased expression relative to the mock-treated plants. The decreased transcript abundance of *LuPIN1* in PBZ-treated plants would be in accordance with observations concerning a cross-talk of GA levels on *PIN1* transcription. However, the response of *LuPIN1* to plants that received GA_3 or IAA+GA_3 does not fit this

model. It is proposed that the discrepancy in the responses may be partly explained by the sampling conditions.

Finally, the transcript abundance of *LuGAST1* increased 24 hours after PBZ treatment, suggesting that it may be negatively regulated by GA. The spatial profiling of *LuGAST1* demonstrated that it was more strongly expressed in mid-stem tissues, where bast fibre secondary cell wall expansion occurs, than at the stem apex, where bast fibre elongation occurs (Figure 3-9 D). The temporal profiling of *LuGAST1* also demonstrated that its transcript abundance in the stem dramatically increases approximately as a snap-point becomes apparent on the plant (Figure 3-9 E), which marks a transition zone between a region of the stem where fibres elongate and a region where elongation ceases and secondary cell wall expansion commences. Taken together with the observation that a putative *Arabidopsis* orthologue of LuGAST1, AtGASA5, is spatially localized to the cell wall/extracellular matrix and that *AtGASA5* has been characterized as a negative regulator of cell elongation, it is proposed that LuGAST1 may have role(s) either as a negative regulator of fibre elongation or as a regulator of secondary cell wall expansion. Future work, potentially including the characterization of *LuGAST1* loss-of-function or gain-of-function lines, should help to confirm whether LuGAST1 has a role in bast fibre differentiation in flax.

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3.7 Tables and Figures

Table 3-1. Sequences of quantitative real-time PCR primers used for all genes investigated in this study. The two closest matches among sequences of flax genomic scaffolds (Michael Deyholos, unpublished) were identified through a BLASTX search of the nucleotide sequence for each gene against predicted flax protein sequences. The e-value of the closest Arabidopsis and *Populus* orthologues were determined through a BLASTX search of the nucleotide sequence of each gene against Genbank.

Gene	Flax Genome Scaffolds	BLASTX e-value against Genbank	F primer sequence (5' to 3')	R primer sequence (5' to 3')	qRTPCR amplicon length
EF1α	scaffold298:51287..53934 (g12711)	2e-82 to Arabidopsis *EF1α* gene	TTG GAT ACA ACC CCG ACA AAA	GGG CCC TTG TAC CAG TCA AG	99
LuGAST1	scaffold587:941128..941812 (g25461)	6e-30 to *AtGASA5*	CTT TCT CCG CCA CCG TTT C	TCG CCG AGC ATC TGT AGT TG	90
LuGA2ox1	scaffold247:319374..320943 g10860)	3e-124 to *Pt2ox1*; 7e-109 to *At2ox1*	CCC GCA ATC GGA GAA AGA C	GAT TCG CTT GTT GCC ATA ACC	64
LuGA3ox1	scaffold2:366036..368753 (g171)	2e-136 to *Pt3ox1* 2e-111 to *AtGA3ox1*	AAC CGG TCC CTG TCA TCG A	CTG CAT GCA TGT CCG ATC TC	64

Gene	Flax Genome Scaffolds	BLASTX e-value against Genbank	F primer sequence (5' to 3')	R primer sequence (5' to 3')	qRTPCR amplicon length
LuPIN1	scaffold845:65426..69501 (g32347)	0.0 to *PttPIN1* and *AtPIN1*	GTT TCC TTC AGG TGG CAC GTA	AGG CCA GCG TCT GAC AGA AT	74
LuIAA1	scaffold212:272504..273978 (g9704)	4e-61 (o *AtIAA4*	CCC TGC TGC TAA GGC ACA GAT	GGC TGC AAG CTA TTC TTC CTG TAG	69

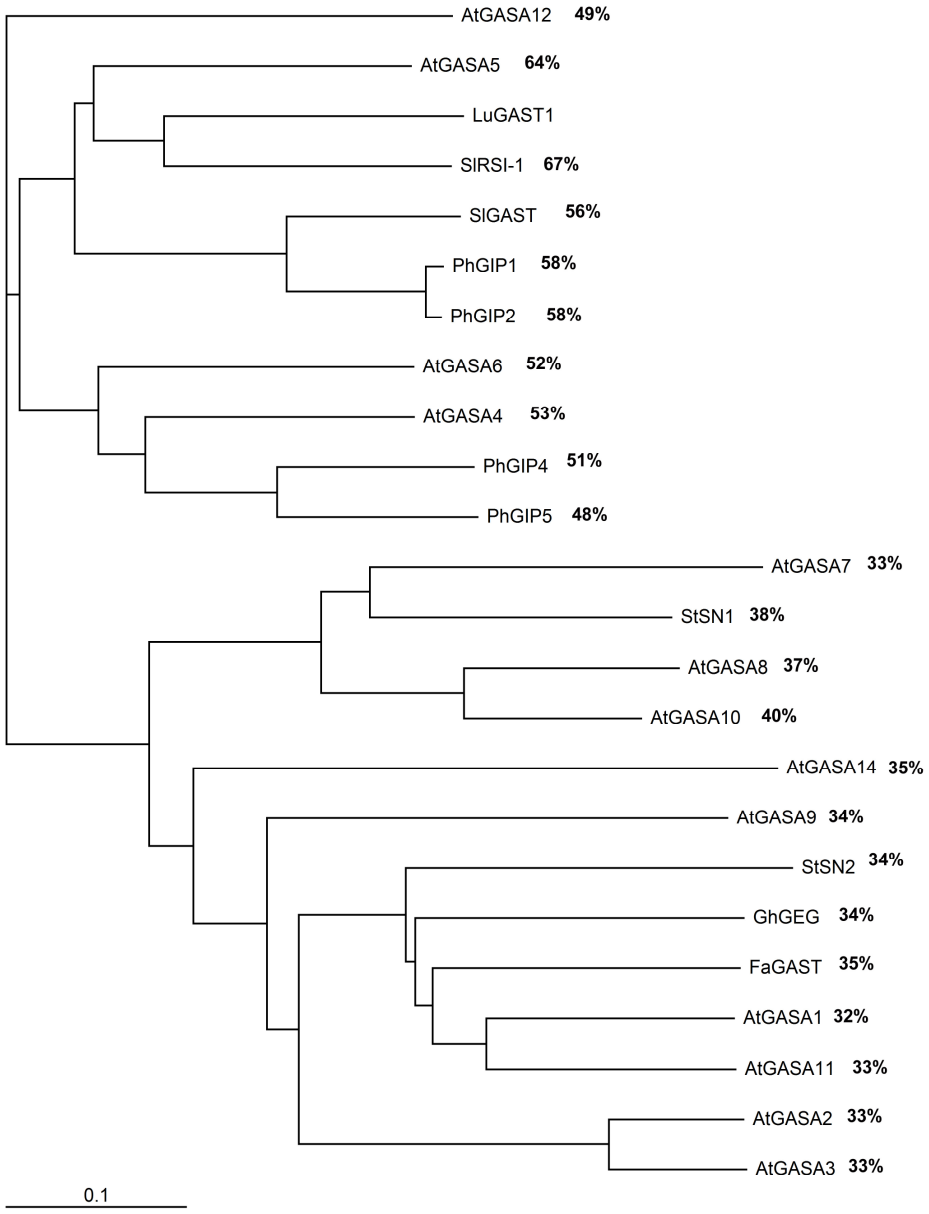
AtGASA12 49%
AtGASA5 64%
LuGAST1
SlRSI-1 67%
SlGAST 56%
PhGIP1 58%
PhGIP2 58%
AtGASA6 52%
AtGASA4 53%
PhGIP4 51%
PhGIP5 48%
AtGASA7 33%
StSN1 38%
AtGASA8 37%
AtGASA10 40%
AtGASA14 35%
AtGASA9 34%
StSN2 34%
GhGEG 34%
FaGAST 35%
AtGASA1 32%
AtGASA11 33%
AtGASA2 33%
AtGASA3 33%
0.1

Figure 3-1. Phylogram for alignments of LuGAST1 with selected GAST/GASA sequences from different plant species. Genbank accession numbers are as follows: *Arabidopsis thaliana*: AtGASA1, NP_001185407; AtGASA2, NP_192699; AtGASA3, NP_192698; AtGASA4, NP_001078587; AtGASA5, NP_566186; AtGASA6, AAS47605; AtGASA7, AEC06348; AtGASA8, AEC09692; AtGASA9, NP_001185066; AtGASA10, NP_568914; AtGASA11, AEC06768; AtGASA12, AAU05509; AtGASA14, BAH56985; *Frageria x ananassa* (strawberry): FaGAST, AAB97006; *Gerbera hybrida*: GhGEG, CAB45241; *Petunia x hybrida*: PhGIP1, CAA60677; PhGIP2, CAD10103; PhGIP4, CAD10105; PhGIP5, CAD10106; *Solanum lycopersicum* (tomato): SlGAST, CAA44807; SlRSI-1, AAA20129; *Solanum tuberosum* (potato): StSN1, ACZ04322; StSN2, ABL74292. The percentage values beside each sequence indicate the percentage of amino acid identity sequence with LuGAST1.

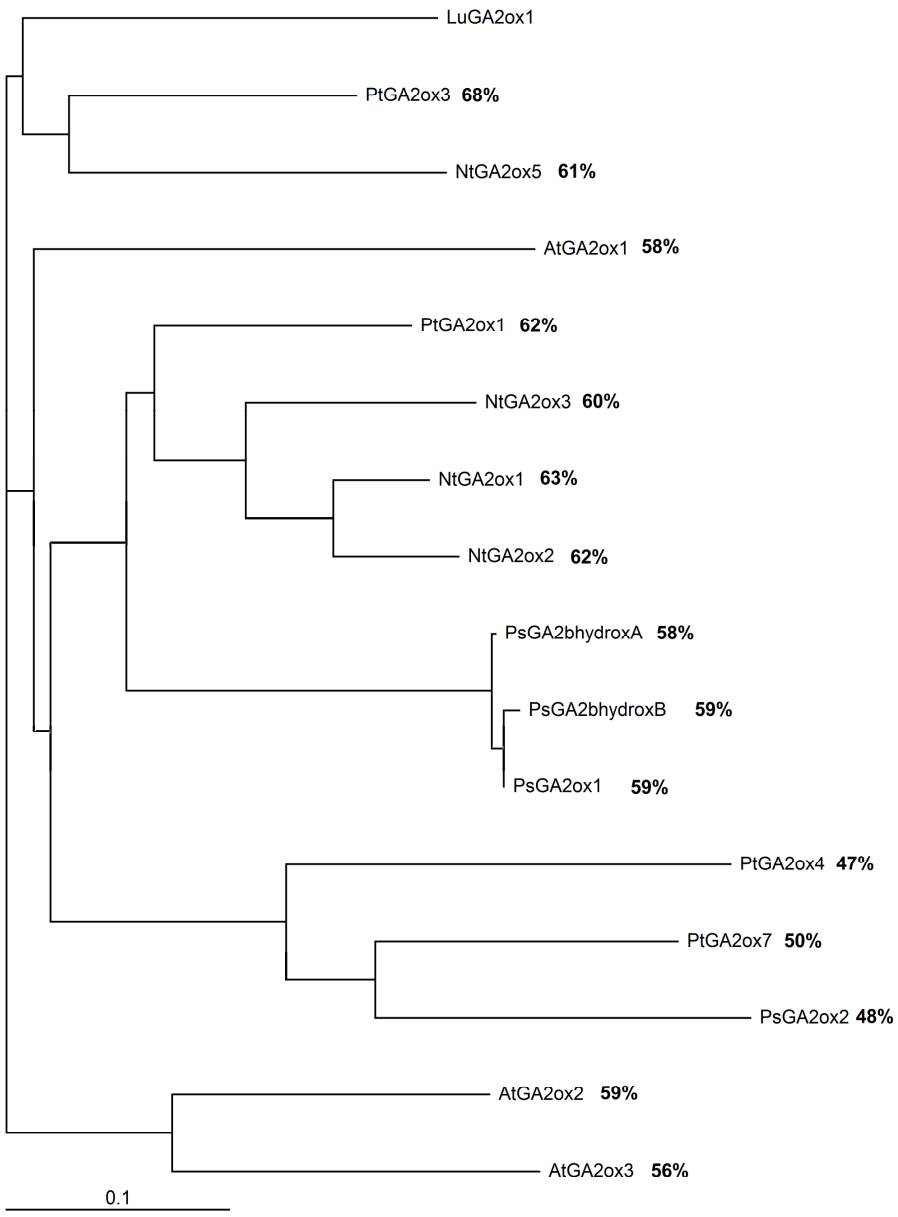
LuGA2ox1
PtGA2ox3 68%
NtGA2ox5 61%
AtGA2ox1 58%
PtGA2ox1 62%
NtGA2ox3 60%
NtGA2ox1 63%
NtGA2ox2 62%
PsGA2bhydroxA 58%
PsGA2bhydroxB 59%
PsGA2ox1 59%
PtGA2ox4 47%
PtGA2ox7 50%
PsGA2ox2 48%
AtGA2ox2 59%
AtGA2ox3 56%
0.1

Figure 3-2. Phylogram for alignments of LuGA2ox1 with selected GA 2 oxidase protein sequences from different plant species. Genbank accession numbers are as follows: *Arabidopsis thaliana*: GA2ox1, CAB41007; GA2ox2, CAB41008; GA2ox3, CAB41009; *Nicotiana tabacum* (tobacco): NtGA2ox1, BAD17855; NtGA2ox2, BAD17856; NtGA2ox3, ABO70985; NtGA2ox5, ABO70986; *Pisum sativum* (pea): PsGA2bhydroxA,AAF08609; PSGA2bhydroxB, AAF13734; PsGA2ox1, AAD45425; *Populus trichocarpa*: PtGA2ox1, XP_002300430; PtGA2ox2, XP_002320960; PtGA2ox3, XP_002305704; PtGA2ox4, XP_002312310; PtGA2ox7, XP_002320960. The percentage values beside each sequence indicate the percentage of amino acid identity sequence with LuGA2ox1.

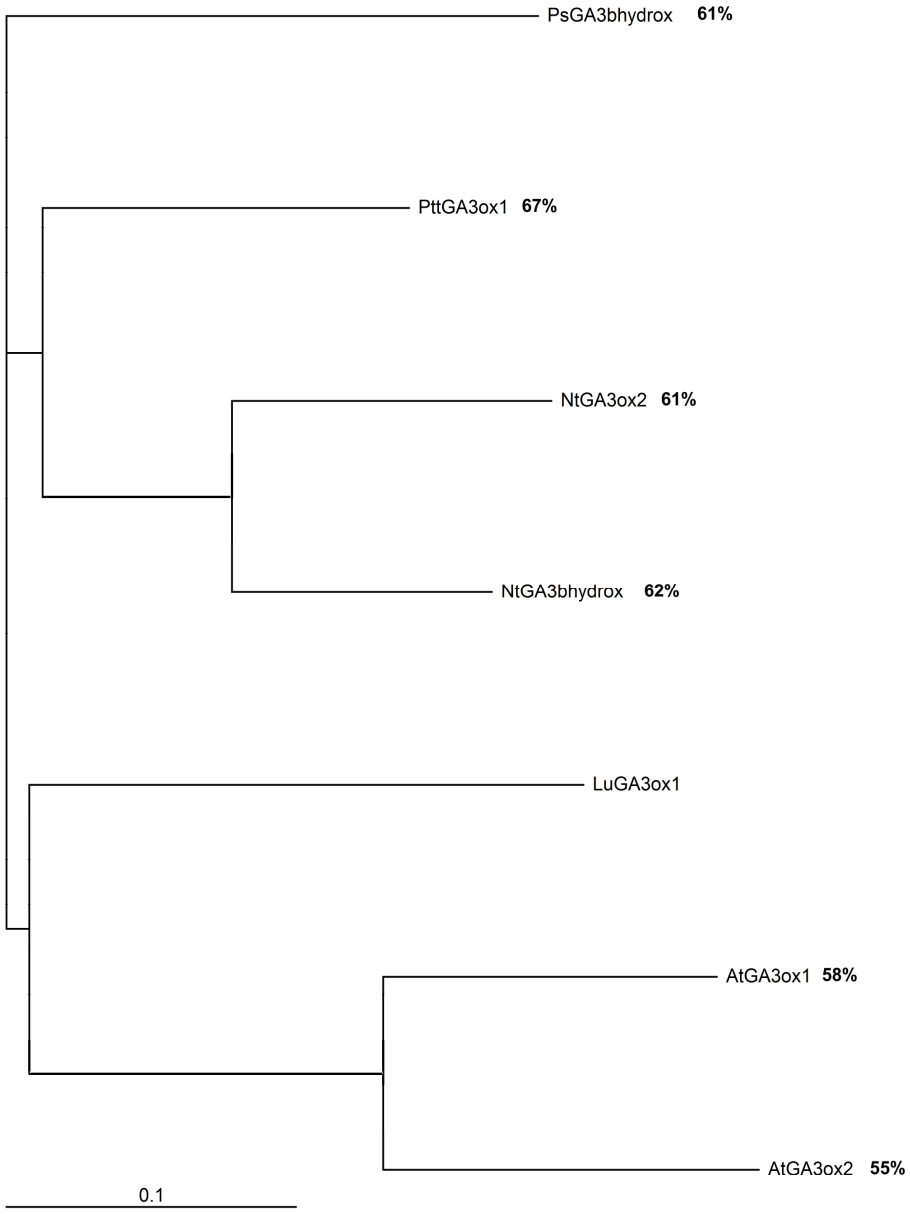
PsGA3bhydrox 61%
PttGA3ox1 67%
NtGA3ox2 61%
NtGA3bhydrox 62%
LuGA3ox1
AtGA3ox1 58%
AtGA3ox2 55%
0.1

Figure 3-3. Phylogram for alignments of LuGA3ox1 with selected GA 3 oxidase protein sequences from different plant species. Genbank accession numbers are as follows: *Arabidopsis thaliana*: AtGA3ox1, NP_173008; AtGA3ox2, NP_178150; *Nicotiana tabacum* (tobacco): NtGA3bhydrox, BAA89316; NtGA3ox2, ABO70984; *Pisum sativum* (pea): PsGA3bhydrox, AAC86820; *Populus tremula x P. tremuloides* (hybrid aspen): PttGA3ox1, AAR12160. The percentage values beside each sequence indicate the percentage of amino acid identity sequence with LuGA3ox1.

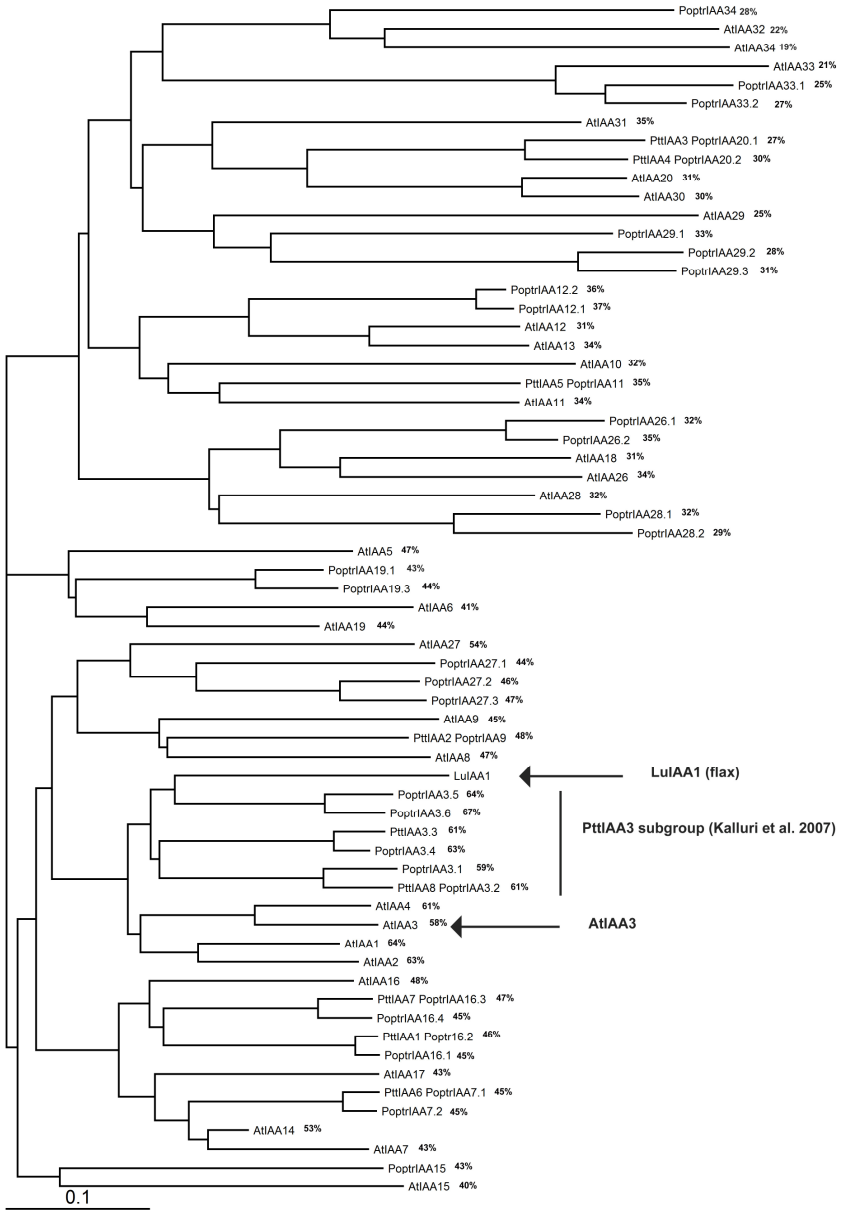
PoptrIAA34 28%
AtIAA32 22%
AtIAA34 19%
AtIAA33 21%
PoptrIAA33.1 25%
PoptrIAA33.2 27%
AtIAA31 35%
PttIAA3 PoptrIAA20.1 27%
PttIAA4 PoptrIAA20.2 30%
AtIAA20 31%
AtIAA30 30%
AtIAA29 25%
PoptrIAA29.1 33%
PoptrIAA29.2 28%
PoptrIAA29.3 31%
PoptrIAA12.2 36%
PoptrIAA12.1 37%
AtIAA12 31%
AtIAA13 34%
AtIAA10 32%
PttIAA5 PoptrIAA11 35%
AtIAA11 34%
PoptrIAA26.1 32%
PoptrIAA26.2 35%
AtIAA18 31%
AtIAA26 34%
AtIAA28 32%
PoptrIAA28.1 32%
PoptrIAA28.2 29%
AtIAA5 47%
PoptrIAA19.1 43%
PoptrIAA19.3 44%
AtIAA6 41%
AtIAA19 44%
AtIAA27 54%
PoptrIAA27.1 44%
PoptrIAA27.2 46%
PoptrIAA27.3 47%
AtIAA9 45%
PttIAA2 PoptrIAA9 48%
AtIAA8 47%
LulAA1
LulAA1 (flax)
PoptrIAA3.5 64%
PoptrIAA3.6 67%
PttIAA3.3 61%
PoptrIAA3.4 63%
PoptrIAA3.1 59%
PttIAA8 PoptrIAA3.2 61%
PttIAA3 subgroup (Kalluri et al. 2007)
AtIAA4 61%
AtIAA3 58%
AtIAA3
AtIAA1 64%
AtIAA2 63%
AtIAA16 48%
PttIAA7 PoptrIAA16.3 47%
PoptrIAA16.4 45%
PttIAA1 Poptr16.2 46%
PoptrIAA16.1 45%
AtIAA17 43%
PttIAA6 PoptrIAA7.1 45%
PoptrIAA7.2 45%
AtIAA14 53%
AtIAA7 43%
PoptrIAA15 43%
AtIAA15 40%
0.1

Figure 3-4. Phylogram for alignments of LuIAA1 with Aux/IAA protein sequences from *Arabidopsis* and *Populus*. Poptr designates names for *Populus trichocarpa* sequences by Kalluri et al. (2008), while Ptt designates names for the homologous hybrid aspen (*P. tremula x P. tremuloides*) sequence described by Moyle et al. (2002). The percentage values beside each sequence represent the percentage of amino acid identity for the sequence with LuIAA1. Sequence data for *Arabidopsis* Aux/IAA proteins can be found at TAIR (The Arabidopsis Information Resource; http://www.tair.org) under the accession numbers IAA1 (AT4G14560), IAA2 (AT3G23030), IAA3 (AT1G04240), IAA4 (AT5G43700), IAA5 (AT1G155809), IAA6 (AT1G52830), IAA7 (AT3G23050), IAA8 (AT2G22670), IAA9 (AT5G65670), IAA10 (AT1G04100), IAA11 (AT4G28640), IAA12 (AT1G04550), IAA13 (AT2G33310), IAA14 (AT4G14550), IAA15 (AT1G80390), IAA16 (AT3G04730), IAA17 (AT1G04250), IAA18 (AT1G51950), IAA19 (AT3G15540), IAA20 (AT2G46990), IAA26 (AT3G16500), IAA27 (AT4G29080), IAA28 (AT5G25890), IAA29 (AT4G32280), IAA30 (AT3G62100), IAA31 (AT3G17600), IAA32 (AT2G01200), IAA33 (AT5G57420), and IAA34 (At1g15050). Sequence data for Poptr Aux/IAAs is reported by Kalluri et al. (2008). Genbank accession numbers for hybrid aspen Aux/IAAs are as follows: PttIAA1, AAM21317; PttIAA2, CAC84706; PttIAA3, CAC84707; PttIAA4, CAC84708; PttIAA5, CAC84709; PttIAA6, CAC84710; PttIAA7, CAC84711; PttIAA8, CAC84712.

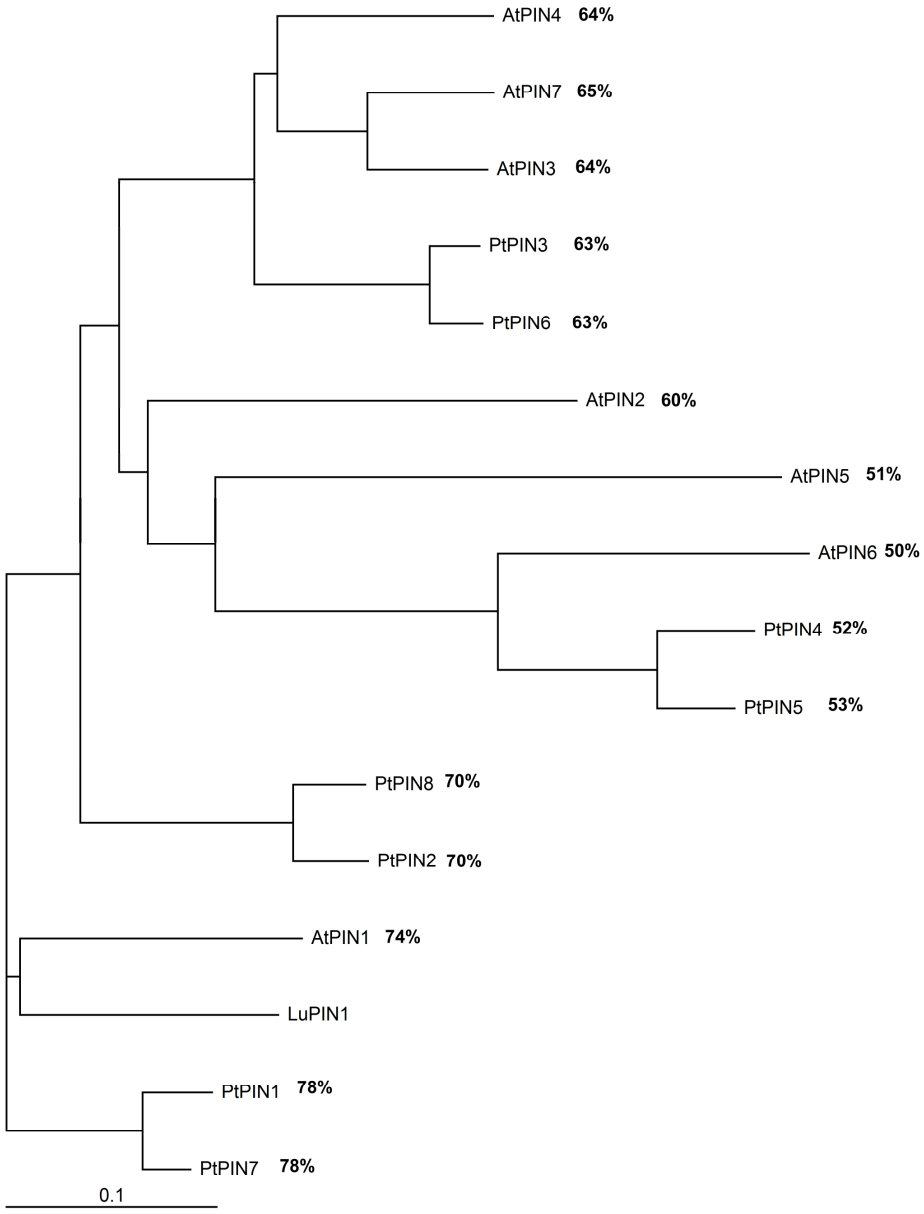
AtPIN4 64%
AtPIN7 65%
AtPIN3 64%
PtPIN3 63%
PtPIN6 63%
AtPIN2 60%
AtPIN5 51%
AtPIN6 50%
PtPIN4 52%
PtPIN5 53%
PtPIN8 70%
PtPIN2 70%
AtPIN1 74%
LuPIN1
PtPIN1 78%
PtPIN7 78%
0.1

Figure 3-5. Phylogram for alignments of LuPIN1 with selected PIN protein sequences from *Arabidopsis* and *Populus*. Genbank accession numbers are as follows: *Arabidopsis thaliana*: AtPIN1, NP_177500; AtPIN2, NP_568848; AtPIN3, NP_177250; AtPIN4, NP_565261; AtPIN5, Q9LFP6; AtPIN6, Q9SQH6; AtPIN7, NP_849700; *Populus trichocarpa*, PtPIN1, XP_002322104; PtPIN2, XP_002322614; PtPIN3, AAM54033; PtPIN4, EEE93674; PtPIN5, EEE81469; PtPIN6, EEE89803; PtPIN7, EEE96094; PtPIN8, XP_002307966. The percentage values beside each sequence indicate the percentage of amino acid identity sequence with LuPIN1.

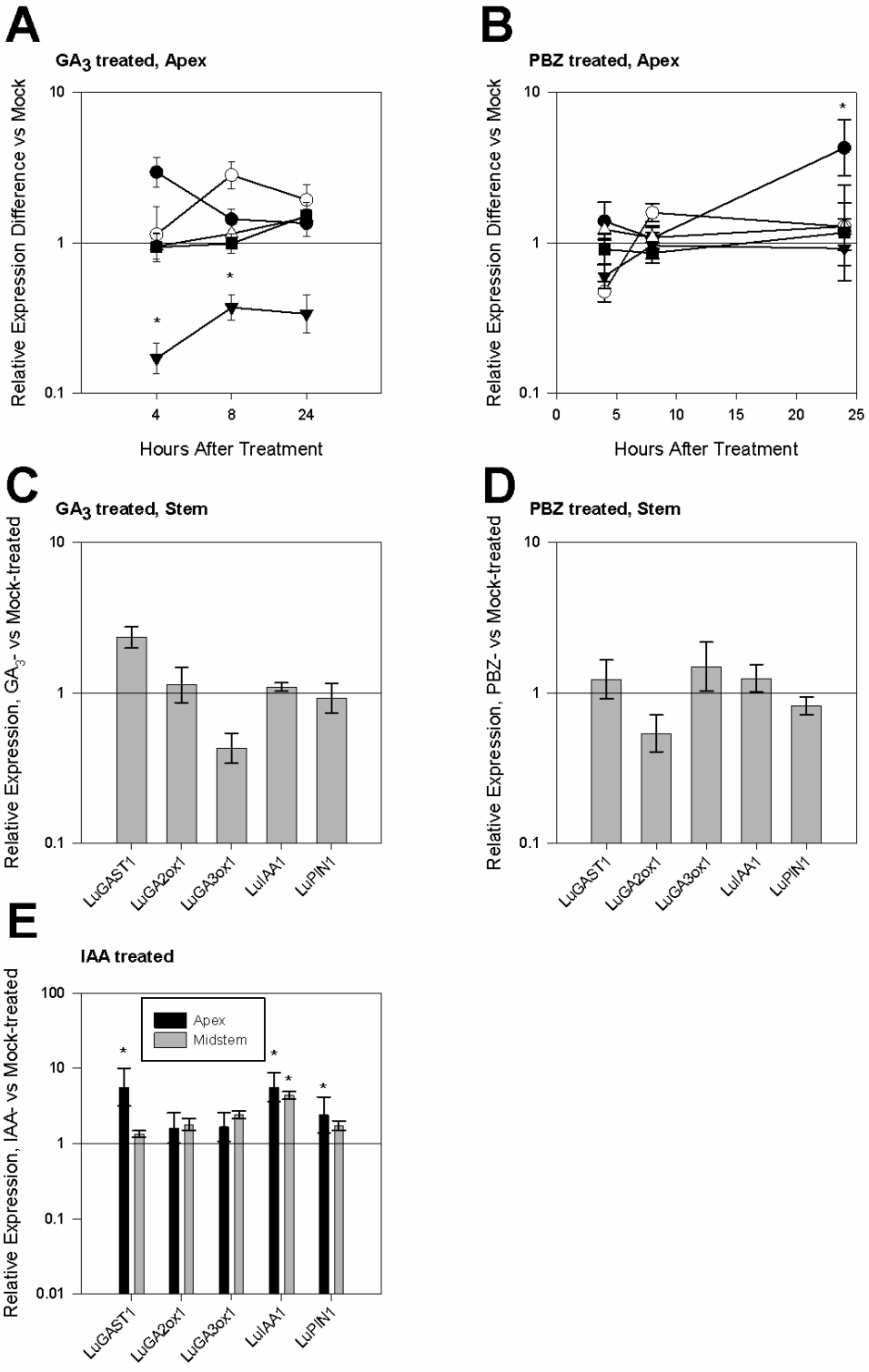
A
GA3 treated, Apex
Relative Expression Difference vs Mock
10
1
0.1
4
8
24
Hours After Treatment
B
PBZ treated, Apex
Relative Expression Difference vs Mock
10
1
0.1
0
5
10
15
20
25
Hours After Treatment
C
GA3 treated, Stem
Relative Expression, GA3- vs Mock-treated
10
1
0.1
LuGAST1
LuGA2ox1
LuGA3ox1
LuIAA1
LuPIN1
D
PBZ treated, Stem
Relative Expression, PBZ- vs Mock-treated
10
1
0.1
LuGAST1
LuGA2ox1
LuGA3ox1
LuIAA1
LuPIN1
E
IAA treated
Relative Expression, IAA- vs Mock-treated
100
10
1
0.1
0.01
Apex
Midstem
LuGAST1
LuGA2ox1
LuGA3ox1
LuIAA1
LuPIN1

Figure 3-6. Short term responses to plant growth regulators. Transcript abundance was measured in stem apex or midstem tissues at varying time periods following treatment. Expression levels shown for treated plants are relative to mock-treated plants sampled at equivalent timepoints. (A) Stem apex tissues of GA_3-treated plants, sampled 4, 8 and 24 hours after treatment. (B) Stem apex tissues of PBZ-treated plants, sampled 4, 8 and 24 hours after treatment. (C) Midstem tissue of GA_3-treated plants, sampled 6 hours after treatment. (D) Midstem tissue of PBZ-treated plants, sampled 6 hours after treatment. (E) Stem apex and midstem tissues of IAA-treated plants, sampled 1 hour after treatment. For (A) and (B), ● = *LuGAST1*, ○ = *LuGA2ox1*, ▼= *LuGA3ox1*, Δ = *LuIAA1*, ■ = *LuPIN1*. * indicates that expression levels in treated plants significantly differ (p<0.05) from mock-treated plants sampled at the equivalent timepoint. Error bars represent the standard deviation for each measurement.

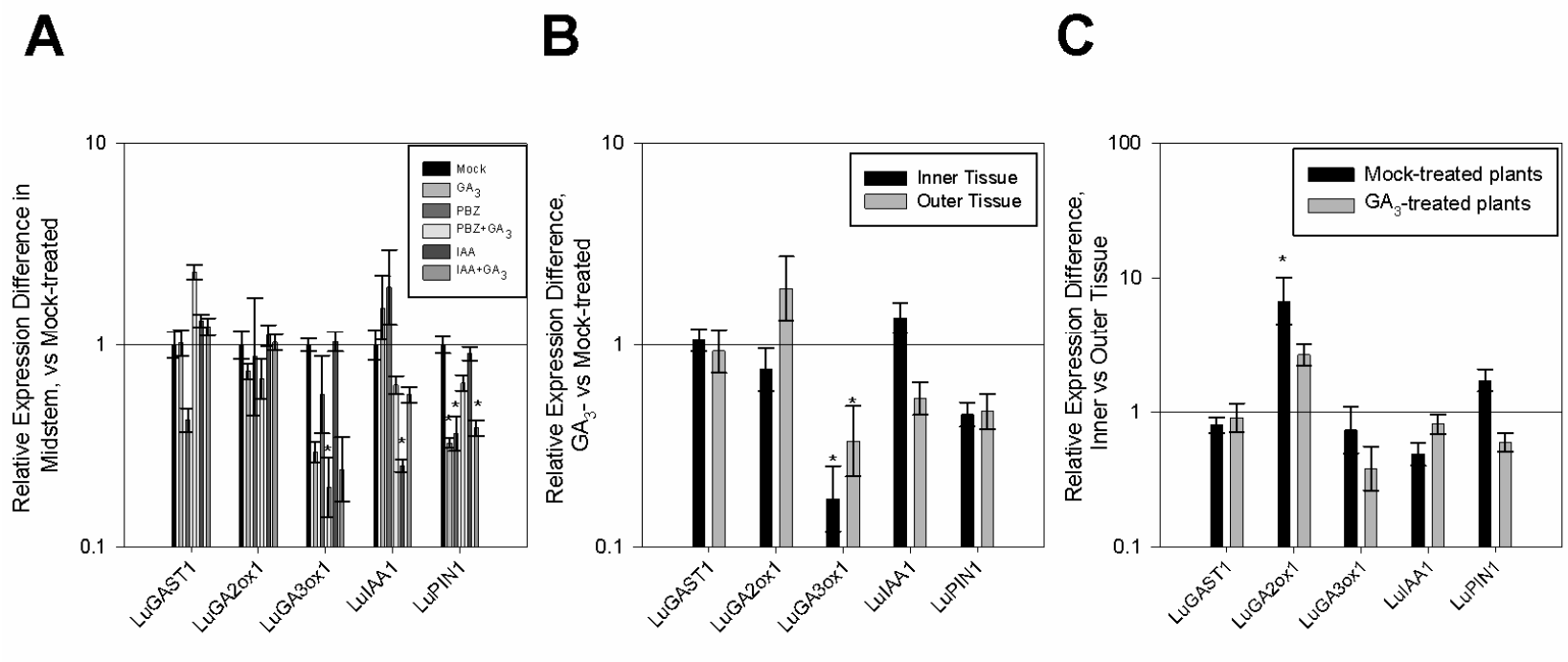
A
B
C
Relative Expression Difference in Midstem, vs Mock-treated
Relative Expression Difference, GA3- vs Mock-treated
Relative Expression Difference, Inner vs Outer Tissue
10
1
0.1
100
Mock
GA3
PBZ
PBZ+GA3
IAA
IAA+GA3
Inner Tissue
Outer Tissue
Mock-treated plants
GA3-treated plants
LuGAST1
LuGA2ox1
LuGA3ox1
LuIAA1
LuPIN1
*

Figure 3-7. Long term responses to plant growth regulators and spatial difference in transcript abundance for inner vs outer tissues. (A) Relative expression levels (fold) in treated midstem tissues. The expression levels of each gene are relative to EF1α and to the value of each gene in the mock-treated plants (set at 1). (B) Relative expression differences for GA_3- vs mock-treated plants for inner tissues and outer tissues. (C) Relative expression differences for inner vs outer stem tissues sampled from mock- and GA_3-treated plants. The symbol * indicates that transcript abundance significantly differed ($p<0.05$) relative to the mock-treated plants (A) or for the comparison shown (B and C). Error bars represent the standard deviation for each measurement.

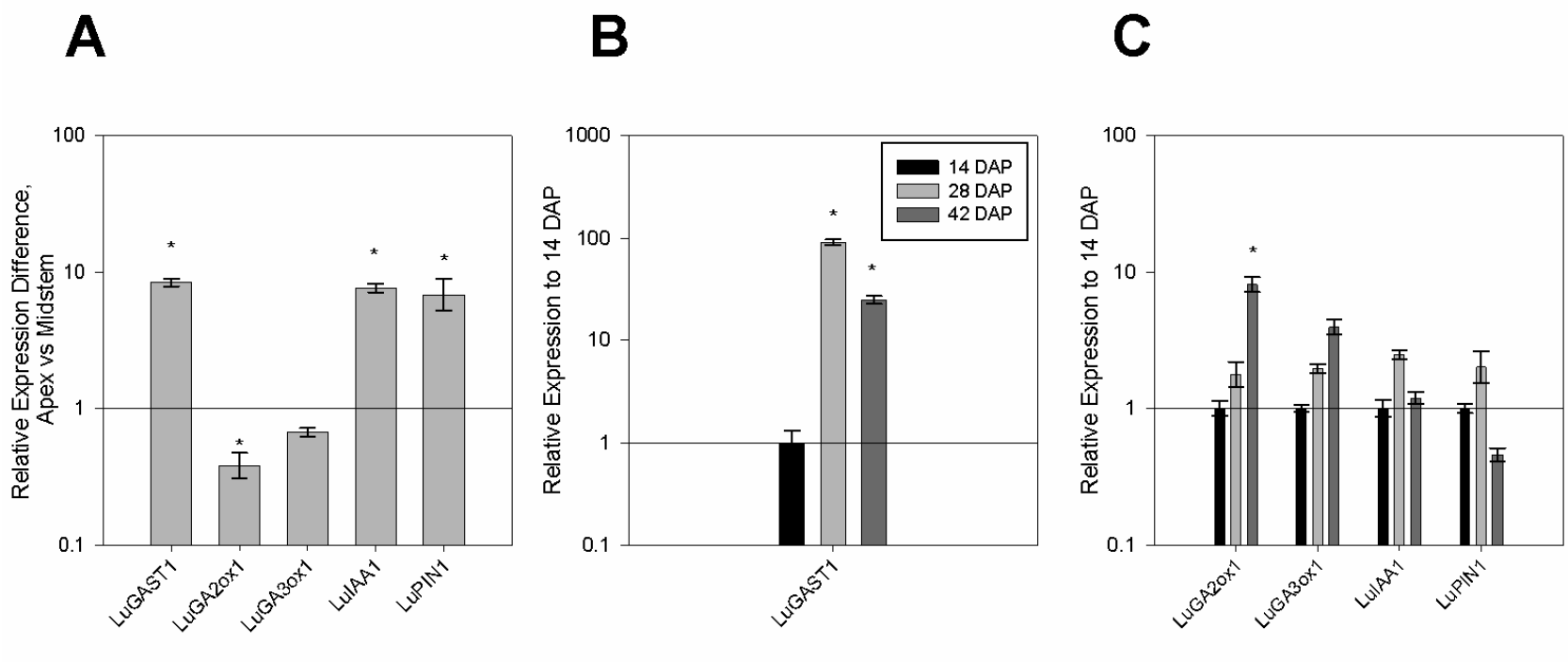
A
B
C
Relative Expression Difference, Apex vs Midstem
100
10
1
0.1
LuGAST1
LuGA2ox1
LuGA3ox1
LuIAA1
LuPIN1
Relative Expression to 14 DAP
1000
14 DAP
28 DAP
42 DAP
LuGAST1
LuGA2ox1
LuGA3ox1
LuIAA1
LuPIN1

Figure 3-8. Temporal and spatial variation in expression in untreated stems. (A) Relative expression differences (fold) for midstem vs stem apex tissues. (B) and (C) Temporal variation in expression. The expression levels for each gene are relative to the EF1α gene and to the value at 14 days after planting (DAP) (set at 1). Note that data for *LuGAST1* is presented in a separate graph (B) from the other four genes (C) because of the differences in scale. * indicates that transcript abundance significantly differed ($p<0.05$) for the midstem and apex tissues (A) or as compared with the 14 DAP timepoint (B). Error bars represent standard deviations for each measurement.

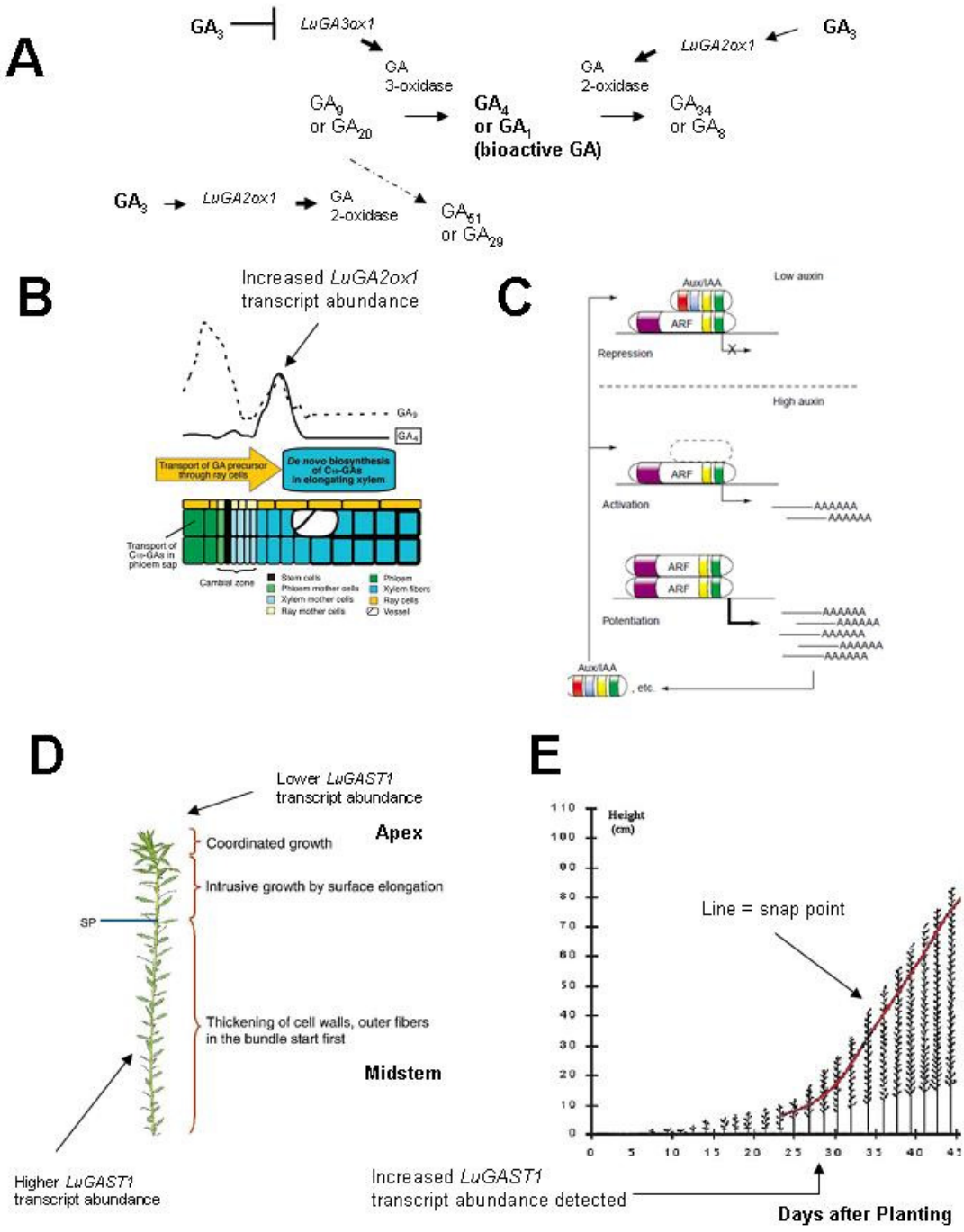
A
GA3
LuGA3ox1
GA 3-oxidase
GA9 or GA20
GA4 or GA1 (bioactive GA)
LuGA2ox1
GA3
GA 2-oxidase
GA34 or GA8
GA3
LuGA2ox1
GA 2-oxidase
GA51 or GA29
B
Increased LuGA2ox1 transcript abundance
GA9
GA4
Transport of GA precursor through ray cells
De novo biosynthesis of C19-GAs in elongating xylem
Transport of C19-GAs in phloem sap
Cambial zone
Stem cells
Phloem mother cells
Xylem mother cells
Ray mother cells
Phloem
Xylem fibers
Ray cells
Vessel
C
Low auxin
Aux/IAA
ARF
Repression
X
High auxin
ARF
Activation
AAAAAA
AAAAAA
ARF
ARF
Potentiation
AAAAAA
AAAAAA
AAAAAA
AAAAAA
AAAAAA
Aux/IAA
, etc.
D
Lower LuGAST1 transcript abundance
Apex
Coordinated growth
Intrusive growth by surface elongation
SP
Thickening of cell walls, outer fibers in the bundle start first
Midstem
Higher LuGAST1 transcript abundance
E
Height (cm)
110
100
90
80
70
60
50
40
30
20
10
0
Line = snap point
0
5
10
15
20
25
30
35
40
45
Increased LuGAST1 transcript abundance detected
Days after Planting

Figure 3-9. Gene expression hypotheses. (A) GA 3 oxidase converts the biologically-inactive gibberellins GA_9/GA_{20} into GA_4/GA_1. GA 2 oxidase converts GA_4/GA_1 into the biologically-inactive GA_{34}/GA_8 forms. GA 2 oxidase also converts GA_9/GA_{20} into the biologically-inactive gibberellins GA_{51}/GA_{29}. Bioactive GAs feedback on the GA biosynthetic pathway, repressing the transcription of GA 3 oxidase genes (e.g. *LuGA3ox1*) and inducing GA2 oxidase genes (*e.g. LuGA2ox1*). (B) Spatial distribution of GA_9 and GA_4 in *Populus tremula* stems, adapted from Israelsson et al. (2005). Israelsson et al. (2005) propose that *de novo* GA biosynthesis occurs in differentiating xylem. Both *PttGA2ox2* and *LuGA2ox1* were determined to have higher transcript abundance in the inner stem tissue, in accordance with this model. (C) Auxin response pathway, adapted from Berleth et al. (2004). Aux/IAA proteins heterodimerize with auxin response factors (ARFs) to inhibit auxin-responsive transcription. Increased auxin levels stimulate the proteolysis of Aux/IAAs, allowing ARFs to activate the transcription of auxin-responsive genes, including Aux/IAA genes. The newly synthesized Aux/IAAs are rapidly degraded through proteolysis as IAA levels remain high, but their rate of degradation declines with IAA levels. (D) Spatial localization of flax bast fibre differentiation stages during stem growth, adapted from Gorshkova et al. (2003). *LuGAST1* transcript abundance increased in stem regions associated with bast fibre secondary cell wall expansion, as compared with regions associated with fibre elongation. SP = snap-point. (E) Position of the snap-point during stem growth, adapted from Gorshkova et al. (2003). The *LuGAST1* transcriptional abundance substantially increased

approximately as the fast growth stage began and the snap-point appeared. Increased *LuGAST1* transcript abundance relative to 14 DAP was also observed in stems sampled 42 DAP.

3.8 Appendix

3.8.1. Nucleotide Sequences

1. *LuGAST1* cDNA sequence

ccacgcgtccgcaagtaattaaattaggaattaggattcagccggcgatcatggatagccggaaagcaaaacctcagtttctagcaacggtgg
ctctgctgtctctgcttctcttgctggcattctccgccaccgtttccgaggctaactaccacgctaagctccgcccttctgattgtaagccgaagtg
caactacagatgctcggcgacgtcgcacaagaagccgtgcatgttcttctgccagaagtgttgcacgaagtgtctgtgtgttccgccgggaact
tacgggaataagcaggtttgcccttgctacaaccagtggaagaccaaagaaggcggccccaaatgcccatagatttatttattcctacctaccat
tcaaattcaaatattccgtcaggttgaatagtagctaggatataatggaattaattaaggaaataccatttcctacgctgatcatgtgtgttgcatggt
tgatttttcatgagtagtaccaagctacatatatataattcaatatttatcaggtgtagcgcttcatgaatgtattattattcaatgaataaagcggtatt
aaaatggtcaaagattatggataaattataaattttaaagtattgacaaattttagtata

2. *LuGA2ox1* predicted ORF

atggtggttctgtctagaccggcgttagatcacttctcctcaatcaaaacatgcaaacccactcccggattcttcttccccgggatcccggtcgtc
gatttctccgaccccaactccaaattcctaatcgccaaagcttgtaaagacttcggctttttcaaactaaccaaccacggaatcccaaaggaaac
catacgccagctggagtccctggctgtagagttcttcagcctcccgcaatcggagaaagactccgctggcccgcctaacccgttcggttatgg
caacaagcgaatcggccccaatggcgatatcggctggatcgagtacctcctcttgcattccgattcgatcaaatcagccggccctcagaatttc
cggtgtgcggtgaaggagtatgtggcggcggtgaagaagatgacaatggagatgcttgaggcgatggcggaaggggttgggatagagcc
gaggaatgcgctcagccggttggttgatgacgagggcagcgactcgtgccttcggttgaaccattacccgggctgcccagaggttcaaccgt
ccttgtcgggcggtcggatgaatctggtcgggttcggcgaacataccgacccgcagattgtctcggttttgagatccaacaacaccaccggcc
tccaaatctgccttaaagacggcgcgtgggtttccgtgccgccggatcagtcttccttctttgtcatcgtcggcgacgccctccaggtgatgact
aatggcaggtttaagagtgtgaagcatagggtgatgacggaccccgcgaaatcgagagtgtcgatgatttatttcgcgggaccaccgatgag
cgaaaagattgtacctttggaatcgataatggaagtcggggaggaaagcagatacgaggagttcacatggtcggagtacaaacaatcggctt
ataagtcgaggctcgctgattataggctcggccatttcgagaagaagaagatcaagaacaccgacaacaatggagggttttggttgtag

3. *LuGA3ox1* predicted ORF

atgccatccatccaactagccgatgctttcagatcccatccagtcaacctccaccaaaaacaccaactaaacgacttcacctccctcaaagaact
ccccgactcctacaaatggtccacccgaattaaccacgacaatgttcgaaccaacgaaccggtccctgtcatcgacctctcccggccagaag
ccatgagggagatcggacatgcatgcagggagtgggggtgttccaagttgtcaaccacggtgtccccaccgaggttgtcgaccgcataga
gaagaatgtcggggagaacttcttcgggttgcccatgtacgaaaaactcaaggctgcccggtcccccaacggggtttctgggtttgggttggct
cggatttcaaccttcttccctaagctcatgtggtccgaagggttcaccgtggttggctcccccgccgagcatttcatgaaagtttggccggagga
ttacagacaattttgtgacattgtagaagggtacgagaaggaaatgcaaaagctagcatcaaggttgatggacctaatccttggctcccttggga
tcacaacccaagacttgagttgggactatagtacccccaagtcggtttcgtcggctgcgattcagctgaaccactacccggcttgcccagaccc
ggaccaggcgatgggtctgggcccgcacactgactcgaccctggtcaccatactccaccagaacgccaccagcgggctgcaggtgctgag
ggacggaggggcccagtgggttaccgtccctcccatcaagggcgcgctggtggtgaacgtgggcgacctgcttcacatcttgtcgaacggg
ttgtactccagcgtggtccaccgggcggtggtgaaccggaagaagaacaggatgtcgattgcgtacctatacgggccgccttcgaatgttag
gatctcgccggtggggaaactcgtggggccggggcaacctccattgtatagggcggtgacttggagtgagtaccttgggactaaggccaaa
cacttcaacaaagctttgtcttcagttaggttgtgtgctggtgctactcccatgaatggactggttgatgcaagagatggtagcaataataatagag
tgaaagttgggtag

4. *LuIAA1* predicted ORF

atggaaggtgctacatatgaaagtgacttgaacttcgaggcaaccgagctcaggcttggactgcctggctcaggagaagaagaaactgctgtt
aaaagcaacaacaaacgacctatgcctgcagagaccaacgaagaaatcgaagccaagggaagatctggagatcatgttcaagctgcccctg
ctgctaaggcacagattgtgggctggccaccaatccgatcctacaggaagaatagcttgcagccaaagaaaagtactgaagctgctgatggt
gctagcgggatgtacgtgaaagttagcatggatggagcaccatatctcaggaagattgacctcaaggtctatagaggctaccctgaactcctaa
tggctttggagaccatgtttaagtttgctgcgggtgtctattctgagagagaaggctataaagggtccgagcacgtacctacttatgaagacaaa
gatggtgactggatgctcgttggagatgttccttgggacatgttcatgtcatcctagaaacgtggaggtcttttcaggcaaccgggttcaagtctc
aacccccaggaggagtcttttgtccctgcagttggacaaggaaacaaagagaaatcactgtga

5. *LuPIN1* predicted ORF

atgatcaccggaacagacttctaccacgtaatgacggcgatggtcccgctctacgtcgcaatgttcctcgcttacggctccgtcaaatggtgga
agatcttcaccccggaccagtgctccgggatcaaccgattcgtcgctctcttcgccgtcccgctcctctccttccacttcatctcctccaacgacc
cttacgccatgaacttccgcttcatcttcgctgattctctccagaagctcatcatcctcgctgccttggccgtctgggccaatctatcccgccgcgg
gacgcttgaatggtcgattactctgttctccctctctaccctcccgaacactctggttatggggatccctttgctgaaagggatgtacggtgacga
atctggaacgctaatggttcaaatcgtcgttctccagtgtataatctggtatacgctgatgctgttcatgttcgagttccgtggagcgaggattttga
tctccgagcagtttcctgataccgctggttcgattgtttcgattcatgtcgattcggatatcatgtcgctcgacgggcggatgccgctggagacgg
aggcggagattaagagcgacgggaagctccacgtcactgtacggaggtcgaacgcttccagatctgacgttttctcgaggagatccggcacc
ggagcgactcctcggccgtcgaatctgacgaatgcggagatctattcgttgcagtcgtcgaggaatccgactcctcggggttcgagctttaacc
atacggatttctactcgatgatggccgccggtcggagctccaatttcggggcgaacgacggccccactcctaggccgtcaaactacgatgag
gatggtaaaacgacgtcgtctaatgctgcgaggtacgggtacggcggcggtcctgccgggtcgggttatcccgctccgaacccgggaatgtt
ctctccgactactgcagctgggaaagtgaatagtaatgttaagaaaggagggaaagaagatggcggcggtggaaaagatcttcatatgttcgt
ttggagctccagtaattcgccggtttccgacgtgttcggcggccatgagtacgccgccagcgccggcggaaatcatcatcagctggagcaga
gtaaggaagtccgcctaggagtgtctcccgggaaagtggtggagcagaggggagagaccgggtttgtcggccatggaggggaggagtatt
tagtcgagagggacgagttcagcttcgggaacagagaggctgagaatgtgaacggcagcggcggcgagaaggcggcggcgaacggcg
gcggcggagatgtgaagagcaaagtgatgccgccgacgagcgtgatgacaaggctgattctgatcatggtgtggaggaagctgataagga
accctaacacatactccagcttgattggtctaatttggtcactggtttccttcaggtggcacgtaacgatgcctgccattatcgcaaagtccatctc
cattctgtcagacgctggcctcggcatggccatgttcagtctagggttgttcatggcattgcagccgaggataatagcatgtgggaaaagaaca
gcaacgtatgcaatgggggttagattctttgttggaccagctgtcatggctggcgcctctttttttttgttgttggcctcaggggcactctcctccac
atcgccattgtccagtcacaccgatgctttctggtctctactcaagcttggttttctgatgcggagggaactcgtgaggatttggaggtttaacgag
gcagcacttcctcaggggattgttccctttgtcttcgccaaggaatacaatgtacaccctgacattctcagcactggggtgatattcgggatgctg
atcgccttgcccttcacgctagtctactacattttgctcggactatga

3.8.2. Multiple Sequence Alignments

1. LuGAST1

```
LuGAST1         ------------------------------------------------------------
SlRSI-1         ------------------------------------------------------------
PhGIP1          ------------------------------------------------------------
PhGIP2          ------------------------------------------------------------
SlGAST          ------------------------------------------------------------
AtGASA5         ------------------------------------------------------------
PhGIP4          ------------------------------------------------------------
PhGIP5          ------------------------------------------------------------
AtGASA4         ------------------------------------------------------------
AtGASA6         ------------------------------------------------------------
AtGASA12        ------------------------------------------------------------
AtGASA7         ------------------------------------------------------------
StSN1           ------------------------------------------------------------
AtGASA8         ------------------------------------------------------------
AtGASA10        ------------------------------------------------------------
AtGASA1         ------------------------------------------------------------
AtGASA11        ------------------------------------------------------------
FaGAST          ------------------------------------------------------------
GhGEG           ------------------------------------------------------------
StSN2           ------------------------------------------------------------
AtGASA2         ------------------------------------------------------------
AtGASA3         ------------------------------------------------------------
AtGASA9         ------------------------------------------------------------
AtGASA14        MALSLLSVFIFFHVFTNVVFAASNEESNALVSLPTPTLPSPSPATKPPSPALKPPTPSYK
```

```
LuGAST1         ---------------------------MDSRQANP-LL--------------VPTVALLL
SlRSI-1         ---------------------------MAKSGYNASFL--------------LLISMFLI
PhGIP1          ---------------------------MAGKLSIVLFV--------------LLVVLLAQ
PhGIP2          ---------------------------MAGKLSIVLFV--------------LLVVLLAQ
SlGAST          ---------------------------MAGKMSIVLFV--------------LLVVFLTQ
AtGASA5         ---------------------------MANCIRRNALF--------------FLTLLFLL
PhGIP4          ---------------------------MAKLVPIFLLA--------------LFVISMFA
PhGIP5          ---------------------------MAKLASFFLLA--------------LIAISIVA
AtGASA4         ------------------------------------------------------------
AtGASA6         ---------------------------MAKLITSFLL------------------LTILF
AtGASA12        ---------------------------MMKLIVVFVISS-------------LLFATQFS
AtGASA7         ---------------------------MKIIVSILVLAS-------------LLLISSSL
StSN1           ---------------------------MKLFLLTLLLVT--------------LVITPSL
AtGASA8         ---------------------------MKLVVVQFFIIS--------------LLLTSSF
AtGASA10        ---------------------------MKFPAVKVLIIS--------------LLITSSL
AtGASA1         ------------------------MAISKALIASLLIS--------------LLVLQLVQ
AtGASA11        ------------------------MAVFRVLLASLLIS--------------LLVLDFVH
FaGAST          ----------------------------------MMMIS--------------LLVFNPVE
GhGEG           ------------------------MAISKPFLAFALLSM-----------LLLLQLG--Q
StSN2           ------------------------MAISKALFDILLRSV-----------QLLEQVQSIQ
AtGASA2         ------------------------MAVFRSTLVLLLII---------------VCLTTYE
AtGASA3         ------------------------MAIFRSTLVLLLIL---------------FCLTTFE
AtGASA9         ------------------MKKMNVVAFVTLIISFLLLS------------QVLAELSSSS
AtGASA14        PPTLPTTPIKPPTTKPPVKPPTIPVTPVKPPVSTPPIKLPPVQPPTYKPPTPTVKPPSVQ

LuGAST1         -LLA-----FSATVSEANYH--------------------------AKLRPSDCKPKCNY
SlRSI-1         -LLT-----FSNVVEGYN----------------------------KLRPTDCKPRCTY
PhGIP1          NQVS-----RAKMVLDSKVQRRGNDQIYG--------------VSQGSLHPQDCQPKCTY
PhGIP2          NQVS-----MAKKVLDSKVQRRGNDQIYG--------------VSQGSLHPQDCQPKCTY
SlGAST          NQVS-----RANIMRDEQQQQQRNNQLYG--------------VSEGRLHPQDCQPKCTY
AtGASA5         ---S-----VSNLVQAARGG-------------------------GKLKPQQCNSKCSY
PhGIP4          TTVL-----ASHDPKRGHHHKG----------------------YGPGSLKPSQCLPQCTR
PhGIP5          TTAL-----AADGQYHLDAAR----------------------YGPGSLKPTQCLPQCIR
AtGASA4         ---M-----ASSGSNVKWSQKR---------------------YGPGSLKRTQCPSECDR
AtGASA6         TFVC-----LTMSKEAEYHPES---------------------YGPGSLKSYQCGGQCTR
AtGASA12        NGDE-----LESQAQAPAIHKN---------------------GGEGSLKPEECPKACEY
AtGASA7         ASAT-----ISDAFGSGAVAPAPQ-------------------SKDGPALEKWCGQKCEG
StSN1           IQTT-----MA---GS-----------------------------------NFCDSKCKL
AtGASA8         SVLS-----SA---DSS----------------------------------CGGKCNV
AtGASA10        FILS-----TA---DSSP---------------------------------CGGKCNV
AtGASA1         AD-------VENSQKKNGY-----------------------------AKKIDCGSACVA
AtGASA11        AD-------MVTSND---------------------------------APKIDCNSRCQE
FaGAST          ADGV-----VVNYGQHASL-----------------------------LAKIDCGGACKA
GhGEG           AYEM-----VN-KIDEATIA---------------------------ASKINCGAACKA
StSN2           TDQV-----TSNAISEAAYS---------------------------YKKIDCGGACAA
AtGASA2         LHVH-----AADGAKVGEG-----------------------------VVKIDCGGRCKD
AtGASA3         LHVH-----AAEDSQVGEG-----------------------------VVKIDCGGRCKG
AtGASA9         NNETSSVSQTNDENQTAAFKRTYHH----------------------RPRINCGHACAR
AtGASA14        PPTYKPPTPTVKPPTTSPVKPPTTPPVQSPPVQPPTAPPVKPPTPPPVRTRIDCVPLCGT
                                                                *   *
```

```
LuGAST1          RCSATSHKKPCMFFCQKCCTKCL-CVPPGTYGNKQVC-PCYNQWKTKEGAPKCP
SlRSI-1          RCSATSHKKPCMFFCQKCCATCL-CVPKGVYGNKQSC-PCYNNWKTQEGKPKCP
PhGIP1           RCSKTSFKKPCMFFCQKCCAKCL-CVPAGTYGNKQTC-PCYNNWKTKEGGPKCP
PhGIP2           RCSKTSFKKPCMFFCQKCCAKCL-CVPAGTYGNKQTC-PCYNNWKTKEGGPKCP
SlGAST           RCSKTSYKKPCMFFCQKCCAKCL-CVPAGTYGNKQSC-PCYNNWKTKRGGPKCP
AtGASA5          RCSATSHKKPCMFFCLKCCKKCL-CVPPGTFGNKQTC-PCYNNWKTKEGRPKCP
PhGIP4           RCSQTQYHNACMLFCQKCCNKCL-CVPPGFYGNKGVC-PCYNNWKTKEGGPKCP
PhGIP5           RCSHTQYHNACMLFCQKCCKKCL-CVPPGFYGNKGVC-PCYNNWKTKEGGPKCP
AtGASA4          RCKKTQYHKACITFCNKCCRKCL-CVPPGYYGNKQVC-SCYNNWKTQEGGPKCP
AtGASA6          RCSNTKYHKPCMFFCQKCCAKCL-CVPPGTYGNKQVC-PCYNNWKTQQGGPKCP
AtGASA12         RCSATSHRKPCLFFCNKCCNKCL-CVPSGTYGHKEEC-PCYNNWTTKEGGPKCP
AtGASA7          RCKEAGMKDRCLKYCGICCKDCQ-CVPSGTYGNKHEC-ACYRDKLSSKGTPKCP
StSN1            RCSKAGLADRCLKYCGICCEECK-CVPSGTYGNKHEC-PCYRDKKNSKGKSKCP
AtGASA8          RCSKAGQHEECLKYCNICCQKCN-CVPSGTFGHKDEC-PCYRDMKNSKGGSKCP
AtGASA10         RCSKAGRQDRCLKYCNICCEKCNYCVPSGTYGNKDEC-PCYRDMKNSKGTSKCP
AtGASA1          RCRLSRRPRLCHRACGTCCYRCN-CVPPGTYGNYDKC-QCYASLTTHGGRRKCP
AtGASA11         RCSLSSRPNLCHRACGTCCARCN-CVAPGTSGNYDKC-PCYGSLTTHGGRRKCP
FaGAST           RCRLSSRPHLCKRACGTCCQRCS-CVPPGTAGNYDVC-PCYATLTTHGGKRKCP
GhGEG            RCRLSSRPNLCHRACGTCCARCR-CVPPGTSGNQKVC-PCYYNMTTHGGRRKCP
StSN2            RCRLSSRPRLCNRACGTCCARCN-CVPPGTSGNTETC-PCYASL----------
AtGASA2          RCSKSSRTKLCLRACNSCCSRCN-CVPPGTSGNTHLC-PCYASITTHGGRLKCP
AtGASA3          RCSKSSRPNLCLRACNSCCYRCN-CVPPGTAGNHHLC-PCYASITTRGGRLKCP
AtGASA9          RCSKTSRKKVCHRACGSCCAKCQ-CVPPGTSGNTASC-PCYASIRTHGNKLKCP
AtGASA14         RCGQHSRKNVCMRACVTCCYRCK-CVPPGTYGNKEKCGSCYANMKTRGGKSKCP
                 **       *  *  **  *  **. *  *:   *  **
```

2. LuGA2ox1

```
PtGA2ox3            MVVLSQLALEPFSV--IKTCKP--IGLFSEIPVIDLTDPHA--KTLIIKACEEFGFFKLV
NtGA2ox5            MVVLTQPLVETLSH--IKTCKYNNTDVFTGIPVIDLSDPEA--NTLIIKACQEFGFFKVV
LuGA2ox1            MVVLSRPALDHFSS--IKTCKPTPGFFFPGIPVVDFSDPNS--KFLIAKACKDFGFFKLT
NtGA2ox1            MVVLTKPGIDHFPI--VKNCKLSS--FFNGVPLIDLSKPNS--KNLIVKACEEFGFFKVI
NtGA2ox2            --------MDQHFS--KDNCKPTS--FFNNVPLIDLSKPDS--KNLIVKACEEFGFFKVI
NtGA2ox3            MVVLSIPAVEQFSI--VKNCKPISSFFPSNIPIIDISKPDS--KNLLVKAYEEFGFFKII
PtGA2ox1            MVLISKPALEQFSF--IRNRKPTT--VFSGIPLIDLSKPDS--KHLLVEACEEFGFFKVI
PsGA2bhydroxA       MVLLSKPTSEQYTY--VRNNMPIT--FSSSIPLVDLSKPDA--KTLIVKACEDFGFFKVI
PsGA2bhydroxB       MVLLSKPTSEQYTY--VRNNMPIT--FSSSIPLVDLSKPDA--KTLIVKACEDFGFFKVI
PsGA2ox1            -----KPTSEQYTY--VRNNMPIT--FSSSIPLVDLSKPDA--KTLIVKACEDFGFFKVI
AtGA2ox2            MVVLPQPVTLDNHISLIPTYKPVPVLTSHSIPVVNLADPEA--KTRIVKACEEFGFFKVV
AtGA2ox3            MVIVLQPASFDSNLYVNPKCKPRPVL----IPVIDLTDSDA--KTQIVKACEEFGFFKVI
AtGA2ox1            MAVLSKPVAIP---------KSGFSL----IPVIDMSDPES--KHALVKACEDFGFFKVI
PtGA2ox7            MVVPSPTP-IRTKT-----TKALG------IPTVDLSLDNSSVSQLIVRACEEYGFFKVI
PsGA2ox2            MVVPSPTSMIRTKK-----TKAVG------IPTIDLSLERSQLSELVVKACEEYGFFKVV
PtGA2ox4            MVVASPTK-LHSEE-----HLAIE------LPTVDLSGDRSMVSNLIVKACEEYGFFKVK
                                                  :* ::::   :  .  : .* :::****:

PtGA2ox3            NHGVPMEVMTKLEALATNFFNLPQPEKDKAGPPNPFGYGNKKIGPNGDVGWVEYLLLNTN
NtGA2ox5            NHGVPIEIMTKLESEAVNFFNLSQVEKDKAGPANPFGYGNKRIGSNGDVGWVEYLLLTTN
LuGA2ox1            NHGIPKETIRQLESLAVEFFSLPQSEKDSAGPPNPFGYGNKRIGPNGDIGWIEYLLLHSD
NtGA2ox1            NHSVPTEFITKLESEAIKFFSSPLSEKQKAGPADPFGYGNKKIGPNGDVGWVEYILLSTN
NtGA2ox2            NHSVPTEFITKLS-EAIKFFSSPLSEKEKAGPPDPFGYGNKQIGCNGDSGWVEHILVSTN
NtGA2ox3            NHGVPMEFINNLELEAIKFFSSPLNQKEKAGPADPFGYGNRKIGTNGDIGWVEYILLSTN
PtGA2ox1            NHGVPMEFISKLESEAVNFFSLPLSEKEKVGPPSPFGYGNKSIGQNGDVGWVEYLLLTTN
PsGA2bhydroxA       NHGIPLDAISQLESEAFKFFSLPQTEKEKAGPANPFGYGNKRIGLNGDIGWIEYLLLTTN
PsGA2bhydroxB       NHGIPLDAISQLESEAFKFFSLPQTEKEKAGPANPFGYGNKRIGLNGDIGWIEYLLLTTN
PsGA2ox1            NHGIPLDAISQLESEAFKFFSLPQTEKEKAGPANPFGYGNKRIGLNGDIGWIEYLLLTTN
AtGA2ox2            NHGVRPELMTRLEQEAIGFFGLPQSLKNRAGPPEPYGYGNKRIGPNGDVGWIEYLLLNAN
AtGA2ox3            NHGVRPDLLTQLEQEAINFFALHHSLKDKAGPPDPFGYGTKRIGPNGDLGWLEYILLNAN
AtGA2ox1            NHGVSAELVSVLEHETVDFFSLPKSEKTQVAG-YPFGYGNSKIGRNGDVGWVEYLLMNAN
PtGA2ox7            NHGVNKEVVTRLEEEAARFFGKPAAEKQQAGPASPFGYGCKNIGCHGDTGELEYLLLHTN
PsGA2ox2            NHSVPKEVISRLDEEGIEFFSKNSSEKRQAGTSTPFGYGCKNIGPNGDKGELEYLLLHSN
PtGA2ox4            NHGVPHDIIAQMEKESFNFFAKPFDEKQKVEPAKPFGYGCKNIGFNGDMGEVEYLLLNIN
                    **.:  : :  :.     **      *  .    *:***   ** :** * :*::*:  :
```

```
PtGA2ox3          PQISSQK-TSIFQENPQIFRSAVEDYILAVKRMAFEVLELMADGLEIESRNVFSRLLRDD
NtGA2ox5          PDLSYHK-SIAIPGNSHLFWSLVNEYVSAVRNLACLALEKIAEGLRIEPKNVLSKMLRDE
LuGA2ox1          SIKS---------AGPQNFRCAVKEYVAAVKKMTMEMLEAMAEGVGIEPRNALSRLVDDE
NtGA2ox1          SEFNYHKFASILGVNPETIRAAVNDYVSAVKKMACEILEMLAEGLNIHPRNVFSKLLMDE
NtGA2ox2          SEFNYYKFASILGVNPENIRAAVNDYVSAVKKMACEILEMLAEGLKIHPRNVFSKLLMDE
NtGA2ox3          SEFNYQKFASILGLDPENIRVAVNDYVSAMKKMACEILEMLAEGLKIYPKNVFSKLLMDE
PtGA2ox1          QESISQRFSSVFGDNPEKFRCALNDYVSAVKKMACEILEMMADGLKLQQRNVFSKLLMDE
PsGA2bhydroxA     QDHN----FSLYGEDIHKFRGLLKDYKCAMRNMACEILDLMAEGLKIQPKNVFSKLVMDK
PsGA2bhydroxB     QEHN----FSLYGEDIDKFRGLLKDYKCAMRNMACEILDLMAEGLKIQPKNVFSKLVMDK
PsGA2ox1          QEHN----FSLYGEDIDKFRGLLKDYKCAMRNMACEILDLMAEGLKIQPKNVFSKLVMDK
AtGA2ox2          PQLSSPKTSAVFRQTPQIFRESVEEYMKEIKEVSYKVLEMVAEELGIEPRDTLSKMLRDE
AtGA2ox3          LCLESHKTTAIFRHTPAIFREAVEEYIKEMKRMSSKFLEMVEEELKIEPKEKLSRLVKVK
AtGA2ox1          HDSGSGPLFPSLLKSPGTFRNALEEYTTSVRKMTFDVLEKITDGLGIKPRNTLSKLVSDQ
PtGA2ox7          LLSVSERSKTISND-PSGFSCAVSDYIRAVRQLACEILDLAAEGLWVPDKHVFSRLIRDV
PsGA2ox2          PISISERSKTIAKDHPIKFSCIVNDYIKAVKDLTCEILELAAEGLWVPDKSSLSKIIKDE
PtGA2ox4          PLSIAESS-------------AVSAYIEAVRELACELLDLMAEGLRVPDRSVFSRLIRDV
                                        :. *   :: ::   *:   : : :  :  :*:::
PtGA2ox3          KSDSCFRLNHYPPCSEL-QALSGG--------NLIGFGEHTDPQIISVLRSNNTSGLQIC
NtGA2ox5          KSDSCFRLNHYPPFPELLQTLSGR--------NLIGFGEHTDPQVISVVRSNNTSGLQIS
LuGA2ox1          GSDSCLRLNHYPGCPEVQPSLSGGR------MNLVGFGEHTDPQIVSVLRSNNTTGLQIC
NtGA2ox1          KSDSVFRLNHYPPCPEIQQFSDNN---------LIGFGEHTDPQIISVLRSNNTSGLQIL
NtGA2ox2          KSDSVFRLNHYPPCTEIQQFNDNN---------LIGFGEHTDPQIISVLRSNNTSGLQIL
NtGA2ox3          QSDSVFRINHYPPCPEVQEFNGRN---------LIGFGAHTDPQIISLLRSNNTSGLQIS
PtGA2ox1          QSDSVFRLNHYPPCPEIEALTDQN---------MIGFGEHTDPQIISVLRSNNTSGLQIS
PsGA2bhydroxA     QSDCLFRVNHYPACPEL-AINGEN---------LIGFGEHTDPQIISILRSNNTSGFQIS
PsGA2bhydroxB     QSDCLFRVNHYPACPEL-AINGEN---------LIGFGEHTDPQIISILRSNNTSGFQIS
PsGA2ox1          QSDCLFRVNHYPACPEL-AINGEN---------LIGFGEHTDPQIISILRSNNTSGFQIS
AtGA2ox2          KSDSCLRLNHYP----AAEEEAEKM-------VKVGFGEHTDPQIISVLRSNNTAGLQIC
AtGA2ox3          ESDSCLRMNHYP----EKEETPVK--------EEIGFGEHTDPQLISLLRSNDTEGLQIC
AtGA2ox1          NTDSILRLNHYPPCPLSNKKTNGGK-------NVIGFGEHTDPQIISVLRSNNTSGLQIN
PtGA2ox7          HSDSVLRLNHYPAVEEIAD--WDPSPIR------IGFGEHSDPQILTILRSNDVAGLQIC
PsGA2ox2          HSDSLLRINHYPPVKKLGNDNWDPSKIQNSNNNNIGFGEHSDPQILTILRSNNVGGLQIS
PtGA2ox4          DSDSLIRLNHYPPMPLLCKD-EDSSPCNQ---NKVGFGEHSDPQILTILRSNDVGGLQIS
                   :*. :*:**** :*** *:***:::::***:. *:**
PtGA2ox3          LKEGTWVSVPPDQTSFFINVGDALQVMTNGRFRSVKHRVLADPLKPRISMIFFGGPPLSE
NtGA2ox5          LKDGTWLSVPPDPYSFFINVDDSLQVMSNGRFRSVRHRVLADSMKSRVSMIYFGGPPLSE
LuGA2ox1          LKDGAWVSVPPDQSSFFVIVGDALQVMTNGRFKSVKHRVMTDPAKSRVSMIYFAGPPMSE
NtGA2ox1          LKNGHWISVPPDPNSFFINVGDSLQVMTNGRFKSVHRVLANSVKSRLSMIYFGGPPLSE
NtGA2ox2          LKNGHWISVPPDPNSFFVNVGDSLQVMTNGKFKSVKHRVLANSMKSRLSMIYFGGPPLSE
NtGA2ox3          LEDGHWISVPPDQNSFFINVGDSLQVMTNGRFKSVKHRVLANSLKSRLSMIYFGGPPLSE
PtGA2ox1          LSDGSWISVPPDQNSFFVNVGDSLQVMTNGRFKSVRHRVLTNSMKARVSMIYFGGPPLSE
PsGA2bhydroxA     LRDGSWISVPPDHSSFFINVGDSLQVMTNGRFKSVRHRVLANGIDPRLSMIYFCGPPLSE
PsGA2bhydroxB     LRDGSWISVPPDHSSFFINVGDSLQVMTNGRFKV--------------------------
PsGA2ox1          LRDGSWISVPPDHSSFFINVGDSLQVMTNGRFKSVRHRVLANGIDPRLSMIYFCGPPLSE
AtGA2ox2          VKDGSWVAVPPDHSSFFINVGDALQVMTNGRFKSVKHRVLADTRRSRISMIYFGGPPLSQ
AtGA2ox3          VKDGTWVDVTPDHSSFFVLVGDTLQVMTNGRFKSVKHRVVTNTKRSRISMIYFAGPPLSE
AtGA2ox1          LNDGSWISVPPDHTSFFFNVGDSLQVMTNGRFKSVRHRVLANCKKSRVSMIYFAGPSLTQ
PtGA2ox7          LHDGLWVPVPPDSTGFYVIVGDSFQVLTNGRFESVRHRVLTNSSQPRMSMMYFGAPPLTA
PsGA2ox2          THHGLWIPVPPDPSEFYVMVGDALQVLTNGRFVSVRHRVLTNTTKPRMSMMYFAAPPLNW
PtGA2ox4          LNDGAWVPVTPDPATFWVNVGDLLQAMTNGRFVSVRHKALTNSSKSRMSMAYFAGPPPNA
                    .* *: *.**   *:. *.* :*.::**:*
PtGA2ox3          KIAPLPSLMAERGGSLYKEFTWFEYKRSAYKSRLADYRLGLFEK--------TAGQ---
NtGA2ox5          KIAPLSCLMEEGEESLYNEFTWCEYKKSAYKTRLGDNRLALFEKKPQTKPATSAAQ---
LuGA2ox1          KIVPLESIMEVGEESRYEEFTWSEYKQSAYKSRLADYRLGHFEKKKIKNTDNNGGFWL-
NtGA2ox1          KIAPLASLMEGE-ESLYEEFTWFEYKKSAYKTRLADNRLVLFEKVAAS-----------
NtGA2ox2          KIAPLASLMEGE-DSLYKEFTWFEYKKSAYKTRLADNRLILFEKIAAS-----------
NtGA2ox3          KIAPLASLIKGDQDSLYKEFTWFEXKKSAYNSRLADNRLVLFEKVL-------------
PtGA2ox1          KIAPLPSLIKGK-ESLYKEFTWFEYKRSAYSSRLADNRLVLFERIAAS-----------
PsGA2bhydroxA     KIAPLPSLMKGK-ESLYKEFTWFEYKSSTYGSRLADNRLGNYERIAAT-----------
PsGA2bhydroxB     ------------------------------------------------------------
PsGA2ox1          KIAPLPSLMKGK-ESLYKEFTWFEYKSSTYGSRLADNRLGNYERIAAT-----------
AtGA2ox2          KIAPLPCLVPEQDDWLYKEFTWSQYKSSAYKSKLGDYRLGLFEKQPLLNHKTLV-----
AtGA2ox3          KIAPLSCLVPKQDDCLYNEFTWSQYKLSAYKTKLGDYRLGLFEKRPPFSLSNV------
AtGA2ox1          RIAPLTCLIDNEDERLYEEFTWSEYKNSTYNSRLSDNRLQQFERKTIKNLLN-------
PtGA2ox7          WIAPLSHMVSQQNPSLYKPFTWSEFKKAAYSLRLRDTRLDLFK----------------
PsGA2ox2          LISPLSKMVTAHSPCLYRPFTWAQYKQAAYALRLGDTRLDQFKVQKQEDSNDSHSL---
PtGA2ox4          RITVPPEMITPTKPALYKPFTWAEFKKAAYAMRLGDRRLGLFRMEGDEQVA--------
```

3. LuGA3ox1

```
NtGA3ox2          MPSR-ISDDV-------HQKQL--DLYTIKELPESHAWRSSLDHDYQC-----NDSLESI
NtGA3bhydrox      MPSR-ISDSFRAH----SQKHL--DLNSIKELPESHAWTSSNDYPSEN-----SCNFESI
PttGA3ox1         MPSRSLADAFRSHPVHLHQKHL--DFSSLQEIPDSHKWTQLDDIEQQHPS-VESFITESV
PsGA3bhydrox      MPSL--SEAYRAHPVHVNHKHP--DFNSLQELPESYNWTHLDDHTLIDSNNIMKESTTTV
LuGA3ox1          MPSIQLADAFRSHPVNLHQKHQLNDFTSLKELPDSYKWSTRINHDNVR-------TNEPV
AtGA3ox1          MPAM-LTDVFRGHPIHLPHSHI-PDFTSLRELPDSYKWTPKDDLLFSAAP-SPPATGENI
AtGA3ox2          MSST-LSDVFRSHPIHIPLSNP-PDFKSL---PDSYTWTPKDDLLFSAS-----ASDETL
                  *.:   ::           .:   *: ::   *:*: *    :                :
NtGA3ox2          PVIDLN-----DEKFAIENIGHACKTWGAFQIINHNISQRLLDNMEE-AGKRLFSLPMQQ
NtGA3bhydrox      PVIDLDNYINNNNINVLEHIGQACKKWGAFQIINHNISERLLQDIEL-AGKSLFSLPMQQ
PttGA3ox1         PVIDLL------DPNVLQNIGNACKTWGVLQVTNHGIPISLLESVEG-VSRSLFSLPVQQ
PsGA3bhydrox      PVIDLN------DPNASKLIGLACKTWGVYQVMNHGIPLSLLEDIQW-LGQTLFSLPSHQ
LuGA3ox1          PVIDLS------RPEAMREIGHACREWGVFQVVNHGVPTEVVDRIEKNVGENFFGLPMYE
AtGA3ox1          PLIDLD------HPDATNQIGHACRTWGAFQISNHGVPLGLLQDIEF-LTGSLFGLPVQR
AtGA3ox2          PLIDLS------DIHVATLVGHACTTWGAFQITNHGVPSRLLDDIEF-LTGSLFRLPVQR
                  *:***          .   :* **  **. *: **.:.  ::: ::      :* **  .
NtGA3ox2          KLKAARSADGIAGYGVARISSFFSKLMWSEGFTIVGSPFDHARQLWPHDYKKFCDVIEEY
NtGA3bhydrox      KLKAARSPEGVTGYGVARISSFFSKLMWSEGFTIVGSPLEHARQIWPHDYQKFCDVIEEY
PttGA3ox1         KLKAARSPDGVSGYGVARISSFFSKLMWSEGFTIVGSPLEHFRQLWPQDYTKFCDVIEEH
PsGA3bhydrox      KHKATRSPDGVSGYGIARISSFFPKLMWYEGFTIVGSPLDHFRELWPQDYTRFCDIVVQY
LuGA3ox1          KLKAARSPNGVSGFGLARISTFFPKLMWSEGFTVVGSPAEHFMKVWPEDYRQFCDIVEGY
AtGA3ox1          KLKSARSETGVSGYGVARIASFFNKQMWSEGFTITGSPLNDFRKLWPQHHLNYCDIVEEY
AtGA3ox2          KLKAARSENGVSGYGVARIASFFNKKMWSEGFTVIGSPLHDFRKLWPSHHLKYCEIIEEY
                  * *::**  *::*:*:***::** * ** ****: *** ..  ::** .: .:*:::  :
NtGA3ox2          EKEMEKLAGRLMWLMLGSLGISKDDMKWACCG---PRGECSALQLNSYPACPDPDRAMGL
NtGA3bhydrox      EREMEKLAGRLMWLMLGSLGITKEEVKWAVCPKGESKGGSAALQLNSYPACPDPDRAMGL
PttGA3ox1         EKEMQKLARRLTWLMLGSLGITKKDLNWAGPK-GESKEGGAALQLNSYPACPDPDLAMGL
PsGA3bhydrox      DETMKKLAGTLMCLMLDSLGITKEDIKWAGSK-AQFEKACAALQLNSYPSCPDPDHAMGL
LuGA3ox1          EKEMQKLASRLMDLILGSLGITTQDLSWDYST--PKSVSSAAIQLNHYPACPDPDQAMGL
AtGA3ox1          EEHMKKLASKLMWLALNSLGVSEEDIEWASLS-SDLNWAQAALQLNHYPVCPEPDRAMGL
AtGA3ox2          EEHMQKLAAKLMWFALGSLGVEEKDIQWAGPN-SDFQGTQAVIQLNHYPKCPEPDRAMGL
                  :. *:***  *  : *.***:  .::.*            :.:*** ** **:** ****
NtGA3ox2          AAHTDSTILTILHQNNTSGLQVFKEG-NGWVTVPPLSGALVINVGDLLHILSNGLYPSVL
NtGA3bhydrox      AAHTDSTILTILHQNNTSGLQVFKEG-SGWVTVPPFPGALVVNVGDLLHILSNGLYPSVL
PttGA3ox1         AAHTDSTLLTILYQNNTSGLQVLKEG-IGWVTVPPIPGGLVVNVGDLLHILSNGLYPSVL
PsGA3bhydrox      APHTDSTFLTILSQNDISGLQVNREG-SGWITVPPLQGGLVVNVGDLFHILSNGLYPSVL
LuGA3ox1          GPHTDSTLVTILHQNATSGLQVLRDGGAQWVTVPPIKGALVVNVGDLLHILSNGLYSSVV
AtGA3ox1          AAHTDSTLLTILYQNNTAGLQVFRDD-LGWVTVPPFPGSLVVNVGDLFHILSNGLFKSVL
AtGA3ox2          AAHTDSTLMTILYQNNTAGLQVFRDD-VGWVTAPPVPGSLVVNVGDLLHILTNGIFPSVL
                  ..*****::*** **  :**** ::.   *:*.**. *.**:*****:***:**:: **:
NtGA3ox2          HRAVVNRTRHRLSVAYLYGPPSGVKISPLSKLVDQGHPPLYRSVTWSEYLGTKAKHFDKA
NtGA3bhydrox      HRAVVNRTRHRLSVAYLYGPPSRVKVSPLAKLVDQRHPPLYRAVTWSEYLGTKARHFDKA
PttGA3ox1         HRAVVNRTKHRLSIAYLYGPPSSVQISPIQKLVGPNHPPLYRPITWNEYLVAKAKHFNKA
PsGA3bhydrox      HRVLVNRTRQRFSVAYLYGPPSNVEICPHAKLIGPTKPPLYRSVTWNEYLGTKAKHFNKA
LuGA3ox1          HRAVVNRKKNRMSIAYLYGPPSNVRISPVGKLVGPGQPPLYRAVTWSEYLGTKAKHFNKA
AtGA3ox1          HRARVNQTRARLSVAFLWGPQSDIKISPVPKLVSPVESPLYQSVTWKEYLRTKATHFNKA
AtGA3ox2          HRARVNHVRSRFSMAYLWGPPSDIMISPLPKLVDPLQSPLYPSLTWKQYLATKATHFNQS
                  **. **: : *:*:*:*:** * : :.*  **:.  ..*** .:**.:** :** **:::
NtGA3ox2          LSSFQLCA---PRIGFANPKD---RNSVQVG
NtGA3bhydrox      LSSVRLCA---PLSGFTDAKD---HNGVQVG
PttGA3ox1         LSSVRICA---PLNGLVDVND---HNSVKVG
PsGA3bhydrox      LSSVRLCT---PINGLFDVNDS-NKNSVQVG
LuGA3ox1          LSSVRLCAGATPMNGLVDARDGSNNNRVKVG
AtGA3ox1          LSMIRNHR-----------EE----------
AtGA3ox2          LSIIRN-------------------------
                  ** .:
```

4. LuIAA1

```
AtIAA1            ----MEVTNGLNLKDTELRLGLPGAQE--EQQLELS-CVRSNNKRKNNDS-TEESA----
AtIAA2            --MAYEKVNELNLKDTELCLGLPGRTEKIKEEQEVS-CVKSNNKRLFEETRDEEES----
AtIAA4            MEKVDVYDELVNLKATELRLGLPGTE------ETVSCG-KSN-KRVLPEDTEKEIE----
AtIAA3            ------MDEFVNLKETELRLGLPGTDNVCEAKERVSCC-NNNNKRVLSTDTEKEIE----
PoptrIAA3.4       MERSMAYESDLNLKATELRLGLPGSDEPEKPSTTPS---VRSNKRASPEISEESRS---K
PttIAA3.3         MERSMAYERHLNLKATELRLGLPGSDEPEKPSTTPS---VRSNKRASPEISEESRS---K
PoptrIAA3.1       ----MEFERDLNLEATELRLGLPGTATEQLEKQTPNSNVTKSNKRSLPDMNEDSAG--RR
PoptrIAA3.2       ----MEFERDLNLDATELRLGLPGTATKQSEKQTPNSNLAKSNKRSLPDMNEEPAGSSRE
PoptrIAA3.5       MEGGVAYENDLNLKETELRLGLPGTGCTNE-KGVSG---ARNNKRPFPETREEGGA---N
PoptrIAA3.6       MEGGVAYENDLNLKATELRLGLPGTSCTNEEQAVSG---ARNNKRPLPETREERGA---K
LuIAA1            -MEGATYESDLNFEATELRLGLPGSGE---EETAVK----SNNKRPMPAETNEEIEAK--
                            :*:. *** *****                . **       .
```

```
AtIAA1          ------------PPPA-----------KTQIVGWPPVRSNRKNNNNK----------NVS
AtIAA2          ------------TPPT-----------KTQIVGWPPVRSSRKNNNS------------VS
AtIAA4          ----STG-KTETAPPP-----------KAQIVGWPPVRSYRKNNIQTKKNES--E-GQGN
AtIAA3          ----SSSRKTETSPPR-----------KAQIVGWPPVRSYRKNNIQSKKNESEHE-GQGI
PoptrIAA3.4     GSSSVSSN-VENGERDS------APPAKAQVVGWPPIRSYRKNCLQPKKNDQ-VD-GAGM
PttIAA3.3       GSSSLSSN-VENSEGDD------APPAKAQVVGWPPIRSYRKNCLQPKKNDR-VD-GAGM
PoptrIAA3.1     ESSSVSSNDKKSHEQET------APPTKTQVVGWPPIRSYRKNCLQARKLE--AE-AAGL
PoptrIAA3.2     NSSTVSSNDKKSHDQET------APPIKAQVVGWPPIRSYRKNCLQAKKLE--AE-AAGL
PoptrIAA3.5     GKSDAQHDDQETASAPNTYSFDMHATCRVQIVGWPPIRSYRKNSLQPKKAED-EA-AAGM
PoptrIAA3.6     GKSDPRHDDQETAPAP-----------KAQIVGWPPIRSYRKNTLQPKKAEA-EA-AAGM
LuIAA1          ---GRSGDHVQAAPAA-----------KAQIVGWPPIRSYRKNSLQPKKSTEAADGASGM
                                           :.*:*****:** ***  .
AtIAA1          YVKVSMDGAPYLRKIDLKMYKNYPELLKALENMFKFTVGEYSEREGYKGSGFVPTYEDKD
AtIAA2          YVKVSMDGAPYLRKIDLKTYKNYPELLKALENMFKVMIGEYCEREGYKGSGFVPTYEDKD
AtIAA4          YVKVSMDGAPYLRKIDLTMYKQYPELMKSLENMFKFSVGEYFEREGYKGSDFVPTYEDKD
AtIAA3          YVKVSMDGAPYLRKIDLSCYKGYSELLKALEVMFKFSVGEYFERDGYKGSDFVPTYEDKD
PoptrIAA3.4     YVKVSVDGAPYLRKIDLKVYKSYPELLKALENMFKLTIGEYSENEGYNGSEFAPTYEDKD
PttIAA3.3       YVKVSVDGAPYLRKIDLKVYRSYPELLKALEDMFKLTIGEYSEKEGYNGSDFAPTYEDKD
PoptrIAA3.1     YVKVSMDGAPYLRKIDLKVYKGYPELLEVVEEMFKFKVGEYSEREGYNGSEYVPTYEDKD
PoptrIAA3.2     YVKVSMDGAPYLRKIDLKVYKGYPELLKALEEMFKSKVGEYSEREGYNGSEHVPTYEDKD
PoptrIAA3.5     YVKVSMDGAPYLRKIDLKVYKGYPELLKALENMFKLTIGEYSEREGYKGSEYAPTYEDKD
PoptrIAA3.6     YVKVSMDGAPYLRKIDLKVYKGYPELLKALENMFKLTIGEYSEREGYKGSEYAPTYEDKD
LuIAA1          YVKVSMDGAPYLRKIDLKVYRGYPELLMALETMFKFAAGVYSEREGYKGSEHVPTYEDKD
                *****:***********. *: *.**:  :* ***   * * *.:**:** ..*******
AtIAA1          GDWMLVGDVPWDMFSSSCQKLRIMK--GS----------EAP---TAL----
AtIAA2          GDWMLVGDVPWDMFSSSCKRLRIMK--GS----------DAPALDSSL----
AtIAA4          GDWMLVGDVPWEMFVSSCKRLRIMK--GS----------EVKGLGCGGV---
AtIAA3          GDWMLIGDVPWEMFICTCKRLRIMK--GS----------EAKG---------
PoptrIAA3.4     GDWMLVGDVPWDMFISSCKRLRIMK--GS----------EARGLGC------
PttIAA3.3       GDWMLVGDVPWDMFISTCKRLRIMK--GS----------EARGLGC------
PoptrIAA3.1     GDWMLVGDVPWEMFINSCKRLRIMK--ES----------EARGLGCAV----
PoptrIAA3.2     GDWMLVGDVPWDMFINSCKRLRIMK--ES----------EARGLGCAV----
PoptrIAA3.5     GDWMLVGDVPWDMFLSSCKKLRIMK--GS----------EAIGLGCGA----
PoptrIAA3.6     GDWMLIGDVPWDMFLSSCKKLRIIK--GS----------EATG---------
LuIAA1          GDWMLVGDVPWDMFMSS-KRGGLFRQPGSSLNPQEESFVPAVGQGNKEKSL-
                *****:*****:**  : ::  :::   *           .
```

5. LuPIN1

```
AtPIN7          MITWHDLYTVLTAVIPLYVAMILAYGSVRWWKIFSPDQCSGINRFVAIFAVPLLSFHFIS
AtPIN3          MISWHDLYTVLTAVIPLYVAMILAYGSVRWWKIFSPDQCSGINRFVAIFAVPLLSFHFIS
AtPIN4          MITWHDLYTVLTAVVPLYVAMILAYGSVQWWKIFSPDQCSGINRFVAIFAVPLLSFHFIS
PtPIN3          MISWNDLYNVLSAVIPLYVAMILAYGSVRWWKIFSPDQCSGINRFVAIFAVPLLSFHFIS
PtPIN6          MISWNDLYNVLSAVIPLYVAMILAYGSVRWWKIFSPDQCSGINRFVAIFAVPLLSFHFIS
AtPIN1          MITAADFYHVMTAMVPLYVAMILAYGSVKWWKIFTPDQCSGINRFVALFAVPLLSFHFIA
LuPIN1          MITGTDFYHVMTAMVPLYVAMFLAYGSVKWWKIFTPDQCSGINRFVALFAVPLLSFHFIS
PtPIN1          MISLLDFYHVMTAMVPLYVAMILAYGSVKWWKIFTPDQCSGINRFVALFAVPLLSFHFIS
PtPIN7          MISLLDFYHVMTAMVPLYVAMILAYGSVKWWKIFTPDQCSGINRFVALFAVPLLSFHFIS
PtPIN8          MISLTDLYHVLTAVVPLYVAMILAYGSVKWWKIFSPDQCSGINRFVALFAVPLLSFHFIS
PtPIN2          MISIGDLYHVLTAVVPLYVAMILAYGSVKWWKIFSPDQCSGINRFVALFAVPLLSFHFIS
AtPIN2          MITGKDMYDVLAAMVPLYVAMILAYGSVRWWGIFTPDQCSGINRFVAVFAVPLLSFHFIS
AtPIN5          MISWLDIYHVVSATVPLYVSMTLGFLSARHLKLFSPEQCAGINKFVAKFSIPLLSFQIIS
PtPIN4          MITADDFYKVMCAMVPLYFAMLVAYGSVKWYKIFTPEQCSGINRFVAVFAVPVLSFHFIA
PtPIN5          MITAGDFYKVMCAMVPLYFAMLVAYGSVKRYKIFTPEQCSGINRFVAVFAVPVLSFHFIA
AtPIN6          MITGNEFYTVMCAMAPLYFAMFVAYGSVKWCKIFTPAQCSGINRFVSVFAVPVLSFHFIS
                **:  ::* *: *  ***.:* :.: *.:   :*:* **:***:**: *::*:***::*:
```

```
AtPIN7          SNNPYAMNLRFIAADTLQKLIMLTLLIIWANFT-----RSGSLEWSITIFSLSTLPNTLV
AtPIN3          TNNPYAMNLRFIAADTLQKIIMLSLLVLWANFT-----RSGSLEWSITIFSLSTLPNTLV
AtPIN4          TNDPYAMNFRFVAADTLQKIIMLVLLALWANLT-----KNGSLEWMITIFSLSTLPNTLV
PtPIN3          TNDPYAMNFRFIAADTLQKIIMLIALGIWTNFT-----KNGSLEWMITIFSVSTLPNTLV
PtPIN6          TNDPYSMNFRFIAADTLQKIIMLFALGIWTNFT-----KNGSLEWMITIFSLSTLPNTLV
AtPIN1          ANNPYAMNLRFLAADSLQKVIVLSLLFLWCKLS-----RNGSLDWTITLFSLSTLPNTLV
LuPIN1          SNDPYAMNFRFIFADSLQKLIILAALAVWANLS-----RRGTLEWSITLFSLSTLPNTLV
PtPIN1          TNDPYNMNLRFITADSLQKIIVLVVLALWTKLS-----KRGCLEWTITLFSLSTLPNTLV
PtPIN7          TNDPYKMNFRFIAADTLQKIIVLVVLAFWTMFS-----KRGCLEWTITLFSLSTLPNTLV
PtPIN8          TNNPYAMNYRFIAADTLQKIIVLVVLAIWTRVI-----SRGSLEWSITLFSLSTLPNTLV
PtPIN2          TNNPYAMNLRFIAADTLQKIIVLVVLAIWSRAS-----SRGSLEWSITLFSLSSLPNTLV
AtPIN2          SNDPYAMNYHFLAADSLQKVVILAALFLWQAFS-----RRGSLEWMITLFSLSTLPNTLV
AtPIN5          ENNPFKMSPKLILSDILQKFLVVVVLAMVLRFWHPTGGRGGKLGWVITGLSISVLPNTLI
PtPIN4          QNNPYQMDTKFILADTLSKVLALVLLSVWAVFFN------GEFDWLITLFSVATLPNTLV
PtPIN5          QNNPYQMDTRFIMADTLSKVLVLVLLSIWVIFFN------GGLDWLITLFSIATLPNTLV
AtPIN6          QNNPYKMDTMFILADTLSKIFVFVLLSLWAVFFKA-----GGLDWLITLFSIATLPNTLV
                 *:*: *.  :: :* *.*.. .  * .            * : * ** :*:: *****:
AtPIN7          MGIPLLIAMYGEYSGSLMVQIVVLQCIIWYTLLLFLFEYRGAKILIMEQFPET-GASIVS
AtPIN3          MGIPLLIAMYGEYSGSLMVQIVVLQCIIWYTLLLFLFEFRGAKMLIMEQFPET-AASIVS
AtPIN4          MGIPLLIAMYGTYAGSLMVQVVVLQCIIWYTLLLFLFEYRGAKLLIMEQFPET-GASIVS
PtPIN3          MGIPLLTAMYGTYSGSLMVQIVVLQCIIWYTLLLFLFEYRGAKMLIMEQFPET-AASIVS
PtPIN6          MGIPLLIAMYDDYSGSLMVQVVVLQCIIWYTLLLFLFEYRGAKMLIMEQFPET-AASIVS
AtPIN1          MGIPLLKGMYGNFSGDLMVQIVVLQCIIWYTLMLFLFEYRGAKLLISEQFPDT-AGSIVS
LuPIN1          MGIPLLKGMYGDESGTLMVQIVVLQCIIWYTLMLFMFEFRGARILISEQFPDT-AGSIVS
PtPIN1          MGIPLLKGMYGDYSGSLMVQVVVLQCIIWYTLMLFMFEYRGAKLLISEQFPDT-AGSIVS
PtPIN7          MGIPLLKGMYGDYSGSLMVQVVVLQCIIWYTLMLFLFEYRGAKLLISEQFPDT-AGSIVS
PtPIN8          MGIPLLKGMYGEASGSLMVQIVVLQCIIWYTLMLFLFEYRGARILIGEQFPDT-AGSIIS
PtPIN2          MGIPLLKGMYGHSSGSLMVQIVVLQCIIWYTLMLFLFEYRGARILIGEQFPDT-AGSIIS
AtPIN2          MGIPLLRAMYGDFSGNLMVQIVVLQSIIWYTLMLFLFEFRGAKLLISEQFPET-AGSITS
AtPIN5          LGMPILSAIYGDEAASILEQIVVLQSLIWYTILLFLFELNAAR-----ALPSS-GASLEH
PtPIN4          MGIPLLKAMYGDFTQSLMVQVVVLQCIIWYTLLLFLFEYRAATLLIKAQFPGPTAATISK
PtPIN5          MGIPLLKAMYGDFTQSLMVQVVVLQCIIWYTLLLFLFEYRAATLLIKTQFPGPKAASISK
AtPIN6          MGIPLLQAMYGDYTQTLMVQLVVLQCIIWYTLLLFLFELRAARLLIRAEFPGQAAGSIAK
                :*:*:* .:*.  :  :: *:****.:****::**:** ..*       :*   ..::
AtPIN7          FKVESDVVSLDGHDFLETDAQIGDDGKLHVTVRKSNASRRSFYGG---GGTNMTPR----
AtPIN3          FKVESDVVSLDGHDFLETDAEIGDDGKLHVTVRKSNASRRSFCG------PNMTPR----
AtPIN4          FKVESDVVSLDGHDFLETDAEIGNDGKLHVTVRKSNASRR---------SLMMTPR----
PtPIN3          FKVDSDVVSLDGRDFLETDAEIGDDGKLHVTVRKSNASRRSLGPG---SFSGMTPR----
PtPIN6          FKVDSDVVSLDGRDFLETDAEIGDDGKLHVTVRKSNASRRSLGPG---SFSGLTPR----
AtPIN1          IHVDSDIMSLDGRQPLETEAEIKEDGKLHVTVRRSNASRSDIYSR---RSQGLSAT----
LuPIN1          IHVDSDIMSLDGRMPLETEAEIKSDGKLHVTVRRSNASRSDVFSR---RS-GTGAT----
PtPIN1          IHVDSDIMSLDGRQPLETEAAIKEDGKLHVTVRKSNASRSDIFSR---RSQGLSSTT---
PtPIN7          IHVDSDIMSLDGRQPLETEAEIKEDGKLHVTVRKSNASRSDIFSR---RSQGLSSTT---
PtPIN8          FRVDSDILSLDGREPLQTDAEVGEDGKLHVTVRKSTSSRSDVFSR---MSHGLNSGLSMT
PtPIN2          FRVDSDILSLDGREPLQTEAEVGEDGKLHVTVRKSTSSRSEVFSH---MSHGLNSGLSLT
AtPIN2          FRVDSDVISLNGREPLQTDAEIGDDGKLHVVVRRSSAASSMISSFNKSHGGGLNSSMIT-
AtPIN5          TGNDQEEANIE-------DEPKEEEDEEEVAIVRTR-------------------------
PtPIN4          IELDDDVISLDGRDPLRTESETDGNGRIRVRIRRSTSSAPDSALS---SSICLTPR----
PtPIN5          IELDNDVISLDGRDPLRTESETDGNGRLRVRIRRSTSSAPDSALS---SSICLTPR----
AtPIN6          IQVDDDVISLDGMDPLRTETETDVNGRIRLRIRRSVSSVPDSVMS---SSLCLTPR----
                   :.:  .::       :     :.. .: : ::
AtPIN7          --PSNLTGAEIYSLNT----TPRGSNFNHSDFYSMMGFPG------GRLSNFGPA-----
AtPIN3          --PSNLTGAEIYSLST----TPRGSNFNHSDFYNMMGFPG------GRLSNFGPA-----
AtPIN4          --PSNLTGAEIYSLSS----TPRGSNFNHSDFYSVMGFPG------GRLSNFGPA-----
PtPIN3          --PSNLTGAEIYSLSSSRNPTPRGSNFNPSDFYSMMGVQGFP----GRHSNLGPA-----
PtPIN6          --PSNLTGAEIYSLSSSRNPTPRGSNFNPSDFYSMMGVQG------GRHSNLGPA-----
AtPIN1          PRPSNLTNAEIYSLQSSRNPTPRGSSFNHTDFYSMMASGG------GRNSNFGPG-----
LuPIN1          PRPSNLTNAEIYSLQSSRNPTPRGSSFNHTDFYSMMAAG--------RSSNFG-------
PtPIN1          PRPSNLTNAEIYSLQSSRNPTPRGSSFNHTDFYSMMAAG--------RNSNFGAS-----
PtPIN7          PRPSNLTNAEIYSLQSSRNPTPRGSSFNHTDFYSMMAAG--------RNSNFGAS-----
PtPIN8          PRPSNLTNAEIYSLQSSRNPTPRASSFNHTDFYS-KNAS----------------------
PtPIN2          PRPSNLTNAEIYSLQSSRNPTPRASSFNHTDFYSMVNGK----------------------
AtPIN2          PRASNLTGVEIYSVQSSREPTPRASSFNQTDFYAMFNASKAPSPRHGYTNSYGGAGAGPG
AtPIN5          ------------------------------------------------------------
PtPIN4          --PSNLSNAEVFSVN-----TPVPLHEYHGYNG---------------------------
PtPIN5          --PSNLSNAEVFSVS-----TPAPLQEYHGYNG---------------------------
AtPIN6          --ASNLSNAEIFSVN-----TPN-NRFFHGGGGSGT------------------------
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AtPIN7          -DMYSVQSSRGPTPRPSNFEESC---------AMASSPRFGYYPGG-APGS---YPAP-N
AtPIN3          -DMYSVQSSRGPTPRPSNFEENC---------AMASSPRFGYYPGG-GAGS---YPAP-N
AtPIN4          -DLYSVQSSRGPTPRPSNFEENN---------AVKYGFYNNTNSSVPAAGS---YPAP-N
PtPIN3          -DLYSVQSSRGPTPRPSNFEENCAP------TATLSSPRFGFYPAQTVPTS---YPAP-N
PtPIN6          -DLYSVQSSRGPTPRPSNFEENCAP------MATITSPRFGFYPAQTVPTS---YPAP-N
AtPIN1          -EAV--FGSKGPTPRPSNYEEDGGPAKPTAAGTAAGAGRFHYQSGGSGGGGGAHYPAP-N
LuPIN1          -------ANDGPTPRPSNYDEDG-------KTTSSNAARYGYGGGPAGSG----YPAP-N
PtPIN1          -DVYGLSASRGPTPRPSNFEEEH---------GGSNKPRFHHYHAPGGAT---HYPAP-N
PtPIN7          -DVYGLSASRGPTPRPSNFEEEN---------GGSNKPRFH-YHAPGGAT---HYPAP-N
PtPIN8          ----------NASPRHSNFSNLQFD------EESGGLGVFGNVPRANGSA----YPTPPN
PtPIN2          ----------NASPRHSNFTNLQFD------EESGGLGVFGNVPRANGSA----YPAPPN
AtPIN2          GDVYSLQSSKGVTPRTSNFDEEVMKT-----AKKAGRGGRSMSGELYNNNSVPSYPPP-N
AtPIN5          ------------------------------------------------------------
PtPIN4          ------HFSHGPNNEIVLCNGDLG----LAY-RSGTSP---RLSGYASSDAYSLQPTPRA
PtPIN5          ------RFSHGPNNEIMLCNGDLG----FGYHRSGTSP---RLSGYASSDAYSLQPTPRT
AtPIN6          -----LQFYNG-SNEIMFCNGDLGG---FGFTRPGLGASPRRLSGYASSDAYSLQPTPRA

AtPIN7          PEFS-----TGNKTGSKAPKENHH-------HVGKSNSNDAKELHMFVWGSNGSPVSDRA
AtPIN3          PEFSSTTTSTANKSVNKNPKDVNTNQQTTLPTGGKSNSHDAKELHMFVWSSNGSPVSDRA
AtPIN4          PEFSTG--TGVSTKPNKIPKENQQQLQ----EKDSKASHDAKELHMFVWSSSASPVSDVF
PtPIN3          PEFAST--VTTKTAKNQQQQ-------------NSKANHDAKELHMFVWSSSASPVSEGG
PtPIN6          PELAST--ITSKTTKNQQQQNHQQQLLQPQPQQNSKVNHDAKELHMFVWSSSASPVSEGG
AtPIN1          PGMFSPNTGGGGGTAAKGNAP--------VVGGKRQDGNG-RDLHMFVWSSSASPVSD--
LuPIN1          PGMFSPTTAAG-----KVNSN--------VKKGGKEDGGGGKDLHMFVWSSSNSPVSD--
PtPIN1          PGMFSPTTAASKGVSANANNTAAAAAKK-PNGQAQQKAEDGRDLHMFVWSSSASPVSD--
PtPIN7          PGMFSPTTAS-KGVAANANN---AAAKK-PNGQAQQKAEDGRDLHMFVWSSSASPVSD--
PtPIN8          AGIFSP----------------------GGKKKANGTENGKDLHMFVWSSSASPVSEGG
PtPIN2          AGIFSP----------------------GGKKKANGAENGKDLHMFVWSSSASPVSEGG
AtPIN2          PMFTGSTSGASGVKKKESGGG--------GSGGGVGVGGQNKEMNMFVWSSSASPVSEAN
AtPIN5          ------------------------------------------------------------
PtPIN4          SNFN-----------------------------ELDLTNATNTPFWVRSPVAGKIYR--
PtPIN5          SNFN-----------------------------EWDLTNATNTPFWARSPVAGKISR--
AtPIN6          SNFN-----------------------------ELDVN-GNGTPVWMKSPAAGRIYR--

AtPIN7          GLQVDNGA----NEQVGKSDQGGAKEIRMLISDHTQNGENK--AGPMNGDYGGE------
AtPIN3          GLNVFGGAPD--NDQGGRSDQG-AKEIRMLVPDQSHNGETKAVAHPASGDFGGEQQFSFA
AtPIN4          G----GGAGD--NVATEQSEQG-AKEIRMVVSDQPRKSGGDDIGGLDSG-----------
PtPIN3          GLHVFGGTDFGASEQSGRSDQG-AKEIRMLVADHPQNGETKTIPQQDGDFAGEDFSFAGR
PtPIN6          GLHVFGGTDFGASEQSGRSDQG-AKEIRMLVADHPQNGDSKTIP-QAGNFAGEDFSFAGR
AtPIN1          ---VFGGGGGNHHADYSTATNDHQKDVKISVPQG----------------------------
LuPIN1          ---VFGGHEYAASAGGNHHQLEQSKEVRLGVSPGKVVEQRGE---------------TGF
PtPIN1          ---VFGGHDYGAHD---------LKDVRVAVSPGKVEGQR--------------------
PtPIN7          ---VFGGHDYGAHD---------QKDVRLAVSPGKVEGHT--------------------
PtPIN8          L-HVFRGGDYGNDLGGVANQKDYEEFGRDEFSFG---------------------------
PtPIN2          L-HVFKGGDYGNDLGGVAHHKDYDEFGRDEFSFG---------------------------
AtPIN2          AKNAMTRGSSTDVSTDPKVSIPPHDNLATKAMQNLIENMS--------------------
AtPIN5          ------------------------------------------------------------
PtPIN4          --------QPSPATLM-------------MFESK----------------------------
PtPIN5          --------HPSPA----------------ISGEK----------------------------
AtPIN6          --------QSSPKMMWESGQRHAAKDINGSVPEK----------------------------

AtPIN7          ----EESERVKEVPNGLHKLRCNSTAELNPKEAIETG---ETVPVKHMPPASVMTRLILI
AtPIN3          G-KEEEAERPKDAENGLNKLAPNSTAALQSKTGLGGA---EASQRKNMPPASVMTRLILI
AtPIN4          ----EGEREIEKATAGLNKMGSNSTAELEAAGGDGGG---NNG--THMPPTSVMTRLILI
PtPIN3          GEGDDVDQREKEGPTGLNKLGSSSTAELQPK-AAEAP---DSGGSRKMPPASVMTRLILI
PtPIN6          GEGED-DQREKEGPTGLNKLGSSSTAELHPK-AVGAP---DSGGSKQMPPASVMTRLILI
AtPIN1          --NSNDNQYVEREEFSFGNKDDDSKVLATDGGNNISN---KTTQAKVMPPTSVMTRLILI
LuPIN1          VGHGGEEYLVERDEFSFGNREAEN-VNGSGGEKAAANGGGGDVKSKVMPPTSVMTRLILI
PtPIN1          --ENQEDYNLERDDFSFGNRGLDRERNSHEGEKVGFD-----GKPKPMPPTSVMTRLILI
PtPIN7          --ENQEDYNLERDGFSFGNRGMDREMNNPEGEKVGAA-----GKPKPMPPTSVMTRLILI
PtPIN8          --NRPVPNGVDRDGPVLSKLASSSTAELHPKSAANGE-----PKPTAMPPTSVVTRLILI
PtPIN2          --NRPGPNGVDRDGPVLSKLGSSSTAELHPKSAANGE-----AKPTVMPPTSVMTRLILI
AtPIN2          ---PGRKGHVEMDQDGNNGGKSPYMGKKGSDVEDGGP----GPRKQQMPPASVMTRLILI
AtPIN5          ------------------------------------------------SVGTMKILL
PtPIN4          --EISFRDSCKMP----------------APEESNSK---ESVSNQEMPHAIVMIRLILV
PtPIN5          --EISFRDSCKMP----------------APEETNSK---EAVTSQEMPHAIVMLRLILV
AtPIN6          --EISFRDALKAAPQATAAGGGASMEEGAAGKDTTPV---AAIGKQEMPSAIVMMRLILT
                                                          *    **
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AtPIN7          MVWRKLIRNPNTYSSLIGLIWALVAFRWDVAMPKIIQQSISILSDAGLGMAMFSLGLFMA
AtPIN3          MVWRKLIRNPNTYSSLIGLIWALVAFRWHVAMPKIIQQSISILSDAGLGMAMFSLGLFMA
AtPIN4          MVWRKLIRNPNTYSSLIGLIWALVAYRWHVAMPKILQQSISILSDAGLGMAMFSLGLFMA
PtPIN3          MVWRKLIRNPNTYSSLIGLTWSLVAFRWHVEMPKIIKQSISILSDAGLGMAMFSLGLFMA
PtPIN6          MVWRKLIRNPNTYSSLIGLIWSLIAFRWHVEMPKIIKQSISILSDAGLGMAMFSLGLFMA
AtPIN1          MVWRKLIRNPNSYSSLFGITWSLISFKWNIEMPALIAKSISILSDAGLGMAMFSLGLFMA
LuPIN1          MVWRKLIRNPNTYSSLIGLIWSLVSFRWHVTMPAIIAKSISILSDAGLGMAMFSLGLFMA
PtPIN1          MVWRKLIRNPNTYSSLIGLIWSLVSFRWNVKMPLIIAKSISILSDAGLGMAMFSLGLFMA
PtPIN7          MVWRKLIRNPNTYSSLIGLTWSLVSFRWDVQMPAIIAKSISILSDAGLGMAMFSLGLFMA
PtPIN8          MVWRKLIRNPNTYSSLIGLTWSLVSFKWDLEMPQIIAHSISILSDAGLGMAMFSLGLFMA
PtPIN2          MVWRKLIRNPNTYSSLIGLTWSLVSFKWNIMMPAIIADSIAILSNAGLGMAMFSLGLFMA
AtPIN2          MVWRKLIRNPNTYSSLFGLAWSLVSFKWNIKMPTIMSGSISILSDAGLGMAMFSLGLFMA
AtPIN5          KAWRKLIINPNTYATLIGIIWATLHFRLGWNLPEMIDKSIHLLSDGGLGMAMFSLGLFMA
PtPIN4          VVGRKLSRNPNTYSSVLGLLWSLASFKWNVGMPSLVKYSIKIISDAGLGMAMFSLGLFMA
PtPIN5          VVGRKLSRNPNTYSSVLGLLWSLVSFKWNVGMPSLVKYSIKIISDAGLGMAMFSLGLFMA
AtPIN6          VVGRKLSRNPNTYSSLLGLVWSLISFKWNIPMPNIVDFSIKIISDAGLGMAMFSLGLFMA
                 . ***  ***:*::::*: *:   ::    :* ::  ** ::*:.**************
AtPIN7          LQPKLIACGNSTATFAMAVRFFTGPAVMAVAAMAIGLR----------------------
AtPIN3          LQPKLIACGNSVATFAMAVRFLTGPAVMAVAAIAIGLR----------------------
AtPIN4          LQPKIIACGNSVATFAMAVRFITGPAIMAVAGIAIGLH----------------------
PtPIN3          LQPKLIACGNSVATFAMAVRFLTGPAVMAAASIAVGLR----------------------
PtPIN6          LQPKIIACGNSVATFAMAVRFLTGPAVMAAASIAVGLR----------------------
AtPIN1          LNPRIIACGNRRAAFAAAMRFVVGPAVMLVASYAVGLR----------------------
LuPIN1          LQPRIIACGKRTATYAMGVRFFVGPAVMAGASFFLLLASGALSSTSPLSSHTDAFWSLLK
PtPIN1          LQPRIIACGNSIAAFSMAVRFLTGPAIMAAASIAVGIR----------------------
PtPIN7          LQPRIIACGNSVAAFAMAVRFLTGPAVMAAASIAVGLR----------------------
PtPIN8          LQPRIIACGNSVAAFAMSVRFLTGPAVMAAASFAVGLR----------------------
PtPIN2          LQPRIIACGNSIASFAMGVRFLTGPAVMAAASFAVGLR----------------------
AtPIN2          LQPKIIACGKSVAGFAMAVRFLTGPAVIAATSIAIGIR----------------------
AtPIN5          SQSSIIACGTKMAIITMLLKFVLGPALMIASAYCIRLK----------------------
PtPIN4          LQPRIIVCGKKRATMAMAIRFICGPIVMSTTSVAVGMR----------------------
PtPIN5          LQPRIIVCGKKRATMAMAIRFICGPVVMSTASLAVGMR----------------------
AtPIN6          LQPKMIPCGAKKATMGMLIRFISGPLFMAGASLLVGLR----------------------
                 :. :* **   *     ::*. ** .:  :.  : :
AtPIN7          -GDLLRVAIV------QAALPQGIVPFVFAKEYNVHPAILSTGVIFGMLIALPITLVYYI
AtPIN3          -GDLLRVAIV------QAALPQGIVPFVFAKEYNVHPAILSTGVIFGMLIALPITLVYYI
AtPIN4          -GDLLRIAIV------QAALPQGIVPFVFAKEYNVHPTILSTGVIFGMLIALPITLVYYI
PtPIN3          -GTLLHVAIV------QAALPQGIVPFVFAKEYNVHPAILSTAVIFGMLIALPITLVYYI
PtPIN6          -GTLLHVAIV------QAALPQGIVPFVFAKEYNVHPAILSTGVIFGMLIALPITLVYYI
AtPIN1          -GVLLHVAII------QAALPQGIVPFVFAKEYNVHPDILSTAVIFGMLIALPITLLYYI
LuPIN1          LGFLMRRELVRIWRFNEAALPQGIVPFVFAKEYNVHPDILSTGVIFGMLIALPFTLVYYI
PtPIN1          -GTLLHIAIV------QAALPQGIVPFVFAKEYNVHPEILSTGVIFGMLIALPITLVYYI
PtPIN7          -GTLLHIAIV------QAALPQGIVPFVFAKEYNVHPDILSTAVIFGMLIALPITLVYYI
PtPIN8          -GVLLHIAIV------QSALPQGIVPFVFAKEYNVHPDILSTGVIFGMLIALPITLVYYI
PtPIN2          -GDLLRIAIV------QAALPQGIVPFVFAKEYNVHPDILSTGVIFGMLIALPITLVYYI
AtPIN2          -GDLLHIAIV------QAALPQGIVPFVFAKEYNVHPDILSTAVIFGMLVALPVTVLYYV
AtPIN5          -STLFKVAIL------QAALPQGVVPFVFAKEYNLHPEIISTGVIFGMLIALPTTLAYYF
PtPIN4          -GVRLRAAIV------QAALSQGIVPFVFAREYGLHPDIMSTGVIFGMLVSLPVTLIYYI
PtPIN5          -GERLRAAIV------QAALSQGIVPFVFAREYGLHPDIMSTGVIFGMLVSLPVTLLYYI
AtPIN6          -GSRLHAAIV------QAALPQGIVPFVFAREYNLHPDLLSTLVIFGMIVSLPVTILYYV
                 .  ::  ::      ::**.**:******:**.:** ::** *****:::** *: **.
AtPIN7          LLGL-------------------------------------------------
AtPIN3          LLGL-------------------------------------------------
AtPIN4          LLGL-------------------------------------------------
PtPIN3          FLGL-------------------------------------------------
PtPIN6          LLGL-------------------------------------------------
AtPIN1          LLGL-------------------------------------------------
LuPIN1          LLGL-------------------------------------------------
PtPIN1          LLGL-SGKKNTYDIGSHADPN-ATS-RMEKLEIKI-KDREKSSSHGESF-
PtPIN7          LMGL-------------------------------------------------
PtPIN8          LLGL-------------------------------------------------
PtPIN2          LLGL-------------------------------------------------
AtPIN2          LLGL-------------------------------------------------
AtPIN5          LLDL-------------------------------------------------
PtPIN4          FLGL-------------------------------------------------
PtPIN5          FIGL-------------------------------------------------
AtPIN6          LLGL-------------------------------------------------
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4. Mutational Analysis of Flax (*Linum usitatissimum*) Bast (Phloem) Fibre Development

4.1 Introduction

Mutant screening has long been an important tool in genetics. The characterization of mutants provides a clear means of associating one or more biological functions with a specific gene, providing valuable insight into the nature of developmental and physiological processes (Page and Grossniklaus, 2002; Malmberg, 2004). To date, few studies have been conducted that have reported on the use of mutant screening to dissect the genetic basis for stem development – or, for that matter, most other aspects of flax development and physiology - in flax. As of 2003, only 44 genes and their functions had reportedly been characterized by mutation in flax: 20 genes were known to affect floral colour; 4 affect seed colour; 2 affect male sterility; 2 affect oil quality; 11 affect disease resistance; and, 5 were characterized as affecting other morphological traits (Diederichsen and Richards, 2003). A pilot genetic screen of an EMS-mutagenised population of flax plants for cell-wall mutants has been reported, employing Fourier-Transform Infrared Spectroscopy (FT-IR) to detect potential mutants (Chen et al., 1998). However, to date there are no reports available which have describe the further detailed characterization of any of the 59 potential flax cell wall mutants that were identified in that screen. Besides the above cell wall screen, reports are available in the literature describing the isolation of a flax dwarf mutant (George and Nayar, 1973), mutants with altered seed fatty acid

profiles (Rowland and Bhatty, 1990), seedling albinism mutants (Bretagne-Sagnard et al., 1996), and a curly stem mutant (Tejklova, 2002).

Ethyl methyl sulfonate (EMS) is a widely used chemical mutagen employed for many types of mutant screens, favoured due to its high probability of inducing mutagenicity but relatively low probability of causing mortality (Maple and Møller, 2007). EMS mutates DNA by alkylating guanine bases, primarily inducing G/C to A/T nucleotide transitions (Maple and Møller, 2007). In a typical plant EMS mutagenesis screen in plants, seed of the parental genotype is treated with the mutagen. The plants subsequently grown from mutated seeds are referred to as the M_1 generation. The progeny derived from the self-fertilization of the M_1 plants are referred to as the M_2 generation. The M_2 generation is the first generation at which homozygous recessive mutants can be detected, and for most screens the M_2 is the generation at which phenotypic screening is conducted (Maple and Møller, 2007). The frequency of mutant segregation in the M_2 generation is dependent on the genetically effective cell number (GECN): the number of cells in the shoot meristem of the embryo that will contribute to the seed output (Page and Grossniklaus, 2002). For flax, the GECN has been determined to be four cells (Bretagne-Sagnard et al., 1996), meaning that homozygous recessive individuals should segregate at a rate of 15 normal individuals:1 displaying the recessive phenotype within the M2 generation. If that recessive mutant individual is identified, 2 of those 15 phenotypically normal siblings would be expected to be heterozygous for that mutant allele.

Once a potential mutant has been identified in a screen, either its M_3 progeny or the progeny of siblings derived from the same M_3 family are screened to confirm that the mutant allele is heritable. Once the segregation of the mutant allele is confirmed, standard practice is to backcross the mutant to its parental line several times in order to remove unlinked second-site mutations (Wiegel and Glazebrook, 2002). Each round of backcrossing removes half of these unlinked secondary mutations (Wiegel and Glazebrook, 2002). Typically, at least three rounds of backcrossing are considered sufficient for most mutant screens, with the mutant phenotype followed through to the F_2 generation after each backcross (Østergaard and Yanofsky, 2004). At some point during the mutant screening and/or backcrossing period, complementation tests might also be employed to determine whether independently isolated mutant lines showing similar phenotypes are actually alleles of the same gene (Wiegel and Glazebrook, 2002).

In this chapter, observations are reported from the genetic screening of an EMS-mutagenized population of flax. An elite linseed variety, CDC Bethune, was used as the parental genotype. 5-10 plants from approximately 850 independent M_2 families were screened for abnormalities in their development. Lines that showed potential mutations in stem vascular tissue development were followed through additional generations to confirm the heritability of each mutation. Several of the mutant genotypes were backcrossed one- to two-times back to CDC Bethune. Among the most interesting mutants identified from this screen were a mutant displaying a distortion in its xylem cell wall thickening (*distorted walls*)

and a mutant demonstrating a reduction in the number of fully differentiated fibres in its stem (*reduced fibre1*).

4.2 Materials and Methods

4.2.1 Mutagenesis

Mutagenesis was conducted by Mike Deyholos. Approximately 10,000 seeds from the CDC Bethune linseed variety (Rowland et al., 2002) were provided by Gordon Rowland (University of Saskatchewan, Saskatoon, SK). Seeds were mutagenized in a 3 L total volume of 0.5% ethyl methyl sulfonate (EMS) in 25 mM phosphate buffer, pH 7.6, for 4 hours at room temperature. The mutagenized seed was rinsed three times with distilled water, dried, and then stored until planting. M_1 plants were cultivated in a field plot at the Edmonton Research Station Experimental Farm. Seed was harvested by hand from individual M_1 plants.

4.2.2 Screening of M_2 Families

Plants were grown in Metromix 360 (Scotts, Maryland, OH), planted either in round pots (7 cm height, 9.5 cm diameter at the top) or square pots (approximately 6.35 cm^3). Approximately 7-10 seeds from each M_2 family were sown in each pot. Plants were observed periodically throughout their development for any morphological abnormalities. Once they had flowered and gone to seed, plants were screened for vascular tissue mutations following the scheme outlined in Figure 4-1. In brief, the stem of each plant was manually broken and the stem was visually inspected for the presence or absence of bast fibers. Any plants that

could not be screened in this manner, or plants in which the bast fibre content appeared potentially altered, were cross-sectioned by hand and viewed under the microscope, so that that the stem vascular tissue organization could be observed.

4.2.3 Characterization and Backcrossing of Potential Mutants

Most of the potential mutants identified in the M_2 screen were sterile. As such, the heritability of these mutant phenotypes was determined by sowing the M_3 progeny of up to 5 phenotypically-normal siblings derived from the same M_2 family. A process of backcrossing potentially interesting mutants with the CDC Bethune parental line was initiated; again, because many of the mutants were sterile, their phenotypically-normal siblings were used for these backcrosses. For mutants of interest, five backcross lines were ultimately identified among which a vascular tissue abnormality was found to segregate in the F_2 generation after a first backcross (BC_1, F_2). One line, *reduced fibre1*, has been successfully recovered following a second backcross; F_2 progeny from a third backcross for this line is available and is undergoing characterization.

4.2.4 Sample Preparation for Light Microscopy

Tissues were immersed in a formalin-alcohol-acetic acid (FAA) fixative (50% (v/v) ethanol; 5% (v/v) glacial acetic acid; 10% (v/v) formalin). Following a minimum of 24 hours fixation at room temperature, tissue pieces were rinsed for 5 minutes in 70% (v/v) ethanol, further rinsed for 5 minutes in double-distilled water, then cross-sectioned by hand. Cross-sections were rinsed in double-distilled water and stained in 0.05% (w/v) Toluidine Blue O (Sigma, St. Louis,

MO; TBO was dissolved in double distilled water). During early phases of the screen, semi-permanent storage of cross-sections was assured by mounting tissue sections on slides in glycerine jelly (Ruzin, 1999). Otherwise, sections were mounted in water on a microscope slide. Cross-sections were viewed using an Olympus BX51 microscope (Olympus Corporation, Tokyo, Japan). Photographs were taken with a Photometrix CoolSnap fx digital camera (Roper Scientific, Trenton, NJ) and a MicroColor liquid crystal tunable RGB filter (Cambridge Research and Instrumentation, Inc., Woburn, MA).

4.3 Results

Approximately 6000 plants from 851 independently mutagenized M_2 families were screened for abnormalities in their development. Several of the M_2 families screened produced individuals with apparent morphological abnormalities, including fasciation and dwarfism (Table 4-1). These lines will be referred to as potential mutant lines; however, in most cases the heritability of these morphological abnormalities has not been tested.

The main intent of the mutant screen was to identify mutants that showed abnormalities in the development of stem vascular tissues, and particularly in the differentiation of the stem phloem (bast) fibres. In order to do this, a strategy had to be designed that would allow the largest number of M_2 families to be screened. The most direct approach conceivable for the screening would have involved growing the plants to a suitable stage, sacrificing the stem, and visually inspecting stem cross-sections under the microscope. However, cross-sectioning the stems from all of the approximately 7000 plants grown appeared to be impractical.

Therefore, the screening for fibre mutants was conducted instead by breaking the stem of each plant and visually inspecting for an absence of fibres. Stem tissue from potential candidates was then set aside for cross-sectioning, so that it could be determined whether vascular patterning in the stem was altered. 31% of the M_2 families screened included one or more plants that appeared to be stunted, and in most cases these plants were too small to successfully pre-screen for fibre content abnormalities; thus, these potential dwarf mutants were cross-sections. Besides the original 851 M_2 families listed in Table 4-1, sections were also made from 35 individuals selected from 300-400 M_2 families that had been independently screened by an undergraduate student who worked in the Deyholos lab in 2006-2007.

18 lines were identified in which both dwarfism and possible vascular tissue developmental abnormalities appeared to segregate into at least the M_3 generation (Figure 4-2; Table 4-2). Of these potential mutants, 16/18 lines demonstrated a potential reduction in the number of differentiated fibres in cross-section (i.e. reduced fibre content), while the fibre shapes appeared altered in 6 of the 18 lines. One line appeared to show abnormal organization of its xylem. However, it should be noted that all of the observations listed above were qualitative observations, and were based on a very limited number of individuals. Attempts were made to backcross each of these mutants with the CDC Bethune (wild-type) flax variety.

Five lines were identified in which the vascular developmental phenotype reappeared in the F_2 progeny from a backcross (Figure 4-3). For three of these

lines – lines 74 (*doc*), 298 (*grumpy*) and 530 (*happy*) – slight reductions were observed in the area of xylem tissue within the stunted individuals. Because the apparent vascular tissue abnormalities observed in the *doc*, *grumpy* and *happy* mutant lines were not particularly distinctive these lines were not characterized any further. More severe abnormalities were evident in lines 294 (*reduced fibre1*) and B361 (*distorted walls*). For the *reduced fibre1* line, a substantial reduction in bast fibre content was evident, with mutant individuals demonstrating ~25% of the bast fibre content of the parental line. For the *distorted walls* mutant, cell wall thickening appeared to be enhanced in the xylem. Backcrosses were made for *reduced fibre1* and *distorted walls*. The detailed characterization of *reduced fibre1* will be described in further detail in Chapter 5. Although a second backcross for *distorted walls* was completed, it has not yet been determined whether the mutant phenotype can be recovered from these crosses.

While *distorted walls* has been successfully recovered in the F_2 and F_3 generations from the first backcross, it occurs at a lower frequency than expected from simple Mendelian ratios. In the F_2 generation, the phenotype was observed in 1 of 12 individuals of a single family, while it was recovered in 2 out of 26 individuals from a separate backcross line. Although dwarfed plants were recovered from both lines, the vascular tissue phenotype was recovered only among two F_3 individuals derived from one of the two backcross lines that have been screened. The phenotype has always been observed to appear in stunted plants. However, not all stunted plants show the phenotype. It can be hypothesized that rather than one mutation being present which has pleiotropic

effects on both stem elongation and vascular tissue patterning there may instead be two linked mutations segregating in these lines and the observation of a mutant phenotype may depend on the presence of the mutant alleles for both genes. If both genes are recessive, it should be anticipated that approximately one of every sixteen individuals segregating among the F_2 progeny from the backcross should be expected to show the mutant phenotype. With respect to the second back-cross line, the chi-square test shows a 2/26 segregation ratio for the vascular phenotype, which does not fit the 3:1 ratio for wild-type to mutant that would be anticipated for a segregating recessive mutant ($\chi^2 = 4.15$, df = 1, $p < 0.05$). However, the segregation ratios would fit a 15:1 ratio for wild-type to mutant (for the vascular phenotype, $\chi^2 = 0.09$, df = 1, $p = 0.76$; for the dwarfism, $\chi^2 = 1.24$, df = 1, $p = 0.27$). Given the complexity that this skewed segregation imposes on the analysis of the *distorted walls* mutant, further characterization of the mutant was not attempted prior to the preparation of this thesis.

4.4 Discussion

Saturating plant mutant screens typically involved the screening of M_2 seedlings, as they can be grown at high density and screened very early in their development for mutant phenotypes (Jürgens et al., 1991). In contrast, the main strategy used in this screen to identify potential mutants impaired in bast fibre formation was to break the mature M_2 plants apart and then to visually inspect the broken stems for the potential absence of fibres (Figure 4-1). Any potential mutants identified through this pre-screening were set aside for cross-sectioning, which was used to confirm any abnormalities in fibre organization in the stem.

These approaches were modeled from the strategies that have been successfully employed in screens for stem vascular tissue mutants in *Arabidopsis* (e.g. Turner and Somerville, 1997; Zhong et al., 1997). However, the strategy of breaking the stems apart yielded few candidates.

A large number of lines potentially segregated dwarf mutants (Table 4-1), and as the dwarfed plants could not be easily screened for fibre content using the approach described above, they were also cross-sectioned by hand. Several potential dwarf mutants (Figure 4-2; Table 4-2) were indeed identified that also demonstrated potential bast fibre abnormalities. For all 18 lines, the potential bast fibre phenotype reappeared in the M_3 generation, further supporting that the observed phenotype was more likely to have been caused by a mutation, rather than being an artifact that was introduced as the plants were cross-sectioned. However, the reproducibility of each mutant was still relatively low at the M_3 generation, and quantitative information was not obtained that could support the preliminary conclusions that were qualitatively made regarding the potential mutant phenotypes. Thus, the potential mutants were not sub-categorized, and it could not be determined whether the observed bast fibre phenotype might be an effect of the dwarfism.

The primary screening method of breaking stems and examining them for a potential reduction in fibre content did not prove to be reliable. A more tedious alternative: methodically sectioning every M_2 plant, including normal and dwarfed plants, might ultimately be the best strategy for identifying fibre mutants. However, it would certainly be worth determining whether methods to

quantitatively measure stem mechanical properties might be useful for more efficiently identifying potential flax bast fibre mutants. Using a tensiometer, Burk et al. (2001) screened an EMS-mutagenized *Arabidopsis* M_2 population and identified a potential mutant, *fragile fiber2* (*fra2*). The reduced tensile strength of the *fra2* mutant was subsequently determined to be caused by a decrease in xylem fibre length and xylem cell wall thickness (Burk et al., 2001). Measurements with a tensiometer on stems from one of the mutants identified in the screen described here, *rdf,* demonstrated that it shows decreased tensile strength (Chapter 5). This observation supports the suggestion that measuring for altered tensile strength might be an effective and relatively simple means of efficiently screening for flax stem bast fibre mutants.

Observations were also recorded for M_2 lines that segregated individuals potentially showing morphological abnormalities, such as unusual cotyledon or leaf morphologies, albinism, etc. (Table 4-1). As the primary goal of the screen was to identify bast fibre mutants, these morphological mutants were not followed further, although it cannot be discounted that some of these lines might prove interesting for investigations carried out in the future by others.

Perhaps the strangest morphological abnormality observed during the primary screening were several plants that exhibited fasciation: an unusual broadening, flattening and curling of portions of the stem. Fasciation has been found to spontaneously occur in a number of different plant species, including flax (Riddle, 1903). Abundant examples exist of mutants that have been identified which exhibit fasciation, such as the *clavata* (Clark et al., 1993, 1995; Kayes and

Clark, 1998) and *fasciata* mutants of *Arabidopsis* (Kaya et al., 2001), the *fasciation* mutant of soybean (Tang and Knapp, 1998) and the *fasciata* mutant of pea (Sinjushin and Gostimskii, 2008). The pea *fasciata* mutant was originally studied by Mendel, who demonstrated that the fasciation phenotype was inherited as a recessive character (Sinjushin and Gostimskii, 2008).

Fasciated flax plants have been examined in two previous studies. Shibuya (1939) observed that fasciation spontaneously occurred more frequently when seeds were more thinly sown compared with when seeds were sown more closely together. Shibuya (1939) also observed that fertilizer application increased the incidence of fasciation. D'Amato (1957) investigated the effects of growing the Bison linseed flax variety in the presence of a gamma radiation source, determining that the incidence of fasciation was inversely proportional to the distance from the radiation source. Both Shibuya (1939) and D'Amato (1957) observed that fasciation potentially affected vascular development. Shibuya (1939) observed that the number of fibre bundles in transverse stem sections increased in fasciated stem portions; this effect was not apparent in cross-sections through non-fasciated stem portions from the same plant. D'Amato (1957) observed that the xylem ring thickness had decreased in fasciated stem portions. D'Amato (1957) also observed that bast fibre length was unaffected by fasciation, although bast fibres in stem portions demonstrated increased variability in cell wall thickness and cell diameter.

Fasciated plants were observed in 9 M_2 families in our mutant screen (Table 4-1). The progeny from the M_2 families that formed fasciated stems were

not re-grown, and thus it remains uncertain whether these are true mutants; this is an important consideration given that the fasciated plants studied by Shibuya (1939) and D'Amato (1957) apparently formed spontaneously. In our hands, fasciation has never been observed to spontaneously form in any plant derived from the CDC Bethune parental variety in the 5 years since the M_2 screen, although plants were grown in the same growth chamber under similar environmental conditions. Thus, it would seem more likely that the fasciated plants that were observed in our M_2 screen probably had a genetic cause.

Transverse sections through fasciated stem portions from all of the fasciated plants that formed in the screen demonstrated that they had an ovate, rather than round, shape (data not shown). Consisent with the previous observations by Shibuya (1939), the number of fibre bundles that formed in fasciated stem portions also appeared to have increased, possibly due to an increase in the circumference of the stem (data not shown). However, sections through non-fasciated stem portions from the same plants were indistinguishable from the parental variety. Apart from the increase in stem circumference in the fasciated stem portions and an increase in the number of fibre bundles, vascular tissue properties appeared relatively normal. Hence, further study of these potential mutants was not pursued.

4.5 Conclusions

This work describes a screen that was conducted on an EMS-mutagenized mutant population derived from an elite linseed variety, CDC Bethune. Mature M_2 plants were screened for a potential reduction in fibre content by breaking the

stems apart and visually observing them. Potential mutants, as well as dwarfed plants that could not be screened as above, were cross-sectioned and viewed under the microscope. Lines that showed potential mutations in stem vascular tissue development were followed through additional generations to confirm the heritability of each mutation, and several of the mutant genotypes were backcrossed one- to two-times back to CDC Bethune. In the process of completing this screen, a number of potential morphological mutant lines were also identified which may be of interest to others.

Among the most interesting mutants identified from this screen were a mutant displaying a distortion in its xylem cell wall thickening (*distorted walls*) and a mutant demonstrating a reduction in the number of fully differentiated fibres in its stem (*reduced fibre1*). More detailed characterization of the *reduced fibre1* is presented in Chapter 5.

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4.7 Tables and Figures

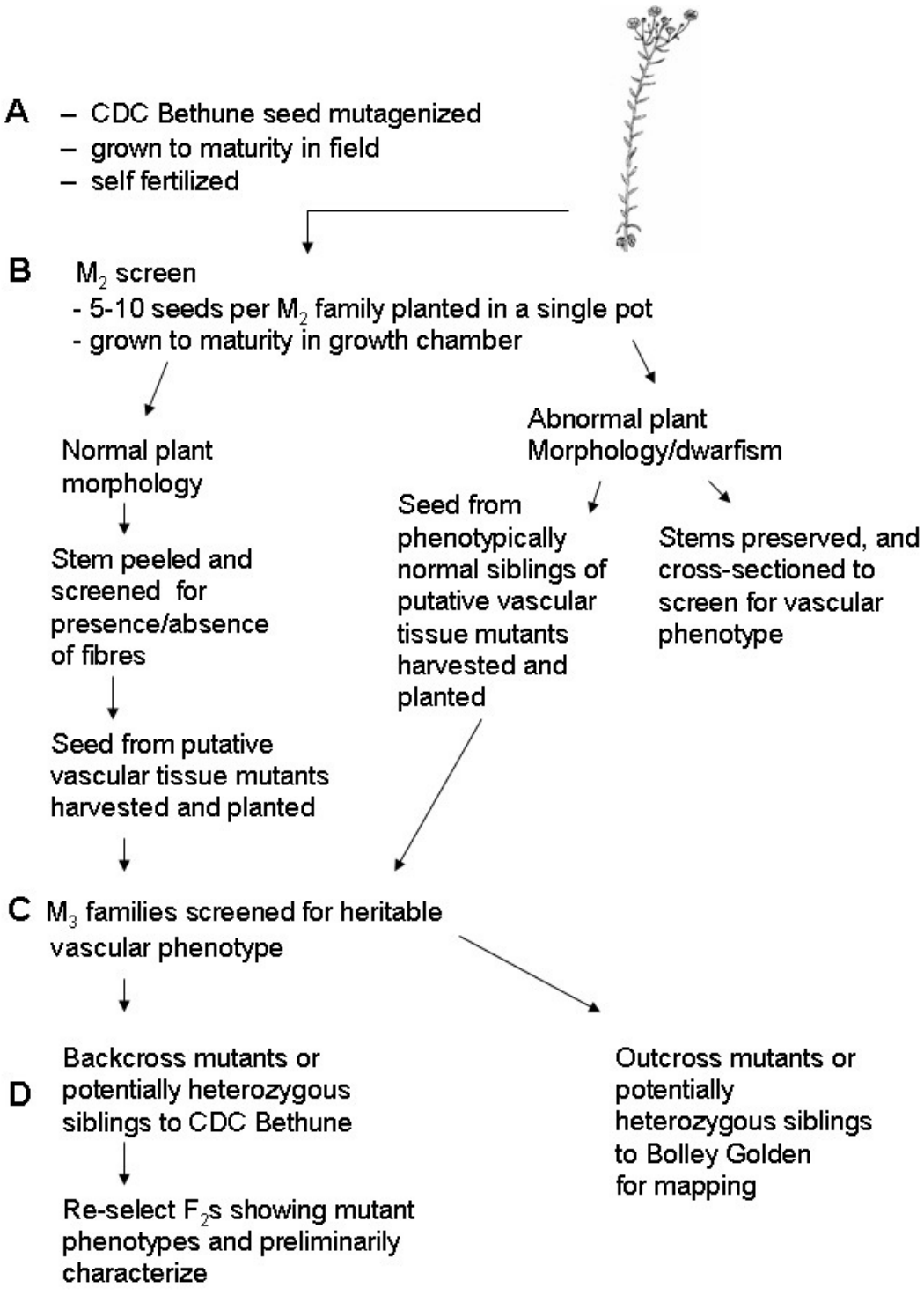

Figure 4-1. Mutant screening strategy followed in this project.

A) M_1 generation. Seeds were chemically mutagenized, planted in soil, and allowed to self-pollinate in order to bring induced mutations to homozygosity in the M_2 generation.

B) M_2 generation. 5-10 seeds from each M_1 family were planted in soil. Plants were monitored throughout their development for any abnormalities. Mature, normal-looking plants were screened for the presence of fibres by tearing the stems apart and visually inspecting them. Potentially abnormal stems, including stems of stunted plants that could not be visually inspected for fibres, were cross-sectioned and inspected.

C) M_3 generation. Seeds from either potential mutants or potentially heterozygous seed from normal-looking siblings of potential mutants were planted in soil and re-screened for a heritable mutant phenotype.

D) Post M_3 generation. Mutants, or potentially heterozygous siblings of potential mutants, were backcrossed to CDC Bethune. F_2 plants from this backcross were re-screened for a segregating mutant phenotype. In cases where the mutant phenotype re-appeared, detailed preliminary characterization of the phenotype was initiated. Mutants, or potentially heterozygous siblings of potential mutants, were also outcrossed to Bolley Golden, in preparation for future mapping of the mutations with SSRs.

Table 4-1. Abnormal morphological characteristics observed among the first 851 M_2 families scored during the mutant screen.

Characteristic	Number of M_2 Families With Putative Mutants	Percentage of M_2 Families With Putative Mutants
	Seedling Phenotypes	
Unusual cotyledon morphology	69	8
Seedling discolouration	32	4
	Mature Plant Phenotypes	
Unusual phyllotaxis/stem branching	12	1
Unusual leaf morphology	26	3
Fasciated stems	9	1
Stunted plants	261	31
Large plants (gigantism)	7	1

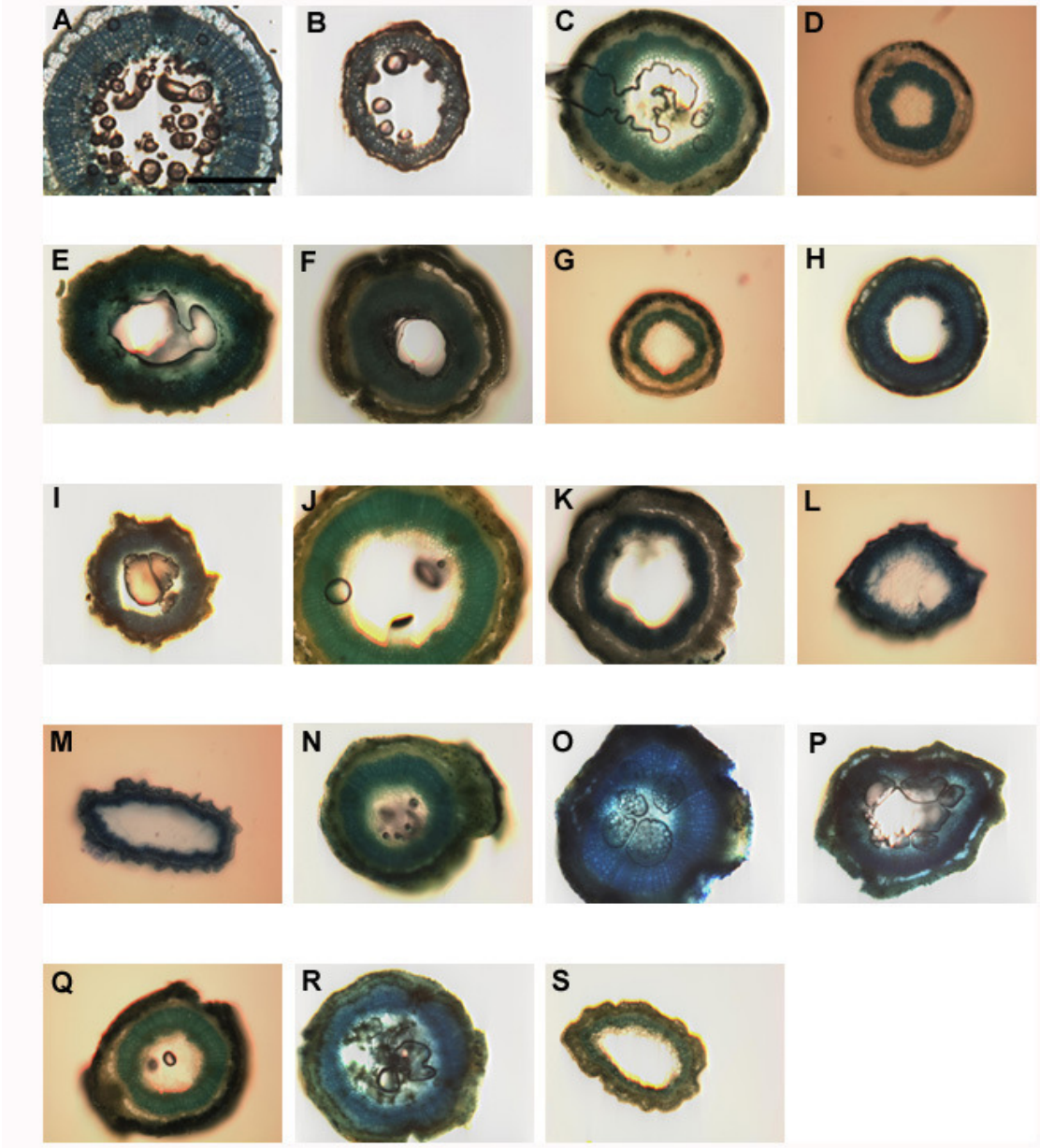
A
B
C
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Figure 4-2. Representative examples of putative flax vascular tissue mutant lines. Transverse sections of FAA-fixed stem tissue from mature, senescent plants were made by hand from the mid-stem, stained in 0.05% Toluidine Blue O, and mounted in glycerine jelly. Bar shown in (A) = 50 μm; this applies to all tissue sections shown in this figure. (A) Bethune (wild-type); (B) line 74, which shows a possible reduction in fibre content; (C) line 185, which shows a possible reduction in fibre content; (D) line 234, which shows a possible reduction in fibre content; (E) line 294, which shows a possible reduction in fibre content; (F) line 298, which shows a possible reduction in fibre content; (G) line 530, which shows a possible reduction in fibre content; (H) line 675, which shows a possible reduction in fibre content; (I) line 678, which shows a possible reduction in fibre content; (J) line 763, which shows fibres with irregular shapes; (K) line 822, which shows a possible reduction in fibre content; (L) line B57, which shows unusually thin fibres; (M) line B76, which shows a convoluted stem with thin fibres; (N) line B96, which shows a possible reduction in fibre content; (O) line B235, which shows a possible reduction in fibre content; (P) line B247, which shows irregularly shaped fibres; (Q) line B249, which shows a possible reduction in fibre content; (R) line B270, which shows a possible reduction in fibre content; (S) line B361, which shows disorganized vascular tissues and a possible reduction in fibre content.

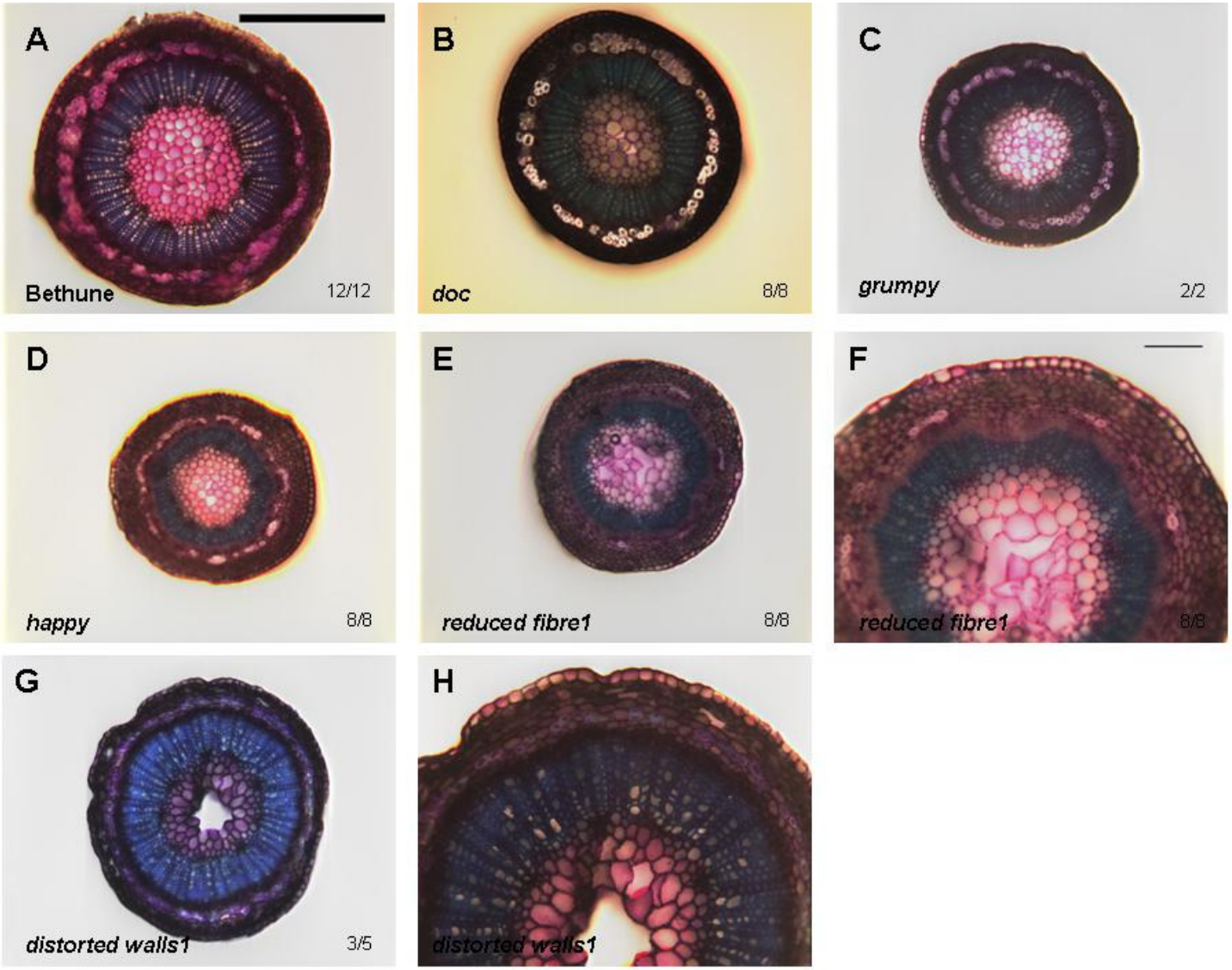
A
Bethune
12/12
B
doc
8/8
C
grumpy
2/2
D
happy
8/8
E
reduced fibre1
8/8
F
reduced fibre1
8/8
G
distorted walls1
3/5
H
distorted walls1

Figure 4-3. Representative examples for stems of mutants which displayed potential vascular tissue abnormalities, sampled 8 weeks after planting. The plants shown segregated among the F_2 generation following a backcross of the originally isolated mutant with the wild-type line, CDC Bethune. The number displayed at the bottom right of each cross-section indicates the number of stunted individuals tested which displayed the vascular tissue phenotype. The *doc* (B), *grumpy* (C) and *happy* (D) lines showed weak reductions in the degree of xylogenesis. The *reduced fibre1* (*rdf*) mutant (E, F) showed substantial reductions in bast fibre content. The *distorted walls* mutant (G, H) displayed thickening of cell walls within its xylem. The scalebar on A represents 500 µm, and equivalently applies to B, C, D, E and G. The scalebar on F represents 100 µm and equivalently applies to H.

Table 4-2. Segregation data for mutant lines displaying potential vascular tissue abnormalities. Families marked in grey have not been successfully backcrossed.

Family	Gross Phenotype	Vascular Phenotype	Direct Descent Progeny		F2s from First Backcross	
			Segregation (Gross Phenotype)	Segregation (Vascular Phenotype)	Segregation (Gross Phenotype)	Segregation (Vascular Phenotype)
74 (*doc)*	Dwarfism	Direct descent from M2: potential fibre content reduction F2s from backcross: evidence for slight reduction in secondary xylem but fibre content normal	M4: 1/8 stunted for 74cb; 6/12 stunted for 74cc; 3/12 stunted for 74cd; 2/14 stunted for 74ce; 2/16 stunted for 74cl M3: 4/17 stunted for 74c	M4: reduced fibre content appears in 1/1 plants sectioned for 74cb; 4/5 sectioned for 74cc; 2/3 sectioned for 74cd; 1/2 for 74ce M3: reduced fibre content appears in 4/4 stunted plants sectioned for 74c	Line 74cl1-2 x Bethune: Undetermined, as height is highly variable. Average height (n=24) 12.1 +/- 4.1 cm.	Line 74cl1-2 x Bethune: 8 most stunted plants tested, 8/8 show indication of reduced xylem and increased phloem. Fibre content in stem shows weakly significant ($p<0.05$) reduction in fibre content compared to wild-type.
185	Dwarfism	Possible reduction in fibre content	M3: 2/12 stunted for 185(R)a; 1/8 stunted for 185(R)b; 1/9 stunted for 185(R)c; 1/7 stunted for 185(R)d; 3/12 stunted for 185(R)e M2: 2/11 stunted	M3: 5/7 stunted plants sectioned for 185a show reduced fibre content; appears in 1/2 plants for 185(R)a; 0/1 plants for 185(R)b; 1/1 plants for 184(R)c; 1/1 plants for 185(R)d M2: 2/2 sectioned show reduced fibre content	n/a	n/a

Family	Gross Phenotype	Vascular Phenotype	Direct Descent Progeny		F2s from First Backcross	
			Segregation (Gross Phenotype)	**Segregation (Vascular Phenotype)**	**Segregation (Gross Phenotype)**	**Segregation (Vascular Phenotype)**
234	Dwarfism	Possible reduction in fibre content	M4: 0/10 stunted for 234aa; 1/9 stunted for 234ac; 3/8 stunted for 234ae; 1/1 stunted for 234af M3: 1/8 stunted for 234a	M4: reduced fibre content observed in 1/1 plants for 234ac M3: reduced fibre content observed in 1/1 stunted plants for 234a M2: reduced fibre content observed in 3 stunted M2 plants	n/a	n/a
294 (*reduced fibre1*)	Dwarfism	Reduction in fibre content, and slight reduction in secondary xylem content	M3: 6/14 stunted for 294(R)a; 3/16 stunted for 294(R)b; 0/13 stunted for 294(R)c; 4/4 stunted for 294(R)d; 4/10 stunted for 294(R)e M2: 3/22 stunted	M3: 2/6 show reduced fibre content for 294(R)a; 1/3 show reduced fibre content for 294(R)b; 2/4 show reduced fibre content for 294(R)d; 1/4 show reduced fibre content for 294(R)e M2: 1/2 stunted plants show reduced fibre content	Undetermined, as height is highly variable. Average height (n=37) 9.2 +/- 2.6 cm.	Line 294ab1-1 x Bethune: 12 most stunted plants tested, 12/12 show indication of reduced xylem and increased phloem.

Family	Gross Phenotype	Vascular Phenotype	Direct Descent Progeny		F2s from First Backcross	
			Segregation (Gross Phenotype)	**Segregation (Vascular Phenotype)**	**Segregation (Gross Phenotype)**	**Segregation (Vascular Phenotype)**
298 (*grumpy)*	Dwarfism	Direct descent from M2: potential fibre content reduction F2s from backcross: evidence for slight reduction in secondary xylem but fibre content normal	M3: 5/16 stunted for 298(R)a; 8/12 stunted for 298(R)b; 1/12 stunted for 298(R)c; 0/1 stunted for 298(R)d; 3/12 stunted for 298(R)e M2: 4/21 stunted	M3: 0/4 plants for 298(R)a show phenotype; 2/8 plants sectioned for 298(R)b show reduced fibre; 1/1 plants sectioned for 298(R)c show reduced fibre; 2/3 plants sectioned for 298(R)e show reduced fibre content and thin fibre shapes M2: 1/4 stunted plants show reduced fibre and irregular fibre shapes	Undetermined, as height is highly variable, and low numbers tested. For 298(R)a, average height (n=4) 7.5 +/- 3.1 cm. For 298(R)e, average height (n=2) 6.2 +/- 0.5 cm.	2/4 plants tested for 298(R)a x Bethune show extreme reduction in stem xylem content; other plants tested show normal xylem content. Fibre content not significantly different than wild-type.
530 (*happy)*	Dwarfism	Direct descent from M2: potential fibre content reduction F2s from backcross: evidence for slight reduction in secondary xylem but fibre content normal	M3: 3/17 for 530(R)a; 1/15 for 530(R)b; 4/22 for 530(R)c; 3/11 for 530(R)d M2: 1/15 stunted	M3: 1/3 plants underdeveloped (not fibreless) for 530(R)a; 1/1 plants normal for 530(R)b; 3/3 show reduced fibre content for 530(R)d; 3/3 normal for 530(R)e M2: 1/1 stunted plants fibreless	Undetermined, as height is highly variable. Average height (n=28) 9.3 +/- 3.5 cm.	Line 530ca1-4 x Bethune: 8 most stunted plants tested, 8/8 show indication of reduced xylem and increased phloem. Fibre content not significantly different than in wild-type.

Family	Gross Phenotype	Vascular Phenotype	Direct Descent Progeny		F2s from First Backcross	
			Segregation (Gross Phenotype)	Segregation (Vascular Phenotype)	Segregation (Gross Phenotype)	Segregation (Vascular Phenotype)
675	Dwarfism	Possible reduction in fibre content	M3: 1/6 stunted for 675a; 1/7 stunted for 675b; 0/2 stunted for 675c; 1/6 stunted for 675d; 3/14 stunted for 675e; 0/11 stunted for 675f'; 4/9 stunted for 675g M2: 4/25 stunted	M3: 0/1 plants for 675a show phenotype; 1/1 plants for 675b fibreless; 0/1 plants for 675d show phenotype; 2/3 plants show reduced fibre content for 675e; 4/4 plants fibreless for 675g M2: 1/3 stunted plants show reduced fibre content	n/a	n/a
678	Dwarfism	Direct descent from M2: possible reduction in fibre content	M4: 2/16 stunted for 678aa; 3/6 stunted for 678ab M3: segregation data for 678a not recorded M2: 7/27 stunted	M4: 2/2 plants for 678aa and 3/3 plants for 678ab show reduced fibre content M3: 2/2 plants for 678a show reduced fibre contents M2: 1/2 plants sectioned show reduced fibre content	undetermined	Undetermined

Family	Gross Phenotype	Vascular Phenotype	Direct Descent Progeny		F2s from First Backcross	
			Segregation (Gross Phenotype)	Segregation (Vascular Phenotype)	Segregation (Gross Phenotype)	Segregation (Vascular Phenotype)
763	Dwarfism	Direct descent from M2: possible reduction in fibre content, unusual fibre cell shapes	M3: 2/5 stunted for 763(R)b; 2/14 stunted for 763(R)c; 2/12 stunted for 763(R)d M2: 1/9 stunted	M3: 2/2 plants for 763(R)b show distorted fibre shape and reduced fibre content; 1/2 plants for 763(R)c show enlarged fibres (more normal than 763(R)b; 2/2 plants for 763(R)d normal M2: 1/1 plants show distorted fibre shapes	undetermined	Undetermined
822	Dwarfism	Direct descent from M2: possible reduction in fibre content, unusual fibre cell shapes	M3: 7/12 stunted for 822a; 1/1 stunted for 822b; 4/11 stunted for 822c; 0/13 stunted for 822d; 3/8 stunted for 822f M2: 4/17 stunted	M3: 1/6 fibreless for 822a; 1/1 show reduced fibre content for 822b; 1/6 show reduced fibre content for 822c; 0/3 show phenotype for 822f M2: 3/4 show reduced/thin fibres	undetermined	Undetermined
B057	Dwarfism	Direct descent from M2: possible reduction in fibre content, unusual fibre cell shapes	M3: 3/8 stunted for B57-1; 0/8 stunted for B57-2; 2/9 stunted for B57-3 M2: 2/9 stunted for B57	M3: 3/3 normal for B57-1; 1/2 show thin fibres for B57-3 M2: 2/3 show thin/reduced fibre contents	n/a	n/a

Family	Gross Phenotype	Vascular Phenotype	Direct Descent Progeny		F2s from First Backcross	
			Segregation (Gross Phenotype)	**Segregation (Vascular Phenotype)**	**Segregation (Gross Phenotype)**	**Segregation (Vascular Phenotype)**
B076	Dwarfism	Direct descent from M2: convoluted stem with very thin fibres	M3: 0/10 stunted for B76-1.; 2/12 stunted for B76-2; 3/9 stunted for B76-3 M2: 4/10 stunted for B76	M3: 1/2 show convoluted stem/thin fibres for B76-2; 1/3 show convoluted stem/thin fibres for B76-3 M2: 3/3 show convoluted stem with very thin fibres	1/17 stunted for B76-4-3	1/1 stunted plants sectioned show normal stem morphology
B096	Dwarfism	Direct descent from M2: reduced fibre content	M3: 1/8 stunted for B96-1 M2: 2/7 stunted for B96	M3: 1/1 shows reduced fibre content for B96-1 M2: 2/2 shows reduced fibre content for B96	undetermined	Undetermined
B235	Dwarfism	Direct descent from M2: reduced fibre content	M3: 6/11 stunted for B235-1 M2: 2/6 stunted for B235	M3: 2/6 show reduced fibre content for B235-1 M2: 1/2 show reduced fibre content for B235	2/22 stunted for B235-2-1	2/2 stunted plants sectioned show normal stem morphology
B247	Dwarfism	Direct descent from M2: irregular fibre shapes	M2: 2/7 stunted for B247	M2: 2/2 show distorted fibre shape	n/a	n/a
B249	Dwarfism	Direct descent from M2: reduced fibre content	M2: 2/8 stunted for B249	M2: 3/3 show reduced fibre content	1/8 stunted for B249-2-1	1/1 stunted plants shows reduced xylem and increased outer tissue content

Family	Gross Phenotype	Vascular Phenotype	Direct Descent Progeny		F2s from First Backcross	
			Segregation (Gross Phenotype)	**Segregation (Vascular Phenotype)**	**Segregation (Gross Phenotype)**	**Segregation (Vascular Phenotype)**
B270	Dwarfism	Direct descent from M2: reduced fibre content	M3: 2/4 stunted for B270-1 M2: 2/6 stunted for B270	M3: 1/2 show reduced fibre content for B270-1 M2: 2/3 show reduced fibre content for B270	n/a	n/a
B361 (*distorted walls*)	Dwarfism	Direct descent from M2: disorganized xylem and reduced fibre content	M3: 0/9 stunted for B361-1; 6/15 stunted for B361-2 M2: 1/7 stunted for B361	M3: 1/6 shows reduced fibre content & disorganized vascular tissues for 361-2 M2: 2/2 show reduced fibre contents and very disorganized vascular tissues (xylem differentiation appears very unusual)	2/26 stunted for 1	

5. Isolation and Characterization of the Flax (*Linum usitatissimum* L.) *reduced fibre1* Mutant

5.1 Introduction

Flax (*Linum usitatissimum* L.) has been an important source of textile fibre for thousands of years (Lev-Yadun et al., 2000). Accordingly, given the importance of flax as a fibre crop, the properties of its fibres have been studied by plant biologists for nearly 150 years (reviewed in Anderson, 1927). Anderson (1927) determined that bast fibres elongate through a combination of coordinate growth, defined as elongation in coordination with surrounding tissues, and intrusive growth, whereby the elongating cell grows between other cells (Anderson, 1927). Esau (1943) further determined that flax bast fibres form as a primary vascular tissue, as a component of the protophloem. Coordinate growth of flax bast fibres has been shown to occur in the first few millimetres below the stem apex, and occurs through intercalary (diffuse) growth along the entire fibre cell (Ageeva et al., 2005). In the growing stem, flax bast fibre elongation has been determined to be restricted to near the apex of the stem and completed in a few days, whereas cell wall thickening and fibre expansion occur in lower portions of the stem (Gorshkova et al., 1996). A region termed the 'snap-point' can be identified in the growing stem below which the tensile strength of the stem dramatically increases. The snap point was shown to mark the location at which bast fibre elongation ceases (Gorshkova et al., 2003). Whereas primary growth of the stem occurs through cell divisions at the shoot apex, the size and patterning of the hypocotyl is established during embryogenesis and, at least in *Arabidopsis*, is

restricted to a finite number of cells (Gendreau et al., 1997; Busse and Evert, 1999a, 1999b). Developing bast fibres in the hypocotyl have similar transcriptional expression patterns as in stems (Roach and Deyholos, 2008). The stages of bast fibre differentiation in the hypocotyl vary depending on the age of the plant, rather than being spatially restricted as in the stem (Roach and Deyholos, 2008).

To date, very few fibre-related mutants have been reported in flax or any other plant species. Primary phloem fibres and secondary phloem fibre-sclereids form in the *Arabidopsis* stem and root (Lev-Yadun, 1997). However, no phloem fibre-specific *Arabidopsis* mutants have been reported. In the normal jute stem, secondary phloem tissues are normally organized in a bundle in cross-section, flanked by ray cells (Mitra, 1984). Alternating bands of lignified fibres and soft tissues (parenchyma, sieve tubes and companion cells) are typically observed in the fibre bundle (Mitra, 1984). In the *undulating stem* mutant of jute, although a phloem pyramid is observed in the mutant, secondary phloem fibre bundles are absent (Mitra, 1984). Secondary phloem fibre differentiation is also reduced in the *deficient lignified phloem fibre* (*dlpf*) mutant of jute, while the secondary phloem fibres which do form were found to show a 50% reduction in lignin content (Sengupta and Palit, 2004). It is unclear from the literature whether complementation tests have been conducted between the *undulating stem* and *dlpf* mutants.

In this chapter, the isolation and characterization of *reduced fibre1* (*rdf*) is reported. This novel flax mutant demonstrates a substantial reduction in the

number of differentiated phloem fibres observed in stem cross-sections. The *rdf* mutant was identified in an M_2 family derived from an EMS-mutagenized population of seeds of an elite linseed variety (Chapter 4). The characterization reported here demonstrates that *RDF* may play a significant role in the regulation of bast fibre cell differentiation and elongation.

5.2 Materials and Methods

5.2.1 Plant Material

The *reduced fibre1* line was isolated from an EMS-mutagenized population of linseed variety CDC Bethune, as described in Chapter 4 of this thesis. Mutant lines were backcrossed twice to the wild-type line, CDC Bethune and the mutant phenotype was recovered in the F_2 generation from each backcross. Plants were grown in Metromix 360 (Scotts, Maryland, OH), planted in round pots (7 cm height, 9.5 cm diameter at the top) to a depth of approximately 1 cm, at a density of 4-6 seeds per pot. The plants were grown in controlled environment chambers at 24°C with 50% humidity, and a light intensity of 200 µE supplied by high output fluorescent bulbs (CRI of 85, colour temperature of 3,500 K) on a 16 h light/8 h dark cycle. While most measurements presented here were based on F_3 individuals from the second backcross, the bast fibre counts in cross-section were obtained from F_2 individuals segregating from the first backcross and measurements of fibre cross-sectional size were obtained from F_4 individuals segregating from the first backcross.

5.2.2 Stem Tensile Strength Measurements

Four five-month-old stems each from the CDC Bethune line and from the *rdf* line were measured for tensile strength using an Instron 5565 Compression and Tension Tester (Instron, Norwood, MA). Clamps were on the tension tester were spaced 5 cm apart.

5.2.3 Sample Preparation for Light Microscopy

For light microscopy, stem pieces approximately 5 mm in length were fixed and stored in FAA (50% (v/v) 95% ethanol; 5% (v/v) glacial acetic acid; 10% (v/v) formalin). Stem pieces were processed through an ethanol-toluene-paraffin series, overnight, using a Leica TP1020 Tissue Processor (Leica Microsystems, Wetzlar, Germany; see Table A1 in Appendix for processing schedule). Processed samples were mounted in paraffin wax using a Tissue Tek II Processor (Sakura Finetek, Torrence, CA). Wax ribbons of 8 µm thickness were cut using a Spencer 820 rotary microtome (Spencer Scientific, Derry, NH), stretched in water, mounted on frosted microscope slides and dried overnight at 37ºC. The slides were deparaffinized through an ethanol-toluene series (see Table A2 in appendix for processing schedule), stained with safranin-fast green, and mounted in DPX mounting medium (Electron Microscopy Sciences, Hatfield, PA). Sections were viewed using an Olympus BX51 microscope (Olympus Corporation, Tokyo, Japan). Photographs were taken with a Photometrix CoolSnap fx digital camera (Roper Scientific, Trenton, NJ) and a MicroColor liquid crystal tunable RGB filter (Cambridge Research and Instrumentation, Inc., Woburn, MA).

5.2.4 Sample Preparation for Scanning Electron Microscopy

Stems were cross-sectioned near the stem base. Samples of approximately 1 mm in length were fixed in FAA. Samples were dehydrated in a graded ethanol solution series (Table A3 in Appendix). Hexamethyldisilazene (HMDS; Electron Microscopy Sciences, Hatfield, PA) was gradually introduced through a graded ethanol-HMDS series. After HMDS removal the samples were left to air dry overnight, mounted on SEM stubs and sputter-coated with gold/palladium using a Ladd/Hummer 6.2 Sputter Coater (Ladd Research, Williston, VT). The stem samples were viewed using a Philips/FEI LaB6 Environmental Scanning Electron Microscope (FEI, Hillsboro, OR).

5.2.5 Tissue Measurements

Measurements of the xylem and outer tissue radii, stem radius, bast fibre cell wall thickness and bast fibre lumen area were determined from photographs of stem cross-sections, taken from within the first internode at the base of the primary stem. Measurements were made using ImageJ, a freeware image analysis software (Abramoff et al., 2004).

5.2.6 Fibre Length Measurements

To measure the lengths of bast and xylem fibres, stems were macerated using Franklin's maceration method (Chaffey, 2002). In brief, stem pieces were boiled in a macerating solution composed of equal parts glacial acetic acid and 6% (v/v) hydrogen peroxide. After 3-4 hours of boiling, the bleached tissues were thoroughly rinsed several times in Milli-Q water. Sodium carbonate was added, as

needed, to neutralize any remaining acid. Once effervescence due to the addition of the sodium carbonate had stopped, the samples were rinsed again in Milli-Q water, and the tubes containing the samples were shaken thoroughly to break up the stem tissue. The macerated material was viewed under the microscope, and xylem and bast fibres from each sample were manually measured.

5.2.7 Quantitative Real-Time PCR

RNA was extracted from stem tissue of 6-week-old *rdf* and CDC Bethune plants using the Qiagen RNeasy Mini Kit (Qiagen, Germantown, MD), following the manufacturer's instructions. The optional on-column DNase digestion steps were included during the RNA extraction procedure. cDNA was synthesized using Revertaid® H Minus M-MuLV reverse transcriptase (Fermentas, Glen Burie, MD) and oligo-dT_{18} primers (Fermentas), following the manufacturer's protocol. Real-time PCR was conducted in an Applied Biosystems 7500 Fast Real-Time PCR System.2.5 µL of a 1/400 dilution of the reverse transcription reaction was used in a total volume of 10 µL, with 0.4 µM of each forward and reverse gene-specific primer, 0.2 µM dNTPs, 0.25X SYBR Green, 1X ROX and 0.075U Platinum Taq (Invitrogen). Threshold values (C_T) were determined using 7500 Fast software. Primer sequences for the *EF1α* (reference gene), *LuGAST1*, *LuGA2ox1*, *LuGA3ox1*, *LuIAA1* and *LuPIN1* genes are provided in Chapter 3. A two-way t-test was used to determine whether the raw C_T values for *EF1α* significantly varied between CDC Bethune and *rdf*; the absence of significant variation ($p>0.05$) supported the suitability of *EF1α* as a reference gene. The primers for *LuGAST1*, *LuGA2ox1*, *LuGA3ox1*, *LuIAA1* and *LuPIN1* were also

quantified over a range of cDNA dilutions to confirm that their primer efficiencies amplified at an equivalent efficiency to primers for *EF1α*.

5.2.8 Statistical Analyses

Statistical analyses were conducted in Microsoft Excel 2003. Two-sample F-tests were conducted to determine whether variances were equal. When the variance was found to be significantly different ($p<0.05$ according to the F-test), measurements were compared using a two-sample *t*-test that assumes unequal variances. In cases where significant differences in the variances were not determined, a two-sample *t*-test that assumed equal variances was used.

5.3 Results

5.3.1 Segregation Pattern for the *rdf* Mutant

To identify mutants with defects in fibre development and/or GA metabolism and perception, a visual screen of EMS-mutagenized flax (linseed) variety CDC Bethune was undertaken (Chapter 4). The *rdf* mutant was initially identified as a putative dwarf mutant demonstrating a reduction in the phloem fibre content of its stem, and has subsequently been backcrossed twice. The dwarfism phenotype segregated away from the *rdf* phenotype in the progeny from the first backcross. Segregation ratios among the F_2 progenies of two backcross and two outcross lines (with Bolley Golden) were consistent with the 3:1 wild-type to mutant ratio that would be expected in the F_2 generation if *rdf* is inherited as a recessive trait of a single locus (for backcross 2 line 1, $\chi^2 = 0.33$, df=1,

p=0.57; for backcross 2 line 2, χ^2 = 1.26, df=1, p=0.26; for outcross line 1, χ^2 = 0.87, df=1, p=0.35; for outcross line 2, χ^2 = 0.01, df=1, p=0.92).

5.3.2 External Appearance of the *rdf* Mutant

To investigate whether the *rdf* phenotype had any association with stem height, the heights of *rdf* mutants were measured along with CDC Bethune plants grown side-by-side. The mutants were all derived from presumed homozygous *rdf* lines, and all individuals were cross-sectioned to confirm the reduced fibre phenotype in their stem. The stem height of the *rdf* plants did not significantly differ from the height of plants derived from the parental line (Figure 5-1A).

5.3.3 Tensile Strength of *rdf* Mutant Stems

In order to determine whether the *rdf* phenotype affected the tensile strength of the stem, the tensile strength 5 cm portions of mature *rdf* and CDC Bethune stems were measured using a tensiometer. The *rdf* stems were determined to have significantly less tensile strength than CDC Bethune plants (Figure 5-1B).

5.3.4 Anatomy of *rdf* Mutant Stems

The number of bast fibres observed in cross-section was reduced by approximately 75% in the primary stem of the *rdf* mutant, as compared with the parental variety (Figure 5-2, Figure 5-3 A). Bast fibre counts were similarly reduced by approximately 75% in hypocotyls (Figure 5-3 A).

In transverse section, bast fibres in the parental CDC Bethune stem formed a dense layer around the stem, positioned between the cortex and the secondary

phloem. The fibres were typically grouped into bundles (Figure 5-2 A, E). In the *rdf* stem, bast fibres often appeared in bundles, but there were fewer fibres in each bundle (Figure 5-2 B, F). However, isolated fibres were sometimes observed in the mutant (Figure 5-2 B, F). The lumen area and cell wall thickness of the bast fibres in *rdf* was found to be decreased (Figure 5-2, Figure 5-3 B, C). Bast fibres were also less frequently observed in longitudinal sections of CDC Bethune and *rdf* stems (Figure 5-2 C, D), and bast fibres in *rdf* typically appeared to be shortened in length.

Because the boundaries of the phloem were often unclear, measurements were made of the "outer tissues", meaning all tissues between the cambium and epidermis (i.e. phloem and cortex), inclusively. The stem, xylem and outer tissue radii of the *rdf* mutant stems were not found to differ from the parental line (Figure 5-4 A). Likewise, the number of cells forming a file across the xylem radius was not altered (Figure 5-4 B).

Fibre development in flax follows a basipetal gradient along the stem axis. To determine whether there was any evidence for a reduction in the numbers of bast fibres at the earliest stages of of differentiation, cross-sections were also made through tissues near the stem apex. The vascular bundles in CDC Bethune and *rdf* were largely indistinguishable in their appearance (Figure 5-5).

To measure the lengths of individual fibres, xylem and bast fibres were isolated from stems by maceration. Both the mean xylem fibre (Figure 5-6 A) and mean bast fibre (Figure 5-6 B) lengths were significantly shorter in the *rdf* mutant,

with the xylem fibres decreased in length by approximately 30% and bast fibres decreased in length by approximately 50%.

5.3.5 Expression of Hormonal Biosynthesis and Response Genes in *rdf* Mutant Stems

In Chapter 3 of this thesis, expression profiles were presented for five genes, *LuGAST1, LuGA2ox1, LuGA3ox1, LuIAA1 and LuPIN1*, all considered to be putative orthologues of well-characterized hormonal biosynthesis or signalling genes. In order to investigate whether the expression of any of these genes might have been altered in *rdf*, transcript levels were measured in mid-stem tissues (i.e. approximately midway from stem base to snap-point) of 6-week-old CDC Bethune and *rdf* mutant plants. While the transcript abundance of the *LuGA2ox1*, *LuGA3ox1*, *LuIAA1* and *LuPIN1* genes did not significantly different between *rdf* and CDC Bethune, the transcript abundance of *LuGAST1* was determined to be significantly increased, by 6.8-fold, in *rdf* stems (Figure 5-7).

5.4 Discussion

5.4.1 Anatomical Differences in *rdf* Stems

The recessive *rdf* mutant trait was characterized by a dramatic reduction in the number of fully differentiated fibres that could be detected in stem and hypocotyl cross-sections (Figure 5-2, Figure 5-3 A). That this effect was evident in both the stem and hypocotyl indicates that it is not directly related to either patterning at the shoot apex, or stem or fibre elongation. Instead, it appears that some aspect of the early stages of fibre secondary wall development may be impaired. Secondary growth (i.e. xylem growth) of the mutant was unaffected, as

demonstrated by the equivalent xylem and outer tissue radius in the mutant and CDC Bethune wild-type stem and the absence of any difference in the number of xylem cells that formed across the stem radius; this supports the long-held view that flax bast fibres are a primary vascular tissue and that their properties do not derive from the vascular cambium (Esau, 1943, 1977).

Based on external appearance, plants that showed the *rdf* phenotype could not be easily distinguished from the parental variety. The height of the *rdf* stems was not significantly altered (Figure 5-1 A). Preliminary measurements showed that the tensile strength of *rdf* stems was reduced (Figure 5-1 B). Although stem diameter was not normalized in these tests, the *rdf* mutation did not affect the stem radius (Figure 5-4 A), and therefore it should be reasonable to assume that the cross-sectional area of the tested stems were approximately equal, making the observations comparable. However, tensile strength measurements can be subject to high variability, and data are often obtained from a much larger sample size than was used for these preliminary measurements (e.g. using 40-50 plants; Jane Batcheller, University of Alberta Department of Human Ecology, personal communication).

The lengths of the bast and xylem fibres were reduced in *rdf* (Figure 5-2 C, Figure 5-6). The cell wall thickness and lumen area of the bast fibres was also found to be reduced (Figure 5-3 B and C), implying a possible decrease in cellular dimensions. The ends of fibres are tapered, and when viewed in cross-section would show a decreased area compared with a cross-section taken through a middle portion of the fibre. Therefore, any reductions in fibre cross-sectional area

and fibre length might actually be related, as a reduced length might increase the likelihood of observing tapered fibre ends. In general, tensile strength is proportional to fibre length (Peel, 1999). Thus, the reduced length of the fibres might be contributing to the reduced tensile strength of the *rdf* stems.

It has also been proposed that a change in bast fibre length proportionately affects the number of fibres that may be viewed in cross-section (Gorshkova et al., 2003). While this effect could be a contributor to the decreased number of fibres observed in *rdf* cross-sections, it does not explain the *rdf* phenotype entirely: bast fibre lengths were similarly reduced in stems of paclobutrazol-treated plants relative to mock-treated plants by approximately the same proportion as for *rdf* compared with CDC Bethune plants (McKenzie and Deyholos, 2011; Chapter 2). However, the number of fibres in transverse section was reduced to a much smaller degree in the paclobutrazol-treated plants: there was a 30% reduction in number of bast fibres for paclobutrazol-treated compared with the mock-treated plants, compared with a 75% reduction for *rdf* compared with CDC Bethune.

Like *rdf*, An *Arabidopsis* mutant*, interfascicular fibereless1/revoluta*, fails to form a normal content of schlerenchyma cells in its stem (Zhong et al., 1997). The mutation responsible for the *ifl1/rev* phenotype is in the *AtREVOLUTA* gene, which encodes a homeodomain leucine zipper (HD-ZIP) Class III transcription factor (Zhong and Ye, 1999; Ratcliffe et al., 2000). Zhong et al. (1997) concluded the potential for fibre intiation is not compromised in *ifl1/rev*, but rather that the spatial positioning of fibre formation is disrupted. However, by using polarizing light to better distinguish cells that are capable of forming secondary cell walls,

Lev-Yadun et al. (2005) demonstrated instead that while *ifl1/rev* mutants that appear 'fibreless' under bright-field microscopy actually form fibres where expected, these fibres cannot be easily distinguished because the mutation disrupts the lignification of their secondary cell walls. The characterization of *ifl1/rev* provides lessons worth applying to the characterization of *rdf*. It should not be assumed that a reduction in the number of fibres that can be identified in a stem cross-section implies that fibres simply fail to form. In order to determine whether the *rdf* phenotype might be potentially explained by the presence of fibres that have elongated but failed to undergo cell wall expansion below the snap-point, longitudinal sections were taken through *rdf* stems (Figure 5-2 C and D). All of the fibres observed in these sections appeared to have undergone normal cell wall expansion; the fibres were simply observed to be shorter in length as compared with fibres in the parental CDC Bethune variety, and to have formed less frequently.

Esau (1943) traces the origin of flax bast fibres to a group of larger cells that appear alongside smaller primary sieve tube elements and companion cells. Continued enlargement of the bast fibre initials crowds out and destroys the primary sieve tube elements and their companion cells (Esau, 1943). The patterning of the vascular bundles in *rdf* was indistinguishable from that of CDC Bethune, lacking obvious differences in the size and number of fibre initials (Figure 5-5). *RDF* function therefore does not appear to be required to establish the patterning of the vascular bundle.

5.4.2 Expression of Hormonal Biosynthetic and Response Genes in *rdf* Stems

The relative transcript abundance of *LuGAST1* increased in *rdf* compared to CDC Bethune (Figure 5-6). The biochemical function of members of the GAST/GASA protein family remains unknown, although *GAST/GASA* genes in several plant species have been determined to be GA_3-responsive (Shi et al., 1992; Herzog et al., 1995; Ben-Nissan and Weiss, 1996; Aubert et al., 1998). As reported in chapter 3, *LuGAST1* may be negatively regulated by GA (Chapter 3), consistent with observations for its closest putative *Arabidopsis* orthologue, AtGASA5 (Zhang et al., 2009). The transcript abundance of *LuGAST1* (referred to by Roach and Deyholos as a 'GASA5-like protein') was enriched in hypocotyls sampled 15 days after planting (DAP) relative to 7 DAP and 9 DAP hypocotyls (Roach and Deyholos, 2008). The three hypocotyl developmental timepoints examined by Roach and Deyholos (2008) represent different stages in bast fibre development (7 DAP, fibre elongation; 9 DAP completion of fibre elongation and onset of cell wall expansion; 15 DAP late cell wall expansion).

Further expression analyses of *LuGAST1* reported in Chapter 3 demonstrated that its transcript abundance in CDC Bethune was higher in the midstem region as compared to the stem apex. The midstem region was characterized by Gorshokova et al. (2003) as the location of the onset of bast fibre secondary cell wall biosynthesis. The *LuGAST1* transcript abundance was also observed to have substantially increased in the midstem in 28 DAP relative to 14 DAP CDC Bethune plants (Chapter 3), suggesting that its expression in the midstem may increase in accordance with the appearance of the snap-point. These

data suggests LuGAST1 might act as a negative regulator of fibre elongation and/or might act as a positive regulator of secondary cell wall biosynthesis. This hypothesis is supported by observations by Zhang et al. (2009), who showed that AtGASA5 is localized to the cell wall and extracellular matrix, and who characterized AtGASA5 as a negative regulator of stem elongation. The increased transcript abundance of *LuGAST1* in *rdf*, where fibre differentiation is impaired, further supports a hypothetical role for LuGAST1 as a negative regulator of fibre differentiation. The relationship between *RDF* and *LuGAST1* function remains unclear, but the available data suggests that *LuGAST1* might act downstream of *RDF* in a pathway affecting bast fibre differentiation.

5.5 Conclusions and Future Perspectives

The most obvious effect of the *rdf* mutation was a dramatic reduction in the number of fully differentiated fibres that could be observed in cross-section, both in the stem and hypocotyl. Measurements of xylem and bast fibres also demonstrated that the mutation suppressed cell elongation. Based on observations that paclobutrazol treatment inhibited bast fibre elongation to a relatively equivalent degree as the *rdf* mutation but affected the number of fibres observed in transverse sections of the stem to a much lesser degree, it can be concluded that the reduction in the number of differentiated fibres observed in cross-sections of *rdf* is not fully explained by the reduction in the fibre length.

Esau (1943) observed that the bast fibre initials are first identifiable as a group of larger cells that form at the stem apex on the abaxial side of the primary vascular bundles, occurring in association with smaller cells that are destined to

form into primary phloem elements and their companion cells. At the onset of their development, the bast fibre initials are not easily distinguished from surrounding cells (Esau, 1943). However, after elongating the bast fibres become longer than adjacent phloem elements and substantially longer than nearby cortical and parenchyma cells, and later form a secondary cell wall (Esau, 1943).

Transverse sections of *rdf* near the stem apex (Figure 5-5) revealed that cells which resemble the expected fibre initials described by Esau (1943) do form as normal in *rdf*. However, in transverse sections taken below the snap-point (Figure 5-2), fully differentiated bast fibres were observed to have formed less frequently in *rdf*. Longitudinal sections of *rdf* also demonstrate an apparent reduction in the number of bast fibres. Thus, although the formation of fibre initials occurs as normal in *rdf*, some of the fibre initials apparently fail to begin elongating, suggesting that *RDF* may be required for the onset of fibre elongation (Figure 5-8). The bast fibres that did form in *rdf*, although shorter than in the CDC Bethune parental variety, were longer than any of the adjacent cells, suggesting that they had elongated through intrusive growth. Thus, it was not apparent that *RDF* function is required for intrusive growth of the bast fibres to occur.

Like the bast fibres, xylem fibres were also less elongated in *rdf*. However, differences in the degree of lignification or patterning of the xylem were not apparent in *rdf*. As an equivalent number of secondary xylem cells were observed to form across *rdf* stems as in CDC Bethune stems that were grown concurrently, it can be concluded that *RDF* is not required for divisions of the vascular cambium. Bast fibres form from the procambium, whereas most of the xylem

fibres are secondary fibres that form from divisions of the vascular cambium, and this difference in origin may explain why *rdf* has a greater effect on bast fibre patterning than on xylem patterning.

The transcript abundance of *LuGAST1*, a gene that putatively acts as a negative regulator of bast fibre elongation and/or secondary cell wall biosynthesis (Chapter 3), increased in stems of the mutant, suggesting that *LuGAST1* may act downstream of *RDF* in the same genetic pathway. The available evidence therefore suggests that *RDF* is required for fibre elongation, both in bast and xylem fibres, perhaps acting by negatively regulating the expression of *LuGAST1*, which functions as a negative regulator of fibre elongation (Figure 5-8).

Microtubules (MTs) have been shown to control the orientation of cellulose microfibrils (CMFs), controlling the elongation and expansion of plant cells (Lloyd and Chan, 2004). In flax bast fibre cells, CMTs re-orient from generally perpendicular to generally parallel to the long axis of the cell before and after the fibre cell elongates (van Lammeren et al., 2003). Several known genes regulate MT and CMF dynamics in *Arabidopsis* and other plant species (for reviews see Lloyd and Chan, 2004; Somerville, 2006) and a mutation in a flax orthologue of any one of these genes, or in a regulator of these genes, could potentially underly the *rdf* phenotype. For example, like *rdf*, an *Arabidopsis* mutant, *fragile fiber2* (*fra2*), has shortened xylem fibres in its stem (Burk et al., 2001). The *fra2* mutant is mutated at the *AtKSS1* gene, which encodes a katanin-like microtubule severing protein (Burk and Ye, 2002). *fra2* and several independently-isolated allelic mutants have been shown to exhibit disorganized

CMT orientations (Bichet et al., 2001; Burk et al., 2001; Webb et al., 2002), while CMF orientations have also been shown to be disorganized in *fra2* and an allelic mutant (Burk and Ye, 2002; Bouquin et al., 2003).

Linseed flax is a crop that is grown primarily for its seed and seed oils, while bast fibres are considered a nuisance for farmers as they tend to clog agricultural equipment and persist in the field longer than other types of straw (Deyholos, 2006). That the *rdf* phenotype, which is derived from an elite linseed variety, is not associated with any other discernible changes in the appearance of the plants is an exciting observation, as it suggests that this mutant may be useful in further crop development, although the possibility of increased lodging on *rdf* mutants remains a primary concern. *rdf* seed is presently being evaluated by flax breeders at the University of Saskatchewan.

The *rdf* phenotype is also exciting from a biological standpoint, as the mutant phenotype suggests that *RDF* may play an important role in regulating bast fibre differentiation. The factors that regulate later stages of plant fibre differentiation are largely unknown (Lev-Yadun, 2010). Further characterization, including the cloning and molecular characterization of the *RDF* gene, may lead to important insight into how the properties of schlerenchyma cells, such as phloem bast fibres, are regulated in plants.

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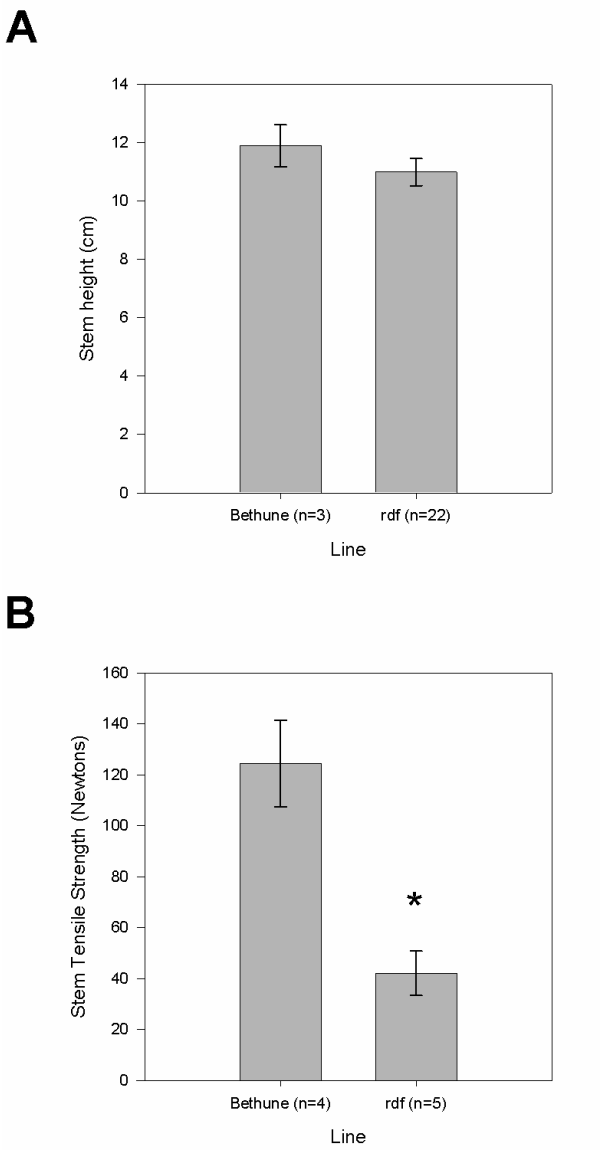


Figure 5-1. Stem height and tensile strength of CDC Bethune plants and rdf mutants. Stem heights (A) were measured 6-weeks after planting. Tensile strength (B) was measured in plants that had completed flowering, approximately 5 months after planting. Measurements from the two groups were compared using two-way *t*-tests. The symbol * denotes that the tensile strength significantly differed in the *rdf* plants ($p<0.05$).

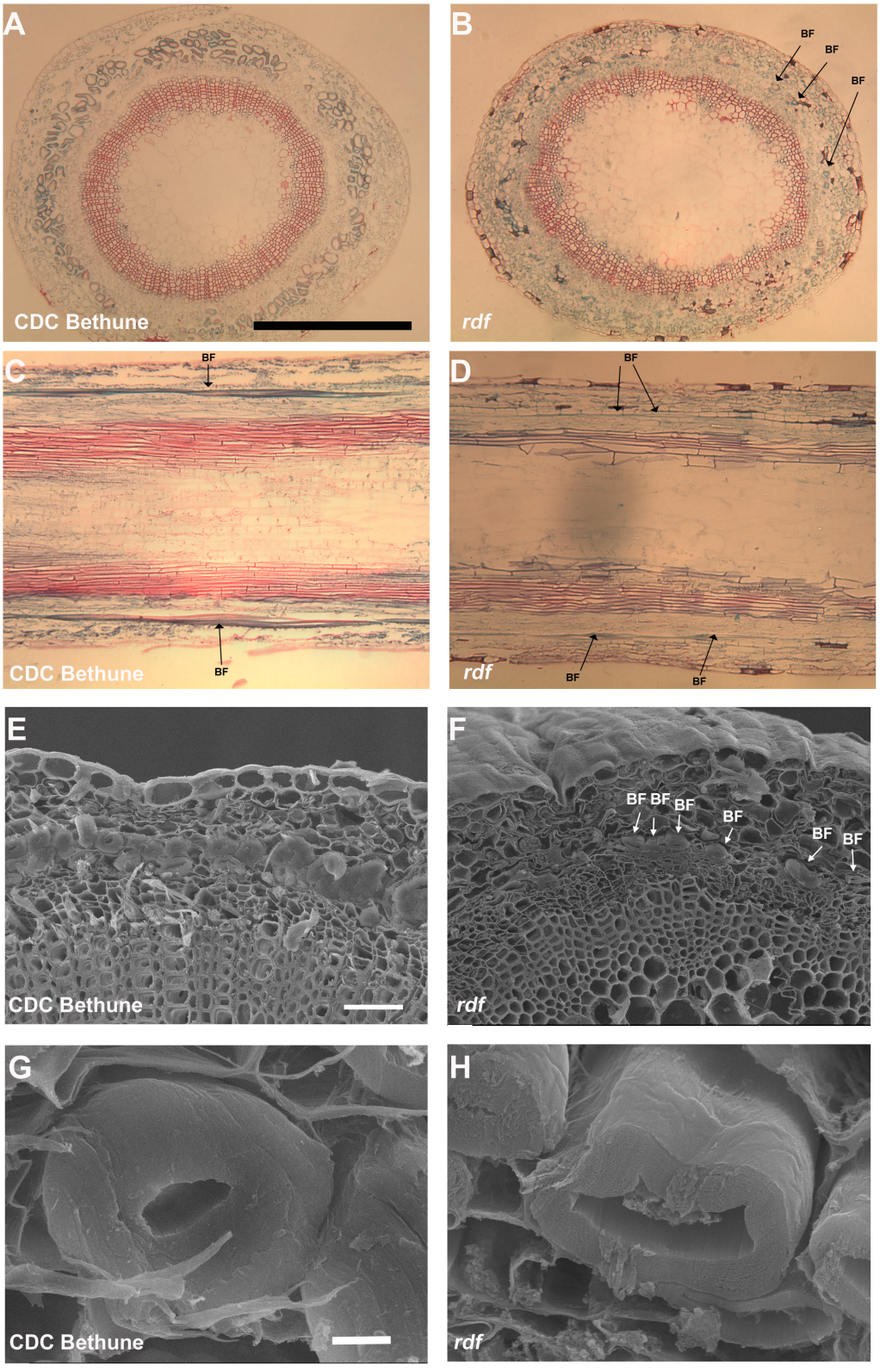

A
CDC Bethune
B
BF
BF
BF
rdf
C
BF
BF
CDC Bethune
D
BF
BF
BF
rdf
E
CDC Bethune
F
BF BF BF
BF
BF
BF
rdf
G
CDC Bethune
H
rdf

Figure 5-2. Transverse and longitudinal sections of CDC Bethune and *rdf* stems. (A-D) Paraffin-embedded CDC Bethune (A, C) and *rdf* (B, D) stems. (E-H) Stems viewed using scanning electron microscopy. (E, F) Vascular tissues in CDC Bethune and *rdf* stems. (G, H) Detailed view of a single bast fibre from CDC Bethune (G) and *rdf* (H) stems. Stems were sampled 6-weeks after planting below the 7^{th} internode above the base of the stem. Bar in A = 500 µm and applies to B, C and D; Bar in E = 50 µm and applies to F; Bar in G = 5 µm and applies to H.

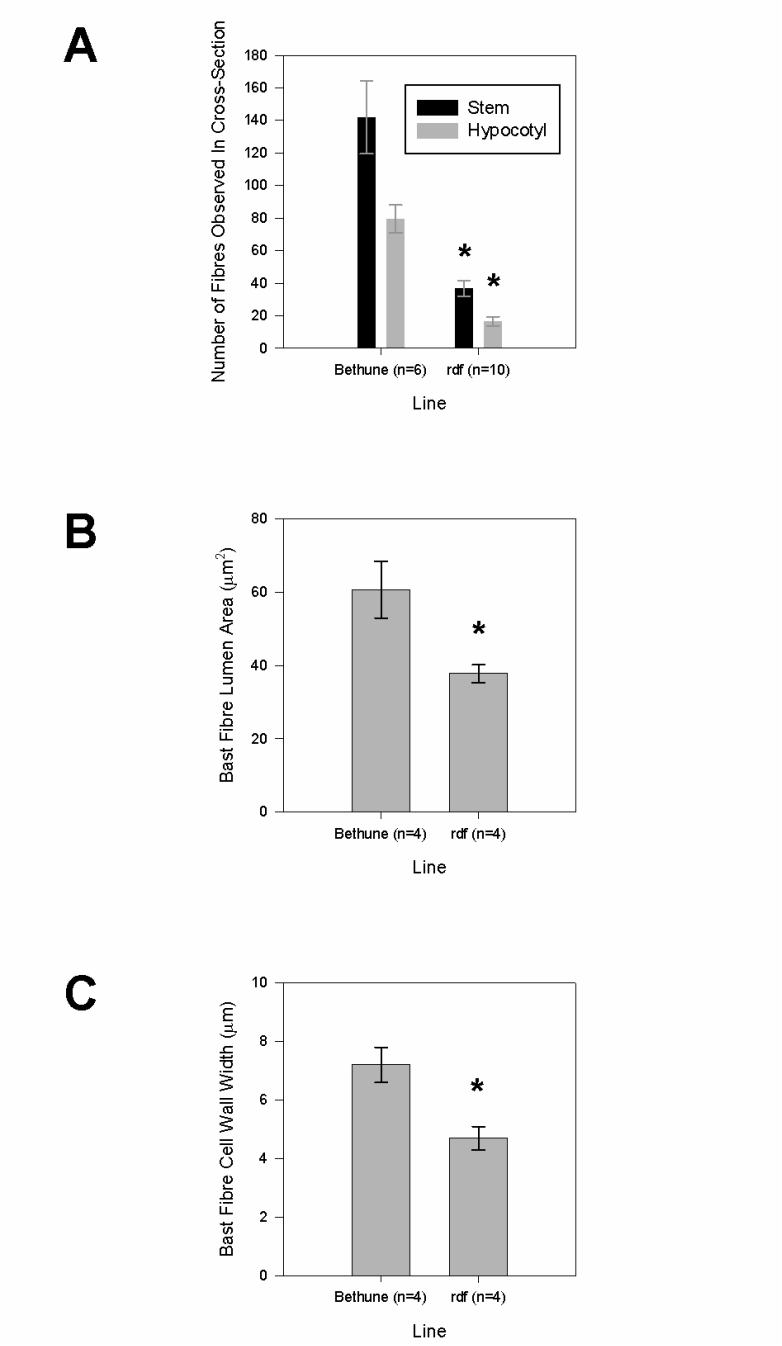


Figure 5-3. Bast fibre properties. (A) Number of fibres observed in cross-section, (B) average bast fibre lumen area and (C) average bast fibre cell wall width for CDC Bethune and *rdf* mutants. The symbol * indicates that a measurement significantly differed in *rdf* compared with Bethune ($p<0.05$), based on a *t*-test.

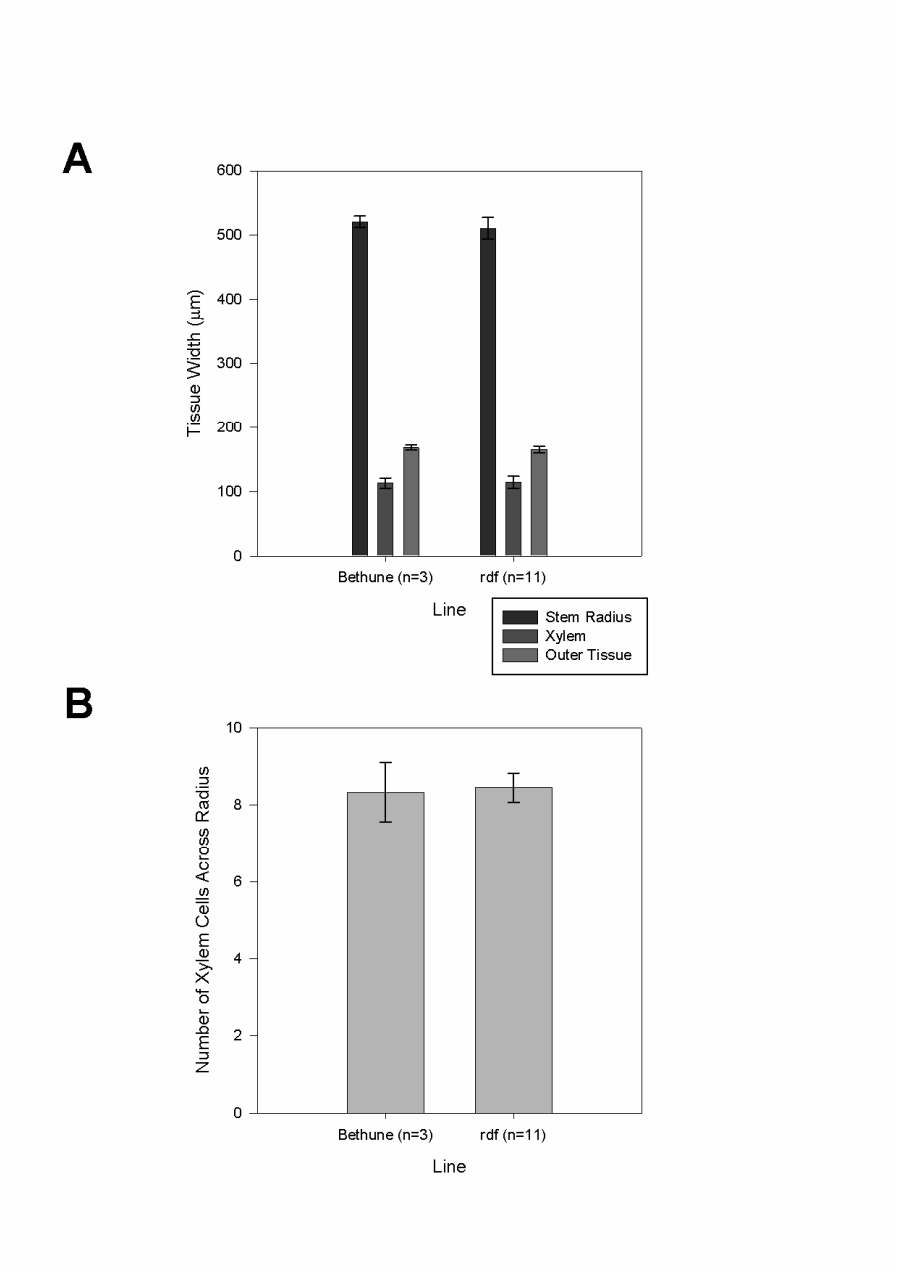
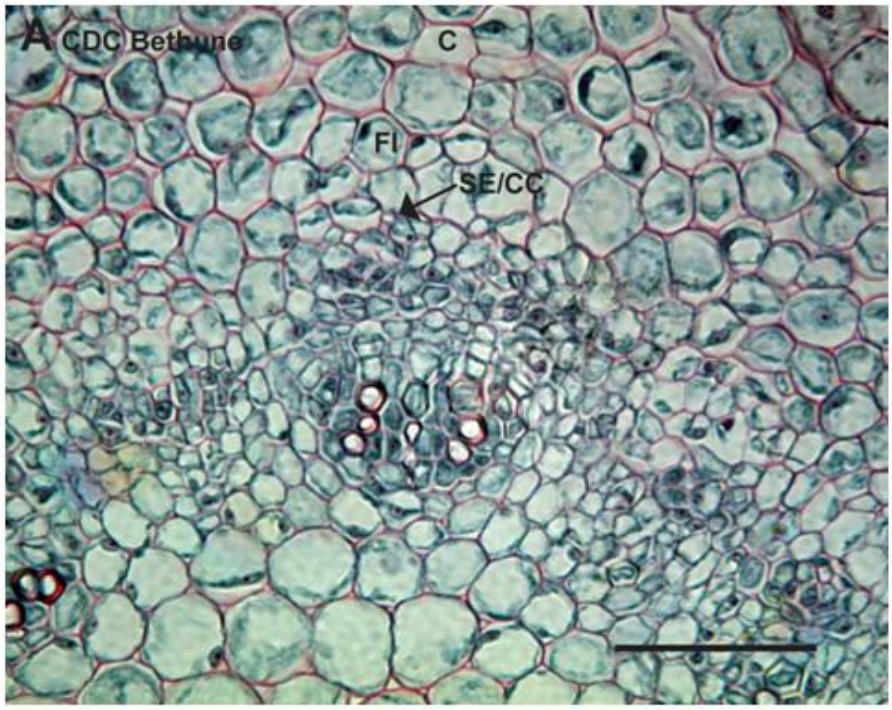


Figure 5-4. Stem tissue properties in 6-week-old CDC Bethune and *rdf* plants, measured in plants grown concurrently. (A) Stem radius, xylem radius and outer tissue radius. (B) Number of cells forming a file across the xylem.


A CDC Bethune
C
Fl
SE/CC

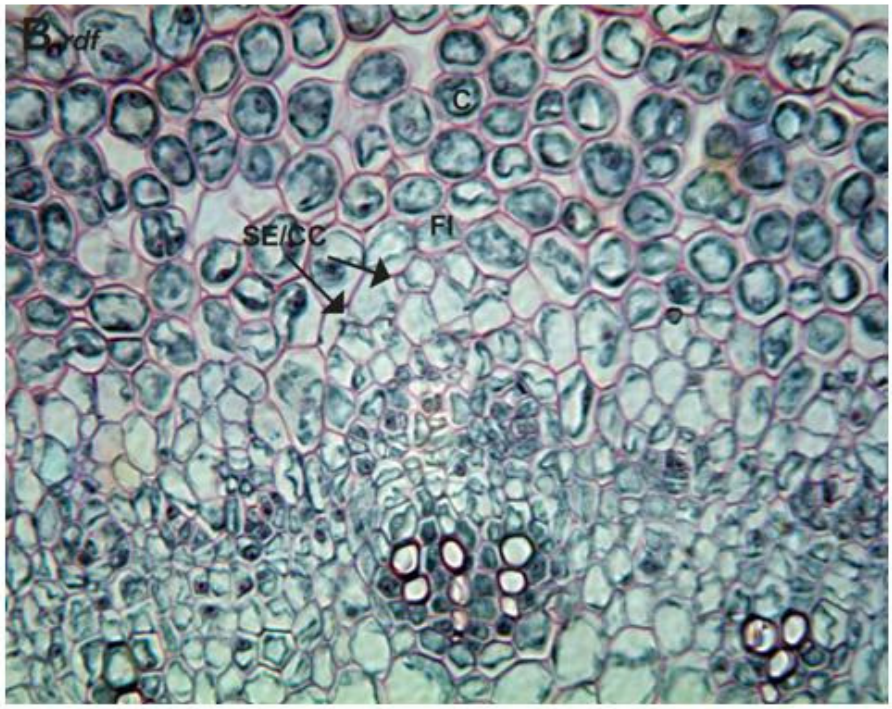

B rdf
C
SE/CC
Fl

Figure 5-5. Transverse sections from CDC Bethune and *rdf* stem apices. Layers of fibre initials (FI) were observed to have formed towards the outside of the vascular bundle (adjacent to the cortex) in both the CDC Bethune (A) and *rdf* (B). Pairs of smaller cells, presumed to be the sieve tube elements and companion cells (SE/CC), were also observed in both genotypes. Note that the right-most sieve element/companion cell pair in the transverse section from *rdf* (B) appears to have been partially compressed. Symbols: FI = fibre initials, SE/CC = sieve tube elements/companion ceels; C = cortex. Bar in A = 50 μm and equivalently applies to B.

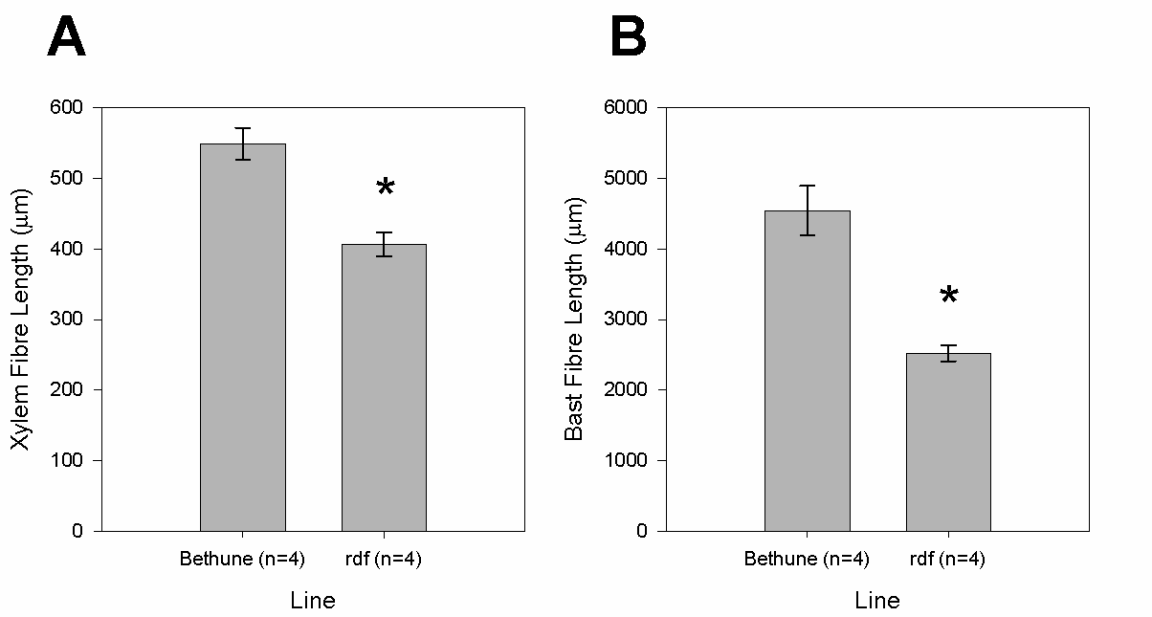


Figure 5-6. Fibre length properties. Xylem (A) and bast (B) fibre lengths were measured in stem macerates from CDC Bethune and *rdf* plants. Samples were taken from mature (post-flowering) plants that had been grown side-by-side. At least 25 xylem and 9 bast fibres were measured in each plant. The symbol * indicates that a measurement from *rdf* significantly differed from the wild-type measurement ($p<0.05$), based on a *t*-test.

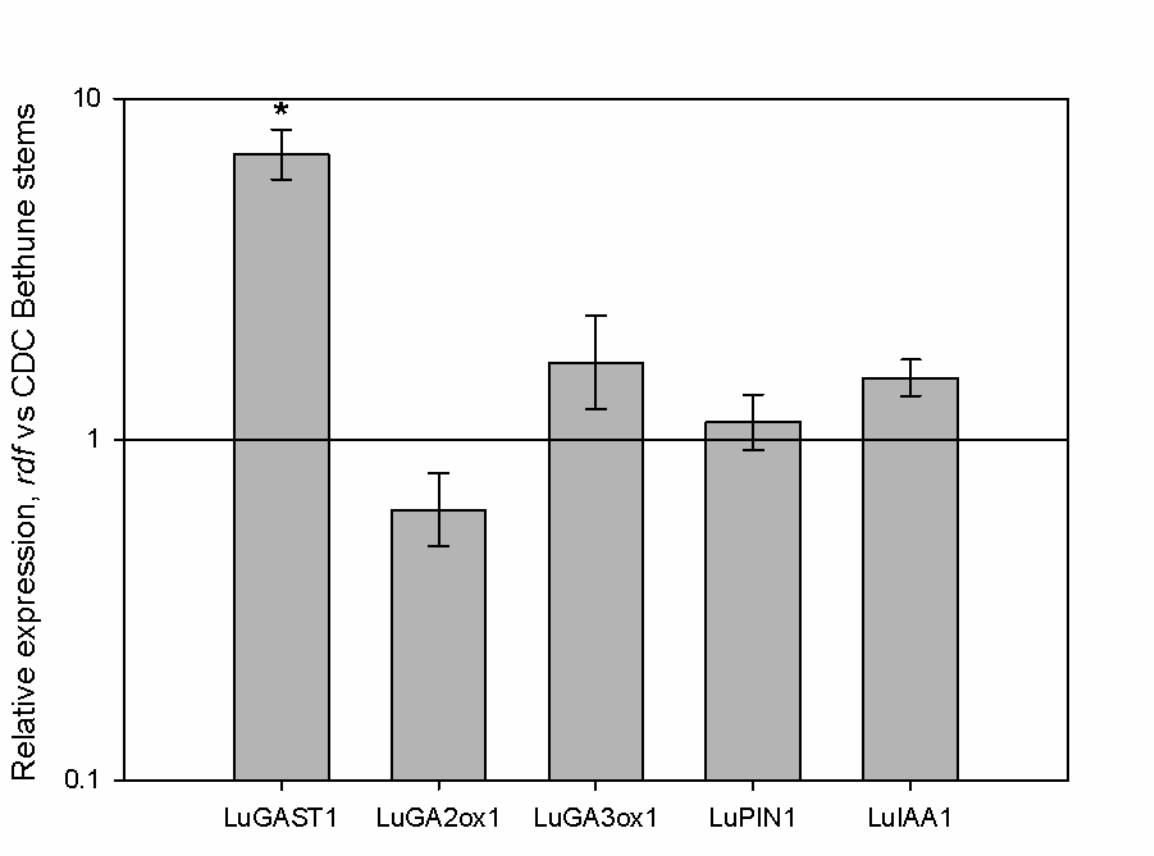


Figure 5-7. Relative expression of putative GA and IAA signalling genes in *rdf* vs CDC Bethune stem tissue, measured by quantitative real-time PCR. Midstem tissues were sampled 6-weeks after planting, n=4 plants per genotype. The symbol * indicates that a expression difference was found to be statistically significant, based on a *t*-test ($p<0.05$).

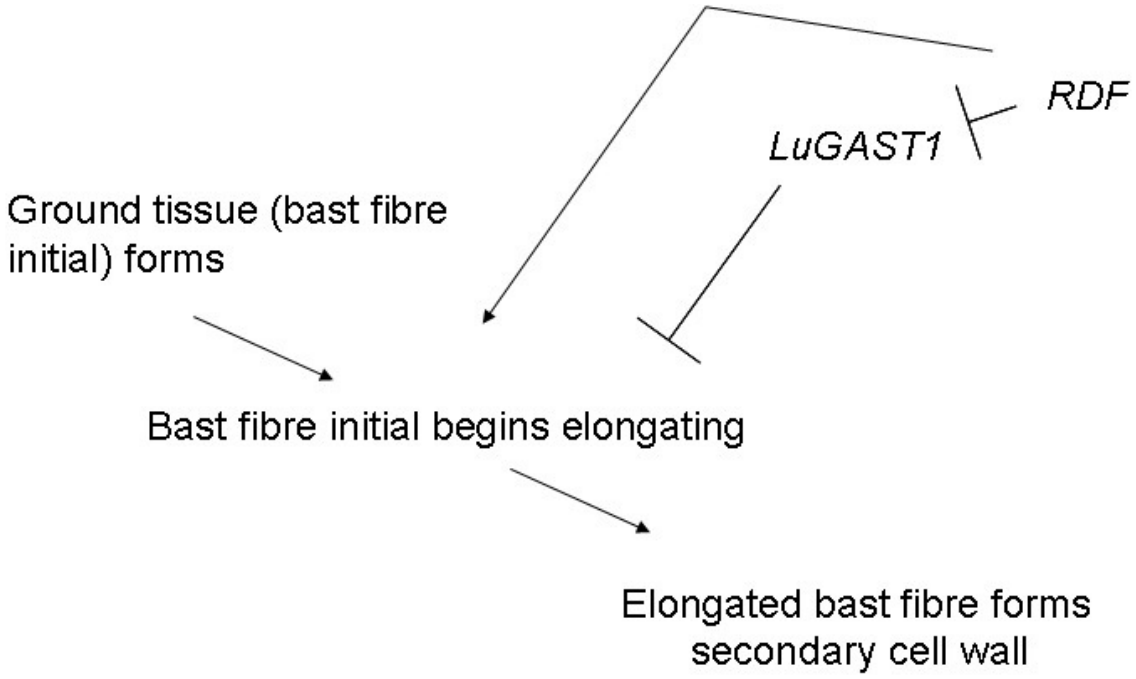


Figure 5-8. Hypothesized role of *RDF* in fibre differentiation. The *RDF* gene is proposed to regulate the elongation and/or formation of the cell wall in differentiating fibres. The function of *RDF* may at least partially mediated by *LuGAST1*.

5.8 Appendix

Table 5-A1. Tissue processing schedule for paraffin embedding.

Treatment	Time (Hours)
50% (v/v) ethanol	~3
70% (v/v) ethanol	~2
90% (v/v) ethanol	2
100% (v/v) ethanol	2
100% (v/v) ethanol	2
1:1 ethanol:toluene	1
Toluene	0.5
Toluene	0.5
molten paraffin wax	4
molten paraffin wax	4

Table 5-A2. Deparaffinization and safranin-fast green staining schedule.

Treatment	Time (Minutes)
Toluene	5
toluene	5
100% (v/v) ethanol	5
100% (v/v) ethanol	5
95% (v/v) ethanol	2
Safranin O (1% (w/v) in 95% ethanol)	>60
95% (v/v) ethanol	~1 (10 dips)
Fast Green (0.8% (w/v) in 95% ethanol)	
100% (v/v) ethanol	~1 (10 dips)
100% (v/v) ethanol	2
toluene	2
toluene	2 plus coverslipping time

Table 5-A3. Tissue processing schedule for scanning electron microscopy.

Treatment	Time (Minutes)
50% (v/v) ethanol	30
70% (v/v) ethanol	30
90% (v/v) ethanol	20-30
100% (v/v) ethanol	20-30
100% (v/v) ethanol	20-30
75% (v/v) ethanol: 25% (v/v) HMDS	20-30
50% (v/v) ethanol: 50% (v/v) HMDS	20-30
25% (v/v) ethanol: 75% (v/v) HMDS	20-30
100% (v/v) HMDS	20
100% (v/v) HMDS	20

6. Characterization and Mapping of Flax (*Linum usitatissimum* L.) SSR markers

6.1 Introduction

Distinct linseed and bast fibre types of flax (*Linum usitatissimum* L.) have been cultivated since flax was first domesticated as a crop, approximately 10,000 years ago (Zohary and Hopf, 2000). Then as now, bast fibres extracted from retted flax stems were used primarily to produce linen cloth. Fibre flax cultivars are grown in some regions of northern Europe and Russia, while distinct linseed flax varieties are widely grown in cool temperate regions of several countries, including Canada (Millam et al., 2005). Linseed oil has a high content of alpha-linolenic acid (ALA), a polyunsaturated fatty acid, and is widely used for industrial applications (Millam et al., 2005). As an omega-3 fatty acid, ALA is considered beneficial to human health, potentially reducing the risk of cancer and heart disease (Cunnane, 2003).

In addition to its value as a commodity, flax is a useful model system for investigating the biochemical and developmental cues underlying plant cell wall formation (Gorshkova et al., 1996; Chen et al., 1998; Ebskamp, 2002; Morvan et al., 2003; Day et al., 2005). Flax has attributes that make it an attractive genetic system. A self-pollinating diploid, flax can be grown to maturity in the laboratory. Flax is genetically transformable using *Agrobacterium tumefaciens* (Millam et al., 2005). The flax genome has been measured at 0.7 pg of DNA/1C nucleus (Bennett and Smith, 1976), corresponding to approximately 700 Mbp (Dolezel et al., 2003), slightly larger than the sequenced 480 Mbp genome of poplar (*Populus trichocarpa*) and 430 Mbp rice (*Oryza sativa*) genome (Pennisi, 2007). The

genome of the CDC Bethune linseed variety has recently been sequenced (M. Deyholos, manuscript in preparation).

The identification of molecular markers is an effective first step towards the map-based cloning of mutations, the mapping of quantitative trait loci (QTLs), the application of marker-assisted breeding and the determination of evolutionary relationships within and between species (Oh et al., 2000; Semagn et al., 2006). Flax mutants have been identified that exhibit dwarfism (George and Nayar, 1973), albinism (Bretagne-Sagnard et al., 1996), altered fatty acid content (Rowland and Bhatty, 1990; Ntiamoah and Rowland, 1997), altered cell wall properties (Chen et al., 1998), a curly stem shape (Tejklova, 2002), and impaired bast fibre differentiation (Chapter 5). Systematic efforts have not been conducted to map or molecularly characterize these and other loci associated with important traits in flax.

Amplified fragment length polymorphism (AFLP) markers and random amplified polymorphic DNA (RAPD) markers have been developed that can be used to determine flax genotypes (Cullis, 2007). RAPDs and AFLPs have many reputed limitations, including poor reproducibility (Weising et al., 2005). AFLP and RAPD markers are not co-dominant, generally making them unsuitable for mapping mutations (Weising et al., 2005). Simple sequence repeats (SSRs) are 2-6 bp tandemly repeated DNA sequence motifs (Weising et al., 2005). PCR can be used to amplify a genomic region containing an SSR locus, and variations in the PCR product length can be identified as polymorphisms (Semagn et al., 2006). Unlike RAPDs and AFLPs, SSRs have a co-dominant nature (Weising et al.,

2005; Semagn et al., 2006). Collections of flax SSRs have been reported by several groups (Roose-Amsaleg et al., 2006; Cloutier et al., 2009; Deng et al., 2010; Soto-Cerda et al., 2011), although this work reported in this chapter was completed when only the report by Roose-Amsaleg et al. (2006) was available.

In this chapter, 335 markers, including 299 markers that were identified at the University of Alberta, were screened for polymorphisms between the linseed cultivars CDC Bethune and Bolley Golden. 36 polymorphic markers were mapped in a F_2 population derived from a cross of CDC Bethune with Bolley Golden. Polymorphic markers were also tested on DNA samples from 22 flax cultivars and 4 additional *Linum* species in order to investigate their suitability for applications in mapping, breeding, and phylogenetic diversity studies.

6.2 Materials and Methods

6.2.1 Plant Material

The *Linum usitatissimum* accessions tested in this study are listed in Table 6-1. Seed from these accessions was predominantly obtained from the Plant Gene Resources of Canada germplasm collection. Seed samples for the Bolley Golden and E1747 varieties were provided by Gordon Rowland (University of Saskatchewan) (Rowland and Bhatty, 1990; Rowland et al., 2002). Seed samples for *Linum corymbiferum*, *Linum decumbens*, *Linum lewisii* and *Linum perenne* were obtained from Linda Hall (University of Alberta, Department of Agricultural, Food and Nutritional Science).

6.2.2 DNA Extraction Procedures

Fresh tissue samples of approximately 100 mg were used for genomic DNA extractions. DNA was extracted either using the Fermentas Genomic DNA Extraction Kit (Fermentas Inc., Glen Burie, Maryland, USA), or a standard CTAB DNA extraction protocol (Sambrook and Russell, 2001), and quantified using a spectrophotometer (Nanodrop® ND-1000, Nanodrop Technologies LLC, Wilmington, Deleware, USA).

6.2.3 Preparation of SSR-enriched Genomic Library

The SSR-enriched library was prepared by Corey Davis (University of Alberta, Department of Biological Sciences Molecular Biology Service Unit), using genomic DNA extracted from a single CDC Bethune plant. An SSR-enriched genomic library was prepared using a previously described protocol (Hamilton et al., 1999). Briefly, 15 µg of DNA was digested using AluI, RsaI and NheI to produce DNA fragments in the 200-1000 bp size range. DNA fragments were ligated to double stranded SNX linkers, and enriched by hybridization to 5' biotinylated $(GT)_{14}$, $(GACA)_7$, $(CT)_{14}$ and $(GATC)_7$ oligonucleotides. Genomic DNA-oligonucleotide hybrids were captured using streptavidin-coated beads, from which SSR-enriched genomic DNA fragments were eluted. The SSR-enriched fragments were cloned into pBLUESCRIPT II SK(+) (Stratagene, La Jolla, CA, USA), and transformed into competent *Escherischia coli* cells using standard techniques. Inserts were PCR-amplified with T7 and T3 primers and sequenced on an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA). From this, 19 novel SSR markers were identified.

6.2.4 Development of SSR Primers

SSR primers were tested on DNA samples from CDC Bethune and Bolley Golden to assess polymorphism. 23 of the tested primers sets were previously published (Roose-Amsaleg et al., 2006). 9 unpublished SSR primer sets were developed by I. Wiesner and D. Wiesnerova (Institute of Plant Molecular Biology, Czech Republic). 280 novel SSR primers were developed by Michael Deyholos, starting with 7000 *L. usitatissimum* EST sequences available in NCBI's nucleotide databases as well as EST libraries from developing flax seeds comprising >150,000 sequences (Raju Datla, University of Saskatchewan). SSR regions were identified in both singlets and contigs using the SSRIT tool (Temnykh et al., 2001). Sequences were assembled into contigs using CAP3 (Huang and Madan, 1999). Separately, 384 inserts from the SSR-enriched genomic library described above were sequenced, from which 19 non-redundant primer pairs that reliably amplified SSR loci from genomic DNA were identified. Primers for SSR regions were designed using Primer3 (Rozen and Skaletsky, 2000).

6.2.5 SSR Genotyping

Genotyping procedures were based on previously described methods (Schuelke, 2000). The M13(–21) universal sequence (18 bp) was fused to the 5′ end of the forward primer for each SSR primer set, and the M13(–21) universal primer was labelled with either 6-FAM, VIC, NED, or PET (Applied Biosystems). PCR amplifications contained 25 ng genomic DNA, 1.5 µL of 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl pH 9.0, 30 mM $MgCl_2$, 1% (v/v)

Triton X-100), 0.1 mM dNTPs, approximately 1.25 U of Taq DNA polymerase, 8 pmol forward and universal primer and 2 pmol reverse primer. Conditions of the PCR amplification were: 94 °C (3 min), 15 cycles of touchdown PCR conditions at 94 °C (30 s)/ 67-52 °C (30s)/ 72 °C (30s), 20 cycles at 94 °C (30 s)/ 52 °C (30s)/ 72 °C (20s), followed by a final extension at 72 °C (3 min). 2 µL of 10 times diluted PCR product was added to 7.95 µL of Hi-Di Formamide and 0.35 µL of 500 LIZ Size Standard (Applied Biosystems) and applied to an ABI 3730 DNA Analyzer (Applied Biosystems). Data was analysed using ABI GeneMapper software.

6.2.6 Linkage Map Construction

Bolley Golden served as the pollen donor and CDC Bethune as the pollen recipient for crosses. A mapping population consisting of 99 F_2 progeny was scored for the occurrence of purple flowers and/or brown F_3 seed (characteristic of CDC Bethune), vs. white flowers and/or golden F_3 seed (characteristic of Bolley Golden). F_2 progeny were genotyped using SSR markers found to be polymorphic between CDC Bethune and Bolley Golden. Two-point linkage analysis was performed and the map constructed using MAPMAKER 3.0 software (Lander et al., 1987). A LOD score of 3.0 was used as minimal criteria for linkage. Recombination frequencies were converted into map distances in centimorgans using the Kosambi mapping function (Kosambi, 1944). A graphical representation of the linkage map was prepared using MapChart 2.2 software (Voorrips, 2002).

6.2.7 Allelic Diversity Analysis

22 markers, chosen from within our collection of markers known to be polymorphic between CDC Bethune and Bolley Golden, were also evaluated on DNA samples from single individuals representing 22 additional *Linum usitatissimum* varieties, as well as four additional *Linum* species (*Linum corymbiferum, Linum decumbens*, *Linum lewisii* and *Linum perenne*). The allelic diversity for the *L. usitatissimum* varieties was analyzed using GENEPOP 4.0 (Raymond and Rousset, 1995). Tests for Hardy-Weinberg equilibrium and linkage disequilibrium were calculated using Fisher's exact test and the Markov chain algorithm with 100 batches, 1000 iterations per batch and 1000 dememorization steps. The critical significance values for both tests were evaluated using the Bonferroni correction (0.05/22 = 0.002) (Rice, 1989).

6.3 Results

By computational analysis of over 150,000 ESTs and 384 genomic sequences obtained from an SSR-enriched library (Temnykh et al., 2001), 299 primer pairs were identified that amplified SSR-containing PCR fragments from genomic DNA (*L. usitatissimum*). These 299 novel primer pairs, plus 32 previously described primer pairs, were tested for SSR length polymorphism between the CDC Bethune and Bolley Golden linseed varieties. CDC Bethune is one of the most widely grown varieties of flax (brown seeded linseed) in Canada, while Bolley Golden (yellow seeded linseed) was chosen as the second variety both because it was determined to be among the more polymorphic from CDC Bethune of 24 varieties tested in a pilot screen (Mary de Pauw, data not shown) and because it could be easily distinguished from CDC Bethune by its distinct

seed and flower colour. The primer sequences and SSR characteristics of the 36 polymorphic primers are listed in Table 6-2, while information on the full set of 299 unpublished SSR markers that were evaluated is provided in Table A1 (Appendix 1).

The distribution of most markers used to construct the map fits the 1:2:1 ratio expected for co-dominant genetic traits, according to the χ^2-test (Table 6-3). However, several markers failed to fit this ratio ($p<0.05$ according to the χ^2-test). At the most extreme, only 1 of the 97 individuals scored for UALU2001 was heterozygous. With 21 additional samples included, the genotypic distribution for UALU2025, which failed to fit a 1:2:1 ratio when 97 individuals were genotyped, fit the 1:2:1 ratio ($N = 116$, $\chi^2 = 4.46$, $p > 0.05$). The segregation of the flower and F_3 seed colour phenotypes fit expected 3:1 ratios for single gene traits, with the golden seed and white flower colour of CDC Bethune determined to be recessive. 16 of the 36 SSR markers, as well as both morphological characters, were mapped into 6 linkage groups (Figure 6-1).

To further assess the degree of polymorphism for the SSR markers, 22 markers chosen from among the 36 polymorphic markers were tested on DNA from 22 additional *L. usitatissimum* cultivars. Also, to investigate whether markers could potentially be used to genotype other *Linum* species, these markers were tested for their ability to amplify DNA from four other organisms: *L. corymbiferum*, *L. decumbens*, *L. lewisii* and *L. perenne* (Table 6-4). The *L. usitatissimum* cultivars tested included both linseed and fibre varieties, from diverse geographic origin. *L. corymbiferum* and *L. decumbens* have been reported

to successfully hybridize with flax, while the two other species tested, *L. lewisii* and *L. perenne*, reportedly are unable to hybridize with flax (Jhala et al., 2008). Among the *L. usitatissimum* cultivars, polymorphism was found to range from 2 to 6 alleles, with an average of 3.3 alleles per locus (Table 6-4). The expected heterozygosity averaged 0.50, while the observed heterozygosity averaged 0.14. All but 5 loci showed departure from Hardy-Weinberg equilibrium (HWE) in the population. Significant deviation from random association ($p < 0.002$) was found in 5 pairs of loci out of 231 pairs analyzed. 19/22 markers successfully amplified DNA from *L. corymbiferum*. 6/22 loci amplified DNA from *L. decumbens*, 6/22 amplified DNA from *L. lewisii*, and 5/22 amplified DNA from *L. perenne.*

Flax has been bred traditionally as a single-purpose crop, with varieties grown either for oil or bast fibre, exclusively. In this study, marker genotypes for 10 oil (i.e. linseed) varieties and 9 fibre varieties of flax were compared (Table 6-1 and data not shown). A significant correlation was not observed between the purpose for which the variety was developed and the genotype at 21 of 22 loci tested. However, for marker UALU2001, it was noteworthy that of the five alleles identified, at least one copy of the 159 bp allele was present in 6 of 10 linseed varieties, and that 5 of those 6 varieties were homozygous for the 159 bp allele. Likewise, the 161 bp allele was present in 6 of 9 fibre varieties, and 5 of those 6 varieties were homozygous for the 161 bp allele.

6.4 Discussion

Of 331 flax SSR primer pairs tested, the number detecting polymorphisms (11%) was relatively low in comparison to other reports of 20-30% polymorphism

for SSRs that have been developed for and tested in other crop plants (Hendre et al., 2008; Lewers et al., 2008). Furthermore, in those other crop plants 20-30% polymorphism was considered to be a relatively low percentage (Hendre et al., 2008; Lewers et al., 2008). The apparently poor abundance of polymorphic primer pairs for flax is consistent with previous observations of low heterozygosity among flax SSRs, and can be explained by the autogamy (self-fertilization) of flax (Roose-Amsaleg et al., 2006).

The SSR map that is reported in this study (Figure 6-1) covers 200.8 cM over six linkage groups. It provides relatively small coverage of the flax genome in comparison to the two >1000 cM maps published for flax that are based on AFLP, RFLP and RAPD markers (Spielmeyer et al., 1998; Oh et al., 2000), or the 833.8 cM SSR-based map for flax that was recently published by Cloutier et al. (2010). The 20 unlinked polymorphic SSR markers that were identified might either be the sole representatives for the other 9 linkage groups, or markers that are found on the same chromosome as other markers, but distantly spaced. Nonetheless, the polymorphic markers characterized here will be very useful for many purposes, including the mapping of mutated loci, such as the *reduced fibre1* mutant that was described in Chapter 5.

Several markers failed to fit the 1:2:1 ratio expected for co-dominant genetic markers. Segregation distortion is frequently observed in mapping studies, occurring, for example, in 29% of the markers in an F_2 population from a cross between two oilseed rape cultivars (Cloutier et al., 1995). UALU2025 showed segregation distortion when 97 F_2 individuals were genotyped. However, when

additional samples were genotyped, UALU2025 fit a 1:2:1 ratio ($N = 116$, $\chi^2 = 4.46$, $p > 0.05$). Therefore, it appears likely that the small population size is at least a contributor to the segregation distortion observed for these markers. A population size of 200 individuals would be preferable for constructing a more accurate map (Ferreira et al., 2006); however, the wide spacing of markers on the map suggests that it would be unlikely that a larger mapping population would influence the reported marker order to a significant degree. It was intriguing that only 1 of the 97 individuals genotyped for UALU2001 was scored as a heterozygote. Dominant SSR markers are unusual, but not impossible: null alleles may be observed if a mutation in a primer binding site prevents amplification (Weising et al., 2005). As segregation distortion has been found to have little effect on marker orders and map length (Hackett and Broadfoot, 2003; Semagn et al., 2006), these markers were included on the map, although these markers are unlikely to be useful for other applications.

In addition to the SSR markers, two loci affecting flower colour and seed colour, denoted as the *C''* and *D* genes, were mapped. Tammes (1922) found that the absence of either of two interacting factors, *B* and *C''*, causes flowers to be white. Seed colour is influenced by the *B*, *D* and *G* factors, with *G* considered the basic factor and the other two modifying factors (Tammes, 1922). Due to the complexity of the genetic basis for flower and seed colour in flax, linkage relationships among these genes have not been conclusively established. The yellow seed colour of the Bolley Golden cultivar has been determined to be due to a mutation in the *D* gene (Mittapalli and Rowland, 2003), while the white flower

colour might be due to a mutation in the *C‘‘* gene, as a mutation in the *B* factor reportedly induces developmental abnormalities (Tammes, 1922). By scoring the flower colour of individuals in the mapping population, and the colour of the F_3 seed produced by each plant, it was established that the presumed *C’’* and *D* genes are located in the same linkage group and linked to an SSR marker, UALU1023.

At least 8,387 accessions of flax are stored in 13 seed banks from 11 countries (Cullis, 2007). The enormous diversity of flax germplasm makes the documentation and distinction of flax cultivars a complex task. Molecular markers can assist in this process, allowing related accessions to be distinguished (Cullis, 2007). In order to investigate whether they could be useful for this purpose, 22 SSR markers in the collection were evaluated on DNA samples from 24 flax cultivars (Table 6-4). The cultivars that were selected for this purpose included both linseed and fibre flax accessions that originated throughout the world. The relatively low heterozygosity and predominant departure from Hardy-Weinberg equilibrium that were observed (Table 6-4) were consistent with the low heterozygosity and frequent linkage disequilibrium among the flax SSRs evaluated by Roose-Amsaleg et al (2006). Therefore, SSRs would be unreliable for discriminating close relatives, such as flax plants from the same cultivar. However, SSRs provide sufficient polymorphism for the discrimination of cultivars.

Many species from the *Linum* genus have been found to grow in sympatry, or within the same geographic range, as flax (Jhala et al., 2008). Furthermore, several species have been shown to successfully hybridize with flax, producing

viable F_1 offspring (Jhala et al., 2008). The hybridization of flax (2n=30) with *L. corymbiferum* (2n=30) has been determined to be successful, producing F_1 progeny exhibiting 80 to 90% germination success (Gill, 1966). Successful crosses with *L. decumbens* have also been reported (Gill, 1966). Conversely, successful hybridization between flax and either *L. perenne* (2n=18) or *L. lewisii* (2n=18) has not been reported (Jhala et al., 2008). Overall it has been found that only species with equal chromosome numbers to flax are able to successfully hybridize with it to produce fertile F_1 progeny (Jhala et al., 2008).

In order to determine whether the SSRs could distinguish flax from its close relatives, 22 of the SSRs were tested on DNA samples from four *Linum* species. 19 of these markers successfully cross-amplified at least one of these four species – in all cases from *L. corymbiferum*. Amplification was much less successful from *L decumbens*, *L. lewisii* and *L. perenne.* The results reported in the present study are consistent with a previous characterization of flax SSRs which demonstrated that SSR markers cross-amplified species with karyotypes that were more similar to flax, such as *L. corymbiferum* (Roose-Amsaleg et al., 2006). Thus, flax SSRs may be useful for determining potential introgression rates between these organisms.

6.5 Conclusions

335 SSR markers were tested for polymorphism between the CDC Bethune and Bolley Golden linseed varieties. 36 markers were determined to be polymorphic, and were mapped in a 99 individual F_2 population derived from a cross between CDC Bethune and Bolley Golden. The SSR-based linkage map

presented here provides coverage of 200.8 cM of the flax genome over 6 linkage groups, and is superseded by a recently available SSR-based map for flax that provides 833.8 cM of coverage (Cloutier et al., 2010). The map presented here has not yet been integrated with the map by Cloutier et al. (2010). However, software, such as JoinMap (Stam, 1993), is available that could facilitate the integration of the maps in the future.

The polymorphic SSRs are potentially useful for several purposes. By testing these markers on 24 different flax accessions, it was demonstrated that they demonstrate sufficient polymorphism supporting a potential use for differentiating flax cultivars. Furthermore, by testing these markers on four related species from the *Linum* genus, it was determined that they cross-amplify from some of their closely related relatives and may be useful for investigating rates of introgression between flax and its relatives. Finally, a goal of this work was to identify markers that could be used to facilitate the mapping of newly isolated mutants, and as such the polymorphic markers reported here are presently being employed to map the *reduced fibre1* mutant (Chapter 5).

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6.7 Tables and Figures

Table 6-1. *Linum usitassimum* cultivars genotyped in this study.

Cultivar	Plant Gene Resources of Canada Accession Number	Country of Origin	Plant Type
CDC Bethune	N/A	Canada	Linseed
Bolley Golden	CN 97291	Hungary	Linseed
NorLin	CN 52732	Canada	Linseed
Sel of N.D.R. 114	CN 97463	United States	Linseed
Victory B	CN 97907	United States	Linseed
Indian Commercial	CN 97607	India	Linseed
Morocco (Cili 376)	CN 97434	Egypt	Linseed
Roman Winter	CN 97459	Netherlands	Linseed
Timbu	CN 97339	Argentina	Linseed
E1747 (McGregor)	N/A	Canada	Linseed
Atlas (Fibre)	CN 97871	Sweden	Fibre
Viking	Unknown	France	Fibre
Pinnacle	CN 97590	United States	Fibre
Unryu	CN 98072	Japan	Fibre
Krislima Spanadslin	CN 98075	Sweden	Fibre
Beatall	CN 97590	Ireland	Fibre
Cili1633	CN 98162	Iran	Fibre
Rolin	CN 101233	Romania	Fibre
Lin de Tunise	CN 97033	Tunisia	Fibre
Lin de Tunisi	CN 100966	Tunisia	Unknown
El Barco	CN 100891	Spain	Unknown
Erythrea	CN 100914	Ethiopia	Unknown
Hollander Weiss	CN 100822	Netherlands	Unknown
Absynnian (Brown)	CN 97410	Ethiopia	Unknown

Table 6-2. Characteristics of SSR determined to be polymorphic between CDC Bethune and Bolley Golden. Markers labelled with a * were reported by Roose-Amsaleg et al. (2006).

Marker	Forward Primer Sequence (5'to 3')	Reverse Primer Sequence (5'to 3')	Repeat Motif	Sequence Type
LU8*	ACACTTGCTATTAGCTACAAGAGAG	CAGCATCCAGAGGTTCTCAC	$(AG)_{24}$	EST-SSR
LU17*	ATGATCGCATGAGCAAATTG	GTTTGTGAGGTGACGGTGAG	$(GA)_{26}$	EST-SSR
LU21*	CCGAGTCCGAAAGAATCTGG	CAGCTCCCATTGTTGTTCCC	$(GA)_{15}A_4$	EST-SSR
LU23*	CATGACCATGTGATTAGCATCG	CATAGGAGGTGGGTTGCTGC	$(CA)_8(GA)_{22}$	EST-SSR
LU37*	GTTCATTATCAAACATTTGATCTTATTTG	CCCGTACTTGAGTTAAGTACGTCC	$(TC)_8$	EST-SSR
SSR_1F11	TCCGGATTGACTCTTCCTTC	AAGATTTCAGACTTTTCAGTGGTTT	$(AGAC)_6$	Genomic
SSR_3A1	CCAACAAGAGCACATGGTCA	TTGGAATTTTGGAATCTGA	$(GT)_{11}$	Genomic
SSR_4D9	TCCGGATTGACTCTTCCTTC	GATTTCAGACTTTTCAGTGGTTT	$(CAGA)_7$	Genomic
SSR_5B6	TGAGTGGGTTAGTGGGATCTG	AAGCAGCACGAACCTGTTTT	$(TGTC)_6$	Genomic
SSR_6E7	CACAGAAATGCCAGAAGAAAAG	CGTTTCTAACATGAGGGCGTTG	$(ACA)_8$	Genomic
SSR_6F11	GACCATCGTCGTCATTTCCT	AATGCGAAAATCGCCAATAC	$(TC)_{13}$	Genomic
SSR_10G3	GCAAGGAGATGGAAATGTTA	TGCCGTCTCTTCAATAAATAA	$(AG)_{13}$	Genomic
UALU1001	TCCAAACACTCTCGTTTATTTATTTTT	ATCAGCAATCAATCGCATCA	$(AT)_9$	EST-SSR
UALU1023	GAAACTCAGTGAATCACCGCT	GTAGCAGCAGCAGCTTCCC	$(AGA)_4$,$(AGA)_8$, $(GCT)_4$, $(AATTAG)_3$	EST-SSR
UALU1028	CTTCCATGTATTTTGCGATCA	GGAAAGCCCAGTCTGAACCT	$(AGC)_8$	EST-SSR
UALU1030	TCGATTAGAGATTCGCAGCA	ACGAATGAACGAAATGAGCC	$(TCT)_7$	EST-SSR
UALU1031	GCTGCTGCACCATTCCTC	TTGAGCATTGGCACTCAAAG	$(TAG)_{10}$	EST-SSR
UALU1044	GGAAGAAGAAGACGACGAAGAA	ACTACAATGTCCGTTTCGGC	$(GAA)_6$	EST-SSR
UALU1063	TTTCAATAGTGCTAAAGTGGTAGAGAA	TGAATTTCTTCACCCTCTATATTCC	$(TC)_{11}$	EST-SSR
UALU1075	AAATCCTTTCAAATTTAATACAACAGA	AGTGGGAGTTACGCTGTTTG	$(CATC)_5$	EST-SSR
UALU1077	CATCACAAACTGACACACCCA	TTTAACGACATGGAATCCCTA	$(ACA)_7$	EST-SSR
UALU1086	GGAGAATGATGGAAGGTGATG	TGGTGGGTTTAATTTAATTGTCTG	$(GAT)_9$	EST-SSR
UALU1088	CGACATTTTCTTCCCCTTCA	AAGAAAATCAACCCGAGGCT	$(CTT)_6$	EST-SSR
UALU1120	TGCTGCAATAAGTTAAGCTAGGG	CCGCACTTTCATCCAAGTCT	$(GA)_{12}$	EST-SSR
UALU1123	TCCAATGAAGCAGTAAAAGGC	GGACACTCCTTCCATCATCAA	$(ATC)_6$	EST-SSR
UALU1154	CATGTTGCGTTGAGATGACA	AGCAACAGGCACAACTTCG	$(TGC)_6$	EST-SSR
UALU1178	GAACCAGGCCTTCTCCTCTG	GAACATAGAGGCCGGTACGA	$(TAG)_7$	EST-SSR
UALU1198	ATCATTTCCCAAACTTCCCC	CGACCAACGAGAGGGATCTA	$(TTC)_8$	EST-SSR
UALU1199	TGCTGAGTGCTCACCACTTT	AAAGCTTGAAAACTGGAATAGAAA	$(TC)_6$,$(TC)_{13}$	EST-SSR
UALU1215	CGTCGGTTTCTGGTTCAGTT	CCGGGAAAGAGGTCAATGTA	$(CCG)_6$	EST-SSR

Marker	Forward Primer Sequence (5'to 3')	Reverse Primer Sequence (5'to 3')	Repeat Motif	Sequence Type
UALU1223	TTTCTCCCAACAAAATACCCC	AATGATTTCATCGGCGACTC	$(CAA)_6$	EST-SSR
UALU1241	CCTCCAACATAAAAGGAGGC	TTGTTTTGAAAGAATGGTCGG	$(CAA)_7$	EST-SSR
UALU2001	AACATACATAACATTCCATCAGCC	TCGTACAAAGACACTGCTAGGTG	$(CT)_{14}(ATT)_5$	EST-SSR
UALU2023	ACATGAAGATGAAGCCACCC	TTCACAGAATCCATTGCCAG	$(CTT)_8$	EST-SSR
UALU2025	CCCAACAAAACTCCAGAGTCA	AATTCTTCTCTGTTGGCGCA	$(TTC)_8$	EST-SSR
UALU2031	AACACACATACAGATGGGCG	TCGGGTTAGGGTATTTGAGTC	$(TA)_9$	EST-SSR

Table 6-3. Markers tested to construct the linkage map. Markers were tested on an F_2 population derived from a cross of the CDC Bethune and Bolley Golden cultivars. The flower colour and colour of the F_3 seed produced was also determined. Distributions of italicized markers did not fit a 1:2:1 ratio according to the Chi-square test. *N* refers to the number of individuals tested that successfully amplified each marker.

SSR Markers					
Marker	**Dye**	**CDC Bethune Allele (bp)**	**Bolley Golden Allele (bp)**	***N***	**Allele Distribution Fits 1:2:1 Ratio? (*df = 2*)**
LU8	6-FAM	242	212	97	Yes: χ^2=0.28, p > 0.05
LU17	PET	301	287	86	Yes: χ^2= 5.23, p > 0.05
LU21	VIC	232	227	89	Yes: χ^2=1.47, p > 0.05
LU23	6-FAM	265	267	84	Yes: χ^2=0.64, p > 0.05
LU37	PET	280	277	63	Yes: χ^2=5.38, p > 0.05
SSR_1F11	VIC	118	102	94	Yes: χ^2=5.15, p > 0.05
SSR_3A1	*PET*	*150*	*148*	*61*	*No: χ^2=8.84, 0.05 > p > 0.01*
SSR_4D9	6-FAM	115	99	95	Yes: χ^2=1.19, p > 0.05
SSR_5B6	VIC	124	128	98	Yes: χ^2=0.84, p > 0.05
SSR_6E7	NED	113	107	99	Yes: χ^2=5.04, p > 0.05
SSR_6F11	6-FAM	136	140	98	Yes: χ^2=0.78, p > 0.05
SSR_10G3	PET	119	121	99	Yes: χ^2=2.94, p > 0.05
UALU1001	VIC	166	172	93	Yes: χ^2=1.84, p > 0.05
UALU1023	6-FAM	134	131	98	Yes: χ^2=0.37, p > 0.05
UALU1028	6-FAM	123	117	96	Yes: χ^2=0.40, p > 0.05
UALU1030	NED	121	125	90	Yes: χ^2=2.98, p > 0.05
UALU1031	VIC	123	118	97	Yes: χ^2=0.42, p > 0.05
UALU1044	VIC	112	110	95	Yes: χ^2=1.44, p > 0.05
UALU1063	VIC	115	117	45	Yes: χ^2=2.91, p > 0.05
UALU1075	NED	108	112	98	Yes: χ^2=1.49, p > 0.05
UALU1077	PET	113	116	99	Yes: χ^2=0.58, p > 0.05
UALU1086	NED	114	111	95	Yes: χ^2=2.20, p > 0.05
UALU1088	PET	116	120	82	Yes: χ^2=5.10, p > 0.05
UALU1120	*6-FAM*	*111*	*107*	*97*	*No: χ^2=8.03, 0.05 > p > 0.01*
UALU1123	*VIC*	*84*	*81*	*44*	*No: χ^2=6.68, 0.05 > p > 0.01*
UALU1154	VIC	461	458	90	Yes: χ^2=5.73, p > 0.05
UALU1178	6-FAM	238	250	98	Yes: χ^2=1.82, p > 0.05
UALU1198	NED	298	292	37	Yes: χ^2=1.11, p > 0.05
UALU1199	*NED*	*101*	*105*	*78*	*No: χ^2=40.4, p < 0.001*
UALU1215	*PET*	*100*	*103*	*91*	*No: χ^2=94.9, p < 0.001*
UALU1223	PET	483	486	77	Yes: χ^2=6.38, p > 0.05
UALU1241	*NED*	*74*	*71*	*99*	*No: χ^2=9.46, 0.01 p > 0.001*
UALU2001	*VIC*	*159*	*175*	*97*	*No: χ^2=126.0, p < 0.001*
UALU2023	PET	117	111	92	Yes: χ^2=0.35, p > 0.05

SSR Markers					
Marker	**Dye**	**CDC Bethune Allele (bp)**	**Bolley Golden Allele (bp)**	***N***	**Allele Distribution Fits 1:2:1 Ratio? (*df = 2*)**
UALU2025	*NED*	*115*	*112*	*97*	*No: χ^2=9.33, 0.01 > p > 0.001*
UALU2031	PET	112	116	86	Yes: χ^2=4.49, p > 0.05

Phenotypic Markers				
Trait	**CDC Bethune Characteristic**	**Bolley Golden Characteristic**	***N***	**Allele Distribution Fits 3:1 Ratio? (*df = 1*)**
Flower Colour (*C''*)	Purple (dominant)	White (recessive)	85	Yes: χ^2=0.19, p > 0.05
F_3 Seed Colour (*D)*	Brown (dominant)	Golden (recessive)	54	Yes: χ^2=0.22, p > 0.05

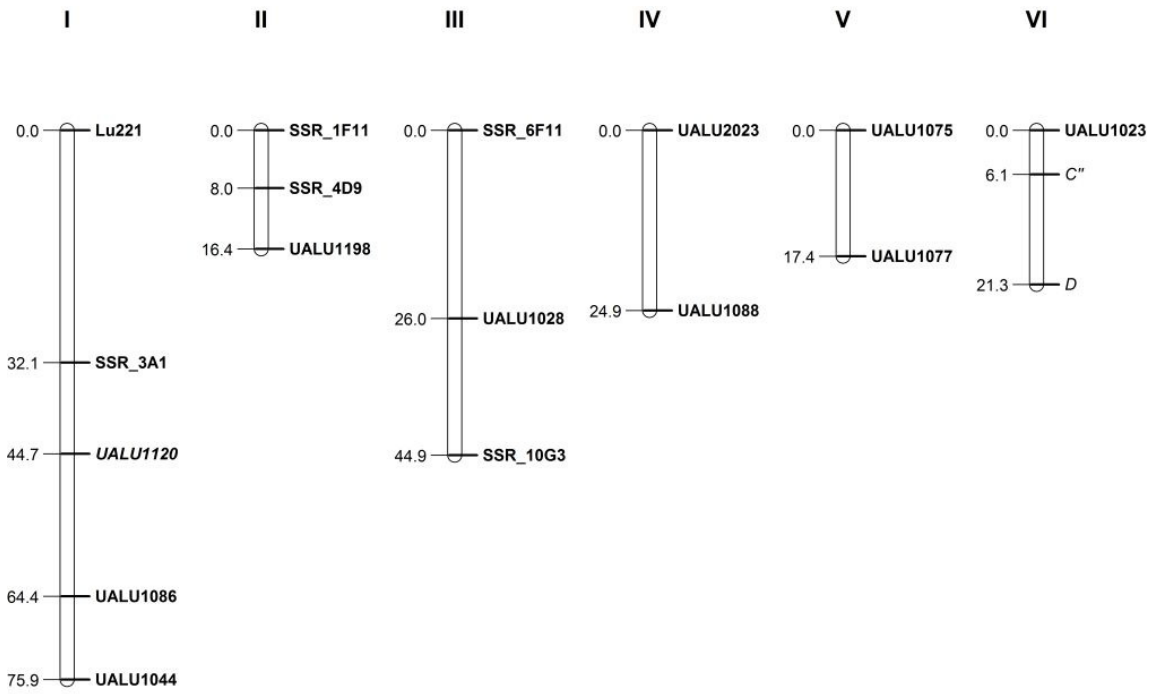

Figure 6-1. Linkage map for flax, based on 16 polymorphic SSR markers. The map was based on 99 F_2 progeny from a cross between the CDC Bethune and Bolley Golden linseed cultivars. Mapping data was also obtained for 20 additional SSR markers, but none of these loci could be mapped into any of the linkage groups. The value at the base of each linkage group refers to its relative value in centimorgans (cM). Italicized markers on the linkage map failed to fit the predicted 1:2:1 genotypic ratios, according to the Chi-square (χ^2) test.

Table 6-4. Allelic diversity attributes of SSR markers. Markers were genotyped on DNA from 24 *Linum usitatissimum* cultivars and 4 *Linum* species. *N* = number of *L. usitatissimum* cultivars genotyped; Ho = observed heterozygosity; H_E = expected heterozygosity; HWE = Hardy-Weinberg Equilibrium; N_A = number of alleles; L.c. = *Linum corymbiferum;* L.d. = *Linum decumbens*; L.l. = *Linum lewisii*; L.p. = *Linum perenne*. * denotes that a marker fit HWE (α = 0.002 after the Bonferroni correction).

Marker	**Amplicon Size Range (bp)**	***N***	**Ho**	**H_E**	**HWE *p*-value**	**N_A**	**Amplification from other *Linum* species (Y = yes, N = no)**			
							L.c.	**L.d.**	**L.l**	**L.p**
SSR_1F11	102-118	20	0.35	0.48	0.024*	3	Y	N	N	Y
SSR_4D9	102-122	24	0	0.16	0.001	3	Y	N	N	N
SSR_5B6	108-128	23	0.17	0.71	0.000	6	Y	N	N	N
SSR_6E7	107-115	22	0.14	0.46	0.000	5	Y	N	N	N
SSR_6F11	133-140	15	0.07	0.60	0.000	3	Y	Y	Y	Y
SSR_10G3	119-121	23	0	0.29	0.000	2	Y	N	Y	N
UALU1001	160-192	21	0.05	0.74	0.000	6	N	N	N	N
UALU1023	122-134	15	0.13	0.67	0.000	5	Y	N	N	N
UALU1028	117-123	23	0	0.51	0.000	2	Y	N	N	N
UALU1031	118-121	24	0	0.08	0.021*	2	Y	N	N	N
UALU1044	108-112	22	0.27	0.47	0.008*	3	Y	Y	N	N
UALU1075	108-112	18	0	0.41	0.000	2	Y	Y	Y	N
UALU1077	113-119	22	0.86	0.54	0.001	3	Y	Y	Y	Y
UALU1086	99-114	22	0.05	0.61	0.000	4	Y	Y	Y	Y
UALU1088	116-120	23	0.17	0.49	0.003*	2	Y	Y	Y	Y
UALU1120	107-111	20	0.55	0.50	0.053*	4	Y	N	N	N

Marker	Amplicon Size Range (bp)	*N*	Ho	H_E	HWE *p*-value	N_A	Amplification from other *Linum* species (Y = yes, N = no)			
							L.c.	L.d.	L.l	L.p
UALU1198	293-299	13	0	0.39	0.000	3	N	N	N	N
UALU1223	483-486	21	0	0.50	0.000	2	Y	N	N	N
UALU2001	159-178	22	0.14	0.68	0.000	5	Y	N	N	N
UALU2023	111-117	23	0.09	0.54	0.000	3	N	N	N	N
UALU2025	109-115	22	0	0.55	0.000	3	Y	N	N	N
UALU2031	112-116	22	0	0.58	0.000	2	Y	N	N	N

Table 6-A1. Primer sequences and repeat motifs for all 299 novel SSR markers that were developed and evaluated in this study. The majority of the markers in this collection were found to be monomorphic between the CDC Bethune and Bolley Golden cultivars and were not tested further, nor were they evaluated on other flax germplasm. Markers labelled in bold were found to be polymorphic between CDC Bethune and Bolley Golden.

Marker	SSR Motif	L primer (5' to 3')	R primer (5' to 3')	Predicted Amplicon Length (bp)
UALU1000	GA(19)	GGTCACTCCTTCCATACATTCA	ATCAGCAATCAATCGCATCA	180
UALU1001	**AT(9)**	**TCCAAACACTCTCGTTTATTTATTTTT**	**TGATCCTAAGATATCGCCAACA**	**150**
UALU1002	CTT(6)	TCCAAGTTCCCAGTACATTTACAA	AGTTTCGCTATTCCGTCTGC	150
UALU1003	AGA(6)	CTATCGATACTTTGACTAAAAGGGA	GTGGATCCCGAGAGAGGAA	149
UALU1004	TA(9)	AACTAATACTCCAACAAAATAACCATT	TCGCCTCCTCCTCTCTGTAA	149
UALU1005	TAT(11)	AAACTTTGATTTGAATAGCGGG	CAATGGTTTGTAAAAGCATCTCA	147
UALU1006	CTT(10)	CCTACAAACTAAAACGGGACCA	GGAAGGAAACGAAAGCACAA	146
UALU1007	TTA(4), TAT(4), AAG(6)	GCACATGAGAAAAGGCAGAA	CACGTGCCATATGGTAGAAATG	145
UALU1008	ACA(7)	TCAAAAGCACAACCCACAAG	TTGCAGTTGGTCTTTTCTGG	144
UALU1009	CT(14)	AACCTGAACCAGACGAGCAT	TGAGATGGATGTGTTTTAAGTTAATG	144
UALU1010	CT(9)	CAAAACCCACCAACCAAAAG	ATGCAGTTTGTGTCGAGCTG	141
UALU1011	GCT(6)	GGAAATTTGCCTCCAAATCA	ACCTTAGCCTCTGGCCTTTT	141
UALU1012	TCT(9)	GTTCGTTTGCTCGTTTCCAT	AGGATTAGACGGGGAGCTTG	141
UALU1013	CTG(6)	TGCTGCTGTCCGTACATACC	CGAGCAGCTGGTGGTTATATG	140
UALU1014	TTC(6)	TCTTTCTGATGATCCTCATCCAT	AGCAAAGAATCCGAAGACGA	135
UALU1015	CTT(6)	CCTGACACCGAAAAGCTGA	CGCAGTACTCCTTTTCACCA	130
UALU1016	CTT(10), CTT(6)	GGTAGAGAGGGAAGAAGTAGCAAA	TGAAGAAGTTGAAGAAGAAGAAGAA	130
UALU1017	AAAC(5)	CAAAATGCTCCTCCGATAGA	CTCTTGGGGTGGTGGAAGTA	129
UALU1018	CA(9)	GCCACTGGGAATCATTCATAC	TGCTGTCGGCTTCTTGAATA	126
UALU1019	AAG(8)	CGGCTCCCTGAATCCTTAC	TTGTTGGGGATGAATCTCGT	126

Marker	SSR Motif	L primer (5' to 3')	R primer (5' to 3')	Predicted Amplicon Length (bp)
UALU1020	GCT(6)	CAGACCAAACAAAAGTTGAAAGAA	CAAAAGGTCCTACGGGTGTT	125
UALU1021	TCC(6)	TGCATTCATTTCTATCTTTGATGA	GAGAAGGAGGATGAGGAGGG	124
UALU1022	TTC(9)	CCACCATCCAACTGGAAAAT	AAGGGATGAAGGGCACTGTA	124
UALU1023	**AGA(4), AGA(8), GCT(4), AATTAG(3)**	**GAAACTCAGTGAATCACCGCT**	**GTAGCAGCAGCAGCTTCCC**	**123**
UALU1024	TC(9)	TGGGTATCAAACAAAATCCGA	CTCCACGCAAAAAGAAATCC	117
UALU1025	ACC(6)	GCAAAACCAGAGGCGTCA	AACTTGTCCCGCGAGATTC	114
UALU1026	TCT(7), CTT(5)	GGATTGTGCAAATTAGGAGGA	GAAAGAAGAAAGAAGAAGAAGAAGGA	114
UALU1027	TCT(13), TTC(4)	CCCAGTACTCCTCATCCTCCT	GATGCTGAATTGAGTGGGGA	114
UALU1028	**AGC(8)**	**CTTCCATGTATTTTGCGATCA**	**GGAAAGCCCAGTCTGAACCT**	**112**
UALU1029	CTG(6)	GCAAAGGAGCTTTCGTTCC	AGATTCAATCACCCCGTCG	111
UALU1030	**TCT(7)**	**TCGATTAGAGATTCGCAGCA**	**ACGAATGAACGAAATGAGCC**	**109**
UALU1031	**TAG(10)**	**GCTGCTGCACCATTCCTC**	**TTGAGCATTGGCACTCAAAG**	**108**
UALU1032	TCC(6)	CCTTCTTGCCTTTCCCATC	ATGAACGACGGCAACGTG	100
UALU1033	GTG(6)	AGGAGGCAAGGCCAAAGT	TCCTTATCAACAACACGCCA	100
UALU1034	ACC(6)	CAAATCCTTCCCCATTCCA	GGCGATGGAAGTTTTGGAT	100
UALU1035	AAG(6)	CCTTACATTTCACGCACAAGA	TTGGGGATAAATCTCGTCAGTT	100
UALU1036	TAC(6)	GAGACCATAGCCAGGCATTT	CTTGACAGGCTGGCTGGTTA	100
UALU1037	AG(3), GAA(3), TCCG(2)	CAGCTGCAGCCATTGAAG	ATGCATATCTCATTATCTATTCCTCTT	100
UALU1038	TTC(9), CTT(6)	TTCTTCTTCTTCTTCTTCTTCTT	GATCCTTTCATGCACCTAAAAT	100
UALU1039	GAT(6)	TGCACAAACACAAATCTCCAA	TTCGCAAGTGGATGATACG	100
UALU1040	AAG(6)	TGAAAGGAAATGCAGGATGA	AGGGTTCTGGGATTTGCATC	100
UALU1041	GTA(6)	TCATCATTAAGTCCAAGCAACTTT	TGATAGGGGAGGATAACCAGA	100
UALU1042	TC(9)	CAAGGGAGTGCTGCTCTGAT	CAGTTGCAGAAATGGAAGCA	100

Marker	SSR Motif	L primer (5' to 3')	R primer (5' to 3')	Predicted Amplicon Length (bp)
UALU1043	AAG(6), AAT(4)	CCATTTTTCATTCATTCCAACA	CCAAAGACCCTCATCATCATC	100
UALU1044	**GAA(6)**	**GGAAGAAGAAGACGACGAAGAA**	**ACTACAATGTCCGTTTCGGC**	**100**
UALU1045	GAA(6)	ACCTGCTAACTTGCATGGCT	AATCCAAACCGTCTGTTTCAG	100
UALU1046	GCT(6)	AGCCTTCTTGTTCGGGTTTC	TCAGGAAGAACCTGGCGA	100
UALU1047	CAA(6)	CATAACCATCGTTAATTCCCA	AAGGCTATCTCCGCTTTACTCA	100
UALU1048	AAGA(5)	GAAATGACAGCAAAACTAAAATAAAA	GCGATCTGATTAGGGCTACTAAA	100
UALU1049	AAAG(5)	AGAGAGTTTGGCGGTTTGC	ATGGACGGTTGACAGAAAGG	100
UALU1050	GCT(7)	AATCTTTCAACAAGCCTTTTTATTT	AGTGGATTCCCAGGCGTT	100
UALU1051	CTT(7)	AACCTTCTTGGTGCCTTCG	GCACCGATCCCATAAGCAG	100
UALU1052	TCA(7)	CAGTGGCCCTCATCATCG	GAGTTGGATAGCGAAGATAAAAGT	100
UALU1053	TCC(7), AATT(3)	GGGAATCAAAGTGCCTCTCTT	TGAGATGGTAATTTTTGAGGATGA	100
UALU1054	TCT(7)	GAAAACCAGAAGAAATCAACAAA	CAATTGCTTTTGCTGTGGC	100
UALU1055	AGT(5), AGT(7)	GCAAACTTGAATTCTGCAATAA	GTTGGTGAAACAATCGTGGG	100
UALU1056	TTC(7)	TCCATGCCAACTTGTTACATTT	AACGCACATAAGCTAGAAAGGTT	100
UALU1057	TTC(7)	CCTCCTAACAAACAGCCCCT	TGGACCAAAAGAACACCAAG	100
UALU1058	CTT(7)	CCACGCACCATTATTTTCTG	ATCGTGATCGAAGGAGCAG	100
UALU1059	ATC(7)	TGATGATGGGATGTTGATGA	CAGCTGAAGCAGAAGATAGTGAA	100
UALU1060	AGT(7)	TGACTTCGACTCTGGCTCTG	GGCACTATCAACCACCACAA	100
UALU1061	GA(11)	AGGCCTTTCCCTTTAAACCA	GGTTGCTGCAAGGAATTGTAA	100
UALU1062	TC(11)	TTGCTCAGTGGGATATAGAACA	CAAACAGAACAAAGTAGGAGAGGA	100
UALU1063	**TC(11)**	**TTTCAATAGTGCTAAAGTGGTAGAGAA**	**TGAATTTCTTCACCCTCTATATTCC**	**100**
UALU1064	AAT(8), AGAGC(3)	GCATCCTTTTTCTTTTATGTTGG	TGCTCTGCTCTGCTCTTTTAGA	100
UALU1065	TCC(4), CTT(8)	TCTTCTTCCTCCTCCTCCTC	CTCAGGCTCCGGCTTACC	100
UALU1066	TCA(9)	GGTCATGCTGCTGAAATAAAAA	CGTGGGCTATGAAATACCATGT	100
UALU1067	AGT(9)	TGGCAGTGTTTAAAAAGCAA	CCATGGGGACCAACCATAAT	100

Marker	SSR Motif	L primer (5' to 3')	R primer (5' to 3')	Predicted Amplicon Length (bp)
UALU1068	AAG(9), AGA(4)	CCTCTTTGTTTCATCCCAAAA	CGTGTTGGTGTCTTCTTCTTCTC	100
UALU1069	GTT(6)	TGATGATCACCGCTTCTTGA	CGGGAATGAGAGAAACGAGA	99
UALU1070	AGA(6), ACA(4)	CGGTTTTGCAGAAGATTAAACA	TTTGCGACTGTTGTTGTTGT	99
UALU1071	GTT(6)	CGATGCTTTCAGCGATTTCT	GCCTGATACCCTTGTTGTTGA	99
UALU1072	CTT(6)	GAAAGGGGAATCTCTGCAAC	GGGGGAAGAAGAGGAGTTTTT	99
UALU1073	ATG(6)	AAAGATACAACAGCAACAACATCA	GAGCTCCTTTTGTTTTGCCC	99
UALU1074	AGAA(5)	TTATTGAGGAATGGCAAAAGC	AACTTCTCAGCGCTTCCAAC	99
UALU1075	**CATC(5)**	**AAATCCTTTCAAATTTAATACAACAGA**	**AGTGGGAGTTACGCTGTTTG**	**99**
UALU1076	GGA(7)	CTCGGTTTCCTCAAATCCAA	GTTTCTCCGTCGATTCTTGC	99
UALU1077	**ACA(7)**	**CATCACAAACTGACACACCCA**	**TTTAACGACATGGAATCCCTA**	**99**
UALU1078	AGA(7)	GGAGATACTGCGGCGACC	AGCGAAGCTGATGAGAAGAGA	99
UALU1079	GCT(7)	TGTTTCTCAGCTTTCCGACC	AGTGGCTGTGATGTGGATGT	99
UALU1080	TCC(7)	GCCAAGAACATGATCAAACG	GACGGTGGAAGAATGGCTC	99
UALU1081	CCA(7)	CAAACAACGTCTCCTACAGTAATTTT	TACTCTAGTAGTTGCCACTTGACAG	99
UALU1082	CT(11)	GGCCCTTCTCTCTTCTTCCT	TTGATTCAATTTGACCCCAAC	99
UALU1083	AAAG(6)	TGAACTATATCTTCGCTTCCTCAA	TTCGGATCTGACCTAATGATACTT	99
UALU1084	AGG(8)	GCCAGCAGCTACAACCTCTC	TTACCAAGGAGACTCGTCCG	99
UALU1085	CTG(9)	TGTGGTCATCGTCAACTCTCA	GATGGCATTTCTGGTCACAG	99
UALU1086	**GAT(9)**	**GGAGAATGATGGAAGGTGATG**	**TGGTGGGTTTAATTTAATTGTCTG**	**99**
UALU1087	TCT(6)	CTCCTCCTGCCATCTCTCTC	CAATCAGTGAAGCGGAAGAAG	98
UALU1088	**CTT(6)**	**CGACATTTTCTTCCCCTTCA**	**AAGAAAATCAACCCGAGGCT**	**98**
UALU1089	GAG(4), GTG(6)	TGGAGGGGATCGACTGAA	GAGCCCTCGTGGTAGGAAGT	98
UALU1090	TCA(6)	TGCTGTTCTGGCTACCACTTT	CCAACCATTGAGAAGGATTCA	98
UALU1091	TGA(6)	GTCATGCCCTTTCTTCCAAA	AGTCATGGTCACGTTCACGA	98
UALU1092	ATC(6)	AAGCAATTGTCTATCTGATTCGG	TGAGGCTACAAAAATTCTCAACA	98

Marker	SSR Motif	L primer (5' to 3')	R primer (5' to 3')	Predicted Amplicon Length (bp)
UALU1093	CTG(6)	TGCTGTCTTTGTCTTTCCTGG	GATGTGTACGATTCCAGTCCC	98
UALU1094	GAT(6)	GGCAAAACCAGTAGTAGTTACTTCAAA	GGCCAAGGCTTTTGTATGTC	98
UALU1095	AC(9)	GCTAACAGTAGCAGCACCAAGT	TGTTTGTTTCTCGCTTTCTCTT	98
UALU1096	CGG(6)	GACGGCGACAGAGGTGAA	GGAGTTCGGGACTCAAAACA	98
UALU1097	CAT(6)	TCCAACTTTATTATCTGACGATCAC	TTCAAAAATGAGGATGGAAGAGA	98
UALU1098	GAG(6)	TCGAGTTCGAACGGCTCTTA	CGTTCATGAGGACGACAATG	98
UALU1099	GAA(6)	TGATATTTTCCACCGGGAAG	AAAGGCATCCCCTTTATGCT	98
UALU1100	TA(9)	CAGTCCCTTTCCTAACTAGCTTT	AAAGTCGGTTTGCGGTAGTG	98
UALU1101	TTTC(5)	AGATTCAGCGGCCAAAAGT	CCCATCAACGAAAAGGAAAA	98
UALU1102	CAT(7), AGAA(3)	CATCATCTCCCTTTCCCAAA	TTTTTCTTTCTTTCTCTTTGGATGA	98
UALU1103	TTA(7), ATA(4)	AAGAACAACCATCTTCATCTTCC	TCATCCAGCTCGAATGTAGG	98
UALU1104	TTC(7)	CCTTTTCACGCAAAGGCTAC	TCCAAGGAAGAGCTGGAAGA	98
UALU1105	TA(11)	AAATTAAAGGGCTGGCAACA	AGGGTTCTTTTGGCTTTGCT	98
UALU1106	ATG(8)	TCCAGCAGCTTCAAATGTCA	TTCATGGACAAGCTCCCTCT	98
UALU1107	TTC(6)	TTCATATTCCAGCGACTGATG	TCATCAGGAAGAAGGCCG	97
UALU1108	GAA(6)	GCTGAAGTTTTATACATGATACGAGAA	TGAAATACTACGGAGGTTCTTGC	97
UALU1109	CTC(6)	TCCTCCACTGAAACCTTCTGA	CAAAGATGACGAGGGAGGAG	97
UALU1110	TGC(6)	TTGGAGCAAATCCCTATACGA	CGAGGCTGGTGGTTGATATT	97
UALU1111	TCC(6)	TGATCATCCATCCAGTTCCA	AGGAGGATGAGGAGGGGAC	97
UALU1112	CAG(6)	CACACTTCGGACGAAAGTTAC	TCCATACTCTGGCATCAATTTTT	97
UALU1113	AGC(6)	CTGACGGTCCTGCATTCTC	TGGGGAAATACATGTGGAGG	97
UALU1114	ATG(3)	GGCATGACACTACCGCAAC	TCCCTCCTATTTTGCTTTGC	97
UALU1115	TC(10)	AAACTAACAACTCTTCCTGTTTTTATC	TTAGGCTTAATAATTGTGTACATAGCA	97
UALU1116	AAG(7)	GAATTACATCAAACCCCCAAAA	CAATGGGTGTATTTATGTTGACTTG	97
UALU1117	GA(11)	CGACCACTGTAGTACCACAAAGTTTA	TGAGAGGGTTTTTGTATTTCGG	97

Marker	SSR Motif	L primer (5' to 3')	R primer (5' to 3')	Predicted Amplicon Length (bp)
UALU1118	CTC(8)	GAATGTTCGCCCTTCTCATC	GAAGCAATGCTGGAAGTCGT	97
UALU1119	AG(12)	GCTGGAGAAAAAGGCTAAGTG	GGCTCTCTCTTCCTGCCTT	97
UALU1120	**GA(12)**	**TGCTGCAATAAGTTAAGCTAGGG**	**CCGCACTTTCATCCAAGTCT**	**97**
UALU1121	CAG(13)	CATCCAGAATAGATGCACGG	AGACGGGAACTTACTTTGGG	97
UALU1122	TGC(6)	CAGAGCCCTCCACTACCAAG	TTGTTGGGTGGACAATGATG	96
UALU1123	**ATC(6)**	**GGACACTCCTTCCATCATCAA**	**TCCAATGAAGCAGTAAAAGGC**	**96**
UALU1124	AT(9)	TCCCCACAGAAGTCCAAGTT	TGTGCTTTGTGATTATTGGACC	96
UALU1125	GCA(6), CTT(6)	GCCATCTTCTTTCCCATTGT	CGACACAGAGAAGTCGGAGA	96
UALU1126	TA(10)	ATCGTTTGCTCTGGTTCCTG	TGGTTTGATTGATTTAAGTTGAAAGA	96
UALU1127	CTG(7)	AGCCCACGTATCTCTCCG	ATGGAACGAGACGAGAATCA	96
UALU1128	ATC(7)	TCCAAACCAAATGTGTGGAC	TGTGACGCTGTTCTATCTGGA	96
UALU1129	CTT(7)	TCCAGCCATCTTTCCCTATC	AGTAGAGGGCGACGAAAAGG	96
UALU1130	TTC(6)	TCGTCACCATTGTCAGAGTTG	GCTTCAAATGGTGTTGCTGA	95
UALU1131	ATT(6)	CACCAACAACAACGCCACTA	TTTGGTATGACAGCTTGGGA	95
UALU1132	CTT(6)	CCTCACCCTTCAAAACCCAT	AGCTTGTGGAAGAACATGGC	95
UALU1133	TCT(5), TCT(6), TTC(4)	CACGGCAGTGGATGGACA	GGAGAAGAAGAAGAACAAGGAGA	95
UALU1134	CAA(6)	TCACACACAAACACACATCTTCA	AGACTTGAGACAATGAGACTGCT	95
UALU1135	GCTG(5)	ACACAGTGGTCAACTTTCCG	CGACGGGTCTGTGCCTAATA	95
UALU1136	AGC(7)	CCCAACAAAACACAGCAAGA	AATATATATGAGAAAGCTGGAAACAGT	95
UALU1137	GGA(7)	GGCCACTCTTTTTCTTGCTG	AAGACGAGTGCCCATTCCTA	95
UALU1138	GCT(7)	TGCACCTTACCCATGGTTTC	GTTTCTCTCAGGTCCGCATC	95
UALU1139	CT(11),TC(5)	CATGGAATGGAAAGGAACAGA	TCTCTGAGAGGACTGCTGCT	95
UALU1140	ACA(9)	ACTTTCAAACACTGTCATCCCT	CCGCAGTGGTGAAGATTAAA	95
UALU1141	TCT(6)	GGAAAAACATCAACTAAAGAAAACG	CATGTTGAAGAACTCGGAGGA	94
UALU1142	CAT(6)	CCTCTCCTTCCCCTCTTAACAA	TGTGATTGGAAGCTGCTCAC	94

Marker	SSR Motif	L primer (5' to 3')	R primer (5' to 3')	Predicted Amplicon Length (bp)
UALU1143	TCT(6)	AGCAGCCTCCGGTGTTTAT	TCTCTGTTCACCGACATGGA	94
UALU1144	TAA(7)	TTCCGGAGCTCTTCTAAACAA	GGAAAGGGAACATCGAACAA	273
UALU1145	CTT(10)	GGGCGAATCACTAGCCCTAT	GAATCGGACGATTTTTGGAA	211
UALU1146	TCA(6), GCT(6)	CTAAAGAACACGGGGGATCA	GGCATTGCTACAAATTTAACCC	307
UALU1147	GCT(6)	GACGAGCTCCTCTCCACAG	GCAGTAACAACAGCAACAGCA	202
UALU1148	CTT(6)	CGTCTTCTCCTTAACCGGC	GCAGTAACAACAGCAACAGCA	202
UALU1149	CTT(7), TTTA(3)	CACACATTACATCCACAACCC	GCAGTAACAACAGCAACAGCA	202
UALU1150	TCT(7)	ACGACGACGTCATGCTTTC	TTCAACAAGCGGAGGAGAAT	206
UALU1151	CTT(8), TCT(6)	TCACATCAACCTTCTCTTCTTCTT	CGGAGGTGTTCTTTCCATGT	231
UALU1152	GCT(9)	GGCTAGATTTCAGGCAGTGG	ATCCTCCGGTTCATCATCAG	269
UALU1153	TCA(6)	TGCCTAAACCACCCACTTCT	ATTGAGTCTGCTGCCCAAAG	164
UALU1154	**TGC(6)**	**CATGTTGCGTTGAGATGACA**	**AGCAACAGGCACAACTTCG**	**200**
UALU1155	GCT(6)	TGTCTGAAAACCCAGAGGGA	AGCAACAGGCACAACTTCG	200
UALU1156	CAG(7), TCT(4)	TTATCATGATCCGACTGGGC	CCTCGTTGAAGTGGGAGAAG	238
UALU1157	TA(11)	GGAAAATTGGATACCGAACG	TTGGATCCCGAAGAGCATAC	331
UALU1158	CAA(8)	GGAATTGATACAATGTCGACCC	TTCTTGAACGGGAGGTATGG	285
UALU1159	TCCT(7)	AACTCGACGCTTTTGGGTTT	GGTCACCGGCTATGACTGTT	320
UALU1160	ACC(6)	CCTGGTAACTGCTCCCTCTC	ACTGCACAAACGACAAAGCA	200
UALU1161	CAT(6), TCC(4)	TCAGTGGGAAGGGAGAGTTG	CTATAACATGCGGGGTTGCT	152
UALU1162	GCTTC(2)	CCTCTTTGTTGGTTGGTGCT	CTATAACATGCGGGGTTGCT	152
UALU1163	CT(13)	AACAACCAAAAAGGGGGAAC	TTTGCTTGCATAAAACAGCG	333
UALU1164	TC(20)	ATTGTGTGGGTTTGGGTGAT	GCTGGATTCTTGTTGCATGA	292
UALU1165	ACCA(2)	AAAACCCTGCAACGACTGAT	GCTGGATTCTTGTTGCATGA	292
UALU1166	TCG(6)	TTTCTCCATTTCCATCAGGG	GCTGGATTCTTGTTGCATGA	292
UALU1167	CT(9)	TTCCAATCAAAATCAAACTAGCC	AAAGCTGCAATTACAGGGGA	261
UALU1168	AAC(9),	TCTCGAAGTAAAATGAGCACAAC	CCCATGTCTTGGTGGAGAGT	338

Marker	SSR Motif	L primer (5' to 3')	R primer (5' to 3')	Predicted Amplicon Length (bp)
	AACAAG(4), AACAAG(5)			
UALU1169	TCA(6), AGTGGT(3)	CAGCGAAAAACTCCTTCCAC	CATCATTTTCACCACCACCA	302
UALU1170	TC(10)	CACCTCCTCAAGTTTCTGCC	TCCAGGATACAGATGGGAGC	267
UALU1171	GGTT(5)	CAACGACCAGACTATTCCAACA	AGGATTGCACTGAGCCAGAT	303
UALU1172	TCA(7)	GATAACCGCCCTCGTCGTAT	TCAGAAGGCCAAAAATGTCC	205
UALU1173	GCT(7)	GAAGACGGATCACTTCTCCTG	GTCGGTTGGAGATCGAGAAG	217
UALU1174	GCT(6), CGA(4)	ACTTGACGGTGATGTTGCTG	GACAGTACCATGTCACCGGA	224
UALU1175	GCT(6)	CCGTTGATCTTCTGCTCGAC	GGGCATTGAGGTTCTCAAAA	228
UALU1176	ATC(6)	TATCGTGACGTCCGTTTTCA	GGGTAAAAGGAATTCCGAGG	134
UALU1177	GCA(6), TTC(4)	CTCTTATCAGCCTCGCCATC	GGGTAAAAGGAATTCCGAGG	134
UALU1178	**TAG(7)**	**GAACCAGGCCTTCTCCTCTG**	**GAACATAGAGGCCGGTACGA**	**221**
UALU1179	TTC(6)	CCAAATATTCTCCAGCCACG	AGGGAACACAATCTGCGAAA	202
UALU1180	TGC(6), TCA(4), CCT(4)	CATGTAAGGATATGGCCTGGA	AGGGAATGCCTGTGATGAAC	231
UALU1181	TC(10)	GTGTCCTGGTTGGGAGAAAA	TTGATTCCAGAAAGCGGAAG	120
UALU1182	CTTT(6)	AAAACACAACTGCCCTTTCC	TGTAACGGCTGTGGGATGTA	297
UALU1183	TTC(6)	TTCATTCCCACTTCCTCCAC	ACTTTCATGGTTGCCGAAAG	142
UALU1184	GGT(6)	GCCTCTACCCCTTGTAGGGA	TCGTTCTCTCCAACCACCTC	207
UALU1185	GCT(6)	CTGCGGCTATAGCTTCCAAC	GGACCATCTTCTTTCAGGCA	239
UALU1186	AG(10)	CATTTCAAGAAAGCATATGGCA	TCGACAATAACACAGGCTGC	250
UALU1187	GAT(7)	CAACAACAAAGACATCCATGC	ACGCGGTGAACGTTTCTATC	306
UALU1188	TTC(9)	GATGTGCCTCCCATCTCTTC	GGCTGGCTGCTGATTTATGT	276
UALU1189	GAA(9)	AACTGGGAGCAAGCAACAAG	GGCTGGCTGCTGATTTATGT	276
UALU1190	TAG(14)	GGTTGTTTGCCAACGGAGTA	GTGAAGGTCGGTGGAGTCAT	336
UALU1191	ATC(6), TCAA(4)	ACGAAGGGCACAATTTAACG	ACGCTTGCTTCTGGTTCACT	278

Marker	SSR Motif	L primer (5' to 3')	R primer (5' to 3')	Predicted Amplicon Length (bp)
UALU1192	CTG(6)	AAACCTCAAGCGCCCTTAC	GATCGTAGTGGTGGTGGCTT	259
UALU1193	ATC(6)	AGCAGCAGGAGTTGACCAAT	TGGGTATGGGAAGCAGTAGG	243
UALU1194	GAA(7)	AGCGGTTCAAGGTAAGCAAC	GGTGATGGCCAGATCAAACT	338
UALU1195	TCT(8)	TTCTTCTTCACCACATCACCA	CCCCTTACAAAGACGGTGAA	326
UALU1196	AGA(6), AGAA(3)	TCCTACCACCCTCCCACATA	ACAGCAGCAACAACAACTGC	350
UALU1197	TCT(7), TTC(4)	CATCCACATCCATCGTCTGA	CCATTGGAGGAGAAGGATGA	346
UALU1198	**TTC(8)**	**ATCATTTCCCAAACTTCCCC**	**CGACCAACGAGAGGGATCTA**	**286**
UALU1199	**TC(6),TC(13)**	**TGCTGAGTGCTCACCACTTT**	**AAAGCTTGAAAACTGGAATAGAAA**	**84**
UALU1200	TAC(6)	CACGAGTTTCAAAACAAACAACA	GACAGATTGGAGCTTGCACA	326
UALU1201	CAC(7)	CACCAGAAAAACTGCCAACA	GAATTTCTCATCCTTGCCCA	284
UALU1202	AAAG(6)	CCAAGGCTTTGAAAAAGGGT	TTGGCTTCAAGAAAGGGAGA	327
UALU1203	CTT(6)	GATTAACAAGTAAAACGGGGG	CGTTGCTTCCCTTGTACCTC	283
UALU1204	TTA(7), ATA(4), AGAC(5), AGGG(3)	CCCAGAAACAAATCAATCAAAA	CTAAGCCTCCAGCAAAGTGG	325
UALU1205	AGCT(6)	TCTTGAATCTGAGCGTTCCA	TGACTGGGATTTCTTCGACC	223
UALU1206	CTT(9)	TCCTCTTCAAAATCAACGGG	TGACTGGGATTTCTTCGACC	223
UALU1207	AAG(10)	TGAAAGAGTACCATATTCTAGTCGTAA	TGACTGGGATTTCTTCGACC	223
UALU1208	CAG(4), TTG(6), GTT(4), CTG(4)	AAGTAGCAACAGCAGCAGCA	TGACTGGGATTTCTTCGACC	223
UALU1209	TCT(6)	CTCTTTATCAGAGCGGTGGG	GCTCACTGTTGGCAGAGATG	219
UALU1210	TAT(6), CCT(4), CGC(4), GCAT(3)	CCAATTACCTTCAAGTGATGAACA	AGTGAAAGCTGCCGTCTCAT	297

Marker	SSR Motif	L primer (5' to 3')	R primer (5' to 3')	Predicted Amplicon Length (bp)
UALU1211	TGA(7)	CCCAAATGTCGTCTTCTTGA	CTACTCATTCTCCCCCACCA	264
UALU1212	TCT(7)	ATAATGCTCTCCCACCCTTG	AAGCCAATGCAGAAGGAGAA	281
UALU1213	TTTC(6)	AAATCTTGAACAACTTCCCCC	GGGATGATGAATTGTGGGAC	215
UALU1214	TTC(3), TTC(3), CAT(3)	TTCACTTCATCGTCACCGTC	AGAGGAAGTGGAGGAAAAGGA	81
UALU1215	**CCG(6)**	**CGTCGGTTTCTGGTTCAGTT**	**CCGGGAAAGAGGTCAATGTA**	**226**
UALU1216	CAG(6)	CAGTTTGTATGGAGAGCCGC	CCGGGAAAGAGGTCAATGTA	226
UALU1217	TTA(6)	CCCTTCCCCTCATTCTCACT	ATGTACTGGGCTAACGCCTG	238
UALU1218	TCT(6)	AGGTAGACTACACCTTCGCCA	GAGCAGCCCTCAAATACAGC	276
UALU1219	CCT(6)	TTGCTCTCCATCATGCAAAC	AGCTTGGACTCGAACAAGGA	228
UALU1220	AGC(6)	TTGCAATAGGAAAAGCTCACA	GTGCAAGTCAGCGATTACGA	245
UALU1221	AAAC(7), ACAA(3), ATCC(4)	AGACAAGCAAAACCCCACAC	CCTCTGGCTACACTTGCTCC	228
UALU1222	TC(9)	TCACCAACCACCTCATGACT	AGTCAGCTTTCAGGGGTCAA	275
UALU1223	**CAA(6)**	**TTTCTCCCAACAAAATACCCC**	**AATGATTTCATCGGCGACTC**	**279**
UALU1224	GA(3), ACC(3), TTCTT(2)	CCTTATTTTCTGCTGAGCCC	AATGATTTCATCGGCGACTC	279
UALU1225	AGT(6), TCT(5)	GCCGAAATCCGTATGGTAAG	CGAAGGATTAGACGTGGAGC	201
UALU1226	AGT(7)	TGTTGCTGCAGACACCGT	CAACCTTAGTTACCCCGCC	287
UALU1227	TCG(4), GTC(8), TCG(4)	TCCTTGTTCTTCGTCGTCGT	AGACGAAGATGATGCCGC	211
UALU1228	CCT(6), TCT(5)	GTGAGAGGGGACTCTGCTTG	GAAGCTGAATGGCTAAAGCG	307
UALU1229	TTC(4)	CCATCTTCTTTCCCATTGTCA	GAAGCTGAATGGCTAAAGCG	307
UALU1230	GCG(6), GGA(4)	TAGGCGGCAGAATACTGGAC	TTCCTTGTCCTTCTTCAATCC	152
UALU1231	TTC(6),	TCATCATCCTTTGTTTAATCCTTG	TTCCTTGTCCTTCTTCAATCC	152

Marker	SSR Motif	L primer (5' to 3')	R primer (5' to 3')	Predicted Amplicon Length (bp)
	TTC(6)			
UALU1232	CCT(6)	ACTTCAACCGGATTCTTCCC	GGTTGCGTTGGGTTGATTAT	254
UALU1233	GAT(9)	AAATCTGGTACGAGAACATCAGG	TGTGTCGACCATTCCAAAGA	231
UALU1234	TTC(6)	TCGCTCTCTTCATCCGAATC	CGAAGTTGATCCCGAGATGG	125
UALU1235	CTT(8)	AGTTCAGTGATCTTTGGGGG	ACGAGCAAGTTGGAAGAGGA	207
UALU1236	TC(11)	AGCTCATCACATGGGGTTTC	CATGTTTGGTGGTCTTGGTG	345
UALU1237	GAG(6), AGG(4)	GGATTGTTAAACGACGGAGG	TGTCTTTTGATTGCTGCTGG	302
UALU1238	GTA(6)	TTGTTCATAATATTGGACTTCAAAGG	AATCGTGTCGGTGATTGGTT	282
UALU1239	ACG(6)	GCCTTTTTCCCGGAGGATA	ACAGTCCTCCTCCGATTCCT	331
UALU1240	TCT(6)	CCCTGATCATTTCCATGCTT	TCTCCAAATGAGGAGGAGGA	63
UALU1241	**CAA(7)**	**CCTCCAACATAAAAGGAGGC**	**TTGTTTTGAAAGAATGGTCGG**	**63**
UALU1242	GGA(6)	GAGCCAGTTGGATTGGAGAC	CTTCTCCGGCTTCCGATCT	60
UALU1243	CTT(6)	CAGCAGCCTCCTTCTCTAGC	CGACACAGAGAAGTCGGAGA	57
UALU2000	TC(9)	TCGTACAAAGACACTGCTAGGTG	TGCTGCTCTGCTACCAATTTT	69
UALU2001	**CT(14), ATT(5)**	**AACATACATAACATTCCATCAGCC**	**TCGTACAAAGACACTGCTAGGTG**	**141**
UALU2002	CTA(9)	TTCGTGGGATTTCATATTTTCC	GGAAGTGAAGTACTAGAGAATGACCA	100
UALU2003	AGC(6)	CACGAAAGAAAGCAGCTTGA	TCGTTGCTGTTTGCAACTTC	97
UALU2004	CT(10)	AACCCAGAAATCAATTCACTCAA	TGAGGCTGAGAGTTCACACAC	98
UALU2005	CTT(7)	TTATACAAAATGAAGCAAGCCC	CCAGGAAAGCTGGAAATAGG	97
UALU2006	CGC(6), AATT(3)	GACCAGTAATAATTAAATGGGGACA	TTCCGACACTTTCCAACTCC	92
UALU2007	TCA(6)	GTCCGTCCATCTTCTTCCAC	AGCTGCATCGAATGGATTG	73
UALU2008	GGA(6)	AGCTGTGAAGAGTCCTCCGA	CCGGAAAGAGAACAGGGAC	80
UALU2009	TCA(6)	CTTCCTTGTGGGAAGCTTTG	GCTGCTCGTGAGAAGAAAGTG	90
UALU2010	CTC(5), CTT(6)	GGGAGGATCTACTGCTGCTG	TTTCCTTTTCATCATCCCCA	96
UALU2011	TACA(5)	ATTCACAAAACCGAGTTCCG	TGTGTTCCTTTTTGGCTTCC	69

Marker	SSR Motif	L primer (5' to 3')	R primer (5' to 3')	Predicted Amplicon Length (bp)
UALU2012	TTTC(5)	CAGCCAAAAAGCCAACATCT	AGAGTGTATTGCGCAGGAAG	96
UALU2013	AGC(6)	TGGCATTTCTTGCCATTACA	TGCATCATTCATTAGTGTGCCT	90
UALU2014	TA(9)	TCAACCACCGATTTTGTAGGA	GGACTGGCTTCACTTAACTGCT	98
UALU2015	GCG(6)	GGCTGTGGTGGATAGGAAGA	CACCTTCTACGGCTCTCCAC	86
UALU2016	TCA(6)	ATTTCGACCTTCCACATCCA	AAGTTTCCCTTCCCGAAGTT	98
UALU2017	ACA(8)	GGGACTTGCTTCTTTCCAATC	CTCCATTGCTAGAAACAGGGA	123
UALU2018	CTT(11)	CCATTACAACACCAACCTACCA	ACGACAAACAAGGCTCCAGT	94
UALU2019	TTA(6)	CATTACACATTTCCCCTTCCC	GCGGGGAAATAAGAACGGTA	88
UALU2020	CAT(7)	CAACATCAACAAATGGCACAG	TTGGGTCACAGTCTTCATGG	98
UALU2021	TGA(6), GTTT(4)	TGGTGGAATTTGTTGTCTTTCTT	TGAAACTGTCCCTCCTGTTCTT	100
UALU2022	GA(12)	CCCAGAAAATGAGGTCAACAA	CAACTCCAAAATCCAATCAACTT	100
UALU2023	**CTT(8)**	**ACATGAAGATGAAGCCACCC**	**TTCACAGAATCCATTGCCAG**	**99**
UALU2024	CTA(6)	CCCTTGGTCGACTTTCCTTT	AACAGCAGGCAACTGGAGAT	86
UALU2025	**TTC(8)**	**CCCAACAAAACTCCAGAGTCA**	**AATTCTTCTCTGTTGGCGCA**	**100**
UALU2026	CGG(5), GAA(6)	TAGGCCAGCTACCAGAAGGA	GTTTGAGTCGTCGTCGTTTG	83
UALU2027	TCA(6)	CGAAAACCCTCTTCACCAGA	TGCCTTAGCTCGGAGACTTC	99
UALU2028	CT(11),TA(7)	CATCAAACCACAACATCTCCC	TGAGGGGAGTTTCAGAATACA	97
UALU2029	GCA(6)	CAGCTTTATGGGCAGAAGGT	GGGAGTCGGGTAAGTCTAGTTTC	97
UALU2030	GGA(7)	ATATCCAATTGGGCAACAGC	CCCCTCCTCTTAGACAGCCT	94
UALU2031	**TA(9)**	**AACACACATACAGATGGGCG**	**TCGGGTTAGGGTATTTGAGTC**	**100**
UALU2032	TCA(6)	CGCTTTTTATGGGCTGGATA	ACGCACTCGAGCTTTTGTTC	82
UALU2033	TCT(8)	ATATTCCTCGACCTCCTCCG	GGAAGAAGTTGTTGAAGAAGAGG	85
UALU2034	GAA(8)	TGAAATAGAGTGAGCACATGAGTG	GGCCCATGAACTCCACTTAG	100
UALU2035	CTG(6)	CGAGCGATTTCGACTTTTTC	AGGAGCATTCACCAGAGCAT	99
SSR_1F11	**AGAC(6)**	**TCCGGATTGACTCTTCCTTC**	**AAGATTTCAGACTTTTCAGTGGTTT**	**102**
SSR_1F12	TG(11)	GAGGAGATTTGTTGCCAAAGA	ATCTGAAGTTTTCCAGGGCCTTT	100

Marker	SSR Motif	L primer (5' to 3')	R primer (5' to 3')	Predicted Amplicon Length (bp)
SSR_2B3	TCA(9)	TCTCCATTCTTCTTCCTCAAACA	GGGAGAAGATGGTGGTGATG	108
SSR_2D2	TC(17)	CATCCAACAACTTTTCGAGGA	GCCTCAGATAATCGAAGAATCA	101
SSR_3A1	**GT(11)**	**TTGGAATTTTGGAATCTGA**	**CCAACAAGAGCACATGGTCA**	**126**
SSR_3A10	GT(17)	TCCTTTGTTTGTCCTCCCTTA	CAAACTCCACACAACCAGCA	102
SSR_3B10	TGTC(7)	AAATAAGACTTCAGACTTTTCAGTGG	TTGACTCTTCCTTCCTCCGATG	100
SSR_3B5	TTAG(3), GACA(5)	CGAGATATATTGGGATGGATCA	TACTGGGGTTTCGTTTCCTG	138
SSR_4D9	**CAGA(7)**	**TCCGGATTGACTCTTCCTTC**	**GATTTCAGACTTTTCAGTGGTTT**	**100**
SSR_5B6	**TGTC(6)**	**TGAGTGGGTTAGTGGGATCTG**	**AAGCAGCACGAACCTGTTTT**	**106**
SSR_6C6	TC(5),CT(11)	TTTGACCAAGCATACACATACA	AAACAACACAACGCAACAGC	107
SSR_6E7	**ACA(8)**	**CACAGAAATGCCAGAAGAAAAG**	**CGTTTCTAACATGAGGGCGTTG**	**100**
SSR_6F11	**TC(13)**	**GACCATCGTCGTCATTTCCT**	**AATGCGAAAATCGCCAATAC**	**115**
SSR_7A2	GT(14),TG(5)	GCAATTCTTTTGTCCTCAAACA	CCGACGAGCCATGTGAAC	100
SSR_7C12	TCTT(6)	TGTTGATAACCTATCTGGTTCAGG	TGGTTGCGTTAACTAACAGAGA	103
SSR_7F2	GACA(5)	TTGGGATGGATCAATTCAGTT	CCTTTCATGCAAATGCTTTC	105
SSR_8F9	TC(18)	AACTCCCCTTCATCCTTTCT	TGATAGTATGATTTGGTTGGAAGG	104
SSR_10D10	GT(11)	TTTTGGAATCTGATTCGTTT	TTCACCACCTAAATCCCTAA	100
SSR_10G3	**AG(13)**	**GCAAGGAGATGGAAATGTTA**	**TGCCGTCTCTTCAATAAATAA**	**100**

7. Concluding Remarks

7.1 Hormonal Regulation of Flax Stem Properties

The first data chapter of this dissertation (Chapter 2) characterized the effects of GA and IAA on flax stem tissue properties. Two previous studies, by El-Shourbagny et al. (1995) and Ayala-Silva et al. (2005), have reported positive effects of GA_3 and IAA treatments on several properties of flax stems, including bast fibre abundance and tensile strength, but neither study determined whether these effects could be directly related to changes in the cellular morphology of the bast fibres that formed in these plants. By testing the effects of exogenously applying GA_3 and a GA biosynthesis inhibitor (paclobutrazol), I demonstrated that GA positively regulates stem elongation and xylogenesis. GA_3-treated plants showed an increased number of bast fibres in transverse-section. I proposed that this effect may be at least partly related to the moderate increase in bast fibre elongation that was also observed in the GA_3-treated plants. GA was also observed to positively regulate bast fibre cell wall expansion. Unlike GA, exogenous IAA treatments had more subtle effects on stem properties. The bast fibre cell walls in plants treated with GA_3 + IAA were significantly increased, suggesting a potential synergistic relationship between the two hormones. However, properties that have been observed to be affected by IAA in other plant species, such as the degree of secondary growth, were unaffected.

The second data chapter (Chapter 3) presented gene expression profiles for five genes, *LuGAST1, LuGA2ox1*, *LuGA3ox1*, *LuIAA1* and *LuPIN1*. *LuGAST1* is a putative member of the *GAST/GASA* gene family; the first member of this family

that was described – the tomato *SlGAST* gene – was identified due to its conspicuous GA responsiveness (Shi et al., 1992). By aligning the predicted amino acid sequence LuGAST1 to 13 AtGASA genes, I determined that the closest putative orthologue of LuGAST1 is AtGASA5. Zhang et al. (2009) determined that *AtGASA5* is negatively regulated by GA and acts as a potential negative regulator of cell elongation in the stem. Roach and Deyholos (2008) observed that the transcript abundance of *LuGAST1* increases in hypocotyls that have been sampled as bast fibres are undergoing secondary cell wall expansion relative to hypocotyls that have been sampled as fibres are elongating, suggesting that *LuGAST1* may have a role in bast fibre differentiation. In Chapter 3, it was reported that the further expression profiling of *LuGAST1* revealed that its transcript abundance substantially increases 28 days after planting (DAP) relative to 14 DAP, as the snap point forms on elongating flax stems, and that its transcript abundance was higher below the snap point relative to above the snap point in 28 DAP stems. Taken together, this data supports the observations made Roach and Deyholos (2008), suggesting that *LuGAST1* may act as a positive regulator of secondary cell wall expansion and/or a negative regulator of bast fibre elongation.

The expression profiling of *LuGA2ox1* and *LuGA3ox1* was initially pursued because both genes were expected to act as markers for GA responsiveness. Likewise, *LuIAA1* was profiled because Aux/IAA genes are markers for short-term IAA responsiveness. The transcript abundance of *LuGA3ox1* decreased at the stem apex shortly following GA_3 treatment, as expected, while the transcript abundance of *LuIAA1* increased at the stem apex

and midstem, as expected. Thus, experimental conditions were identified in which positive short-term responses to IAA can be documented, but not where positive short-term responses to GA_3 can be documented. However, as responses to GA are typically near saturation in wild-type backgrounds (Nemhauser et al., 2006), it may be necessary in future to either identify a GA-deficient flax mutant or to down-regulate the transcription of a component of the GA biosynthetic pathway (e.g. via RNAi) in order to adequately test GA-responsiveness.

As with GA_3-responsiveness, conclusive observations generally could not be made with respect to short term responses to PBZ. The only exception to this was *LuGAST1*, whose transcript abundance was observed to significantly increase 24 hours after PBZ treatment. This observation suggests that *LuGAST1* might be negatively regulated by GA; such a response would be consistent with the positive response to PBZ that was reported for *AtGASA5* by Zhang et al. (2009). It was presumed as the experiments were planned that a response to PBZ should occur relatively quickly. However, responses to PBZ are typically more delayed than responses to GA_3, as more time would be required for PBZ to inhibit GA biosynthesis, as well as time for GA catabolism to reduce the levels of bioactive GAs (Silverstone et al., 2001). Further measurements of the transcript abundance of all five genes 24-72 hours following the PBZ treatment might be required in the future in order to conclusively determine whether PBZ affects the transcription of these genes.

In plants treated repeatedly with GA_3, it was observed that the *LuGA3ox1* transcript abundance remained significantly decreased, even though the most

recent treatment was applied one week prior to sampling. This observation suggests that the negative feedback of GA_3 on GA biosynthesis continues to persist after treatment, and this effect might be explained by preliminary hormonal content profiling data which demonstrated a high GA_3 concentration in the stems of GA_3-treated plants. Conversely, whereas the transcript abundance of *LuIAA1* was significantly increased in short term IAA- relative to mock-treated plants, as has typically been observed for Aux/IAA genes, the transcript abundance of *LuIAA1* was determined to be significantly decreased in the long term IAA-treated plants. I proposed that this decreased transcript abundance may be consistent with a negative feedback of LuIAA1 on its own transcription that occurs as the IAA concentration in the treated plants declines to near-endogenous levels.

The last of the five genes examined, *LuPIN1*, putatively encodes an auxin efflux carrier. jörklund et al. (2007) determined that local GA_3 application to hybrid aspen (*Populus tremula x tremuloides*) stems weakly stimulated *PttPIN1* transcription, and they they proposed that this response serves as evidence for an increase in polar auxin transport in the stem following GA_3 application. Short-term responsivness of *LuPIN1* to GA_3 or PBZ was not reported in Chapter 2. However, *LuPIN1* transcript abundance did increase at the stem apex following IAA treatment, in accordance with observations from other studies. In stems treated over a long term period with GA_3, PBZ and IAA+GA_3, the transcript abundance of *LuPIN1* was decreased relative to mock-treated plants. I proposed that the discrepancy in the observations for *LuPIN1* relative to those by Björklund

et al. (2007) for *PttPIN1* might relate to the context under which the experiments were completed. Nonetheless, it was not apparent that the increased secondary growth in GA_3-treated plants can be explained by increased transcription of *LuPIN1*.

7.2 Mutational Analysis of Bast Fibre Development

The bast fibre mutant screening, described in Chapter 4, was a slow process, and the necessary follow-up work – such as backcrossing – took several years to complete. With respect to the fibre mutant screening strategy, the main method that was employed to identify potential mutants – snapping the mature plants apart and visually inspecting for any potential reduction in bast fibre content – did not prove reliable. Most potential mutants were identified following transverse sections of dwarfed plants that segregated among the M_2 families. However, it was difficult to determine during these early stages of the screen whether any potential phenotype represented an actual mutation, or was simply a consequence of an arrested development and/or premature death that followed abiotic or biotic stress. Furthermore, most of the dwarfed potential mutants were sterile, and thus in order to determine whether the mutant segregated into the M_3 generation the progeny from several siblings of the potential mutant had to be sown, in the hopes that at least one of these siblings was a heterozygote. Many potential mutants failed to segregate and had to be abandoned in the course of completing the screen.

As noted in Chapter 4, adjustments to the screening strategy may be useful in future in order to more efficiently identify fibre mutants. Chen et al. (1998)

reported that they identified several flax and *Arabidopsis* cell wall mutants by screening leaf tissue with Fourier transform infrared spectroscopy. An even simpler screening method was reported by Burk et al. (2001), who screened for *Arabidopsis* xylem fibre mutants using a tensiometer.

Nonetheless, the exciting outcome of all of this work was that two very interesting mutants were isolated: *distorted walls* and *reduced fibre1* (*rdf*). The *distorted walls* mutant appears to resemble members of the *irregular xylem* (*irx*) family of *Arabidopsis* mutants, demonstrating misshapen xylem cell walls. d*istorted walls* was demonstrated to segregate among the progeny of a single backcross with the CDC Bethune parental variety, but further work on *distorted walls* was not pursued.

The *reduced fibre1* (*rdf*) mutant appears to lacks normal numbers of fully differentiated bast fibres in its stem. As described in Chapter 5, the stem height and degree of xylogenesis in the *rdf* stem are unaffected by the mutation. However, bast and xylem fibre lengths were determined to be reduced in *rdf* stems. Based on observations that paclobutrazol treatment inhibited bast fibre elongation to a relatively equivalent degree as in *rdf* but affected fibre number in cross-section to a much lesser degree, it was concluded that the reduction in the number of differentiated fibres observed in cross-sections of *rdf* is not fully explained by the reduction in fibre length.

One possibility is that *RDF* function may be required for the establishment of bast fibre identity. Transverse sections through the stem apex revealed that the earliest stages of bast fibre differentiation were unaffected in the mutant, as the

patterning of phloem within the primary vascular bundles appeared normal. Below the snap point, longitudinal and transverse sections show that the bast fibres have a normal morphology, but simply form less frequently than normal. One possibility would be that some of the fibres elongated in *rdf* but simply fail to form secondary cell walls, thereby causing fewer fully differentiated fibres to be observed in cross-section. However, all of the fibres that were observed in the longitudinal sections appeared to have formed undergone normal secondary cell wall expansion, and therefore the above hypothesis was rejected. I proposed instead that *RDF* might trigger the elongation of the bast fibre initials; in *rdf*, the failure of some of these initials to elongate might cause them to remain as phloem parenchyma. As the bast and xylem fibres that did form were shorter in length than normal, in addition to triggering further bast fibre differentiation, *RDF* might positively regulate cell elongation.

Interestingly, the relative transcript abundance of *LuGAST1* was determined to be higher in the midstem of the *rdf* mutant relative to its parental variety, suggesting that *LuGAST1* may function downstream of *RDF* in the same genetic pathway. This observation suggests that *RDF* might negatively regulate *LuGAST1*, which in turn acts as a negative regulator of fibre elongation. As a consequence, the decreased fibre length in *rdf* might occur because *LuGAST1* transcript abundance increases to an abnormally high level.

7.3 Screening for Polymorphic SSR Markers and Linkage Mapping

The work describing the identification of simple sequence repeat (SSR) polymorphisms between CDC Bethune and Bolley Golden (Chapter 6) was

completed by mid-2008. The F_2 linkage map that was produced from this data was the first available linkage map for flax containing SSR marker data. However, that the map only provided limited coverage of the flax genome was very disappointing, as it made it difficult to publish this data. I proposed in the chapter that screening additional F_2 individuals might have allowed more of the markers to be placed on the map. Another possibility would have been to screen for more polymorphic markers so that the map could have been expanded and/or linked with pre-existing linkage maps based on other types of molecular markers. I considered screening for polymorphisms between CDC Bethune and Bolley Golden for the RAPD (Random Amplification of Polymorphic DNA) markers mapped in flax by Oh et al. (2000), and then converting polymorphic markers into potentially co-dominant SCAR (Sequence Characterized Amplified Regions) markers using a technique introduced by Paran and Michelmore (1993). We were aware that other groups were mapping SSRs in flax and that a denser SSR-based linkage map, ultimately published by Cloutier et al. (2010), was under development. Thus, identifying more SSRs or expanding the size of the F_2 population for mapping purposes was not pursued.

7.4 Proposed Future Research

The work presented in Chapter 2 revealed that GA has a role in bast fibre differentiation. It would be interesting in future to investigate how GA actually affects these properties. GA has been shown to regulate microtubule dynamics, while microtubules have been shown to reorient during the elongation of many types of cells, including flax bast fibres (van Lammeren et al., 2003), and to

regulate cellulose deposition into cell walls (Shibaoka, 1993). Thus, variations in GA levels might directly affect the structure of the cell wall and/or the elongation of the bast fibres. Arabinogalactan (AGP) proteins, which are enriched in flax bast fibres, have also been hypothesized to aid in establishing cellulose microfibril axial orientations (Hobson et al., 2010). A possible connection has been proposed in the literature between gibberellin signalling and the expression of AGPs (Suzuki et al., 2002; Masiguchi et al., 2008). It might be interesting to examine whether the expression of AGP genes is affected by GA.

The spatial and temporal expression patterns for *LuGAST1* that were described in Chapter 3 suggest a potential association of *LuGAST1* with later stages of bast fibre differentiation. A role for *LuGAST1* in bast fibre differentiation is further supported by the observation that its expression increased in the midstem of the *rdf* mutant relative to the parental variety. It would be very interesting to investigate whether the overexpression of *LuGAST1* and/or suppression of *LuGAST1* expression affects bast fibre properties, as this might also provide insight into how GA affects bast fibre differentiation. Transgenic *RNAi* and/or overexpression lines might be very useful for demonstrating whether *LuGAST1* has a specific role in bast fibre differentiation.

The cell walls of developing flax fibres have been observed to have a bipartite structure; transmission electron microscopy studies have demonstrated that the inner (Gn) layer is loosely-packed and heterogeneous, while the outer (G) layer is tightly-packed and homogeneous (Gorshkova et al., 2004). As secondary cell wall development proceeds, newly deposited Gn layers are gradually

converted in G-layers (Gorshkova et al., 2004). The Gn-layer is enriched in a tissue-specific galactan (Gorshkova et al., 2004), while β-galactosidase activity and the expression of genes encoding β-galactosidases increases at and below the snap point (Roach and Deyholos, 2007; Hotte and Deyholos, 2008; Roach and Deyholos, 2008; Roach et al., 2011). Bast fibres from transgenic lines in which the *LuBGAL1* gene had been downregulated using an RNAi construct were determined to show reduced β-galactosidase activity and to form a thinner G-layer in their cell wall (Roach et al., 2011). The tensile strength of the *LuBGAL1-RNAi* lines was also decreased, demonstrating that β-galactosidase activity is required to provide strength to the fibres (Roach et al., 2011). It would be interesting to determine whether *LuGAST1* and/or *RDF* regulate the expression of β-galactosidases.

In addition to *rdf*, several other potentially interesting mutants were identified in the mutant screen. Although there are potential pitfalls that need to be overcome – in particular its apparently low penetrance – I would expect that the further characterization of the *distorted walls* mutant might provide interesting insight into the genetic regulation of cell wall differentiation in flax. Likewise, several of the potential morphological mutants that were indirectly identified during the mutant screen may be useful. For example, several lines were identified with altered stem branching and/or giganticism. A primary difference between linseed and fibre flax varieties is that the former tend to be more branched while the latter tend to be less branched. Taller linseed mutants with less branching might potentially form longer fibres, potentially making these mutants

useful as dual-purpose fibre and linseed varieties. More branched mutants, conversely, may have an increased seed content, also potentially making them useful for breeders.

Finally, determining the molecular identity of *RDF* should prove very interesting, and thus cloning *RDF* should be given high priority. Advances in genomic sequencing technologies potentially allow a mutated genetic locus to be rapidly isolated through a whole-genome sequencing approach, whereby a sequence is obtained from a pooled population of homozygous F_2 individuals, following an outcross of the mutant with another variety. By comparing the sequence of the homozygous mutants to the sequences of each of the parental varieties, single nucleotide polymorphisms (SNPs) that distinguish the parental varieties can be isolated from SNPs that distinguish the mutant from both parental varieties, and the location of the latter would suggest the genomic position of the mutation (Lister et al., 2009). This approach might also be complemented by traditional map-based cloning approaches, whereby the *rdf* mutant is outcrossed to another variety and polymorphic molecular markers tested for potential in the F_2 generation for genetic linkage to *rdf*. I have obtained DNA samples from F_2 individuals showing the *rdf* phenotype. The samples were obtained from two separate crosses of *rdf* with the Bolley Golden linseed variety. In the near future, these individuals could be easily screened for linkage with the polymorphic markers described in Chapter 6. The identification of a linked SSR locus would then provide a molecular marker that could be screened against available bacterial artificial chromosome (BAC) libraries.

7.5 References

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