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Genetics of Seed Coat and Stem Development in Flax (*Linum usitatissimum* L.)

by

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Abstract

Flax (linseed, *Linum usitatissimum L*) is cultivated either for its seeds or its stem (bast fiber). With the goal of contributing to development of novel flax germplasm, a forward genetics screen of EMS mutagenized flax (var. CDC Bethune) was conducted to identify seeds with abnormal pigmentation or mucilage. Seeds of 16,764 M_4 families were examined visually, with and without staining by Toluidine Blue. A total of 86 putative seed color mutants were identified, with the most common phenotype being yellow seeds. There were 1,975 identified putative mucilage mutants, with a range of phenotypes, including Blue Seed, No Mucilage Released, and Abnormal Mucilage Released. A subset of the seed colors and mucilage phenotypes were tested for heritability. The seed colors were generally heritable, but the mucilage phenotypes appeared to show variable expressivity that is probably highly dependent on the environment. Histological analysis revealed how seed coat anatomy changes affected seed color, but did not explain the mucilage phenotypes tested. The lines have been provided to flax breeders other collaborators for further analysis and potential use in breeding programs.

GASA (Gibberellic Acid Stimulated in *Arabidopsis*) genes encode a family of small proteins of unknown biochemical function, whose expression has been correlated with various processes in plants including hormone response, defense, and development. GASA genes have also been reported in our lab to be expressed in developing flax stems. To better characterize the GASA-like gene family in flax, I identified 20 predicted GASA-like genes in the flax whole genome assembly. Using microarray and qRT-PCR analysis, seven of these genes showed differential transcript expression during stem

development. I cloned and verified the sequences of these genes. Based on these studies, I was able to define groups of flax GASA-like genes whose expression was well-correlated with stem segments in either the elongation or cell wall deposition stage. These results provide further evidence that specific GASA genes are involved in different processes in flax stem and/or fiber development.

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ALA	alpha-linolenic acid
ABA	abscisic acid
ap2	*apetala2*
BAC	bacterial artificial chromosome
BLAST	basic local alignment search tool
BuOH-HCl	n-butanol-HCl
cDNA	complimentary DNA
DNA	deoxyribonucleic acid
dcr	*defective in cuticular ridges*
ESTs	expressed sequence tags
EMS	Ethyl methane sulfonate
Fuc	Fucose
gl2-1	*glabra2*
GASA	Gibberellic Acid Stimulated in *Arabidopsis*
GA	Gibberellic Acid
GAST	GA stimulated transcript
GUS	beta-glucuronidase
G-type	gelatinous type
Gn-layer	galactan-enrich matrix layer
GalUA	Galacturonic Acid

Chapter 1 Literature Review

1.1 Why Study Flax?

Flax (*Linum usitatissimum* L.) is an important crop in temperate regions. There are two distinct types of flax, which are linseed and fiber flax. Linseed is grown for its seed, which has nutritional and industrial uses. Fiber flax is cultivated for its phloem (bast) fibers, which are traditionally used in textiles. Canada does not produce fiber flax, because this crop has specialized requirements for growth and fiber extraction (Foster et al., 1997). Linseed cultivars are grown in Canada on about 700,000 to 900,000 hectares. Canada is the world's largest exporter of linseed, with up to 1,100,000 tons of seed exported annually (Mackiewicz-Talarczyk et al., 2009).

Flax fibers are biodegradable and recyclable. Attempts are now being made to replace glass fibers in composite materials with bast fibers (Kalia et al., 2011). In current agricultural practice, linseed straw is not used as a source of bast fibers, since the yield and quality of these fibers is insufficient for textile production. However, fibers of linseed straw may be suitable for other industrial applications (Foster et al., 1997). Therefore, efforts are being made to improve the value of linseed fiber to make flax a dual-purpose crop. A better understanding of the genes that influence fiber development would therefore be useful in crop improvement.

The seeds of linseed are rich in alpha-linolenic acid (ALA), which is an omega-3 fatty acid. ALA has nutritional benefits and is also used for industrial purposes, including painting and flooring (Vaisey-Genser & Morris, 2003). The composition of flax protein, dietary fiber and its oil can be affected by growth conditions, breeding, or seed processing. For example, the ALA

content and the quality of linseed oil improves when cultivated under the cool weather of northern Canada (Morris, 2007). To satisfy the requirements of various industries, cultivars of linseed have been developed by mutation breeding to have different fatty acid composition (Daun et al., 2003). For example, linseed cultivars with low ALA content (<5%) have been developed, which have higher oxidative and thermal stability (Saeidi & Rowland, 1997). Altered seed colors, which can be used to identify cultivars with unique seed composition, have been developed through mutagenesis.

One of the components of the seed coat is mucilage, which is a proteo-heteroglycan, frequently containing uronic acids, that occurs in plants (Cammack et al., 2006). Linseed mucilage has reported functions in promoting seed hydration and germination (Esau, 1977; Fahn, 1982). In poultry and fish feed, however, the presence of mucilage is considered undesirable because it inhibits digestion and absorption of dietary nutrients (Rodriguez et al., 2001; Alzueta et al., 2003). Therefore the development of flax seeds with reduced mucilage is of interest to producers. Because flax mucilage contains a mixture of complex carbohydrates (Fekri et al., 2008), it is also an interesting subject for the study of polysaccharide synthesis.

In this literature review, the structure, development, and function of the seed coat will be described, with special focus on the pigments and mucilage of flax seed. Following this, the development of fibers in the flax stem will be briefly described.

1.2 The Anatomy of Mature Seed Coat

In seed plants, the embryo sac is surrounded by one or more protective structures called integuments from which the mature seed coat develops. In some species, the inner integument is eliminated and only the outer integument persists in the mature seed. In other species, including

flax, the seed coat is derived from both the inner and outer integuments (Bhojwani & Bhatnagar, 1974). Endosperm (or aleurone) may be present between the seed coat and the embryo (Hayward, 1938), and is often highly compressed (Venglat et al., 2011).

The mature seed coat consists of multiple distinct layers. From the outermost layer inwards, these are classified as: epidermis; round cells or ring cells; fiber cells or palisade cells; cross cells or transversal cells and pigment cells (Hayward, 1938; Freeman, 1995).

The epidermal layer is derived from the outer integument, and is often covered by a thin cuticle layer consisting of waxy hemicellulose and pectinaceous substances (Hayward, 1938). This epidermis layer can become gelatinous or mucilaginous when hydrated, due to release of pectins and hemicelluloses (Arsovski et al., 2009). Some seed coat epidermal cells differentiate to form mucilage secretory cells (MSCs), which have a secondary cell wall (Beeckman et al., 2000; Western et al., 2000; Windsor et al., 2000). Mucilage release occurs because of the pressure generated between the swelling of secondary layers and the inelastic middle lamella, following water absorption (Hayward, 1938). The cells in this layer are usually polygonal in transverse section and rectangular in longitudinal section.

Underlying the epidermis are the round cells (ring-cells), which are also derived from the outer integument (Boesewinkel, 1980; Hayward, 1938). As their name suggests, these cells have a circular appearance in transverse section, with conspicuous intercellular spaces. These parenchymatous cells may be present in one or two layers, with uneven thickening of their walls (Hayward, 1938).

The third layer is made of fiber cells, which are a type of sclerenchyma, and is made from the inner integument. The cells in this layer are elongated, with their long axis perpendicular to

the surface of the seed (Hayward, 1938). Fiber cells may vary in size within the same seed coat, with larger cells located closer to the micropylar ends, or at the narrow lateral edges of the seed (Freeman, 1995; Boesewinkel, 1980).

Cross cells or transversal-cells are derived from the middle layer of the inner integument. Their long axes are oriented perpendicular to the long axis of the fiber cells. Cross cells are colorless, with thin cell parenchymatous walls. In mature seeds, these cells become obliterated (Hayward, 1938).

The pigment layer is the innermost component of the seed coat, and thus is derived from the inner most layer of the inner integument (Hayward, 1938). This pigment layer is a single layer of square or polygonal cells with a thick porous wall. The color of the seed coat is determined by the density of the pigments, which are composed of tannin, as the brown seeds contain high amount of pigments compared to the yellow seeds (Freeman, 1995).

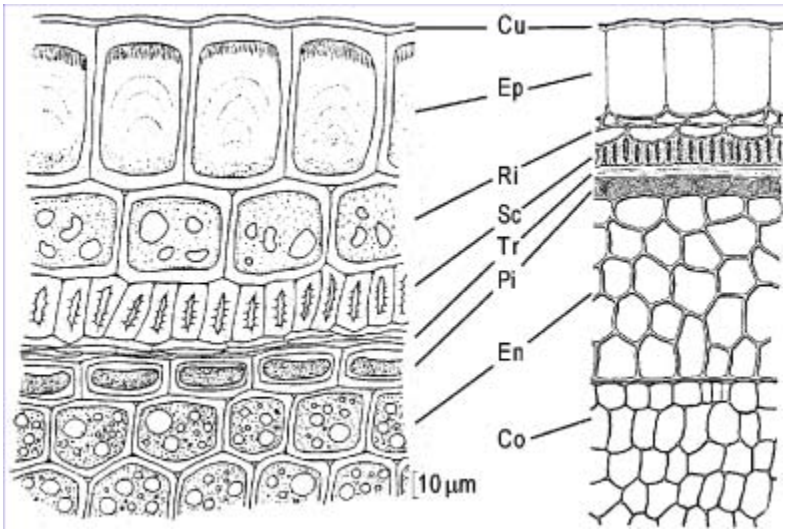

Figure 1-1 Cross-sections of flax seed coat at the early and mature stages. Cu: cuticula; Ep: epidermal layer; Ri: ring-cell layer; Sc: sclerenchymatous layer; Tr: transversal-cell layer; Pi: Pigment layer; En: endosperm; Co: cotyledon (Diederichsen & Richards, 2003)

1.3 Seed Coat Development

The flax seed is composed of three main parts including a diploid embryo, a triploid endosperm and the seed coat (Venglat et al., 2011). The seed coat has a maternal, sporophytic origin and is derived from both inner and outer integuments (Hayward, 1938). The inner integument develops into the tegmen and the outer integument into the testa (Corner, 1976). As described above, the mature flax seed coat is comprised of five structural layers and has a similar developmental program as Arabidopsis (Venglat et al., 2011). All seed coat layers initially undergo enlargement and division, and then each layer follows a distinct program of differentiation (Dean et al., 2011).

Beeckman et al. (2000) conducted a study of seed coat development using light and transmission electron microscopy. Differentiation of seed coat layers occurs primarily during the globular and torpedo stages of embryo development (Venglat et al., 2011). At globular stage, the differentiation of sub-epidermal and epidermal layers can be distinguished by the formation of starch grains within the sub-epidermal cells. Their inner periclinal cell walls become thickened by the torpedo stage. Another cytological change in the seed coat is the formation of mucilage, which is synthesized within the epidermis, and is mainly composed of pectinaceous substances. From the torpedo stage until maturation of the seed coat, the sub-epidermal cells collapse, but remain as a distinctive layer due to their thickened inner periclinal walls known as the palisade layer. This layer may eventually contribute to the brown pigment layer.

In the inner integument, the innermost layer (called the endothelium or pigment layer), is composed of small vacuolated cells. At the onset of embryogenesis, the composition of the endothelium changes by the synthesis of proanthocyanidin (PA) flavonoid compounds that condense to form tannin. The accumulation of tannin, followed by oxidization to form pigments, occurs during the torpedo stage. Pigments are initially deposited in the central vacuole and fill most of the cell including cytoplasm and other organelles. From late torpedo stage onward, the pigment disappears from the central part and accumulates in the cell periphery and consequently cells become dead at the desiccation stage. The remnants are incorporated into a brown pigment layer of the mature seed coat. There is no cellular differentiation occurring in the two other layers of the inner integument, which eventually become compressed and die (Nakaune et al., 2005). At maturity, all the seed coat cells die and are crushed together, except for the epidermal cells, as has been demonstrated in Arabidopsis (Haughn & Chaudhury, 2005).

1.4 Seed Coat Pigments

1.4.1 Biochemistry of Seed Coat Pigments

Pigments are typically present in the inner layer of the inner integument of seed coats (Hayward, 1938). Pigments include compounds of three main classes: phlobaphenes, condensed tannins and anthocyanins. Phlobaphenes are red-brown pigments such as those seen in pericarp of maize (*Zea mays* L.) (Hahlbrock & Grisebach, 1979; Styles & Ceska, 1989). Anthocyanins (red-purple) are the major contributors of pigments of legume seed coats (Harborne & Marby, 1975). In flax and Arabidopsis, tannins contribute to the dark color of the seed coat (Oomah et al., 1995).

Condensed tannins accumulate in the vacuoles of endothelial cells. During the biosynthesis pathway, the precursor dihydroflavonol reductase uses the dihydroflavonols to synthesize flavan-3,4-diols (leucoanthocyanidin). Production of the subsequent intermediate, flavan-3-ol, is catalyzed by leucoanthocyanidin reductase (Jende-Strid, 1991; 2004), and the condensed tannins are formed by the polymerization and condensation. Tannin compounds are usually colorless in immature tissues (Harborne, 1967; Marby et al., 1975). In flax, during seed germination, the phenolic compounds become visible pigments in the seed coat through the oxidation and dehydration processes. Brown linseed cultivars are reported to contain more phenolics in their pigments than the yellow seed lines (Oomah et al., 1995).

Anthocyanin, a sub-family of flavonoids, is synthesized from the same precursor (leucoanthocyanidin) used in condensed tannin biosynthesis (Marby et al., 1975; Jackman at el., 1987). In seed tissue, condensed tannins and anthocyanins can be distinguished by a histochemical stain, n-butanol-HCl (BuOH-HCl), in which condensed tannins gradually become red (> 30 minutes), while anthocyanins immediately turn red (< 2 minutes) (Lees et al., 1993). It has also been reported that the accumulation of anthocyanin occurs in the vacuoles along with many other flavonoid constituents, most of which are colorless and some of those are yellow (Harborne & Williams, 1995). The pigmentation is also enhanced by colorless flavonoids and phenylpropanoids compounds in the seed coat (Harborne, 1998; Harborne & Williams, 1995).

Diederichsen & Richards (2003) explained how anatomical changes in the seed coat structure resulted in differences in seed coat coloration. The presence or absence of a pigment layer and pigment cells had the greatest impact on seed coat color. When present, the pigment cells in the pigment layer contribute to dark brown coloration of the seed. Colorless seed coats usually lack either the pigment layer or pigment cells within this layer. It has been suggested that

the absence of pigments from the seed coat causes the transparent testa phenotype in which the yellow cotyledons are visible through the colorless seed coat. Parenchyma cells distributed in the ring-cell layer also contribute to the darker seed phenotypes. These cells may accumulate a dark tannin-liked component or chlorophyll, which can be seen especially through the colorless seed coat, resulting in the appearance of greenish or dark green seeds. Another possible anatomical effect is the presence of a dark yellow component in sclerenchymatous cells. Seed coat structure therefore has a strong effect on seed color.

In flax seed, the amount of pigment cells in the inner seed coat determines the seed coat color, which can be modified through plant breeding practices (Morris, 2007). Wild type flax seed is brown, but breeders have a preference for yellow seeds under some circumstances as described below. It is also known that linseed color has no significant effect on germination (Saeidi & Mirik, 2006).

Currently, the flax cultivars registered in Canada use seed colors as phenotypic marker to distinguish between seeds with different qualities. There are two broad categories of seed color: brown seed, which contains high levels of α-linolenic fatty acid (ALA) and Solin cultivars (yellow seeds) with lower ALA (<5%), which produces a polyunsaturated edible oil. As described by Rowland et al. (2003), the high ALA content of conventional linseed cultivars (brown seed) can lead to rancidity due to oxidization during crushing and food production. Thus, a new low ALA cultivar, Solin, was produced for its utility as a health-food additive and the yellow color seed was reserved as a marker for this cultivar. The yellow seeds had a thinner seed coat, thus seed coat damage (e.g. the split seed phenomenon) occurred in Solin more frequently compared to brown seed cultivars. The yellow seed cultivars also had a lower proportion of seed fiber compared to conventional flax (Flax Council of Canada, 1966, 2003).

Mutation breeding programs also produced a new line with variegated seed color that contained a high level of palmitic acid. The palmitic acid content in this cultivar is quadruple what is typically found in linseed, and almost no linolenic acid is present (Saeidi & Rowland, 1970). They also determined that the variegated seed coat trait is controlled by a single recessive gene and it segregates independently from the loci affecting palmitic acid content.

1.4.2 Genetics of Seed Color

Flax seed color was initially classified into just two major phenotypic groups: yellow and brown. Dillman proposed a more detailed classification that included different intermediate colors: yellow, chamois yellow, greenish yellow, mottled or brown, mummy brown, cinnamon brown (as cited by Barnes et al., 1960). Seed pigmentation phenotypes were also characterized in the world collection of flax germplasm at the Plant Gene Resources Canada (PGRC) seed bank at Saskatoon, SK. A range of seed colors were observed including yellow, yellowish brown, olive, light brown, medium brown, dark brown (Diederichsen & Richards, 2003).

The brown seed color was indicated as dominant over yellow color (Sood et al., 2012). Mittapalli & Rowland (2003) reviewed the studies in the inheritance of seed color in flax, which was mostly contributed by Tammes. Tammes described the anatomical basis of flax seed color. Yellow cotyledons can show through a colorless seed coat, making the seed appear yellow. Darker colored seeds are produced by pigments in the seed coat. Tammes also proposed a genetic framework for seed coat pigmentation. She identified three loci (*G*, *D* and *B'*) that regulate seed color. *G* is considered the major factor determining the color of the seed coat. In the presence of the dominant allele *G*, the seed has a colored seed coat, whereas genotype *gg*

seeds that lack normal function of this gene are colorless. The other two genes (*D* and *B'*), act as modifying factors as shown in Table 1-1.

A second allele of $B^{'}$ was found by Kappert (as cited by Barnes et al., 1960), and $B^{'}$ was renamed $B_{1.}$

Later studies by Barnes et al., (1960) and Groth et al., (1970) partly contradicted Tammes' original results. They reported that the homozygous recessive alleles at any of three loci (*G, D* or B_1) resulted in the yellow color, while the brown seed was produced in the presence of at least one dominant allele at all three loci ($G\_D\_B_1\_$). This model was also tested in different cultivars of flax. In the Minerva and four spontaneous recessive yellow seed mutants named: YSED2, YSED4, S95407, and S96071, the yellow seed color was caused by a *gg* genotype (Comstock et al., 1963; Mittapalli & Rowland, 2003), while in Crystal cultivar and one European yellow line G-1186/94, the recessive allele at b_1 was responsible for the yellow seed color (Culbertson & Kommedahl, 1955; Mittapalli & Rowland, 2003).

Furthermore, in partial disagreement with Tammes, Shaw proposed a model of three factors: *M*, *D* and *G* (Table 1-1) (as cited by Beard & Comstock, 1965). In this model, *G* was considered as an "additional factor". In the absence of factor *G*, the seed coat retained a new color "fawn", which was different from what was described by Tammes. According to Shaw, the factor *B* described by Tammes had no effect on seed color, while the appearance of new factor *M* contributed to seed coloration. The influence of locus *M* was also observed by Afzal Naz (as cited by Mittapalli & Rowland, 2003). Beard & Comstock (1965) suggested the symbol *Sc* be used for *M.*

Table 1- 1 Genetics models of flax seed colors

Genotype		Phenotype
Genetics model provided by Tammes	**Genetics model provided by Shaw**	
(*G_ddb'b'*); (*G_ddB'_*); (*G_D_B'_*)	(*M_D_G_*); (*mmD_G_*)	Brown
G_D_b'b'	(*mmddG_*); (*M_ddG_*); (*M_D_gg)*	Grayish-green; Grey; Fawn
gg____	(*mmddgg*); (*mmD_gg*); (*M_ddgg*)	Yellow

Furthermore, other studies have identified a modifying factor called *X*, which affects the intensity of seed color (as cited by Mittapalli & Rowland, 2003). Genotype *XX* results in a darker appearance, changing fawn to dark fawn and yellow to dark yellow. In its recessive state (*xx*), the color was reported to be weakened (Beard & Comstock, 1965). Still more genetic factors involved in seed color were identified when studied in different flax cultivars. The yellow phenotype was determined by a dominant allele *Y1* (*Y1_*) in the Australian plant introduction CPI84495 (Mittapalli & Rowland, 2003), or in a German flax variety Bionda (Popescu & Marinescu, 1996). Finally, Mittapalli & Rowland (2003) studied the genetics of yellow seeds in different flax cultivars. They found that a variegated seed color was also controlled by a second recessive allele of the *b1* locus ($b1^{vg}$). The homozygous recessive in *g*, *d*, and $b1^{vg}$ loci were epistatic to other loci.

In molecular genetics, defects in pigment (flavonoids) biosynthesis have been identified by changes in seed color. To date, 21 *transparent testa* (*tt*) mutants have been detected in

Arabidopsis. Two of them also have a hairless phenotype, known as *transparent testa glabra* (*ttg*), and six mutants are *tannin-deficient seed* (*tds*) (Abrahams et al., 2002). Some of the mutants that affect seed pigmentation of Arabidopsis are described in the table below.

Table 1- 2 Selected mutants in pigmentation biosynthesis in *Arabidopsis* (Moïse et al., 2005).

Mutants	Phenotype
ban (encodes anthocyanidin reductase)	Purple seed color.
tt1 and *tt16*	Aberrant morphology of endothelial cells.
ttg2	Seed size is reduced.
aba10 (encodes plasma membrane H^+ -ATPase)	Seed coat PAs is reduced.

1.5 Seed Coat Mucilage

1.5.1 Composition and Production of Mucilage

The major component of mucilage is pectin. The pectic polysaccharide in plant cell walls includes three major components: homogalacturonan (HG), rhamnogalacturonan I (RGI), and RGII (Somerville et al., 2004). In the seed coat mucilage, the pectin is characterized by RGI, which contains 1->2-linked rhamnose and 1->4-linked galacturonic acid in the backbone substituted with 1->5-linked arabinose, 1->3-linked galactose and arabinogalactan side-chains

linked to the rhamnose residues (Caffall & Mohnen, 2009). The hemicellulose known as arabinoxylan is also prevalent in seed coat mucilage (Lerouxel et al., 2006).

Linseed mucilage is extruded by the hydration of the seed coat, and comprises up to 8% of the seed mass (Bhatty & Cherdkiatgumchai, 1990; BeMiller & Whistler, 1993). The raw mucilage components can be divided into two groups: neutrals (arabinoxylan) and acidic (pectin-like) fractions. The yellow seeds often have lower gum content (Naqvi et al., 1987; Cui et al., 1996), which is higher in neutral polysaccharides and lower in acidic fractions compared to those from brown seeds (Cui et al., 1996). The constituent polysaccharides of mucilage vary due to different genotypes of flaxseed (Cui et al., 1996). Mucilage is not only released from the seed coat; it has also been found surrounding the root cap and in the transmitting tract of the pistil (Esau, 1977, as cited by Western et al., 2000). Mucilage extruded from different tissues possesses different properties, as demonstrated for example in comparisons of mucilage of the Arabidopsis seed coat with mucilage from the Arabidopsis root cap (Willats et al., 2001). The composition of mucilage also varies slightly between species. While Arabidopsis seed mucilage accumulates mostly unbranched RGI (Goto, 1985; Western et al., 2000; Penfield et al., 2001) and cellulose microfibrils (Mühlethaler, 1950; Fahn, 1982; Kreitschitz, 2009), flax mucilage is characterized by the presence of branched RGI and arabinoxylan (Naran et al., 2008).

During mucilage accumulation, the number of Golgi stacks increases approximately two-fold. These are concentrated at the site of mucilage production and supply energy for their synthesis. The associated secretory vesicles transfer mucilage to the apoplast between the plasma membrane and cell wall (McFarlane et al., 2008; Young et al., 2008). As mucilage accumulates, the stacks and vesicles are reduced and are redistributed throughout the cytoplasm. This general

pattern of mucilage synthesis is typical of most myxospermous (i.e. mucilage producing) species (Western, 2012).

1.5.2 The Secretion and Extrusion of Mucilage

The complex carbohydrate in the secretory vesicles is secreted via exocytosis to form part of the extracellular matrix (Staehelin & Moore, 1995; Dupree & Sherrier, 1998). The cytoplasmic rearrangement that occurs during mucilage secretion has been divided into three stages by Young et al. (2008). Prior to the secretion of mucilage, the volume of the epidermal cell is occupied by a large central vacuole, which is surrounded by Golgi stacks. The Golgi appears to be cup-shaped. During mucilage release, the vacuole becomes smaller, leaving space for the formation of mucilage pockets under the apical surface of the epidermal cell. Well-developed mucilage pockets accumulate between the plasma membrane and primary cell wall, which shrink the vacuole to the bottom of the cell. The Golgi stacks become shorter and have a more pronounced cis-trans polarity.

Following secretion, mucilage is extruded as a result of hydration when a dry seed is exposed to an aqueous environment. Mucilage is released as a bipartite structure: an outer, diffuse layer and an inner, dense layer (Western et al., 2000; Macquet et al., 2007; Huang et al., 2011; Walker et al., 2011). The mechanism of mucilage release in flax has been described by Haberlandt (as cited by Hayward, 1938). During development of the seed coat, a thin middle lamella forms between adjacent cells, but not on the external surface covered by a cuticle layer. When a high volume of water is absorbed, the internal pressure of the cell increases. This pressure forces the cell to start swelling outward towards the cuticle layer, because the middle lamella is inelastic. As the wall of swelling mucilage pushes up against the cuticle layer, the

outer wall of the epidermal cell is disrupted and fissured. In Arabidopsis, the mucilage is formed in a volcano-shaped columella (Beeckman et al., 2000; Western et al., 2000; Windsor et al., 2001). Mucilage is released as the enlarged columella breaks the upper portion of the radial cell wall (Western et al., 2000; Windsor et al., 2001)

Microtubules lining the membrane of mucilage pockets can also be detected (McFarlane et al., 2008). After the secretion of mucilage ends, a thick secondary wall is deposited in the upper portion of the cell between the plasma membrane and the mucilage pockets. The volume occupied by the vacuole is reduced although it is still located in the lower portion of the cell. The microtubules become less prominent and the Golgi stack morphology does not change in this stage.

The presence of the microtubules during the mucilage secretion contributes to establishing the physical properties of mucilage (McFarlane et al., 2008). Lining of cortical microtubules would change the mucilage pocket morphology, and that promotes more mucilage released. However, the exact function of the cytoskeleton in mucilage secretion is still unclear (McFarlane et al., 2008).

1.5.3 Mutants of Mucilage Secretion

Mutants of the seed coat and especially the cells that secrete mucilage have been studied in Arabidopsis (Western et al., 2000; Western et al., 2001, 2004; Young et al., 2008). These mutations affect synthesis or secretion of mucilage, and may affect differentiation of the outer integument, mucilage secretory cells (MSCs), mucilage and post-deposition cell wall modification (Western et al., 2001). The mutant phenotypes can be divided into two categories:

mutants completely lacking mucilage and those with a reduced amount of mucilage production (Western, 2012).

Young et al. (2008) described the developmental program that occurs during mucilage production in *mucilage-modified4* (*mum4*)/*rhamnose synthase2* (*rhm2*), mutants, in which the number of Golgi stacks double. The modification of Golgi stack morphology in *mum4/rhm2* disrupts normal mucilage extrusion. However, this mutant is able to release a very small amount of mucilage when treated with a metal chelator to weaken the primary cell wall. One possibility is that less mucilage is produced in the absence of normal microtubule (MT) organization during mucilage synthesis (McFarlane et al., 2008). The MT structure affects the physical properties of mucilage by leading to re-arrangement of the Golgi apparatus or secretory vesicles (McFarlane et al., 2008). The *microtubule organization1-1* (*mor1-1*) mutation, however, had no effect on the MSC morphology and the organization of internal organelles that would resulted in reduced amount of mucilage produced compare to those in wild type under restrictive temperatures (McFarlane et al., 2008).

Mutations in seed coat development may result in the physiological defects in seed germination, vigour and dormancy (Debeaujon et al., 2000). By screening for defects in seed coat mucilage, mutations have been detected in altered processes occurring during the epidermal differentiation including mucilage synthesis, secretion, and epidermal cellular division (Western et al., 2001). A screen for mutants affected in mucilage extrusion identified mutants that showed a complete lack of a mucilage capsule surrounding after hydration. It was known that de-methylation of carboxyl groups in poly-GalUA in the mucilage coincided with mucilage release (Brett & Waldron, 1996; Carpita & Gibeaut, 1993). An increase in the proportion of methyl esterification was detected in *mum1* and *mum2* mutant seeds, so that mucilage was retained

within the primary cell wall of the seed coat epidermal cells upon maturity. Panikashvili et al. (2009) described defects in seed coat differentiation in the cuticle layer or subtending cell walls, which led to a failure of mucilage release. Defective mucilage extrusion was also detected in *defective in cuticular ridges* (*dcr*) mutants, in which MSCs were observed having a cuticle-like osmophilic layer covering their outer cell walls (Panikashvili et al., 2009).

Studies have revealed alterations in pectin composition that are consistent with a reduced amount of mucilage production. The mutants *transparent testa glabra1-1* (*ttg1-1*), *glabra2* (*gl2-1*), and *mum4-1* all have decreases in galacturonic acid (GalUA) and L-rhamnose (Rha). Reduced content of Rha and Fucose (Fuc) in *mum3-1* and *mum5-1* both resulted in less mucilage being produced compared to wild type seeds. Lack of the hormone abscisic acid (ABA), which regulates mucilage production, results in only a small amount of mucilage observed in *abscisic acid deficient1* mutant seeds (Karssen et al., 1983).

The identification of mutants defective in seed mucilage revealed defects in differentiation of seed coat cells affecting both the mucilage extrusion and seed phenotype (Bowman & Koornneef, 1994; Jofuku et al., 1994). In cross-sections of mature seed coats, an absence of epidermal and sub-epidermal palisade cell types was observed in *APETALA2* (*ap2*) mutants. The seed had a heart-shaped appearance rather than typical oval shape in wild type, and the epidermal cells became rectangular with a thin cell wall which differed from the hexagonal,thick cell walls of the wild type (Leon-Kloosterziel et al., 1994; Mcabee et al., 2006). It was also reported that the seed shape defects delayed developmental processes (Kunieda et al., 2008).

1.5.4 The Roles of Mucilage

1.5.4.1 Functions of Mucilage in Natural Systems

Mucilage, which is extruded from the seed coat, supports the hydration and germination of the seed, and helps attach it to the soil (Esau, 1977; Fahn, 1982).

The physiological and ecological roles of mucilage are a subject of study and speculation. As described above, mucilage is believed to facilitate seed hydration because mucilage forms a gel capsule around the seed. This hydrogel helps the seed resist desiccation during occurrences of low water potential after imbibition (Esau, 1977; Franz, 1989; Gutterman & Shem-Tov, 1996).

Comparisons of germination rates between seeds from mucilaginous and non-mucilaginous species have shown poorer germination of seeds without mucilage (Harper & Benton, 1966). The presence of mucilage also promotes germination of *Artemisia sphaerocephala* seeds under drought and salt-stress (Yang et al., 2010). However, in spinach seeds, mucilage may inhibit germination by limiting the uptake of oxygen by the embryo. This viscous layer slows down the passage of oxygen to the embryo approximately 10,000 times compared to through the air (Heydecker & Orphanos, 1968). In summary, the roles of mucilage in seed germination appear to be complex and dependent on the species and environment in which it is present.

Mucilage is adhesive, so it sticks easily to animal fur or bird feathers. Thus, it is expected to aid seed dispersal (Young & Evans, 1973). Expanded dry mucilage significantly increased wind dispersal of seeds (Sun et al., 2012). The seed may be transported far away from the source by the moving of animals and field workers. This may help to expand the species range.

Another possible function of seed coat mucilage is in defense against insects. The mucilage in seed of Shepherd's purse (*Capsella bursa- pastoris*) contains proteolytic enzymes which digest larvae (Barber, 1978).

1.5.4.2 Potential Uses of Mucilage in Health and Animal Feed

The viscous mucilage of flax seed has been used in both traditional and commercial applications. Mucilage is a rich source of soluble dietary fiber (Kozlowska et al., 2008). Flax seed mucilage has been reported to reduce the risk of diabetes and heart disease and the incidence of obesity (Mazza & Oomah, 1995; Jenkins et al., 1985). A study was conducted to define the ability of mucilage used in pharmaceuticals to improve glucose tolerance (Jenkins & Wolever, 2001). When adding 25 g flaxseed mucilage to a 400 ml solution containing 50g glucose, the risk of blood glucose response reduced 27% (Wolever, 1995). It was also proposed that dietary fiber in flax-seeded supplemented diets functions to reduce the total serum cholesterol and low-density lipoprotein (LDL), known as a bad cholesterol (Watkins et al., 1995;Cunnane et al., 1993; 1995).

The uses of mucilage extracted from flax seeds include its addition to medicinal products like laxatives or sore throat syrups (Muir & Westcott, 2003). The mucilage acts as soluble fiber to absorb water from the gastrointestinal tract and increase stool bulk and so acts effectively as laxative. The thickening function of mucilage helps to protect the lining of the stomach and intestine from irritation. Thus, adding mucilage to sore throat syrups is a common practice. Kishk et al. (2011) have demonstrated an effective replacement of flaxseed for wheat flour in noodles producing. They proved that using flaxseed mucilage, a non-starch polysaccharide, improves both the cooking quality and sensory attributes of noodles (Kishk et al., 2011).

In addition, there are some inconsistent results about using flaxseed in poultry industry. As described above, mucilage is believed to inhibit the digestion and uptake of carbohydrates by increasing the viscosity of the contents of the small intestine (Jenkins et al., 1978; Edwards et al., 1987). However, Jiang et al. (1991) and Caston et al. (1994) reported that feeding flaxseed has no effect on egg production, while others indicate flaxseed feeding has a negative effect on egg production (Aymond & Van Elswyk, 1995; Leeson et al., 2000). The negative effect may result from low nutrient utilization or other anti-nutritional effects of mucilage (Bhatty, 1995).

1.6 Flax Bast (Phloem) Fibers

1.6.1 Fiber Development

Fibers are a specific cell type defined by thick walls and a slender threadlike structure with extreme length compared to width (Esau, 1977; Fahn, 1982). Xylem fibers and phloem fibers are two distinct types of fibers; phloem fibers are also known as bast fibers (Esau, 1943). In the flax stem, bast fibers begin their differentiation at the earliest stages of vascular tissue development, within procambial strands just a few cell layers below the apex of a growing flax shoot. All phloem fibers in flax are derived from primary growth and are single cells that are often multinucleate (Anderson, 1927; Deyholos, 2007). Bast fibers are organized in bundles that occur between the epidermis and the vascular cambium, and provide mechanical support to the plant (Huis et al., 2010).

Phloem fibers are distinguished from xylem fibers by their extensive length and the composition of their secondary walls, which have a distinct pattern of development (Roach & Deyholos, 2007; Gorshkova et al., 2012). Following the specification of fiber identity near the shoot apical meristem (200 μm from the shoot apex) (Esau, 1943; Snegireva et al., 2010), the

extreme fiber length is achieved through two types of cell elongation: coordinated growth during the early stages of differentiation and intrusive growth (Gorshkova et al., 2003). Along the flax stem, coordinated (i.e. symplastic) growth occurs within the first one millimeter of stem below the shoot apex, in which the primary cell wall expansion has the same rate as surrounding non-fiber cells (Esau, 1943). The subsequent elongation of fiber cells occurs through intrusive growth, during which fibers continue to elongate while surrounding tissues have slowed or stopped elongation (Lev-Yadun, 2001). Generally, there are two mechanisms of cell elongation in intrusively growing cells: tip growth and diffuse/intercalary growth (Wasteneys & Galway, 2003). During tip growth, new cell wall material is added only at the termini of the fiber cells. In diffuse (or intercalary) growth, new material is added along the surface of the entire cell (Ageeva et al., 2005). The intrusive elongation of flax phloem fibers occurs through intercalary growth while secondary xylem fiber elongation is characterized by tip growth (Ageeva et al., 2005; Gorshkova et al., 2012). Elongation of individual phloem fiber cells was estimated to take only 2-4 days at a rate of 1-2 cm per day (Gorshkova et al., 2003).

After fiber cell elongation is complete, secondary cell wall formation begins (Gorshkova et al., 2003; Ageeva et al., 2005; Snegireva et al., 2010). Flax bast fibers have very thick walls (up to 10 μm) compared to most other cell types (0.1-1.0 μm) (Gorshkova et al., 2003). The developmental transition from elongation to secondary cell wall thickening is marked by a transition point called the snap point, which is a region of the stem in which a sharp increase in mechanical strength occurs, protecting it from tearing when manually pulled (Gorshkova et al., 2012). At this point, most of the fiber cells have gained sufficient mechanical strength to resist breaking; therefore, the cross section at the snap point should contain only a few cells that are still elongating (Gorshkova et al., 2003). The snap point is usually located between 45 mm and

90 mm below the shoot apex. The snap point is present during the fast growth phase and disappears as stem growth is completed (Gorshkova et al., 2003).

1.6.2 Fiber Composition

There are two major types of secondary cell wall structure: xylan type and gelatinous type (G-type). The xylan type is most common outside of bast fibers (Carafa et al., 2005). G-type cell walls occur only in bast fibers and tension cell walls (Mokshina et al., 2012; Anderson, 1927; Crônier et al., 2005; Gorshkova & Morvan, 2006).

Cellulose microfibrills are the foundation of all cell walls (Müller et al., 2006; Mellerowicz & Sundberg, 2008). Xylan type walls also contain abundant β-D-(1→4)-xylan and lignin (Mokshina et al., 2012). Those components are arranged in several layers (S1, S2, S3) (S-layer) (Gorshkova et al., 2012). In S-layer type, the cellulose microfibrills arrange in helicoidal orientation and different angle also found between microfibrills and the longitudinal cell axis (Groshkova et al., 2012).

In contrast, G-type secondary walls contain very little xylan or lignin (Mellerowicz et al., 2001; Pilate et al., 2004; Gorshkova et al., 2010). Furthermore, the cellulose microfibrills of G-type walls lay almost parallel to the fiber's longitudinal axis throughout a single layer called the G-layer. The different orientation of cellulose microfibrills in each type of cell wall has an impact on the contractile ability of the secondary cell wall (Goswami et al., 2008; Burgert & Fratzl, 2009).

An important aspect of development of the G-type phloem fibers is the modification of the initial, galactan-enrich matrix Gn-layer to a denser, cellulosic G-layer of the mature fiber.

This is dependent on the expression of β-galactosidase (Gorshkova et al., 2010). Studies indicate that β-galactosidase impacts the metabolism of cell wall associated galactans, and shows high expression during the formation of the cellulosic G-layer (Roach & Deyholos, 2007; Mokshina et al., 2012; Roach et al., 2011). A bipartite structure is often seen in developing bast fibers: a striated Gn-layer that appears loosely-packed is surrounded by a more homogenous G-layer of comparable thickness (Gorshkova et al., 2003; Gorshkova & Morvan, 2006). In case of reduced transcription of β-galactosidase, the thickness of the G-layer is greatly reduced and is difficult to detect, and shows a reduction in stem strength of up to 30% (Roach et al, 2011).

Flax phloem fiber walls consist of 70-75% cellulose, 15% hemicellulose, 10-15% pectin, and small amounts (1.5- 4.2%) of lignin (Gorshkova et al., 2000; Mooney et al., 2001; Day et al., 2005). Lignin is a highly complex polymer composed of hydroxycinnamyl alcohols or "monolignols" [the phenylpropane units p-hydroxyphenyl-propane (H), guaiacyl (G) and syringyl (S)], and indicated that both flax xylem- and bast fiber-lignins were rich in guaiacyl (G) (Day et al., 2005). In bast fibers, the condensed guaiacyl-type (G) lignins have been detected in the middle lamella, cell wall junctions, and in the S1 layer of the secondary wall (Day et al., 2005). The chemical analyses suggested that flax bast fiber lignin is more condensed than the correspondent xylem lignin (Day et al., 2005). The cellulose crystallinity was determined to be four-fold higher in gelatinous layers than in xylan layers (Müller et al., 2006). In flax fibers, there are also various other phenolic compounds which are variously defined as flavonoids, anthocyanins or cinnamic acids (Love et al., 1994; Gamble et al., 2000; Morrison et al., 2003).

The amount of cellulose and noncellulosic constituents in a fiber determines the structural properties and influences its crystallinity and moisture retention (Reddy & Yang, 2005). Fibers

with higher cellulose content, higher degree of polymerization of cellulose and lower microfibrillar angle have stronger mechanical properties (Reddy & Yang, 2005).

1.6.3 Previous Studies of Gene Expression in Flax Stems

A whole genome-sequence of flax has been recently obtained by *de novo* assembly of short, shotgun reads using the Illumina Genome Analyzer (Wang et al., 2012). In the whole-genome shortgun assembly, there are 43,384 predicted protein-coding genes (Wang et al., 2012). The contig assembly contains 302 Mb of non-redundant sequence, which is 81% genome coverage in comparison to the published value of the nuclear DNA content of flax by reassociation kinetics analyses (estimated C = 350 Mb) (Cullis, 1981; Wang et al., 2012). The flax genome has been characterized with 50% low-copy-number sequences, with 35% highly repetitive sequences, which are arranged in a long period interspersion pattern, and the remaining 15% in the middle-repetitive fraction (Cullis, 1981). According to public databases, genomic sequence resources for L. *usitatissimum* include 286,895 ESTs (http://www.ncbi.nlm.nih.gov/nuccore?term=linum%20usitatissimum), and 96% of those aligned to the whole-genome shotgun scaffold (Wang et al., 2012). In CDC Bethune, a linkage map based on SSRs and a 368 Mb BAC physical map have been published (Cloutier et al., 2009; Ragupathy et al., 2011).

Moreover, some studies about microarray gene expression and proteomics of flax have also been reported (Lynch & Conery, 2000; Roach & Deyholos, 2007; Fenart et al., 2010). Using the library of 9,600 cDNA clones from cortical tissues of 4 week-old flax stems, stem-enriched transcripts involved in the processes of polysaccharide and cell wall metabolism have been identified when comparing transcript abundance in stem-peels and leaves (Roach & Deyholos,

2007). The microarray compared three segments (TOP, MID, BOT) of the vertical stem axis which constituted a developmental series for phloem fibers and other cell types (Roach & Deyholos, 2007). Because of the low proportion of ESTs within the randomly selected clones that were related to phloem fiber development, the putative cell wall-related transcripts represented the smallest functional class (1%). Up to 34% of the randomly sequenced ESTs had no significant match (BLASTx E-value < 10^{-10}) to any proteins in the GenBank peptide database. Using the previous cDNA transcript library from flax stem-peel, microarray analysis of developing flax hypocotyls at 7 d (elongation phase), 9 d (onset of secondary cell wall deposition) and 15 d (late cell wall deposition) was conducted to identify novel transcripts correlated with specific stages of phloem fiber differentiation (Roach & Deyholos, 2008). In total, from 1,568 probesets, 660 were enriched by at least 2-fold in comparisons of one or more stem segments. Of the sequenced clones, 43 did not align to any known proteins in GenBank.

Another study used expressed sequence tags (ESTs) that were obtained from the outer, fiber-bearing tissues of flax stems (cv Hermes) harvested at the mid-flowering stage (Day et al., 2005). In total 927 ESTs were described, of which 4.4% (41) were potentially related to cell wall formation and maturation. In addition, in a microarray described by Fenart et al. (2010), nine different RNA samples obtained from flax inner- and outer-stems, seeds, leaves and roots were used to generate a collection of 1,066,481 ESTs by massive parallel pyrosequencing. Sequences were assembled into 59,626 unigenes and 48,021 sequences were selected for oligo design and high-density microarray. Using 454 sequencing and contig assembly, a total of 59,626 unigenes were assembled, and identified 609 root-specific genes, 599 leaf-specific genes, 79 shoot-specific genes and 70 seed-specific genes.

Further understanding of the proteins that contribute to the unique properties of flax bast fibers came from studies of ultimate fibers (i.e. individual cells) dissected from the snap-point region of vegetative stems (21-24 days post germination). The abundance of many proteins in fiber was notably different from the surrounding non-fiber cells of the cortex, with approximately 13% of the 1,850 detectable spots being significantly (> 1.5 fold, $p < 0.05$) enriched in fibers. Following mass spectrometry, there were 114 identified spots, of which 51 were significantly enriched in fibers (Hotte & Deyholos, 2008).

1.7 *GASA*/*GAST* Gene Family

Members of the *GASA* (*GIBBERELLIC ACID STIMULATED IN ARABIDOPSIS*) gene family encode small polypeptides that comprise three domains: an N-terminal domain containing 18 to 29 amino acid residues, a hydrophilic domain with a diverse number of polar amino acid residues, and a highly conserved 60 amino acid C-terminal domain (GASA domain) (Zhang & Wang, 2008; Peng et al., 2008). *GA STIMULATED TRANSCRIPT 1* (*GAST1*) is the first member of *GASA* gene family, and was cloned from the tomato GA-deficient mutant *gib*1 (Shi et al., 1992). Since then, putative homologs of *GAST1* were isolated from tomato (*Lycopersicon esculentum*) (Taylor & Scheuring, 1994), petunia (*Petunia hybrida*) (Ben-Nissan & Weiss, 1996; Ben-Nissan et al., 2004), potato (*Solanum tuberosum*) (Berrocal-Lobo et al., 2002; Segura et al., 1999), gerber daisy (*Gerbera hybrida*) (Kotilainen et al., 1999), and *Arabidopsis thaliana* (Herzog et al., 1995; Aubert et al., 1998; Roxrud et al., 2007).

Most of the GASAs described to date are localized in apoplasm or cell wall, and their expression is regulated by gibberellins (GAs) (Peng et al., 2008). The potential functions of GASA members are diverse, and include roles reported in cell division, cell elongation, and

flower, root and fruit development (Ben-Nissan et al., 2004; José et al., 2006; Kotilainen et al., 1999; Roxrud et al., 2007).

The *GASA* family identified in *Arabidopsis* includes 15 genes, named as *GASA1* to *GASA15* shown in Table 1- 3 (Zhang & Wang, 2008).

Table 1- 3 Members of *GASA* gene family in *Arabidopsis*

Gene	***Arabidopsis* gene number**	**Amino acid length**	**GASA domain**
GASA1	At1g75750	98	39-98
GASA2	At4g09610	99	39-98
GASA3	At4g09600	99	40-99
GASA4	At5g15230	106	46-106
GASA5	At3g02885	97	38-97
GASA6	At1g74670	101	42-101
GASA7	At2g14900	108	49-108
GASA8	At2g39540	87	29-87
GASA9	At1g22690	119	60-119
GASA10	At5g59845	89	29-89
GASA11	At2g18420	88	28-88

GASA12	At2g30810	106	47-106
GASA13	At5g15230	275	215-275
GASA14	At1g10588	90	31-90
GASA15	At3g10185	103	44-103

The expression of several members of the *GASA* gene family in *Arabidopsis* has been analyzed (Herzog et al., 1995): *GASA1* transcripts were detected in flower buds and immature siliques; *GASA2* and *GASA3* localized in dry seeds, and *GASA4* was in meristematic regions (roots and flower buds) (Aubert et al., 1998). *GASA5* is a negative regulator of GA-induced flowering and stem growth (Zhang et al., 2009); otherwise, overexpression of this gene increases the sensitivity of *Arabidopsis* to heat stress (Zhang & Wang, 2011). GUS reporter gene assays using the presumptive promoter regions of *GASA6*, *GASA7*, *GASA8*, *GASA9*, *GASA10*, *GASA11*, and *GASA12* showed that all *GASA* genes are expressed in growing and differentiating organs and abscission zones, suggesting a role for these genes in cell growth and differentiation (Zhang & Wang, 2008).

The objective of the research outlined in the following chapters is to identify mutants of linseed with abnormal mucilage and seed coat color, because these seeds may be useful in both agriculture, and in further scientific research.

This study also pursues in identification and characterization of GASA-like gene family in flax stem development. The putative functions of this gene family will be inferred, which may contribute to further understanding the developmental process of bast fiber in flax.

All the findings in this research will help to improve both seed and stem products in linseed varieties, making it possible to develop a true dual purpose crop, which will be useful to flax producers.

1.8 Bibliography

Abrahams, S., Tanner, G. J., Larkin, P. J., & Ashton, A. R. (2002). Identification and biochemical characterization of mutants in the proanthocyanidin pathway in *Arabidopsis*. *Plant Physiology*, *130*(2), 561–576.

Ageeva, M. V, Petrovska, B., Kieft, H., Sal'nikov, V. V, Snegireva, A. V, Van Dam, J. E. G., Van Veenendaal, W. L. H., Emons, A. M. C., Gorshkova, T. A., Van Lammeren, A. A. M. (2005). Intrusive growth of flax phloem fibers is of intercalary type. *Planta*, *222*(4), 565–574.

Alzueta, C., Rodriguez, M. L., Cutuli, M. T., Rebole, A., Ortiz, L. T., Centeno, C., & Trevino, J. (2003). Effect of whole and demucilaged linseed in broiler chicken diets on digesta viscosity, nutrient utilisation and intestinal microflora. *British Poultry Science*, *44*(1), 67–74.

Anderson, D. B. (1927). A microchemical study of the structure and development of flax fibers. *American Journal of Botany, 14(4)*, 187–211.

Arsovski, A. A., Popma, T. M., Haughn, G. W., Carpita, N. C., McCann, M. C., & Western, T. L. (2009). AtBXL1 encodes a bifunctional β-d-xylosidase/α-l-arabinofuranosidase required for pectic arabinan modification in Arabidopsis mucilage secretory cells. *Plant Physiology, 150(3),*1219-1234.

Aubert, D., Chevillard, M., Dorne, A. M., Arlaud, G., & Herzog, M. (1998). Expression patterns of *GASA* genes in *Arabidopsis thaliana*: the *GASA4* gene is up-regulated by gibberellins in meristematic regions. *Plant Molecular Biology*, *36*(6), 871–883.

Aymond, W. M., & Van Elswyk, M. E. (1995). Yolk thiobarbituric acid reactive substances and n-3 fatty acids in response to whole and ground flaxseed. *Poultry Science*, *74*(8), 1388–1394.

Barber, J. T. (1978). Capsella bursa-pastoris seeds. Are they 'carnivorous'. *Carniv. Pl. Newsl*, *7*(2), 39–42.

Barnes, D. K., Culbertson, J. O., & Lambert, J. W. (1960). Inheritance of seed and flower colors in flax. *Agronomy Journal*, *52*(8), 456–459.

Beard, B. H., & Comstock, V. E. (1965). Flax genetics and gene symbolism. *Crop Science, 5(2)*, 151–155.

Beeckman, T., De Rycke, R., Viane, R., & Inzé, D. (2000). Histological study of seed coat development in *Arabidopsis thaliana*. *Journal of Plant Research*, *113*(2), 139–148.

BeMiller, J. N., & Whistler, R. L. (1993). Industrial gums: polysaccharides and their derivatives (Ed.). Academic Press Limited, London.

Ben-Nissan, G., Lee, J. Y., Borohov, A., & Weiss, D. (2004). GIP, a *Petunia hybrida* GA-induced cysteine-rich protein: a possible role in shoot elongation and transition to flowering. *The Plant Journal*, *37*(2), 229–238.

Ben-Nissan, G., & Weiss, D. (1996). The petunia homologue of tomato *gast1*: transcript accumulation coincides with gibberellin-induced corolla cell elongation. *Plant Molecular Biology*, *32*(6), 1067–1074.

Berrocal-Lobo, M., Segura, A., Moreno, M., López, G., García-Olmedo, F., & Molina, A. (2002). *Snakin*-2, an antimicrobial peptide from potato whose gene is locally induced by wounding and responds to pathogen infection. *Plant Physiology*, *128*(3), 951–961.

Bhatty, R S, & Cherdkiatgumchai, P. (1990). Compositional analysis of laboratory-prepared and commercial samples of Linseed meal and of hull isolated from flax. *Medicine*, *67*(2), 79–84.

Bhatty, R. S. (1995). Nutrient composition of whole flaxseed and flaxseed meal. Flaxseed in human nutrition (Ed.). AOCS Press Champaign, USA.

Bhojwani, S. S., & Bhatnagar, S. P. (1974). The embryology of angiospermae. Vikas Publishing House PVT LTD, New Delhi.

Boesewinkel, F. D. (1980). Development of ovule and testa of *Linum usitatissimum* L. *Acta Botanica Neerlandica*, *29*(1), 17–32.

Bowman, J. L., & Koornneef, M. (1994). Seed morphology. *Arabidopsis:* an atlas of morphology and development. Springer-Verlag, New York.

Brett, C. T., & Waldron, K. W. (1996). Physiology and biochemistry of plant cell walls (2nd Ed.). Chapman & Hall, London, UK.

Burgert, I., & Fratzl, P. (2009). Plants control the properties and actuation of their organs through the orientation of cellulose fibrils in their cell walls. *Integrative and Comparative Biology*, *49*(1), 69–79.

Caffall, K. H., & Mohnen, D. (2009). The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. *Carbohydrate Research*, *344*(14), 1879.

Cammack, R., Attwood, T. K., Campbell, P. N., Parish, J. H., Smith, A. D., Stirling, J. L., & Vella, F. (2006). Oxford dictionary of biochemistry and molecular biology (2nd Ed.). Oxford University Press.

Carafa, A., Duckett, J. G., Knox, J. P., & Ligrone, R. (2005). Distribution of cell-wall xylans in bryophytes and tracheophytes: new insights into basal interrelationships of land plants. *New Phytologist*, *168*(1), 231–240.

Carpita, N. C., & Gibeaut, D. M. (1993). Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *The Plant Journal*, *3*(1), 1–30.

Caston, L. J., Squires, E. J., & Leeson, S. (1994). Hen performance, egg quality, and the sensory evaluation of eggs from SCWL hens fed dietary flax. *Canadian Journal of Animal Science*, *74*(2), 347–353.

Cloutier, S., Niu, Z., Datla, R., & Duguid, S. (2009). Development and analysis of EST-SSRs for flax (*Linum usitatissimum* L.). *Theoretical and Applied Genetics*, *119*(1), 53–63.

Crônier, D., Monties, B., & Chabbert, B. (2005). Structure and chemical composition of bast fibers isolated from developing hemp stem. *Journal of Agricultural and Food Chemistry*, *53*(21), 8279–8289.

Comstock, V.E., Ford, J.H. and Beard, B.H. (1963). Association among seed and agronomic characteristics in isogenic lines of flax. *Crop Science*, *3(2)*, 171–172.

Corner, E. J. H. (1976). The seeds of dicotyledons. Cambridge University Press. The Edinburgh Building, Cambridge, UK.

Cui, W., Kenaschuk, E., & Mazza, G. (1996). Influence of genotype on chemical composition and rheological properties of flaxseed gums. *Food Hydrocolloids*, *10*(2), 221–227.

Culbertson, J. O., & Kommedahl, T. (1955). The effect of seed coat color upon agronomic and chemical characters and seed injury in flax. *Agronomy Journal*, 48(1), 25-28.

Cunnane, S. C., Hamadeh, M. J., Liede, A. C., Thompson, L. U., Wolever, T. M., & Jenkins, D. J. (1995). Nutritional attributes of traditional flaxseed in healthy young adults. *The American Journal of Clinical Nutrition*, *61*(1), 62–68.

Cunnane, S., Gaguli, S., Menard, C., Wolever, T., & Jenkins, A. (1993). High a-linolenic acid flaxseed (*Linum usitatissimum*): some nutritional properties in humans. *British Journal of Nutrition*, *69*, 443–453.

Cullis, C. A. (1981). DNA sequence organisation in the flax genome. *Biochimica et Biophysica Acta (BBA)-Nucleic Acids and Protein Synthesis*, *652*(1), 1–15.

Daun, J. K., Barthet, V. J., Chornick, T. L., Duguid, S., Thompson, L. U., Cunnane, S. C. (2003). Structure, composition, and variety development of flaxseed. Flaxseed in human nutrition (Ed.). AOCS Press, USA.

Day, A., Ruel, K., Neutelings, G., Crônier, D., David, H., Hawkins, S., & Chabbert, B. (2005). Lignification in the flax stem: evidence for an unusual lignin in bast fibers. *Planta*, *222*(2), 234–245.

Day, A., Addi, M., Kim, W., David, H., Bert, F., Mesnage, P., Rolando, C., Chabbert, B., Neutelings, G., Hawkins, S. (2005). ESTs from the fibre-bearing stem tissues of flax (*Linum usitatissimum* L.): expression analyses of sequences related to cell wall development. *Plant Biology*, *7*(1), 23–32.

Dean, G., Cao, Y., Xiang, D., Provart, N. J., Ramsay, L., Ahad, A., White, R., Selvaraj, G., Haughn, G. (2011). Analysis of gene expression patterns during seed coat development in *Arabidopsis*. *Molecular Plant*, *4*(6), 1074–1091.

Debeaujon, I., Léon-Kloosterziel, K. M., & Koornneef, M. (2000). Influence of the testa on seed dormancy, germination, and longevity in *Arabidopsis*. *Plant Physiology*, *122*(2), 403–414.

Deyholos, M. K. (2007). Bast fiber of flax (*Linum usitatissimum* L.): Biological foundations of its ancient and modern uses. *Israel Journal of Plant Sciences*, *54*(4), 273–280.

Diederichsen, A., & Richards, K. (2003). Cultivated flax and the genus Linum. Flax: the genus Linum. Taylor & Francis. New York, NY, USA.

Dupree, P., & Sherrier, D. J. (1998). The plant golgi apparatus. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1404*(1), 259–270.

Edwards, C. A., Blackburn, N. A., Craigen, L., Davison, P., Tomlin, J., Sugden, K., Johnson, I. T., & Read, N. W. (1987). Viscosity of food gums determined in vitro related to their hypoglycemic actions. *The American Journal of Clinical Nutrition*, *46*(1), 72–77.

Esau, K. (1977). Anatomy of seed plants. John Wiley and Sons. *New York, NY, USA.*

Esau, K. (1943). Vascular differentiation in the vegetative shoot of Linum. III. The origin of the bast fibers. *American Journal of Botany*, *30(8)*, 579–586.

Fahn, A. (1982). Plant anatomy. Pergamon Press. Oxford, UK.

Fekri, N., Khayami, M., Heidari, R., & Jamee, R. (2008). Chemical analysis of flaxseed, sweet basil, dragon head and quince seed mucilages. *Research Journal of Biological Sciences*, *3(2)*, 166–170.

Fenart, S., Ndong, Y. P., Duarte, J., Rivière, N., Wilmer, J., Van Wuytswinkel, O., Lucau, A., Cariou, E., Neutelings, G., Gutierrez, L., Chabbert, B., Guillot, X., Tavernier, R., Hawkins, S., Thomasset, B. (2010). Development and validation of a flax (*Linum usitatissimum* L.) gene expression oligo microarray. *BMC Genomics*, *11(1)*, 592-606.

Flax Council of Canada, & Saskatchewan Flax Development Commission. (2003). Growing flax: production, management & diagnostic guide. Flax Council of Canada, Saskatechewan Flax Development Commission.

Foster, R., Pooni, H. S., & Mackay, I. J. (1997). Quantitative evaluation of *Linum usitatissimum* varieties for dual-purpose traits. *The Journal of Agricultural Science*, *129*(2), 179–185.

Franz, G., & others. (1989). Polysaccharides in pharmacy: current applications and future concepts. *Planta Medica*, *55*(6), 493-497.

Freeman, T. P. (1995). Structure of flaxseed. Flaxseed in human nutrition. AOCS Press, USA.

Gamble, G. R., Snook, M. E., Henriksson, G., & Akin, D. E. (2000). Phenolic constituents in flax bast tissue and inhibition of cellulase and pectinase. *Biotechnology letters*, *22*(9), 741–746.

Gorshkova, T. A., Gurjanov, O. P., Mikshina, P. V, Ibragimova, N. N., Mokshina, N. E., Salnikov, V. V, Ageeva, M. V., Amenitskii, S. I., Chernova, T. E. & Chemikosova, S. B. (2010). Specific type of secondary cell wall formed by plant fibers. *Russian Journal of Plant Physiology*, *57*(3), 328–341.

Gorshkova, T. A., Salnikov, V. V, Pogodina, N. M., Chemikosova, S. B., Yablokova, E. V, Ulanov, A. V., Ageeva, M. V., Van Dam, J. E. G. & Lozovaya, V. V. (2000). Composition and distribution of cell wall phenolic compounds in flax (*Linum usitatissimum* L.) stem tissues. *Annals of Botany*, *85*(4), 477–486.

Gorshkova, T. A., Sal'nikov, V. V, Chemikosova, S. B., Ageeva, M. V, Pavlencheva, N. V, & Van Dam, J. E. G. (2003). The snap point: a transition point in *Linum usitatissimum* bast fiber development. *Industrial Crops and Products*, *18*(3), 213–221.

Gorshkova, T., Brutch, N., Chabbert, B., Deyholos, M., Hayashi, T., Lev-Yadun, S.,…& Pilate, G. (2012). Plant fiber formation: state of the art, recent and expected progress, and open questions. *Critical Reviews in Plant Sciences*, *31*(3), 201–228.

Gorshkova, T., & Morvan, C. (2006). Secondary cell-wall assembly in flax phloem fibres: role of galactans. *Planta*, *223*(2), 149–158.

Goswami, L., Dunlop, J. W. C., Jungnikl, K., Eder, M., Gierlinger, N., Coutand, C., …& Burgert, I. (2008). Stress generation in the tension wood of poplar is based on the lateral swelling power of the G-layer. *The Plant Journal*, *56*(4), 531–538.

Groth, J. V, Comstock, V. E., Anderson, N. A. (1970). Effect of seed color on tolerance of flax to seedling blight caused by Rhizoctonia solani. *Phytopathology*, *60(6809)*, 379–380.

Gutterman, Y., & Shem-Tov, S. (1996). Structure and function of the mucilaginous seed coats of Plantago coronopus inhabiting the Negev Desert of Israel. *Israel Journal of Plant Sciences*, *44*(2), 125–133.

Hahlbrock, K., & Grisebach, H. (1979). Enzymic controls in the biosynthesis of lignin and flavonoids. *Annual Review of Plant Physiology*, *30*(1), 105–130.

Harborne, J. B. (1998). Phytochemical methods a guide to modern techniques of plant analysis. Thomson Science, London, UK.

Harborne, J. B. (1967). Comparative biochemistry of the flavonoids. Academic Press, London, UK.

Harborne, J. B., & Williams, C. A. (1995). Anthocyanins and other flavonoids. *Natural Product Reports*, *12*(6), 639–657.

Harper, J. L., & Benton, R. A. (1966). The behaviour of seeds in soil: II. The germination of seeds on the surface of a water supplying substrate. *The Journal of Ecology*, *54(1)*, 151–166.

Haughn, G., & Chaudhury, A. (2005). Genetic analysis of seed coat development in *Arabidopsis*. *Trends in Plant Science*, *10*(10), 472–477.

Hayward, H. E. (1938). The structure of economic plants. Macmillan Company. London/New york.

Heydecker, W., & Orphanos, P. I. (1968). The effect of excess moisture on the germination of *Spinacia oleracea* L. *Planta*, *83*(3), 237–247.

Herzog, M., Dorne, A. M., & Grellet, F. (1995). *GASA*, a gibberellin-regulated gene family from *Arabidopsis thaliana* related to the tomato *GAST1* gene. *Plant Molecular Biology*, *27*(4), 743–752.

Hotte, N. S. C., & Deyholos, M. K. (2008). A flax fibre proteome: identification of proteins enriched in bast fibres. *BMC Plant Biology*, *8(1)*, 52-65.

Huis, R., Hawkins, S., & Neutelings, G. (2010). Selection of reference genes for quantitative gene expression normalization in flax (*Linum usitatissimum* L.). *BMC Plant Biology*, *10*(1), 71-85.

Jende-Strid, B. (1991). Gene-enzyme relations in the pathway of flavonoid biosynthesis in barley. *TAG Theoretical and Applied Genetics*, *81*(5), 668–674.

Jende-Strid, B. (2004). Genetic control of flavonoid biosynthesis in barley. *Hereditas*, *119*(2), 187–204.

Jenkins, D. J., Wolever, T. M., Kalmusky, J., Giudici, S., Giordano, C., Wong, G. S., Bird, J. N., Patten, R., Hall, M., Buckley, G. (1985). Low glycemic index carbohydrate foods in the management of hyperlipidemia. *The American Journal of Clinical Nutrition*, *42*(4), 604–617.

Jenkins, D. J., Wolever, T. M., Leeds, A. R., Gassull, M. A., Haisman, P., Dilawari, J., Goff, D. V, Metz, G. L., Alberti, G. K. (1978). Dietary fibres, fibre analogues, and glucose tolerance: importance of viscosity. *British Medical Journal*, *1*(6124), 1392–1394.

Jenkins, D., & Wolever, T. (2001). Effect of dietary fiber and foods on carbohydrate metabolism. CRC Handbook of Dietary Fiber in Human Nutrition, (3rd Ed.). CRC Press. Florida, US.

Jiang, Z., Ahn, D. U., & Sim, J. S. (1991). Effects of feeding flax and two types of sunflower seeds on fatty acid compositions of yolk lipid classes. *Poultry Science*, *70*(12), 2467–2475.

Jofuku, K. D., Den Boer, B. G., Van Montagu, M., & Okamuro, J. K. (1994). Control of Arabidopsis flower and seed development by the homeotic gene *apetala2*. *The Plant Cell*, *6*(9), 1211–1225.

José, I., Amaya, I., Castillejo, C., Sánchez-Sevilla, J. F., Quesada, M. A., Botella, M. A., & Valpuesta, V. (2006). The strawberry gene *FaGAST* affects plant growth through inhibition of cell elongation. *Journal of Experimental Botany*, *57*(10), 2401–2411.

Karssen, C. M., Swan, D. L. C. B. Der, Breekland, A. E., & Koornneef, M. (1983). Induction of dormancy during seed development by endogenous abscisic acid : studies on abscisic acid deficient genotypes of *Arabidopsis thaliana* (L .) Heynh. *Planta, 157(2)*, 158–165.

Kishk, Y. F. M., Elsheshetawy, H. E., & Mahmoud, E. A. M. (2011). Influence of isolated flaxseed mucilage as a non-starch polysaccharide on noodle quality. *International Journal of Food Science & Technology*, *46*(3), 661–668.

Kotilainen, M., Helariutta, Y., Mehto, M., Pöllänen, E., Albert, V. A., Elomaa, P., & Teeri, T. H. (1999). GEG participates in the regulation of cell and organ shape during corolla and carpel development in *Gerbera hybrida*. *The Plant Cell Online*, *11*(6), 1093–1104.

Kozlowska, J., Muñoz, G. A., & Kolodziejczyk, P. P. (2008). Food and feed applications for flaxseed components. In *International Conference on Flax and other Bast Plants*. Saskatchewan.

Kreitschitz, A. (2009). Biological properties of fruit and seed slime envelope: How to live, fly, and not die. *Functional Surfaces in Biology*. Springer Dordrecht Heidelberg, London.

Kunieda, T., Mitsuda, N., Ohme-Takagi, M., Takeda, S., Aida, M., Tasaka, M., Kondo, M., et al. (2008). NAC family proteins NARS1/NAC2 and NARS2/NAM in the outer integument regulate embryogenesis in *Arabidopsis*. *The Plant Cell*, *20*(10), 2631–2642.

Lees, G. L., Suttill, N. H., & Gruber, M. Y. (1993). Condensed tannins in sainfoin. 1. A histological and cytological survey of plant tissues. *Canadian Journal of Botany*, *71*(9), 1147–1152.

Leeson, S., Summers, J. D., & Caston, L. J. (2000). Response of layers to dietary flaxseed according to body weight classification at maturity. *The Journal of Applied Poultry Research*, *9*(3), 297–302.

Leon-Kloosterziel, K. M., Keijzer, C. J., & Koornneef, M. (1994). A seed shape mutant of *Arabidopsis* that is affected in integument development. *The Plant Cell*, *6*(3), 385–392.

Lerouxel, O., Cavalier, D. M., Liepman, A. H., & Keegstra, K. (2006). Biosynthesis of plant cell wall polysaccharides - a complex process. *Current Opinion in Plant Biology*, *9*(6), 621–630.

Lev-Yadun, S. (2001). Aposematic (warning) coloration associated with thorns in higher plants. *Journal of Theoretical Biology*, *210*(3), 385–388.

Love, G. D., Snape, C. E., Jarvis, M. C., & Morrison, I. M. (1994). Determination of phenolic structures in flax fibre by solid-state ^{13}C NMR. *Phytochemistry*, *35*(2), 489–491.

Lynch, M., & Conery, J. S. (2000). The evolutionary fate and consequences of duplicate genes. *Science*, *290*(5494), 1151–1155.

Mackiewicz-Talarczyk, M.,Barriga-Bedoya, J., Manskowski, J., & Pniewska, I. (2009). Global flax market situation. In *2008 International Conference on Flax & Other Bast Plants*, Saskatoon, Canada.

Marby, J. T., Harborne, J. B., Marby, H., (1975). The flavonoids. Academic Press. New York, US.

Mazza, G. and Oomah, B. D. (1995). Flaxseed, dietary fiber, and cyanogens. Flaxseed in human nutrition. AOCS Press, USA.

McAbee, J. M., Hill, T. A., Skinner, D. J., Izhaki, A., Hauser, B. A., Meister, R. J., Reddy, G. V., Meyerowitz, E. M., Bowman, J. L., Gasser, C. S. (2006). Aberrant testa shape encodes a kanadi family member, linking polarity determination to separation and growth of Arabidopsis ovule integuments. *The Plant Journal*, *46*(3), 522–531.

McFarlane, H. E., Young, R. E., Wasteneys, G. O., & Samuels, A. L. (2008). Cortical microtubules mark the mucilage secretion domain of the plasma membrane in *Arabidopsis* seed coat cells. *Planta*, *227*(6), 1363–1375.

Mittapalli, O., & Rowland, G. (2003). Inheritance of seed color in flax. *Crop Science*, *43(6)*, 1945–1951.

Mellerowicz, E. J., Baucher, M., Sundberg, B., & Boerjan, W. (2001). Unravelling cell wall formation in the woody dicot stem. *Plant Molecular Biology*, *47*(1), 239–274.

Mellerowicz, E. J., & Sundberg, B. (2008). Wood cell walls: biosynthesis, developmental dynamics and their implications for wood properties. *Current Opinion in Plant Biology*, *11*(3), 293–300.

Mokshina, N. E., Ibragimova, N. N., Salnikov, V. V, Amenitskii, S. I., & Gorshkova, T. A. (2012). Galactosidase of plant fibers with gelatinous cell wall: Identification and localization. *Russian Journal of Plant Physiology*, *59*(2), 246–254.

Mooney, C., Stolle-Smits, T., Schols, H., & De Jong, E. (2001). Analysis of retted and non retted flax fibres by chemical and enzymatic means. *Journal of Biotechnology*, *89*(2), 205–216.

Morris, D. H. (2007). Flax: A health and nutrition primer. Flax Council of Canada.

Moïse, J. a., Han, S., Gudynaitę-Savitch, L., Johnson, D. a., & Miki, B. L. a. (2005). Seed coats: Structure, development, composition, and biotechnology. *In Vitro Cellular & Developmental Biology - Plant*, *41*(5), 620–644.

Morrison III, W. H., Himmelsbach, D. S., Akin, D. E., & Evans, J. D. (2003). Chemical and spectroscopic analysis of lignin in isolated flax fibers. *Journal of Agricultural and Food Chemistry*, *51*(9), 2565–2568.

Muir, A. D., & Westcott, N. D. (2003). Flax: the genus Linum. Taylor & Francis, New York, US.

Mühlethaler, K. (1950). The structure of plant slimes. *Experimental Cell Research*, *1*(2), 341–350.

Müller, M., Burghammer, M., & Sugiyama, J. (2006). Direct investigation of the structural properties of tension wood cellulose microfibrils using microbeam X-ray fibre diffraction. *Holzforschung*, *60*(5), 474–479.

Nakaune, S., Yamada, K., Kondo, M., Kato, T., Tabata, S., Nishimura, M., & Hara-Nishimura, I. (2005). A vacuolar processing enzyme, δVPE, is involved in seed coat formation at the early stage of seed development. *The Plant Cell*, 17(3), 876-887.

Naqvi, P. A., Rai, M., & Vasishtha, V. (1987). Association of different components of seed and oil in linseed. *Indian Agricultural Science*, *57*(4), 231–236.

Oomah, B. D., Kenaschuk, E. O., & Mazza, G. (1995). Phenolic acids in flaxseed. *Journal of Agricultural and Food Chemistry*, *43*(8), 2016–2019.

Panikashvili, D., Shi, J. X., Schreiber, L., & Aharoni, A. (2009). The *Arabidopsis* DCR encoding a soluble BAHD acyltransferase is required for cutin polyester formation and seed hydration properties. *Plant Physiology*, *151*(4), 1773–1789.

Popescu, F., & Marinescu, I. (1996). *Y1-a* dominant gene for yellow colour of oil flax seeds. *Probleme de genetica teoretica si aplicata*. *28(2)*, 99-106.

Peng, J. Z., Lai, L. J., & Wang, X. J. (2008). PRGL: A cell wall proline-rich protein containning GASA domain in *Gerbera hybrida*. *Science in China Series C: Life Sciences*, *51*(6), 520–525.

Pilate, G., Déjardin, A., Laurans, F., & Leplé, J. C. (2004). Tension wood as a model for functional genomics of wood formation. *New Phytologist*, *164*(1), 63–72.

Ragupathy, R., Rathinavelu, R., & Cloutier, S. (2011). Physical mapping and BAC-end sequence analysis provide initial insights into the flax (*Linum usitatissimum* L.) genome. *BMC Genomics*, *12*(1), 217.

Reddy, N. & Yang, Y. (2005). Biofibers from agricultural byproducts for industrial applications Biofibers from agricultural byproducts for industrial applications. *Trends in Biotechnology, 23(1)*, 22-27.

Roach, M. J., & Deyholos, M. K. (2007). Microarray analysis of flax (*Linum usitatissimum* L.) stems identifies transcripts enriched in fibre-bearing phloem tissues. *Molecular Genetics and Genomics*, *278*(2), 149–165.

Roach, M. J., & Deyholos, M. K. (2008). Microarray analysis of developing flax hypocotyls identifies novel transcripts correlated with specific stages of phloem fibre differentiation. *Annals of Botany*, *102*(3), 317–330.

Roach, M. J., Mokshina, N. Y., Badhan, A., Snegireva, A. V, Hobson, N., Deyholos, M. K., & Gorshkova, T. A. (2011). Development of cellulosic secondary walls in flax fibers requires β-galactosidase. *Plant Physiology*, *156*(3), 1351–1363.

Rodriguez, M. L., Alzueta, C., Rebole, A., Ortiz, L. T., Centeno, C., & Trevino, J. (2001). Effect of inclusion level of linseed on the nutrient utilisation of diets for growing broiler chickens. *British Poultry Science*, *42*(3), 368–375.

Rowland, G. G., Wilen, R., Hormis, Y., Saeidi, G., & Ntiamoah, C. (2003). Modification of flax (*Linum usitatissimum*) by induced mutagenesis and transformation. *Improvement of new and traditional industrial crops by induced mutations and related biotechnology*, IAEA, Vienna, Austria.

Roxrud, I., Lid, S. E., Fletcher, J. C., Schmidt, E. D. L., & Opsahl-Sorteberg, H. G. (2007). *GASA4*, one of the 14-member *Arabidopsis* GASA family of small polypeptides, regulates flowering and seed development. *Plant and Cell Physiology*, *48*(3), 471–483.

Saeidi, G., & Mirik, A. A. M. (2006). Fungicide seed treatment and seed colour effects on seed vigour and emergence in flax. *International Journal of Agriculture & Biology*, *8*(6), 732–735.

Saeidi, G., & Rowland, G. G. (1997). The inheritance of variegated seed color and palmitic acid in flax. *The Journal of Heredity, 88(6),* 466-468.

Segura, A., Moreno, M., Madueño, F., Molina, A., & García-Olmedo, F. (1999). Snakin-1, a peptide from potato that is active against plant pathogens. *Molecular Plant-Microbe Interactions*, *12*(1), 16–23.

Shi, L., Gast, R. T., Gopalraj, M., & Olszewski, N. E. (1992). Characterization of a shoot-specific, *GA3*-and *ABA-Regulated* gene from tomato. *The Plant Journal*, *2*(2), 153–159.

Snegireva, A. V, Ageeva, M. V, Amenitskii, S. I., Chernova, T. E., Ebskamp, M., & Gorshkova, T. A. (2010). Intrusive growth of sclerenchyma fibers. *Russian Journal of Plant Physiology*, *57*(3), 342–355.

Somerville, C., Bauer, S., Brininstool, G., Facette, M., Hamann, T., Milne, J., Osborne, E., et al. (2004). Toward a systems approach to understanding plant cell walls. *Science Signalling*, *306*(5705), 2206-2211.

Sood, S. P., Bhateria, S., Sood, S. (2012). Inheritance of flower & seed colour in Flax (*Linum usitatissimum* L .) over environments in north western Himalaya. *National Academy Science Letters*, *35*(3), 127–130.

Sun, Y., Tan, D. Y., Baskin, C. C., & Baskin, J. M. (2012). Role of mucilage in seed dispersal and germination of the annual ephemeral Alyssum minus (Brassicaceae). *Australian Journal of Botany*, *60(5)*, 439–449.

Staehelin, L. A., & Moore, I. (1995). The plant Golgi apparatus: structure, functional organization and trafficking mechanisms. *Annual Review of Plant Biology*, *46*(1), 261–288.

Styles, E. D., & Ceska, O. (1989). Pericarp flavonoids in genetic strains of Zea mays. *Maydica*, *34(3)*, 227-237.

Taylor, B. H., & Scheuring, C. F. (1994). A molecular marker for lateral root initiation: the *RSI-1* gene of tomato (*Lycopersicon esculentum* Mill) is activated in early lateral root primordia. *Molecular and General Genetics MGG*, *243*(2), 148–157.

Vaisey-genser, M., & Morris, D. H. (2003). Introduction History of the cultivation and uses of flaxseed. Flax: the genus linum. Taylor & Francis, New York.

Venglat, P., Xiang, D., Qiu, S., Stone, S. L., Tibiche, C., Cram, D., Alting-Mees, M., Nowak, J., Cloutier, S., Deyholos, M. K., Bekkaoui, F., Sharpe, A., Wang, E., Rowland, G., Selvaraj, G., Datla, R. (2011). Gene expression analysis of flax seed development. *BMC Plant Biology*, *11*(1), 74-89.

Wang, Z., Hobson, N., Galindo, L., Zhu, S., Shi, D., McDill, J., Yang, L., Hawkins, S., Neutelings, G., Datla, R., Lambert, G., Galbraith, D. W., Grassa, C. J., Geraldes, A., Cronk, Q. C., Cullis, C., Dash, P. K., Kumar, P. A., Cloutier, S., Sharpe, A. G., Wong, J. K. S., Wang, J., Deyholos, M. K. (2012). The genome of flax (*Linum usitatissimum*) assembled de novo from short shotgun sequence reads. *The Plant Journal*. 72(3), 461-473.

Wasteneys, G. O., & Galway, M. E. (2003). Remodeling the cytoskeleton for growth and form: an overview with some new views. *Annual Review of Plant Biology*, *54*(1), 691–722.

Watkins, T. R., Tomeo, A. C., Struck, M. L., Palumbo, L., & Bierenbaum, M. L. (1995). Improving atherogenic risk factors with flax seed bread. *Developments in Food Science*, *37*, 649–658.

Western, T. L., Skinner, D. J., & Haughn, G. W. (2000). Differentiation of mucilage secretory cells of the Arabidopsis seed coat. *Plant Physiology*, *122*(2), 345–356.

Western, T. L. (2012). The sticky tale of seed coat mucilages : production, genetics, and role in seed germination and dispersal. *Seed Science Research*, 22(1), 1-25.

Western, T. L., Burn, J., Tan, W. L., Skinner, D. J., Martin-mccaffrey, L., Moffatt, B. A., & Haughn, G. W. (2001). Isolation and characterization of mutants defective in seed coat mucilage secretory cell development in *Arabidopsis*. *Plant Physiology*, 127(3), 998-1011.

Western, T. L., Skinner, D. J., & Haughn, G. W. (2000). Differentiation of mucilage secretory cells of the *Arabidopsis* seed coat. *Plant Physiology*, *122*(2), 345–356.

Western, T. L., Young, D. S., Dean, G. H., Tan, W. L., Samuels, A. L., & Haughn, G. W. (2004). Mucilage-modified4 encodes a putative pectin biosynthetic enzyme developmentally regulated by *apelata2, transparent testa glabra1*, and *glabra2* in the *Arabidopsis* seed coat, *Plant Physiology*, *134*(1), 296–306.

Windsor, J. B., Symonds, V. V, Mendenhall, J., & Lloyd, A. M. (2001). *Arabidopsis* seed coat development: morphological differentiation of the outer integument. *The Plant Journal*, *22*(6), 483–493.

Wolever, T. M. S. (1995). Flaxseed and glucose metabolism. Flaxseed in human nutrition. AOCS Press, USA.

Yang, X., Dong, M., & Huang, Z. (2010). Role of mucilage in the germination of *Artemisia sphaerocephala* (Asteraceae) achenes exposed to osmotic stress and salinity. *Plant Physiology and Biochemistry*, *48*(2), 131–135.

Young, J. A., & Evans, R. A. (1973). Mucilaginous seed coats. *Weed Science*, *21(1)*, 52–54.

Young, R. E, McFarlane, H. E., Hahn, M. G., Western, T. L., Haughn, G. W., & Samuels, A. L. (2008). Analysis of the golgi apparatus in *Arabidopsis* seed coat cells during polarized secretion of pectin-rich mucilage. *The Plant Cell Online*, *20*(6), 1623–1638.

Zhang, S. C., & Wang, X. J. (2008). Expression pattern of *GASA*, downstream genes of DELLA, in *Arabidopsis*. *Chinese Science Bulletin*, *53*(24), 3839–3846.

Zhang, S., & Wang, X. (2011). Overexpression of *GASA5* increases the sensitivity of *Arabidopsis* to heat stress. *Journal of Plant Physiology*, *168*(17), 2093–2101.

Zhang, S., Yang, C., Peng, J., Sun, S., & Wang, X. (2009). *GASA5*, a regulator of flowering time and stem growth in *Arabidopsis thaliana*. *Plant Molecular Biology*, *69*(6), 745–759.

Chapter 2 Seed Coat & Mucilage Mutant Screen

2.1 Introduction

To better understand the functions of seed mucilage in plant development, and to provide novel germplasm to flax breeders, my objective was to identify flax mutants that produced less mucilage or had otherwise abnormal mucilage. Seeds with abnormal pigmentation may also be useful for some applications including use as varietal identification markers. Therefore, I performed a phenotypic screen of seed color and mucilage production in an EMS mutant population of the elite linseed cultivar CDC Bethune.

2.2 Methods and Materials

2.2.1 Mutant Population Development

Seeds of *Linum usitatissimum* L. cultivar CDC Bethune, received from Gordon Rowland (University of Saskatchewan, Saskatoon, SK). Seeds were mutagenized in 5 volumes of 0.5% ethyl methyl sulfonate (EMS) in 25 mM phosphate buffer (pH 7.6) for 4 hours at room temperature and then were rinsed with distilled water (three times), and then air dried prior to planting. These M_1 seeds were sown at the University of Alberta farm (Edmonton, AB), and their M_2 progeny were harvested as individual families. One seed from each M_2 family was grown in growth chambers at the University of Alberta, and their progeny (i.e. M_3 families) were harvested on individual plant basic. The M_3 generation seeds were sown in rows at Kernen Farm (Saskatoon, SK) in summer 2010, with approximately six seeds planted from each M_3 family. The M_4 seeds were harvested from individual M_3 plants, then threshed and stored at ambient temperature in envelopes until screening.

2.2.2 Screening of M_4 Generation

All of the seeds (30 seeds in average) in each M_4 family were visually inspected for abnormalities for seed color and morphology. The mucilage screening was conducted using a protocol modified from Arsovski et al. (2009). Five seeds from each M_4 family were immersed in distilled water in a 48-well culture plate, and then agitated for 1 hour at 750 rpm using a Heidolph Titramax 100. The water was replaced with 0.05% (w/v in distilled water) Toluidine Blue O dye solution, and agitated on a shaker for another 30 minutes. Seeds were observed under a stereo dissecting microscope and photographed using a digital camera. After observation, seeds were rinsed with water and dried by incubating at 28°C overnight, and returned to their original envelope to allow them to be germinated later, if needed. For field-grown seeds from the 2012 season, the standard protocol was modified as follows: ten seeds from each family were incubated in distilled water for 1 hour but without agitation.

2.2.3 Plant Growth Conditions

Except for field grown plants, seeds from M_4 family were sown in Metromix 360 (Scotts, Maryland, OH), planted in 10 cm pots, with four seeds per pot. Plants were grown in a glass house at the University of Alberta, under environmental conditions of 20^{o}C, 50% humidity, and a light intensity ranging from 500 μE to 1000 μE depending on cloud cover. The light cycle was controlled as 16 h light/ 8 h dark cycle with supplementation by artificial light. Seeds from M_4 family were harvested at maturity and stored at ambient temperature.

2.2.4 Paraffin Embedding for Bright-Field Microscopy

Ten mature seeds were randomly selected from the wild-type cultivar CDC Bethune and each of four different M_4 mutant families representing the following pigment and mucilage phenotypic classes: Yellow, Cream, No Mucilage Released, and Blue Seed Staining. Seeds were fixed in FAA (4% [v/v] paraformaldehyde, 15% [v/v] acetic acid, and 50% [v/v] ethanol) at room temperature overnight. Seeds were dehydrated in a Leica TP1020 Tissue Processor, and then embedded in paraffin using a Tissue TEK II. Sections of 8 μm size were cut on a Leica RM 2125 RTS microtome, and these sections were mounted on glass slides then incubated overnight. Tissues were stained for 20 seconds with 0.5% [w/v] TBO in water. Sections were photographed using a Zeiss AX10 light microscope. Photographs were edited with Abode Photoshop CS to prepare composite figures.

2.3 Results

2.3.1 Seed Color Screen

I screened seeds from a total of 16,764 field-grown M_4 mutant families. Generally, six M_4 families were harvested from each M_3 family. Thus approximately 2,794 M_3 mutant families were represented in the population I screened. On average, 30 seeds of each M_4 families were screened for seed color, and five seeds from each family were screened for mucilage.

The phenotype of wild-type CDC Bethune flax seed was uniformly brown (Figure 2-1a). The seeds were oval shaped with a pointed tip, and were approximately 5 mm x 2.5 mm in size. Their seed coats were shiny and smooth in texture. Five classes of abnormal seed color phenotypes were observed in the mutant population: Yellow, Green, Cream, Brown-Gradient and Brown-Variegated (Figure 2-1b to 2-1f). As shown in Table 2-1, I identified 56 M_4 families with yellow seeds. In 54/56 of these families, all seeds had the same yellow phenotype whereas in the

other 2/56 families, approximately 80% of the seeds were yellow and the remainder were brown. In the Green phenotypic class, 25 families with green seeds were identified; in 16/25 of these families all of the seeds were green while in 9/25 families, a portion of seeds (10% to 90%) were green. In the Cream class, all seeds from two M_4 families had a cream colored seed coat with irregular areas of pale brown pigment on the seed coat surface. In the Brown-Gradient class, all seeds of two M_4 families were dark brown at the micropylar end of the seed, and this coloration gradually diminished towards the chalazal end of the seed. Finally, in the Brown-Variegated class, all of the seeds of one M_4 family had a distinct border between an irregularly shaped brown region at the micropylar end, and a yellow region at the chalazal end.

2.3.2 Mucilage Screening

In wild-type CDC Bethune linseed, mucilage was released from the surface of the seed upon hydration. After wild-type seeds were stained with Toluidine Blue O (TBO, a meta-chromatic dye), these seeds displayed a uniform halo of mucilage with a light-purple color, and a dark TBO-stained seed surface (Figure 2-2a). While screening five seeds from each of 16,764 M_4 families**,** three classes of abnormal phenotypes were observed after TBO staining: Blue Seed, No Mucilage Released, and Abnormal Mucilage Released (Figure 2-2b to 2-2d).

In the 885 M_4 families classified in the Blue Seed class (Table 2-2), parts of the seed (excluding the mucilage) were stained blue. This phenotype was seen in at least 4/5 of these seeds tested from 825/885 families in the Blue Seed class. In the remaining 54/885 families, the mutant phenotype was seen only in 3/5 seeds. The Blue Seed phenotype was occasionally observed in 1/5 or 2/5 seeds from other M_4 families, but these were not recorded as putative mutants. The blue staining was varied from dark blue to light blue, or green-blue. The

distribution of the blue pigments also varied, appearing either thickly and evenly over the surface, or intensely in the micropylar end of the seed coat. The mucilage was present as a thin layer covering the seed surface, and appeared as a diffuse halo surrounding the seed. The TBO-staining of the mucilage varied from light to heavy, with the heaviest staining generally observed near the edge of the halo. Interestingly, there was considerable overlap between one of the mucilage phenotypes and one of the seed coat color phenotypes: 56/885 blue-staining families were yellow-seeded, and all yellow-seeded families stained blue with TBO.

In the second mucilage-related phenotypic class, No Mucilage Released, 838 M_4 families did not produce a halo of mucilage (this putative mutant phenotype was observed in at least 4/5 seeds per family). Instead, in these putative mutants, the seed coat was partly stained with purple patches, and no halo was seen. In some cases, little mucilage content was released from the micropylar end, or presented as small distinct pieces attached to the seed coat.

Finally, in the Abnormal Mucilage Released phenotypic class, 5/5 tested seeds from 252 M_4 families released mucilage, but the halo of mucilage in these putative mutants appeared to have irregular morphology (Table 2-2). These mutants exhibited a similar seed coat phenotype to the wild type. The level of mucilage was dramatically increased, and presented as a double thickness of halo of mucilage in most of the cases. In some instances, a clump of mucilage was either intensely accumulated at the seed edge, exposed the seed surface, or totally enveloped the seed.

2.3.3 Progeny Test for Seed Color and Mucilage Phenotypes in Glasshouse Grown Plants

Because of limitations on growth space, I selected a subset of putative seed color mutants for further characterization. I grew eight M_4 families from the Yellow phenotypic class and 19

M_4 families from the Green phenotypic class. The selection of these families was based in part on preference for families that had a large number of available seeds. Vegetative and floral morphology of all M_4 individuals was indistinguishable from wild-type, except for petal color, which was white (rather than blue) in three of the Yellow families. All seeds produced by all M_4 Yellow families were yellow (Table 2-3), showing that the Yellow phenotype was heritable in all families tested. In contrast, the Green seed phenotype was not inherited in any of the families tested.

To determine the stability of mucilage phenotypes, four seeds from each of 19 M_4 families of the Blue Seed class and four seeds from each of 48 M_4 families with No Mucilage Released were grown in the glasshouse. The number of families that could be tested was limited by space availability and these particular families were selected based on several criteria including having well-defined phenotypes with a large number of available M_4 seeds. Because the Abnormal mucilage phenotype was highly variable, further study of these mutants was not pursued. Plants were allowed to self-pollinate, and 108 days after planting, the mature, dry seeds of the M_5 generation were harvested. Ten seeds from each M_5 family were stained with TBO. As shown in Table 2-4, within the No Mucilage Released phenotypic category, 33 M_4 families (33/48) produced the same mutant phenotype among all of their M_5 seed, and 10/48 showed the mutant phenotype among only some of their M_5 seed (Table 2-5). The No Mucilage Released phenotype was not stability in any of the seed of 4/48 families, and one family (line 3219) produced the Abnormal Mucilage phenotype (Table 2-4). Among the Blue Seed M_4 families, 12/19 showed the same mutant phenotype among all of their M_5 seeds, 4/19 had the wild-type purple staining among all of their seeds and no mucilage released. There were 3/19 blue staining and surprisingly also had no mucilage (Table 2-6).

2.3.4 Mucilage Phenotypes in M_6 Seed From Field Grown Plants

Having demonstrated that the phenotypes initially observed during the screen of M_4 field-grown seeds were generally heritable when M_5 seed was collected from the glass house, I wanted to test heritability of seed coat phenotypes in a subsequent field-grown generation. I randomly selected 25 families of M_5 seed. When grown in the glasshouse, 20 of these families showed the involved No Mucilage Released phenotype (15 families homozygous No Mucilage Released, 5 families segregated with wild-type homozygous), one family showed the Abnormal Mucilage Released phenotype, and four families with wild-type phenotype. I was interested to know whether the mutant phenotype in these five segregated families might appear again if they were planted in the field.

The M_5 seeds were planted in May 2012 in short rows at Kernen Farm, Saskatoon, by field staff working in the flax breeding program. CDC Bethune was used as a check and was planted in alternating rows with the mutants. For unexplained reasons, the mutants and the CDC Bethune checks did not initially grow well, and by July appeared stunted compared to flax in other rows and plots at the farm. By August, the plants had recovered somewhat (mostly through the increased production of lateral branches). Seeds that were harvested from these field-grown plants appeared normal, prior to staining. However, even in non-mutagenized CDC Bethune from the 2012 field-grown rows, mucilage could not be detected when using our standard staining protocol (Section 2.2.3). Many of the field grown seeds floated during the agitation, which may have limited water absorption and the release of mucilage. Thus, I modified the staining protocol by first imbibing seeds in water without agitation to increase hydration, and then I followed the same staining procedure with TBO as in the standard protocol. Using this

modified procedure, mutant phenotypes could be distinguished in some of the families. Ten seeds from each family were tested to determine the putative mutant of those families. Within 20 M_5 families with the No Mucilage Released phenotype (Table 2-7), six families showed stability of the phenotype in M_6 seeds, two lines showed Blue Seed Staining phenotype, and 12 families showed the wild type phenotype. In four wild-type M_5, two families were Blue Seed phenotype, one family was detected with No Mucilage Released, and one family showed wild-type phenotype. The Abnormal Mucilage Released (line 103(3219)) reproduced within its M_6 seeds.

2.3.5 Microscopy of Seed Coat

Transverse sections of the testa of mature seeds were analyzed to detect changes in anatomy that might explain the observed abnormalities in pigmentation or mucilage production. Wild-type (CDC Bethune) and representative mutants in the following phenotypic classes were studied: Yellow, Cream, No Mucilage Released, and Blue Seeds (Figure 2-4). In the mature wild-type seed coat, four layers could be distinguished (listed from the outside to the inside): (Ep) the mucilage producing epidermal layer; (Sc) the sclerenchymatous fiber layer; (Tr) the transversal-cells (or cross cells) layer; and (Pi) a pigment layer. Mucilage was present as an extrusion surrounding the outer layer of seed coat. The mucilage and remnants of epidermis were stained dark purple. The thick cell wall of the fiber layer and the thin walls of the cross layer stained dark blue. Cells of the pigment layer appeared to be filled with a brown substance.

In the mucilage mutants (Figure 2-4a, b), the cellular organization of No Mucilage Released seeds appeared a distinguished thin transversal-layer, while no difference was observed in the Blue Seed testa compared to wild-type. All cell layers were present with normal structure and organization. The outer cell wall of the epidermal cells has burst and mucilage was excreted,

and the cell wall material attached to the mucilage sometimes formed a distinctive ring-shaped mucilage pocket.

In the pigmentation mutants from the Yellow and Cream classes (Figure 2-4d, e), the absence of pigment layer, and a thin transversal-layer made the testa was overall thinner than wild type. The sclerenchymatous fiber layer was present, but had cell walls that appeared thinner than wild-type. A distinct transversal-layer was visible in the mutant from the Yellow class, and this layer remained attached to adjacent layers in all sections examined. In contrast, in the Cream class mutant, the transversal cell layer was less prominent than in any of the other genotypes and was easily separated from adjacent layers.

2.4 Discussion

A forward genetic screen of field-grown flax seeds collected from individual plants was designed to identify mutants with abnormal seed coat morphology. The specific phenotypes targeted were defects in pigmentation or mucilage. Because seed coat is a maternal, sporophytic tissue, and all of the seeds screened were progeny of at least M_3 generation, I expected that all of the seed coats produced by a given plant would have the same genotype and phenotype. In total, 2,062 M_4 families derived from 1,577 M_3 lines with putative mutated pigmentation and/or mucilage were identified, of which 53/94 families tested showed heritable phenotypes. Because of time constraints, complementation tests were not completed, and so the number of distinct loci identified has not been determined. Nevertheless, the large number of mutants observed and recovered suggests that the mutation frequency in this EMS population was high, and therefore this population can also be used effectively in other forward and reverse genetics screens.

2.4.1 Variation of Seed Coat Color

The screen for seed pigmentation defects identified five different phenotypic classes of seed coat colors. Four of these were within the range of seed color phenotypes (ranging from yellow to dark brown) reported by the Plant Gene Resources Canada seed bank at Saskatoon (Diederichsen & Richards, 2003). The darker brown seeds were due to more pigment cells in the seed coat, and generally reported to be dominant to the lighter yellow seeds (Barnes et al., 1960; Mittapalli & Rowland, 2003). The seeds with green pigmentation could be due to the accumulation of a dark tannin-liked component or chlorophyll in the ring-cell layer of seed coat (Diederichsen & Richards, 2003), but because the green seed phenotype did not transmit to the progenies, it is more likely that this trait was due to immaturity of the seed, possibly due to environmental factors or mutations that affect seed maturation. At early stages of seed development, flax seeds are green due to the chlorophyll in the developing embryo and lack of pigments in the immature seed coat. As the embryo matures and the seed becomes desiccated, seeds start to lose their green color and turn brown (Western, Skinner, & Haughn, 2000).

A set of confirmed yellow seed mutants (lines 4524(6), 5091(2), 3252(4), 848(4), 4334(2), 515(5), 755(4), 755(1)) were passed to collaborators at the National Research Council – Saskatoon (Dr. Gopalan Selvaraj), who will further characterize them and use them in development of yellow seeded linseed varieties.

The variegated seed line (line 361), which had a distinctive appearance will be provided to breeders at the Crop Diversification Center (Dr. Helen Booker) for preliminary field testing. If the yield and performance of these mutant seeds is close to the elite parental variety (CDC Bethune), it may be possible to use the variegated seed coat trait in the future as a distinctive marker for seeds with other unique traits, e.g. modified oils.

2.4.2 Mucilage Screening

I identified 1,970 putative mutants with defects in mucilage production, which I categorized into three different phenotypic classes. Thirty-three confirmed, heritable mutant families in the glass house (2812(2), 3219(4), 3112(5), 3160(3), 2667(1), 3067(4), 3181(1), 3176(1), 3232(6), 2712(1), 3220(6), 3204(2), 3200(5), 3092(6), 3050(3), 3218(4), 3017(4), 3046(4), 2607(6), 2811(1), 2829(1), 2754(4), 3064(2), 3020(3), 3049(4), 3049(5), 3186(3), 3100(1), 2814(5), 4984(1), 2694(6), 3224(2), 3215(3)) in the phenotypic class No Mucilage Released, revealed a lack of mucilage compared to wild-type seeds. The visible phenotype of No Mucilage Released mutants was similar in character to the *mucilage modified* (*mum*) mutants in Arabidopsis (Western et al., 2001, 2004). Three types of *mum* mutants have been described: mutants of pectin modification, mutants affecting cytoplasmic rearrangement, and mutants of mucilage biosynthesis (Western et al., 2001). As cell wall or mucilage modification can also cause defects in mucilage release (Western, 2006), further microscopic analysis of epidermal wall structure will be conducted in those seeds to infer whether a reduced quantity of mucilage or changes in the epidermal cells led to disruptions in mucilage release. The composition of the mucilage can also be tested using for example, quantitative colorimetric assays for uronic acids (which are characteristic of pectin), or GC/MS to measure the monosaccharide composition of the mucilage. This novel germplasm may be useful in future efforts to breed linseed with modified feed and meal properties.

In 12 confirmed mutants of the Blue Seed phenotypic class (line 5045(5), 755(4), 4936(3), 2591(2), 2729(2), 4334(2), 848(4), 1193(5), 3252(4) 5091(2), 3143(2), 4780(2)), the seed surface was covered with only a thin mucilage layer that led to the blue-staining of round

cells and sclerenchymatous layer within the seed coat being clearly visible. Based on the staining properties of toluidine blue O (O'brien et al., 1964), the blue or green-blue staining is probably produced in the presence of lignified compound. The changing in the color of seed coat cells could be due to either the increased permeability of the outer tangential cell wall, or specific mutant genes encoded for modifications of the cell wall. Further study using thin plastic sections of those seeds stained with TBO will help to reveal the structure and composition of the seed coat.

Alterative morphologies of halo of mucilage released have been observed in the Abnormal Mucilage Released phenotype, which is a novel phenotype not been reported in previous studies. It suggests a potentially modification in mucilage composition. However, this mutant phenotype appeared to have highly variable expressivity and was not pursued further.

2.4.3 The Stability of Putative Mucilage Mutants

EMS treatment introduces base substitutions (especially G/C to A/T) or small deletions in the genome, and the mutation within each seed will give rise and be carried forward into future generation (Østergaard & Yanofsky, 2004). Generally, the M_2 seeds are planted and screened for the desired phenotypes, the frequency of mutant segregation in this generation is dependent on the genetically effective cell number (GECN) (Page, Grossniklaus, & others, 2002). In flax, the GECN is reported to be four (Bretagne-Sagnard, Fouilloux, & Chupeau, 1996), so the segregation of homozygous recessive individuals should be 15 normal individuals: 1 recessive phenotype within the M_2 generation, and two of the 15 phenotypically normal siblings should be heterozygous. I screened 16,764 M_4 families, which were derived from approximately 2,794

different M_2 plants. Because the GECN in flax is at least 4 cells; that means the likely more than 2,794 different independent genotypes are represented among the 16,764 M_4 families I screened.

The overlap between the yellow seed pigmentation phenotype and the Blue Seed TBO-staining mutants might result from defective seed coat development, and those defects may affect the release of seed coat mucilage (section 2.3.3). Those putative mutants also showed some phenotypic instability after two subsequent generations (M_5 in glass house and M_6 in field trial). This indicates considerable influence of the growing condition on the ability of mucilage to be released.

The confirmation of those putative mutant phenotypes is still in the progress. Test for allelism could also be employed to determine whether those mutants with similar phenotypes are represent alleles of the same gene (Weigel & Glazebrook, 2008). Individual mutants could be analyzed in further detail so that corresponding genes could be identified by positional cloning and sequencing analysis. The first step in this process is to cross a mutant with a plant of different genetic background for analysis of linkage of the mutant locus with polymorphic molecular markers. This involved genotyping a large number of F_2 or later generation recombinants. After linkage analysis narrows down to the chromosomal region containing the mutation, the whole genome assembly of flax can be used to find genes that are near to the linked marker, and candidate genes can be cloned and sequenced from the mutant plants to identify the gene that was mutated (Uchida et al., 2011).

2.4.4 Microscopy of Seed Coat

In an attempt to establish the basis of seed color and mucilage phenotypes of mutants that I identified, a histological analysis of seed testa from representative lines was performed. The

cellular organization was examined by polychromatic dye TBO staining. For wild-type seeds and the mucilage mutants, four distinct layers could be distinguished. This observation was consistent with the flax seed coat model as described by Hayward (1938), with the fifth layer (ring cells) being obliterated in the mature seed coat.

The main defects that I could detect in the testa of pigmentation mutants was an absence of the pigment layer, a reduction in the thickness of the transversal-layer, and thin sclerenchymatous cell wall in the yellow and cream seeds. According to Flax Council of Canada 2003, yellow seeds have a thinner seed coat compared to brown seeds, and my observations are consistent with this generalization. This is consistent with the theory proposed by Diederichsen & Richards (2003), which described how anatomical changes in the seed coat structure resulted in different appearance of seed coat color. The Cream phenotype might be associated with a the thin and ruptured transversal cell layer, which was thicker in Yellow seeds and in wild-type and other mutant classes, as compared to Cream.

The mutant from the Blue Seed class showed a wild-type structure and staining. The No Mucilage Released seeds were distinguished by the complete lack of extruded mucilage when hydrated. However, sectioning of those seeds showed the mutant to have no obvious changes in terms of its testa structure, except perhaps in the transversal-layer thickness.

It appeared that the fixation and embedding process allows similar expansion of mucilage in both wild-type and mutants. In Arabidopsis, the *mum1* and *mum2* mutants are unable to release their mucilage upon seed hydration due to defects in the primary cell wall or mucilage modification (Western, 2006). Thus, the abnormal mucilage phenotypes observed in intact seeds from these mutant lines do not appear to be due to any abnormalities in the structure or

anatomical development of the seed coat, but may instead relate to the physical and biochemical properties of the mucilage and/or epidermal cell walls.

2.5 Conclusions

I have described a forward genetics screen for pigmentation and mucilage mutants in an EMS-mutagenized linseed cultivar. Five different pigmentation phenotypes have been characterized, in which the yellow and variegated seed families have potential applications in plant breeding. The stability of these phenotypes has been confirmed. The screen also identified three classes of mucilage mutants. Further studies have been undertaken on the two most homogeneous phenotypes: No Mucilage Released, and Blue Seed staining. The variability in mucilage phenotypes of some mutants under different growing conditions (glasshouse and field-grown) indicates a considered influence of environment on the mucilage phenotype. I also performed the histological analysis in representative mutant lines: Yellow, Cream, No Mucilage Released, and Blue Seeds. There was an overall thinner seed coat in seed color mutants resulted from reduce/lack of pigment layer, a reduced thickness of the transversal layer, and they were also characterized by thin cell walls of sclerenchymatous cells. The anatomy of the mucilage mutants was generally indistinguishable from wild-type. Together, the defined mutants and the putative mutants that have been identified in this study provide an initial material for further genetic analysis and development of novel germplasm.

2.6 Figures and Tables

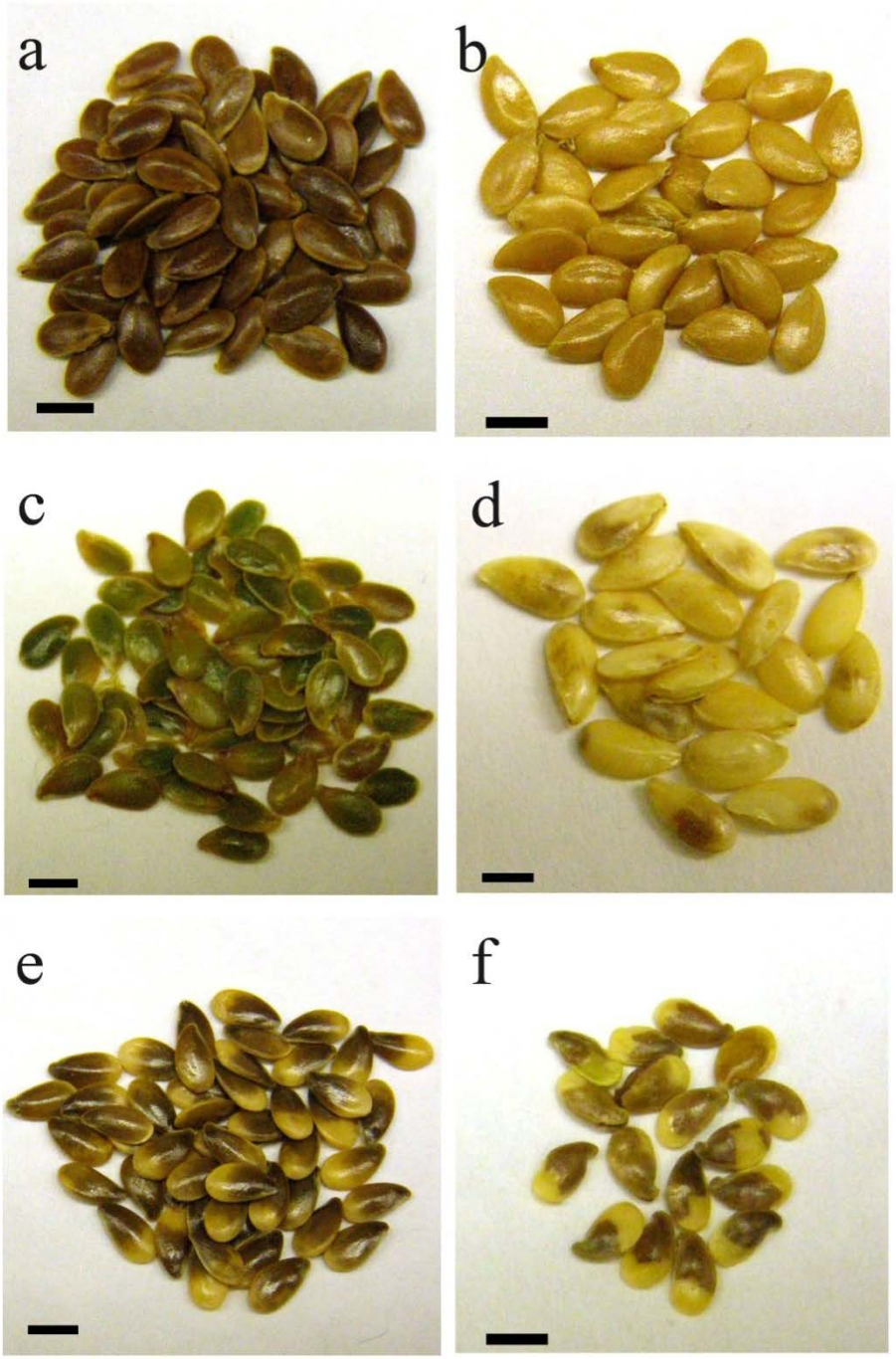

Figure 2-1 Phenotypes of seed color mutants in M_4 seeds CDC Bethune cultivar. a: Wild type ; b: Yellow ; c: Green ; d: Cream; e: Brown-Gradient; f: Brown-Variegated

Table 2- 2 Seed color phenotypes of M_4 seeds CDC Bethune cultivar observed in a screen of gross morphology.

Phenotype	**Number of M_3 families**	**Number of M_4 families**	**Number of M_4 families**		**Remark**
			Non-segregating	**Segregating**	
Brown (wild-type)	2,728	16,677	16,677	0	Seeds were uniformly brown.
Yellow (56 families)	43	56	54	2	Seeds were uniformly yellow and ranged from light yellow to golden.
Green (25 families)	18	25	16	9	Seeds were uniformly green or irregularly green-brown.
Cream (2 families)	1	2	2	0	Seeds were cream with irregular patches of light brown pigment
Brown-Gradient (2 families)	2	2	2	0	Seeds were dark brown at the micropylar end, and yellow at the opposite end with a gradient between the two colors

Brown-Variegated (1 family)	1	1	1	0	Seeds were brown at the micropylar end, and yellow at the opposite end with a distinct but irregular border between the two colors

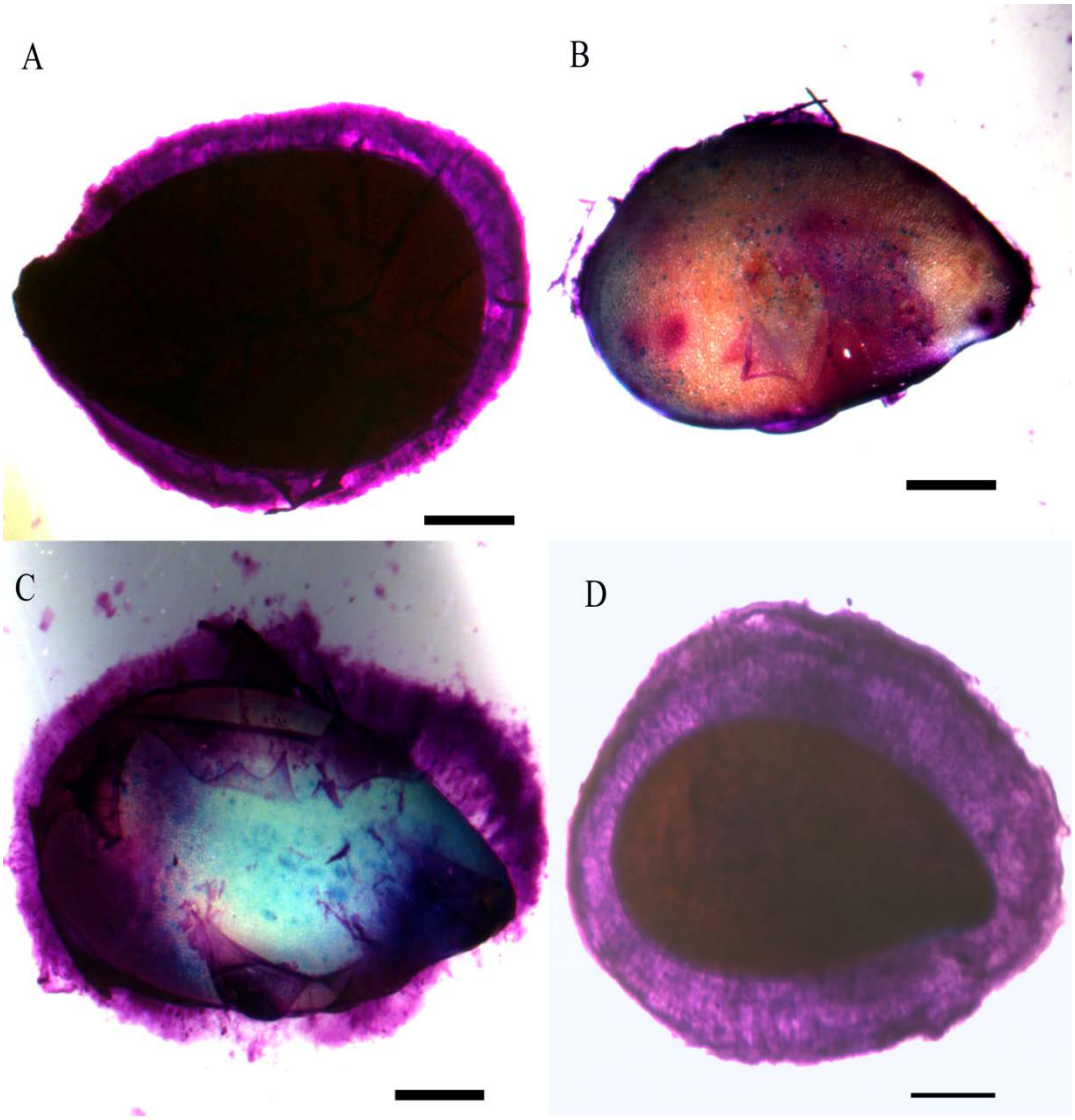

Figure 2-2 Phenotypes of mucilage mutants in M_4 seeds CDC Bethune cultivar A: wild type; B: No Mucilage Released; C: Blue Seed; D: Abnormal Mucilage Released. The scale bar is 1 mm.

Table 2-2 Mutant mucilage phenotypes of M_4 seeds CDC Bethune cultivar after TBO staining

Abnormal phenotypes after TBO staining	**M_4**	**M_3**	**Observed/seeds tested**
Blue	885	638	5/5, 4/5 and 3/5
No Mucilage Released	838	661	5/5 and 4/5
Abnormal Mucilage Released	252	212	5/5

Table 2-3 Progeny test of abnormal seed color phenotypes of M_4 seeds CDC Bethune cultivar

M_4		**M_5**
pigmentation	**Number of tested lines**	**pigmentation**
Yellow	8	All Yellow
Green	19	All Brown

Table 2-4 Stability of No Mucilage Released phenotypic class of M_4 seeds CDC Bethune cultivar tested in glass house

M_4 phenotype	**M_5 families and phenotype**				
No Mucilage Released	**Total**	**Wild-type**	**Abnormal Mucilage**	**No Mucilage Released (segregate)**	**No Mucilage Released (non-segregate)**
48 families	48	4	1	10	33

Table 2-5 Frequency of staining phenotypes among M_5 seeds in the No Mucilage Released phenotypic class, CDC Bethune cultivar.

M_4 family	**M_5 family**					
	Total number of seeds tested	**Seeds lacking a normal mucilage halo**	**Seeds with a normal mucilage halo**	**Ratio**	**Chi square value**	**P value**
3179(5)	10	2	8	1:3	0.13	0.71
2658(5)	10	2	8	1:3	0.13	0.71
3237(5)	10	4	6	1:3	1.2	0.27
2740(3)	10	3	7	1:3	0.13	0.71
3135(3)	10	3	7	1:3	0.13	0.71
2650(1)	10	2	8	1:3	0.13	0.71
2742(4)	10	4	6	1:3	1.2	0.27
2977(4)	10	4	6	1:3	1.2	0.27
4939(5)	10	3	7	1:3	0.13	0.71
2755(2)	10	4	6	1:3	1.2	0.27

Table 2-6 Stability of Blue Seed phenotypic class M_4 seeds CDC Bethune cultivar tested in glass house

M_4 phenotype	**M_5 families and phenotype**			
Blue Seed	**Total**	**Blue Seed, Wild-type mucilage**	**Blue Seed, No Mucilage Released**	**Wild-type staining and No Mucilage Released**
19 families	19	12	3	4

Table 2-7 Mucilage phenotypes of M_6 seeds from 25 field-grown families (NMR. = No Mucilage Released phenotypic class; Blue = Blue Seed phenotypic class; wt = wild-type mucilage phenotype)

	Family	**M_4 phenotype**	**M_5 phenotype (ratio)**	**M_6 phenotype (ratio)**
1	146(3067)	NMR	NMR	NMR
2	127(3092)	NMR	NMR	NMR
3	122(3176)	NMR	NMR	NMR(8/10)
4	112(3050)	NMR	NMR	NMR
5	139(4984)	NMR	NMR	NMR
6	106(3219)(1)	NMR	NMR	Blue (9/10)
7	113(3046)	NMR	NMR	Blue (5/10)
8	115(3232)	NMR	NMR	wt
9	148(3100)	NMR	NMR	wt
10	107(3017)	NMR	NMR	wt
11	137(3160)	NMR	NMR	wt
12	109(2814)	NMR	NMR	wt
13	110(3049)	NMR	NMR	wt
14	124(3218)	NMR	NMR	wt
15	104(3181)	NMR	NMR	NMR (5/10)
16	133(4766)	Blue	wt	Blue
17	101(5038)	Blue	wt	Blue (6/10)
18	121(3112)	NMR	wt	NMR
19	116(3135)	NMR	NMR (7/10)	wt
20	143(3179)	NMR	NMR (2/10)	wt
21	131(2650)	NMR	NMR (2/10)	wt

22	145(2740)	NMR	NMR (3/10)	wt
23	125(4939)	NMR	NMR (7/10)	wt
24	118(3233)	NMR	wt	wt
25	103(3219)	NMR	Abnormal Mucilage Released	Abnormal Mucilage Released

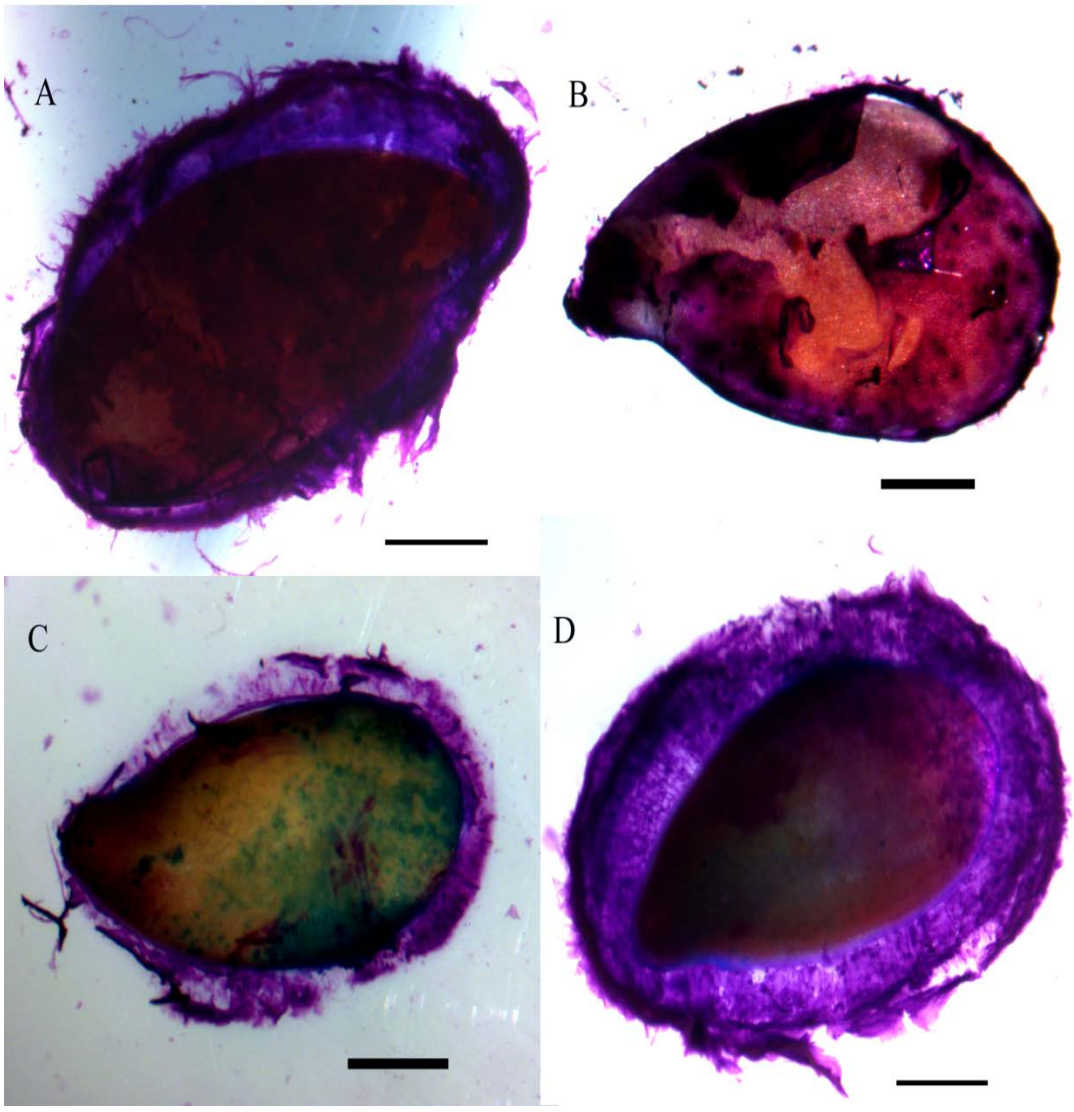

Figure 2-3 Phenotypes of mucilage mutants in field-grow M_6 seeds CDC Bethune cultivar. A: wild type; B: No Mucilage Released; C: Blue Seed; D: Abnormal Mucilage Released. The scale bar is 1 mm.

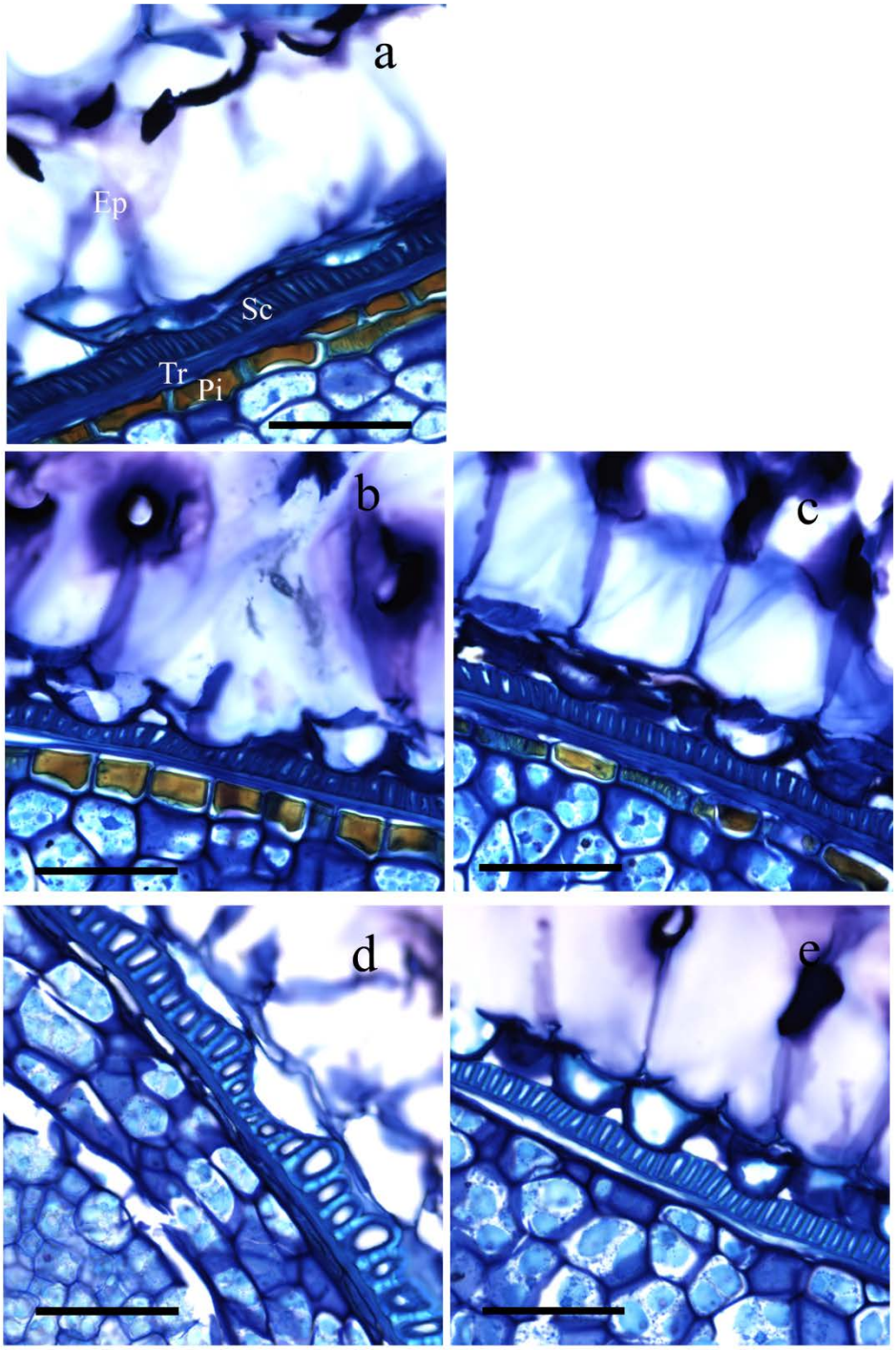

Figure 2-4 The histological organization of flax seed coat (M_4 families CDC Bethune cultivar) stained with TBO. a: wild type; b: No Mucilage Released; c: Blue Seed; d: Yellow; e: Cream. Ep: epidermal layer; Sc: sclerenchymatous layer; Tr: transversal-cell layer; Pi: pigment layer. The scale bar is 50µm.

2.7 Bibliography

Arsovski, A. A., Villota, M. M., Rowland, O., Subramaniam, R., & Western, T. L. (2009). *Mum enhancers* are important for seed coat mucilage production and mucilage secretory cell differentiation in *Arabidopsis thaliana*. *Journal of Experimental Botany*, *60*(9), 2601–2612.

Barnes, D. K., Culbertson, J. O., & Lambert, J. W. (1960). Inheritance of seed and flower colors in flax. *Agronomy Journal*, *52*(8), 456–459.

Bretagne-Sagnard, B., Fouilloux, G., & Chupeau, Y. (1996). Induced albina mutations as a tool for genetic analysis and cell biology in flax (*Linum usitatissimum*). *Journal of Experimental Botany*, *47*(2), 189–194.

Diederichsen, A., & Richards, K. (2003). Cultivated flax and the genus Linum. Flax: the genus Linum, Taylor & Francis. New York, NY, USA.

Flax Council of Canada, & Saskatchewan Flax Development Commission. (2003). Growing Flax: Production, Management & Diagnostic Guide. Flax Council of Canada, Saskatechewan Flax Development Commission.

Hayward, H. E. (1938). The structure of economic plants. Macmillan Company. London/New york.

Mittapalli, O., & Rowland, G. (2003). Inheritance of seed color in flax. *Crop Science*, *43*(6), 1945–1951.

O'brien, T. P., Feder, N., & McCully, M. E. (1964). Polychromatic staining of plant cell walls by toluidine blue O. *Protoplasma*, *59*(2), 368–373.

Page, D. R., Grossniklaus, U. (2002). The art and design of genetic screens: *Arabidopsis thaliana*. *Nature Reviews Genetics*, *3*(2), 124–136.

Uchida, N., Sakamoto, T., Kurata, T., & Tasaka, M. (2011). Identification of EMS-induced causal mutations in a non-reference *Arabidopsis thaliana* accession by whole genome sequencing. *Plant and Cell Physiology*, *52*(4), 716–722.

Weigel, D., & Glazebrook, J. (2008). Genetic analysis of *Arabidopsis* mutants. *Cold Spring Harbor Laboratory Press*, NY, USA.

Western, T. L. (2006). Changing spaces: the *Arabidopsis* mucilage secretory cells as a novel system to dissect cell wall production in differentiating cells. *Canadian Journal of Botany*, *84*(4), 622–630.

Western, T. L., Burn, J., Tan, W. L., Skinner, D. J., Martin-McCaffrey, L., Moffatt, B. A., & Haughn, G. W. (2001). Isolation and characterization of mutants defective in seed coat mucilage secretory cell development in *Arabidopsis*. *Plant Physiology*, *127*(3), 998–1011.

Western, T. L., Skinner, D. J., & Haughn, G. W. (2000). Differentiation of mucilage secretory cells of the *Arabidopsis* seed coat. *Plant Physiology*, *122*(2), 345–356.

Western, T. L., Young, D. S., Dean, G. H., Tan, W. L., Samuels, A. L., & Haughn, G. W. (2004). *mucilage-modified4* encodes a putative pectin biosynthetic enzyme developmentally regulated by *apetala2*, *transparent testa glabra1*, and *glabra2* in the *Arabidopsis* seed coat. *Plant Physiology*, *134*(1), 296–306.

Østergaard, L., & Yanofsky, M. F. (2004). Establishing gene function by mutagenesis in *Arabidopsis thaliana*. *The Plant Journal*, *39*(5), 682–696.

Chapter 3 Analysis of GASA Expression in the Developing Flax Stem

3.1 Introduction

The *GASA* (*GIBBERELLIC ACID STIMULATED ARABIDOPSIS*; also called *GAST* (*GIBBERELLIC ACID STIMULATED TRANSCRIPT*) like genes form multigene families in diverse plant species encode proteins with a unique cysteine-rich domain (GASA domain). Insight about *GASA* gene members has been established from studies mostly in the model plant *Arabidopsis thaliana*. Many *GASA* genes have been reported to be GA stimulated, especially when GA primarily acts as regulator of fiber cell elongation (Björklund et al., 2007; Mauriat & Moritz, 2009). In general, members of this family have been associated with cell division or cell elongation during flower, root, and fruit development, defense and stress responses (Zhang et al., 2009).

The first GASA-like gene described in flax (*Linum usitatitssimum*), *LuGAST1*, was identified in an EST library of outer stem tissues, and its possible involvement in bast fiber development was suggested as its transcript abundance increased during later stages of cell expansion (Roach & Deyholos, 2007; 2008). Moreover, unpublished observations made in our lab showed that transcript expression of flax GASA-like gene was negatively correlated with bast fiber development (Mckenzie, 2011, personal communication). Based on this observation, I decided to characterize the GASA-like gene family in flax, including examining the transcript expression pattern of GASA-like genes at various stages of stem development, identifying candidate members related to bast fiber development, and predicting their putative functions in this biological process. The experiments described in this chapter was designed so that the relationship between GASA-transcript expression and fiber development could be compared in the context of phylogenetic analyses based on the flax whole genome shotgun assembly.

3.2 Materials and Methods

3.2.1 Plant Growth, Tissue Isolation, and Microscopy

Seeds of *Linum usitatissimum* L. linseed variety CDC Bethune were sown 1 cm deep in Metromix 360 (Scotts, Maryland, OH) in square pots (approximately 10 cm x 10 cm x 10 cm) and were grown in an growth chamber set at 24 °C with 50% humidity, and a light intensity of 300 μE on a 16 h light/8 h dark cycle.

The stem tissues were collected from plants 3 weeks after sowing. The leaves were removed, and then a ruler was used to mark along the stem axis the positions that needed to be collected. Segments of 1cm length were cut from whole stems at five different positions along the stem axis (1 cm, 3 cm, 4 cm, 5 cm, and 9 cm from shoot apex) using a blade. Segments from different positions were stored in separate 1.5 ml tubes, and were immediately frozen in liquid Nitrogen. Segments from corresponding positions on four individual plants were pooled to constitute a sample. This process was repeated on four different occasions to constitute four independent biological replicates of each of five samples for a total of 20 samples. All tissues were stored at -80^{o}C prior to RNA extraction.

For microscopy, transverse sections were cut by hand from positions along the stem. These were stained with Toluidine Blue 0.05 % (w/v) for about 30 seconds, and then rinsed in distilled water. Sections were mounted in water on a microscope slide, and examined microscopically using a transmitted light Olympus BX51 microscope to assess stem development.

For DNA extraction, leaves tissues were collected from a new set of 2 week-aged-plants after germination, and then ground in liquid nitrogen. Total DNA was extracted using DNeasy Plant Mini kit (Qiagen). DNA concentration was estimated from the Nanodrop absorbance at 260 nm.

3.2.2 RNA Extraction

Total RNA was extracted from each of the stem segments using Sigma TRIreagent (Invitrogen), following the manufacturer's instructions (MacRae, 2007). Total isolated RNA was further cleaned using On-Column DNase Digestion set (Sigma-Aldrich). RNA integrity and purity were justified by examining 260/280 and 260/230 ratios for protein and solvent contamination.

3.2.3 Microarray Analysis

Mean raw pixel intensities were processed in the R Bioconductor package limma using background correction ("backgroundCorrect") method "normexp", with offset=50, followed by normalization ("normalizeBetweenArrays") with method "quantile". Each of the five different sampling zones was used as an independent factor ("coefficient") in fitting multiple linear models with function lmFit. Subsequently, the eBayes function was used to calculate empirical Bayes statistics for all possible contrasts between the five different tissue zones sampled. Log-normalized signal intensities for array elements that showed significant differences (adjusted p-value <0.05) in at least one contrast were used as input for the STEM clustering program with a maximum of 48 model profiles, maximum 1 unit change, minimum absolute expression change 0.5 (maximum-minimum), and all other settings as default.

Microarray probes were originally designed based on similarity to EST sequences (Venglat et al., 2011) and to unpublished drafts of the whole genome shotgun (WGS) assembly of flax. Probes were subsequently mapped to the published release of the WGS assembly (version 1.0) by BLASTN alignment with probes as query sequences against WGS scaffolds. Probes that aligned (>95% of probe length, >95% identity) to WGS were assigned to annotated genes by comparing the co-ordinates of the probe alignment with exon co-ordinates using BEDTools, allowing up to 300 bp separation between the exon of a CDS and an aligned probe to account for possible UTRs.

3.2.4 qRT-PCR

Three biologically independent replicates of qRT-PCR were conducted on seven genes. All replicates used aliquots of the same RNA samples used for microarrays. cDNA synthesis was conducted using RevertAid H Minus Reverse Transcriptase (Fermentas, Glen Burie, MD) and oligo-dT_{18} primers (Fermentas), following the manufacturer's protocol. Real time PCR was performed in an Applied Biosystems 7500 Fast Real-Time PCR System. For quantitative PCR reactions, 2.5 µl of a one-fiftieth 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, with 0.2 µM dNTPs, 0.25X SYBR Green, 1X ROX and 0.075U Platinum Taq (Invitrogen). Threshold cycles (C_T) were determined using 7500 Fast Software. C_T values were normalized using three genes ETIF1 (Lus10002264 or Lus10000920), GAPDH (Lus10006435 or Lus10011375), ETIF5A (Lus10037129) as endogenous controls (Table 3-2). The GASA primers used in the qRT-PCR analysis (Table 3-3) were designed using Primer Express Software version 3.0.1, followed by BLASTN alignments against the predicted flax gene CDS sequences and whole genome assembly (version 1.0). Flax_gene CDS and scaffold library. Specific primers were quantified

over a range of cDNA dilutions to confirm that their primer efficiencies amplified at an equivalent efficiency to primers for three endogenous controls.

3.2.5 Cloning of Genomic DNA Fragments

The gDNA sequences of seven predicted genes were obtained from the linum.ca database (Version 1.0), and gene-specific primers (Table 3-4) were designed using Primer3 Input (Version 0.4.0). Total flax leaf gDNA was PCR amplified with gene-specific primers, and the products were analyzed on a 1% agarose electrophoresis gel. The PCR products were gel purified according to protocol in the Wizard® SV Gel and PCR Clean-Up system (Promega). Cloning was conducted using the InsTAclone PCR Cloning kit (Fermentas). PCR fragments were ligated with Vector pTZ57R/T (the vector/insert ratio is around 1:3), and incubated overnight at 4°C. 2.5 µl ligations were transformed into 50 µl of chemically competent *E. coli* (DH5α) cells. The transformations were incubated on ice for 30 minutes, then held in a water bath 42°C for 90 seconds, and placed on ice for 1-2 minutes. Approximately 800 µl of LB medium was added to the transformed cells, and proceed with incubating at 37°C in the shaker for 45 minutes. And then, 50 µl of this transformation plated on the LB medium. The concentration of antibiotic used in the medium was 100 µg/ml for Ampicilin, IPTG 0.2 µM, and X-gal 0.004%. After incubation overnight at 37°C, white colonies were selected and analyzed by PCR with gene-specific primers.

3.2.6 DNA Sequencing and Sequence Analysis

Plasmid DNA was extracted from white colonies following the user guide of the GenEluteTM Plasmid Miniprep Kit (Sigma). Colony PCR and sequencing were conducted with M13 primers, after that the BigDye Terminator V3.1 cycle Sequencing kit (3730 Genetic Analyzer, Applied Biosystems) was used for sequencing. Vector and poor quality sequences at

the 3’ end of the sequencing output reads were eliminated by manual editing. Similarity analysis for each sequence was performed using alignments in CLC Genomics workbench.

3.2.7 Phylogenetic Analysis

A GASA domain (PF02704) consensus model from the Pfam 26.0 database was used to identify *GASA* gene sequences in different species, and amino acid sequences for the full-length proteins were obtained from the Phytozome (version 9.0) database. One gene model for each locus was collected from *Linum usitatissimum*, *Arabidopsis thaliana*, and *Populus trichocarpa.* Amino acid sequences were imported into MEGA5.05, aligned using ClustalW with default protein alignment parameters. Full-length protein sequences were used in the analysis, and a consensus maximum likelihood tree of 1000 bootstraps was constructed using MEGA5.05.

3.3 Results

3.3.1 Microarray Analysis

To identify genes that are associated with development of stem tissues, I extracted RNA from five segments of whole flax stems (Figure 3-1). Each segment was 1cm long and was made by cutting the stem transversely relative to the shoot axis. The first segment (S1), which included the shoot apex, contained tissues at their earliest stages of differentiation. The next three samples (S2, S3, S4) were adjacent segments of the stem region between 2 cm and 5 cm below the shoot apex. The final segment (S5), which contained most of the mature tissues, was from the stem region between 8 cm and 9 cm below the shoot apex. A complete set of five segments was isolated from four pools of biologically independent plants to produce four replicate samples for analysis by microarray.

Microscopic sections from the middle of each segment showed details of the developmental stages represented in that segment. In S1, vascular bundles were already well-defined, with tracheary elements present in files of two or more cells, and several distinct sieve cells and companion cells evident within each bundle. No thickening of phloem fibers cells was yet evident in S1. S2 was anatomically similar to S1, although it could be inferred based on knowledge from previous studies that phloem fiber cells were elongating intrusively in S2 (and in part in S1 and S3). In S3, the formation of fascicular cambium was evident, although the transverse anatomy of vascular tissues remained largely unchanged as compared to S1 and S2. In S4, the first thickenings of phloem fiber cell walls could be seen, which were expected to coincide with the cessation of intrusive elongation in these cells, lignified secondary xylems developed in individual vascular bundles. In S5, the gelatinous-type secondary wall thickenings of phloem fiber cells were nearly complete, and the vascular cambium and secondary xylem had formed a contiguous cylinder.

RNA from each of the five segments was converted to cDNA, and after fluorescent labeling, was hybridized to a Combimatrix oligonucleotide microarray containing probes for 47,361 predicted genes from the flax genome. The results of the complete microarray experiment are described in another manuscript in preparation (Pinzon, To, et al.). Here I will focus only on the expression of the *GASA* genes in this microarray experiment.

In total, 39 probes for 20 GASA-like genes were on the microarray. By using the k-Means/Medians clustering, the transcript abundance values were clustered into three general patterns (Table 3-1a, b, c). There were 10 (26%) probes that had a pattern of high expression in the S1 segment and then gradually decreased through S2, S3, S4, and S5 (Table 3-1b, Figure 3-2b). The transcript intensities of four probes were generally similar between S1 and S2, and were

at least threefold more abundant when S1 was compared to S3, and more than fivefold abundant in S1 compared to either S4 or S5. In the converse pattern, the transcript abundance of 21 (54%) probes were low in the S1 and more enriched in S2, S3, S4, S5, respectively (Table 3-1c, Figure 3-2c). Four transcripts were enriched specifically in the S5 segment, greater than twofold compared to the S4, and dramatically low expression in S1 segment. Eight (20%) probes had a small variation or an even expression pattern in five segment comparison (Table 3-1a, Figure 3-2a). Among these was one probe for high expression (more than twofold) compared to the remaining, which were low expression detected in all stem segments.

3.3.2 Validation of Selected GASA Transcript Expression Using qRT-PCR

Quantitative real-time PCR (qRT-PCR) was used to measure relative transcript abundance of seven GASA-like genes. The objective was to confirm that the RNA expression patterns observed in the microarray experiment could be reproduced with an independent analytical method. These seven cDNAs were selected to represent a variety of expression patterns in specific stem segments. In Figure 3-3, the expression patterns of these genes as measured using qRT-PCR and microarray were compared. The expression patterns in the five stem segments (S1, S2, S3, S4, and S5, respectively) were qualitatively consistent between qRT-PCR and microarray analyses for each of the genes analyzed, although the observed expression ratios differed quantitatively.

3.3.3 Cloning and Sequencing of GASA gDNA

I cloned the genomic DNA sequences of the seven selected GASA-like genes, which had been used in qRT-PCR and which represented three different expression patterns related to stem development. The genes were sequenced individually using the Sanger method and the sequences were aligned with the predicted genes from the published whole genome assembly

(Version 1.0) (Wang et al., 2012). Six of the seven gene sequences (Lus10008612, Lus10042203, Lus10002059, Lus10017212, Lus10039443, and Lus10021098) were identical (100% nucleotide identity) between the cloned sequences and the WGS assembly. Gene Lus10004048 was 99% nucleotide identical. The sequence of each gene was attached in Table 3-A1.

3.3.4 Phylogenetic Analysis of *GASA* Genes in Flax

To gain insight into the function of GASA-like genes in flax, a phylogenetic tree was constructed to compare the structure flax GASA-like genes with those from two model plants: Arabidopsis (*Arabidopsis thaliana*) and poplar (*Populus trichocarpa*). The GASA-like genes were defined by the presence of a conserved GASA domain in their amino acid sequences (pfam, gathering cutoff value 21.6) (Peng et al., 2008). On this basis, 22 genes in flax, 15 genes in Arabidopsis, and 22 genes in poplar were defined as GASA-like genes. Alignment, followed by clustering, of the amino acids sequences showed there were three main clades in the phylogenetic tree (Figure 3-4). In the alignment (Figure 3-A1), the 22 GASA-like genes of flax had a generally similar structure as described in Arabidopsis. In flax, the number of Cysteine residues in the C-terminus varied from 9 to 12; while a putative signal peptide in N-terminus was present in 20 out of 22 genes, except Lus10009421 and Lus10024216. There was a difference in the polypeptide of Lus10039443, which had a proline-rich region in the N-terminus. In phylogenetic analysis, the seven GASA-like genes that I focused on in this study (Figure 3-2) were distributed in various clades. Lus10042203 and Lus10008612, which were apex enriched transcripts, were clustered with the gene had a low expression pattern in all developmental stages (Lus10002059). Genes in this cluster were closely related to *GASA6* and *GASA4* in Arabidopsis, which were reported to have the expression in all meristematic regions, and function in cell division (Aubert et al., 1998). Another apex enriched transcript Lus10039443 was grouped with

GASA14 (62% identity). Two genes with the same expression pattern (bottom enriched transcripts) Lus10017212 and Lus10021098 were homologous (96%), but not with another gene Lus10004048, which was identical to *GASA5* (66%).

3.4 Discussion

This is the first use of transcriptomics to study flax stem development since the publication of the whole genome shotgun assembly of flax. The Combimatrix microarray study I conducted was therefore more advanced compared to previous microarray studies of flax stem peel and hypocotyl (Roach & Deyholos, 2007; 2008), which contained 9600 cDNA probes representing approximately 1000 unique genes. Some important technical errors appear to have been introduced during the Combimatrix array design by the service provider, which has delayed the analysis of the global transcriptome (data not shown). Therefore, here I have focused only on the transcript expression of GASA-like genes as verified by qRT-PCR analysis.

3.4.1 *GASA* Gene Sequences in Flax

The *GASA* gene family has been well-described in Arabidopsis as a small polypeptide characterized with an N-terminal putative signal sequence, a highly divergent intermediate region and a conserved 60 amino acid C-terminal domain containing 12 conserved cysteine residues (Roxrud et al., 2007; Peng et al., 2008). As previously reported, the molecular and biochemical functions of *GASA* genes are unknown, but based on their expression patterns, member of this gene family are understood to participate in various aspects of plant development and stress responses (Aubert et al., 1998; Roxrud et al., 2007; Zhang & Wang, 2008; Sun et al., 2013).

The flax Lus10039443 gene shared common sequence features with *GASA14*, including a proline-rich region in its N-terminus, which was consistent with the observations made in the phylogenetic analysis, which showed a close relationship between Lus10039443 and *GASA14*. Recently, *GASA14* was reported to regulate leaf expansion and abiotic stress resistance (Sun et al., 2013). Further studies should focus on obtaining full length genomic sequences for the remaining flax GASA-like genes to facilitate their heterologous expression and *in planta* analysis to further characterize the expression and evolution of GASA-like genes in flax.

3.4.2 *GASA* Gene Expression

To begin to interpret the functions of the *GASA* gene family in flax stem development, their transcript abundances were analyzed by qRT-PCR in stem tissues representative of five different developmental stages.

In the plant vascular system, stem development was divided into different stages of the formation of fiber system, and each stage was characterized by anatomical, chemical and molecular genetics changes (Chemikosova et al., 2006). In flax, the phloem (or bast) fiber formation is distinguished and has been well-defined along the stem development, and the snap point is a boundary between the elongation and secondary cell wall formation in phloem fiber above and below the snap point respectively (Gorshkova et al., 2003). In this study, as described in the transversal sections, the S3 was defined as the transition point (snap point). The qRT-PCR analysis did not show a remarkable difference in the GASA transcript expression when S1 was compared to S2. Neither was there a difference S4 was compared to S5. Thus the putative functions of GASA-like genes in either the shoot apex (S1, S2) or the bottom (S4, S5) were discussed below.

In the shoot apex, phloem fibers are expected to undergo extensive elongation by coordinated and intrusive growth, which occurs mostly in the 1-cm tip region (Ageeva et al., 2005; Gorshkova et al., 2003). The transcript abundances of GASA-like genes (Lus10042203, Lus10008612, Lus10039443) enriched in this region (Figure 3-2) might be involved in regulating different functional enzymes related to aquaporin or cell wall loosening. The fiber elongated in response to internal turgor pressure, which was regulated by the water absorption mechanism. As demonstrated in flax, in osmotic stress, the elongation of the upper part of the stem is retarded (Chemikosova et al. 2006). Moreover, these GASA transcripts might initiate the expression of cell wall loosening and expansion enzymes, which function in dissociating a polysaccharide complex that links microfibrils together (Cosgrove, 2005).

As indicated in the transverse sections below the snap point (S4, S5), anatomical changes were demonstrated by the formation of secondary walls in phloem fibers as well as the development of lignified secondary xylem. Thus enriched transcript abundance in this stage could indicate involvement in a wide range of functions in phloem and xylem development. However, auxin, glucosinolates, cytokinin, and gibberellic acid, have been implicated in the synthesis of secondary phloem, and GASA is a hormone-regulated gene family (Zhao et al., 2005; Roxrud et al., 2007). The transcript abundance of *LuGAST1* (GASA5-like protein) was reportedly increased the cell wall expansion stage (Roach & Deyholos, 2008), and was also dramatically increased in abundance in the middle of the stem longitudinal axis compared to the apex (Mckenzie, 2011). *LuGAST1* transcript expression was consistent with the expression of Lus10004048 in this study. Therefore, I believe that the transcripts of GASA-like genes (Lus10017212, Lus10021098, and Lus10004048) enriched at this stage (Figure 3-2) were potentially involved in the modification of cell wall composition and structure in phloem fibers.

In flax, the cell wall deposition in phloem fibers is marked by the fiber cell-specific occurrences of a β-1,4-galactan epitope, which were believed to be involved in either orienting cellulose microfibrils early in wall deposition or remodeling and cross-linking the cell wall later in development (Gorshkova et al., 2004; Gorshkova & Morvan, 2006; Chemikosova et al., 2006, Roach et al., 2011). Therefore, the genes that were activated at this stage must encode enzymes required for metabolism of tissue-specific galactan. Moreover, the transition of fiber elongation into cell wall deposition coincided with the changes in microtubule orientation from transversal to longitudinal (Ageeva et al. 2005).

3.5 Conclusions

I have described a functional and comparative genomics approach to investigate the transcript expression of candidate GASA-like genes in flax stem development. The sequences of those putative genes have been characterized with small polypeptides consisting of a Cysteine-riche domain (GASA domain). The correlations between *GASA* gene families in Arabidopsis and poplar were analyzed. The putative functions of specific transcripts were inferred based on their expression pattern in the elongation or secondary wall deposition of bast fibers. Overall, the data in this study provides valuable information of putative function GASA-like genes that will aid future attempts to understand the unique developmental process of bast fiber in flax.

3.6 Tables and Figures

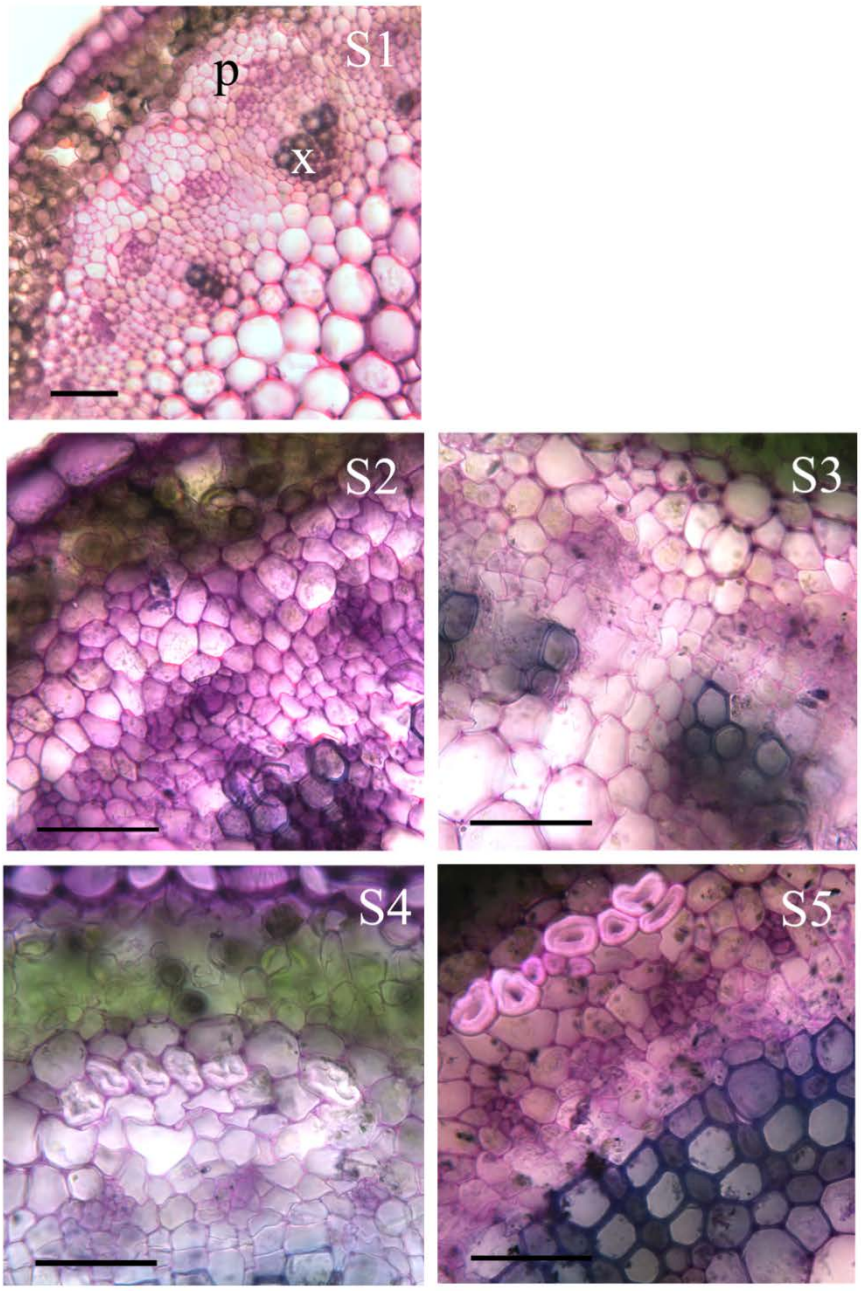

Figure 3-1 Representative tissue cross sections for microarray analysis. S1: Shoot Apex, S2: Above Snap-point; S3: Snap-point; S4: Below Snap-point; S5: Bottom. P: phloem, X: xylem. The scale bar is 50 µm.

Table 3-1a Probes with unchanged expression pattern

Probe name	Gene name	S1	S2	S3	S4	S5
g12114.t1|sl-138-173	Lus10030680	0.186	0.247	0.414	0.233	0.135
g23645.t1|sl-191-231	Lus10009421	0.601	0.709	0.965	0.863	0.772
g30140.t1|sl-153-188	Lus10014262 Lus10042203	4.430	5.026	4.953	4.380	4.075
g31766.t1|sl-196-236	Lus10024791	0.124	0.156	0.180	0.166	0.151
g33488.t1|sl-266-301	Lus10030680	0.196	0.252	0.247	0.246	0.225
g42761.t1|sl-167-205	Lus10018708	0.833	0.987	1.088	1.166	0.709
g4919.t1|sl-227-263	Lus10002059	0.171	0.171	0.158	0.166	0.167
g4919.t1|sl-210-245	Lus10002059	0.059	0.056	0.071	0.065	0.085

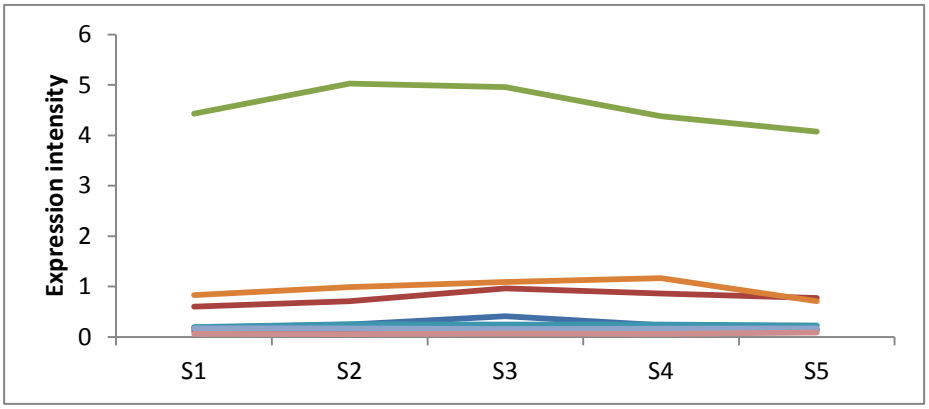

Figure 3-2a Probes with unchanged expression pattern in S1, S2, S3, S4, and S5 comparison. S1: Shoot Apex, S2: Above Snap-point; S3: Snap-point; S4: Below Snap-point; S5: Bottom.

Table 3-1b Probes with Apex enriched expression pattern

Probe name	Gene name	S1	S2	S3	S4	S5
g10552.t1|sl-207-242	Lus10024216 Lus10002059	3.146	2.607	1.922	1.571	1.523
g1362.t1|sl-0-37	Lus10042203 Lus10008612	9.135	8.272	3.565	1.084	1.065
g1362.t1|sl-138-173	Lus10042203	11.924	10.222	3.872	1.315	1.007
g23645.t1|sl-34-69	Lus10009421	0.073	0.0746	0.068	0.070	0.081
g27907.t1|sl-428-466	Lus10039443	2.795	0.309	0.189	0.137	0.280
g27907.t1|sl-464-499	Lus10039443	3.909	0.663	0.426	0.312	0.539
g29163.t1|sl-138-173	Lus10008612	12.285	12.577	4.866	1.481	1.152
g29163.t1|sl-36-71	Lus10008612	7.574	6.724	3.772	1.970	1.705
g38474.t1|sl-169-204	Lus10018016	0.304	0.211	0.258	0.266	0.262
g38474.t2|sl-77-112	Lus10018016	0.164	0.155	0.147	0.152	0.170

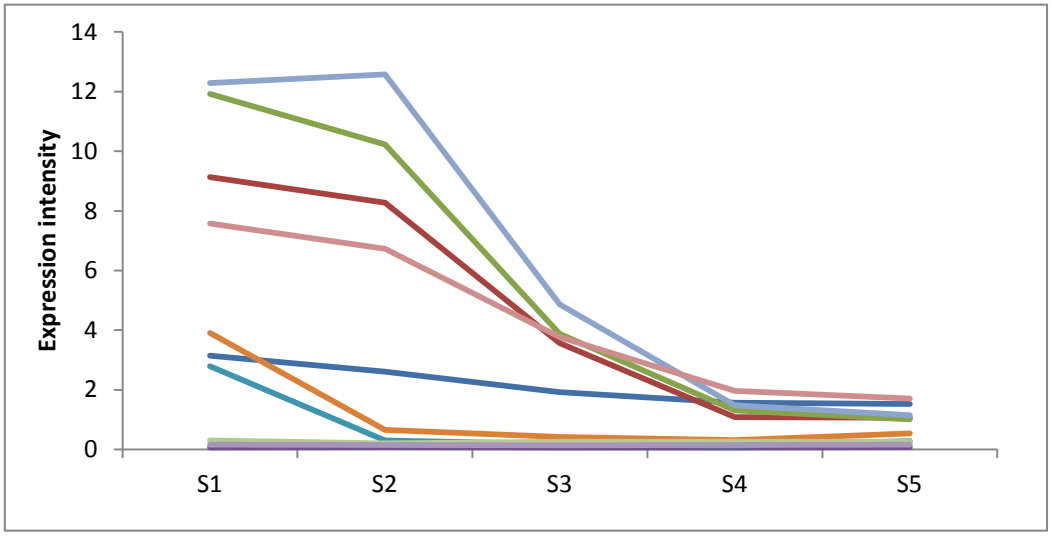

Figure 3-2b Probes with Apex enriched expression pattern in S1, S2, S3, S4, and S5 comparison.

S1: Shoot Apex, S2: Above Snap-point; S3: Snap-point; S4: Below Snap-point; S5: Bottom.

Table 3-1c Probes with Bottom enriched expression pattern

Probe name	Gene name	S1	S2	S3	S4	S5
g511.t1|sl-161-196	Lus10029340	0.071	0.086	0.124	0.137	0.142
g511.t1|sl-229-264	Lus10029340	0.123	0.144	0.150	0.146	0.142
g10552.t1|sl-122-157	Lus10002059	0.235	0.203	0.386	0.626	0.443
g14175.t1|sl-122-157	Lus10017212	0.180	1.088	2.852	4.265	5.997
g14175.t1|sl-224-260	Lus10017212	0.259	1.626	3.582	3.863	6.198
g21292.t1|sl-189-224	Lus10004048	0.059	0.091	0.156	0.184	0.348
g21292.t1|sl-240-275	Lus10004048	0.171	0.212	0.209	0.180	0.215
g24030.t1|sl-221-256	Lus10021098	0.142	0.165	0.174	0.163	0.185
g24030.t1|sl-238-273	Lus10021098	0.129	0.143	0.150	0.132	0.148
g25461.t1|sl-241-276	Lus10004048	0.600	1.960	3.397	5.911	10.126
g25461.t1|sl-258-293	Lus10004048 Lus10017212	0.189	0.660	1.511	2.066	4.704
g30140.t1|sl-17-52	Lus10014262 Lus10042203	0.378	0.390	0.420	0.433	0.386
g3056.t1|sl-153-188	Lus10033145	0.530	0.688	0.822	0.766	0.713
g30781.t1|sl-204-241	Lus10025962 Lus10025962	0.062	0.064	0.065	0.058	0.074
g30781.t1|sl-222-257	Lus10025962	0.170	0.176	0.224	0.222	0.174
g31293.t1|sl-275-311	Lus10034524	0.071	0.090	0.121	0.097	0.107
g31293.t1|sl-293-328	Lus10034524	0.103	0.109	0.109	0.099	0.118
g31766.t1|sl-216-256	Lus10024791	0.118	0.135	0.159	0.152	0.149
g33488.t1|sl-283-318	Lus10030680 Lus10018708	0.086	0.079	0.091	0.086	0.112
g41090.t1|sl-236-276	Lus10001407 Lus10042012	0.071	0.084	0.096	0.090	0.103

g41090.t1|sl-256-296	Lus10001407	0.066	0.063	0.078	0.073	0.087

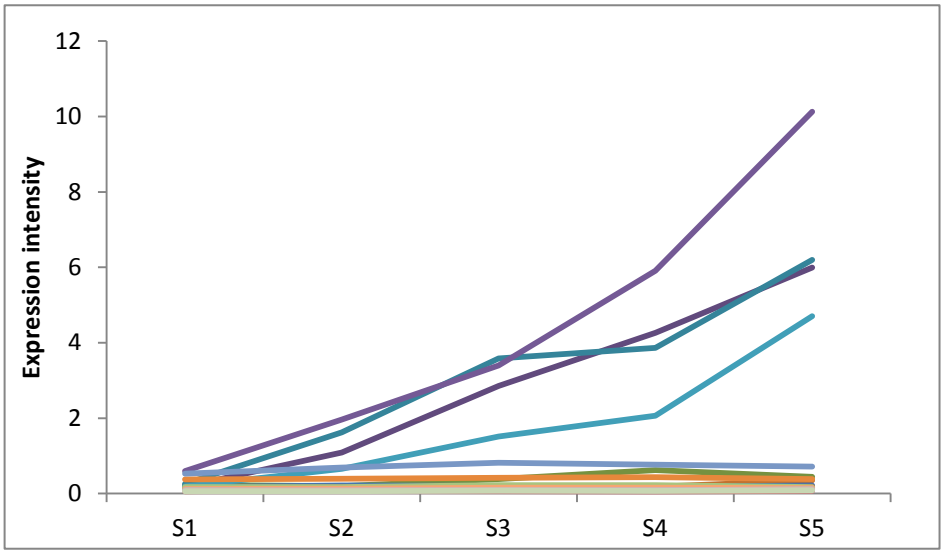

Figure 3-2b Probes with Bottom enriched expression pattern in S1, S2, S3, S4, and S5 comparison. S1: Shoot Apex, S2: Above Snap-point; S3: Snap-point; S4: Below Snap-point; S5: Bottom.

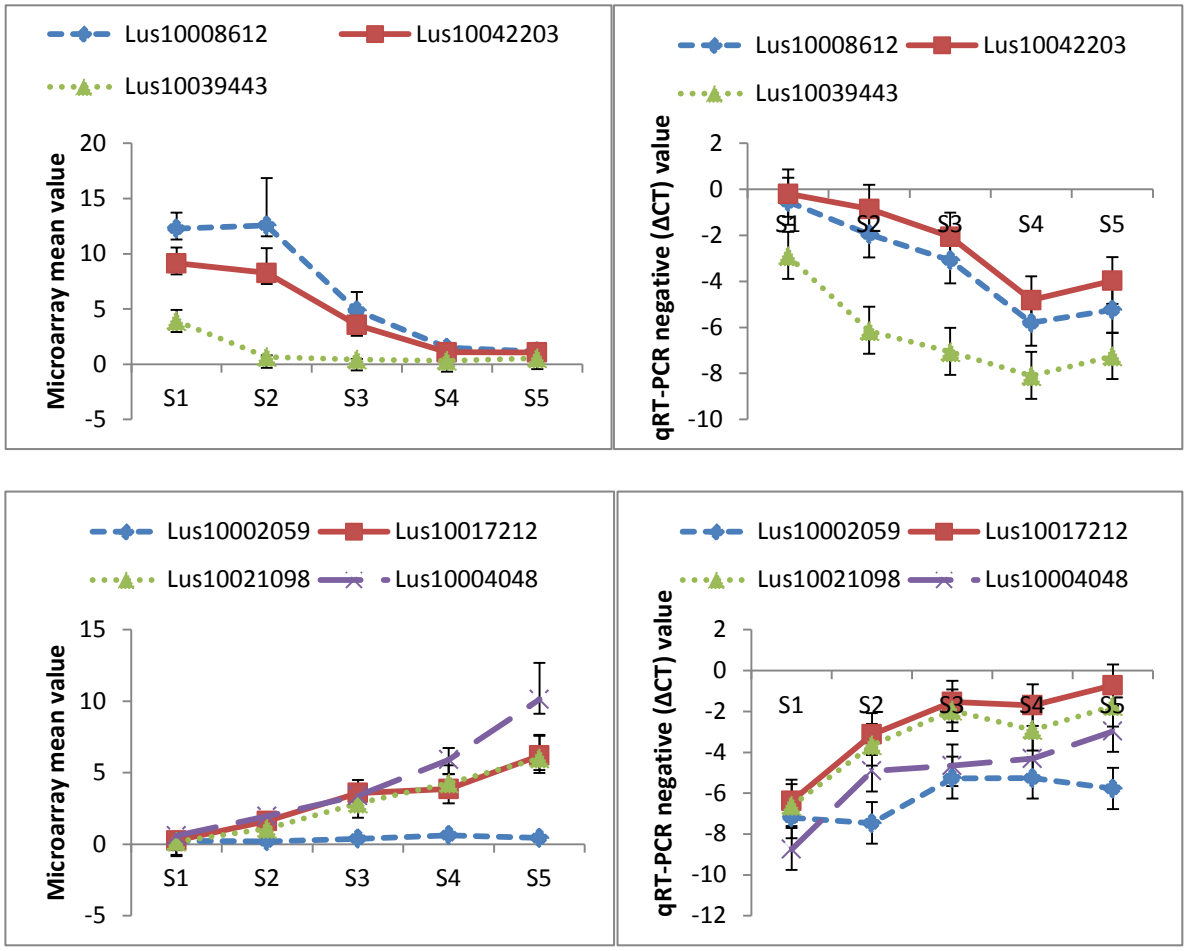

Figure 3-3 Relative transcript abundance of 7 GASA-like genes in five different stem segments as measured by (a) microarray analysis and (b) qRT-PCR. The mean signal intensity in microarray and $(\Delta C_T)^{-1}$ value for each probe set were plotted as continuous line between S1, S2, S3, S4 and S5 segments, respectively. Abbreviations: S1 (apex); S2 (above the snap point); S3 (the snap point); S4 (below the snap point); S5 (bottom).

Table 3-2 Primer sequences of endogenous control genes

Gene abbreviation	**Gene description**	**Primer Sequence (Forward/Reverse primer)**
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	5'-GACCATCAAACAAGGACTGGA-3' 5'-TGCTGCTGGGAATGATGTT-3'
ETIF1	Eukaryotic translation initiation Factor 1	5'-CTCAGGTGATGCGAATGCT-3' 5'-AATCCCTCAGCCCTACAAGG-3'
ETIF5A	Eukaryotic translation initiation Factor 5 A	5'-CCGGAGCCTCCAAGACTTA-3' 5'-TGACGATGTATCCGTTCTTACG-3'

Table 3-3 Primer sequences of 7 GASA-like genes for qRT-PCR analysis

Gene name	**Primer Sequence** **(Forward/Reverse primer)**
>Lus10008612	5'-GGTCGCCACTGAGGTCATG-3' 5'-GCATCTCCTCGTGCATTGC-3'
>Lus10042203	5'-GGAACTATGGACCTGGAAGTCTGA-3' 5'-ACAAGCATGGCTTCCTGTACTG-3'
>Lus10002059	5'-TGCGGGTCGCAGTGTACTC-3' 5'-ACTTTGCGCAGCACTTGTTG-3'
>Lus10017212	5'-TGTCGAGCAGGCCAAACC-3' 5'-GCGTAGCAAGCACACTTGTCA-3'
>Lus10021098	5'-TCCAGCTTACCCTGCATGCT-3' 5'-TTGCACGCGCCCTTACA-3'
>Lus10039443	5'-CTTGCTACTGCTGTTCTAGCTCTTTC-3' 5'-ATTTAGTGGTAACAGCCTCATCGAA-3'
>Lus10004048	5'-GATAGCCGGCAAGCAAACC-3' 5'-CATGGTAATTAGCTTCGGAAACG-3'

Table 3-4 Primer sequences of seven gDNA for cloning and sequencing analyses

Gene name	**Primer Sequence** **(Forward/Reverse primer)**
>Lus10008612	5’-AATGGCTGCTAAGCAACTCC-3’ 5’-TACAGCATTGGTGAGCTTGG-3’
>Lus10042203	5’-TACTCGTACGGGGACAAAGC-3’ 5’-TTGCAGAAGAACAAGCATGG-3’
>Lus10002059	5’-CACTTGCAGACCCAGTTCAA-3’ 5’-ACAATTTCTGAGGGGGCTTT-3’
>Lus10017212	5’-CTCGTCGTCCTCCAGCTTAC-3’ 5’-TTTGCAGCAAGTTCCACAAG-3’
>Lus10021098	5’-CCCTTCTTCTCTCCCTCGTC-3’ 5’-CTTTTGCAGCAAGTTCCACA-3’
>Lus10039443	5’-ATCTCCACCACCTCAACTGG-3’ 5’-AGTCCCGCACTTTTCCCTAT-3’
>Lus10004048	5’-GTGGCTCTGCTTCTCTTGCT-3’ 5’-TCTTTGGTCTTCCACTGGTTG-3’

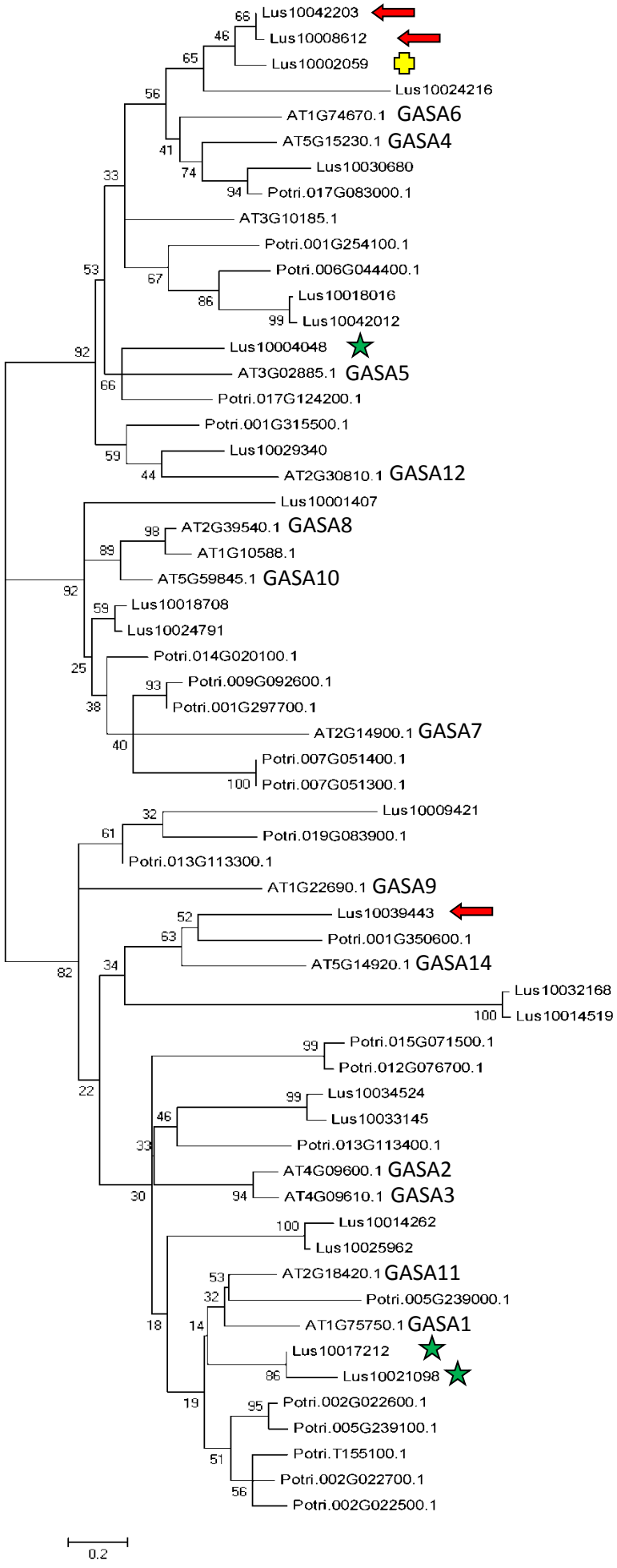
Lus10042203
Lus10008612
Lus10002059
Lus10024216
AT1G74670.1 GASA6
AT5G15230.1 GASA4
Lus10030680
Potri.017G083000.1
AT3G10185.1
Potri.001G254100.1
Potri.006G044400.1
Lus10018016
Lus10042012
Lus10004048
AT3G02885.1 GASA5
Potri.017G124200.1
Potri.001G315500.1
Lus10029340
AT2G30810.1 GASA12
Lus10001407
AT2G39540.1 GASA8
AT1G10588.1
AT5G59845.1 GASA10
Lus10018708
Lus10024791
Potri.014G020100.1
Potri.009G092600.1
Potri.001G297700.1
AT2G14900.1 GASA7
Potri.007G051400.1
Potri.007G051300.1
Lus10009421
Potri.019G083900.1
Potri.013G113300.1
AT1G22690.1 GASA9
Lus10039443
Potri.001G350600.1
AT5G14920.1 GASA14
Lus10032168
Lus10014519
Potri.015G071500.1
Potri.012G076700.1
Lus10034524
Lus10033145
Potri.013G113400.1
AT4G09600.1 GASA2
AT4G09610.1 GASA3
Lus10014262
Lus10025962
AT2G18420.1 GASA11
Potri.005G239000.1
AT1G75750.1 GASA1
Lus10017212
Lus10021098
Potri.002G022600.1
Potri.005G239100.1
Potri.T155100.1
Potri.002G022700.1
Potri.002G022500.1
0.2

Figure 3-4 Phylogenetic analysis of GASA/GAST amino acid sequences. The GASA/GAST sequences, identified by GASA domain, were aligned. These included GASA/GAST sequences in *Linum usitatissimum* (Lus-), *Arabidopsis thaliana* (AT-) and *Populus trichocarpa* (Potri-). Alignment was conducted using ClustalW, and then a maximum likelihood phylogenetic tree was constructed using MEGA5.05. The arrow indicated for genes had apex enriched pattern, the star showed genes with bottom enriched pattern, the cross showed gene with unchanged expression pattern.

3.7 Bibliography

Ageeva, M. V, Petrovska, B., Kieft, H., Sal'nikov, V. V, Snegireva, A. V, Van Dam, J. E. G., Van Veenendaal, W. L. H., Emons, A. M. C., Gorshkova, T. A., Van Lammeren, A. A. M. (2005). Intrusive growth of flax phloem fibers is of intercalary type. *Planta*, *222*(4), 565–574.

Aubert, D., Chevillard, M., Dorne, A. M., Arlaud, G., & Herzog, M. (1998). Expression patterns of *GASA* genes in *Arabidopsis thaliana*: the *GASA4* gene is up-regulated by gibberellins in meristematic regions. *Plant Molecular Biology*, *36*(6), 871–883.

Björklund, S., Antti, H., Uddestrand, I., Moritz, T., & Sundberg, B. (2007). Cross-talk between gibberellin and auxin in development of Populus wood: gibberellin stimulates polar auxin transport and has a common transcriptome with auxin. *The Plant Journal*, *52*(3), 499–511.

Chemikosova, S. B., Pavlencheva, N. V., Gur'yanov, O. P., & Gorshkova, T. A. (2006). The effect of soil drought on the phloem fiber development in long-fiber flax. *Russian Journal of Plant Physiology*, *53*(5), 656–662.

Cosgrove, D. J. (2005). Growth of the plant cell wall. *Nature Reviews Molecular Cell Biology*, *6*(11), 850–861.

Gorshkova, T. A., Sal’nikov, V. V, Chemikosova, S. B., Ageeva, M. V, Pavlencheva, N. V, & Van Dam, J. E. G. (2003). The snap point: a transition point in *Linum usitatissimum* bast fiber development. *Industrial Crops and Products*, *18*(3), 213–221.

Gorshkova, T. A., Chemikosova, S. B., Sal'nikov, V. V., Pavlencheva, N. V., Gur'janov, O. P., Stolle-Smits, T., & Van Dam, J. E. G. (2004). Occurrence of cell-specific galactan is coinciding with bast fiber developmental transition in flax. *Industrial Crops and Products*, *19*(3), 217–224.

Gorshkova, T., & Morvan, C. (2006). Secondary cell-wall assembly in flax phloem fibres: role of galactans. *Planta*, *223*(2), 149–158.

MacRae, E. (2007). Extraction of plant RNA. Protocols for Nucleic Acid Analysis by Nonradioactive Probes (pp. 15–24). Springer.

Mauriat, M., & Moritz, T. (2009). Analyses of GA20ox-and GID1-over-expressing aspen suggest that gibberellins play two distinct roles in wood formation. *The Plant Journal*, *58*(6), 989–1003.

Mckenzie, R. (2011). Genetic and hormonal regulation of stem vascular tissue development in flax *(Linum usitatissimum L.)*. Unpublished doctoral dissertation, University of Alberta, Alberta, Canada.

Peng, J. Z., Lai, L. J., & Wang, X. J. (2008). PRGL: A cell wall proline-rich protein containning GASA domain in *Gerbera hybrida*. *Science in China Series C: Life Sciences*, *51*(6), 520–525.

Roach, M. J., & Deyholos, M. K. (2007). Microarray analysis of flax (*Linum usitatissimum* L.) stems identifies transcripts enriched in fibre-bearing phloem tissues. *Molecular Genetics and Genomics*, *278*(2), 149–165.

Roach, M. J., & Deyholos, M. K. (2008). Microarray analysis of developing flax hypocotyls identifies novel transcripts correlated with specific stages of phloem fibre differentiation. *Annals of botany*, *102*(3), 317–330.

Roach, M. J., Mokshina, N. Y., Badhan, A., Snegireva, A. V, Hobson, N., Deyholos, M. K., & Gorshkova, T. A. (2011). Development of cellulosic secondary walls in flax fibers requires β-galactosidase. *Plant Physiology*, *156*(3), 1351–1363.

Roxrud, I., Lid, S. E., Fletcher, J. C., Schmidt, E. D. L., & Opsahl-Sorteberg, H. G. (2007). *GASA4*, one of the 14-member Arabidopsis GASA family of small polypeptides, regulates flowering and seed development. *Plant and Cell Physiology*, *48*(3), 471–483.

Sun, S., Wang, H., Yu, H., Zhong, C., Zhang, X., Peng, J., & Wang, X. (2013). *GASA14* regulates leaf expansion and abiotic stress resistance by modulating reactive oxygen species accumulation. *Journal of Experimental Botany*.

Venglat, P., Xiang, D., Qiu, S., Stone, S. L., Tibiche, C., Cram, D., Alting-Mees, M.,..& Datla, R. (2011). Gene expression analysis of flax seed development. *BMC Plant Biology*, *11*(1), 74-89.

Wang, Z., Hobson, N., Galindo, L., Zhu, S., Shi, D., McDill, J., Yang, L., …& Deyholos, M. K. (2012). The genome of flax (*Linum usitatissimum*) assembled de novo from short shotgun sequence reads. *The Plant Journal*. 72(3), 461-473.

Zhang, S. C., & Wang, X. J. (2008). Expression pattern of *GASA*, downstream genes of DELLA, in *Arabidopsis*. *Chinese Science Bulletin*, *53*(24), 3839–3846.

Zhang, S., Yang, C., Peng, J., Sun, S., & Wang, X. (2009). *GASA5*, a regulator of flowering time and stem growth in *Arabidopsis thaliana*. *Plant Molecular Biology*, *69*(6), 745–759.

Zhao, C., Craig, J. C., Petzold, H. E., Dickerman, A. W., & Beers, E. P. (2005). The xylem and phloem transcriptomes from secondary tissues of the *Arabidopsis* root-hypocotyl. *Plant Physiology*, *138*(2), 803–818.

3.8 Appendix

Table 3-A1 Sequences of seven GASA genes identified.

Gene name	**Sequence**
>Lus10008612	GCAACTCCTCTGTCTATTGATCCTCGCCATTCTCGGCCTCTCCATGGTCGCCACT GAGGTCAGTAATTTCAAAGCTCAATCTTCATGATATATATCCCTAATCTGGTTA TTTGCATTACTGATGAATTAAGTTAACCCTTGGGTTTTTGTCAATGTGCAGGTC ATGGCTAGGGTATGTCGAAACAATGCTTCTACTTCTTCTGCTGTTGATACGCTT CCGAATTTGTGTTCATAATCTTGTTTGTTCTTGCAGGGGAAGGGGGGGAACTTT GGACCTGGAAGTCTGAAGAGCTACCGTAAGTCATATTAGTTAATCAATACTCAT TGATCTCTTCGCTTAATTCCCTTACTAGATATTATAAAATACTCGTACTGGACA AAGCTGCTGCTTGCTGCACATGAGCACAGTAAACCAAGCTCA
>Lus10042203	CCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGG TTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGA GTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCC TATCTCGGTCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTCGGCCTATTGG TTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTTTAACAAAATATT AACGCTTACAATTTCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGA TCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGC AAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAA CGACGGCCAGTGAATTCGAGCTCGGTACCTCGCGAATGCATCTAGATTGGCTA GGGTACGTCGAAACAATGCTTCTACTTCTTCTGCTGTTGATTCGCTGCCGAATC TGTGTTCCTAATCTTGTTTGTTCTTGCAGGGGAAGGGGGGGAACTATGGACCTG GAAGTCTGAAGGGCTACCGTAAGTCCTATTAGTTAATCAATACTACTCATTGAT CTATTCGCTTAACTCCCCTTACTAGATATTATTAAATACTCGTACGGGGACAAA GCTGCTGGTTGCTGCACATGAGCACAGTAAACCAAGCTCATCAATGCAGCTGC

	AACCAAAAACCACTGATCAAATGGAACAATAGATGAATACCCATTTTAAATTG TGGCAAAAGAGTGAAAACCCACCAATCTATTCACCAAATCTAGGTTGAATAAT AACAAAAGGTTTAGACTTTAGAGAGGACCAAGTAGACAATCGATTGTCGGTGC GTGTCCGGTAGATAAGTAACATTATTTTTGGGGGGGTTGCAGAATGCGGGGGG CAATGCACGAGGAGATGCAGCAGGACGCAGTACAGGAAGCCATGCTTGTTCTT CTGCAAAATCGGATCCCGGGCCCGTCGACTGCAGAGGCCTGCATGCAAGCTTT CCCTATAGTGAGTCGTATTAGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCT GTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCAT AAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGT TGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAAT GAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCT TCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCA GCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGG AAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCC GCGTTGCTGGCGTTTT
>Lus10002059	GTTTCTTCAACTGCAGGGGAATCATGGGCCTGGAAGTCTCAAGAGCTACCGTA AGTATGATTACTTGATGATCTCTTCTATTCCCAACTTAAAAATCATTTTACAAA AAATTTGATATTAATTTACAGAGTATAGTGAGGAATTGAATCTTGTTAAGTGCA ACTTGGTCTCTTAGTTACAATGCTCTGTATCATTTTAACCATAGCTTGTGTATAA AATATGCCTGATAATACTCACCCATCTAACTCGGTTTGGAGCAACTCTGTTTGG GTCTGACAAGTATGAATGTGTAAATTTTGGCACATGCAGTCCCCTTAAATAACA CCATAAATGCTTTAACCAACCCAAACGGTCCGTCTATCT
>Lus10017212	CTGATAACTACCCCAGTACTCCGACCATCGGTAACTACTTCTCTCTTCGTCTCCC TGCAAATTAATCAAAACTTGTCCTCGAGCTAATCATGTTGTGGTCGAGTTGTTG TAGCACCTGACTTCGTTCCCAGAGAATCAGGGTTCGAAGCCTGAAATGCGCAA TCATTTCGATTCTCATATTACGGCATCACCGGTCTCACCTGACATAACTAAATG TGGTGCTCGTCGAAAGAGTTTTTCGAAGAGAAAAACTAGCTAGATCAATCATT

	AATTTATATATGGATAGTGGCGGAACCAGCAGTGGGCCAACCTGAGAAATCGC TCAGGGTATTTTGGTGCCGAATTGATAAAAAAAATTGTAAAATTGTTTCACTCG ATGTGCTTTAGCATTCGCCGCAAATTTGAAATTTTGACCACGATGTCTGACCAT CTGATTTCCGCTATTTGTGTGGATTTCAGATTGTAAGGGGGCATGCAAGGCGAG GTGTCAGCTGTCGAGCAGGCCAAACCTGTGCCACAGGGCTTGTG
>Lus10021098	TCCCTTCTTCTNTCCCTCGTCGTCCTCCAGCTTACCCTGCATGCTGCTGCTGCTG CTGCTGCTGATAGCTACCCCGGTACTCCAACCACCGGTAATTACTTAACTTCCC TGCAAAAGATTAATGAAATTTTGTGTAAGATTTGATTGATAAAGTGTTGTAACA AATAATTTCAGACTGTAAGGGCGCGTGCAAGGCGAGGTGTCGGCTGTCGAGCA GGCCAAACCTATGCCACAGGGCTTGTGGAACTTGCTGCAAAAGA
>Lus10039443	CCTCAACTGGCACCGGTAGTGCCGCCGGTGAAGCCACTTCCAAGTCCCTCTCCT CCACCGTCACCGCCGTACAGCAAGCCACCACCAGCAGCTCCAACTCCACACTA CCCACCACCGGTCGCCAAACCGCCTACTCCTGCCCCTGCCCCTGCCCCACTTCC AACGCCACCACCCCCGATCAGGTCCATTAAAGGTAATTAAAAGAAAGAAAATG TAGTCGATTGGAGATCTCGATTTTATGATAGATATATCGAATGAATAATTACAT GTTGGTTGGTTGGTTGTGTACGTGCAGACTGCGTTCCATTGTGCAGCGAGAGGT GCAAGCTACACTCGAGGCAAAATGTGTGCAACAGAGCATGTATAACTTGCTGT GGTAGGTGCAAATGCGTGCCTCCTGGAACTTATGGGAATAGGGAA
>Lus10004048	CCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGT CGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTT ATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTC GTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGT TCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGAT TCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGC CGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAA TACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACG

	ACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGT TAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGT TGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGACCAT GATTACGCCAAGCTCTAATACGACTCACTATAGGGAAAGCTTGCATGCAGGCC TCTGCAGTCGACGGGCCCGGGATCCGATTTACGGTGGCTCTGCTTCTCTGCTTC TCTTGCTGGCATTCTCCGCCACCGTTTCCGAAGCTAATTACCATGCTAAGCTCC GCCCTTCTGGTAATTAAATAACTGTCACTGTAGTACTAATTTCTTCCCAAATAT AATCCCATTACTAATTAATAACGTTGAATGCGTATAGATTGTAAGCCGAAGTGC AACTACAGATGCTCGGCGACGTCGCACAAGAAGCCGTGCATGTTCTTCTGCCA GAAGTGTTGCACAAAGTGTCTGTGTGTTCCGCCGGGAACTTACGGGAACAAGC AGGTGTGCCCTTGCTACAACAATCTAGATGCATTCGCGAGGTACCGAGCTCGA ATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCC AACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAG AGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGG AAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCA GCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAG AATAGACCGAGATAGGGTTGAGTGTTGTTCCAGTTTGGAACAAGAGTCCACTA TTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTATCAGGGCGA TGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAGGTGCCG TAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGG GAAAGCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGG GCGCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCGTAACCACCACACCC GCCGCGCTTAATGCGCCGCTACAGGGCGCGTCAGGTGGCACTTTTCGGGGAAA TGTGCGCGGAACCCCTATTTGTTTATTTTTCTAA

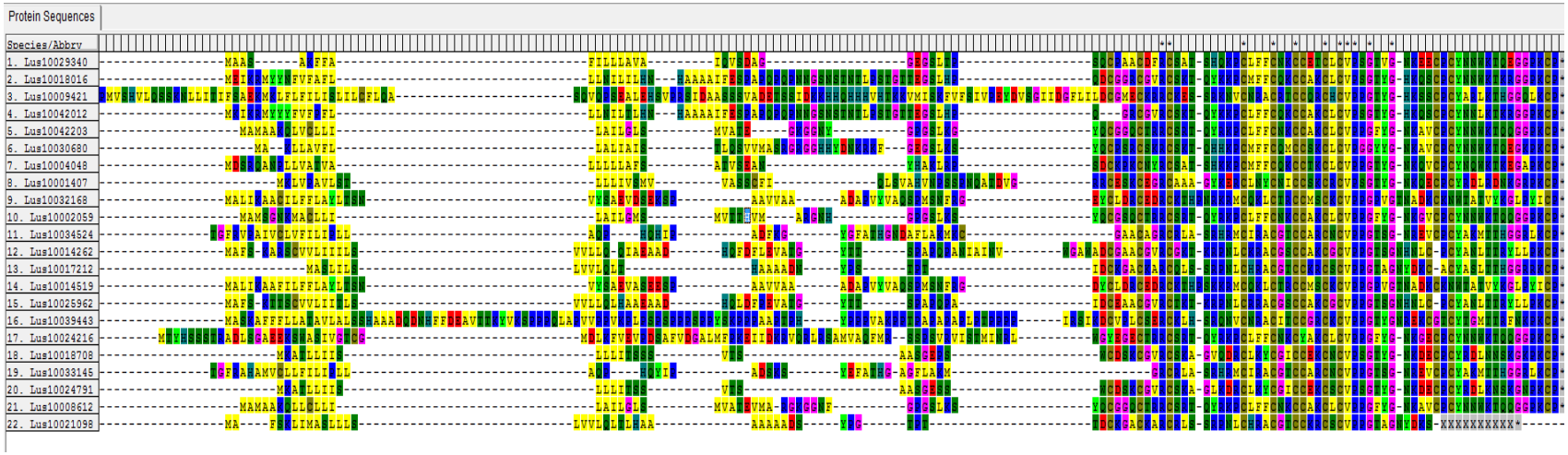

Figure 3-A1 Alignment of 22 GASA-like genes in flax

Chapter 4 Conclusions

4.1 Concluding Remarks

4.1.1 Mutational Analysis of Seed Coat

The screen for seed coat color and mucilage defects resulted in identification of germplasm with novel traits, which provides a foundation for further crop scientific research and use in breeding for cultivar development. The study was conducted in an elite linseed cultivar, which is popular grown in Canada; the identified mutants are possibly suitable for direct incorporation into breeding programs for germplasm improvement. The abnormal seed coat color mutants (yellow or variegated seeds) may be useful for example as markers to distinguish seeds with altered fatty acid contents. The study has also identified a few families out of the 16,764 might deserve further investigation when looking for germplasm with high or low mucilage content.

The seed coat of selected mutants was also studied using histological analysis, which confirmed the flax seed coat structural model and provided explanation for some of the altered pigmentation phenotypes. The seeds with the Yellow phenotype lacked a pigment layer, and a additional ruptured crossed layer appeared to results in the Cream seed coat. No certain conclusions could be made about the relationship between the mutant mucilage phenotypes and the structural change of the testa.

4.1.2 GASA-like Genes in Flax Stem Development

The content presented in Chapter III broadened our understanding of the roles of GASA-like genes in flax. Candidate members of this gene family are well-associated with stem elongation and secondary wall deposition. Confirmation of expression patterns was conducted

for seven GASA transcripts of interest, and their genomic sequences were cloned and sequenced to confirm from the whole-genome shotgun assembly. Using comparative genomic approach I found a shared protein motif in a flax apex enriched transcript and *Arabidopsis thaliana GASA14*; while the bottom enriched transcripts including Lus10004048 were identified as *LuGAST1*, which was previously described as being involved in the secondary cell wall biosynthesis.

The candidate GASA-like genes with putative roles in distinct stages of bast fiber development have been identified, and the genes were chosen to follow up in this study that were identified to be involved in both fiber elongation and secondary wall deposition. This study also gives more consistent evidences suggesting the role of *LuGAST1* (Lus10004048) in cell wall expansion. The findings in this study contribute to potential hypotheses for future research related to *GASA* gene function in bast fiber development.

4.2 Future Perspectives

4.2.1 Further Analyses in Putative Seed Coat Mutants

A set of putative mutants has been identified with well-defined phenotype including No Mucilage Released and Blue Seed staining. Similar to No Mucilage Released category, some mutants of Arabidopsis have been isolated and characterized in a mutant screen of mucilage. Some Arabidopsis mutants showed a no mucilage phenotype. These were found to be due to various defects in seed coat morphology, mucilage synthesis and secretion (Willats, et al., 2001). In the current histological study of those mutants, mucilage accumulates and expands from ruptured epidermal layer, and no obvious changes in the seed testa were observed. Further study should determine the precise mucilage composition of mutants, using GC/MS to reveal the basic

neutral sugar profile and relative uronic acid levels. The outer cell wall weakening or removing treatment with hot hydrochloric acid (0.05 M, 85^{o}c) followed by sodium hydroxide (0.3 M) was proposed by Arsovski et al. (2010) should be useful to know whether the defect in outer epidermal cell wall prevents the extrusion of mucilage. If a little mucilage blisters slightly out of the epidermal cells, this indicates that either less mucilage is in fact present in the epidermal cells or that its hygroscopic properties are altered.

The overlap or interact of some phenotypes between yellow and mucilage phenotypes in subsequent generations proposes potential relation between seed coat structural modification and the phenotypic mucilage. It would be interesting to know whether the reduced-pigment layer in the yellow seeds does have any effect on mucilage release.

The influence of growth conditions on the mucilage phenotype was found to be very large in this report, thus the heritability of those mutant phenotypes is still being determined. An understanding of the influences of the environment conditions on this trait and the interactions between genotype and environment would also be important.

As noted in Chapter II, adjustments to the screening strategy were taken with seeds grown in different conditions, so refining the screening strategy may be useful in future in order to more efficiently identify mucilage mutants. In Arabidopsis mutant screens, Ruthenium Red was effectively used without agitation (Western et al., 2001). Debeaujon et al. (2000) also suggested a simple mutant detection method by incubating the seed for 15 min in an aqueous solution of 0.03% (w/v) Ruthenium Red at room temperature and rinsed with water before observation under a stereomicroscope.

The analysis of mutants with detectable phenotypes will give insight into how the function of a gene is carried out in a biological mechanism. Such forward-genetic mutant screens also have limitations caused by redundancy and difficulties in identifying subtle phenotypes (Dean et al., 2011). Analysis of global gene expression offers an alternative approach to finding additional genes that are expressed and involved in specific biological process, as studies gene expression in seed coat development have been done in Arabidopsis (Le et al., 2010), barley (Pang et al., 2004), and canola (Jiang & Deyholos, 2010). Genome-wide microarray analysis can be used as a powerful tool to provide information on which genes are involved in the formation of mucilage, in which the seed coat tissue can be collected at the mucilage formation duration about seven days after pollination (Western et al., 2000). Mucilage is also an interesting subject to study polysaccharide synthesis. The candidate genes for roles in pectin biosynthesis and secretion can be tested using both gene knockouts and expression analyses.

4.2.2 Function of GASA-like Gene in Bast Fiber Formation

From previous studies I knew that *LuGAST1*, the first GASA-like family member described in flax, might be involved in secondary cell wall formation in phloem fibers (Roach & Deyholos, 2007; 2008; Mckenzie, 2011). Together, in this study, a consistent result in the transcript expression of Lus10004048 (or *LuGAST1*) suggests that the group of bottom enrich transcripts is dominant for further studies in phloem fiber development. Secondary cell wall biosynthesis is involved in various mechanisms of modifying microtubule orientation, cell wall structure and composition (Ageeva et al., 2005; Roach et al., 2011). Therefore, a challenge for future research will be to elucidate specific roles of the putative GASA transcripts.

GA has been shown to be involved in modifying microtubule orientation, which changes from transverse to longitudinal orient coincide to the fiber development, and implicated in

regulating cellulose deposition into cell walls (Ageeva et al., 2005; Lammeren et al., 2003; Shibaoka, 1993). Thus, GA level might have a vital role in fiber differentiation. It would be interesting to know which genes among the GASA genes I described are GA-inducible genes.

To address the function of GASA-like genes in bast fiber properties, the combination between expression data and reverse genetics should be investigated. The creation of T-DNA insertion lines or transgenic overexpression lines would be useful to determine whether suppression or overexpression of GASA-like gene has an effect on bast fiber development in flax.

4.3 Conclusion

Breeding programs with a focus on seed coat mucilage are limited. Not many investigation made in the chemical and physical properties of the mucilage. This thesis represents the starting point, and it will be certainly continued for the years to come for pursuing further insight into flax seed mucilage function development, and possibly use in flax crop development.

The transcript profile of GASA-like gene family in flax stem and fiber development has been established in this study. Our identification and validation of the major GASA transcripts in stem development will facilitate future research of this interesting gene family in this economically-important plant.

4.4 Bibliography

Ageeva, M. V, Petrovska, B., Kieft, H., Sal'nikov, V. V, Snegireva, A. V, Van Dam, J. E. G., Van Veenendaal, W. L. H., Emons, A. M. C., Gorshkova, T. A., Van Lammeren, A. A. M. (2005). Intrusive growth of flax phloem fibers is of intercalary type. *Planta*, *222*(4), 565–574.

Arsovski, A. A., Haughn, G. W., & Western, T. L. (2010). Seed coat mucilage cells of *Arabidopsis thaliana* as a model for plant cell wall research. *Plant Signaling & Behavior*, *5*(7), 796–801.

Dean, G., Cao, Y., Xiang, D., Provart, N. J., Ramsay, L., Ahad, A., White, R., Selvaraj, G., Haughn, G. (2011). Analysis of gene expression patterns during seed coat development in *Arabidopsis*. *Molecular Plant*, *4*(6), 1074–1091.

Debeaujon, I., Léon-Kloosterziel, K. M., & Koornneef, M. (2000). Influence of the testa on seed dormancy, germination, and longevity in *Arabidopsis*. *Plant Physiology*, *122*(2), 403–414.

Jiang, Y., & Deyholos, M. K. (2010). Transcriptome analysis of secondary-wall-enriched seed coat tissues of canola (*Brassica napus* L.). *Plant Cell Reports*, *29*(4), 327–342.

Lammeren, A., Ageeva, M., Kieft, H., Lhuissier, F., Vos, J., Gorshkova, T., & Emons, A. (2003). Configuration of the microtubule cytoskeleton in elongating fibers of flax (*Linum usitatissimum* L.). *Cell Biology International*, *27*(3), 225.

Le, B. H., Cheng, C., Bui, A. Q., Wagmaister, J. A., Henry, K. F., Pelletier, J., Kwong, L., Belmonte, M., Kirkbride, R., Horvath, S., Drews, G. N., Fischer, R. L., Okamuro, J. K., Harada, J. J., Goldberg, R. B. (2010). Global analysis of gene activity during *Arabidopsis* seed development and identification of seed-specific transcription factors. *Proceedings of the National Academy of Sciences*, *107*(18), 8063–8070.

Mckenzie, R. (2011). Genetic and hormonal regulation of stem vascular tissue development in flax *(Linum usitatissimum* L.*)*. Unpublished doctoral dissertation, University of Alberta, Alberta, Canada.

Pang, J.-S., He, M.-Y., & Liu, B. (2004). Construction of the seed-coat cDNA microarray and screening of differentially expressed genes in Barley. *Acta Biochimica et Biophysica Sinica*, *36*(10), 695–700.

Roach, M. J., & Deyholos, M. K. (2007). Microarray analysis of flax (*Linum usitatissimum* L.) stems identifies transcripts enriched in fibre-bearing phloem tissues. *Molecular Genetics and Genomics*, *278*(2), 149–165.

Roach, M. J., & Deyholos, M. K. (2008). Microarray analysis of developing flax hypocotyls identifies novel transcripts correlated with specific stages of phloem fibre differentiation. *Annals of Botany*, *102*(3), 317–330.

Roach, M. J., Mokshina, N. Y., Badhan, A., Snegireva, A. V, Hobson, N., Deyholos, M. K., & Gorshkova, T. A. (2011). Development of cellulosic secondary walls in flax fibers requires β-galactosidase. *Plant Physiology*, *156*(3), 1351–1363.

Shibaoka, H. (1993). Regulation by gibberellins of the orientation of cortical microtubules in plant cells. *Functional Plant Biology*, *20*(5), 461–470.

Western, T. L., Burn, J., Tan, W. L., Skinner, D. J., Martin-McCaffrey, L., Moffatt, B. A., & Haughn, G. W. (2001). Isolation and characterization of mutants defective in seed coat mucilage secretory cell development in *Arabidopsis*. *Plant Physiology*, *127*(3), 998–1011.

Western, T. L., Skinner, D. J., & Haughn, G. W. (2000). Differentiation of mucilage secretory cells of the *Arabidopsis* seed coat. *Plant Physiology*, *122*(2), 345–356.

Willats, W. G. T., McCartney, L., & Knox, J. P. (2001). In-situ analysis of pectic polysaccharides in seed mucilage and at the root surface of *Arabidopsis thaliana*. *Planta*, *213*(1), 37–44.