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A Versatile Soybean Recombinant Inbred Line Population Segregating for Low Linolenic Acid and Lipoxygenase Nulls - Molecular Characterization and Utility for Soymilk and Bioproduct Production

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1. Introduction

The chapter aims to: (a) review the development of a soybean recombinant inbred line (RIL) population obtained from the RG10 x OX948 cross that is segregating for low linolenic acid (LLA) and lipoxygenase nulls (3lx), (b) present the molecular characterization of the LLA and 3lx traits and (c) discuss the applicability of the population for soymilk and biocomposite production.

The chapter is organized as follows. Section 2 describes development and evaluation of the RG10 x OX948 RIL population, inheritance of LLA and 3lx traits and the development of novel low linolenic acid, lipoxygenase free (LLA.3lx) germplasm. Section 3 describes the molecular characterization of the LLA and 3lx traits derived from RG10 and OX948, respectively. The versatility of the RIL population for different applications, including soymilk and bioproduct production are presented in Section 4, which illustrates that there is potential for utilizing the whole soybean plant. The conclusion summarizes the results and discusses possible future uses of the RG10 x OX948 RIL population.

Problem - Oxidation of linolenic acid (LA) is catalyzed by lipoxygenase (LX; linoleate: oxygen oxidoreductase; EC 1.13.11.12) and is associated with off-flavours of soybean products. The hydroperoxides that are produced and their breakdown products impart undesirable grassy-beany and bitter flavours on soybean products (Rackis et al., 1979). Most soybean cultivars contain 70 to 90 g kg^{-1} LA and approximately 20 g kg^{-1} of the total seed protein is LX. A possible solution to soybean stability problems is to genetically eliminate seed LX and reduce the content of LA. A number of soybean cultivars with low LA content (LLA; < 40 g kg^{-1} LA) or seed LX nulls (Hammond et al., 1972; Hildebrand & Hymowitz, 1982; Wilcox, 1985; White, 2000) have been developed. It was shown that oil extracted from LLA lines is more stable than oil extracted from conventional soybean, needs less hydrogenation and consequently, contains less unhealthy trans fatty acids (Mounts et al., 1988), which may reduce the risk of heart disease (Hayakawa et al., 2000). Similarly, protein products from lx3 soybean have more favourable flavour profiles (King et al., 1998). It has been suggested that a combination of seed LX nulls with LLA content might lead to even

better tasting soybean protein products and oil with improved oxidative stability (Davies & Nielsen, 1986). However, oil extracted from a line combining LX2 and LX3 nulls with less than 30 g kg^{-1} LA content did not show improved oxidative stability (Shen et al., 1996). To explore the potential for improving oil stability in soybean further we combined LX triple null (*lx*1*lx*2*lx*3) from line OX948 with LLA content from lines RG10 (*fan*-b) and PI 361088B [*fan*(PI 361088B)] into new LLA.3lx soybean lines (Reinprecht et al., 2005; 2006a).

Inheritance - Different patterns of inheritance for LA have been reported including quantitative inheritance (Graef et al., 1988) or a combination of major and minor genes (Fehr et al., 1992). The LLA content in RG10 is simply inherited and controlled by homozygous recessive alleles with additive effects at the *Fan* locus (Stojsin et al., 1998). Different types of gene action for LA content, ranging from codominance to complete dominance, have been reported for different crosses (Rahman et al., 1998). Also, depending of cross, complete maternal inheritance of LA content (Martin et al., 1983), partial maternal effects (Graef et al., 1988), or the lack of a maternal effect (Rahman et al., 1998) have been observed. In addition, environmental conditions, especially temperature were reported to have effect on unsaturated fatty acid contents (Wolf et al., 1982).

Similarly, inheritance of seed LX is simple. A single distinct gene encodes each of the three seed isozymes. Nulls for LX are controlled by single recessive loci, named *lx*1, *lx*2 and *lx*3 (Hildebrand & Hymowitz, 1982; Kitamura et al., 1983; Davies & Nielsen, 1986). *Lx*1 and *Lx*2 are tightly linked whereas the inheritance of *Lx*3 is independent of *Lx*1 and *Lx*2 (Davies & Nielsen, 1986), so that in 3lx mutants, a two-gene inheritance pattern has been observed (Hajika et al., 1992). No maternal or cytoplasmic effects were reported (Hildebrand & Hymowitz, 1982). The new LLA.3lx soybean lines were used to study the inheritance of LA and LX genes and to test for possible interactions between these traits (Reinprecht et al., 2005; 2006a).

Molecular basis of LLA and lx3 traits - In soybean seed, LA is synthesized predominantly in the endoplasmic reticulum (ER) by desaturation of linoleic acid (Cherif et al., 1975). The reaction is catalyzed by cytoplasmic, membrane-bound, ω-3 fatty acid desaturase (E.C. 1.14.99.-), which introduces the third double bond in linoleic acid at the ω-3 position. It was suggested that three microsomal ω-3 fatty acid desaturase genes (*GmFad*3*A*, *GmFad3B* and *GmFad3C*) contribute to LA levels in soybean seed, with the *GmFad3A* (*GmFad3-1b*) being the most abundant (Bilyeu et al., 2003; Anai et al., 2005). Therefore, mutations in these genes could reduce the LA concentration in soybean seeds (Byrum et al., 1997; Bilyeu et al., 2005). A number of soybean breeding lines and cultivars with LLA content have been developed by mutagenesis and conventional breeding. The LA content in an ethyl methane sulfonate (EMS) mutant line C1640 (~34 g kg^{-1}, Wilcox et al., 1984) is controlled by a single recessive *fan*(C1640) allele at a major *Fan* locus (Wilcox & Cavins, 1985), which encodes ω-3 fatty acid desaturase (Brummer et al., 1995). The *Fad3* gene, coding for ω-3 fatty acid desaturase, was mapped to the same region (Byrum et al., 1995). The molecular basis of the LLA trait has been described in different backgrounds. The *fan* allele from the LLA line A5, initially associated with the full or partial deletion of an unknown microsomal ω-3 desaturase gene (Byrum et al., 1997), was later assigned to the *GmFad3A* gene (Bilyeu et al., 2003). The mutated gene from the line M24 (X-ray mutant) corresponds to *GmFad3B* (*GmFad3-1a*) at the *Fanx* locus (Anai et al., 2005). Soybean line A29 has mutations in all three ω-3 fatty acid desaturases (*GmFad*3*A*, *GmFad3B* and *GmFad3C*) and has a LA content of 10 g kg^{-1} (Bilyeu et al., 2006). RG10 is a LLA mutant line produced by EMS treatment of C1640. The LLA content in RG10 (less than 25 g kg^{-1}) is simply inherited and controlled by homozygous

recessive alleles at the *Fan* locus with additive effects. The RG10 allele was designated as *fan-b* (for a very low content of LA) by Stojsin et al. (1998). The objective of this study was to determine the molecular basis of the LLA trait in soybean mutant line RG10, developed by two rounds of EMS treatment.

Mature soybean seeds contain three LX isozymes, namely, LX1, LX2 and LX3 (Axelrod et al., 1981). The isozymes have different pH optima, substrate specificities and reaction products (Feussner & Wasternack, 2002). Spontaneous mutants for single LX isozymes have been identified (Hildebrand and Hymowitz 1981; Kitamura, 1984) and a number of single, double (Kitamura et al., 1985) and 3lx mutants were developed by gamma (γ)-irradiation (Hajika et al., 1991). The molecular basis for three seed LX nulls has been described in different backgrounds. Wang et al. (1994) identified a single amino acid replacement at H532Q (corresponds to H504 in LX1) in two triple null γ-mutant lines (Kyushu 111 and the K line) that resulted in an inactive LX2 isozyme. Two null alleles have been identified for the *Lx3* gene. Two single nucleotide substitutions in the promoter region of *Lx3* (mutation 2: C to T at -636 and mutation 1: T to A at -585) in Kyushu 111 and the K line resulted in a complete null for that isozyme (Wang et al., 1995). The lines PI 205085, PI 417458 (*Lx1Lx2lx3*) and Jinpumkong 2 (*lx1lx2lx3*) have an *lx3-a* allele, which is a frame shift mutation (single G deletion at the position 101 in exon 1) that introduces a stop codon and results in premature termination of protein translation (Lenis et al., 2010). Three null alleles have been reported for the *Lx1* gene. The *lx1-a* allele (PI 408251) is a 74 bp deletion in exon 8 at position 2752, which introduces a stop codon and terminates protein synthesis after 524 amino acids. In addition to the same 74 bp deletion, the *lx1-c* allele (Jinpumkong 2) contains seven substitutions and 3 bp deletion at the 5′ end of the gene. A nonsense mutation C2880A in the *lx1-b* allele (PI 133226) results in an S568STOP change (Lenis et al., 2010). The objective of this work was to determine the molecular basis of the seed LX nulls in soybean mutant line OX948.

Many GenBank records of ω-3 fatty acid desaturase (*Fad3*) and seed LX *(Lx1*, *Lx2* and *Lx3*) genes exist. Recent release of a complete draft of soybean genome sequence opens new insights into genome organization of this ancient paleopolyploid (Schmutz et al., 2010). Four homologous regions containing 19 LX genes are present in the soybean genome (Shin et al., 2008). Similarly, homology between *GmFad3-1a* (*Fad3B*) and *GmFad3-1b* (*Fad3A*) and between *GmFad3-2a* (*Fad3C*) and *GmFad3-2b* (‘*Fad3D*’) suggests that the ω-3 fatty acid desaturase gene family in soybean may have developed through gene duplication (Anai et al., 2005).

LA and LX markers - Conventional methods like gas liquid chromatography (Bannon et al., 1987) and colorimetric assays (Suda et al., 1995), are convenient for screening lines for LA content and seed LX status in soybean breeding programs focussed on selecting cultivars with improved flavour. Both procedures can be performed on a small piece of the seed, leaving the rest of the seed for planting. However, the assays are time consuming and the colorimetric LX assays are subjective and cannot distinguish heterozygotes. In contrast, screening procedures based on codominant, gene-specific, markers for LLA and 3lx traits could increase the precision and efficiency of breeding for LLA.3lx soybean since the markers, which are environmentally neutral, can be determined quickly and accurately using DNA extracted from any small tissue sample. The use of marker-assisted selection (MAS) can accelerate and simplify breeding soybean with improved flavour. Kim et al. (2004) developed a single nucleotide polymorphism (SNP) marker for the *Lx2* gene. Spencer et al. (2004) developed a simple sequence repeat (SSR) marker Satt534, while Sauer et al. (2008) developed *Fad3A* gene-based markers for LA content. Working with the LLA.3lx RG10 x OX948 population, our group developed markers for LLA (SSR Satt534 and gene-

based Fad3i6) and 3lx (Lox1-3 and Lox3-$_{HaeIII}$) (Reinprecht, 2002; Reinprecht et al., 2006b), which have been used to simultaneously select for LLA and 3lx (*lx1*/*lx2* and *lx3*) phenotypes, thus accelerating selection for soybean lines for improved soymilk stability and flavour (Luk, 2006). The objectives of this work were to characterize previously developed gene-based markers and to develop additional markers based on mutations in newly sequenced ω-3 fatty acid desaturase and LX genes.

Use of RIL population in soymilk study - Soymilk is a highly nutritious beverage but faces some consumer acceptance barriers because of objectionable flavours and odours (Torres-Penaranda & Reitmeier, 2001). It is made by grinding water-soaked soybeans with additional water. The liquid is separated from the okara (ground soybean) by filtration and then heat processed (Liu, 1997). The compounds that give soy its poor taste are believed to be breakdown products of the hydroperoxides formed during oxidation of polyunsaturated fatty acids, like linolenic acid (Dutton et al., 1951). Hydroperoxide formation is catalyzed by seed LX (Furata et al., 1996; Wilson, 1996). Hydrogenation has been used to improve both the flavour and stability of soybean oil (Dutton et al., 1951; Mounts et al., 1994) but studies have shown that oils produced by this process contain *trans* fatty acid isomers, which are associated with an increased risk of coronary heart disease when consumed for an extended period of time (Zock & Katan, 1997). Studies on oil stability and flavour show that soybean oils with reduced LA content compared favourably to oils derived from conventional soybean cultivars (Mounts et al., 1988; Mounts et al., 1994). LX-free lines have also been shown to produce less of the volatiles responsible for the generation of off-flavours (Kobayashi et al., 1995; Furata et al., 1996).

The level of LA in soybean oil is typically 8% (or 80 g kg^{-1}) but LLA soybean lines (~ 2%) have been produced that are comparable to conventional soybean lines for their agronomic and seed traits (Ross *et al*., 2000; Reinprecht et al 2005). It has been also suggested that the elimination of lipoxygenase from soy products would make them blander and more acceptable to consumers (Wilson, 1996). Lines from the LLA.3lx soybean RIL population were used to test the effects of LLA and lx3 traits on lipid oxidation and soymilk flavour (Luk, 2006).

Use of RIL population in composite study - Soybean is grown for it high value oil (20%) and protein (40%) seed. However, residues (stem and leaf) left after harvesting the seed are potential sources of significant amounts of low cost plant fibers. For every ton of grain harvested there is 3-5 times that weight in stem fibers. Although annually renewable and available in abundance, agricultural residues (including soybean) are of limited value at present and are usually discarded and left in the field to decompose (Sticklen, 2006). The use of crop residues for industrial applications could be an additional source of revenue for farmers. If only a portion (approximately 30%) of the stems are collected this can be done without adversely affecting soil fertility (Lindstrom, 1986). Properties such as good mechanical performance, good formability, biodegradability, high sound absorption, low abrasiveness, low dermal and respiratory irritability and low density make plant fibers suitable for use as fillers and reinforcements in composite materials. Use of these materials has the potential to reduce the environmental impact of composite manufacture and reduce fuel consumption. Demands for lightweight car parts and good recycling possibilities ("green" car) are the main reasons for investigating the potential of using biofibers in the automotive industry. However, because of weak bonding between the fibers (hydrophilic) and the polymer matrices (hydrophobic), moisture sensitivity, odour emission, the relatively low processing temperatures that can be used and variation in the quality of biofibers the use of composites with plant fibers currently has limited applications to the car interior.

Plant fibers are composed of cell wall materials. As a dicot, soybean has a type I primary cell wall, which consists of cellulose microfibrils buried in a matrix composed of hemicellulose (xyloglucan), pectin (homogalacturonan and rhamnogalacturonan) and protein (extensins and atabinogalactans). The interactions between the different polysaccharides provide the strength and flexibility of the cell wall (Hartholt et al., 2010). Secondary wall (synthesized after cell stops growing) contains mainly cellulose, hemicellulose and lignin (Yokoyama & Nishitani, 2004).

Cellulose is a linear polymer of hydrogen-bonded β-(1,4)-linked glucose molecules organized in parallel crystalline layers, which form 3 nm thick microfibrils. Hydrogen bonds between different layers of the polysaccharides contribute to the resistance of crystalline cellulose to degradation (Sticklen, 2008). Primary cell wall microfibrils consist of approximately 8000 glucose molecules, while secondary cell wall may contain 15000 glucose molecules (Mutwil et al., 2008). Physical properties such as the crystalline state, degree of crystallinity and molecular weight are highly variable. Cellulose is synthesized in the plasma membrane by cellulose synthases (CesA) multimeric complexes arranged as hexagonal rosettes, each producing one microfibril of 36 glucan chains. *Arabidopsis thaliana* has at least 10 CesA genes with cell-specific expression patterns and functions. *CesA1*, *CesA3* and *CesA6* encode enzymes for primary wall cellulose biosynthesis, while *CesA4*, *CesA7* and *CesA8* are expressed during secondary wall cellulose biosynthesis. Interactions between *CesA* and the cytoskeleton have important effects on cellulose fibril orientation and length (Somerville, 2006; Mutwil et al., 2008; Penning et al., 2009).

While cellulose has a relatively simple chemical structure, which is same for all plant species, hemicellulose is more complex. Hemicelluloses encompass a heterogeneous group of polysaccharides (including xylans, xyloglucans and (gluco)mannans) that have β-(1,4)-linked backbones (composed of various 5- and 6-carbon sugars such as arabinose, galactose, glucose, mannose and xylose) with equatorial configurations. Its structure differs among plant species and cell types. Dicot primary cell walls are composed mainly of xyloglucan (20-25% w/w) with small amounts of glucuronoarabinoxylan (5%) and (gluco)mannan (3-5%). Glucuronoxylan is the major component (20-30%) of secondary cell walls with the small amounts of xyloglucan, (gluco)mannan (2-5%) and galactoglucomannan (0-3%) (Scheller & Ulvskov, 2010). Hemicelluloses are synthesized in the Golgi membranes by glycosyltransferases. The enzymes required for xyloglucan biosynthesis include α-L-arabinofuranosidase, xyloglucan endotransglucosylase, endo-xyloglucan transferase, β-mannosidase and β-galactosidase.

Pectin is structurally and functionally the most complex polysaccharide and it forms a gel matrix in the plant primary cell wall. Pectins are composed of highly complex family of polysaccharides rich in D-galacuronic acid. Cell walls contain different pectic polysaccharides including homogalacturonan (HG), xylogalacturonan (XGA), apiogalacturonan, rhamnogalacturonan (RGI) and rhamnogalacturonan II (RGII). Pectin is synthesized in the Golgi membranes and about 67 different glycosyltransferases, methyltransferases and acetyltransferases are required for these processes (Mohnen et al., 2008).

Lignin is a complex aromatic polymer composed of three major phenolic compounds, namely: *p*-coumoryl alcohol (H lignin), coniferyl alcohol (G lignin) and sinapyl alcohol (S lignin). Monolignol biosynthesis starts from the amino acid phenylalanine and proceeds through number of ring and side-chain modifications catalyzed by dozens of enzymes. The three monolignols differ from each other only by their degree of metoxylation. Peroxidase, laccase and dirigent proteins are involved in monolignol polymerization. The ratio of

monolignols within the polymer varies between plant species, different tissues and cell wall layers (Boerjan et al., 2003).

Cell walls also contain proline-rich proteins, hydroxyproline-rich glycoproteins, arabinogalactan proteins, expansins, germin-like proteins, extensins, actin, 14-3-3-like proteins and laccase. Some of the cell wall modifying enzymes include: endo-1,4-β-D-glucanase, endo-1,3 1,4-β-D-glucanase, β-1,3-glucanase, β-galactosidase and cellulase.

Regulation of cell wall biosynthesis is complex. To build secondary cell walls, genes encoding cellulose, hemicellulose and lignin biosynthetic enzymes need to be coordinately expressed in a cell type specific manner. Secondary wall-associated NAC domain protein 1 (SND1) acts as a master switch turning on a subset of MYB and NAC transcription factors, which then activate the developmental machinery of secondary wall biosynthesis in fibers of Arabidopsis (Zhong & Ye, 2007; Zhong et al., 2008).

Lignin and hemicellulose reduce the value of plant fibers for composite manufacture because, compared to cellulose, they degrade at different temperatures. Hemicellulose degrades at a lower temperature compared to cellulose while lignins with different monolignol components degrade at different temperatures. However, it has been shown that lignin compositions and amounts can be engineered to improve the processing efficiency of plant biomass (Vanholme et al., 2008). The interactions between lignin biosynthesis and other metabolic pathways as well as the cellulose and hemicellulose networks can also influence fiber traits. As pectin has roles in both primary and secondary cell walls, manipulation of pectin synthesis is expected to have diverse impacts on plant properties, including plant biomass (Mohnen, 2008). The principal aim of this work was to analyze the structure and properties of soybean stem fibers with a view to assessing their potential as additions for composites for the automotive industry. In order to simplify and accelerate future selection and/or molecular manipulation of plant fiber characteristics, the first step is to increase the information about the structural and regulatory genes that control the synthesis and modification of cell wall components (Reinprecht et al., 2010).

2. RG10 x OX948 – LLA.3lx RIL population

2.1 RIL population development and evaluation

The soybean RIL population from the RG10 x OX948 cross was developed for a study to improve soybean oil and protein flavour. The population was derived from reciprocal crosses between a LLA line (RG10) and a 3lx line (OX948) and consists of 169 RILs (Reinprecht et al., 2005). Mutant line RG10, used as the source for the LLA trait, was developed at the University of Guelph, Ridgetown Campus, Ridgetown, Ontario, Canada by chemical mutation of the LLA line C1640 (an ENS mutant of cultivar Century) with EMS (an EMS mutant of cultivar Century). The allele at the *Fan* locus in RG10 is designated as *fan*-b, for a very low content (< 25 g kg^{-1}) of LA (Stojsin et al., 1998). The 3lx mutant line OX948 was developed at Agriculture and Agri-Food Canada, Greenhouse and Processing Crops Research Centre, Harrow, Ontario, Canada (Buzzell, unpublished). OX948 is a selection from a cross between Harovinton and a 3lx F_2 plant [produced by γ-irradiation; F_1 seed was obtained from Dr. Kitamura (National Agricultural Research Centre, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Japan)]. The 3lx line was selected at the F_4 stage and included in yield trials. Both RG10 and OX948 were evaluated for yield and other agronomic characteristics. The parental lines were lower yielding compared to adapted commercial cultivars.

Reciprocal crosses between RG10 (*fan-bLx1Lx2Lx3*) and OX948 (*Fanlx1lx2lx3*) were made in the growth room at the Department of Plant Agriculture, University of Guelph. The F_1 plants, segregating F_2 plants and parents were evaluated under controlled indoor environments [16 h light (200 μmol m^{-2} s^{-1}) and 8 h dark, 26 and 22°C, respectively] in order to reduce possible environmental effects on fatty acid content. Single seed descent was used to accelerate inbreeding. A portion of a single seed (~2/3 of the seed with embryo axis remaining after 1/3 of the seed was removed and used to determine the fatty acid composition and presence of LX) from each F_2 line was planted in a growth room using a 12 h day length until flowering, followed by a 16 h day length (26°C day and 22°C night) with a light intensity of 500 μmol m^{-2} s^{-1}, to produce F_3 seeds. The identity of each F_2 line and parent seeds was maintained during the inbreeding. The F_3 seed was advanced to the F_5 generation in Belize and the F_5 lines were increased in single rows at Ridgetown and Harrow during the summer of 1999 (Reinprecht et al., 2006a).

The RIL populations [at the F_6 and F_7 stages together with parents and two check cultivars (OAC Bright and OAC Glencoe)] were evaluated for a number of seed and agronomic traits at three Ontario locations (Harrow, Ridgetown and Woodslee) in 2000 and 2001. The seed and agronomic characteristics of six LLA.3lx (<30 g kg^{-1} LA and all three seed LX enzymes absent) F_7 lines together with the parents (RG10 and OX948) and eight lines/cultivars commonly grown in Ontario (OAC Bayfield, OAC Bright, OAC Glencoe, OAC Kent, OAC Oxford, 9163, RCAT Legacy and RCAT Staples) were evaluated in separate experiments at four Ontario locations (Harrow, Ridgetown, Woodslee and Woodstock) in 2001. Traits that were evaluated include: fatty acid composition (palmitic, stearic, oleic, linoleic and linolenic acid), presence of seed lipoxygenases (LX1, LX2 and LX3), days to flowering, days to maturity, plant height, plant lodging, seed yield, seed weight, oil and protein content. LX and fatty acid compositions were determined using a half-seed technique (Wilcox & Cavins, 1985). Approximately one third of the cotyledon tissue distal from embryonic axis was used for fatty acid and LX analyses and the rest of seed was planted. Individual LX was assayed for two seeds of the parents, check cultivars and RILs by visually judging the outcome of a slightly modified colorimetric method (Suda et al., 1995). Fatty acid compositions were obtained by analyzing 10-seed bulks of parental, check cultivars, and F_6 lines by gas liquid chromatography of fatty acid methyl esters according to the modified method of Bannon et al. (1987) using a Hewlett Packard 6890 Series gas chromatograph (Mississauga, ON), equipped with the J&W BB-23 15 m x 250 μm x 0.25 μm capillary column (J & W Scientific, Folsom, CA).

Some seed and agronomic characteristics [averaged across six environments (three locations and two years)] for 169 RILs of the RG10 x OX948 population are shown in Table 1. Reduction of LA and removal of seed LX did not cause any obvious detrimental effects on agronomic traits including yield, indicating the possibility of developing productive soybean cultivars with high protein quality and oil stability (Reinprecht, 2002; Reinprecht et al., 2006b).

2.2 Inheritance of LA and LX traits

Reciprocal crosses between RG10 and OX948 were made and populations derived from them were evaluated for their fatty acid profiles and seed LX at the F_2, F_5 and F_6 generations. RG10 contains a single gene at a *Fan* locus that controls LA content with alleles acting in an additive manner (Stojsin et al., 1998). OX948 is normal line with more than 90 g kg^{-1} LA. In a RG10 x OX948 cross LA levels were determined by a single major gene with alleles acting in

Line	LA	LX			H/L	Oil	Protein	Yield
		1	2	3				
RG10	53.5	+	+	+	38.1	188.3	401.6	2481.8
OX948	146.1	-	-	-	29.2	176.6	402.2	2874.0
RO1	58.2	+	+	+	38.9	180.2	428.7	2872.0
RO2	78.7	-	-	-	30.4	186.7	420.2	2604.0
RO3	116.2	+	+	+	34.2	181.3	410.7	2714.3
RO4	138.3	+	+	+	25.3	184.8	414.5	3213.5
RO5	62.1	-	-	+	30.0	182.0	424.3	2997.5
RO6	120.8	-	-	+	40.7	187.8	410.0	2463.5
RO7	64.1	-	-	+	37.7	180.7	429.8	2797.0
RO8	136.7	-	-	+	35.8	181.8	420.8	2501.0
RO9	64.4	-	-	+	25.0	180.2	424.5	2383.8
RO10	143.0	-	-	+	26.2	182.7	411.7	1625.7
RO11	84.7	-	-	+	35.4	176.3	430.2	3217.0
RO12	64.1	+	+	+	35.7	174.7	440.7	2835.3
RO13	65.8	-	-	+	55.8	180.0	389.5	2248.0
RO14	62.0	+	+	+	36.4	182.0	428.8	2378.3
RO15	138.0	+	+	+	33.2	181.5	414.0	2940.2
RO16	61.0	+	+	+	41.9	183.7	408.0	3549.8
RO17	NA	+	+	+	38.0	195.0	366.0	1505.0
RO19	48.4	-	-	+	32.2	183.3	418.3	2876.5
RO20	72.6	+	+	+	30.8	171.5	422.3	2599.2
RO21	141.0	+	+	+	36.6	183.5	405.0	3205.2
RO22	63.0	+	+	+	26.7	173.5	425.8	2462.0
RO23	41.4	+	+	+	32.0	177.0	411.2	3355.8
RO24	57.8	+	-	+	30.0	192.8	414.0	3532.3
RO25	74.2	+	+	+	32.2	180.7	416.0	2740.7
RO26	123.8	-	-	+	27.7	173.8	425.8	3210.3
RO27	68.2	+	+	+	34.5	176.5	416.8	2960.8
RO28	122.2	+	+	+	39.0	173.2	402.3	3806.2
RO29	143.2	+	+	+	30.9	186.7	409.3	2815.7
RO30	116.8	+	+	+	33.5	175.2	423.8	2796.3
RO31	48.3	+	+	+	40.1	176.8	437.0	2606.0
RO32	101.4	-	-	-	36.2	185.2	405.5	2633.2
RO33	130.7	+	+	+	33.4	188.0	415.8	2512.0
RO34	139.0	+	+	+	26.7	187.8	395.8	2826.8
RO35	63.0	+	+	+	29.2	183.8	429.5	3219.2
RO36	72.1	-	-	+	31.4	176.8	432.5	3151.5
RO37	107.0	-	-	-	25.4	174.5	430.0	2561.7
RO38	33.5	+	+	+	37.6	181.2	417.0	3208.5
RO39	88.5	+	+	+	45.8	180.3	422.0	2818.2
RO40	37.4	+	+	+	52.4	179.3	439.8	2729.7
RO41	61.4	+	+	+	39.0	180.7	418.2	2976.3
RO42	98.9	-	-	-	43.1	180.7	415.8	2216.8
RO43	38.6	+	+	+	32.0	172.5	420.0	3121.2
RO44	122.2	+	+	+	37.9	178.8	419.2	2611.3
RO45	NA	+	+	+	NA	NA	NA	NA
RO46	41.2	-	-	+	34.7	185.7	422.7	2124.0
RO47	82.5	-	-	+	32.9	180.0	410.5	2562.5
RO48	70.3	-	-	-	37.1	184.0	415.0	2979.3
RO49	60.4	+	+	+	30.6	185.5	410.3	2423.5

Line	LA	LX			H/L	Oil	Protein	Yield
		1	2	3				
RO88	119.7	+	+	+	24.8	179.0	406.8	1553.5
RO89	65.2	-	-	-	33.6	187.2	428.3	2758.8
RO90	109.8	-	-	-	28.8	177.5	415.8	2821.7
RO91	124.9	+	+	+	37.6	182.3	419.7	2425.3
RO92	67.8	+	+	+	32.2	171.7	426.0	3298.7
RO93	56.0	+	+	+	35.6	182.5	415.2	2917.3
RO94	114.1	+	+	+	33.4	183.7	411.8	3590.5
RO95	128.7	-	-	+	37.2	186.0	420.7	3123.8
RO96	134.6	+	+	+	24.5	179.8	418.3	1234.7
RO97	35.4	+	+	+	34.3	186.7	407.5	3180.5
RO98	120.8	-	-	-	23.8	189.3	402.0	2386.0
RO99	NA	-	-	+	NA	NA	NA	NA
RO100	69.9	+	+	+	32.5	178.2	422.5	3876.2
RO101	59.5	+	+	+	28.0	185.5	415.7	3322.0
RO102	134.4	+	+	+	37.6	181.8	408.0	2600.3
RO103	72.6	-	-	-	42.4	180.3	437.7	2820.3
RO104	147.7	-	-	-	26.7	184.2	408.2	2391.7
RO105	NA	+	+	+	NA	NA	NA	NA
RO106	42.9	+	+	+	52.8	193.8	389.2	3607.2
RO107	120.3	+	+	+	39.6	183.0	414.0	2608.5
RO108	55.0	+	+	+	29.8	179.0	413.2	2860.8
RO109	49.4	-	-	+	32.6	178.3	417.0	3750.8
RO110	124.4	+	+	+	37.2	179.8	410.8	3806.2
RO111	38.4	+	+	+	31.5	174.0	413.5	3537.3
RO112	65.8	+	+	+	26.8	179.0	429.3	2496.3
RO113	46.5	-	-	-	32.9	185.5	416.2	2669.2
RO114	36.1	+	+	+	37.5	175.7	430.8	3024.2
RO115	50.9	-	-	-	27.4	178.0	434.0	2550.3
RO117	108.4	-	-	+	41.5	180.3	427.7	3699.8
RO118	65.0	+	+	+	27.6	182.0	423.3	3259.7
RO120	104.8	-	-	+	39.7	185.3	417.3	2268.3
RO121	118.7	-	-	+	34.6	186.7	408.8	2747.5
RO122	126.7	+	+	+	34.4	177.5	417.5	2537.5
RO123	116.8	-	-	+	35.6	188.0	414.8	2915.3
RO124	115.4	+	+	+	32.0	183.3	406.8	2553.3
RO125	73.1	+	+	+	28.3	177.7	427.7	2308.7
RO126	93.7	-	-	+	21.4	169.0	433.7	1910.8
RO127	34.0	+	+	+	33.5	184.8	399.8	2375.5
RO128	62.8	+	+	+	33.9	178.3	426.7	2377.0
RO129	38.8	+	+	+	36.0	174.5	417.3	2792.0
RO130	122.3	-	-	-	36.6	184.0	405.8	2575.2
RO131	103.2	-	-	+	35.5	179.8	414.5	3408.7
RO132	133.4	-	-	-	30.1	186.5	415.2	2478.5
RO133	85.8	-	-	+	27.3	181.7	418.5	2235.3
RO134	143.7	+	-	+	36.6	176.2	420.7	3027.8
RO135	62.6	+	+	+	27.1	183.8	420.7	3201.5
RO136	91.0	-	-	-	25.2	180.5	419.7	2893.7
RO137	66.2	+	+	+	33.4	179.3	432.8	3116.3
RO138	81.0	-	-	+	28.4	185.2	405.8	2518.0
RO139	63.1	+	+	+	21.7	178.5	424.7	1462.8

Line	LA	LX			H/L	Oil	Protein	Yield	Line	LA	LX			H/L	Oil	Protein	Yield
		1	2	3							1	2	3				
RO50	54.7	+	+	+	30.5	183.5	420.5	2813.7	RO140	57.6	-	-	+	38.6	188.0	419.7	2477.2
RO51	72.5	+	+	+	25.8	182.5	415.7	2673.2	RO141	61.1	-	-	+	35.4	181.8	423.0	2954.2
RO52	66.0	+	+	+	33.5	181.0	426.8	2403.0	RO142	90.8	-	-	+	28.4	179.7	421.8	2986.0
RO53	132.6	-	-	+	29.4	186.2	418.7	3159.0	RO143	133.8	+	+	+	32.1	185.8	426.2	3096.7
RO54	114.9	+	+	+	35.0	191.0	414.2	2835.0	RO144	47.0	+	+	+	21.0	181.2	424.0	1570.8
RO55	107.0	+	+	+	45.0	177.7	409.7	3426.5	RO145	64.4	+	+	+	36.1	180.5	419.8	2629.0
RO56	142.3	+	+	+	32.2	185.7	411.5	2670.8	RO146	83.5	-	-	+	29.1	179.3	422.2	2868.8
RO57	47.1	+	+	+	29.0	180.7	418.3	2677.0	RO147	60.4	+	+	+	51.0	177.7	427.2	3401.0
RO58	37.9	+	+	+	25.8	181.5	412.2	2371.0	RO148	127.2	+	+	+	32.7	184.5	410.7	3400.7
RO59	NA	+	+	+	NA	NA	NA	NA	RO149	105.9	+	+	+	30.6	181.3	421.5	3601.0
RO60	55.9	+	-	+	34.9	178.5	417.7	3378.8	RO150	35.1	-	-	+	35.5	178.0	433.0	2978.3
RO61	142.1	+	+	+	30.3	185.3	415.8	2954.5	RO151	125.3	+	+	+	30.2	184.2	402.5	2693.5
RO62	60.9	+	+	+	48.8	182.0	421.3	3705.5	RO152	114.2	+	+	+	31.0	180.2	423.3	2993.7
RO63	133.6	+	+	+	25.7	180.8	516.8	2099.3	RO153	136.9	+	+	+	33.7	175.7	405.7	3116.7
RO64	38.2	+	+	+	38.2	180.2	409.8	3302.2	RO154	67.8	+	+	+	38.3	176.3	425.7	3414.8
RO66	139.3	+	+	+	27.5	172.8	438.2	2236.0	RO155	89.3	-	-	+	31.9	179.5	428.5	3133.0
RO67	72.8	+	+	+	28.1	177.7	418.3	3296.7	RO156	101.4	-	-	+	29.7	182.3	416.5	3063.8
RO68	122.1	+	+	+	23.9	182.5	411.0	3031.3	RO157	119.5	+	+	+	28.4	186.3	420.8	2000.8
RO69	36.3	+	+	+	32.7	175.0	418.7	3246.0	RO158	121.0	+	+	+	31.5	181.7	414.5	2477.7
RO70	76.3	-	-	-	28.4	176.8	438.7	2191.2	RO159	126.1	+	+	+	41.5	182.5	426.8	2905.3
RO71	118.8	+	+	+	42.8	187.3	417.5	2338.8	RO160	59.1	-	-	+	31.5	184.3	419.5	2600.5
RO72	120.0	-	-	+	29.4	175.7	420.8	2273.5	RO161	67.2	-	-	+	26.0	176.2	431.8	2453.7
RO73	40.5	-	-	+	27.6	175.0	432.8	2718.8	RO162	35.0	-	-	-	37.8	184.0	416.0	2934.0
RO75	108.0	+	+	+	29.3	181.3	411.7	3044.7	RO163	38.9	-	-	+	37.8	179.0	427.0	3211.5
RO76	76.1	+	+	+	29.7	173.0	434.5	2297.0	RO164	61.6	+	+	+	33.0	178.8	403.5	3130.2
RO77	119.8	-	-	-	34.1	179.7	421.2	2565.0	RO165	34.0	+	+	+	49.9	180.0	413.3	3395.3
RO78	115.1	+	+	+	35.0	174.8	418.0	3465.4	RO166	50.3	-	-	+	34.3	181.8	416.7	2652.2
RO79	150.6	+	+	+	36.7	176.2	413.2	3224.5	RO167	63.8	-	-	+	42.8	185.2	407.7	3525.3
RO80	127.7	-	-	-	39.5	185.7	417.5	2569.7	RO168	135.0	+	+	+	32.2	179.2	421.5	2879.7
RO81	139.1	-	-	+	35.3	183.2	410.7	3211.5	RO169	74.3	+	+	+	42.3	176.3	423.2	2921.8
RO82	124.2	-	-	-	31.7	180.7	403.5	2608.8	RO170	63.6	+	+	+	31.1	183.5	431.8	2335.7
RO83	142.7	+	+	+	24.3	180.3	413.7	1921.8	RO171	41.7	+	+	+	36.7	184.3	414.8	2666.3
RO84	143.6	+	+	+	34.3	180.3	429.3	3163.7	RO172	38.4	+	+	+	37.9	174.2	416.7	2818.5
RO85	61.3	-	-	+	32.7	173.8	429.5	3552.5	RO173	124.9	-	-	+	31.6	179.0	419.5	2369.7
RO86	85.0	-	-	+	24.5	191.2	418.8	2022.5	RO174	93.1	-	-	+	30.1	180.8	423.8	2557.2
RO87	63.0	-	-	+	36.7	174.3	417.7	2365.7									

LA, linolenic acid content (g kg^{-1}); LX, seed lipoxygenase (+, present; - , absent); H/L, height/lodging index trait (cm/unit of lodging); Oil, oil content (g kg^{-1}, on dry weight basis); Protein content (g kg^{-1}, on dry weight basis); Yield (kg ha^{-1}, based on 13% moisture)

Table 1. Seed and agronomic characteristics of RG10 x OX948 population (averaged across three locations and two years)

an additive manner. However, evidence for the presence of minor genes in this material interacting with the major gene was observed. These results might explain the transgressive segregants that were observed at the high end of the LA content distribution for the F_2 population from this cross (Reinprecht et al., 2005). No significant cytoplasmic effects were observed on LA content. The LLA trait was highly heritable and stable in different environments (Belize and Ontario, Canada). The three seed LX were each controlled by

single genes in RG10 x OX948 crosses. No significant correlations were observed between LA content and seed LX.

2.3 Novel LLA.3lx germplasm

Several RILs that combine LLA content (<35 g kg^{-1}) with 3lx were obtained from RG10 x OX948 reciprocal crosses and evaluated for a number of seed and agronomic traits at four Ontario (Canada) locations (Harrow, Ridgetown, Woodslee and Woodstock) in 2001 (Reinprecht et al., 2006a). The LLA.3lx lines (RO115, RO162, OR265, OR297, OR315 and OR322) had higher than average seed weight and protein content, but were 21 to 31% lower yielding than typical cultivars for these regions. Compared with parental lines, the LLA.3lx lines were 5 to 18% lower yielding. However, comparisons of LLA.3lx lines with lines combining high LA and all three seed LX (HLA.3LX) indicated no yield difference between the two groups of lines in these populations. The results suggest that it should be possible to use this novel germplasm to develop competitive soybean cultivars that are resistant to oxidative degradation (Reinprecht et al., 2006a).

3. ω-3 fatty acid desaturase (*Fad3*) genes and three LX genes in RG10 and OX948

3.1 Linkage/QTL map

F_5 RILs from the RG10 x OX948 cross were genotyped for simple sequence repeats (SSR), random amplified polymorphic DNA (RAPD), sequence tagged sites (STS), and cleaved amplified polymorphic sequences (CAPS) markers and evaluated for seed and agronomic traits at three Ontario locations in two years. Linkage analysis was performed using Mapmaker/EXP version 3.0b (Lander et al., 1987). A minimum LOD score of 3.0 and maximum distance between two markers of 50.0 cM were used to assign loci into linkage groups. Recombination frequencies were converted to cM distances using Kosambi's mapping function (Kosambi, 1944). The RG10 x OX948 map was compared with soybean composite_2003 genetic map (http://soybase.org). QTL analysis was performed with composite interval mapping (CIM) using QTL Cartographer version 2.00 (Wang et al., 2001-2004). Genome-wide scans were performed for each trait and QTL were declared significant if their LOD threshold values were ≥ 2.0.

One hundred and twenty markers covering 1247.5 cM were mapped to 18 linkage groups (LG) in the soybean composite genetic map (Reinprecht, 2002; Reinprecht et al., 2006b). Seed LX *Lx1* and *Lx2* mapped as single major genes to adjacent locations 0.2 cM apart on chromosome 13 (LG F), which confirms their previous location (Davies & Nielsen, 1986). *Lx3* mapped to chromosome 15 (LG E). A major quantitative trait locus (QTL) associated with reduced LA content was identified on chromosome 14 (LG B2). QTL for 12 additional seed and agronomic traits were detected. LA content, linoleic acid content, yield, seed weight, protein content and plant height QTL were present in at least four of six environments. Three to eight QTL per trait were detected that accounted for up to 78% of total traits variation (Reinprecht, 2002; Reinprecht et al., 2006b). LA and LX loci did not overlap yield QTL, suggesting that it should be possible to develop high yielding cultivars that are resistant to oxidative degradation by MAS.

3.2 Molecular basis of LLA and 3lx traits

An understanding of the molecular bases of the LLA trait in RG10 and the 3lx trait in OX948 can facilitate breeding efforts of soybean with RG10-type and/or OX948-type of oxidative

stability. In order to determine the molecular bases of the LLA and 3lx traits, four ω-3 fatty acid desaturases (*Fad3A*, *Fad3B*, *Fad3C* and *Fad3D*) and three LX genes (*Lx1*, *Lx2* and *Lx3*) were sequenced in RG10 and OX948. The mutations in *Fad3A* gene (same as C1640) and the first characterized mutation in *Fad3B* gene, make RG10 an attractive source of the LLA trait (Reinprecht et al., 2009). A potential immediate use of RG10 (or its derivatives) is to produce very low LA content soybeans by crossing with lines containing the *fan* mutation only. Alternatively, extremely low LA (10 g kg^{-1} or less) lines could be derived from RG10 directly by mutating the *Fad3C* gene it carries with an additional EMS treatment. Also, since we have identified the molecular bases of the mutations in three LX genes in OX948 it is becoming more attractive source of the 3lx trait. Furthermore, because the simple and independent inheritance of these traits has been demonstrated in the LLA.3lx RIL population these lines should be an attractive breeding material for soybean cultivars with high oxidative stability.

3.2.1 Mutation in *Fad3* genes

The LLA line RG10 has reduced level of linolenic acid (<25 g kg^{-1}), increased level of linoleic acid (18:2, 650 g kg^{-1}) and decreased level of relative ω-3 (18:2D) desaturation {3%, calculated as 18:2D = [(18:3) / (18:2 + 18:3)] x 100} compared to normal line OX948 (>60 g kg^{-1}, 510 g kg^{-1}, 20%). A strong negative correlation between the linolenic and linoleic acid, consistent with the decrease in relative 18:2D activity was also observed in F_2, F_5 and F_6 generations of the RG10 x OX948 crosses (Reinprecht et al. 2005). These results suggest that the lower LA content in RG10 and its derivatives resulted from partial inactivation of ω-3 fatty acid desaturase and provides an additional evidence of the role of the *Fad3* genes in the expression of RG10 phenotype.

RG10 was developed by a two-step EMS mutagenesis process. The initial EMS treatment of the cultivar Century (approximately 70 g kg^{-1} of linolenic acid) produced line C1640 (Wilcox et al., 1984) with the mutation at the *Fad3A* ω-3 fatty acid deasturase gene (Chappel & Bilyeu, 2006) resulting in about 50% reduction of LA content (34 g kg^{-1}). Further reduction of LA (approximately 30%) to less then 25 g kg^{-1}, was achieved by mutating the *Fad3B* gene of C1640 by the EMS treatment to produce RG10 (Reinprecht et al., 2009). Therefore, RG10 has the first mutation in the *Fad3A* gene [same as C1640, *fan*(C1640) allele] and the second mutation in the *Fad3B* gene (*fan-b* allele).

Sequence analyses of mutant RG10 and wild-type OX948 ω-3 fatty acid desaturase genes showed that the low level of linolenic acid in RG10 is likely a result of mutations in two *Fad3* genes. For *Fad3A* the premature stop at the 798 position of the coding sequence (cDNA GenBank accession AY204710) would lead to a truncated protein that would not likely function as an active enzyme because it would be missing the third histidine box involved in the catalytic site of the enzyme (Schmidt et al., 1994) and C-terminus for ER localization. Similarly, the incomplete translation of the *Fad3B* transcript that would result from the splicing disruption could also lead to an inactive enzyme. This would leave only a functional *Fad3C* gene product in RG10 seeds [since *Fad3-2b* (*Fad3D*) is not generally active in seeds] and is likely the reason it contains less than 25 g kg^{-1}.

Mutations in both *Fad3A* and *Fad3B* genes contribute to the reduced level of linolenic acid in the RG10 line. In the RG10 x OX948 population, both *Fad3A* and *Fad3B* gene mutations were present only in lines with less than 35 g kg^{-1} of linolenic acid (data not shown). These support results of our previous genetic studies of the LLA trait in RG10 that suggested additive gene action (Reinprecht et al., 2005). In particular, the detection of the mutation in

the *Fad3B* gene in RG10 can explain the presence of additional minor gene(s) in RG10 x OX948 population that interact with the major *Fan* (*Fad3A*) allele (Reinprecht et al., 2005). This is further supported by the presence of minor LA QTL on chromosomes 9 (LG K) and 15 (LG K) in addition to the major QTL mapped to the *Fan* region on chromosome 14 (LG B2) (Reinprecht et al., 2006a). In addition, a *Fad3A* mutation-based marker developed in the current study explain the majority (approximately 60%) of the variability for the LA level in RG10 x OX948 population and maps in a major LA QTL in *Fan* region on chromosome 14 (LG B2). On the other hand, the *Fad3B*-based mutation identified in this study explains only 7% variability for the trait. This may explain why a cross between RG10 and C1640 indicated that a single gene controls the mutation in RG10 and that C1640 and RG10 have different alleles acting in an additive manner (Stojsin et al., 1998) and a second cross between RG10 and Century confirmed that the mutation in RG10 occurred at the *Fan* locus (Stojsin et al., 1998). Determination of the map location of *Fad3B* gene is underway.

3.2.2 Mutation in LX genes

The pedigree of OX948 includes an unnamed 3lx source produced by γ-irradiation. The status of LX null alleles in OX948 has not been previously investigated. This study identified molecular differences in three LX genes between OX948 and a soybean genotype with normal LX level (RG10) that explain the null LX phenotypes in the mutant. Sequence analyses of mutant OX948 and wild-type RG10 *Lx1* and *Lx2* genes showed that the mutations were affecting highly conserved group of 6 histidines necessary for enzymatic activity. The mutation in *Lx1* gene is a 74 bp deletion in exon 8, which introduces stop codon that would prematurely terminate translation. A single T to A substitution in *Lx2* gene changes histidine H532 (one of the iron-binding ligands essential for LX2 activity) to glutamine. The mutation in *Lx3* gene is in the promoter region and represents two single base substitutions in a cis-acting AAATAC paired-box. All three mutations would result in the loss of LX in mature seed. In conclusion, the seed 3lx trait in OX948 is caused by the *lx1-a* (Lenis et al., 2010), *lx2-a* (Wang et al., 1994) and *lx3* (Wang et al., 1995) null alleles. However, when complete sequences for all three LX genes were compared to the published null allele sequences, the OX948 alleles also contain some unique features: *Lx1* - 5′ UTR and 3′ UTR; *Lx2* - 5′ UTR, 3′ UTR and SNPs in several introns; Lx3 - one deletion and five SNPs at 5′ UTR (Reinprecht et al., 2011).

3.3 Gene-based markers for LLA and lx3 traits

The results from these studies were also an excellent starting point for the development of gene-specific molecular markers. Several markers were developed for ω-3 fatty acid desaturase alleles leading to low levels of LA (Reinprecht et al., 2009) and three seed LX nulls in soybean seed (Reinprecht et al., 2011). These markers are useful to distinguish between the presence and absence of seed LX (LX1 + LX2 and LX3) as well between the high and low LA levels in a wide range of Ontario soybean germplasm.

Two single nucleotide polymorphism (SNP) markers were developed for LLA (Fad3A-e6SNP and Fad3B-i5SNP) and two STS and one CAPS markers were developed for LX null (Lox1-3, Lox2-P1 and Lox3-$_{HaeIII}$) mutations (Table 2). Selection of cultivars with low levels of LA and free of seed LX but independent of screening with gas chromatography and colorimetric assays is an important parameter in breeding soybean cultivars with improved flavour stability. Rapid gel-based LLA and 3lx marker assays developed in these studies

should simplify and accelerate introgression of the RG10-based LLA trait and/or OX948-based 3lx (or their LLA.3lx derivatives) into elite soybean cultivars.

Trait	Gene	Marker	Primer		PCR product (bp)	
			Sequence 5′ to 3′	T (°C)	RG10	OX948
LA	*Fad3A*	Fad3A-e6SNP	CATCATCAGAAACTGCCTTGA CGAGGTGATAATGAGGAATTT	66	1250	missing
	Fad3B	Fad3B-i5SNP	GCTCTATGGAATTCCATATTGGA GTGACCATGGTGATGCAAGT	65	2100	missing
LX	*Lx1*	Lox1-3	GCAATCTATGGTGATCAAAG ATAGTCCTCTATCAGAAGACGAAC	60	1032	958
	Lx2	Lox2-P1	TTTCGATCTTGGCGTTCTTC CCTCATCAACACCACTGTCC	60	1158	1270
	Lx3	Lox3-$_{HAEIII}$	AGTTCCCTCCACGAAGCAAG CTTGTCTCCATGACCCACCT	60	754+469	374/380+469

Table 2. Linolenic acid (LA) and seed lipoxygenase (LX) gene-specific markers

4. Use of RG10 x OX948 RILs for soymilk and bioproduct production

4.1 Use of LLA.3lx lines from RG10 x OX948 reciprocal crosses for soymilk production

To evaluate the effects of the combination of LLA and 3lx traits on soymilk quality the biochemical and sensory characteristics of soymilk made from OR297, a LLa.3lx soybean, was compared to soymilk made from its parents (OX948, a 3lx line, and RG10, a LLA line), a standard soymilk cultivar (Harovinton) and a standard oilseed cultivar (OAC Kent).

4.1.1 Soymilk palatability

An evaluation of the soymilk samples produced from OX948, RG10, OR297, Harovinton, and OAC Kent was conducted by a 28-member sensory panel. The protocol for soymilk production was obtained from J. Jenkinson (First Line Seeds). Two methods of soymilk production were employed. A 'hot' method, which involves an incubation of finely ground soybeans in 1L of 96°C water for 30 minutes and a 'cold' method, which involves the incubation of finely ground soybeans in 200mL of 22°C water for 20 minutes followed by an incubation of the mixture in 800mL of 96°C water for 30 minutes. Both methods were used to prepare soymilk from OAC Kent. Only the cold method was used to prepare soymilk from OX948, RG10, OR297, Harovinton and OAC Kent. The hot method was used to inactivate LX activity, thus giving a more palatable soymilk control. The cold method tends to accentuate differences in stability between samples. OAC Kent was prepared using both methods and served as a control sample to aid in the identification of panelists who were not able to distinguish between soymilk samples.

Panelists were recruited from the University of Guelph community. Following a training session, panelists were asked to evaluate the six soymilk samples at three separate sessions over a three-week period. For each sample evaluated, panelists provided a written description as well as rankings for individual characteristics (i.e., beaniness, sourness, sweetness, rancidity, bitterness and nuttiness). Figure 1 depicts the panel's average ranking

of the five cultivars/ lines of soymilk, for a variety of descriptors. For Green/Beany and Grainy/Nutty the LLA.3lx line (OR297) ranked significantly better than the standard soymilk cultivar included in the test (Harovinton).

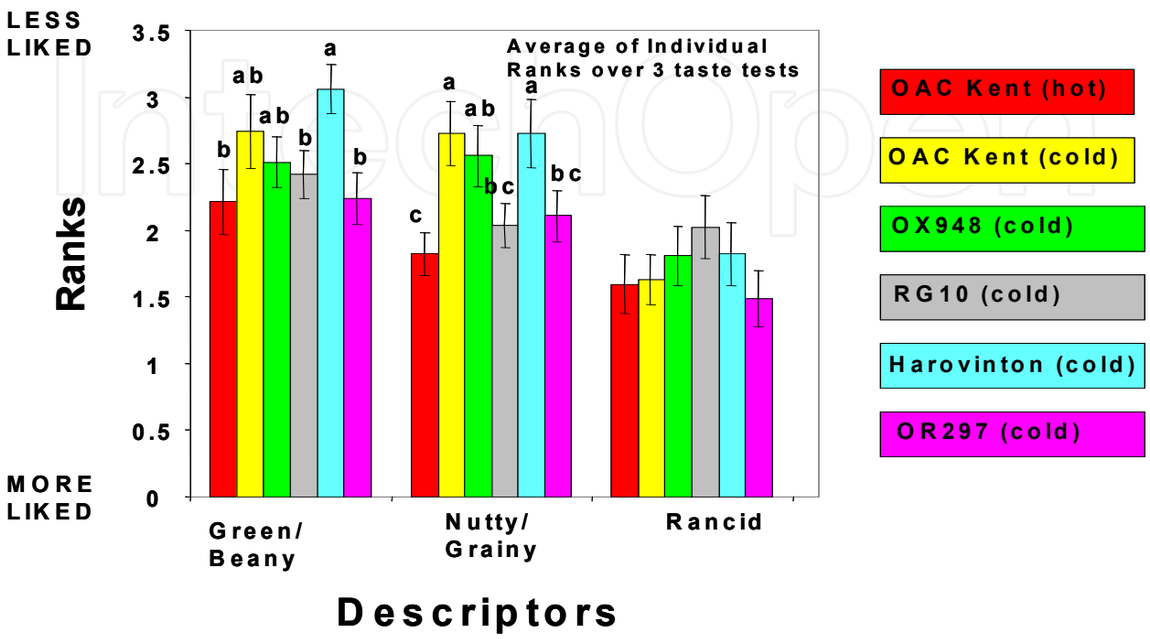

Fig. 1. Ranking of the soymilk samples prepared from five soybean cultivars over three tastings by 28 panelists for various descriptors. Means+/- SE are shown. Soymilk samples labelled with the same letter were not significantly different according to a LSD (P=0.05).

4.1.2 Soybean and soymilk compositions

To test for correlations between sensory evaluations and chemical compositions of the five soybeans cultivars/ lines used in the taste evaluation they were analyzed for total free sugar, total oil, and total protein compositions and lipid oxidation. Raw bean samples of OAC Kent, OX948, RG10, OR297 and Harovinton were analyzed prior to their use for soymilk preparation.

The LA levels of RG10 and OR297 were one-quarter of those measured for Harovinton, which is a tofu-type cultivar. OX948, the 3lx parent of OR297, had the highest LA level (11.0%) among the cultivars/ lines examined. OAC Kent, an oilseed cultivar, had the lowest protein content (42.5%) but had the second highest oil content of all of the cultivars/ lines examined. The sucrose and total free sugar contents in OAC Kent and OR297 were approximately 10% higher than in all of the other cultivars/ lines that were tested.

The largest differences among soymilks at the time of serving to the taste panel (20.5 hrs after preparation) were observed in their malondialdehyde (MDA) levels. The amount of fatty acid oxidation that occurred in the soymilk samples was measured using the thiobarbituric acid-reactive-substances (TBARS) assay described by Wills (1964) and Pedersen et al. (1973) with modifications from Buege & Aust (1978) and Woodrow & Luk (unpublished) to adapt the test to soymilk samples. The oxidation of polyunsaturated fatty acids produces MDA.

Sample ID	OAC Kent	Harovinton	OX948	RG10	OR297
LX status [a]	+/+	+/+	-/-	+/+	-/-
% LA [a]	6.2 ± 0.31	9.5± 0.35	11.0 ± 0.34	2.1± 0.08	2.6± 0.06
% protein [b]	42.5	46.7	46.6	45.9	46.7
% oil [b]	20.6	20.0	19.2	20.9	19.8
% dry matter [b]	94.3	93.5	93.5	93.8	93.7
% moisture [b]	5.7	6.5	6.5	6.2	6.3
% sucrose [b]	6.8	6.1	6.6	5.6	6.9
% raffinose [b]	1.1	1.2	1.0	0.9	0.8
% stachyose [b]	4.0	3.7	4.2	4.1	4.2
% raffinose + stachyose [b]	5.4	5.2	4.8	5.0	5.1
% total free sugars [b]	11.9	11.1	11.7	10.6	12.1
% hydrolysable carbohydrates [b]	18.5	16.7	18.3	16.7	18.2

[a] performed on seed extracts (n = 5, ± SEM)
[b] performed on whole seeds (n = 1)

Table 3. Raw bean composition of OAC Kent, OX948, RG10, OR297 and Harovinton. Lipoxygenase (LX) status and percent of linolenic acid (LA) were determined by colorimetric assay and gas liquid chromatography, respectively. Protein, oil, dry matter, moisture, sucrose, raffinose, stachyose, raffinose and stachyose, total sugars and hydrolysable carbohydrates (free and bound sugars) were determined in whole seed samples using the FOSS NIRSystems (Soy Analyzer, Model No. 6500).

The interaction of MDA with two molecules of TBA generates a red TBA-MDA chromagen absorbing at 535 nm. Polyunsaturated fatty acid oxidation activity can therefore be inferred by measuring the absorbance of the red compound after the reaction of MDA with TBA has taken place (Buege & Aust, 1978; Hodges et al., 1999).

The soymilk prepared from OAC Kent using the hot method had approximately one-third the MDA levels of all the other samples. The reduction in lipid oxidation activity was expected since the heat treatment was used to denature proteins like LX, which catalyze the lipid oxidation process. In the current study, the soymilk prepared by the hot method was included as a control sample with relatively low lipid oxidation activity. Heat treatment is used in commercial soymilk production to reduce the generation of rancid flavours (Wolf, 1975; Davies et al., 1987; Kitamura, 1993; Wilson, 1996). The limitation with this approach is that it leads to other changes in the composition of the soymilk, such as protein denaturation and lipid hydrolysis. This was observed in the current study as large drops in the quantities of these compounds in the hot preparation of OAC Kent soymilk compared to the cold preparation of OAC Kent soymilk (data not shown). It is noteworthy that OR297, the LLA.3lx line, had the lowest lipid oxidation activity of all the cold produced soymilk samples, thus suggesting improved oxidative stability (although there were no significant differences among the cold samples).

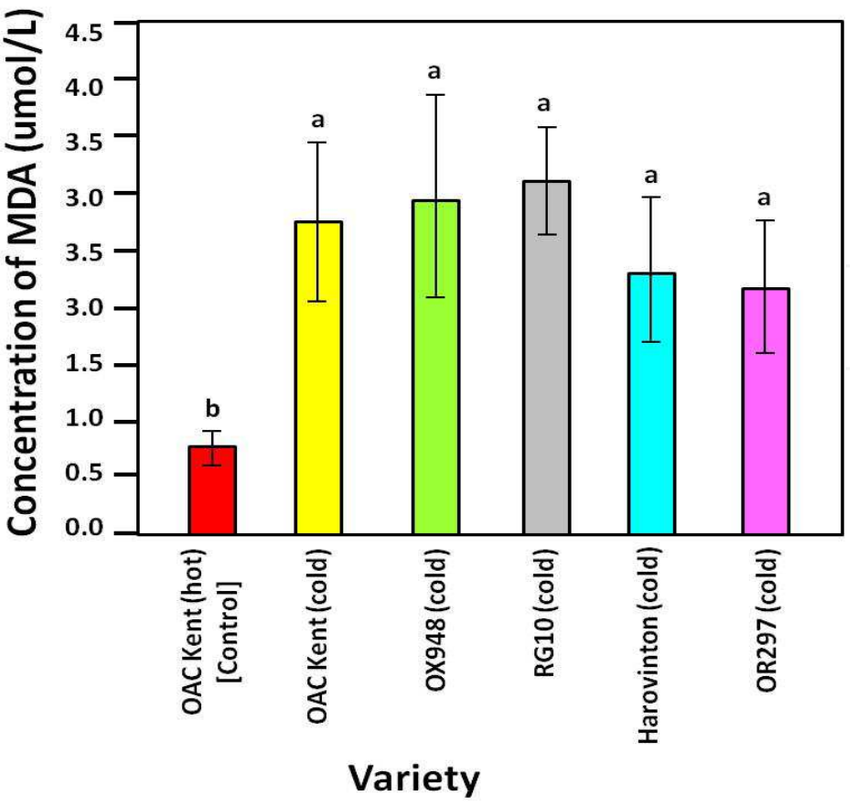

Fig. 2. Lipid oxidation activity, measured as µmol/L of malondialdehyde (MDA) in soymilk samples made from OAC Kent (hot method), OAC Kent (cold method), Harovinton (cold method), OX948 (cold method), RG10 (cold method), and OR297 (cold method) at t = 0 hrs (time of preparation) and 20.5 hrs (time of service to panelists) averaged over 4 repetitions. Cultivars/lines identified by the same letter are not significantly different at p = 0.05; t = 0 hrs series identified by uppercase letters, t = 20.5 hrs series identified by lowercase letters.

4.1.1 Relationship between lipid oxidation and taste in soymilk

Correlation and regression analyses were performed using the 3-week averaged ranks for a number of taste descriptors and composition measurements. These analyses revealed that the taste panel was discriminating and reliable. For example, all the panelists could distinguish between the hot and cold preparations of OAC Kent soymilk (data not shown). In addition, the sensory evaluation for sweetness was highly correlated with the measured free sugar levels in the samples (Figure 3). Interestingly, a positive correlation between the MDA levels in the soymilk samples and their rancidity rankings (r = 0.81, p = 0.05; Figure 3) was determined, thus verifying the connection between the improvement in flavour and reduction in lipid oxidation.

For most taste test descriptors, OR297 was more preferred than its parents OX948 and RG10, or Harovinton, a standard tofu cultivar, although statistical significance was lacking in some cases. In many instances, the ranking of OR297 was similar to the ranking of OAC Kent (hot method), which was included as a low lipid oxidation sample. These finding suggests that the reduction in LA and the removal of LX produced a detectable difference in terms of soymilk flavour and use of this material would improve soymilk flavour to the consumer.

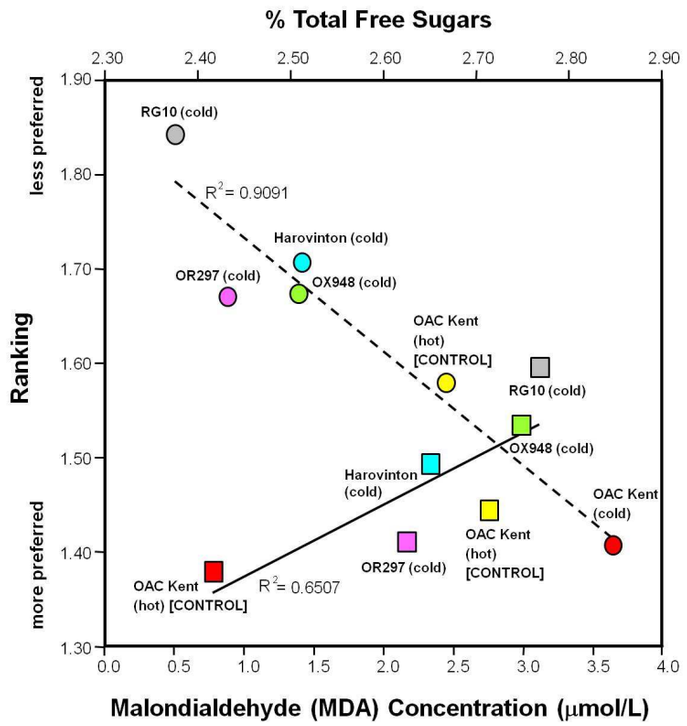

Fig. 3. Regressions for ranking for the rancid taste test descriptor on malondialdehyde (MDA) concentration (μmol/L) in the soymilk samples (————) (not labeled) at t = 20.5 hrs and for the ranking for the sweet taste test descriptor on % total free sugars in the soymilk samples (- - - - - - -). The rankings for the taste descriptors were determined by 28 panelists over 3 evaluation sessions with unlabeled samples prepared 20.5 hrs previously.

4.2 Use of RILs from the RG10 x OX948 cross to identify QTL for bioproduct manufacture

The F_5-derived RILs from the RG10 x OX948 cross are being used to identify genes that contribute to soybean fiber performance in composites, develop fiber gene-specific markers and map Quantitative Trait Loci (QTL) for fiber traits.

4.2.1 Stem fiber genes and markers

To identify fiber genes, we were using a candidate gene approach and a microarray based screen. These two approaches complement each other. The availability of cell wall gene sequences from numerous plant databases to design PCR primers makes a candidate gene approach attractive for relatively fast (and cheap) development of gene-specific markers. Moreover, the availability of the whole soybean genome sequence facilitates the development of a fiber gene *in silico* (sequence) map. Alternatively, the soybean oligo array, developed from 80 libraries and different tissues by University of Illinois, contains approximately 38,000 genes. Hybridization with DNA or total RNA can potentially identify many single feature polymorphism (SFP) markers or expressed QTL (eQTL), simultaneously. However, the microarray approach is more expensive (cost of array and hybridization) and more complex (work and data analysis). For the RNA work, sample preparations from stems at different stages of development would be necessary.

Databases (NCBI, DFCI, SoyBase and Maizewall) and microarray literature were searched for genes associated with the cell wall biosynthesis and modification (cellulose, hemicellulose, pectin and lignin biosynthesis, cell wall proteins, modifying enzymes and regulatory proteins). Over 100 candidate genes were selected and more than 200 gene-based primers were designed and tested with genomic DNA of parents (RG10 and OX948) of a RIL mapping population. The majority of the PCR products were monomorphic and required an additional sequencing step to develop SNP markers. An example of a peroxidase (involved in monolignol polymerization) STS marker is shown in Fig. 4.

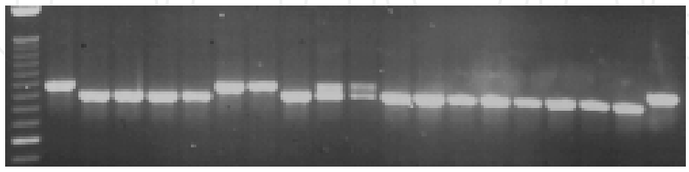

Fig. 4. Segregation pattern of peroxidase (Pox1148) STS marker

Currently, more than 30 gene-specific STS and SNP markers for key enzymes in lignin (PAL2, NAD, and LAC), hemicellulose (XE776), cellulose (CesA3, COBL4) and pectin (PECLYca) biosynthetic pathways as well as regulatory proteins (LIM1, IFL1, KNAT7 and ATHB8) were developed using the candidate gene approach. Some of the fiber genes were placed on the existing RG10 x OX948 linkage map (Reinprecht et al., 2006b). Figure 5 shows partial alignment of linkage map (right) and *in silico* (sequence) map (left). Only chromosomes with (currently) mapped fiber genes were shown. The soybean fiber gene *in silico* map was developed by blasting all gene sequences used to design primers against soybean genome. Peroxidase (Pox1148, lignin biosynthesis) was mapped on chromosome Gm09 (LG K) and glycine-rich protein (GRP) mapped on Gm12 (LG H). Transcription factor NST2 and 4-coumarate: CoA ligase 2 (4CL2, which activates hydroxycinamic acids involved in flavonoid and lignin biosynthesis) mapped on Gm13 (LG F). 4CL2 was placed close to a height QTL for the RILs in the population. Laccase (LAC, a polyphenoloxidase) and COBRA homolog COBL4 (which plays a role in cellulose deposition) were mapped on Gm18 (LG G). LAC was placed close to previously mapped height and lodging QTL. Cinnamyl alcohol dehydrogenase (CAD, lignin biosynthesis) was mapped on Gm10 (LG I) (Fig. 5).

It has been estimated that over 1000 genes might be involved in cell wall biosynthesis and modification and a microarray was also used to identify additional fiber genes and develop SFP markers. A soybean oligo microarray was hybridized with the genomic DNA of parents or RILs and RNA from stem tissue. Based on differences in signal intensity between parental (RG10 and OX948) DNA hybridizations, SFP markers for 164 candidate genes were detected. A number of these genes code for proteins with unknown functions. However, some of the genes encode cell wall biosynthesis/modification proteins (such as: ABC transporter, expansin or callose synthase) or regulatory proteins (such as: MYB109, MYB128 or MYR1) (Table 4) (Reinprecht et al., 2010. After verification, these SFPs will be mapped.

Based on the initial results, it seems feasible to use comparative genomic hybridization (CGH) of DNA from high and low height/lodging soybean genotypes to a spotted 70-mer oligonucleotide microarray to map candidate genes for stem cell wall components in soybean and test their associations with fiber quality QTL. The advantage of this procedure is that the genetic constitution of the parents (RG10 and OX948) was obtained with only a few arrays and a short list of candidate genes was quickly generated.

Number	GenBank accession	Annotation	Signal intensity ratio (RG10/OX948)
1	BE805115	*A. thaliana* transcription factor Z46606	2513.8
13	BU549808	*G. max* MYB transcription factor MYB109	164.8
15	BG509363	*S. polyrhiza* PDR5-like ABC transporter	149.5
24	BU981652	*G. barbadense* fiber protein *Fb11*	52.9
25	BU550544	*R. communis* polygalacturonase-like protein	52.1
41	BE821186	*P. cerasus* expansin (EXP2)	24.6
45	BU550921	*V. vinifera* similar to Callose synthase 10	21.2
47	BE474044	*C. sinensis* hexokinase	19.2
55	BE800964	*A. thaliana* MYR1 (Myb-related protein1) transcription factor	14.2
64	BU544938	*G. max* MYB transcription factor MYB 128	11.5
160	BU550857	*T. majus* mRNA for α-D-xylosidase	2.5

Table 4. Microarray-based (genomic DNA) candidate fiber genes and potential SFP markers (partial list)

4.2.2 Fiber trait characterization and QTL mapping

Fifty RILs (selected on the basis of a height/lodging index) and parents (RG10 and OX948) were evaluated (selective phenotyping) in controlled (growth room) and field environments (Harrow, Ridgetown and Woodstock in 2008 and 2009). Mature stems were collected, ground in a Wiley mill (Arthur Thomas Co., Philadelphia, Penn.) to pass a 2.0 mm screen and stored at -20°C for further fiber characterization. Selection and optimization of protocols for determination of fiber chemical, physical and functional properties were performed on the parents (RG10 and OX948).

Thermal degradation profiles of stem fibers are affected by variation in the chemical composition of the stem material because different cell wall components have different thermal behaviours. Thermogravimetric analysis (TGA) was performed with the TGA Q500 instrument (TA Instruments, New Castle, DE) by heating RG10 and OX948 stem samples from ambient temperature to 600°C at heating rate of 20°C/min in a nitrogen environment. Under these conditions, RG10 and OX948 had similar degradation characteristics. Stems (untreated containing ~4% moisture) started to decompose at 187°C in both parental lines, which is lower than untreated soybean hulls, which decompose at 209°C (Alemdar & Sain, 2008). In addition, about 34% of the carbonaceous material in the stems remained after heating to 600°C. The amount of lignocellulosic materials in RG10 and OX948 stems could not be assessed with the current TGA conditions. In both lines, hemicellulose (230°C), cellulose (325°C) and lignin (150-600°C) degradation resulted in a broad peak with a number of shoulders (Fig. 6).

Fourier transform spectroscopy (FTIR) was performed with the Nicolet 6700 FTIR spectrometer (Thermo Fischer Scientific, Waltham, MA). The data were processed by the Omnic software (version 8.0) and recorded in the transmittance mode as a function of wavenumber after air background subtraction. FTIR spectra of each sample were obtained in the range of 4000–400 cm^{-1}. FTIR analysis was useful to distinguish parental genotypes. Several polysaccharide peaks dominate in the FTIR spectra of RG10 and OX948 stems (Fig. 7) and parental lines show differences in intensities for major peaks in the lignin and

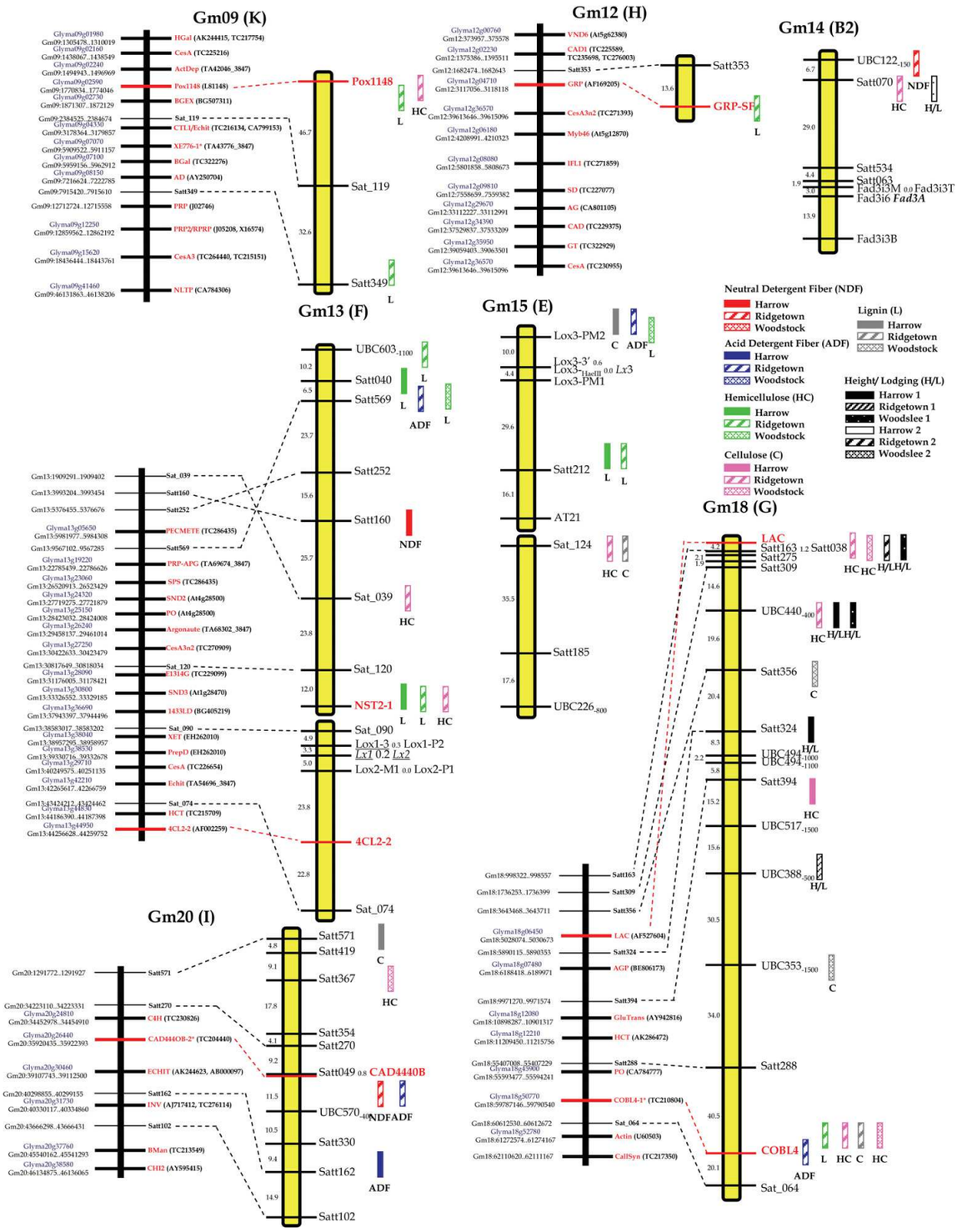

Fig. 5. The alignment of RG10 x OX948 linkage/QTL map (right) with an *in silico* (sequence) map (left). Fiber genes are labelled in red.

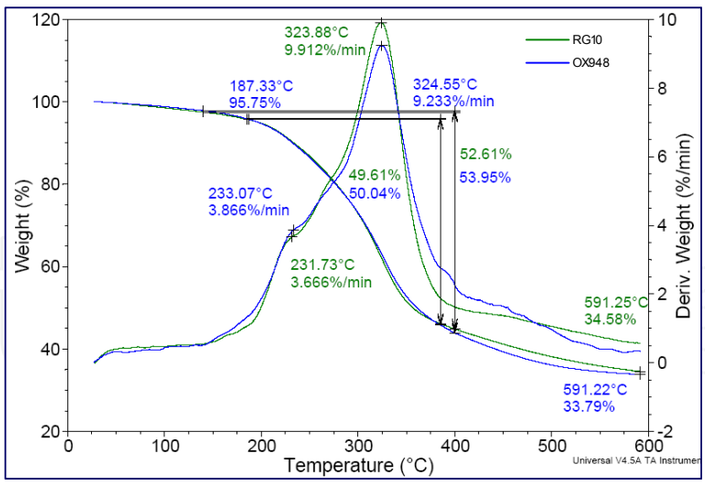

Fig. 6. TGA thermograms of RG10 and OX948 stems heated at 20°C/min

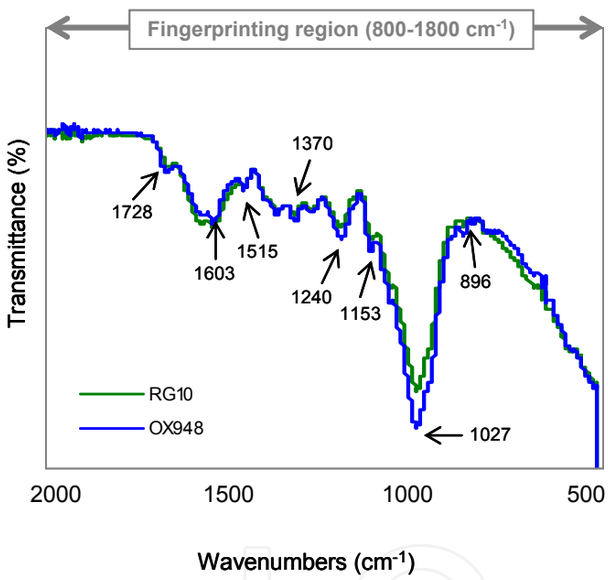

Fig. 7. FTIR spectra of RG10 and OX948 stems

polysaccharide spectral regions (peaks at 896, 1027, 1240 and 1632 wavenumbers) that indicate that quantitative (peak area) FTIR analysis might be a suitable technique to determine amount of lignocellulosic material in soybean stems.

Stems were characterized for neutral detergent fibers [NDF, isolates (grass) cell wall (hemicellulose, cellulose and lignin)], acid detergent fibers (ADF, isolates cellulose and lignin) and acid detergent lignin (ADL, isolates lignin) using sequential analysis with the Ankom 200 fiber anlyzer (Ankom technology, Macedon, NY). Hemicellulose and cellulose content were calculated from NDF, ADF and ADL values (hemicellulose = NDF - ADF, cellulose = ADF - ADL). Soybean stem contains approximately 289 g kg^{-1} hemicellulose, 526 g kg^{-1} cellulose and 173 g kg^{-1} lignin (Johnson et al., 2007). The stems of RILs grown in three locations in 2008 contained an average of 365 g kg^{-1} cellulose (range 263-454), 173 g kg^{-1} hemicellulose (range 123-220) and 119 g kg^{-1} lignin (range 32-169). Significant negative

correlations were detected between lignin and cellulose and lignin and hemicellulose, while cellulose and hemicellulose were positively correlated (Table 5). In addition, lignin content was positively correlated with the height/lodging index trait (data not shown).

Location	Trait	Location (2008)							
		Harrow			Ridgetown			Woodstock	
		Lignin	Hemicellulose	Cellulose	Lignin	Hemicellulose	Cellulose	Lignin	Hemicellulose
Harrow	Hemicellulose	0.26							
	Cellulose	-0.61**	-0.01						
Ridgetown	Lignin	0.37	0.26	-0.30					
	Hemicellulose	-0.02	0.10	0.01	0.21				
	Cellulose	-0.10	-0.08	0.40	-0.54**	-0.32			
Woodstock	Lignin	0.01	0.15	-0.01	-0.01	0.14	0.10		
	Hemicellulose	0.13	0.06	0.10	0.02	-0.14	0.18	-0.40**	
	Cellulose	0.08	0.23	0.09	0.60**	0.18	-0.32	-0.20	0.11

Table 5. Correlations among cellulose, hemicellulose and lignin content in soybean stems across different locations

Fifteen stem fiber QTL were detected in Harrow, 33 in Ridgetown and 13 in Woodstock in 2008. Some QTL were associated with fiber genes. For example, a lignin QTL on chromosome Gm09 (LG K) was linked to the peroxidase gene Pox1148 (monolignol polymerization) while a cellulose QTL on Gm18 (LG G) was associated with the COBRA homolog gene COBL4, which is involved in cellulose biosynthesis (Fig. 5).

5. Conclusions

This chapter describes development of a versatile soybean RIL population segregating for LLA and lx3, its molecular characterization and utility for soymilk and composite material production. The 169 F_5-derived RILs from the RG10 x OX948 cross are well characterized for numerous seed, agronomic and stem fiber traits. In addition to the LA and LX traits, the population is segregating for a number of other traits, including: seed traits [such as: other fatty acids (palmitic, steraic, oleic and linoleic), oil and protein content]; developmental traits (such as: flowering, maturity, lodging, plant height and height/lodging index); seed weight and yield and stem fiber traits (such as: cellulose, hemicellulose and lignin content). QTL for these traits were placed on a RG10 x OX948 linkage/QTL map and their relationships were determined. A number of these QTL were stable across many environments so that the markers associated with them would likely be useful for MAS. In the future, the addition of new markers will saturate map and help us to determine whether the regions significant for more than one trait reflect pleiotropy or gene linkage.

The molecular bases of LLA in RG10 and lx3 in OX948 as well as six LLA.3lx RILs (RO115, RO162, OR265, OR297, OR315 and OR322) were determined. The LLA trait in RG10 is caused by the mutation in two *Fad3* genes (*Fad3A* and *Fad3B*). The lx3 trait in OX938 is caused by mutations in all three LX genes. The novel LLA.3lx RILs contain all five mutated alleles (*fanfan-blx1lx2lx3*), which resulted in low levels of LA and lack of all three LX in the seed. When these lines were compared with lines combining high LA and all three LX present, no yield difference between the two groups of lines was detected suggesting that it should be possible to use this novel germplasm to develop competitive soybean cultivars that are resistant to oxidative degradation.

The simple and independent inheritance of LA and seed LX in RG10 x OX948 crosses should simplify breeding for soybean cultivars that are resistant to oxidative degradation. Acceleration and further simplification of breeding for soybean with improved flavour could be accomplished by using molecular markers. Markers developed for mutations in two ω-3 fatty acid desaturase (*Fad3A* and *Fad3B* in RG10) and three LX (*Lx1*, *Lx2* and *Lx3* in OX948) genes will allow more accurate selection for LLA and 3lx phenotypes based on DNA screens rather than relying on phenotypic expression. Some of the markers were already successfully used in MAS for soybean with improved flavour.

Soymilk prepared from OR297 (LLA.3lx), OX948 (3lx), RG10 (LLA), Harovinton (tofu-type) and OAC Kent (oilseed type) were evaluated by a sensory panel. Generally, OR297 was the most preferred and a significant relationship between rancidity and lipid oxidation levels was identified.

Although, work on soybean stem fibers is still in progress, a number of genes coding for key enzymes in cell wall biosynthesis and modification were identified. Markers were developed and some fiber traits were characterized and mapped. When completed, this work will allow identification of key factors in fiber quality and development of quick, marker-based screening method(s) to allow rapid introgression of genes for good fiber quality into elite soybean cultivars. It would be possible to create agriculturally-acceptable cultivars with good fiber performance characteristics in composites such as cultivars with higher cellulose content for products requiring higher strength and modulus, with higher lignin content when higher extensibility of products is needed, or cultivars with reduced hemicellulose content to achieve higher processing temperatures of biocomposites. Furthermore, the newly available sequence information can be used to characterize fiber gene mutations at molecular level.

Different combinations of seed, agronomic and stem fiber traits in RILs derived from the RG10 x OX948 cross, and the availability of a linkage/QTL map and correlations among traits could be further exploited to develop novel or specialty soybeans, both for food and industrial applications. The population is available for research purposes.

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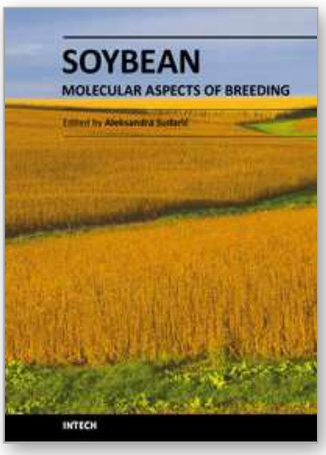

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