# CHARACTERIZATION OF A NOVEL MAIZE *BROWN MIDRIB* MUTANT

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To my family, teachers and friends.

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As part of the National Science Foundation (NSF) Plant Genome project 'Identification and characterization of cell wall mutants in maize and Arabidopsis using novel spectroscopies,' 2,200 $F_2$ families of *Mutator*-tagged maize lines from the Uniform*Mu* population developed at the University of Florida were planted at Purdue University in the summer of 2002-2004. The Uniform*Mu* population has a high mutagenic rate caused by *Mutator (Mu)* transposons that have the capacity to move from one locus to another in the genome. If a *Mu* transposon inserts in a gene, it has the potential to disrupt the function of that gene. Among the mutants identified in the Uniform*Mu* population, was a novel *brown midrib (bm)* mutant that displayed a subtle orange-brown midrib. Currently, there are four *bm* mutants known in maize. These mutants, *bm1, bm2, bm3*, and *bm4,* are Mendelian recessives and are recognized by reddish-brown vascular tissue in the leaves and stems resulting from changes in lignin content and/or composition. This coloration has been observed in the stem, root, leaf, tassel and cob of the plant in several of the four *bm* mutants. The objective of this research was to characterize the novel *brown midrib* mutant and to identify the genetic basis of this mutation.

Several chemical analyses were performed to compare the new *bm* mutant to the other characterized *bm* mutants and to wild-type plants. Stover from the *bm* mutant has similar

biomass conversion efficiency as wild-type stover, and pyrolysis gas-chromatography-mass-spectrometry analysis of wild-type and *bm* midribs showed that the *bm* mutant has identical lignin sub-unit composition to the wild-type. Collectively, these data suggested that the *bm* phenotype is not due to a mutation in the lignin biosynthetic pathway.

Pigment analyses have not provided evidence in favor of carotenoids and flavonoids as the pigments that accumulate in the midrib of the *bm* mutant. Work is in progress to identify the pigment responsible for the orange-brown color in the midribs.

Since the assumption was that this mutation was caused by a *Mu* transposon insertion, obtaining all sequences flanking *Mu* transposons allowed a test of which element caused the mutant phenotype. A total of 192 flanking sequences were obtained using a PCR-based protocol. The sequences were subsequently filtered through a sequence database with *Mu*-flanking DNA from the progenitor and siblings of the *bm* mutant. This analysis resulted in 42 unique candidate sequences. Each of these were tested to see if they co-segregated with the *bm* mutant phenotype. None of the candidate genes co-segregated with the phenotype.

CHAPTER 1
LITERATURE REVIEW

## Cell Walls

Plant cell walls contribute to the functional specialization of cell types (Carpita and McCann, 2000). They are a highly organized composite of many different polysaccharides, proteins, and aromatic substances. The cell wall is composed of a middle lamella, primary cell wall, and in specialized tissues, a secondary cell wall. Primary cell walls are composed of polysaccharides, smaller proportions of glycoproteins and, in some specialized cell types, various non-carbohydrate substances such as lignin, suberin, cutin or silica (Fry, 2003). In contrast to primary cell walls, plant secondary cell walls are deposited once the cell has stopped expanding. Secondary cell walls offer several advantages for genetic analysis of plant cell walls: it is possible to recover severe mutants because the plants usually remain viable (Turner, 2001). The molecular composition and arrangement of the wall polymers differ among species, among individual cells, within a species and even among the regions of the wall around a single protoplast.

## Cell Wall Polysaccharides

Polysaccharides are the main components of the cell wall and form its main structural framework (Carpita and McCann, 2000). There are three different classes of polysaccharides in the cell wall: cellulose, hemicellulose and pectin.

### Cellulose

Cellulose is the principal scaffolding component of all plant cell walls (Carpita and McCann, 2000) (Figure 1-1). It is an essential part of the plant cell wall, where it is vital within the load-bearing network and an important determinant of the orientation of cell expansion (Taylor et al., 2004). It exists in the form of microfibrils, para-crystalline assemblies of several

dozen (1-4)-β-D-glucan chains hydrogen bonded to one another along their length. Cellulose is the most abundant component of plant secondary walls and makes up a large proportion of the dry weight of these secondary cell walls (Taylor et al., 2004). Turner and Sommerville (1997) isolated a series of mutants following a screen of a chemically mutagenized population of Arabidopsis plants that had collapsed xylem cells and that were termed *irregular xylem (irx)* mutants. The collapse of the xylem vessels was attributed to a weakness in the secondary cell wall of the xylem cells which resulted in them being unable to withstand the negative pressure generated during water transport up the stem. Analysis of the cell wall composition of the mutants revealed that they had a severe decrease in the amount of cellulose in the stems. Turner and Sommerville (1997) reported that each mutant contains about a third the amount of cellulose found in the wild-type. Callose, which consists of (1-3)-β-D-glucan chains, is made by a few cell types at specific stages of wall development that include growing pollen tubes and cell plates of dividing cells.

**Hemicellulose**

Cross-linking glycans are a class of polysaccharides that can hydrogen bond to cellulose microfibrils (Carpita and McCann, 2000). They either coat microfibrils or are long enough to span the distance between microfibrils (Figure 1-1), and link them together to form a network (Carpita and McCann, 2000). Major hemicelluloses are the xylans (including arabinoxylans, glucuronoarabinoxylans [GAXs]), xyloglucans (XyGs) (composed mainly of glucose, xylose [Xyl], galactose, fucose, and mixed linkage β-[1-3]), (1-4)-D-glucans only found in the Gramineae and a few related families (Fry, 2003). Xyloglucans consist of linear chains of (1-4)-β-D-glucan with numerous alpha-D-Xyl units linked at regular sites to the *O*-6 position of the glucose unit.

## Pectin

Pectins are a mixture of heterogeneous branched and highly hydrated polysaccharides that are rich in d-galacturonic acid (Carpita and McCann, 2000). They determine wall porosity, provide charged surfaces that modulate wall pH and ion balance, regulate cell-cell adhesion at the middle lamella, provide an environment for the deposition, slippage and extension of the cellulosic-glycan network and serve as recognition molecules that alert the plant cells to the presence of symbiotic organisms, pathogens, and insects. Changes in the structures of these polysaccharides are associated with different developmental stages of plant cells and tissues (O'Neill et al., 2003). There are two main components of pectins, homogalacturonan (HGA) and rhamnogalacturonan I (RG I). Homogalacturonans are structurally modified into xylogalacturonan and rhamnogalacturonan II (RG II). These three pectic polysaccharides are covalently linked to one another to form a pectic macromolecule. Further covalent and non-covalent cross-linking of some glycosyl residues in this macromolecule form a three-dimensional pectic network (O'Neill et al., 2003). RG I is composed of d-galactosyluronic acid, l-rhamnosyl, d-galactosyl, l-arabinosyl, and small amounts of l-fucosyl residues (McNeil, 1982). The backbone of RG I is composed of alternating 2-linked l-rhamnosyl and 4-linked d-galactosyluronic acid residues. Overall, the modulation of pectic structure can be viewed as the fine-tuning of conditions and capacities within the cell wall matrix, providing both mechanical properties and an operating environment for the activities of cell-wall modifying enzymes and other factors (O'Neill et al., 2003).

## Type I vs. Type II Walls

There are two distinct types of cell walls that differ in chemical composition. Type I cells are found in the walls of most dicots and noncommelinoid monocots and are composed of equal amounts of xyloglucans and cellulose (Carpita and McCann, 2000). Xyloglucans bind to the

cellulose microfibrils locking them into proper spatial arrangements. The cross-linking glycans span the distance between two microfibrils and bind to both of them. The XyG – cellulose framework is embedded in a pectin matrix. In contrast, Type II walls of commelinoid monocots contain cellulose microfibrils that are interlocked together by GAXs even though small amounts of XyGs are also present. In addition, Type II cells are pectin-poor compared to Type I walls. There is also little structural protein compared to dicots and other monocots, but they accumulate extensive interconnecting networks of phenylpropanoids as the cells stop expanding.

## Lignin

Lignin is a major constituent of secondary cell walls in all vascular plants, and it plays several important roles including the strengthening and impermeabilization of cell walls and providing a mechanical and chemical barrier against pathogens (Baucher et al., 1999). Lignin is a heterogeneous aromatic polymer that is composed of different phenypropanoids mainly the monolignols *p*-coumaryl, coniferyl and sinapyl alcohols (Figure 1-2). Upon incorporation into lignin, these monolignols are called *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units (Raes et al., 2003).

In addition to these three units, there are other phenylpropanoids that can be incorporated in lignin, including hydroxycinnamyl aldehydes, hydroxycinnamyl acetates, hydroxycinnamyl *p*-hydroxybenzoates, hydroxycinnamyl *p*-coumarates and hydroxycinnamate esters (Ralph et al., 2001; Boerjan et al., 2003). The lignin structure is complex, incorporating ether and carbon-carbon linkages between monomers with extensive cross-links, probably via hydroxycinnamic acid bridges, to other cell wall polymers. The monolignols are linked by way of ester, ether, or carbon-carbon bonds.

The phenylpropanoid pathway is responsible for the biosynthesis of a variety of products that include lignin, flavonoids, and hydroxycinnamic acids conjugates (Humphreys and Chapple,

2002), (Figure 1-3). Most intermediates and end products of this pathway play vital roles in plants as phytoalexins, antiherbivory compounds, antioxidants, UV protectants, pigments and aromatic compounds (Humphreys and Chapple, 2002). Monolignols and hydroxycinnamic acids are the products of the phenylpropanoid pathway, which also supplies intermediates for the synthesis of phytoalexins, flavonoids and tannins (Halpin et al., 1998). Of the many enzymes on this pathway only cinnamoyl coenzyme A-reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) are dedicated solely to monolignol synthesis (Halpin et al., 1998).

## Lignin Biosynthetic Enzymes

### Phenylalanine Ammonia-Lyase

Phenylalanine ammonia-lyase (PAL) catalyzes the deamination of phenylalanine to cinnamate – the committed step in phenylpropanoid metabolism (Whetten and Sederoff, 1995). Considered as the entry point enzyme to the phenylpropanoid pathway, it was hypothesized that PAL served as a rate-determining step. As a result, it was assumed that PAL plays a regulatory role in controlling biosynthesis of all phenylpropanoid compounds, including lignin. However, Anterola et al. (2002) indicated that during the profiling of loblolly pine (*P. taeda L.)*, PAL does not serve as a rate-limiting step. They further argued that there may be distinct phenylalanine pools that exist for specific purposes. That is, there may be a coordinated and specific up-regulation of upstream pathways [leading to phenylalanine] for phenylpropanoid metabolism. If correct, this assumption would remove PAL as being the "entry step", due to the existence of distinct and coordinated metabolic networks beginning from, for example, the pentose phosphate and glycolysis pathways and ending with the monolignols/lignins directly (Anterola and Lewis, 2002). In most plant species, PAL is encoded by a multigene family (Baucher et al., 1998) and it has been speculated that individual genes have distinct metabolic roles such as flavonoids and lignins.

Transgenic plants with modified levels of PAL activity have provided opportunities to test hypotheses about the role of PAL in metabolism and plant development. Bate et al. (1994) analyzed phenylpropanoid metabolites in transgenic tobacco plants with decreasing amounts PAL activity and reported that lignin content is not greatly affected until PAL activity is reduced to 20% of wild-type levels. Anterola and Lewis (2002) agreed with the previous result and concluded that transgenic plants with modified PAL levels result in reductions in lignin, but only if PAL levels have been reduced below half that of the control plants.

**Cinnamate 4-Hydroxylase**

Cinnamate 4-hydroxylase (C4H) is a cytochrome P-450-linked monooxygenase that catalyzes the hydroxylation of cinnamic acid to *para*-coumaric acid (Whetten and Sederoff, 1995). C4H is expressed in all tissues and upon exposure to light, wounding, and fungal infection (Bell-Lelong et al., 1997). However, expression only increases during the later stages of stem development (Raes et al., 2003). C4H exists in at least two forms depending upon the species, C4H-1 and C4H-1/C4H-2 (Anterola and Lewis, 2002). C4H-1 is correlated with lignification (Lewis et al., 1999) and other phenylpropanoid pathway branch points, whereas the physiological role of C4H-2 still needs to be fully established. C4H catalyzes the addition of an oxygen atom obtained from molecular oxygen, with one oxygen atom added to the aromatic ring and the other reduced to water. C4H has been purified and characterized from several plant species (Fahrendorf and Dixon, 1993). C4H cDNAs have been expressed in yeast and the active enzyme has been recovered. Fahrendorf and Dixon (1993) reported that C4H is able to couple effectively with yeast NADPH-cytochrome P-450 reductase and catalyze with high efficiency and capacity, the hydroxylation of cinnamate in microsomes from transformed yeast.

**4-Coumarate: Coenzyme A Ligase**

4-Coumarate: coenzyme A ligase (4CL) catalyzes the formation of coenzyme A thioesters of cinnamic acids, p-coumaric acid, caffeic acid, ferulic acid, 5-hydroxyferulic acid, and sinapic acid, in the biosynthesis of a variety of phenolic derivatives including benzoic acids, condensed tannins, flavonoids and the cinnamyl alcohols (Gross, 1985). The high number of substrates could explain why there are many 4CL isoenzymes in most plants (Raes et al., 2003, Lewis et al., 1999). The isoforms typically have different substrate specificities and spatial/temporal expression patterns, which have suggested distinct physiological roles (Anterola and Lewis, 2002).

In Arabidopsis, At4CL1 and At4CL2 are believed to be involved in lignification, whereas At4CL3 is considered to function in flavonoid biosynthesis (Ehlting et al., 1999). Similarly, in aspen it was proposed that PtCL1 was specifically involved in lignin biosynthesis, whereas PtCL2 participated in flavonoid formation (Hu et al., 1998). Expression analysis showed that 4CL genes are expressed in almost all tissues investigated.

4CL down-regulation has been carried out only with isoforms considered to be involved in lignin biosynthesis using three different species: tobacco, Arabidopsis and aspen. Responses ranged from no visible phenotypic differences in Arabidopsis, to dwarfing in tobacco and enhanced growth in aspen (Anterola and Lewis, 2002). However, the effect of 4CL down-regulation on overall lignin content was consistent in all three species; 4CL down-regulation resulted in significant reductions in lignin content, but only after more than 60% reduction in 4CL activity (Anterola and Lewis, 2002).

**Hydroxycinnamoyl-Coenzyme A Shikimate/Quinate Hydroxycinnamoyltransferase**

Hoffman et al. (2003) showed that hydroxycinnamoyl-coenzyme A shikimate/quinate hydroxycinnamoyltransferase (HCT) is an acyltransferase that uses *p*-coumaroyl-coenzyme A as

an acyl donor and shikimic acid or quinic acid as acceptor, yielding the shikimate or quinate ester, respectively. The enzyme acts both upstream and downstream of the 3-hydroxylation step. HCT also catalyzes the reverse reaction whereby it forms caffeoyl-Co A from chlorogenate (5-*O*-caffeoyl quinate ester). When the *HCT* gene was silenced in Arabidopsis, it resulted in dwarf plants that showed a decrease in syringyl units and an increase in *p*-hydroxyphenyl units (Hoffmann, 2004). Expression analysis in Arabidopsis also showed that *HCT* is expressed in all tissues, but the strongest expression is in the inflorescence stem.

**Coumarate 3-Hydroxylase**

Coumarate 3-hydroxylase (C3H) is a cytochrome P450-dependent monooxygenase (Franke et al., 2002, Schoch et al., 2001). Franke et al. (2002), showed that the Arabidopsis mutant reduced epidermal fluorescence *(ref8)*, which has a defective *C3H* gene, accumulates *p*-coumarate esters in place of the sinapoylmalate found in wild-type plants and has an extremely dwarf phenotype. In addition, the *ref8* mutant also deposits a lignin primarily from *p*-coumaryl alcohol (H units), the lignin subunit which is a minor component in the lignin of normal plants. Furthermore, the *ref8* mutant deposits only minor amounts of G and S units (Franke et al., 2002). These data indicate that both the G and S pathways are blocked in the *ref8* mutant. Therefore, the *ref8* mutant demonstrates that elimination of *C3H* activity restricts carbon flow into the monolignol pathway, as well as growth and developmental processes. In addition, transcriptional profiling of *P. taeda* also showed that this enzyme was directly correlated with induction of monolignol biosynthesis in a rate-determining capacity (Anterola et al., 2002)

Contrary to earlier reports which suggested that *p*-coumaric acid was the main substrate for C3H, Schoch et al. (2001) showed that the C3H enzyme is highly active towards *p*-coumaroyl quinate and *p*-coumaroyl shikimate. Thus, the name coumarate 3-hydroxylase is now a

misnomer. Expression analysis showed that C3H is expressed in all tissues with the highest expression being detected in vascular tissues of stems and roots (Raes et al., 2003).

**Caffeoyl-Coenzyme A *O*-Methyltransferase**

Caffeoyl-coenzyme A *O*-methyltransferase (CCoA-OMT) is distinct from caffeate *O*-methyltransferase (COMT) and has been identified in connection with the defense response in several dicot plant species (Kühnl et al., 1989). Ye et al. (1994) reported that CCoA-OMT plays a role in methylation of both caffeoyl-coenzyme A and 5-hydroxyferuloyl-coenzyme A during monolignol biosynthesis. CCoA-COMT is expressed in all tissues of Arabidopsis (Raes et al., 2003).

**Cinnamoyl-Coenzyme A Reductase**

Reduction of hydroxycinnamoyl-coenzyme A thioesters to the corresponding aldehydes is catalyzed by cinnamoyl-coenzyme A reductase (CCR) (Whetten and Sederoff, 1995). CCR plays a key role in lignin biosynthesis as the first committed step in the production of monolignols from phenylpropanoid metabolites. Down-regulation of the *AtCCR1* gene in Arabidopsis led to plants with a 50% decrease in lignin content, shorter than wild-type plants, and with changes in lignin composition, with ferulic acid being deposited in the cell wall (Goujon et al., 2003).

**Caffeate *O*-Methyltransferase**

Caffeate O-methyltransferase (COMT) has the predominant role of methylating 5-hydroxyconiferaldehyde and 5-hydroxyconiferyl to sinapaldehyde and sinapyl alcohol, respectively (Humphreys et al., 1999). The methylation step limits the reactivity of the 3-hydroxyl group, thereby reducing the number of sites on the aromatic ring that can form bonds to other monolignol molecules during polymerization. Vignols et al. (1995) showed that the maize *bm*3 mutant was a result of two independent mutations that resulted in structural changes in the COMT gene. Down regulation of COMT in maize using the anti-sense method led to a decrease

in lignin content, decrease in syringyl units, lower p-coumaric acid content and the occurrence of unusual 5-hydroxyguaiacyl units (Piquemal et al., 2002). The same method in tall fescue grass, Festuca arundinacea, resulted in almost similar results to those in maize except that there was no reduction in p-coumaryl acid content and the incorporation of 5-hydroxyguaiacyl units (Chen et al., 2004). Furthermore, it was shown that the preferred substrates for the tall fescue recombinant COMT are 5-hydroxyferulic acid and caffeoyl aldehyde in contrast to maize COMT, which preferred 5-hydroxyconiferaldehyde and 5-hydroxyconiferyl (Piquemal et al., 2002). In general, however, it has been observed that down-regulation of COMT results in reduced S/G ratio mainly due to inhibition of S lignin biosynthesis (Guo et al., 2001a).

**Ferulate 5-Hydroxylase**

Ferulate 5-ydroxylase (F5H), correctly named coniferaldehyde 5-hydroxylase (Cald 5-H) is a cytochrome P-450-linked monooxygenase that catalyzes the hydroxylation of ferulate to 5-hydroxyferulate. A mutation in the Arabidopsis gene encoding F5H (*fah-1)* was identified, and mutant plants were shown to lack sinapate-derived residues in lignin (Chapple et al., 1992). Consequently the lignin in *fah-1* mutant Arabidopsis resembles a gymnosperm type of lignin composed of guaiacyl units derived from coniferyl alcohol. Isolation of the gene encoding F5H and expression of the protein product in a heterelogous system will provide material for more intensive characterization of this little studied enzyme. In addition, F5H has been implicated in the differences in lignin composition between angiosperms and gymnosperms. Its activity is necessary for the hydroxylation of coniferaldehyde, an essential step in the formation of sinapyl alcohol. Whetten and Sederoff (1995) hypothesized that F5H is present in all plants except gymnosperms (Baucher et al., 1998) at varying levels and that low levels of F5H activity are sufficient to allow synthesis of significant levels of sinapyl alcohol and siringyl lignin.

## Cinnamyl Alcohol Dehydrogenase

Cinnamyl alcohol dehydrogenase (CAD) catalyzes the reduction of hydroxycinnamaldehydes (cinnamyl aldehydes) to hydroxycinnamyl alcohols, the last stage in monolignol biosynthesis (Whetten and Sederoff, 1995). CAD has been considered to be an indicator of lignin biosynthesis because of its specific role at the end of the monolignol biosynthetic pathway. The maize *bm1* mutant has reduced CAD transcript and protein levels (Halpin et al., 1998). The mutation results in reduced lignin content and altered structure of the lignin polymer. Reduction of CAD activity in *bm1* plants causes incorporation of cinnamyl aldehydes in the growing lignin polymer (Halpin et al., 1998). The gene encoding *CAD* has been a target for modification of lignin content in plants through genetic engineering. Transformed tobacco plants with an antisense CAD construct show varying degrees of reduction in CAD activity and modification of phenolic products (Halpin et al., 1994). Two lines of transgenic plants were investigated: one with 75% of wild-type CAD and the other with 20% of wild type CAD activity. The former had changes in monolignol composition while the latter did not.

The compositional changes included increases in the ratio of aldehyde-to-alcohol-derived products, with a preferential effect on syringyl subunits.

## Sinapyl Alcohol Dehydrogenase

Sinapyl alcohol dehydrogenase (SAD) is a member of the NADP(H)-dependent dehydrogenase family that catalyzes the last reductive step in the formation of monolignols (Bomati et al., 2005). Although SAD and classical cinnamyl alcohol dehydrogenases (CADs) catalyze the same reaction and share some sequence identity, the active site topology of SAD is strikingly different from that predicted for classical CADs (Li et al., 2001).

## Lignin Polymerization

The process by which the structure of lignin is determined is not clearly understood. There are two competing hypotheses to describe this process. The traditional hypothesis is that lignin is a polymer built from the more-or-less random coupling of the three monolignol units into the growing polymer (Hatfield and Ralph, 1997). Under this hypothesis lignin assembly occurs after passage of monolignol monomers into the cell wall, with polymer formation only requiring oxidative enzymes, namely peroxidase and laccase, to generate the corresponding free radicals, which will then undergo random coupling (Adler, 1977). Lewis and Davin (1998) put forward an alternative hypothesis based on the discovery of dirigent proteins. Dirigent proteins (Latin: *dirigere*, to align or guide) are proteins which bind and orientate the coniferyl alcohol-derived free radicals which then undergo stereoselective coupling to lignans. Davin and Lewis (1998) question the random coupling model based on a premise that the formation of approximately 20 - 30% of all plant organic matter should not be left to "chance". They go further and point out that the random coupling does not explain many biological aspects of lignification, including targeting of specific monolignols into discrete regions within the lignifying cell wall and the observed regiospecificity in coupling resulting in approximately 50 -70% of all inter-unit linkages being *β*-*O*-4-bonded. Therefore, Davin and Lewis (1998) concluded that some coupling specificity was being exercised *in planta* – which the dirigent protein model could help explain. This conclusion is based on several reports on the discovery of dirigent proteins (Ralph et al., 2006) as well as reports claiming evidence for the control of β-*O*-4 coupling (Lourith et al., 2005). Davin and Lewis (2005) also cited unpublished results which reportedly show that over expressing dirigent proteins resulted in significant increased levels of lignin constituents. Despite a steady increase in reports of dirigent proteins discovery in a number of species (Chen and Sarkanen, 2003), there has been no evidence which characterizes at the molecular level, the basis

of the various (dirigent) monomer-binding sites for both lignin and lignin formation and on obtaining the primary sequences of the lignins being generated (Davin and Lewis, 2005). If lignin biosynthesis was under the control of the dirigent proteins, there has been, however, no explanation on the flexibility shown by plants under different conditions to allow monomer substitution and significant variation in final lignin structure. For example, COMT-deficient maize mutants do not produce syringyl lignin and instead, they incorporate a novel structure (5-hydroxyguaiacyl units) into the growing lignin polymer (Piquemal et al., 2002).

## Lignin Mutants

Analysis of mutants has been employed successfully to uncover some of the mechanisms involved in the regulation of lignification (Rogers and Campbell, 2004). Many of the lignin mutants that were initially identified were impaired in their ability to synthesize normal lignin, rather than in the control of its deposition. Recently, mutant analysis has begun to reveal the genes that are necessary and sufficient to control the timing and localization of lignin deposition (Rogers and Campbell, 2004). Such genes show a more direct link between the control of cell differentiation and lignin biosynthesis.

Lignin mutants are also important for studying the effects that plants with altered lignin content and/or subunit composition have on the environment. To reduce the concentration of $CO_2$ in the atmosphere, soil carbon (C) sequestration is one of the methods that can be used. This is because plants with modified lignin sub-unit composition and lignin content may have modified decomposition processes that will affect carbon cycling. White et al. (2007) compared C mineralization rates between sorghum *brown midrib* mutant *(bmr)* plants and their normal counterparts. They concluded that the *bmr* mutants had a faster rate of C mineralization compared to the normal isolines. Webster et al. (2005) also compared transgenic tobacco plants with antisense *CAD, COMT* and *CCR* genes and reached the same conclusion. This shows that

alterations in lignin content and sub-unit composition leads to changes in the decomposition rates of plant residues. However, more field studies are required to evaluate how widespread production of plants with altered lignin content affects the ecology.

**Arabidopsis *Ref* Mutants**

Arabidopsis and other members of the *Brassicaceae* accumulate hydroxycinnamic acid esters that are fluorescent when exposed to UV light (Ruegger and Chapple, 2001). These compounds include sinapoylmalate, a leaf-specific ester, sinapoylcholine, a seed-specific ester and a common intermediate ester sinapoylglucose. Therefore, mutants that are defective in leaf sinapate ester biosynthesis can be readily identified since the fluorescent nature of the compounds can be visualized *in vivo* (Chapple et al., 1992). As a result, mutations that lead to quantitative or qualitative changes in sinapate ester content in Arabidopsis either decrease the fluorescence, or reveal the chlorophyll fluorescence that makes the sinapate ester in other mutants appear under a particular color under UV light.

Representatives of one class of mutants, *reduced epidermal fluorescence (ref),* display reductions in the blue-green fluorescence of their cotyledons and/or leaves suggesting the accumulation of lower levels of sinapoylmalate than the wild type (Ruegger and Chapple, 2001). All the *ref* mutants display a UV phenotype that is intermediate between the wild type and the *fah1-2* null mutant. *Bright trichomes (brt),* a second class of mutants, also show less fluorescence when compared to the wild type, but have trichomes that are hyper-fluorescent under UV light. Analysis of three week old Arabidopsis rosettes by high performance liquid chromatography (HPLC) showed that all *ref* mutants contained less sinapoylmalate than wild type plants. When compared to the *ref* mutations, the *brt1* mutations led to modest reductions in sinapoylmalate content.

**Maize *Brown Midrib* Mutants**

Variation in lignin characteristics was first observed in maize *brown midrib (bm)* mutants by Kuc and Nelson (1964). The *brown midrib* mutants of maize show a reddish-brown pigmentation of the leaf midrib and stalk pith. This pigment is associated with tissues that are typically lignified and may result from accumulation of an unknown phenolic derivative where normal lignin biosynthesis is blocked (Vignols et al., 1995). In maize, four spontaneous mutants (*bm1, bm2, bm3* and *bm4*) corresponding to independent genetic loci have been identified (Jorgensen, 1931; Kuc and Nelson, 1964; Kuc et al., 1968). These mutants have low lignin content (Kuc et al., 1968).

The *bm1* mutant gene is closely associated with, and possibly identical to, the gene encoding cinnamyl alcohol dehydrogenase (CAD). Halpin et al. (1998) determined CAD activity in stem tissue (second internode) of wild-type and *bm1* plants at two-week intervals throughout development in three different inbred backgrounds. They showed that in all *bm1* genotypes, CAD activity was significantly reduced at every developmental stage and in all lignified tissues. Guillaumie et al. (2007) performed an in-depth transcriptome analysis of the gene families encoding enzymes of the lignin biosynthetic pathway. They not only confirmed that the *bm1* mutant has reduced *CAD* expression, but when compared to other known *bm* mutants, it had the highest number of differentially expressed genes. The *bm1* mutant had the following under expressed genes: *CAD/SAD*, and several regulatory genes including *MYB, ARGONAUTE* (ortholog of Arabidopsis *ARGONAUTE*) *and HDZip* (Guillaumie et al., 2007). Using acid phloroglucinol, a reagent used to detect cinnamyl aldehydes and lignin, sections of midribs and stems were stained. Wild-type stems showed little staining, whereas *bm1* stems showed strong staining (Halpin et al., 1998). The results suggest an increased content of

cinnamyl aldehydes in *bm1* lignin. Moreover, Klason lignin analysis of *bm1* plants showed a 20% reduction in total lignin content.

The profile of under expressed genes in *bm2* seedlings is nearly similar to that of *bm1* (Guillaumie et al., 2007). The genes fall under several functional categories including phenylpropanoid metabolism, transport and trafficking, transcription factors and regulatory genes. In addition, *bm2* has a 15-25% reduction in lignin content and its mature plants have a 60% decrease in ferulic acid (FA) levels (Barriere et al., 2004). On the other hand, Marita et al. (2003) reported a different conclusion when their study showed that younger *bm2* plants had elevated levels of etherified FA than mature plants. There is, however, no information on the genes that are affected in the *bm2* and *bm4* mutants.

Maize *bm3* is severely deficient in COMT activity, with only 10% of the activity found in normal plants (Halpin et al., 1998) and a lignin content that is reduced by 25-40% (Barriere et al., 2004). Vignols et al. (1995) characterized the first gene encoding a lignin-related *OMT* in maize. Their results showed that the *bm3* gene encodes a bi-functional enzyme that is able to methylate both caffeoyl coenzyme A and 5-hydroxyconiferaldehyde to produce feruloyl coenzyme A and sinapaldehyde, respectively. Guillaumie et al. (2007) performed differential expression studies in young *bm3* mutant plants and their normal counterparts that confirmed the under-expression of the *COMT* gene. To compensate for the modified expression of *COMT*, the differential expression studies showed that two *OMT*'s and two cytochrome P450 genes were significantly over-expressed. The lignin-related *COMT* gene is expressed in tissues that are undergoing lignification and these include elongating roots and vascular bundles in leaves (Collazo et al., 1992).

**Sorghum *Brown Midrib* Mutants**

In sorghum (*Sorghum bicolor* (L.) Moench), 19 independently occurring *bmr* mutants were identified in segregating progenies of chemically treated seeds of two lines after treatment with diethyl sulfate (DES) (Porter et al., 1978). The resultant *brown midrib* plants were identified by the characteristic brown pigmentation (by the 4-6 leaf stage) present in the mid leaf of the stem as well as in the stem, pith, and immature panicle branches of mutant plants. There was variation among mutants in terms of lignin concentration, but there was no consistent reduction among the collection of mutants. This suggested the presence of several different *bmr* genes. Of the 19 mutants, genotypes designated *bmr6*, *bmr12* and *bmr18* were selected for further evaluation.

In the *bmr6* mutant, the CAD and COMT activities were shown to be depressed (Bucholtz et al., 1980). However, the structural modifications of the lignin corresponded only to a reduction in CAD activity with a higher amount of cinnamaldehydes. The *bmr6* mutant is thus, quite similar to that of *bm1* although no cinnamaldehydes were detected in the latter.

Using a candidate-gene-approach, Vermerris and Bout (2003), proved that the *bmr12, bmr18* and *bmr26* mutations are mutant alleles of the gene encoding COMT. In all three mutants there was a reduction in syringyl residues and cell wall bound *p*-coumaric acid. The point mutations in the three cases resulted in a premature stop codon, which resulted in reduced expression levels and reduced COMT activity. These data indicate that these mutants are similar to the *bm3* mutants. An increase in ferulic acid was observed in both *bmr12* and *bmr18.* Allelism tests by Bittinger et al. (1981) indicated that *bmr12* and *bmr18* are allelic and different from *bmr6*.

**Pearl Millet *Brown Midrib* Mutants**

Pearl millet (*Pennisetum glaucum* (L.) R.Br.) is used extensively as forage for livestock. Based on the negative relationship between lignin and digestibility, reducing lignin content is

one way of increasing digestibility of pearl millet. Cherney et al. (1988) chemically induced a *brown midrib* mutation in pearl millet from two inbred lines using ethyl methyl sulfonate (EMS) or diethyl sulfate (DES). A naturally occurring *brown midrib* mutant in pearl millet was also identified at the Coastal Plain Experiment Station in Tifton Georgia (Degenhart, 1991). This *bmr* was originally called orange node and was similar to the *brown midrib* mutant developed by Cherney et al. (1988) using EMS.

## Lignin Modification via Transgenic Approaches

Transgenic plants show gross alterations in lignin amount, composition, primary structure and on phenotypic effects caused by altering the expression of a single gene (Boerjan et al., 2003). The cell wall has a major impact on the utilization of plants by mankind, and has therefore, become a major target for genetic engineering (Grima-Pettenati and Goffner, 1999). Lignin genetic engineering has become an active area of research which has been stimulated within recent years by the characterization of important genes controlling lignification. A significant number of transformed plants exhibiting quantitative changes in their lignins, but also qualitative changes have been obtained using partial sense or antisense constructs corresponding to several enzymes. However, there are drawbacks that are associated with this route. An important aspect of lignin manipulation lies in the potential disturbance of plant development such that reduction or modification of the lignin content of plant cell walls may potentially exert pleiotropic effects on plant functions through changes in the strength of plant organs, sap conduction through the phloem, or permeability of cell wall barriers (Boudet, 2000).

Ralph et al. (2005) reported that the down-regulation of *C3H* gene in alfalfa resulted in an increase of *p*-hydroxyphenyl (H) units in the plants by up to 65% compared to wild-type levels of 1%, and a decrease of G units. C3H lines with 5% residual activity showed delayed flowering activity by 10-20 days and the plants were generally smaller, and grew more slowly than wild-

type controls. The most severely down-regulated C3H line was G-and-S depleted, but was still viable, albeit with stunted growth, suggesting that plants without access to the two primary monolignols could be viable (Ralph et al., 2006). On the other hand, lines with 20% residual activity were of almost normal size and showed delayed flowering by 1-2 days. In contrast, Franke et al. (2002) showed that Arabidopsis *ref8* mutant lacking C3H activity produces lignin at 100% H level and is totally devoid of S and G units. Even though the *ref8* mutant is viable, it has a dwarf phenotype, has collapsed xylem vessel elements is susceptible to fungal attack, and does not flower, compared to the wild-type control.

In transgenic Arabidopsis *(fah1)* mutant plants that over expressed F5H ectopically under the control of the C4H promoter, a novel lignin was generated, composed almost completely of S units (Meyer et al., 1996). This result implicates F5H in determining the lignin monomer composition. Therefore, modifying F5H (Cald5H) and altering the relative amount of S units may offer opportunities for engineering lignin quality in hardwoods and softwoods (Baucher, 2003). A combinatorial down-regulation of 4CL along with an over expression of Cald5H has been achieved by co-transformation of two *Agrobacterium* strains in aspen (Li et al., 2003). This resulted in an additive independent transformation, particularly a 52% reduction in lignin content associated with an increase in cellulose and a higher S/G ratio. The result suggests that transgene stacking allows several beneficial traits to be improved in a single transformation (Li et al., 2003).

For functional analysis in Arabidopsis, acyltransferase (HCT) was silenced by RNA-mediated posttranscriptional gene silencing (Hoffmann et al., 2004). The silenced plant was severely dwarfed, had inhibited root growth, and had a 15% decrease in lignin accumulation. Results on down regulation of CCR in tobacco indicate that the transgenic plants have lower

lignin content and a modified lignin composition. In fact, the transgenic plants with the lowest CCR activity showed the strongest reduction in lignin content indicating that CCR is a rate limiting step in lignin biosynthesis (Lee, 1997). Piquemal (1998) obtained transgenic tobacco plants down-regulated in CCR activity via ectopic expression of homologous antisense genes which resulted in a 53% decrease in lignin content and S and G units. The line with the most severely depressed CCR activity exhibited altered development in the form of reduced size, abnormal morphology of the leaves and collapsed vessels. Similarly, Goujon et al. (2003) reported that down regulation of the *AtCCR1* gene in *Arabidopsis thaliana* resulted in a 50% decrease in lignin accompanied by changes in lignin composition and structure. The transgenic plants also exhibited phenotypic alterations consisting of limited height and collapsing of the xylem vessels. These phenotypic modifications are likely direct consequences of the dramatic lignin depletion in the cell walls. Lignin is essential for maintenance of xylem vessel integrity and it is, therefore, likely that the collapse of the xylem vessels may result in the dysfunction of xylem sap transport. These perturbations may affect water supply and consequently normal growth and plant morphology (Piquemal et al., 1998).

Decreases in lignin content through the manipulation of different lignin biosynthetic pathway genes, do not always lead to developmental oddities. For example, down regulation of one of the major enzymes involved in lignin biosynthesis, 4-coumarate: coenzyme A ligase (*Pt*CL1) in transgenic aspen (*Populus tremuloides),* resulted in a 45% decrease in lignin with a compensation of 15% increase in cellulose, doubling the plant cellulose: lignin ratio without any change in lignin composition and without any harm to plant growth, development, or structural integrity (Dean, 2005). This result indicates that lignin and cellulose deposition are regulated in a compensatory fashion and that a reduced carbon flow toward phenylpropanoid biosynthesis

increases the availability of carbon for cellulose biosynthesis (Li et al., 2003). Similarly, COMT down regulation in alfalfa only had a moderate effect on overall estimated Klason lignin contents even at highly repressed levels (Guo et al., 2001). In the study, transformants were generated by expressing the full-length alfalfa *COMT* gene in the antisense orientation. There was no report of any developmental abnormalities in the transgenic lines even though they had a low *COMT* activity of about 3 – 5 % of the wild-type and almost undetectable COMT transcripts and proteins. These results show that genetic engineering has yielded new insights into how the lignin biosynthetic pathway operates and demonstrates that lignin can be improved.

As shown by the previous examples, the data obtained by genetic engineering or by the analysis of mutants in the lignin biosynthesis pathway are very promising as they show that it is possible to engineer this pathway. However, more research is still needed to improve the stability of sense/antisense constructs. Baucher et al. (1998) suggest large scale experiments to assess the effects of an alteration in the lignin content and lignin composition on the digestibility of forage crops and on pulping. Other characteristics such as calorific value, overall growth and the resistance against diseases have to be examined.

## Agro-Industrial Processes Affected by Cell Wall Composition

The composition and structure of the cell wall has a dramatic impact on the technological value of raw materials. As a result, numerous strategies have been, and are being, developed to optimize the composition of plant cell walls for improved agro-industrial uses (Boudet, 2003). In addition to their critical role in normal plant health and development, high lignin levels are problematic in the agro-industrial exploitation of various plant species. Lignin is considered an undesirable component in paper manufacture, and it has a negative impact on forage crop digestibility (Baucher et al., 1998). On the other hand, the high caloric values of lignin are an important source of energy if the material is to be used for combustion.

**Ethanol Production**

Currently, most ethanol produced in the United States is derived from maize kernels at a level of seven billion gallons per year (Sticklen, 2006). Although the fermentation technologies for such sugar and starch sources are well developed, they have a number of limits (Hamelinck, 2005). Maize kernels have a high value for food applications, and the sugar yield of kernels per hectare is low when compared to the most abundant forms of sugar in nature: cellulose and hemicellulose. Lignocellulosic biomass holds tremendous promise as a feedstock for ethanol production due to its widespread availability and potential for high fuel yields (Brekke, 2005). Furthermore, lignocellulosic biomass is renewable and inexpensive. Several potential sources for cellulosic ethanol include corn stover, cereal straws, sugarcane bagasse, sawdust, paper pulp, and switch grass (an energy crop). All these characteristics help make the case for the USA to develop alternate energy sources, reduce the nation's dependency on foreign oil, and increase the use of excess agricultural feedstock for biofuel.

However, converting cellulosic biomass to ethanol requires first hydrolyzing the biomass into its constitutive sugars. One of the factors contributing to this bottleneck is the presence of lignin and hemicellulose. The combination of hemicellulose and lignin provides a protective sheath around the cellulose (Figure 1-1), which must be modified or removed before efficient hydrolysis of cellulose can occur (Hamelinck, 2005). Thus, to economically hydrolyze cellulose, more advanced technologies are required more than for the processing of sugar or starch crops.

Hydrolysis is most efficiently done enzymatically. However, enzymes are very expensive and the ones currently available have low activity. Over the past few years, the cost of cellulose enzymes to convert cellulose to glucose has been the greatest technical barrier to cost-effective production of cellulosic ethanol. Therefore, the challenge for adoption of stover as a source of ethanol remains that of converting lignocellulosic biomass to ethanol on a commercial scale. In

addition, researchers have expressed disappointment at the lack of specially tailored crops or the slow breeding programs for such crops.

Recently, however, several companies have identified a range of new enzymes with enhanced activities to boost sugar yields, thus reducing the cost of enzymes for making ethanol from corn stover 30-fold, from $5 per gallon in 2001 to 10 cents per gallon in 2005 (Biotechnology Industry Organization, 2006). More importantly, recent genetic engineering advances could reduce biomass conversion costs by developing crop varieties with less lignin (Sticklen, 2006). As an illustration, down-regulation of COMT in maize and alfalfa led to lower and modified lignin and consequently enhanced digestibility (Piquemal, 2002; Guo, 2001). Chen and Dixon (2007) also reported that transgenic alfalfa lines independently down regulated in each of the six lignin biosynthetic enzymes yielded more sugar compared to wild-type plants.

**Paper Production**

Pulp and paper production require the use of costly, energy consuming, and often polluting treatments to separate lignins from cellulose (Boudet, 2000). Mechanical production gives a particularly high pulp yield (90 to 95%) but conserves all wood components (Reid, 1991). However, paper made via mechanical pulping has a low strength and turns yellow on exposure to sunlight due to photochemical oxidation of lignin (Baucher et al., 1998). Chemical pulping on the other hand involves the chemical hydrolysis and solubilization of lignin using acid or alkaline in which lignin is degraded at high temperatures and extreme pH. Alkaline pulping (kraft) is the most commonly utilized method worldwide, but it results in a lower pulp yield (40 – 50 %) compared with mechanical pulping. In addition, kraft pulping releases volatile and toxic mercaptans which pollute the atmosphere. Moreover, a subsequent bleaching step with chlorine causes the formation of highly toxic and chlorinated organic compounds. Genetic engineering can thus play an important role in reducing the use of chemicals for pulping and bleaching by

decreasing the lignin amount or by modifying the lignin composition in trees to substantial industrial and environmental benefits.

**Forage**

Plant cell walls are the major source of dietary fiber for animals (Buxton and Redfearn, 1997). However, several chemical and structural features have been identified that may limit fiber digestibility in most forage crops. Lignin has been prominently mentioned and interferes with microbial degradation of fiber polysaccharides by acting as a physical barrier. In addition, the cross-linking of lignin to polysaccharides by ferulate bridges also contributes to the inhibition of grass fiber digestion (Buxton and Redfearn, 1997).

Lignin is necessary to provide mechanical support for the plant and to impart strength and rigidity to plant cell walls. Also, lignin provides resistance to diseases, insects, pathogens and other biotic and abiotic stresses. The brown midrib plants with reduced lignin produce reduced forage yield, compared with the normal phenotype, by an average of 15% for the first harvest and 30% for the second harvest (Casler et al., 2003). It is suggested that the reduced forage yield was due partly to reduced ground cover resulting from reduced tillering capability of the brown midrib lines.

However, despite the agronomic performance limitations of the brown midrib plants, Casler et al. (2003) reported that brown midrib plants increased relative feed value by seven to 23% and raised predicted milk production by 19 to 50%. The brown midrib plants are more palatable than wild-type plants based on a higher dry matter intake for *bmr* silage (Stallings et al., 1982). Higher intake may have been related to increased digestibility of dry matter and the sweet taste of the *bmr* plants. In conclusion, since the *bmr* genotypes have lower fiber and lignin values, intake and digestibility by animals are higher, leading to better animal performance (Cherney et al., 1991).

## Plant Pigmentation

Pigments are chemical compounds that absorb light in the visible region of the electromagnetic spectrum (Delgado-Valgas, 2000). Plant pigmentation is generated by the electronic structure of the pigment interacting with sunlight to alter the wavelengths that are either transmitted or reflected by the plant tissue (Davies, 2004). Pigments are classified based on their structural characteristics.

### Carotenoids

Carotenoids are compounds comprised of eight isoprenoid units (ip) (Delgado-Valgas, 2000). They can be considered as lycopene ($C_{40}H_{56}$) derivatives that involve: (i) hydrogenation, (ii) dehydrogenation, (iii) cyclization, (iv) oxygen insertion, (v) double bond migration, (vi) methyl migration, (vii) chain elongation, (viii) chain shortening. In general, carotenoids are classified by their chemical structures as: carotenes that are constituted by carbon and hydrogen; oxycarotenoids or xanthophylls that have carbon, hydrogen, and, additionally oxygen.

Carotenoids' main functions are to provide photoprotection during photosynthesis and to serve as precursors for the biosynthesis of the phytohormone abscisic acid (ABA) and vitamin A biosynthesis (Grotewold, 2006). Biosynthesis takes place in plastids where isopentynyl diphosphate provides the five-carbon building block for carotenoids.

As a result of the various important functions that carotenoids play, most of the enzymes in the carotenoid biosynthetic pathway have been identified. Because of the identification of biosynthetic genes for several plant pigment pathways, it has been possible for genetic modification approaches to be used to alter pigment production in transgenic plants. Such metabolic engineering experiments have resulted in stable transgenic plants with improved nutritional quality, increased ability to synthesize high value carotenoids and improved tolerance to abiotic stresses. Phytoene synthase is the first committed step in the carotenoid biosynthetic

pathway and is, thus, considered to be a regulatory point. As a result, several constitutive over expression approaches have been targeted at this gene. Perhaps the best known achievement to date is golden rice (Datta et al., 2003). By using the rice-seed glutein promoter (Gt-1 P) to over-express the *phytoene synthase* gene and the cauliflower mosaic virus 35S promoter (CaMV35S) to control the expression of the *lycopene β-cyclase* (*lcy*) and *phytoene desaturase* (*crtl*) genes. Datta et al. (2003) generated yellow-colored rice grains that contained high levels of β-carotene.

Shewmaker et al. (1999) over-expressed a bacterial *phytoene synthase* gene in rapeseed (*Brassica napus)* resulting in orange embryos and mature seed that contained 50-fold increase in carotenoids. Similarly, constitutive expression of the same enzyme in tomato led to 1.5 – 2-fold increase in carotenoids in tomatoes (Fray et al., 1995). However, over expression of phytoene synthase in other species did not always lead to dramatic increases in carotenoid level. These differences may be due to the source of the *phytoene synthase* (plant or bacterial), the tissue where expression is targeted (embryo versus endosperm versus vegetative), and/or the photosynthetic capability of the tissue, i.e. green versus white (Shewmaker et al., 1999). In contrast, tomato plants transformed with a copy of the fruit-expressed *phytoene synthase* cDNA under the control of the CaMV 35S promoter showed ectopic production of carotenoids and stunted growth (Fray et al., 1995). The dwarf plants also showed a 30-fold reduction in levels of gibberellins $A_1$ ($GA_1$) but normal growth was restored by exogenous application of $GA_3$. This can be explained by the fact that geranylgeranyl diphosphate (GGDP), a substrate for *phytoene synthase*, is a precursor to three different major pathways of plant growth regulators: cytokinins, GAs and abscisic acid (ABA). Thus, the dwarf phenotypes are a result of altered flux through the isoprenoid pathway, with the high levels of phytoene being generated at the expense of *ent*-kaurene synthesis, therefore resulting in reduced levels of GAs (Fray et al., 1995).

Mutant analysis has also provided much information regarding plant pigment biology. One interesting mutation is the cauliflower (*Brassica oleracea* L. var *botrytis*) *Or* gene. A spontaneous single-locus *Or* gene mutation causes a high level accumulation of β-carotene in tissues where it is normally repressed. As a result of the mutation, the white edible curd is turned orange (Crisp et al., 1975).

**Flavonoids**

Flavonoids have a 15-carbon base structure that is made up of two phenyl rings (A and B rings) connected to a three carbon bridge that usually forms a third phenyl ring (C-ring) (Schwinn and Davies, 2004). There are more than 4500 different representatives of flavonoids known with various classes determined by the degree of oxidation of the C-ring (Buchanan et al., 2002; Schwinn and Davies, 2004). Despite the similarity in structure, only some flavonoids have the ability to absorb light in the visible region of the spectrum and are thus pigments.

Flavonoids have been demonstrated to perform a variety of functions in plants: attracting pollinators, conferring resistance to natural enemies, facilitating interaction with symbionts, protection from ultra-violet light (Bieza and Lois, 2001), regulating hormones, mediation of pollen-stigma interactions, ameliorating drought stress and amelioration of adverse effects of heat stress on fertilization and early seed maturation (Coberly and Rausher, 2003; Shirley, 1996; Warren and McKenzie, 2001).

Even though carotenoids play the predominant role in yellow pigmentation, flavonoids are responsible for this color in some instances (Schwinn and Davies, 2004). The yellow pigments are the chalcones, aurones and some flavonols. Chopra et al. (2003) characterized an *Unstable factor for orange1 (Ufo1)*, a dominant allele-specific modifier of expression of the maize *pericarp color1 (p1)* gene. The *p1* gene encodes a Myb-homologous transcriptional activator of genes required for biosynthesis of red phlobaphenes. The plants have red pigmented pericarp and

an intense cob color caused by accumulation of phlobaphenes. Phlobaphenes are one of two major groups of flavonoid pigments, the other being anthocyanins. The latter are water soluble compounds that occur in almost all vascular plants (Stobiecki and Kachlicki, 2006). Anthocyanins are the major cause of orange, red, purple and blue colors of flowers.

Most of the currently known flavonoids can be modified at one or several positions by methylation, acylation or glycosylation. These modifications are believed to provide flavonoids with unique properties. For example flavonoids found on the surface of leaves or flowers are often methylated (Onyilagha and Grotewold, 2004).

## Pyrosequencing

Pyrosequencing, also known as 454 sequencing, is a relatively new DNA sequencing technique that is based on the detection of a released pyrophosphate (PPi) during DNA synthesis (Ronaghi, 2001). Margulies et al. (2005) described a novel and high-throughput method to sequence DNA. The entire genome is isolated, fragmented, ligated to adapters and then separated into single strands. The individual fragments are attached to small beads under conditions that promote one fragment per bead before the beads are captured in an emulsion of PCR-reaction-mixture. PCR amplification occurs within each droplet clonally amplifying the individual fragment resulting in beads carrying millions of copies of a unique DNA template. This is a significant divergence from the Sanger-based sequencing technology which requires sub-cloning of fragments into vectors. The emulsion is broken, releasing the bead-attached fragments which will be subsequently denatured, and beads carrying single-stranded DNA clones are deposited into wells of a fiber-optic slide. A mixture of smaller beads that carry immobilized ATP sulfurylase, luciferin and luciferase necessary to generate light from free pyrophosphate are also loaded into the wells. It is in these wells that sequencing-by-synthesis takes place. Each well has an opening through which sequencing reagents flow while the base of the well is in optical

contact with a fiber optic cable connected to a charge-coupled device (CCD) camera sensor. Each time a particular nucleotide is added onto the growing chain, a pyrophosphate is released, which in turn reacts with ATP sulfurylase to form ATP. The ATP then combines with luciferin and luciferase enzyme giving off a flash of light. In a cascade of enzymatic reactions visible light that is proportional to the number of incorporated nucleotides is generated and is recorded by the CCD sensor.

Pyrosequencing is high-throughput, has high sequencing accuracy, and is fast and sensitive when dealing with small and microbial genomes (Wicker et al., 2006). Cheung et al. (2006) showed that 454 has the power of deep sequencing because it resulted in sequences that generated more gene hits, and revealed rare and novel transcripts. In addition, 454 sequencing can sequence large genomes 10 times faster compared to Sanger sequencing (Wicker et al., 2006). However, it has limitations of read length and capacity to sequence complex genomes that contain high amounts of repetitive DNA. As a result, a whole-genome shotgun approach by 454 sequencing does not seem practical for multi-gigabase plant genomes. Instead, a bacterial artificial chromosome (BAC-by-BAC) approach still remains the best option for genomic sequencing in large and complex genomes.

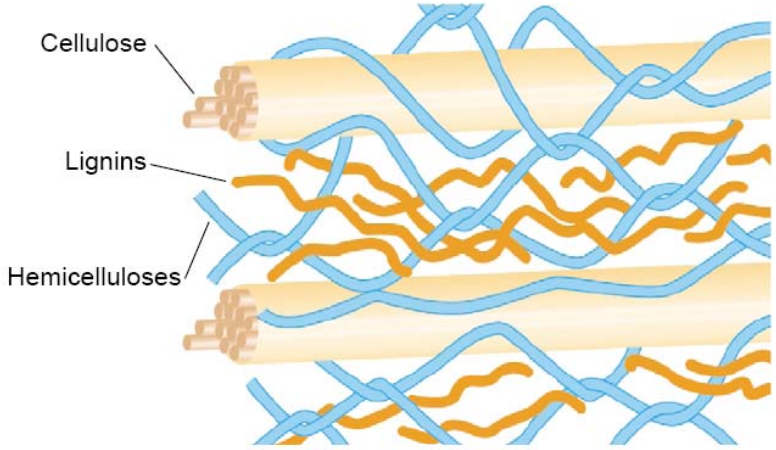



Figure 1-1. Schematic representation of the lignified secondary wall. In addition to cellulose, lignins and hemicelluloses, other cell wall constituents of minor abundance, including proteins and low-molecular weight phenolics, are not indicated on the figure. [Adapted from Boudet, A.M., Kajita, S., Grima-Pettenati, J., Goffner, D., 2003. Lignins and lignocellulosics: a better control of synthesis for new and improved uses. Trends Plant Sci. 8, 576-581].

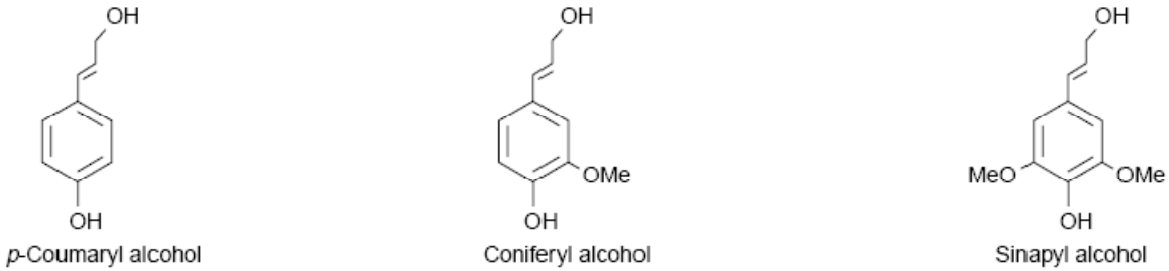



Figure 1-2. Three monolignols that are incorporated into the lignin polymer. [Adapted from Boudet, A.M., Kajita, S., Grima-Pettenati, J., Goffner, D., 2003. Lignins and lignocellulosics: a better control of synthesis for new and improved uses. Trends Plant Sci. 8, 576-581].

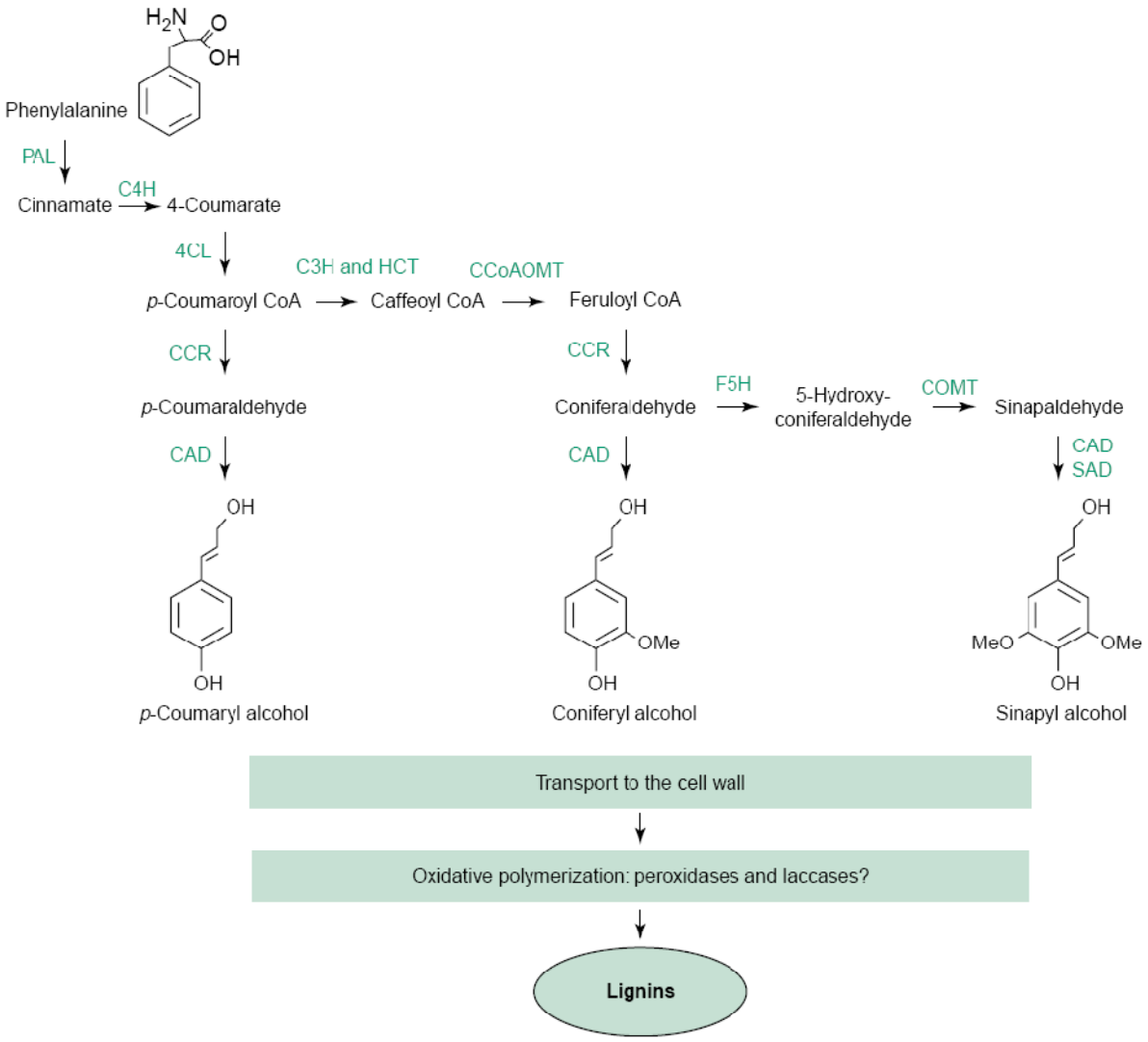



Figure 1-3. The lignin biosynthetic pathway. [Adapted from Boudet, A.M., Kajita, S., Grima-Pettenati, J., Goffner, D., 2003. Lignins and lignocellulosics: a better control of synthesis for new and improved uses. Trends Plant Sci. 8, 576-581].

CHAPTER 2
SCREENING FOR ALTERED CELL WALL COMPOSITION USING NIR SPECTROSCOPY

## Background

One of the goals of the NSF Plant Genome project 'Identification and characterization of cell wall mutants in maize and Arabidopsis using novel spectroscopies' (http://cellwall.genomics.purdue.edu; Yong et al., 2005) is the generation of a large mutant population followed by identification of novel maize mutants with altered cell wall composition. The former was generated by the Uniform*Mu* population and the latter by the use of NIR spectroscopy (Yong et al., 2005). Identification of cell wall mutants will help in the understanding of roles played by various cell wall structural elements. Cell walls contribute to human and animal nutrition in several ways. Products of cell walls provide dietary fiber and calories that influence taste and texture of fruits and vegetables, and they provide human clothing and shelter in the forms of fiber products. Cell walls of plants in the form of wood, lignocellulosic plant material provide fuel for cooking, transportation and electric power. Therefore, identification of mutants is essential in understanding the roles of each structural element in cell walls.

## Introduction

In an effort to create mutations in cell-wall-related genes, 2,200 F2 families (~ 40,000 plants) of *Mu*-tagged maize lines from the Uniform*Mu* population developed at the University of Florida, Gainesville, FL were planted at Purdue University, West Lafayette, IN between 2002 and 2004. In order to screen all 40,000 plants, a method that enabled rapid screening of large numbers of plants was needed. Near infrared reflectance spectroscopy (NIRS), a high-throughput screening method, was an ideal option. Besides being high-throughput, NIRS requires little or no sample preparation, is non-destructive, has no chemical usage and, therefore, does not generate

chemical waste, is portable (can be used in the field), and operators do not require extensive training.

NIRS is a vibrational spectroscopic technique in which the reflectance (R) of light in the near-infrared range of the spectrum (800 – 2500 nm) is quantified (Seisler et al., 2002). Absorbance, which is defined as the logarithm of the inverse of R of light of a given wavelength confers information on the chemical composition of the sample. However, NIR only deals with organic molecules - with water as an exception (Analytical Spectral Devices, 2005). Consequently, the types of materials that can be analyzed with NIR are limited. Thus, metals such as silver, lead, and most inorganics, cannot absorb NIR light because they have electron transitions incapable of absorbing NIR wavelengths. Various molecular arrangements in a sample will give that particular sample its characteristics, the ability to absorb different wavelengths of light. So when a sample is analyzed by NIR spectroscopy, what the instrument will be measuring is the number of photons at a particular wavelength (Analytical Spectral Devices, 2005). The number of photons that are absorbed is proportional to the abundance of a particular functional group present in the sample.

The screening process, as mentioned above, generates large quantities of data, as is common for many techniques of modern analytical chemistry. For example, infrared, nuclear magnetic resonance (NMR), atomic absorption and UV/vis spectroscopies, chromatography and mass spectrometry, all generate large quantities of data (Kemsley, 1998). As a result, special processing and analytical methods are required to handle these complex data. What may appear as a single and simple spectrum can be composed of a large number of discrete values, from several hundred in a low resolution infrared spectrum to as many as several hundred thousand in

a high resolution NMR spectrum (Kemsley, 1998). There are, however, several statistical techniques that can deal with this kind of data, known collectively as chemometrics.

The statistical software package Windows Discriminant Analysis Software (Win-DAS) (Kemsley, 1998) was used in this study. Two common chemometric techniques were used: principal component analysis (PCA) and partial least squares (PLS) (Kemsley, 1998). For the study described in this report a FieldSpec Pro NIR spectrometer (Analytical Spectral Devices, Inc. Boulder, CO; www.asdi.com) was used to acquire spectra from dried maize leaf samples. The resultant spectra were comprised of a large number of discrete data values, which are measurements of a series of spectral properties of each leaf sample. By definition, a spectrum reflects the absorbance at multiple frequencies or wavelengths, which makes the observations 'multivariate'. Win-DAS will only handle spectral data that is ordered sequentially and equally spaced. That is variate values must be plotted sequentially and against a variate number (i.e. an integer 1, 2, 3, d).

**Data Compression**

As mentioned previously, data sets obtained from modern instruments like the FieldSpec Pro NIR spectrometer have a very large number of variates *(d)* that far exceed the number of observations (samples, *n*) and as a result such data sets are classified as 'high-dimensional'. High dimensional data are complex and contain inter-correlated variates. The *n* observations, which are continuous sets of variates that are closely adjacent to each other, can be arranged into a matrix. These can be visualized as *n* points plotted on a set of *d* mutually perpendicular axes. Data compression techniques are used to transform these data into a new set of variates of manageable size. The transformed variates are known as "scores". For example, data compression can reduce a set of 20 pairs of bivariate observations (a total 40 observations) plotted as 'spectral traces' (value versus variate name) into 20 points which can be located on a

plot of two axes in a two-dimensional coordinate system. Further reduction in complexity is obtained by rearranging the axes (rotation) until the 20 points are arranged in a manner that results in the maximum spread or variability (variance). This reduction in complexity is helpful in data exploration, as it unmasks patterns or groups of observations which were not apparent in the original complex data set. The data compression methods used in Win-DAS are PCA and PLS with the latter specifically adapted for dealing with grouped data.

**Principal Component Analysis**

Principal component analysis (PCA) simplifies a data set by reducing the number of variates needed to express relevant information. It is a way of identifying patterns in data, and expressing the data in such a way as to highlight their similarities and differences (Smith, 2002). This is achieved by rearranging the information in a data set by ordering the scores in terms of decreasing variance. This means that PCA takes a cloud of data from, for example, hundred samples, each with about 1,000 variates, and rotate it such that maximum variability between different classes is clearly visible.

**Partial Least Squares**

PCA can effect a reduction in the complexity of a data set (Kemsley, 1998). In addition, PCA is unable to produce scores which conform to a predetermined structure, i.e. it is not a modelling method. Therefore, in cases when there is an idea about the way in which the data might be structured, partial least square (PLS) is preferable. PLS is, however, solely used to analyze data sets that are believed to be structured in groups. Therefore, in cases where PCA transformation does not yield information that is efficiently compressed in the first few PC scores, PLS can be used.

## Materials and Methods

### Uniform*Mu* Population

The Uniform*Mu* population carries active Robertson's *Mutator* transposons that can move from one locus to another within the genome to cause mutations. It was created by introgression of active *Mutator* and the *bronze1 Mu*-induced *mutable9 (bz1-mum9)* gene (Brown and Sundaresan, 1992) to a standard color-converted W22 inbred line which simplifies the identification of parental transposon insertions (McCarty et al., 2005). This *Mu* population was designed to address specific constraints associated with high-copy transposons. These include (i) high mutagenic activity in a homogenous inbred; (ii) genetic control of *Mu* activity for suppression of *Mu* transposition prior to molecular analysis; (iii) screening and removal of parental seed mutations to maximize independence of seed mutation; and (iv) ability to implement high-throughput molecular analyses of high-copy transposon insertions based on *Mu*-TAIL (Settles et al., 2004; McCarty et al., 2005).

Approximately 40,000 plants were grown at Purdue University, West Lafayette, IN between the summers of 2002 and 2004. To accommodate variation in the field as well as variation related to the time of day, W22 controls were planted along the edges and through the middle of the field. Leaf samples were collected from a field of maize plants that were bar-coded when they were eight weeks old. An eight centimeter section of the central part of the fifth leaf blade from the bottom was harvested. The leaf samples along with the barcode were collected in a glassine envelope (#5) and dried at $50^0$C in a drier for a week and subsequently stored at room temperature.

One day before spectral acquisition, leaf samples were re-dried overnight to remove any moisture absorbed during storage. Spectral acquisition was a two-person job: one person arranged the leaves on a tray while the other acquired the spectra in a custom-made structure

covered by a dark cloth to prevent light penetration. A FieldSpec Pro NIR spectrometer (Analytical Spectral Devices, Inc. Boulder, CO; www.asdi.com) was used to acquire spectra. From a handheld probe, light was emitted and directed through a leaf sample sandwiched between a piece of glass and a GoreTex® disk (www.gore-tex.com). The GoreTex® disk is a white disk that serves as the white reference (100 % reflectance). The glass, disk and probe were kept clean by periodically cleaning them with methanol. The RS$^3$ (Analytical Spectral Devices, Inc. Boulder, CO; www.asdi.com) program was used for acquisition. Following acquisition, the ViewSpec program (Analytical Spectral Devices, Inc. Boulder, CO; www.asdi.com) was used to export the data into one JCAMP-DX file (Analytical Spectral Devices, Inc. Boulder, CO; www.asdi.com). Analysis of the data was ultimately performed using the WinDAS program (Kemsley, 1988) after the .dx files were converted to .txt format using custom-designed software (Kemsley, 1988 and Vermerris et al., 2008).

After the NIR data were imported into WinDAS they were visually inspected, and aberrant-looking spectra were deleted. The spectra were then truncated to the 1000 – 2400 nm range, excluding variation in reflectance of visible light. The spectra were baseline-corrected (reflectance at 1000 and 2400 nm set to zero) and then the spectra were area normalized. Baseline correction removes linear shifts in the baseline that could be caused, for example, by instrumental drift during the course of data acquisition (Kemsley, 1998). Normalization corrects for variation in signal strength due to physical effects, such as variation in leaf size and light leaks between the glass surface and contact probe. After data formatting, the spectra were saved in WinDAS format (.wdd file extension) and subjected to statistical analysis.

**Novel *Brown Midrib* Mutant**

A novel *brown midrib* mutant was compared to the wild-type control using NIR (Figure 2-1 and Figure 2-2). Sixty-seven *bm* mutant leaf samples and 39 wild-type samples were compared

for analysis. Spectra were analyzed as described above. Any contrasts in their spectra can help identify potential chemical differences leading to the chemical basis of the mutation, since specific functional group(s) are associated with each particular change in absorbance.

**Class Modelling**

Spectra of putative mutants (putants) were compared to spectra of W22 controls that were grown in the same area of the field. The W22 spectra were used to define a class. The spectra of the putants were tested for fit within the class, and outliers were reported. Based on the assumption that the majority of the mutations would be segregating in a Mendelian recessive manner, we expected at least two out of 15-20 spectra to be different from the W22 controls but similar to each other. Ideally, outlier spectra would consistently and tightly cluster together. The first spectral acquisition was performed by a team of people taking turns. However, since the spectral acquisition process turned out to be sensitive to individual operators, two individuals then independently re-acquired the spectra from all the rows that had putative mutants. Data analysis was then redone on the new set of spectra. Only the samples that were consistently being detected by the two individuals as outliers were selected for the generation of homozygous mutants.

## Results

A total of 39 cell wall mutants were identified. Seeds from these mutants were planted and the plants were selfed to generate homozygous plants. Once at least five individuals representing a mutant were identified, their spectra were compared to those of the W22 controls to identify the chemical basis of the altered spectral properties. Part of the raw data that was generated was further analyzed by Dr. Vermerris and the results are accessible to the public on Purdue University's cell wall genomics website (http://cellwall.genomics.purdue.edu/families/7.html). Two of the 39 putative mutants have been further evaluated in the field and by pyrolysis

molecular-beam mass-spectrometry (Py-MB-MS) analysis. Figures 2-3 and 2-4 show examples of how the two putative mutants differ in their chemical composition as well as in their performance under field conditions from their wild-type counterparts. Further characterizations of the remaining putative mutants are underway.

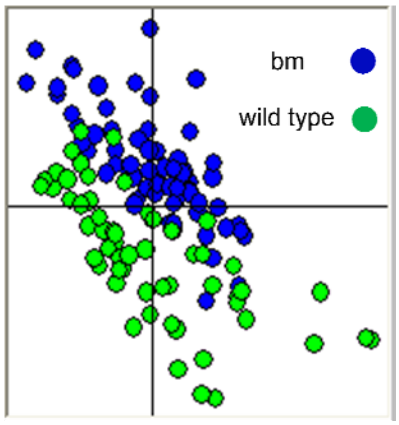



Figure 2-1. Partial Least Squares plot showing separation between *bm* mutant and wild-type leaf samples.

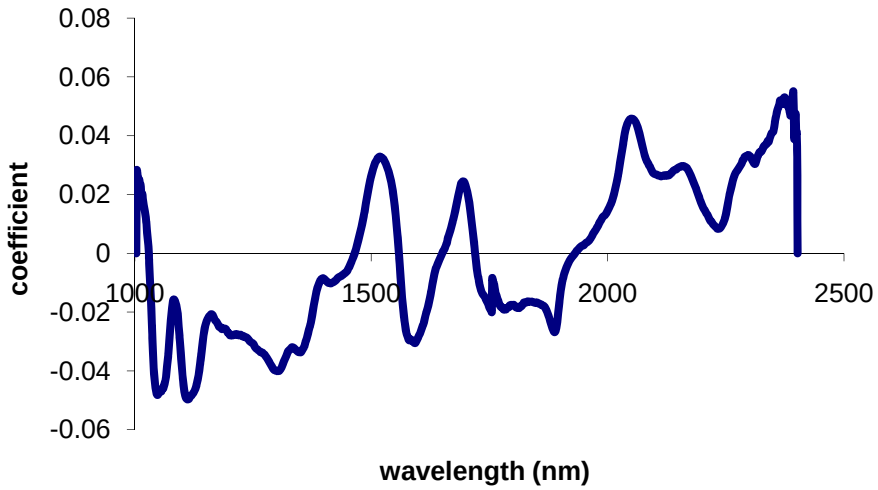



Figure 2-2. Based on NIR data, the mutant appears to have an abundance of proteins (1528 nm, 2061 nm) carbohydrates (1702 nm, 2379 nm).

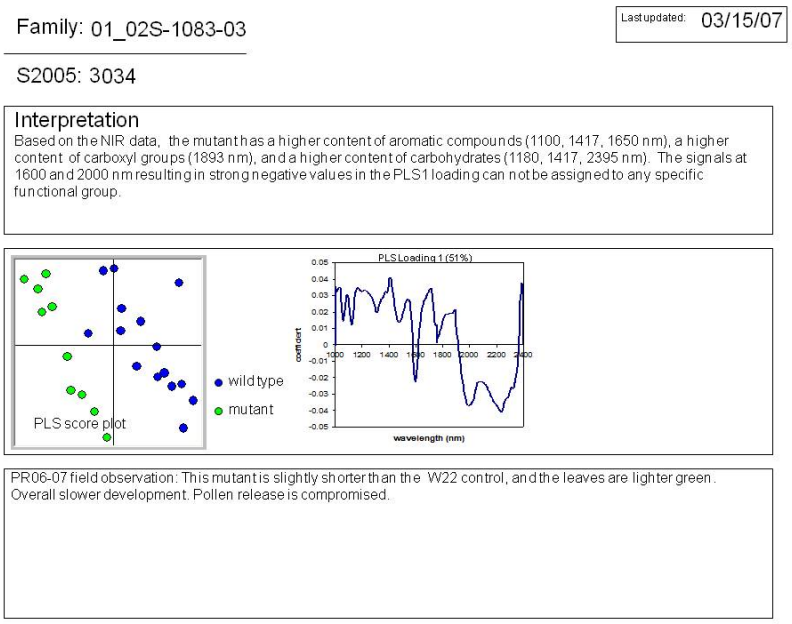



Figure 2-3. Results confirming variation in cell wall composition between putative mutant and wild-type plants following NIR analysis. Observations made in the field also show differences in growth and development.

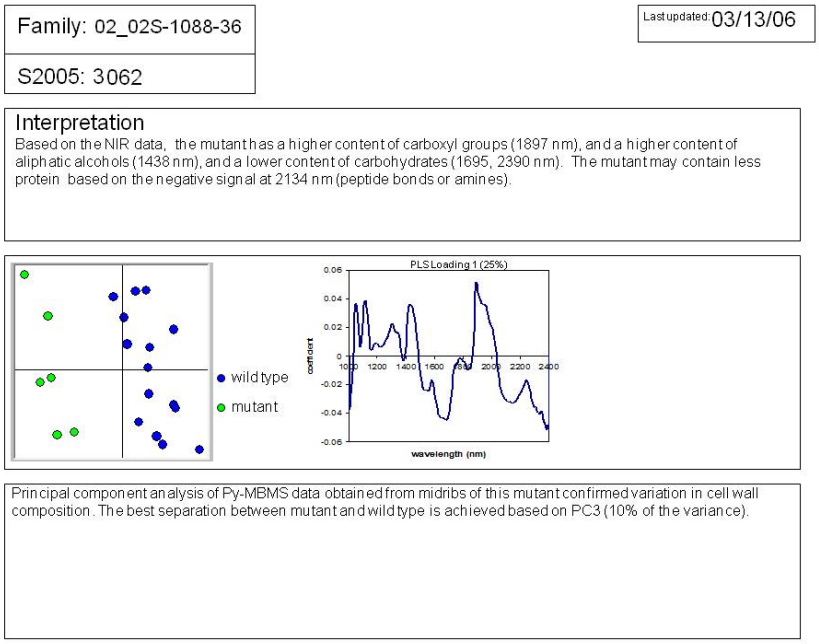



Figure 2-4. Results confirming variation in cell wall composition between putative mutant and wild-type plants. Results were obtained after NIR and Py-MB-MS analyses.

CHAPTER 3
AGRONOMIC AND CHEMICAL ANALYSES OF THE NOVEL *BM* MAIZE MUTANT

## Introduction

### Agronomic Traits

Functional genomics using mutants of maize enables the identification and manipulation of genes to gain understanding of the genetic control of plant genes, including those for quality and plant performance traits. This approach can, therefore, be used to develop and assess novel maize for applications such as enhanced forage feed or as raw materials for biofuel processing (Donnison and Morris, 2003).

The *bm* mutations have been shown to affect yield, plant height, flowering dynamics and biomass conversion efficiencies (Vermerris et al., 2007). Vermerris et al. (2002) showed that *bmr* sorghum and *bm* maize have different flowering dynamics compared to their wild-type counterparts. Oliver et al. (2005) concluded that the *bmr* phenotype is generally associated with reduced vigor and yield. Previous research in maize has also demonstrated that *bm* mutants have reduced grain and stover yield (Lee and Brewbaker, 1984). In a comprehensive study on the effect of the *bm* mutation on *bm* sudan grass, Casler et al. (2003) showed that the *bm* phenotype reduced forage yield by 15 – 30 %. Reduction in forage yield was partly due to reduced ground cover as a result of reduced tillering capability of the *bm* lines.

### Chemical Analysis

To investigate the chemical basis of the mutation, chemical analytical methods were used that enabled the extraction and analysis of the pigment in the midrib, high-throughput leaf screening with NIR spectroscopy, tissue staining experiments and to look for differences between wild-type and *bm* mutant plants. The objective of this study was to determine if the *bm*

mutation had an effect on agronomic developmental traits. We also wanted to investigate any cell wall related chemical differences between the wild-type and *bm* mutant plants.

## Materials and Methods

### Plant Material

The novel *bm* mutant sources 05-06 PRWN 301S, 05-06 PRWN 302ST1, 05-06PRWN 303ST3, 05-06 PRWN 299ST1 and their wild-type counterparts NSF04 360S1, NSF04 324S1, NSF04 309S1, NSF04 309S2 were grown in the field at the University of Florida's Plant Science Research and Education Unit (PSREU) in Citra, FL in the fall of 2006 and again in the spring of 2007. Four replicates of 18 seeds of each genotype were planted in rows. Seeds were planted 30 cm apart.

### Flowering Time

After six weeks, the plants were observed daily for anthesis. Flowering time (emergence of pollen-shedding anthers from the tassel) and silking time (emergence of first silks from the ear) were recorded daily at midday. At physiological maturity, each plant's height was measured and recorded.

### Klason Lignin Content

Because of the heterogeneity of lignin structure, and also because lignins are covalently linked with cell wall carbohydrates, proteins, phenolics, or other compounds, all lignin concentrations are purely empirical. The available analytical procedure for the quantitative determination of lignin fall in two basic categories: those that remove all cell wall constituents except for lignin and quantify it gravimetrically, and those that oxidize the lignin polymer and quantify it spectrophotometrically. In this study, the former was used.

Six *bm* and six wild-type plant stalks were collected at physiological maturity and placed in a $50^0$C drier for one week. The bottom three internodes were ground into a powder to pass

through a 1-mm screen and collected in plastic 50-ml Falcon tubes. All samples were then cleaned in 80% (v/v) ethanol recovered by filtration through a Glass Microfiber Filter GF/A 55mm filter paper (Whatman®, England, UK) and dried at $50^0$C for 48 hours.

Klason lignin content was quantified gravimetrically as the ash-corrected residue remaining after total hydrolysis of cell wall polysaccharides (Hatfield et al., 1994). One hundred mg of the cleaned and dried sample was weighed into a 20 × 125 mm Pyrex glass tube containing five glass beads. The tube was capped with a Teflon-coated screw cap. The samples underwent primary hydrolysis in 1.5 ml 12M $H_2SO_4$ on ice for 30 minutes. The samples were then incubated in a shaker at $30^0$C for two hours, diluted with 9.75 ml of $ddH_2O$ and autoclaved at $120^0$C for one hour for secondary hydrolysis. Samples were cooled and filtered through a glass filter disc placed on a Büchner funnel. The Pyrex glass tube was rinsed three times with 5-ml $60^0$C warm distilled water to make sure that all small pieces of residue were collected on the filter disc. The filter discs where then oven dried at $50^o$C for 48 hours, weighed and then ashed in an Isotemp® Muffle Furnace (Fisher Scientific, Pittsburgh, PA) at $500^0$C for five hours before being carefully reweighed. Klason lignin was calculated as the weight before ashing corrected for the weight after ashing. T-test analysis was performed using Excel.

**Lignin Sub-unit Composition**

Pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS) has been used to characterize the composition in the cell wall of the *bm* mutants in corn (Vermerris and Boon, 2001). Lignins can be pyrolized reproducibly to produce a mixture of relatively simple phenols that result from the cleavage of the ether and carbon-carbon (C-C) bonds (Ralph, 1991). Pyrolysis of the samples results in the formation of a vapor containing fragments that are separated by gas chromatography and identified with mass spectrometry. For lignin, the

substitution pattern of the phenolic ring is typically retained, but only limited information about the inter-unit bonds is obtained.

Midribs from wild-type and *bm* mutant leaf samples were dissected from fully expanded leaves and cut into 3-mm pieces. The fresh pieces were soaked in deionized water for 30 minutes twice, to remove water soluble compounds, then in acetone for one hour twice to remove unbound phenolics (Vermerris and Boon, 2001). Acetone was discarded and the samples rinsed and left to dry in the hood.

Dried samples were analyzed with Py-GC-MS. One milligram midrib samples were placed in a small cup and injected into the Py-GC-MS (Varian 3800 GC, 1200 MS). The samples were separated on a capillary column which was inserted in the pyrolysis outlet set at $325^0$C. Helium gas (1.2ml/min) was used as the carrier gas. For the analysis of midrib samples the GC program is shown in Table 3-1 and Table 3-2. Pyrograms were acquired and analyzed using Varian workstation software (Varian Inc., CA). Qualitative analysis of pyrograms from mutants and their wild-type plants were made by looking for differences in the peaks.

**Wiesner Reaction**

The Wiesner reagent is a 1% (w/v) solution of phloroglucinol dissolved in a 3:1 mixture of ethanol and concentrated HCl (Vermerris and Nicholson, 2006). This reagent reacts with cinnamaldehyde end groups in the lignin, forming a red cationic chromophore. This procedure, which is commonly known as the Wiesner reaction, is also used as a general stain for lignin. Phloroglucinol staining is probably the most common stain used because of its ease of use. The Wiesner reaction has also been used to compare lignin content and composition.

Wild-type and *bm* mutant plants were grown in the field and the stalks were harvested at the tassel emergency (VE) stage. The stalks were cut off from the base and placed in a bucket with water. The sixth internode of each plant was selected for analysis. The stem was sectioned

with a razor blade and the sections were incubated in five drops of 1% (w/v) phloroglucinol in ethanol: concentrated HCl (3:1). After five minutes, the drops were removed and the section was rinsed with distilled water. The sections were viewed on a Leica MZ12.5 stereomicroscope (Leica Microsystems, IL) with a camera attached.

**Enzymatic Saccharification**

In order to evaluate if the coloration of the midrib phenotype of the novel *bm* mutant had an impact on the extent of hydrolysis of cellulose in maize stover, enzymatic saccharification experiments were performed.

The term 'cellulase' refers to a mix of three classes of enzymes that together hydrolyze cellulose to glucose. The cellulases produced by the wit-rot fungus *Trichoderrna reesei* consist of endoglucanase (E.C. 3.2.1.4), cellobiohydrolase (E.C.3.2.1.91), and β-glucosidase (E.C.3.2.1.21) (Gum et al., 1976). The combined synergistic actions of these three enzymes in the cellulase preparation completely hydrolyze cellulose to D-glucose. Lignin interferes with the access of the enzyme complex to the cellulose, probably due to their coating of the cellulose fibers (Boudet, 2003). Furthermore, lignin itself can bind cellulase enzymes thereby rendering them inactive or less effective for digesting cellulose (Berlin et al., 2006).

**Cellulase Assays**

Cellulases are a mixture of enzymes that act synergistically (Mandels et al., 1976). The rates of enzyme reaction are affected by type of substrate. This includes chain length and crystallinity of the cellulose as well as by physical or chemical pretreatments. In order to be able to measure enzyme productivity there was need for an enzyme unit that would be used to predict sugar output. In addition, the enzyme unit would allow comparison of cellulase activities from different sources as well as allow comparison between various systems used in different laboratories. Mandels et al. (1976) proposed a unit of cellulase activity based on the hydrolysis

of a Whatman No. 1 filter paper. They recognized that for quantitative results, the enzyme unit must be based on a fixed percent conversion: a four percent conversion of 50 mg of filter paper unit, which required hydrolysis of some of the crystalline cellulose and not only the loose amorphous portions of the substrate that are easily and quickly hydrolyzed.

Cellulase was assayed using a 50-mg Whatman No. 1 filter paper following the National Renewable Energy Laboratory (NREL) in Golden , CO analytical procedure LAP 006 (http://www.nrel.gov/biomass/analytical_procedures.html#LAP-006). This method describes a procedure for measurement of cellulase activity using International Union of Pure and Applied Chemistry (IUPAC) guidelines. The procedure has been designed to measure cellulase activity in terms of "filter-paper units" (FPU) per milliliter of original (undiluted) enzyme solution. For quantitative results the enzyme preparations must be compared on the basis of significant and equal conversion. The value of 2.0 mg of reducing sugar as glucose from 50 mg of filter paper (4% conversion) in 60 minutes has been designated as the intercept for calculating filter paper cellulase units (FPU) by IUPAC (Ghose, 1987).

An enzyme mix (Table 3-3) containing the two cellulase enzymes was prepared into six different dilutions resulting in different enzyme concentrations which were then used to hydrolyze the Whatman filter paper. After 60-minute incubation, glucose readings from the hydrolysis of the Whatman paper in tubes containing the different enzyme concentrations were recorded. A calibration standard curve was constructed using the actual glucose readings and the measured glucose readings. The standard curve was used to determine the amount of glucose released from each sample tube after subtraction of enzyme blank. Corrected or calculated values were acquired by substituting the measured glucose readings into the equation from the standard curve. The corrected value was converted from mg/dl to mg (glucose yield) by multiplying by

1.5 (1.5 ml total volume) and dividing by 100. From these data, the enzyme dilution is plotted along the y-axis on a logarithmic scale and the glucose yield along x-axis on a regular scale. The two point reflecting readings closest to 2-mg are connected and the enzyme concentration that would yield 2-mg is recorded. Using the filter paper activity formula, the enzyme concentration that yielded 2-mg glucose was used to calculate the filter paper units (Mandels et al., 1987; Ghose, 1987).

Six stalks from wild-type plants and six from *bm* mutant plants were harvested at grain harvesting stage. The stalks were placed into a $50^0$C drier for one week before the bottom three internodes were collected and ground into a fine powder. The ground tissue was cleaned with 80% warm ethanol to remove salts, soluble sugars, small molecules such as hormones and other metabolites before being air-dried in a $50^0$C oven. A 300-mg stover sample was weighed into a 50-ml polypropylene tube. Forty six filter paper units (FPU) of an enzyme cocktail consisting of sodium citrate buffer, distilled water, cellulase enzymes sodium azide were added to each sample and the tubes were vortexed and placed on ice (Table 3-3).

Celluclast 1.5L (Sigma, St Louis, MO), is a cellulase preparation obtained from *Trichoderma reesei.* Novozyme 188 (Sigma, St Louis, MO) is a cellobiase preparation obtained from the fungus *Aspergillus niger.* A 0.5 ml aliquot was taken immediately after the enzyme cocktail was added to the stover samples labeled $t_0$, boiled for 5 minutes in a waterbath, and stored at $-20^0$C. The tubes containing the remainder of the samples were incubated in a shaker operating at 200 rpm at $50^0$C. After exactly 24 hours, the samples were placed on ice, and a 1-ml aliquot was collected labeled $t_{24}$ and boiled in a waterbath. In random order the glucose concentration of the samples was measured using the OneTouch® Ultrasmart® blood glucose

meter. Each sample had three measurements taken before they were averaged. T-test analysis was done using Excel.

**Carotenoid Extraction**

Using a mortar and pestle, frozen midrib tissue from six *bm* plants and six wild-type plants was ground into a fine powder and 0.8 – 1.2 g were weighed into screw-capped 15 ml polypropylene tubes. The tubes and their contents were kept frozen in a container with liquid $N_2$ at all times. Working under low light throughout the whole experiment, the weighed sample was suspended in 10 ml of methanol, vortexed and centrifuged at 5,000 g for 15 minutes at $4^0$C in a 15 ml Polypropylene tube. The supernatant was transferred to a clean 50-ml Polypropylene tube to which 1 ml 60 % (w/v) KOH was added. The mixture was heated at $65^0$C for 20 minutes in a water-bath. Carotenoids were extracted by vortexing the mixture three times in 10% (v/v) diethyl ether in hexane. The organic phase was evaporated under nitrogen to leave an oily residue at the bottom of the tubes which was dissolved in 700 µl methanol. The extracted carotenoids were stored in the dark in $-20^0$C freezer.

For analysis, 100 µl from the six replicates of each genotype (*bm* and wild type) were aliquoted randomly into individual wells in a 96-well plate. A third row was filled with methanol as the control. Carotenoid absorbance readings were measured at 450 nm using a Spectra Max M5 (Molecular Devices, CA) plate reader. Data acquisition and analysis were performed using the Soft Max Pro software v5 (Molecular Devices, CA). Carotenoid samples were also analyzed using the GC-MS. Three micro liters of a carotenoid sample were injected into the GC-MS where separation of carotenoid compounds is based on retention on the column (Table 3-4 and 3-5).

**Thin Layer Chromatography**

Carotenoid samples (100 µl) were applied to silica gel 60-Å, 20 × 20 cm TLC plates (Whatman, Scheicher & Schuell, England, UK) using a pasteur pipette. The application was done in a way that left sharp concentrated spots evenly separated from each other and 1.5-cm from the bottom of the TLC plate. A distance of 1-cm was left between the plate's edge and the first sample spot. Three *bm* and three wild-type samples were analyzed, with one *bm* mutant sample lined up next to a wild-type sample. The TLC plate was run in a 250 ml mixture of 40:10:10 (v/v/v) petroleum ether (PE), diethyl ether (DE) and acetone for 4-5 cm in about 5 minutes.

**Flavonoid Extraction**

Flavonoid biosynthesis has a number of branch pathways that result in nine major subgroups: chalcones, aurones, isoflavonoids, flavones, flavonols, flavondiols, anthocyanins, condensed tannins and phlobaphenes pigments (Winkel-Shirley, 2001). A number of these sub-groups are yellow, orange, red or brown. In order to investigate if the orangish-brown pigment in the *bm* mutant is a flavonoid, we used a flavonoid extraction protocol on the ground-midrib samples.

One gram of the ground powder from three midrib samples from wild-type and three samples from mutant leaves was washed in 10 ml of acetone by vortexing before removing the supernatant. The residue was then washed in 10 ml 95% (w/v) ethanol as in the previous step. Five ml 0.1 N HCl in methanol was added to the remaining residue and left to soak overnight. After 24 hours the color of the pellet and the solution were observed to see if any pigments had been extracted (Figure 3-7).

## Results

When viewed under a compound microscope, the novel *bm* mutant is characterized by the accumulation of an orange pigment in the sclerenchyma cells (Figure 3-8). Sclerenchyma cells are thick, often lignified secondary walls. Functionally, sclerenchyma is the strengthening and supporting element in plant parts. They are located adjacent to the vascular bundle and spongy parenchyma. The orange coloration is restricted only to the sclerenchyma tissue. In contrast, the wild-type sclerenchyma is cream in color (Figure 3-8) and the cells are clearly distinct from the vascular tissue cells as well as the spongy parenchyma cells.

## Agronomic Traits

Wild-type and *bm* mutant plants were grown in fall 2006 and summer 2007. In both seasons, the *bm* plants were shorter than the wild-type controls. Figures 3-1A and 3-1B show that the mean height of the *bm* mutant is statistically different from the wild-type control. In fall 2006 the average height of the wild-type and *bm* mutant plants was 133± SE and 108± SE cm respectively. The differences were also observed in summer 2007 when the average height for the wild-type was 154± SE cm while that of the mutant was 142± SE cm.

Figure 3-2 shows that time to flowering (as measured by the time to anthesis) between the *bm* mutant and the wild-type was significantly different in fall 2006. The mutant flowered earlier by four days. However, in summer 2007 there were no significant differences in time to flowering between the wild-type controls and *bm* mutant. This suggests a seasonal effect on time to flowering. There were no differences in time to silking in both seasons for the two genotypes (Figure 3-3). This suggests that the mutation of the *bm* gene has no effect on time to silking.

## Chemical Analysis

### Wiesner Staining

Following incubation of the stem section samples with the Wiesner reagent, the wild-type and *bm* samples were stained with the same intensity in the vascular tissues. The results indicate equal levels of total lignin content and levels of coniferaldehyde end groups in the two genotypes (Figure 3-4). This result also confirms the pyrolysis-gas chromatography mass-spectrometry outcome, which detects no differences in sub-unit composition.

### Enzymatic Saccharification

Stover samples that were collected from wild-type and mutant plants at physiological maturity were hydrolyzed with a cocktail of cellulase enzymes (Table 3-3). Glucose levels were measured using a OneTouch® Ultrasmart® (LifeScan, Inc. Milpitas, CA) blood glucose meter and both stover samples yielded equal amounts of glucose (Figure 3-5).

### Carotenoid Extraction

Midrib samples from both the wild-type and mutant samples were collected and ground with a mortar and pestle into a powder using liquid nitrogen. Carotenoids were extracted following a protocol by Wurtzel et al. (2001). The optical density (OD) of the carotenoid samples was measured (Table 3-6) using a spectrophotometer and there were no significant differences between the carotenoid samples from the wild-type and mutant samples. Carotenoids were extracted on three different occasions but the outcome remained the same (Table 3-1).

Three microliters of the carotenoid samples were also injected into a GC-MS and separation of the compounds was based on the retention on the column. Comparison of the chromatograms from wild-type and mutant carotenoid samples did not show any differences.

Thin layer chromatography (TLC) is a technique used to separate chemical compounds into individual components based on their solubility in the solvent and their affinity for the

matrix. In order to investigate if the carotenoids extracted from the *bm* mutant and wild-type midribs were different in composition, TLC was performed. One hundred microliter carotenoid samples from both genotypes were separated on a 60-Å, 20×20 $cm^2$ silica gel TLC plate. Following separation samples from the two genotypes did not show any differences in composition or intensity.

### Klason Lignin

Klason lignin analysis is a gravimetric procedure that dissolves all cell wall components except lignin. The procedure's outcome revealed that the stover samples from the wild-type and mutant plants did not have significant differences in the total lignin content (Figure 3-6). This suggests that the *bm* mutation does not have an effect on the Klason lignin content. The results further confirm the outcome from the stem section staining with the Wiesner reagent.

### Pyrolysis-Gas Chromatography-Mass Spectrometry

Wild-type and mutant dissected midribs that are three to five millimeters in length were injected into the Py-GC-MS. Pyrograms obtained from the wild-type and mutant samples were compared and showed no apparent differences in the composition of the midrib samples.

### Near Infrared Reflectance Spectroscopy

Near infrared reflectance spectra were acquired from dried wild-type and *bm* mutant leaf samples. The data were analyzed with WinDAS software. Based on NIR data, the mutant appears to have an abundance of proteins (1528 nm, 2061 nm) and carbohydrates (1702 nm, 2379 nm).

### Efforts to Clone the *Bm* Gene

*Mu*-TAIL PCR products were cloned into a pCR4-TOPO vector and 192 colonies were selected for sequencing at the UF-ICBR genome core facility. One hundred and ninety two single-pass sequence reads from randomly selected *Mu*-TAIL clones in the *bm* library were

obtained. Candidate clones were identified by performing in silico subtraction against a large collection of *Mu*-TAIL sequences by assembling all sequences in the data set into contigs using the PHRAP program (Ewing and Green, 1998; Ewing et al., 1998). Out of the 192 *bm Mu*-TAIL clones, a total of 19 contigs were obtained. Of these, only six contigs were unique to the *bm* library. In addition, 15 unique singletons were identified giving a total of 21 unique candidates for the causative insertion in the *bm* gene.

The 21 unique *Mu*-TAIL clones were analyzed by BLASTN search (Altschul et al., 1990) to extract all available maize sequences that matched the *Mu*-TAIL clone sequences. Co-segregation analysis of a segregating set of plants was performed using *Mu*-TAIL clone-specific primers and a *Mu* terminal inverted repeat (TIR)-specific primer. PCR products were expected from DNA of heterezygotes as well as the homozygous *bm* mutant individuals. None of the 21 *bm* candidate gene sequences co-segregated with the *bm* phenotype.

In a second round of trying to clone the *Bm* gene, the 192 *Mu*-TAIL PCR single- pass sequence reads were filtered through a large collection of private and public *Mu*-TAIL databases. Forty-two unique candidates were identified and these were analyzed by BLASTN search to extract all available maize sequences that matched the *Mu*-TAIL clone sequences, as before. These sequences were used to design *Mu*-TAIL clone-specific primers which were used together with TIR-specific primers to perform co-segregation analysis as described before. None of the 42 candidates co-segregated with the *bm* phenotype

Table 3-1. Py-GC-MS program used to analyze midrib samples

| Rate (°C/min) | Hold (min) | Total (min) |
|---|---|---|
| | 0.25 | 0.25 |
| 200 | 0.5 | 2.6 |

Table 3-2. Py-GC-MS oven program used to analyze midrib samples

| Rate (°C/min) | Hold (min) | Total (min) |
|---|---|---|
| | 3.5 | 3.5 |
| 5 | 0 | 15.5 |
| 4 | 0 | 43 |
| 20 | 1.75 | 50 |

Table 3-3. Cellulase enzyme cocktail

| Reagent | Volume (ml) per tube | |
|---|---|---|
| Sodium citrate (pH 4.5) | 5 | 50mM final conc. |
| $ddH_20$ | 4.47 | |
| Novozyme 188 | 0.065 | 46 FPU |
| Celluclast 1.5L | 0.065 | |
| Sodium azide ($NaN_3$) | 0.1 | 10% |

Table 3-4. GC-MS program used to analyze carotenoid samples

| Rate (°C/min) | Hold (min) | Total (min) |
|---|---|---|
| | 0.5 | 0.5 |
| 100 | 1 | 1.7 |
| 200 | 0.5 | 3.04 |

Table 3-5. GC-MS oven program used to analyze carotenoid samples

| Temperature | Rate (°C/min) | Hold (min) | Total (min) |
|---|---|---|---|
| | | 4 | 4 |
| 180 | 40 | 0 | 7.25 |
| 250 | 5 | 0 | 21.25 |
| 300 | 20 | 0.25 | 24 |

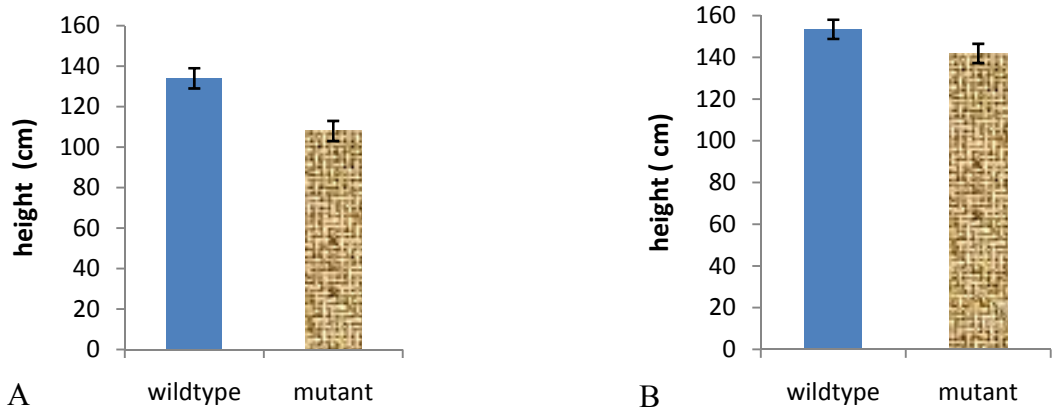



Figure 3-1. Differences in plant height between the *bm* mutant and wild-type in A) Fall 2006 (p-value 0.002) and B) Summer 2007 (p-value = 0.008). Wild-type and mutant plant heights were measured at physiological maturity and averaged.

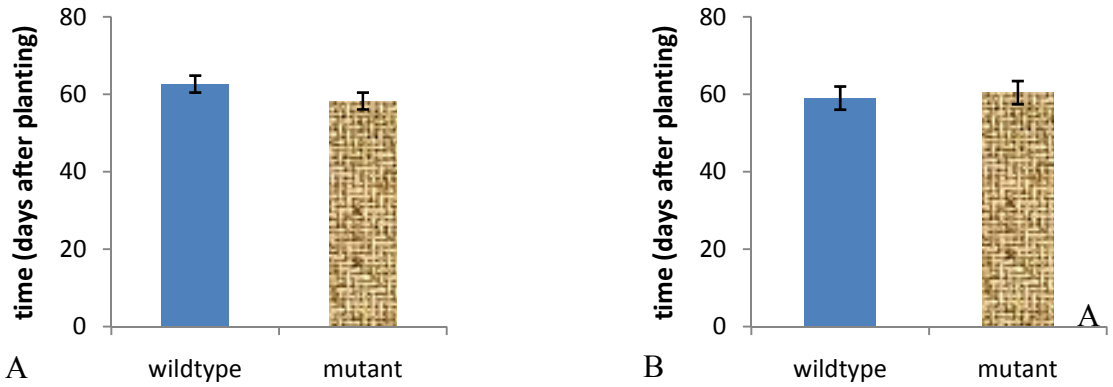



Figure 3-2. Time to flowering, measured as days after planting (averaged across 4 replications), for the wild-type and mutant plants in A) Fall 2006 (p-value = 0.002) and B) summer 2007 (p-value = 0.08).

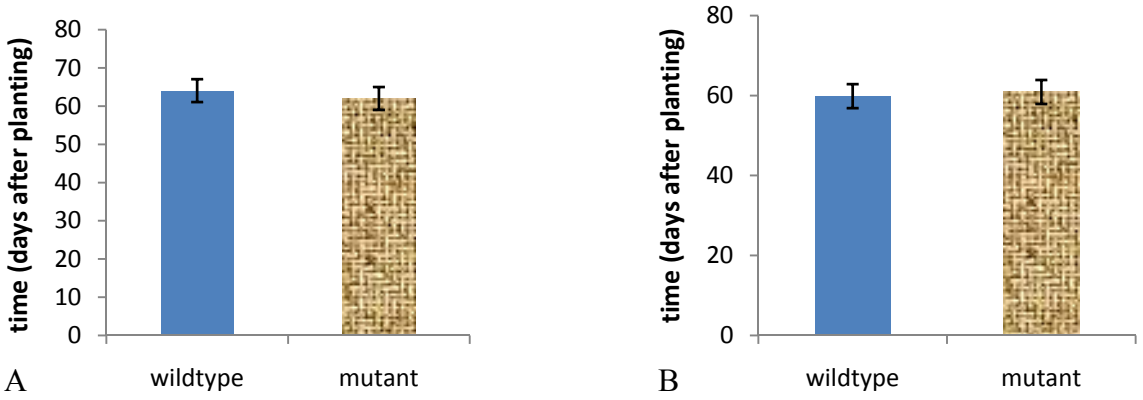



Figure 3-3. Time to silking, measured as days after planting (averaged across 4 replications), for the wild-type and mutant plants in A) Fall 2006 and B) Summer 2007.

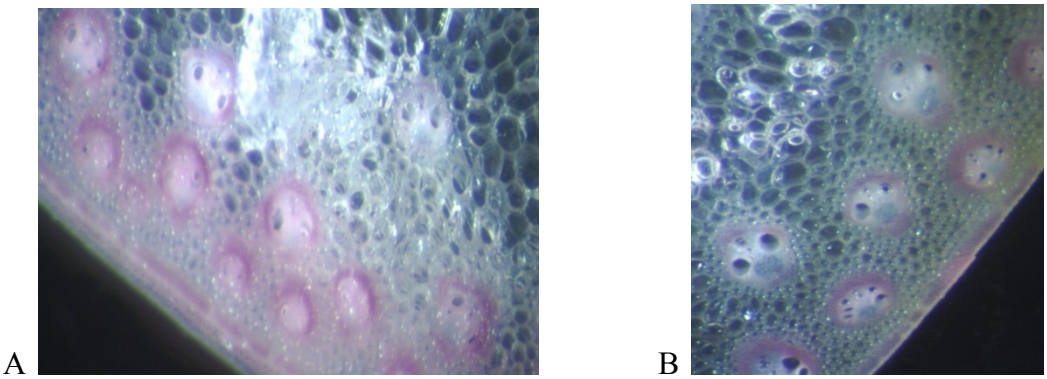



Figure 3-4. Maize stem sections after staining with the Wiesner reagent. A) *bm* mutant plant B) wild-type.

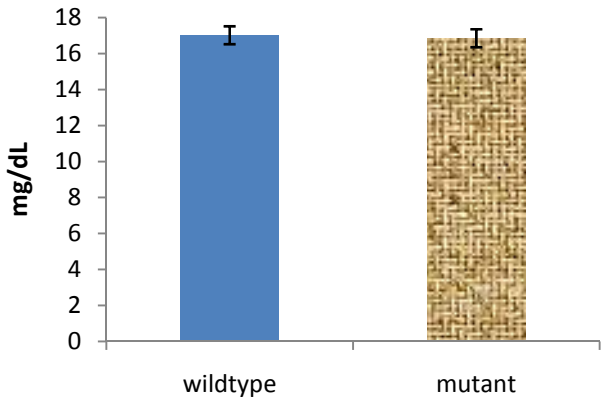



Figure 3-5. Glucose yields (mg glucose /g DW stover) following hydrolysis of wild-type and *bm* mutant stover samples (p-value = 0.85).

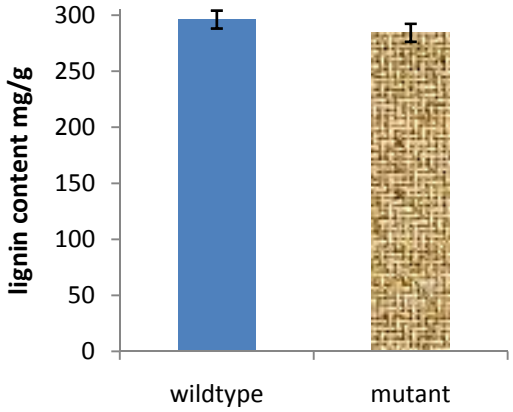



Figure 3-6. Klason lignin content comparison between wild-type and mutant plants averaged across six replications (p-value = 0.57).

Table 3-6. Spectrophotometer absorbance readings of carotenoids extracted from wild-type and *bm* mutant midrib samples

| | wild-type | mutant |
|---|---|---|
| Mean | 1.2 | 1.1 |
| Standard deviation | 0.3 | 0.4 |

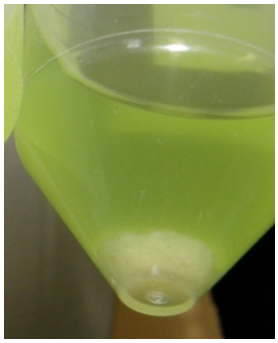
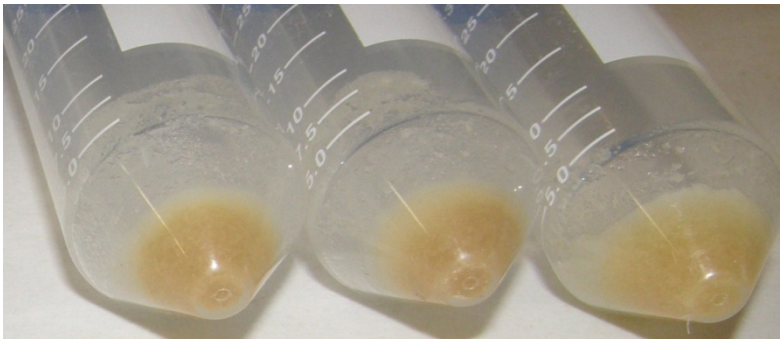
A  B 

Figure 3-7. The residue of the *bm* and wild-type samples after 24 hours of flavonoid extraction. The *bm* samples B) still retain their orangish-brown pigment compared to wild-type samples A).

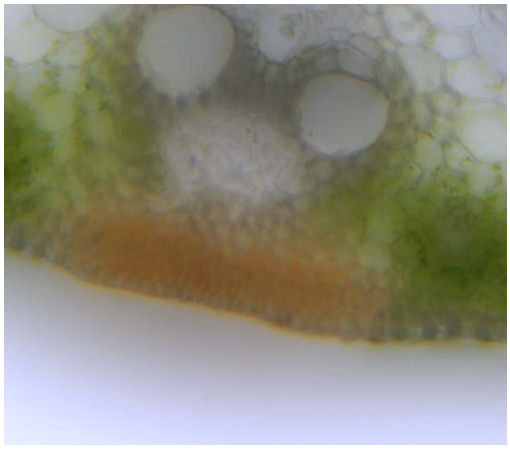
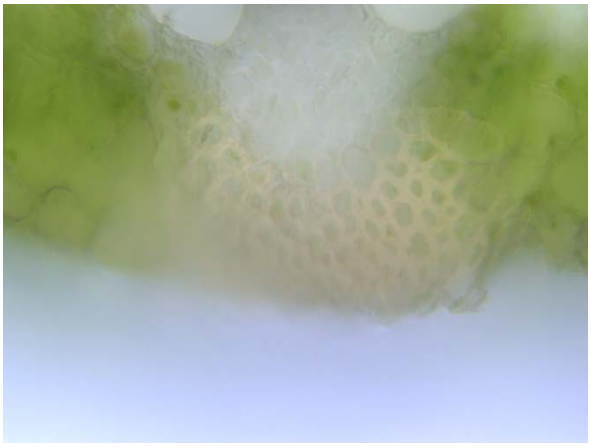
A  B 

Figure 3-8. Midrib sections from 60 day old maize plants grown in a greenhouse. The sections were viewed under a compound microscope (20 ×) showing that the *bm* mutant A) accumulates the orange-brown pigment in the sclerenchyma. B) Wild-type sections show only faint cream coloration in the sclerenchyma cells.

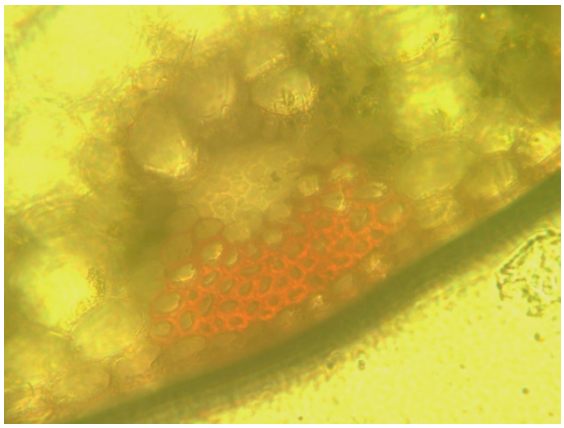
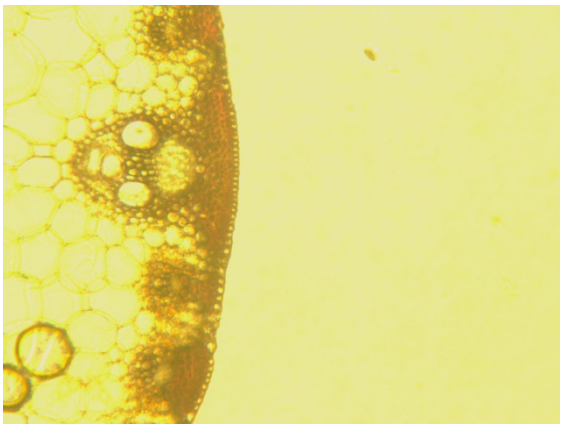
C  D 

Figure 3-9. Midrib stem section C) stained with Wiesner reagent and then viewed under a compound microscope (magnification 40×). D) Dark staining surrounding the vascular tissue and the sclerenchyma confirms the presence of lignin in these tissues.

CHAPTER 4
EFFORTS TO CLONE THE *BM* GENE

## Introduction

Since our *brown midrib* mutant was isolated from a transposon-active, mutagenic population, there is a high probability that a *Mutator (Mu)* element is inserted in the causal gene. Such an insertion will enable identification of the *brown midrib* gene using *Mu*-TAIL PCR (TAIL stands for Thermal Asymmetric InterLaced). *Mu*-TAIL PCR (Table 4-1), produces a population of fragments, each with one end anchored in the terminally inverted repeat (TIR) of the element and the other end at an arbitrary site in the flanking DNA. The *Mu*-Tail PCR products can either be directly sequenced using a *Mu*-anchored 454 protocol, or cloned into a TOPO vector (Invitrogen, Carlsbad, CA) and the resulting microlibrary sequenced.

## DNA Extraction

*Mu*-TAIL PCR amplifies genomic DNA flanking maize Robertson's *Mutator* insertions. DNA was extracted from 100-mg frozen leaf tissues of a *bm* mutant plant using the DNeasy Plant Mini kit (Qiagen, Carlsbad, CA) DNA extraction protocol. The eluted DNA (200 µl) was collected in a 1.5 ml Epperndorf tube and stored at - $20^0$C.

## *Mu*-TAIL PCR

A mutant *bm* DNA sample was used undiluted in the first of two PCR reactions. One microliter of ~50ng/µl DNA was added to a 20 µl primary *Mu*-TAIL PCR reaction. The primary reaction was composed of 1 U native *Taq* polymerase, (Invitrogen, Carlsbad, CA), 10× PCR buffer consisting of 20 mM Tris-HCl pH 8.4, 50 mM KCl, 2 mM $MgCl_2$, 200µM dNTPs, 100 nM primer TIR6, 1 µM arbitrary primer. Twelve arbitrary primers (AP) were used. Water-only negative controls from the primary reaction were diluted and processed identically to reactions containing DNA templates. During the set up of the PCR, all samples were kept cold at all times.

The following order worked efficiently: water (negative control) no-template (NT) PCR tubes placed in a PCR tube cooler. Master mix was prepared then DNA template added to template (T) PCR tubes followed by arbitrary primer (AP) (Table 4-3) addition to NT and T tubes. Tubes were capped, vortexed and spun down while pre-heating the thermal cycler block (Table 4-1). After the primary reaction, all samples were kept cold (4°C). It was imperative to proceed to the secondary reaction on the same day. The secondary *Mu*-TAIL reaction was identical to the primary except that the TIR8 nested primer was used instead of the TIR6 (Table 4-2). *Mu*-TAIL thermocycling conditions were also different (Table 4-1). TIR8 primer is composed of TIR8.1, TIR8.2, TIR8.3 and TIR8.4 primers mixed in a 2:4:4:1 ratio, respectively, to adjust for the degeneracy of each primer synthesis (Settles et al., 2004). PCR products from the primary reaction were used as the templates for the secondary reaction, but after they had been diluted 100 times in double distilled water. PCR products were run on a 1.2% agarose gel in 0.5× TBE for 70 minutes at 100 volts (Figure 4-1).

**Sequencing**

The products from the twelve reactions were divided into two groups of six, based on the strength of their intensity on the gel. From each of the AP products, 15 µl were pooled to make a total of 90 µl. The products were size-selected using a Sephacryl-400 (Promega, Madison, WI) spin column following manufacturer's instructions. Size selection was done to limit sequencing reactions to products above 500 bp. Twenty microliters of the pooled sample were added to the center of the column and spun in a centrifuge at 2800 rpm. The size-selected product was collected in the eluate. The two pooled groups resulted in two-size-selected products which were labeled size-selected-1 and -2 (SS1 and SS2).

## TOPO Cloning Reaction

Following size selection, 8-µl of the eluate was run on a gel to confirm if the size selection had succeeded (reduction in products less than 500 bp). After confirmation, the size selected products were mixed with a TOPO cloning reaction shown in the table below. The vector used was pCR2.1 (Invitrogen, Carlsbad, CA). After mixing, the reaction was mixed gently by stirring with a pipette tip. The reaction was incubated in a PCR machine at 23°C for 30 minutes and then placed on ice.

## Transformation

After incubation on ice 3-µl of the TOPO cloning reaction was added to a vial of One Shot® Electrocompetent *E. coli* (Invitrogen, Carlsbad, CA) and mixed gently with a pipette tip. The mixture was incubated on ice for 10 minutes, heat shocked at 42°C for 30 seconds and immediately transferred to ice. This was followed by addition of 250 µl of room temperature S.O.C medium (Invitrogen, Carlsbad, CA). The vial was tightly capped and placed in a shaker at 37°C, 200 rpm for 30 minutes. The cells were spread on LB agar medium containing 35 µl 50mg/ml X-gal, 20 µl 100mM IPTG and 20 µl ampicilin. Eight sterile glass beads were used to spread the cells on three 100mm petri dishes each containing 10, 50 and 200 µl of the reaction respectively. Petri dishes were incubated overnight at 37°C. White colonies were used to inoculate liquid LB (50 µg/ml) with ampicillin in two 96-well plates which were incubated at 37°C before being sent for sequencing. Sequencing reactions were performed at the University of Florida's Interdisciplinary Center for Biotechnology Research (ICBR) using a Applied Biosystems Model 3130 Genetic analyzer. The T7 primer was used for all sequencing reactions.

## *In Silico* Subtraction

The sequencing exercise returned 192 *Mu*-TAIL sequences from the *Mu*-TAIL clone library. In order to identify candidate clones for the *bm* mutant, *in silico* subtraction was performed against a large collection of parental and sibling *Mu*-TAIL sequences. *In silico* selection compared the *bm* mutant *Mu*-TAIL sequences against a collection of known parental sequences to identify unique *Mu*-TAIL sequences and discard the parental sequences which are presumably not the cause of the mutation (Table 4-4). In a second stage of analysis, the 42 unique *Mu*-TAIL clones were analyzed by BLASTN search of public and private databases to extract all available maize genome sequence and EST that matched the *Mu*-TAIL sequences.

## Co-segregation Analysis

Primers were designed from the maize genomic sequences extracted from the BLASTN (Altschul, 1997) searches of public and private databases (Table 4-5). Depending on genomic sequence length, upper and lower primers that flank the *Mu* insertion site were designed and these were tested on DNA extracted from a segregating population. Twenty one *bm* DNA samples and 19 wild-type DNA samples were tested.

To confirm the presence of a *Mu* insertion in a gene that is causing the *bm* phenotype, PCR products were expected from 21 *bm* mutant individuals and wild-type heterozygotes but not from homozygote wildtypes if we use a TIR8 primer and an upper/lower primer.

Table 4-1. Thermal cycler programs used for *Mu*-TAIL PCR

| TAIL 1 | | | TAIL 2 | | |
|---|---|---|---|---|---|
| Cycle # | Temp (ºCelsius) | Time | Cycle # | Temp (ºCelsius) | Time |
| 1 | 95 | 2 min | 1 | 95 | 2 min |
| 2 | 94 | 30 sec | 2 | 94 | 10 sec |
| 3 | 67 | 1 min | 3 | 64 | 1 min |
| 4 | 72 | 2:30 | 4 | 72 | 2:30 |
| 5 | Go to 2 | 4 times | 5 | 94 | 10 sec |
| 6 | 94 | 30 sec | 6 | 64 | 1 min |
| 7 | 25 | 3 min | 7 | 72 | 2:30 |
| 8 | 72 | 2:30 | 8 | 94 | 10 sec |
| 9 | 25-72 (Ramp to 72) | @ 0.3 degree/sec | 9 | 44 | 1 min |
| 10 | 94 | 10 sec | 10 | 72 | 2:30 |
| 11 | 67 | 1 min | 11 | Go to 2 | 11 times |
| 12 | 72 | 2:30 | 12 | 72 | 5 min |
| 13 | 94 | 10 sec | 13 | 4 | forever |
| 14 | 67 | 1 min | | | |
| 15 | 72 | 2:30 | | | |
| 16 | 94 | 10 sec | | | |
| 17 | 44 | 1 min | | | |
| 18 | 72 | 2:30 | | | |
| 19 | Go to 9 | 14 times | | | |
| 20 | 72 | 5 min | | | |
| 21 | 4 | Forever | | | |

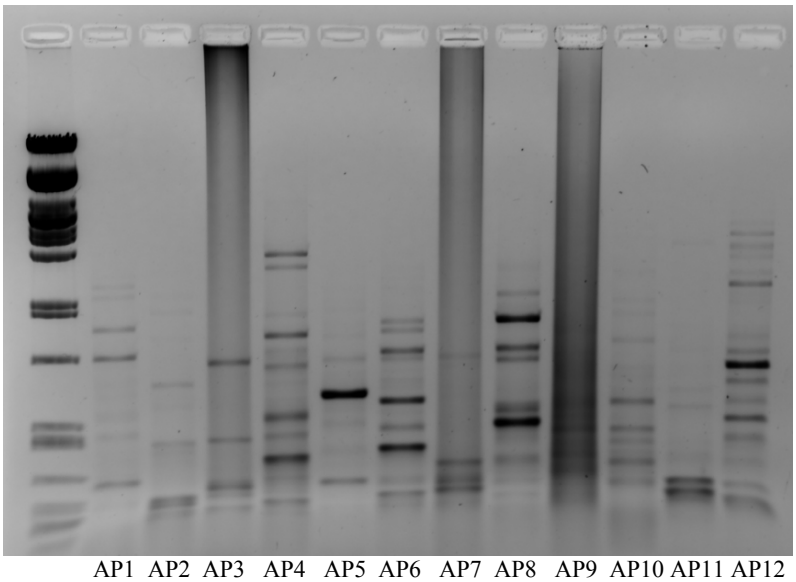



Figure 4-1. Agarose gel image of *Mu*-TAIL PCR products amplified using *Mu*-TIR6 and nested TIR8 primer and 12 arbitrary primers.

Table 4-2. Nested *Mu*-TIR primers used to amplify *Mu*-adjacent sequences

| Primer | Sequence (5' – 3') |
|---|---|
| TIR6 | AGAGAAGCCAACGCCAWCGCCTCYATTTCGTC |
| TIR8.1 | CGCCTCCATTTCGTCGAATCCCCTS |
| TIR8.2 | CGCCTCCATTTCGTCGAATCCSCTT |
| TIR8.3 | SGCCTCCATTTCGTCGAATCCCKT |
| TIR8.4 | CGCCTCCATTTCGTCGAATCACCTC |

Table 4-3. Arbitrary primers used in combination with *Mu*-TIR nested primers in *Mu*-TAIL PCR

| Primer | Sequence (5' – 3') |
|---|---|
| cggc1 | GVCTYCGWSSGC |
| SAD11 | NTCAGSTWTSGWGWT |
| SW41 | AGWGHAGSAHCADAAS |
| BAD5 | WTCCASNTGSNACG |
| DRM-CG2 | GCNGNWCGWCGWG |
| CST1 | GTANTCGWAWNCST |
| CTG1 | GWWGGTSCWASWCTG |
| AMS2-GAG3 | GWSIDRAMSCTGCTC |
| geeky1 | GKYKGCKGCNGC |
| DRM-AG1 | GNGWSASTNGAGC |
| BAD8 | GTGASNTGSWATGG |
| DRM-NC1 | GSCNCSGWNCC |

Table 4-4. Unique *Mu*-TAIL sequence types of the *bm* mutant obtained from *in silico* subtraction

| Type of sequence | Quantity |
|---|---|
| Representative read with TIR | 8 |
| Representative read with no TIR | 12 |
| Unplaced with TIR | 22 |
| Total | 42 |

Table 4-5. Co-segregation primers

| Name | Sequence |
|---|---|
| CONTIG16_U346 | CAACCTTGTGCAGAAGTACAAGTTCCA |
| CONTIG16_U700 | GCACCCCTTAAGTACGCATTAGTGTGT |
| CONTIG3_U168 | CTACAAGTATACAGCAGACTCCAAG |
| CONTIG3_U363 | GCATAATACCTGCTGGGCACCA |
| CONTIG4_U2189 | TGATACGGCGGAGGTGAAGACTTC |
| CONTIG5_U155 | GCAGACCTGGGTAACATGATCC |
| CONTIG5_U230 | GCTCTGGGCTACTCCATACCAA |
| CONTIG5_U823 | CTGATTCTGCTAACTGCCGCTT |
| CONTIG6_L1294 | TCAAGTGCCCAGTACCAGAAGAAG |
| CONTIG6_U862 | AGGACGCCGACCATTTGGTTAGTG |
| SS1_A06-U1-22 | GTGTCAGTTGAGCAAGTTTAGGAC |
| SS1_A09-U1-23 | GGGTCAGTTGAGCAAGTTTAGGA |
| SS1_A12-L789 | TCCGCCACTGATTTCAGGTTCT |
| SS1_A12-L789 | TCCGCCACTGATTTCAGGTTCT |
| SS1_A12-U330 | TCGCCTCCGCATTCTAGGGTTT |
| SS1_A12-U330 | TCGCCTCCGCATTCTAGGGTTT |
| SS1_B02-L1167 | GCGGCTGCGAGCTGAGGTTA |
| SS1_B02-U583 | GGGCCAAAATTGCTCAGCCCA |
| SS1_C05-U1-24 | GTGACACTTGAGCACATTGGATTC |
| SS1_C05-U693-22 | AAGTGGCGACAGCACGAGTGAT |
| SS1_C08-U1-22 | GTGCTCCATTTCCTCTAATCCC |
| SS1_C08-U37-26 | CATAATGGCAATGATCTCGTTCATGC |
| SS1_C12-L645 | CGGCCTAATCAAGTCAAGGTACTCC |
| SS1_C12-U1-22 | GACGGAACCCTGCTCAATGACA |
| SS1_C12-U222-23 | TACGCTTGTAACTGTGCCAGATC |
| SS1_C12-U381 | GGGGCGAGCATACCTGCTGGG |
| SS1_D01-U580 | TTGCGTAAGAGTGATGAGACATAAAT |
| SS1_D02-U182-22 | GCTCTGGGCTACTCCATACCAA |
| SS1_D06-U13-24 | CGCATCAATGGGTTCTCAGAAGCT |
| SS1_D06-U176-24 | TGCTGCCGTAGCTGGTATGTAATG |
| SS1_D08-U290 | GCACGGGCACTTAGCTAGTTAAGG |
| SS1_DO2-U618-23 | TTCGGATGTAGTTGAGCGGGATA |
| SS1_E07-U347-24 | GGAATTGTTCGTGACACCAAGGAC |
| SS1_E07-U588-24 | TAGGAAAGATGTGTAGATCGGTGG |
| SS1_E08-L570 | GCGGGAGGGCACCTTGTCAAAGG |
| SS1_E08-U229 | GCCGGACACAGTAGCACGGAGGT |
| SS1_E08-U229 | GCCGGACACAGTAGCACGGAGGT |
| SS1_E10-U1-22 | GAGTCAGTTGAGCGGGATAAGG |
| SS1_E10-U54-24 | TGTCCTGTTGCCAGTGTCACACTAG |
| SS1_F03-U1-24 | GTGTCAGTTGAGCAAGTTTAGGAC |

Table 4-5. Continued

| Name | Sequence |
|---|---|
| SS1_F03-U75-22 | ATTATGGACGAAGAGGGAAGCG |
| SS1_F06-L849-26 | AATGACGTGGCTCTACGGAATCACAG |
| SS1_F06-U191-23 | GTGTTGGATGTGGTGATTTGCTG |
| SS1_F06-U225-25 | GCATGTAAGTGTACTGAGTTCCTTC |
| SS1_F06-U447-28 | AGTACGCATTAGTGTGTACATACGGTAG |
| SS1_F06-U849-25 | CTGTGATTCCGTAGAGCCACGTCAT |
| SS1_F07_FB | GGAAGCGGCGGGTAGGTTCTTAGTAC |
| SS1_F07_RB | CCGATTGAAGGGCACCGCCTATCAATTA |
| SS1_F07_U71 | TGCGTATCCATAACGGAGAAACC |
| SS1_F07-L1171-23 | TTCGGGTGCTCTAATTGATAGGC |
| SS1_F07-L1344-24 | GGAATCAAGAACCGTCTGCTGGAA |
| SS1_F07-L143-24 | TAGCGGTCTTGTTCAACGTGGTCT |
| SS1_F07-U187 | TCAAAGGGACCGAGATTCATGGG |
| SS1_F07-U206-24 | CAAGAAGCCTAACGGTCGAGTAAG |
| SS1_F07-U284-22 | AGTGATGGGATAGCCACATGGA |
| SS1_F07-U558-24 | AGATCCTGAGCCCTATGAACAACT |
| SS1_F08-U200-23 | CCAAGTCAAGTGAGGATTCACCG |
| SS1_F08-U674-22 | TTGTCCTGTTGCCAGTGTCACA |
| SS1_F11-U | GGAAGCGGATTCGACGAAATGGAG |
| SS1_F11-U4-23 | TCAGTTGAGCGGGATAAGGAGAA |
| SS1_F12-U11-28 | GCATTAGTACCAATTGTTGACTCTAGCC |
| SS1_F12-U1-25 | GGGTCAGTCGAGCATTAGTACCAAT |
| SS1_G03-L926 | CTTGCCCAGCATGTCAGAGAGA |
| SS1_G03-L926 | CTTGCCCAGCATGTCAGAGAGA |
| SS1_G03-U475 | ATGACGGCAGTCCTACAATCAG |
| SS1_G03-U475 | ATGACGGCAGTCCTACAATCAG |
| SS1_G03-U66 | TACGAGCGGAGCAAGAACCAC |
| SS1_G03-U87 | CCACTACCAGGAACAGCACAT |
| SS1_G04-U29-22 | GTTGCGTTGTGGAGCCCAACTA |
| SS1_G04-U66-25 | AGACCGTCGGGTTCCACTAGTTTTA |
| SS1_G05-U177-25 | GCTGCCGTAGCTGGTATGTAATGAC |
| SS1_G05-U91-27 | GGGAATACACAAAGGCTCCTTGAATTG |
| SS1_G09_FA | CTGCAGAGAAGCAGCAGGCATCGG |
| SS1_G09_FB | GTCGCGTTTCCAAACACTCCGACGACTAGG |
| SS1_G09_RA | CTAGTCACAATCCGAAGTGCTGTGCTGTGC |
| SS1_G09_RB | ATCGGAACCTGTCCTAGAGCAAGTAGCAGC |
| SS1_G09_RC | GTGTAGTAGGTCAGACGCCTGCTAATGCC |
| SS1_G09-L1175 | TGGATCGGAACCTGTCCTAGAG |
| SS1_G09-L1175 | TGGATCGGAACCTGTCCTAGAG |
| SS1_G09-L175 | CCGACTGATCCATCCGCACAAG |

Table 4-5. Continued

| Name | Sequence |
|---|---|
| SS1_G09-L291 | CCAATCTAGAGGAGGATTAGAGAGG |
| SS1_G09-L416 | TCTAGTCACAATCCGAAGTGCTGT |
| SS1_G09-L425 | CATGTCATGTCTAGTCACAATCCG |
| SS1_G09-L565 | GAGTACAGTGTAGTAGGTCAGAC |
| SS1_G09-U491 | GAACGGGGAAACACACCTTTATTG |
| SS1_G09-U491 | GAACGGGGAAACACACCTTTATTG |
| SS1_G09-U60 | GAGATAGATAGTTAGGTTTAGCCC |
| SS1_G12-U1-22 | GGGTCAGTTGAGCAAGTTTAGG |
| SS1_G12-U39-24 | TGGGTTCACATTCCTGTTTCAAGG |
| SS1_H11-U377 | TCTTCTTGGGGTTGGCAGCCAG |
| SS1_H12-U231-22 | AGGATTATCACCCCAGGTTCTC |
| SS1_H12-U311-24 | CAGCAAGTCGAGCTCTAGCATGAG |
| SS2_A06-L1564 | AGGTGGTGGTGGAGATGGAGT |
| SS2_A06-L1564 | AGGTGGTGGTGGAGATGGAGT |
| SS2_A06-U952 | GGCACCGCAAGCAGTAAAAACA |
| SS2_A06-U952 | GGCACCGCAAGCAGTAAAAACA |
| SS2_A08-L2045 | CCCCTTAGCATTGAGACTACAAA |
| SS2_A08-L2045 | CCCCTTAGCATTGAGACTACAAA |
| SS2_A08-U1251 | ATCGCAACATCACAAGGCTAACC |
| SS2_A08-U1251 | ATCGCAACATCACAAGGCTAACC |
| SS2_A09 _FA | TACGGTGGCACGGTGCGTATGT |
| SS2_A09_RA | CACAGCTGGCGAACCAAACACCT |
| SS2_A09-L1267-22 | CTGATGAGTTAGACATGAGTGC |
| SS2_A09-L1322-24 | TTGGACCTAGTAGCAGCAGTCATG |
| SS2_A09-L1355-24 | AGAGTGCTCTACACGATAGAGACT |
| SS2_A09-U1 | GAGCAGCAACATAACCACAAAAAACTA |
| SS2_A09-U119 | ACTGCCTATCTCATCGAGCACCTC |
| SS2_A09-U1-24 | CAATGTTGATGTGGGACTTCTCTT |
| SS2_A09-U25 | CTAAGAGAATGGAGACGCAGTAG |
| SS2_A09-U659-23 | ACAGAAGACCGAATCACTTCAGG |
| SS2_A09-U693-26 | AACAGAGCACAGCTTTGAACCACTGC |
| SS2_A11-U130 | AATACCAGTTTCCCCTTTTACGATGC |
| SS2_B02-L1457-23 | CTGGCTACTGGAGCAAATACCTC |
| SS2_B02-U173-23 | TTGTTGGTGGATGATCTGAGCAG |
| SS2_B02-U385-22 | CCGTACAGTCCTCAGCAGAATG |
| SS2_B02-U526-22 | AGGTGCCCAACCAAGAGTGTAG |
| SS2_B02-U64 | ACACGTCCAGAGGTAGAAGAGGCT |
| SS2_B04-L581 | GGCGATCTTAACTCTTCACTAGAA |
| SS2_B04-L581 | GGCGATCTTAACTCTTCACTAGAA |
| SS2_B04-U170 | TCGGCATATCTATACTCTCGTG |

Table 4-5. Continued

| Name | Sequence |
|---|---|
| SS2_B06-L435-22 | ATCACTCGTGCTGTCGCTACTT |
| SS2_B06-U1-22 | GAGCAGCACCATAACCAACTTAATG |
| SS2_B06-U162-22 | ATAGATGACCCTCCACAGACCT |
| SS2_B06-U520 | CGGGCTGCTGGACTGGACCAAATT |
| SS2_B09-U1925 | CGCCGAGCTGCGTCTGTAAAATC |
| SS2_B09-U482 | ATTGCCCGCTTCACCTCCATACG |
| SS2_C07-L218 | CTAACTGTACCGTGCGGATGAGAG |
| SS2_C07-U1 | GCTGCTGCTGAGGTCGATCAC |
| SS2_C11-L4339 | GCTCAGGCATCACCTAATGTGTA |
| SS2_C11-L4339 | GCTCAGGCATCACCTAATGTGTA |
| SS2_C11-U3448 | GACTTCTCCATCCTTGAAATCC |
| SS2_C11-U3448 | GACTTCTCCATCCTTGAAATCC |
| SS2_C12-U765 | CGGGGTATTTTCTCGATATTTTCTTG |
| SS2_D01-U266 | CCACGTTTATTTACTTTCACTTTCAC |
| SS2_D01-U470 | TATTGCGGTGTTGGTTCTACAATGTC |
| SS2_D02-L536-24 | GTGAAGGACCATAAGGCAGATAAG |
| SS2_D02-U186-23 | TGGCAGGGGCATCCATTTCATCT |
| SS2_D02-U368-26 | TTTGCAGGCTCCCTATCCTACATAGC |
| SS2_D02-U426-24 | CTTATAACGTACTGCCGAGAGCGC |
| SS2_D02-U477-25 | CAGCTTCACTACAGCCATGCTCTTC |
| SS2_D09-L1463-24 | CTAGTACCAGCTTATCGCCAACCG |
| SS2_D09-U1094-23 | CACAATCGGTCCAACAAGTCAAC |
| SS2_D09-U205 | ACAATCGGTCCAACAAGTCAACTG |
| SS2_D09-U514-23 | GACCAGTGGCTCTATACAGATTG |
| SS2_E01-L752 | CGCCTCGTCCTTCTCGTCCAGG |
| SS2_E01-U223 | TGGCAAGCCGGACACAGTAGCACG |
| SS2_F02-L1270-23 | CCAACCTACCACGAGCATACTTC |
| SS2_F02-U540-24 | GCATTGATCACGAGGTGAGGTCTC |
| SS2_F02-U691-22 | ATGGGCTCCAACACGATACACG |
| SS2_G06-L4344 | TGGGCAGGGGGTCAACCTATAT |
| SS2_G06-L4620 | ATCATAAGACAGGGCAACGATAAAAA |
| SS2_G06-U3976 | CTGACGCTGCGGTTGTTTCTTG |
| SS2_G06-U4120 | CTCTCTTCCCCGTTCCTCTGTA |
| SS2_G12-L1310-25 | AACATCTTCCTCGCACCAATAACTC |
| SS2_G12-U646-23 | CAAACCGATGAACGACGACACTG |
| SS2_H05-L1234-23 | GGGTACTAGGCTAGAAAGAGTTG |
| SS2_H05-U637-23 | GAGGAAGTGATCCCACCATGTTC |
| SS2_H05-U747-24 | CCACTCTGAGCCATACGTCAGCAT |
| SS2_H06-U318-23 | CCTAACCATACAGGTGACCTTGC |
| SS2_H06-U757-23 | AGGGCTGTTCAACAACATGATCG |

Table 4-5. Continued

| Name | Sequence |
|---|---|
| SS2_H06-U815-23 | TCGTTGGTGCCTTTGAGATTGAC |
| SS2_H09-U131-23 | AAACCAGCAGTTCACCACCTCAA |
| SS2_H09-U278-23 | CCCATCACTGAGCGTGTTCATAT |
| SS2_H09-U508-23 | TACAGGGCATGTTTCGGAAGAGG |
| SS2-B02_FA | ACTGTTGTAGCAGCAGCAAGGAGCATGCT |
| SS2-B02_FB | GACGTTCGGCTGCATCGTCTACGT |
| SS2-B02_RA | TCTGAGCACCTTGTGCTCTCTGCCAGTG |
| SS2-B02_RB | GGAAGATCTGCAAGTGTCCAGGTCTGG T |
| SS2-D09_FA | TAGACTTGTGCGTGCTCTGCCGCT |
| SS2-D09_FB | GGAATCGTCGAGTTCACTGGGAAGAATGGCG |
| SS2-D09_RA | AACTGCTGCCTCTACTCCGCCTACTTC |
| SS2-D09_RB | GCGACAGCTCAACCGAATGAGACATCTC |
| SS2-F02_FA | ATCCGGAGGCACGACCAGACCATCT |
| SS2-F02_RA | AGCTGGCGGCTGAGGTCATCATTCC |
| SS2-G12_FA | TAGAATGGCGAGGCCCGATCTTCC |
| SS2-G12_RA | GACAACACGAGCACGTGCACGACAGCTAA |
| SS2-H05_FA | TGCCGGCTAGTATGTGATGTCCTCTGAATG |
| SS2-H05_FA | AACAATGGCGACTGAGTGCTGAGGAAC |
| SS2-H05_FA | TGCCGGCTAGTATGTGATGTCCTCTGAATG |
| SS2-H05_RA_ | AAGGAGCCGATGAGGATGTCGACATCGA |
| SS2-H05_RB | TGGTAGACCTGTCCACCGAATTGGTGG |
| SS2-H06_FA | GTGCGTTGCATGTGGTCTGGTGGTAAC |
| SS2-H06_FB | CAGACGCGACATCCAGGAGCAGATGT |
| SS2-H06_RA | ATGCACACTGGAGGTCTAGAAGCGCTCAT |
| SS2-H06_RB | CATAGCTGGAGCAGCAGCAGGCTT |
| SS2-H09_FA | CAAGACGTTCGGCTGCATTGTCTATGTCC |
| SS2-H09_FB | TCATCTTCGACGAACAGGCTCAGTGGG |
| SS2-H09_RA | TATCCCTTCACTACCAGCCGTGCC |
| SS2-H09_RB | GAGGAAGATCCACAAGAGTCCAGGTCTGG |

# CHAPTER 5
# DISCUSSION

Results from several experiments have shown that the novel brown midrib mutant phenotype is different from the other known maize and sorghum brown midrib mutants. They show that the novel *bm* mutant does not have altered lignin content or modified lignin sub-unit composition compared to the wild-type controls. However, chemical tests, NIR spectroscopy and thermal analyses have been unable to identify the compound that is causing the orange-brown coloration. The brown coloration is, however, located in the sclerenchyma tissue (Figure 3-9). The most distinctive feature of sclerenchyma cells is their thickened and lignified primary cell walls (Raven et al., 1999).

Enzymatic saccharification experiments show that the *bm* mutant and the wild-type plants yield similar glucose amounts. The result suggests that the *bm* mutation does not impact the yield of glucose obtained after enzymatic saccharification. In contrast, the data from NIR analysis suggest that the mutant has an abundance of carbohydrates. Since cellulase enzymes were used in this experiment, the glucose yield was as a result of the hydrolysis of cellulose microfibrils. These data are, however, not conclusive because cellulase enzymes used in the enzymatic saccharification experiments only hydrolyze cellulose fibers and do not act on hemicelluloses and other carbohydrate sources. In order to get a complete picture, there is need to include hemicellulases in the enzyme cocktail to make sure that all carbohydrates are hydrolyzed to their individual monosaccharides.

Field trials revealed variation in plant height between controls and *bm* mutants, which provides evidence for a relationship between the mutated gene and plant development. The *bm* mutant plants were shorter than their wild-type counterparts when grown in field trials in two consecutive seasons: fall 2006 and summer 2007. Vermerris et al. (1999) showed that *bm1* and

*bm2* not only have an effect on lignin biosynthesis, but also on flowering dynamics and plant height. The height differences between wild-type and *bm* mutant plants suggest that the *bm* mutation has an effect on plant height. However, there were also significant height differences between the wild-type plants grown in fall 2006 and the wild-type plants grown in summer 2007. Fall 2006 wild-type plants were on average 21 cm shorter, across four replications, than the summer grown wild-type plants. Similarly, the fall 2006 *bm* mutant plants were on average 34 cm shorter, across four replications than the summer 2007 *bm* plants. The differences in heights among same genotypes between the seasons could be due to changes in photoperiod or field management. In fall the day length is shorter and, therefore, it is possible that both *bm* mutant and wild-type plants will be shorter compared to the summer crop. On the other hand, time to silking was not statistically different for the same genotypes between seasons, i.e. between fall 2006 and summer 2007. Since maize is a day-neutral plant, the day length differences in fall and summer were not expected to have any effect. The results from this study indicate that there is no effect from the *bm* on silking dynamics. However, while there were no differences in time to flowering in summer 2007, the plants that were grown in fall 2006 showed significant differences. It is possible that the mutant is sensitive to short days in the fall. However, more evidence would be required before drawing any conclusions based on these data.

It can be hypothesized that the reduced height of the *bm* mutant is due to the accumulation of the orange-brown compound in the sclerenchyma tissue in the midrib. Thus, the over-production of the orange-brown compound could be diverting crucial intermediates from a pathway that is vital for producing phytohormones (possibly gibberellins) involved in plant development. Fray et al. (1995) showed that constitutive expression of *phytoene synthase* in tomato resulted in the overproduction of phytoene synthase, which converts geranylgeranyl

diphosphate to phytoene and, thereby, diverts this intermediate away from the gibberellin and phytol biosynthetic pathways. As a result the tomato plants were dwarfs.

However, it is possible that the *bm* phenotype is caused by over-expression of the mutated gene, thereby causing the overproduction of the orange-brown pigment in the sclerenchyma cells. Careful examination of the wild-type intercellular space in the sclerenchyma shows that it is pink in color. We could, therefore, hypothesize that the wild-type gene codes for a protein that represses the production and accumulation of the orange-brown pigment in the sclerenchyma. It is also possible that *bm* is a dominant modifier of expression. Chopra et al. (2003) described a dominant factor named *Ufo1* (Styles et al., 1987) which modifies the organ-specific expression patterns of the *P1-wr* allele. *Ufo1* was originally identified because of its ability to induce orange-red pigmentation (phlobaphenes) in vegetative and floral tissues of maize plants, which normally do not accumulate significant amounts of phlobaphenes. Along the same lines, the phenotype of the *bm* mutant could also be due to a gain-of-function mutation whose gene product becomes constitutively active or gains a novel function normally not found in the wild-type protein. Lu et al. (2006) characterized the *orange* (*or)* gene mutation in cauliflower (*Brassica oleracea* var. *botrytis*) that confers the accumulation of high levels of β-carotene in various tissues normally devoid of carotenoids. The *or* gene mutation is due to the insertion of a long terminal repeat retrotransposon in the *Or* allele.

Since the *bm* mutant was identified from a transposon-active, mutagenic population, there is a high probability that a *Mu* element is inserted in the causal gene. Therefore, the assumption is that the insertion of the *Mu* element disrupts the normal transcription and expression of the gene.

While co-segregation analysis showed that none of the candidate genes segregate with the phenotype, there are several reasons to believe that *Mu*-TAIL PCR did not faithfully amplify all the *Mu*-flanking genomic sequences. According to Settles et al. (2004), *Mu*-TAIL PCR only samples 67-86% of the *Mu* insertion sites in the genomes they tested. This leaves a significant number of inserts potentially unsequenced. The major reason is due to the failure of the 12 arbitrary primers to amplify all maize genic sequences robustly (Settles et al., 2004). Studies showed that the 12 optimized arbitrary primers amplify 95.6% of the *Mu* insertion sites within a genome. These 12 arbitrary primers were designed from the 100 most over-represented sequences found in the maize genome. This potentially excludes 4.4% part of the genome in which *Mu*-adjacent sequences are not amplified. There has been more evidence that has since been accumulated that points to the bias of the arbitrary primers in *Mu*-TAIL PCR (Koch, personal communication). It is, therefore, likely that *Mu*-TAIL PCR might not have amplified all the *Mu* flanking genomic sequences in the *bm* mutant genome.

In addition, Settles et al. (2004) reported that there is a subset of maize gene sequences that appears to be resistant to *Mu*-TAIL analysis. For example, even though in this study they tested a large number of lines that contained the *bz1-mum9* locus, they did not identify the locus in the *Mu*-TAIL sequence libraries. This limitation is not only unique to *Mu*-TAIL analysis, but to all other flanking PCR methods. These include adapter-mediated PCR and inverse PCR methods.

Despite the afore-mentioned limitations, Suzuki et al. (2006) and Porch et al. (2006) successfully cloned the molybdenum cofactor biosynthetic protein gene (*Cnx1)* and the maize *viviparous15* gene (*Vp15)* using the *Mu*-TAIL PCR method. These results show that despite its limitations, *Mu*-TAIL PCR can be successfully used to clone genes where *Mu* has inserted.

Another explanation for the inability to identify a PCR product representing a *Mu*-flanking DNA fragment that co-segregated with the *bm* phenotype is that the mutation is a *Mutator*-suppressible phenotype. Martienssen et al. (1990) showed that the phenotypic effects of *Mu* element insertion sometimes depend on transposon activity. They described a maize mutant, *high chlorophyll fluorescence106 (hcf106),* which conditions a pale; non-photosynthetic phenotype caused by the insertion of a *Mu1* element in the 5′ untranslated region (UTR). The *hcf106* phenotype is only expressed when the *Mu* transposon system is active, where the insertion interferes with the accumulation of *Hcf106* mRNA. When the *Mu* is inactive, the mutant phenotype is suppressed, and plants homozygous for the *hcf106* mutation exhibit a normal phenotype. This is because when *Mu* becomes inactive, the promoter near the end of *Mu1* is activated and it directs transcription outward, into the adjacent *hcf106* gene (Barkan and Martienssen, 1991). They go on to suggest that *hcf106* is a prototype for what may be a frequent class of *Mu*-induced mutations whose phenotypes are modulated by the phase of *Mu* activity. In fact, several *Mutator*-suppressible phenotypes have been described and include maize *knotted1* gene (Greene et al. 1994), *rough sheath1* and *liguleless3* (Girard and Freeling, 2000) and *les28* (Martienssen and Baron, 1994). The mechanism by which phase of *Mu* activity regulates the *hcf106::Mu* promoter is not known. Barkan and Martienssen (1991) hypothesize that there is a factor that is only expressed when *Mu* is active that binds to the *Mu* termini and represses transcription. Since the *bm* mutant phenotype has never been recovered from plants grown from bronze kernels (which are presumed to be *Mu*-inactive) after the *bm* mutant was backcrossed to a color-converted purple W22, it can be hypothesized that orange-brown phenotype is dependent on *Mu*-activity. Consequently, co-segregation analysis would be affected. It will be hard to identify a co-segregating fragment, because a plant homozygous for a *Mu* insertion in the *Bm*

gene would have a wild-type phenotype if *Mu* became inactive, but would also generate a PCR product indicative of the mutation.

# CHAPTER 6
# FUTURE WORK

In order to circumvent the limitations associated with *Mu*-TAIL PCR and other PCR methods that are designed to amplify flanking DNA for that matter, a new method, 454-*Mu*-direct, has been suggested. *Mu*-direct is a highly efficient protocol for sequencing large numbers of germinal *Mu* insertion sites in the Uniform*Mu* population based on the massively-parallel 454 DNA sequencing platform developed by 454 Corp (Margulies et al., 2005) (http://currant.hos.ufl.edu/mutail/454/454_summary.htm). This method is currently being tested and the details are still to be released. It is expected that this new protocol will be able to significantly increase the chances of amplifying all *Mu*-flanking genomic sequences.

Enzymatic saccharification experiments showed that stover material from wild-type plants and *bm* mutant plants yielded equal glucose levels. This process only used a cocktail of cellulase enzymes that hydrolyzed cellulose microfibrils. However, NIR analysis results suggest that the mutant has an abundance of carbohydrates. It is, therefore, a worthy idea to carry out an experiment that hydrolyzes all types of carbohydrates from the wild-type and mutant samples. Saha and Cotta (2007) described an enzymatic hydrolysis protocol that contains cellulases, hemicellulases and xylanases. Such a protocol for maize would be able to give a detailed composition of stover material than the protocol used in our study.

There is a possibility that the mutation causing the *bm* phenotype was a spontaneous event. In that case map-based/positional cloning can be used to identify the causal gene. This method is, however, time consuming.

Microarray analysis can be used to identify up and down-regulated genes that can potentially explain the biological basis of the *bm* mutation. However, Shulze and Downward (2001) concluded that using microarray analysis to try and identify individual genes that are

differentially expressed has not been the most successful use of microarray technology. This is because a given stimulus or phenotypic differences could potentially lead to changes in the mRNA levels of hundreds of genes. However, these limitations can be minimized by careful experimental design. Nonetheless, changes in mRNA levels of hundreds of genes could provide some valuable information that if used in conjunction with information that is now known could provide important clues.

As hypothesized earlier, the *bm* mutant could be a *Mu*-suppressible mutant, whereby, the mutant phenotype is dependent on *Mu*-activity. In this case, the *bm* mutant phenotype will only show when *Mu* is active. However, when *Mu* is inactive, the mutant phenotype is suppressed and the plants homozygous for the *bm* mutation will exhibit a normal phenotype. To test if this is the case, co-segregation analysis results have to be analyzed and identify primers that resulted in amplification in all 21 *bm* individual plants. By selfing the normal plants, which could be homozygous (wild-type or mutant with inactive *Mu*) or heterozygotes, the color of the kernels will reveal whether the *bm* is a *Mu*-suppressible mutant or not. In addition, methylation experiments will have to be done to find out the methylation status of the gene in question. To change the phase of the *Mu* element the *bm* mutant will have to be back-crossed to a *Mu*-off line. If the *bm* mutant is a *Mu*-suppressible mutant phenotype, then it is expected that after backcrossing to a *Mu*-off line, it will no longer show the *bm* phenotype.

It is possible that there is a *Mu*-insertion in or near the *bronze (Bz)* locus that is causing the ectopic accumulation of the brown pigment in the sclerenchyma tissue. This hypothesis is based on an observation made by Rhoades (1952) that in *Bz* plants, no brown pigments are present and the anthocyanin is confined to the vacuoles. However, *bz* plants have deeply-colored, brown cell walls and greatly reduced amounts of anthocyanin in the vacuole. The *bm* mutant came out of a

Uniform*Mu* population that used the *bronze1* mutation as the marker gene and it ectopically accumulates a brown pigment in the cell walls of sclerenchyma cells. To test this hypothesis, upper and lower primers that flank the hypothetical *Mu*-element have to be designed. These will be used in combination with the *Mu*-TIR primer to test if the 21 individual *bm* mutant plants from a segregating population carry a *Mu*-insertion in the *Bz1* gene. PCR products will be expected from all the 21 *bm* mutant individuals if there is a *Mu*-insertion in or near the *bronze* locus but not from homozygous wild-type individuals.

LIST OF REFERENCES

Abbott, J.C., Barakate, A., Pinchon, G., Legrand, M., Lapierre, C., Mila, I., Schuch, W., Halpin, C., 2002. Simultaneous suppression of multiple genes by single transgenes: down-regulation of three unrelated lignin biosynthetic genes in tobacco. Plant Physiol. 128, 844-853.

Adler, E., 1977. Lignin chemistry: past, present and future. Wood Sci. Technol. 11, 169-218.

Altschul, S.F., Madden, T.L., Schafer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389-3402.

Analytical Spectral Devices, Inc. 2005. Introduction to NIR Technology. ASD Document 600510. (http://www.asdi.com/asd-600510_nir-introduction_revc.pdf ). April, 2008.

Anterola, A.M., Jeon, J.H., Davin, L.B., Lewis, N.G., 2002. Transcriptional control of monolignol biosynthesis in *Pinus taeda*: factors affecting monolignol ratios and carbon allocation in phenylpropanoid metabolism. J. Biol. Chem. 277, 18272-18280.

Anterola, A.M., Lewis, N.G., 2002. Trends in lignin modification: a comprehensive analysis of the effects of genetic manipulations/mutations on lignification and vascular integrity. Phytochemistry 61, 221-294.

Arioli, T., Peng, L., Betzner, A.S., Burn, J., Wittke, W., Camilleri, C., Höfte, H., Plazinski, J., Birch, R., 1998. Molecular analysis of cellulose biosynthesis in Arabidopsis. Science 279, 717-720.

Barièrre, Y., Ralph, J., Méchin, V., Guillaumie, S., Grabber, J.H., Argillier, O., Chabbert, B., Lapierre, C., 2004. Genetic and molecular basis of grass cell wall biosynthesis and degradability. II. Lessons from *brown midrib* mutants. C. R. Biol. 327, 847-860.

Barkan, A., Martienssen, R.A., 1991. Inactivation of maize transposon *Mu* suppresses a mutant phenotype by activating an outward-reading promoter near the end of *Mu1*. Proc. Natl. Acad. Sci. U.S.A. 88, 3502-3506.

Bate, N., Orr, J., Ni, W., Meroni, A., Nadler-Hassan, T., Doerner, P.W., Dixon, R.A., Lamb, C.J., Elkind, Y., 1994. Quantitative relationship between phenylalanine ammonia-lyase levels and phenylpropanoid accumulation in transgenic tobacco identifies a rate-determining step in natural product synthesis. Proc. Natl. Acad. Sci. U.S.A. 91, 7608-7612.

Baucher, M., Bernard-Vailhe, M.A., Chabbert, B., Besle, J.M., Opsomer, C., Van Montagu, M., Botterman, J., 1999. Down-regulation of *cinnamyl alcohol dehydrogenase* in transgenic alfalfa and the effect on lignin composition and digestibility. Plant Mol. Biol. 39, 437-447.

Baucher, M., Chabbert, B., Pilate, G., van Doorsselaere, J., Tollier, M.T., Petit-Conil, M., Cornu, D., Monties, B., Van Montagu, M., Inzé, D., Jouanin, L., Boerjan, W., 1996. Red xylem and higher lignin extractability by down-regulating a cinnamyl alcohol dehydrogenase in poplar (*Populus tremula x P. alba).* Plant Physiol. 112, 1479-1490.

Baucher, M., Halpin, C., Petit-Conil, M., Boerjan, W., 2003. Lignin: Genetic engineering and impact on pulping. Crit. Rev. Biochem. Mol. Biol. 38, 305-350.

Baucher, M., Monties, B., Van Montagu, M., Boerjan, W., 1998. Biosynthesis and genetic engineering of lignin. Crit. Rev. Plant Sci. 2, 125-197.

Bell-Lelong, D.A., Cusumano, J.C., Meyer, K., Chapple, C., 1997. Cinnamate-4-hydroxylase expression in Arabidopsis. Plant Physiol. 113, 729-738.

Bennetzen, J.L., Freeling, M., 1997. The unified grass genome: synergy in synteny. Genome Res. 7, 301-306.

Berlin, A., Balakshin, M., Gilkes, N., Kadla, J., Maximenko, V., Kubo, S., Saddler, J., 2006. Inhibition of cellulase, xylanase and β-glucosidase activities by softwood lignin preparations. J. Biotech. 125, 198-209.

Bieza, K., Lois, R., 2001. An Arabidopsis mutant tolerant to lethal ultraviolet-B levels shows constitutively elevated accumulation of flavonoids and other phenolics. Plant Physiol. 126, 1105-1115.

Biotechnology Industry Organization, 2006. Industrial biotechnology is revolutionizing the production of ethanol transportation fuel. (http://www.bio.org/ind/biofuel/CellulosicEthanolIssueBrief.pdf). April, 2008.

Bittinger, T.S., Cantrell, R.P., Axtell, J.D., 1981. Allelism tests of the *brown midrib* mutants of sorghum. J. Hered. 72, 147-148.

Boerjan, W., Ralph, J., Baucher, M., 2003. Lignin biosynthesis. Annu. Rev. Plant Biol. 54, 519-46.

Bomati, E.K., Joseph, P., Noel, J.P., 2005. Structural and kinetic basis for substrate selectivity in sinapyl alcohol dehydrogenase. Plant Cell 17, 1598-1611.

Boudet, A.M., 2000. Lignins and lignification: selected issues. Plant Physiol. Biochem. 38, 81-96.

Boudet, A.M., Kajita, S., Grima-Pettenati, J., Goffner, D., 2003. Lignins and lignocellulosics: a better control of synthesis for new and improved uses. Trends Plant Sci. 8, 576-581.

Bout, S., Vermerris, W., 2003. A candidate gene-approach to clone the sorghum *Brown midrib* gene encoding caffeic acid *O*-methyltransferase. Mol. Gen. Genet. 269, 205-214.

Brekke, K., 2005. The promise of cellulosic ethanol. Ethanol Today. (http://www.ethanol.org/documents/6-05_Cellulosic_Ethanol.pdf). pp 32-36. April, 2008.

Brown, J., Sundaresan, V., 1992. Genetic study of the loss and restoration of *Mutator* transposon activity in maize: evidence against dominant-negative regulator associated with loss of activity. Genetics 130, 889-898.

Brown, L., Torget, R., 1996. Enzymatic saccharification of lignocellulosic biomass. Chemical analysis and testing task – Laboratory Analytical Procedure 009. (http://www.nrel.gov/biomass/pdfs/9578.pdf). April, 2008.

Bucholtz, D.L., Cantrell, R.P., Axtell, J.D., Lechtenberg, V.L., 1980. Lignin biochemistry of normal and *brown midrib* mutant sorghum. J. Agric. Food Chem. 28, 1239-1241.

Buxton, D.R., Redfearn, D.D., 1997. Plant limitations to fiber digestion and utilization. J. Nutr. 127, 814S-818S.

Carpita, N.C., McCann, M., 2000. The cell wall. In: Buchanan, B.B., Gruissem, W., Jones, R.L., (Eds), Biochemistry and molecular biology of plants. John Wiley and Sons, Inc., Rockville, MD, pp. 1367.

Casler, M.D., Pedersen, J.F., Undersander, D.J., 2003. Forage yield and economic losses associated with the *brown midrib* trait in Sudan grass. Crop Sci. 43, 782-789.

Chabbert, B., Tollier, M.T., Schuch, W., 1994. Manipulation of lignin quality by down regulation of cinnamyl alcohol dehydrogenase. Plant J. 6, 339-350.

Chapple, C.C.S., Vogt, T., Ellis, B.E., Sommerville, C.R., 1992. An Arabidopsis mutant defective in the general phenypropanoid pathway. Plant Cell 4, 1413-1424.

Charlotte, S., 2006. Can biofuels finally take center stage? Nat. Biotechnol. 24, 777-784.

Chen, F., Dixon, R.A., 2007. Lignin modification improves fermentable sugar yields for biofuel production. Nat. Biotechnol. 25, 759-761.

Chen, L., Auh, C.K., Dowling, P., Bell J., Lehmann, D., Wang, Z., 2004. Transgenic down-regulation of caffeic acid *O*-methyltransferase (COMT) led to improved digestibility in tall fescue *(Festuca arundinacea)*. Func. Plant Biol. 31, 235-245.

Chen, M., Sanmiguel, P., deOliveira, A.C., Woo, S.S., Zhang, H., Wing, R.A., Bennetzen, J., 1997. Microlinearity of the *sh-2* homologous regions of the maize, rice and sorghum genomes. Proc. Natl. Acad. Sci. U.S.A. 94, 3431-3435.

Chen,Y., Sarkanen, S., 2003. Macromolecular lignin replication: A mechanistic working hypothesis. Phytochem. Rev. 2, 235-255.

Cherney, D.J.R., Patterson, J.A., Johnson, K.D., 1990. Digestibility and feeding value of pearl millet as influenced by the *brown midrib*, low-lignin traits. J. Anim. Sci. 68, 4345-4351.

Cherney, J.H., Axtell, J.D., Hassen, M.M., Anliker, K.S., 1988. Forage quality characterization of a chemically-induced *brown midrib* mutant in pearl millet. Crop Sci. 28, 783-787.

Cherney, J.H., Cherney, D.J.R., Akin, D.E., Axtell, J.D., 1991. Potential of *brown midrib*, low lignin mutants for improving forage quality. Adv. Crop Sci. 46, 157-198.

Cheung, F., Haas, B.J., Goldberg, S.M.D., May, G.D., Xiao, Y., Town, C.D., 2006. Sequencing Medicago truncatula expressed sequenced tags using 454 Life Sciences technology. BMC Genomics 7, 272.

Chopra, S., Cocciolone, S.M., Bushman, S., Sangar, V., McMullen, M.D., 2003. The maize Unstable factor for orange1 is a dominant epigenetic modifier of a tissue specifically silent allele of pericarp color1. Genetics 163, 1135-1146.

Coberly, L.C., Rausher, M.D., 2003. Analysis of a *chalcone synthase* mutant in *Ipomoea purpurea* reveals a novel function for flavonoids: amelioration of heat stress. Mol. Ecol. 12, 1113-1124.

Collazo, P., Montoliu, L., Puigdomenech, P., Rigau, J., 1992. Structure and expression of the lignin *O*-methyltransferase gene from *Zea mays* L. Plant Mol. Biol. 20, 857-867.

Crisp, P., Walkey, D.G.A., Bellman, E., Roberts, E., 1975. A mutation affecting curd color in cauliflower (*Brassica oleracea* L. *botrytis* DC). Euphytica 24, 173-176.

Datta, K., Baisakh, N., Oliver, N., Torrizo, L., Abrigo, E., Tan, J., Rai, M., Rehana, S., Al-Babili, S., Beyer, P., Potrykus, I., Datta, S.K., 2003. Bioengineered 'golden' indica rice cultivars with β-carotene metabolism in the endosperm with hygromycin and mannose selection systems. Plant Biotechnol. J. 1, 81-90.

Davies, K., 2004. Plant pigments and their manipulation. Oxford, CRC, Palmerston North, New Zealand. pp. 356.

Davin, L.B., Lewis, N.G., 2000. Dirigent proteins and dirigent sites explain the mystery of specificity of radical precursor coupling in lignan and lignin biosynthesis. Plant Physiol. 123, 453-461.

Davin, L.B., Lewis, N.G., 2005. Dirigent phenoxy radical coupling: advances and challenges. Curr. Opin. Biotechnol. 16, 398-406.

Davin, L.B., Lewis, N.G., 2005. Lignin primary structures and dirigent sites. Curr. Opin. Biotechnol. 16, 407-415.

Dean, J.F.D., 2005. Synthesis of lignin in transgenic and mutant plants. In: Steinbüchel, A., Doi, Y., (Eds), Biotechnology of biopolymers, from synthesis to patents. Wiley-VCH Verlag, Weinheim, Germany. pp. 4-25.

Degenhart, N.R., Werner, B.K., Burton, G.W., 1995. Forage yield and quality of a *brown midrib* mutant in pearl millet. Crop Sci. 35, 986-988.

Delgado-Vargas, F., Jiménez, A.R., Paredes-López, O., 2000. Natural pigments: carotenoids, anthocyanins, and betalains - characteristics, biosynthesis, processing, and stability. Crit. Rev. Food Sci. Nutr. 40, 173-289.

Donnison, I., Morris, P., 2003. *Mutator* maize – a new tool for functional genomics of grasses. Iger Innovations Newsletter Number 7 2003. (http://www.iger.bbsrc.ac.uk/Publications/Innovations/In2003/Ch1.pdf). April, 2008.

Ehlting, J., Buttner, D., Wang, Q., Douglas, C.J., Somssich, I.E., Kombrink, E., 1999. Three 4-coumarate:coenzyme A ligases in *Arabidopsis thaliana* represent two evolutionarily divergent classes in angiosperms. Plant J. 19, 9-20.

Elkind, Y., Edwards, R., Mavandad, M., Hedrick, S.A., Ribak, O., Dixon, R.A., Lamb, C.J., 1990. Abnormal plant development and down regulation of phenylpropanoid biosynthesis in transgenic tobacco containing a heterelogous *phenylalanine ammonia-lyase* gene. Proc. Natl. Acad. Sci. U.S.A. 87, 9057-9061.

Ewing, B., Green, P., 1998. Base-calling of automated sequencer traces using phred. II. Error possibilities. Genome Res. 8, 186-194.

Ewing, B., Hillier, L., Wendl, M.C., Green, P., 1998. Base-calling of automated sequencer traces using phred. I. Accuracy assessment. Genome Res. 8, 175-185.

Eyster, W.H., 1926. Chromosomes VIII in maize. Science 64, 22.

Fahrendorf, T., Dixon, R.A., 1993. Stress responses in alfalfa (*Medicago sativa* L.) XVIII: Molecular cloning and expression of the elicitor-inducible cinnamic acid 4-hydroxylase cytochrome P450. Arch. Biochem. Biophys. 2, 509-515.

Farrell, A.E., Plevin, R.J., Turner, B.T., Jones, A.D., O'Hare, M., Kammen, D.M., 2006. Ethanol can contribute to energy and environmental goals. Science 311, 506-508.

Franke, R., Humphreys, J.M., Hemm, M.R., Denault, J.W., Ruegger, M.O., Cusumano, J.C., Chapple, C., 2002. The Arabidopsis *REF8* gene encodes the 3-hydroxylase of phenylpropanoid metabolism. Plant J. 1, 33-45.

Fray, R.G., Wallace, A., Fraser, P.D., Valero, D., Hedden, P., Bramley, P.M., Grierson, D., 1995. Constitutive expression of a fruit *phytoene synthase* gene in transgenic tomatoes causes dwarfism by redirecting metabolites from the gibberellins pathway. Plant J. 8, 693-701.

Fry, S.C., 2003. Primary cell wall metabolism: tracking the careers of wall polymers in living plant cells. New Phytol. 161, 641-675.

Ghose, T.K., 1987. Measurement of cellulase activities. Pure Appl. Chem. 59, 257-268.

Ghosh, P., Ghose, T.K., 2003. Bioethanol in India: Recent past and emerging future. Adv. Biochem. Eng. Biotechnol. 85, 1-27.

Girard, L., Freeling, M., 2000. *Mutator*-suppressible mutants of *rough sheath1* and *liguleless3* in maize reveal multiple mechanisms for suppression. Genetics 154, 437-446.

Goujon, T., Ferret, V., Mila, I., Pollet, B., Ruel, K., Burlat, V., Joseleau, J.P., Barriere, Y., Lapierre, C., Jouanin, L., 2003. Down-regulation of the *AtCCR1* gene in *Arabidopsis thaliana*: effects on phenotype, lignins and cell wall degradability. Planta 217, 218-228.

Goujon, T., Sibout, R., Eudes, A., MacKay, J., Jouanin, L., 2003. Genes involved in the biosynthesis of lignin precursors in *Arabidopsis thaliana.* Plant Physiol. Biochem. 41, 677-687.

Grabber, J.H., Ralph, J., Lapierre, C., Barriére, Y., 2004. Genetic and molecular basis of grass cell-wall degradability. I. Lignin-cell wall matrix interactions. C. R. Biol. 327, 455-465.

Greene, B., Walko, R., Hake, S., 1994. *Mutator* insertions in an intron of the maize *knotted1* gene result in dominant suppressible mutations. Genetics 138, 1275-1285.

Grima-Pettenati, J., Goffner, D., 1999. Lignin genetic engineering revisited. Plant Sci. 145, 51-56.

Gross, G., 1985. Biosynthesis and metabolism of phenolic acids and monolignols. In: Higuchi, T., (Ed.), Biosynthesis and biodegradation of wood components, Academic Press, New York, NY. pp. 229-271.

Grotewold, E., 2006. The genetics and biochemistry of floral pigments. Annu. Rev. Plant Biol. 57, 761-80.

Guillaumie, S., Pichon, M., Martinant, J.P., Bosio, M., Goffner, D., Barriere, Y., 2007. Differential expression of phenylpropanoid and related genes in *bm1, bm2, bm3,* and *bm4* young near-isogenic maize plants. Planta 226, 235-250.

Gum, E.K., Brown, R.D., 1976. Structural characterization of a glycoprotein cellulase, 1,4-β-D-glucan cellobiohydrolase C from *Trichoderma viride*. Biochim. Biophys. Acta 446, 371-386.

Guo, D., Chen, F., Inoue, K., Blount, J.W., Dixon, R.A., 2001a. Down regulation of caffeic acid 3-*O*-methyltransferase and caffeoyl coenzyme A 3-*O*-methyltransferase in transgenic alfalfa: Impacts on lignin structure and implications for the biosynthesis of G and S lignin. Plant Cell 13, 73-88.

Halpin, C., Jan, K.H., Duncan, C., Bridgette, O., Monties, C.B., Edwards, K., Barakate, A., Foxon, G.A., 1998. *Brown midrib1* maize a mutation affecting the *cinnamyl alcohol dehydrogenase gene*. Plant J. 14, 545-553.

Halpin, C., Knight, M.E., Foxon, C.A., Campbell, M.M., Boudet, A.M., Boon, J.J., Chabbert B., Tollier, M.T., Schuch, W., 1994. Manipulation of lignin quality by down regulation of cinnamyl alcohol dehydrogenase. Plant J. 6, 339-350.

Hamelinck, C.N., van Hooijdonk, G., Faaij, A.P.C., 2005. Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle- and long-term. Bio. Bioenerg. 28, 384-410.

Hatfield, R.D., Jung, H.G., Ralph, J., Buxton, D.R., Weimer, P. J., 1994. A comparison of the insoluble residues produced by the Klason lignin and acid detergent lignin procedures. J. Sci. Food Agric. 65, 51-58.

Hatfield, R.D., Ralph, J., 1997. Should lignin be redefined? US Dairy Forage Research Centre Research summary. (http://www.dfrc.ars.usda.gov/Research_Summaries/RS97Index.html). April, 2008.

Hatfield, R.D., Vermerris, W., 2001. Lignin formation in plants. The dilemma of linkage specificity. Plant Physiol. 126, 1351-1357.

Hoffmann, L., Besseau, S., Geoffroy, P., Ritzenthaler, C., Meyer, D., Lapierre, C., Pollet, B., Legrand, M., 2004. Silencing of hydroxycinnamoyl-coenzyme A shikimate/quinate hydroxycinnamoyltransferase affects phenylpropanoid biosynthesis. Plant Cell 16, 1446-1465.

Hu, W.J., Kawaoka, A., Tsai, C.J., Lung, J., Osakabe, K., Ebinuma, H., Chiang, V.L., 1998. Compartmentalized expression of two structurally and functionally distinct *4-coumarate:Co A ligase* genes in Aspen (*Populus tremuloides*). Proc. Natl. Acad. Sci. U.S.A. 95, 5407-5412.

Humphreys, J.M., Hemm, M.R., Chapple, C., 1999. New routes for lignin biosynthesis defined by biochemical characterization of recombinant ferulate 5-hydroxylase, a multifunctional cytochrome P450-dependent monooxygenase. Proc. Natl. Acad. Sci. U.S.A. 96, 10045-10050.

Humphreys, M.J., Chapple, C., 2002. Rewriting the lignin roadmap. Curr. Opin. Plant Biol. 5, 224-229.

Jorgensen, L.R., 1931. *Brown midrib* in maize and its linkage relations. J. Am. Soc. Agron. 23, 549-557.

Kalton, R.R., 1988. Overview of the forage sorghums. In: Wilkinson, D., (Ed.), American Seed Trade Association Proceedings. 43rd Corn Sorghum Research Conference. 8–9 Dec. 1988, Chicago, IL. pp. 1-12.

Kemsley, E.K., 1998. Discriminant analysis and class modelling of spectroscopic data. John Wiley and Sons Ltd. West Sussex, England. pp. 179.

Kuc, J., Nelson, O.E., 1964. The abnormal lignins produced the *brown midrib* mutants of maize. Arch. Biochem. Biophys. 105, 103-113.

Kuc, J., Nelson, O.E., Flanagan, P., 1968. Degradation of abnormal lignins in the *brown midrib* mutants and double mutants of maize. Phytochemistry 7, 1435-1436.

Kühnl, T., Koch, U., Heller, W., Wellmann, E., 1989. Elicitor induced *S*-adenosyl-L-methionine: caffeoyl-coenzyme A 3-*O*-methyltransferase from carrot cell suspension cultures. Plant Sci. 60, 21-25.

Lee, D., Meyer, K., Chapple, C., Douglas, C.J., 1997. Antisense suppression of 4-coumarate:coenzyme A ligase activity in Arabidopsis leads to altered lignin subunit composition. Plant Cell 11, 1985-1998.

Lee, M.H., Brewbaker, J.L., 1984. Effects of *brown midrib3* on yields and yield components of maize. Crop Sci. 24, 105-108.

Lewis, N.G., Davin, L.B., 1998. The biochemical control of monolignol coupling and structure during lignan and lignin biosynthesis. In: Lewis, N.G., Sarkanen, S., (Eds), Lignin and Lignan Biosynthesis. ACS Symposium Series 697. American Chemical Society, Washington, DC. pp. 334-361.

Lewis, N.G., Davin, L.B., Sarkanan, S., 1999. The nature and function of lignins. In: Barton, D.H.R., Nakanishi, K., Meth-Cohn, O., (Eds), Comprehensive natural products chemistry, Volume 3, Pergamon, Oxford. pp. 617-745.

Li, L., Cheng, X.F., Leshkevich, J., Umezawa, T., Harding, S.A., Chiang, V.L., 2001. The last step of syringyl monolignol biosynthesis in angiosperms is regulated by a novel gene encoding sinapyl alcohol dehydrogenase. Plant Cell 13, 1567-1585.

Li, L., Lu, S., Cosman, K., Earle, E., Garvin, D.F., O'Neill, J., 2006. β-carotene accumulation induced by the cauliflower *Or* gene is not due to an increased capacity of biosynthesis. Phytochemistry 67, 1177-1184.

Li, L., Zhou, Y., Cheng, X., Sun, J., Marita, J.M., Ralph, J., Chiang, V.L., 2003. Combinatorial modification of multiple lignin traits in trees through multigene co-transformation. Proc. Natl. Acad. Sci. U.S.A. 100, 4939-4944.

Lourith, N., Katayama, T., Suzuki, T., 2005. Stereochemistry and biosynthesis of 8-*0*-4' neolignans in *Eucommia ulmoides*: diastereoselective formation of guaiacyglycerol-8-*0*-4'-(sinapyl alcohol) ether. J. Wood Sci. 51, 370-378.

Lu, S., Van, E.J., Zhou, X., Lopez, A.B., O'Halloran, D.M., Cosman, K.M., Conlin, B.J., Paolillo, D.J., Garvin, D.F., Vrebalov, J., Kochian, L.V., Küpper, H., Earle, E.D., Cao, J., Li, L., 2006. The cauliflower *Or* gene encodes a DnaJ cysteine-rich domain-containing protein that mediates high levels of β-carotene accumulation. Plant Cell 18, 3594-3605.

Maher, E.A., Bate, N.J., Ni, W., Elkind, Y., Dixon, R.A., Lamb, C.J., 1994. Increased disease susceptibility of transgenic of transgenic tobacco plants with suppressed levels of preformed phenylpropanoid products. Proc. Natl. Acad. Sci. U.S.A. 91, 7802-7806.

Mandels, M., Andreotti, R.E., Roche, C., 1976. Measurement of saccharifying cellulase. Biotechnol. Bioeng. Symp. 6, 21-23.

Margulies, M., Egholm, M., Altman, W.E., Attiya, S., Bader, J.S., Bemben, L.A., Berka, J., Braverman, M.S., Chen, Y.J., Chen, Z., Dewell, S.B., Du, L., Fierro, J.M., Gomes, X.V., Godwin, B.C., He, W., Helgesen, S., Ho, C.H., Irzyk, G.P., Jando, S.C., Alenquer, M.L.I., Jarvie, T.P., Jirage, K.B., Kim, J.B., Knight, J.R., Lanza, J.R., Leamon, J.H., Lefkowitz, S.M., Lei, M., Li, J., Lohman, K.L., Lu, H., Makhijani, V.B., McDade, K.E., McKenna, M.P., Myers, E.W., Nickerson, E., Nobile, J.R., Plant, R., Puc, B.P., Ronan, M.T., Roth, G.T., Sarkis, G.J., Simons, J.F., Simpson, J.W., Srinivasan, M., Tartaro K.R., Tomasz, A., Vogt, K.A., Volkmer, G.A., Wang, S.H., Wang, Y., Weiner, M.P., Yu, P., Begley, R.F., Rothberg, J.M., 2005. Genome sequencing in microfabricated high-density picolitre reactors. Nature 437, 376-380.

Marita, J.M., Vermerris, W., Ralph, J., Hatfield, R.D., 2003. Variations in the cell wall composition of maize *brown midrib* mutants. J. Agric. Chem. 51, 1313-1321.

Martienssen, R.A., Barkan, A., Taylor, W.C., Freeling, M., 1990. Somatically heritable switches in DNA modification of *Mu1* transposable elements in maize revealed by a suppressible mutant. Genes Dev. 4, 331-343.

Martienssen, R.A., Baron, A., 1994. Coordinate suppression of mutations caused by Robertson's *Mutator* transposon in maize. Genetics 136, 1157-1170.

McCarty, D.R., Settles, A.M., Suzuki, M., Tan, B.C., Latshaw, S., Porch, T., Robin, K., Baier, J., Avigne, W., Lai, J., Messing, J., Koch, K.E., Hannah, L.C., 2005. Steady-state transposon mutagenesis in inbred maize. Plant J. 44, 52-61.

McNeil, M., Darvil, A.G., Fry, S.C., Albersheim, P., 1984. Structure and function of the primary cell wall of plants. Ann. Rev. Biochem. 53, 625-63.

McNeil, M., Darvill, A,G., Albersheim, P., 1982. Structure of plant cell walls. Plant Physiol. 70, 1586-1591.

Mechin, V., Argillier, O., Rocher, F., Hebert, Y., Mila, I., Pollet, B., Barriere, Y., Lapierre, C., 2005. In search of a maize ideotype for cell wall enzymatic degradability using histological and biochemical lignin characterization. J. Agric. Food Chem. 53, 5872-5881.

Mele, G., Ori, N., Sato, Y., Hake, S., 2003. The *knotted1*-like homeobox gene *BREVIPEDICELLUS* regulates cell differentiation by modulating metabolic pathways. Genes Dev. 17, 2088-2093.

Meyer, K., Cusumano, J.C., Ruegger, M.O., Bell-Lelong, D.A., Chapple, C.C., 1997. Regulation and manipulation of lignin monomer composition by expression and over expression of ferulate 5-hydroxylase, a cytochrome P450-dependent monooxygenase required for syringyl lignin biosynthesis. Abstract presented at the Second International Wood Biotechnology Symposium, Canberra, Australia, March 10-12, 1997, paper 9.

Meyer, K., Cusumano, J.C., Somerville, C., Chapple, C.C., 1996. Ferulate-5-hydroxylase from Arabidopsis thaliana defines a new family of cytochrome P450-dependent monooxygenases. Proc. Natl. Acad. Sci. U.S.A. 93, 6869-6874.

Miller, J.E., Geadelmann, J.L., Marten, G.C., 1983. Effect of the *brown midrib* allele on maize silage and yield. Crop Sci. 23, 493-496.

Miron, J., Zuckerman, E., Sadeh, D., Adin, G., Nikbachat, M., Yosef, E., Ben-Ghedalia, D., Carmi, A., Kipnis, T., Solomon, R., 2005. Yield, composition and *in vitro* digestibility of new forage sorghum varieties and their ensilage characteristics. Anim. Feed Sci. Technol. 120, 17-32.

O'Neill, M.A., Ishii, T., Albersheim, P., Darvill, A.G., 2003. Rhamnogalacturonan II: Structure and function of a borate cross-linked cell wall pectic polysaccharide. Annu. Rev. Plant Biol. 55, 109-139.

Oliver, A.L., Pedersen, J.F., Grant, R.J., Klopfenstein, T.J., 2005. Comparative effects of the sorghum *bmr6* and *bmr12* genes: I. Forage sorghum yield and quality. Crop Sci. 45, 2234-2239.

Onyilagha, J.C., Grotewold, E., 2004. The biology and structural distribution of surface flavonoids. Recent Res. Dev. Plant Sci. 2, 53-71.

Patten, A.M., Cardenas, C.L., Cochrane, F.C., Laskar, D.D., Bedgar, D.L., Davin, L.B., Lewis, N.G., 2005. Reassessment of effects on lignification and vascular development in the *irx4* Arabidopsis mutant. Phytochemistry 66, 2092-2107.

Pedersen, J.F., Vogel, K.P., Funnell, D.L., 2005. Impact of reduced lignin on plant fitness. Crop Sci. 45, 812-819.

Pflieger, S., Lefebvre, V., Causse, M., 2001. The candidate gene approach in plant genetics. Mol. Breed. 7, 275-291.

Piquemal, J., Chamayou, S., Nadaud, I., Beckert, M., Barriére, Y., Mila, I., Lapierre, C., Rigau, J., Puigdomenech, P., Jauneau, A., Digonnet, C., Boudet, A.M., Goffner, D., Pichon, M., 2002. Down regulation of caffeic acid *O*-methytransferase in maize revisited using a transgenic approach. Plant Physiol. 130, 1675-1685.

Piquemal, J., Lapierre, C., Myton, K., O'Connell, A., Schuch, W., Grima-Pettenati, J., Boudet, A.M., 1998. Down-regulation of cinnamoyl Co-A reductase induces significant changes of lignin profiles in transgenic tobacco plants. Plant J. 13, 71-83.

Porch, T.G., Tseung, C., Schmelz, E.A., Settles, M.A., 2006. The maize *Viviparous10/Viviparous13* locus encodes the *Cnx1* gene required for molybdenum cofactor biosynthesis. Plant J. 45, 250-263.

Porter, K.S., Axtell, J.D., Lechtenberg, V.L., Colenbrander, V.F., 1978. Phenotype, fiber composition and *in vitro* dry matter disappearance of chemically induced *brown midrib* mutants of sorghum. Crop Sci. 18, 205.

Raes, J., Rohde, A., Christensen, J.H., van De Peer, Y., Boerjan, W., 2003. Genome-wide characterization of the lignification toolbox in Arabidopsis. Plant Physiol. 133, 1051-1071.

Ralph J., Akiyama, T., Kim, H., Lu, F., Schatz, P.F., Marita, J.M., Ralph, S.A., Reddy, M.S., Chen, F., Dixon, R.A., 2006. Effects of coumarate 3-hydroxylase down-regulation on lignin structure. J. Biol. Chem. 13, 8843-8853.

Ralph, J., Hatfield, R.D., 1991. Pyrolysis-GC-MS characterization of forage materials. J. Agric. Food Chem. 39, 1426-1437.

Ralph, J., Lapierre, C., Marita, J.M., Kim, H., Lu, F., Hatfield, R.D., Ralph, S., Chapple C., Franke, R., Hemm, M.R., 2001. Elucidation of new structures in lignins of CAD – and COMT – deficient plants by NMR. Phytochemistry 57, 993-1003.

Ralph, J., Lu, F., Akiyama, T., Kim, H., Marita, J.M., Reddy, M.S., Dixon, R.A., 2005. Tools for lignin structural analysis; applications to *C3H* down regulation. In: Proceedings of biosynthesis of plant cell walls, August 4-7, 2005, Pacific Grove, California. pp.11.

Ralph, J., Lundquist, K., Brunow, G., Lu, F., Kim, H., Schatz, P.F., Marita, J.M., Hatfield, R.D., Ralph, S.A., Christensen, J.H., Boerjan, W., 2004. Lignins: Natutal polymers from oxidative coupling of 4-hydroxyphenyl-propanoids. Phytochem. Rev. 3, 29-60.

Ralph, S., Park, J., Bohlmann, J., Mansfield, D., 2006. Dirigent proteins in conifer defense: gene discovery, phylogeny, and differential wound- and insect-induced expression of a family of DIR and DIR genes in spruce (*Picea* spp.). Plant Mol. Biol. 60, 21-40.

Rastogi, S., Dwivedi, U.N., 2006. Down-regulation of lignin biosynthesis in transgenic *Leucaena leucocephala* harboring *O*-methyltransferase. Gene. Biotechnol. Prog. 22, 609-616.

Raven, P.H., Evert, R.F., Eichhorn, S.E., 1999. Biology of plants 6th Ed. W.H. Freeman and Company. Worth Publishers. New York, NY. pp 575.

Reid, I.D., 1991. Biological pulping in paper manufacture. Trends in Biotechnol. 9, 262-265.

Rhoades, M.M., 1952. The effect of the *bronze* locus on anthocyanin formation in maize. Am. Nat. 827, 105-108.

Rogers, L.A., Campbell, M.M., 2004. The genetic control of lignin deposition during plant growth and development. New Phytol. 164, 17-30.

Ronaghi, M., 2001. Pyrosequencing sheds light on DNA sequencing. Genome Res. 11, 3-11.

Ruegger, M., Chapple, C., 2001. Mutations that reduce sinapoylmalate accumulation in *Arabidopsis thaliana* define loci with diverse roles in phenylpropanoid metabolism. Genetics 159, 1741-1749.

Saha, B.C., Cotta, M.A., 2007. Production of fuel ethanol from wheat straw. Abstract presented at the Environment, Industrial, and Applied Microbiology Meeting. Seville, Spain. Nov 28, 2007 - Dec 01, 2007. Paper No. 434.

Schoch, G., Goepfet, S., Morant, M., Hehn, A., Meyer, D., Ullmann, P., Werck-Reichhart, D., 2001. CYP98A3 from *Arabidopsis thaliana* is a 3'-hydroxylase of phenolic esters, a missing link in the phenylpropanoid pathway. J. Biochem. Chem. 39, 36566-36574.

Schulze, A., Downward, J., 2001. Navigating gene expression using microarrays - a technology review. Nat. Cell Biol. 3, E190-E195.

Schwinn, K.E., Davies, K.M., 2004. Flavonoids. In: Davies, K.M., (Ed.), Plant pigments and their manipulation. Annual Plant Reviews, Volume 14. Blackwell Publishing, Victoria, Australia.

Seisler, H.W., Ozaki, Y., Kawata, S., Heise, H.M., 2002. Near infrared spectroscopy. Wiley-VCH Verlag GmbH, Weinham, Germany. pp. 348.

Selwat, V.J.H., Ni, W., Blount, J.W., Jung, H.G., Masoud, S.A., Howles, P.A., Lamb, C., Dixon, R.A., 1997. Reduced lignin content and altered lignin composition in transgenic tobacco down-regulated in expression of L-phenylalanine ammonia-lyase or cinnamate 4-hydroxylase. Plant Physiol. 115, 41-50.

Semenov, D.A., Sudachkova, N.E., Khlebopros, R.G., 2001. A new scheme of lignin biosynthesis and the mechanism of its regulation of functional properties. Biochem. Biophys. 382, 50-52.

Settles, A.M., Latshaw, S., McCarty, D.R., 2004. Molecular analysis of high-copy insertion sites in maize. Nucleic Acids Res. 32: e54.

Shewmaker, C.K., Sheehy, J.A., Daley, M., Colburn, S., Yang Ke, D., 1999. Seed specific over expression of *phytoene synthase*: increase in carotenoids and other metabolic effects. Plant J. 20, 401-412.

Shirley, B.W., 1996. Flavonoid biosynthesis: 'new' functions for an 'old' pathway. Trends Plant Sci. 1, 377-381.

Smith, L.I., 2002. A tutorial on principal components analysis. (http://csnet.otago.ac.nz/cosc453/student_tutorials/principal_components.pdf). April, 2008.

Somerville, C., 2006. Cellulose synthesis in higher plants. Annu. Rev. Cell Dev. Biol. 22, 53-78.

Stallings, C.C., Donaldson, B.M., Thomas, J.W., Rossman, E.C., 1982. *In vivo* evaluation of *brown midrib* corn silage by sheep and lactating dairy cows. J. Dairy Sci. 65, 1945-1949.

Sticklen, M., 2006. Plant genetic engineering to improve biomass characteristics for biofuels. Curr. Opin. Biotechnol. 17, 315-319.

Stobiecki, M., Kachlicki, P., 2006. Isolation and identification of flavonoids. In: Grotewold, E., (Ed.), The science of flavonoids. Springer Science + Business Media, Inc., New York, NY. pp. 268.

Styles, E.D., 1982. Unstable pigmenting factors. Maize Genet. Coop. News Lett. 56, 160-161.

Styles, E.D., Erfanifar, B., Ceska, O., 1987. An unstable factor for orange pigment. Maize Genet. Coop. News Lett. 61, 171.

Sun, X.F., Sun, R.C., Fowler, P., Baird, M.S., 2005. Extraction and characterization of original lignin and hemicelluloses from wheat straw. J. Agric. Food Chem. 53, 860-870.

Suzuki, M., Settles, M.A., Tseung, C., Li Q., Latshaw, S., Wu, S., Porch, T.G., Schmelz, E.A., James, M.G., McCarty, D.R., 2006. The maize *viviparous15* locus encodes the molybdopterin synthase small subunit. Plant J. 45, 264-274.

Szyjanowicz, P.M.J., McKinnon, I., Taylor, N.G., Gardiner, J., Jarvis, M.C., Turner, S.R., 2004. The *irregular xylem 2* mutant is an allele of *korrigan* that affects the secondary cell wall of *Arabidopsis thaliana*. Plant J. 37, 730-740.

Taylor, N.G., Gardiner, J.C., Whiteman, R., Turner, S.R., 2004. Cellulose synthesis in the Arabidopsis secondary cell wall. Cellulose 11, 329-338.

Terashima, N., Fukushima, K., He, L.F., Takabe, K. 1993. Comprehensive model of the lignified plant cell wall. In: Jung, H.G., Buxton, D.R., Hatfield, R.D., Ralph, J., (Eds), Forage cell wall structure and digestibility. ASA-CSSA-SSSA, Madison, WI. pp. 247-270.

Tobias, C.M., Chow, E.K., 2005. Structure of the *cinnamyl alcohol dehydrogenase* gene family in rice and promoter activity of a member associated with lignification. Planta 220, 678-688.

Todd, R., 2000. Higher plant cellulose synthases. Gen. Biol. 4, 3001-3006.

Turner, S.R., Sommerville, C.R., 1997. Collapsed xylem phenotype of Arabidopsis identifies mutants deficient in cellulose deposition in the secondary cell wall. Plant Cell 9, 689-701.

Turner, S.R., Taylor, N., Jones, L., 2005. Mutations of the secondary cell wall. Plant Mol. Biol. 47, 209-219.

Varagona, M.J., Purugganan, M., Wessler, S.R., 1992. Alternative splicing induced by insertion of retrotranspozons into the maize *waxy* gene. Plant Cell 4, 811-820.

Venglat, S.P., Dumonceaux, T., Rozwadowski, K., Parnell, L., Babic, V., Keller, W., Martienssen, R., Selvaraj, G., Datla, R., 2002. The homeobox gene *BREVIPEDICELLUS* is a key regulator of inflorescence architecture in Arabidopsis. Proc. Natl. Acad. Sci. U.S.A. 99, 4730-4735.

Vermerris, W., Boon, J.J., 2001. Tissue-specific patterns of lignification are disturbed in the *brown midrib2* mutant of maize (*Zea mays* L.). J. Agric. Food Chem. 49, 721-728.

Vermerris, W., McIntyre, L., 1999. Time to flowering in *brown midrib* mutants of maize: an alternative approach to the analysis of developmental traits. Heredity 83, 171-178.

Vermerris, W., Nicholson, R., 2006. Phenolic compound biochemistry. Springer, Amsterdam, Netherlands. pp. 352.

Vermerris, W., Saballos, A., Ejeta, G., Mosier, N.S., Ladisch, M.R., Carpita, N.C., 2007. Molecular breeding to enhance ethanol production from corn and sorghum stover. Crop Sci. 47, S142-S153.

Vermerris, W., Thompson, K.J., McIntyre, L.M., 2002. The maize *Brown midrib1* locus affects cell wall composition and plant development in a dose-dependent manner. Heredity 88, 450-457.

Vignols, F., Rigau, J., Torres, M.A., Capeliades, M., Puigdomenech, P., 1995. The *brown midrib3* mutation in maize occurs in the gene encoding caffeic acid *O*-methyltransferase. Plant Cell 7, 407-416.

Wardrop, A.B., 1971. Occurrence and formation in plants. In: Sarkanen, K.V., Ludwig, C.H., (Eds), Lignins: occurrence, formation, structure and reactions. Wiley-Interscience, New York. pp. 19-41.

Warren, J., MacKenzie, S., 2001. Why all color combinations not equally represented in as flower-color polymorphisms? New Phytol. 151, 237-241.

Webster, E.A., Halpin, C., Chudek, J.A., Tilston, E.L., Hopkins, D.W., 2005. Decomposition in soil of soluble, insoluble and lignin-rich fractions of plant material from tobacco with genetic modifications to lignin biosynthesis. Soil Biol. Biochem. 37, 751-760.

Weller, R.F., Phipps, R.H, Cooper, A., 1985. The effect of the *brown midrib3* gene on the maturity and yield of forage maize. Grass For. Sci. 3, 335-339.

Whetten, R., Sederoff, R., 1995. Lignin biosynthesis. Plant Cell 7, 1001-1013.

White, P.M., Rice, C.W., Baldock, J.A., Tuinstra, M.R., 2007. Soil biological properties following additions of mutant grain sorghum. Soil Biol. Biochem. 39, 1518-1532.

Wicker, T., Schlagenhauf, E., Andreas-Graner, A., Close, T.J., Keller, B., Stein, N., 2006. 454 sequencing put to the test using the complex genome of barley. BMC Genomics 7, 275.

Winkel-Shirley B., 2001. Flavonoid biosynthesis: A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol. 126, 485-493.

Wurtzel, E.T., Luo, R.B., Yatou, O., 2001. A simple approach to identify the first rice mutants blocked in carotenoid biosynthesis. J. Exp. Bot. 52, 161-166.

Ye, Z.H., Kneusel, R.E., Matern, U., Varner, J.E., 1994. An alternative methylation pathway in lignin biosynthesis in zinnia. Plant Cell 6, 1427-1439.

Yong, W., Link, B., O'Malley, R., Tewari, J., Hunter, C.T., Lu, C.A., Li, X., Bleecker, A.B., Koch, K.E., McCann, M.C., McCarty, D.R., Patterson, S.E., Reiter, W.D., Staiger, C., Thomas, S.R., Vermerris, W., Carpita, N.C., 2005. Genomics of plant cell wall biogenesis. Planta 221, 747-751.

# BIOGRAPHICAL SKETCH

Reuben Tayengwa was born on February 2, 1977 in Harare, Zimbabwe. The fifth child of seven children, he grew up mostly in Harare, graduating from an Anglican High School in 1994. After graduation he was offered a scholarship to attend an international United World College institution in Norway. He earned an International Baccalaureate Diploma in 1997 at the college before returning to Zimbabwe in 1997. He took a year off and travelled to England, Denmark, and Germany to visit friends and relatives. In 1999 he enrolled at the University of Zimbabwe in the capital Harare.

Upon graduating in August 2002 with a BSc. in Crop Science, Reuben joined a private company that specialized in developing and distributing elite plant cultivars to poor rural farmers. Reuben worked in a lab as well as the field, developing disease free sweet potato, Irish potato and cassava planting material using tissue culture methods. Most of the planting material was distributed to families that had members suffering from HIV AIDS. Reuben was involved in instructing the families on the best agronomic methods to grow the crops as well as how to market the yield to their neighbors.

In 2004, Reuben joined a Swedish NGO, Swedish Co-operative Center, to continue working on the same project, but at a national level. He was responsible for a large district in the rural southern part of Zimbabwe.

In early 2005, Reuben decided to go back to school and joined Dr. Wilfred Vermerris' lab at Purdue University to pursue a master's degree. When Dr. Vermerris's was offered a new position at the University of Florida, Reuben followed him to Florida where he completed his studies. In fall 2008, Reuben will join the Molecular Plant Sciences program at Washington State University as a PhD research assistant.