DOWN-REGULATING THE EXPRESSION OF A LIGNIN BIOSYNTHETIC GENE
BY ANTISENSE TECHNOLOGY TO IMPROVE FORAGE QUALITY

By

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LIST OF ABBREVIATIONS

ADL: acid detergent lignin

ADF: acid detergent fiber

bm: brown midrib

bp: base pair

bar: bialaphos resistant

CaMV: cauliflower mosaic virus

dsRNA: double-stranded RNA

GUS: β -glucuronidase

IVOMD: *in vitro* organic matter digestibility

knt: kilo nucleotides

kb: kilo base pairs

NDF: neutral detergent fiber

NOS: nopaline synthase

OMT: caffeic acid O-methyltransferase

PCR: polymerase chain reaction

pg: pico-gram

pkat: pico-katal

TESC: total ethanol-soluble carbohydrate

Ubi-1: ubiquitin-1 gene of maize

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Lignin is a complex aromatic polymer in plant cell walls. It limits cell wall degradability by ruminants, reducing the nutritional value of forages. Genetic engineering using an antisense gene offers the potential to modulate enzymes in the lignin biosynthetic pathway to reduce and or modify lignin and thereby improve forage quality and animal performance.

Maize (*Zea mays* L.) Hi-II was used as a model system to investigate the effectiveness of sorghum [*Sorghum bicolor* (L.) Moench] caffeic acid O-methyltransferase (OMT) antisense constructs to down regulate maize OMT and improve forage quality. Sorghum OMT antisense constructs were assembled with the *bar* selectable gene, which confers glufosinate herbicide resistance, along with the appropriate promoters and terminators. Embryogenic maize callus tissue was transformed by biolistic particle bombardment and 350 plants were regenerated.

These plants were characterized using herbicide resistance, PCR, Southern and northern analyses. About 55% of the 350 plants were resistant to 1% glufosinate and 85.3% of the 204 PCR-tested plants were PCR positive for *bar* gene. About 10% of the transgenic plants expressed the brown midrib phenotype at the 4- to 6-leaf stage and vascular tissue discoloration in mature stems similar to the natural *bm3* maize mutants, which have improved digestibility. Southern analysis of OMT indicated that transgene copy number in transgenic plants varied from 1 to 20, with full-length and rearranged transgenes present. The *bar* gene was more stably transmitted to progeny than the antisense OMT, which exhibited multiple rearrangements.

OMT activity of the transgenic plants was significantly ($p < 0.05$) reduced compared to the controls, with some transgenic plants showing 60% reduction. The distribution of OMT activity and OMT mRNA steady state level showed OMT activity was highly correlated to the OMT mRNA level.

Transgenic plants with down-regulated OMT activity had improved ($p < 0.05$) *in vitro* organic matter digestibility (IVOMD) as compared to controls, with the stems being most improved (7.4%), followed by sheaths (5.2%) and leaves (1.9%). Lignin content was significantly reduced (stems -19.8%, sheaths -18.8%, leaves -11.8%). On a whole plant basis, the transgenic plants increased IVOMD an average of 5.0% with lignin reduction of 16.5%. The highest IVOMD increase was 8.0% with lignin reduction of 30.6%.

CHAPTER 1 INTRODUCTION

Lignin is a complex phenolic polymer in the plant cell wall, essential for mechanical support, water and mineral transport, and defense in vascular terrestrial plants (Taiz and Zeiger, 1991). However, lignin is unfavorable for the utilization of plant biomass, reducing forage digestibility and making paper pulping difficult. Therefore, there is worldwide interest in reducing plant lignin content to improve the utilization of the plant wall.

Lignin is derived from the dehydrogenative polymerization of three monolignols (para-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol), and the general lignin biosynthetic pathway is partially understood. In maize (*Zea mays* L.), sorghum [*Sorghum bicolor* (L.) Moench] and pearl millet [*Pennisetum glaucum* (L.) R. Br.], mutants characterized by a brown midrib phenotype have reduced lignin content and/or altered lignin composition (Cherney et al., 1991). The cloning of genes in the lignin biosynthetic pathway has offered the potential to manipulate lignin. Several genes have been targeted to down-regulate the gene expression involved in lignin biosynthesis (see Table 2-2). Among them, caffeic acid 3-O-methyltransferase (OMT) has been chosen most often because the maize natural brown midrib mutants *bm3* resulting from a deletion of or insertion in the OMT gene have reduced lignin content and altered lignin composition.

Reports in the literature have focused on OMT down-regulation using either tobacco (*Nicotiana tabacum*) (Ni et al., 1994; Vailhe et al., 1996; Sewalt et al., 1997) or poplar (*Populus tremuloides*) (Dwivedi et al., 1994; Legrand et al., 1994; Doorsselaere et al., 1995; Atanassova et al., 1995) as a model system. The endogenous OMT activity level was reduced by varying amounts, from 30% to more than 90% in different studies. In general, lignin chemical composition [the syringyl (S)/guaiacyl (G) ratio] was significantly reduced while lignin content was not significantly changed (Dwivedi et al., 1994; Legrand et al., 1994; Doorsselaere et al., 1995; Atanassova et al., 1995). Only Ni et al. (1994) and Sewalt et al. (1997) reported moderate reduction of lignin content in the transgenic plants. There was no brown midrib phenotype in these transgenic plants except that Atanassova et al. (1995) observed a pale rose color in the xylem of the transgenic poplar trees.

Lignin has been negatively correlated with forage digestibility. Brown midrib mutants have been considered an efficient way to improve forage quality because of their lower lignin content and/or modified lignin composition (Cherney et al., 1991; Barriere and Argiller, 1993). Most of the transgenic research was focused on the molecular characterization and lignin characteristics of transgenic plants. Recently, Vailhe et al. (1996) and Sewalt et al. (1997) reported that *in vitro* cellulase degradability improved in the OMT down-regulated transgenic tobacco.

As tropical grass is generally less digestible than temperate grass, we are using maize as a model system to investigate the potential for forage digestibility improvement resulting from OMT down-regulation by antisense technology, which could be valuable for the forage quality improvement of tropical grass.

Objectives of this project are as follows:

1, Construct a set of sorghum OMT antisense gene vectors, transform maize with these vectors via the microprojectile-mediated gene transfer method, and present molecular evidence (Southern blot and northern blot analyses) for the presence of transgene.

2, Characterize the transgenic plants for OMT down-regulation (OMT activity and endogenous OMT mRNA steady state levels), phenotype changes, and lignin content.

3, Perform forage quality studies on OMT down-regulated transgenic plants, including *in vitro* organic matter digestibility and cell wall analyses.

CHAPTER 2 LITERATURE REVIEW

Plant Cell Wall and Digestibility

Lignin and the Plant Cell Wall

Lignin is the second most abundant organic compound of plant biomass on earth after cellulose (Bouget and Grima-Pettenati, 1996). Lignin is deposited in the cell wall of certain plant cells, such as sclerenchyma, xylem vessels, and tracheids, providing mechanical support, enabling water and mineral transport, and conferring protection against pathogens (Sarkanen and Ludwig, 1971). Lignin is broadly defined as a complex polymer arising from enzyme-initiated, dehydrogenative polymerization of three monolignols (Sarkanen and Ludwig, 1971). The monolignols are para-coumaryl alcohol (H), coniferyl alcohol (G) and sinapyl alcohol (S). Because lignin is a complex and heterogenous macromolecule, there is no exact chemical definition for it. The subunits of lignin are not linked in a repetitive way. The multiple links of C-C and C-O-C between the free radicals of monolignols, result in a highly branched three dimensional structure. Variations in lignin composition occur within plant species, tissues and cell types, and are also depending on developmental stages (Campbell and Sederoff, 1996).

There are three major differences between monocot lignin and dicot lignin. (1) Monocot lignin is more soluble than dicot lignin in alkaline solution. (2) Monocot lignin is more divergent in composition than dicot lignin and has more p-coumaric acid and

ferulic acid esters linked to cell wall polysaccharides. Monocot lignin may have ester linkages in addition to C-C and C-O-C linkages. Ester linkages result from the phenylpropane alcohol polymerization. Dicot lignin does not have ester linkages and is a monolignol alcohol polymer type of lignin. (3) Monocot lignin has more syringyl units and is less condensed; dicot lignin has more guaiacyl units and is more condensed (Van Soest, 1994).

The plant cell wall is a rigid structure around the protoplast. Growth of the plant cell wall can be categorized into two phases: primary growth and secondary growth. Primary cell wall growth is the phase when the plant cell is increasing in size. The primary cell wall consists of polysaccharides, proteins, and phenolic acids. When cell wall elongation ceases, the plant cell wall starts secondary wall growth. The secondary wall contains a higher proportion of cellulose and fewer pectins than the primary cell wall. Lignin deposition begins with the initiation of secondary thickening. Lignin is first deposited in the middle lamella, the primary cell wall region, and then proceeds into the secondary wall. Thus, lignin has a concentration gradient: highest in the middle lamella / primary cell wall and lowest close to the plasma membrane. In addition to the concentration difference, lignin types also shift from H-type lignin (containing more para-coumaryl alcohol units) to S-type lignin (containing more sinapyl alcohol units) (Higuchi, 1985; Terashima et al., 1986; Fukushima and Terashima, 1990; Jung and Allen, 1995; Joseleau and Ruel, 1997).

During secondary wall thickening, cross-linkages occur. Iiyama et al. (1994) reviewed three types of cross-links between lignin and polysaccharides: (1) ester linkages between uronic acids and hydroxyl groups of lignin monomers on lignin surfaces, (2)

ether linkages between polysaccharides and lignin involving glucose or mannose residues, and (3) glycosidic linkages between carbohydrates and terminal phenolic or side chain hydroxyls in lignin. In addition to the above direct linkages, lignin and polysaccharides can be associated through hydroxycinnamic acid ester-ether bridges. For example, in grass cell walls, ferulic acid is esterified to arabinoxylans and etherified to lignin. Moreover, lignin can also crosslink to cell wall proteins (Iiyama et al. 1993).

Plant cell wall components and structure differ from cell types within a plant and differ among taxonomic groups (Jung and Allen, 1995). Not all plant cell types have the secondary wall, and cells such as leaf mesophyll and collenchyma do not have lignified cell walls. Major differences in cell wall composition are listed in Table 2-1.

Lignin and Forage Digestibility

Forage digestibility influences the growth rate of young livestock and milk production of lactating animals (Buxton and Casler, 1993). Improved digestibility is one of the major objectives for improving forage quality. A number of chemical and structural characteristics of the plant cell wall that limit cell wall digestibility have been identified (Buxton and Casler, 1993). Lignin and associated phenolic compounds are the most important factors limiting the digestibility of the wall polysaccharides in the rumen of grazing animals. Lignin seems to exert its negative effect on digestibility by shielding the polysaccharides from enzymatic hydrolysis (Jung and Deetz, 1993). The negative relationship between lignin concentration and digestibility has been recognized for many years (Johnson et al., 1962; Tomlin et al., 1964; Jung, 1989; Jung et al., 1997; Wilman and Moghdam, 1998). Van Soest (1993) pointed out that lignin only affects the

Table 2-1. Composition of lignified cell wall regions in grasses and legumes.

Cell wall region	Forage groups	Wall polymer component			
		Polysaccharides	Lignin	Phenolic acids	Protein
Middle lamella/primary cell wall	Grasses	Cellulose, glucuronarabinoxylans, mixed linkage β -glucans, heteroglucans, pectic polysaccharides (minor)	Guaiacyl (major), syringyl (minor), p-hydroxyphenyl (middle lamella only)	Ferulic acid esters and ethers, p-coumaric acid esters (minor)	Proteins with low or no hydroxyproline, extensin (minor)
	Legumes	Pectic polysaccharides, cellulose, heteroglucans, heteroxylans (minor)	Guaiacyl (major), syringyl (minor)	Ferulic acid ethers and esters (minor), p-coumaric acid esters (minor)	Extensins, other proteins
Secondary cell wall	Grasses	Cellulose, glucuronarabinoxylans, mixed linkage β -glucans, heteroglucans, pectic polysaccharides (minor)	Guaiacyl (major), syringyl (minor)	p-Coumaric acid esters and ethers	None
	Legumes	Cellulose, 4-O-methylglucuronoxylans, glucomannans (minor)	Syringyl (major), guaiacyl (minor)	p-Coumaric acid ethers and esters (minor)	None

digestibility of cell wall rather than the digestibility of total forage organic matter. Lignin subunit composition may also have impact on cell wall degradability. As indicated by Jung and Deetz (1993), S-type lignin might protect more polysaccharides in the cell wall from digestion than G-type lignin. However, there are contradictory reports. Grabber et al. (1997) observed no effect of lignin composition on cell wall digestibility when sinapyl or guaiacyl lignin precursors were added to a maize tissue culture system resulting in artificial lignification of the primary cell wall. Jung et al. (1999) also reported that complete elimination of S-type lignin without reducing total lignin had no effect on cell wall digestibility when mutant *Arabidopsis thaliana* plants were used as a model system.

Phenolic acids in the cell wall may also affect digestibility. Early reports indicated that phenolic acids might be toxic to the ruminal microbes, but a more recent study suggests this may not be true (Jung and Deetz, 1993).

Cross-linkages between lignin and cell wall polysaccharide may have a more important effect on digestibility than lignin or phenolic acid concentration (Jung and Allen, 1995). As indicated by Grabber et al. (1997), digestibility of the maize primary cell wall was significantly reduced by cross-linkage of arabinoxylan to diferulate. In addition to lignin and associated phenolic compounds, cutin, suberin, tannin and biogenic silica also inhibit plant cell wall digestibility (Van Soest, 1993).

The Brown Midrib Phenotype Effect on Forage Digestibility

Natural or induced brown midrib mutant plants have been observed and characterized in several monocot species including maize (*Zea mays* L.), sorghum [*Sorghum bicolor* (L.) Moench], and pearl millet [*Pennisetum glaucum* (L.) R. Br.] (Cherney et al., 1991). The brown midrib phenotype is characterized by a reddish-brown

pigmentation of leaf midribs at the 4 to 6-leaf maturity stage. This pigmentation is also observed in the lignified rind and vascular cross-section of the stem (Fritz et al., 1981; Cherney et al., 1991; Akin and Rigsby, 1991). Brown midrib mutant plants are generally associated with the reduction of lignin content or a change of lignin subunit composition (Muller et al., 1971; Barnes et al., 1971; Lechtenberg et al., 1972; El-Tekriti et al., 1976; Fritz et al., 1981; 1991; Akin and Rigsby, 1991; Barriere and Argillier, 1993; Barriere et al., 1994; Chabbert et al., 1994). Modified lignin content or composition results in an increase in forage digestibility when consumed by ruminant animals suggesting that the brown midrib genes may be useful in improving forage quality (Cherney et al., 1991; Barriere and Argillier, 1993).

A brown midrib mutation was first found in maize (Jorgenson, 1931). Maize has four naturally occurring brown midrib mutant genes: *bm1*, *bm2*, *bm3*, and *bm4*. Each of the genes is located on a different chromosome and each *bm* allele segregates as a recessive Mendelian character (Barriere and Argillier, 1993). *Bm3* has been characterized as a deletion or insertion in the gene encoding caffeic acid O-methyltransferase in the lignin biosynthetic pathway (Vignols et al., 1995; Morrow et al., 1997). The other brown midrib genes have not been characterized. Porter et al. (1978) and Cherney et al. (1988) used chemical mutagenesis to produce brown midrib mutants in sorghum and pearl millet, respectively. Brown midrib mutants in all three species have lower lignin content and generally increase *in vitro* dry matter digestibility (Cherney et al., 1991; Chabbert et al., 1994). In addition, brown midrib mutants also show improved voluntary intake, and animal performance. This information suggests that down regulation of the enzymes in

the lignin biosynthetic pathway could make important improvements in forage quality and animal productivity.

Plant Cell Wall Components and *In Vitro* Digestibility Analysis

Plant cell walls of forages account for 400 to 700g kg⁻¹ of plant dry matter and provide energy for animal production after fermentation by rumen microorganisms (Lagowski et al., 1958; Waite and Garrod, 1959; Barton et al., 1976; Chesson and Forsberg, 1988). In forages, the analysis of the cell wall components is based on fiber analysis. Fiber has nutritional characteristics that describe the forage cell wall components that have low solubility in specific solvent systems (neutral or acid detergent) and are relatively less digestible than starch (Jung and Allen, 1995). Neutral detergent fiber (NDF) is an adequate characterization of the plant cell wall in nutritional studies and for the general description of forage quality (Jung and Allen, 1995).

Several procedures exist for fiber analysis (Theander and Westerlund, 1993). The widely accepted method for feed analysis is the detergent gravimetric method introduced by Van Soest (1963a). This method includes sequential procedures. One procedure is a neutral detergent fiber (NDF) analysis which is based on extraction of the plant material with a hot neutral solution of sodium lauryl sulfate (Van Soest, 1963a; Van Soest and Wine, 1967). The NDF analysis is accepted as a method of estimating the cell wall content. The other procedure is an acid detergent fiber method (ADF) using heat treatment of the sample with sulfuric acid containing cetyltrimethyl-ammonium bromide (Van Soest, 1963b; Goering and Van Soest, 1970). The ADF residue contains mainly cellulose and lignin. Lignin content can be determined by permanganate oxidation or sulfuric acid treatment leaving acid detergent lignin (ADL).

For determining feed digestibility, the Tilley and Terry (1963) *in vitro* rumen method is considered the most reliable method (Van Soest, 1993). This method involves a 48 hr fermentation by rumen microorganisms followed by HCl-pepsin digestion. The difference in dry matter or organic matter between the pre-treatment and post-treatment residues is considered to be degraded.

Genetic Manipulation of Lignin with Biotechnology

Lignin Biosynthetic Pathway

The biosynthesis of lignin involves three pathways (1) the shikimate pathway which supplies phenylalanine and tyrosine (in grasses), (2) the common phenylpropanoid pathway from phenylalanine to the cinnamoyl CoAs, and (3) the lignin specific pathway which leads to monolignols and lignin synthesis (Boudet et al., 1995; Whetten and Sederoff, 1995; Dixon et al., 1996). Several other plant secondary metabolites in addition to lignin are also derived from phenylalanine, such as flavonoids, tannins, and benzoic acid derivatives (Taiz and Zeiger, 1991). The enzymes and their catalyzed reactions are illustrated in Fig. 2-1.

Modification of Gene Expression-Antisense Strategy

There are a number of ways to down-regulate gene expression, such as radiation, chemical mutagenesis, T-DNA or transposon insertion, and sense gene suppression. Compared to these methods, antisense gene suppression is a more direct approach, although the mechanism is not well understood. There are several hypotheses concerning the mechanisms of antisense function: (1) antisense RNA may interfere with endogenous mRNA processing and transport; (2) antisense RNA may inhibit target mRNA translation; (3) antisense RNA and the target mRNA may form dsRNA and trigger RNA

Fig. 2-1. Lignin biosynthetic pathway. The respective enzymes are: PAL, Phe ammonia-lyase; C4H, cinnamate 4-hydroxylase; C3H, 4-hydroxycinnamate 3-hydroxylase; OMT, caffeic acid O-methyltransferase; F5H, ferulate 5-hydroxylase; 4CL, hydroxycinnamate:CoA-ligase; CCoA-3H, 4-hydroxycinnamoyl-CoA 3-hydroxylase; CCoA-OMT, caffeoyl-CoA-O-methyltransferase; CCR, hydroxycinnamoyl-CoA:NADPH oxidoreductase; CAD, hydroxycinnamyl alcohol dehydrogenase (Whetten and Sederoff, 1995; Campbell and Sederoff, 1996).

degradation; and (4) antisense RNA may interact with the target DNA and slow down mRNA transcription (De Lange et al., 1995).

Several factors can affect antisense efficiency in gene suppression. First, antisense RNA amount may influence the efficiency but the presence of antisense RNA does not guarantee the inhibitory effect on targeting gene expression. In some cases, a higher concentration of antisense RNA results in a higher rate of suppression, while in other cases, a high level of antisense RNA was detected but the target mRNA level was not reduced (Robert et al., 1989; Cannon et al., 1990; Van Der Meer et al., 1992; Stockhaus et al., 1990). Secondly, the sequence homology between antisense and the target gene may be another factor. However, the overall nucleotide sequence homology between antisense and the resident gene could be low, because short regions of highly homologous sequences can have a significant inhibitory effect (Mol et al., 1990; Temple et al., 1993). Additionally, different antisense gene fragments may have different and unpredictable inhibitory effects. Sometimes, full-length antisense transgenes cannot inactivate endogenous gene expression, whereas antisense genes containing only the 3'-fragment or the 5'-fragment of the target gene can completely suppress target gene expression (De Lange et al., 1993; Mol et al., 1990; Sandler et al., 1988; Van Der Krol et al., 1990). Also, the relative location of antisense RNA and target mRNA may also contribute to the efficiency of antisense down-regulation (De Lange et al., 1995; Arndt and Rank, 1997). Recent progress showed that coexpression of the sense and antisense gene on the same construct resulted in more effective inhibition of the target gene than that of the simple antisense and sense genes (Waterhouse, et al., 1998).

Table 2-2. Characteristics of different transgenic plants with down-regulated gene expression in the lignin biosynthetic pathway.

Targeted gene	Plant species	Lignin content	Lignin S/G ratio	Phenotype	Reference
PAL	Tobacco	Reduced	Increased	Pleiotrophic	Sewalt et al., 1997
C4H	Tobacco	Reduced	Decreased	Not determined	Sewalt et al., 1997
OMT	Tobacco	Reduced	No change	Not determined	Ni et al., 1994
OMT	Tobacco	No change	Decreased	Pale rose color in xylem	Atanassova et al., 1995
OMT	Poplar	No change	Decreased	Not determined	Van Doorselaere et al., 1995
CCoA-OMT	Tobacco	Reduced	Decreased	Brown color in xylem	Zhong et al., 1998
OMT+ CCoAOMT	Tobacco	Reduced	Decreased	Not described	Zhong et al., 1998
4CL	Tobacco	Reduced	Decreased	Brown color in xylem	Kajita et al., 1997
CCR	Tobacco	Reduced	Increased	Orange-brown color in xylem	Piquemal et al., 1998
CAD	Tobacco	Reduced	Decreased	Red color in xylem	Halpin et al., 1994
CAD	Poplar	No change	No change	Red color in xylem	Baucher et al., 1996
Peroxidase	Tobacco	No change or slightly increased	Not determined	Pleiotrophic	Lagrimini et al., 1997

Target Enzymes in the Lignin Biosynthetic Pathway

Different enzymes in the lignin biosynthetic pathway have been targeted to modify lignin (see summary in Table 2-2). Caffeic acid 3-O-methyltransferase (OMT, E. C. 2.1.1.6) catalyzed the methylation of caffeic acid to form ferulic acid using S-adenosylmethionine as the methyl group donor. It is known that OMT in gymnosperms is monofunctional and only catalyzes caffeate to ferulic acid, while OMT in angiosperms is bifunctional and methylates both caffeic and 5-hydroxyferulic acids to ferulic acid and sinapic acid, respectively (Boudet et al., 1995; Whetten and Sederoff, 1995). OMT has been often selected as target because it plays an important role in monolignol synthesis. It is assumed that decreasing the degree of methoxylation in lignin would improve forage digestibility as evidenced by the *bm3* mutant, or improve the efficiency of wood chemical pulping (Boudet et al., 1995).

OMT Gene Organization and Expression

Different cDNA clones encoding OMT have been isolated from several plant species. Bugos et al. (1991) cloned the first OMT cDNA from aspen (*Populus tremuloides*). Other OMT cDNA clones have been successively obtained from alfalfa (*Medicago sativa*) (Gowri et al., 1991), poplar (*Populus deltoides*) (Dumas et al., 1992), tobacco (*Nicotiana tabacum*) (Jaek et al., 1992), eucalyptus (*Eucalyptus gunnii*) (Poeydomege et al., 1994), and pine (*Pinus taeda*) (Li et al., 1997). Collazo et al. (1992) characterized the structure and expression of maize OMT, which was the first clone from monocots.

OMTs are highly conserved in dicots. It seems that a gene family encodes OMTs in dicots because different OMT cDNAs have been isolated from the same species (Bugos et

al., 1991; Ye and Varner, 1995; Jaeck et al., 1996; Hayakawa et al., 1996). The best-characterized example is tobacco OMT. Tobacco OMTs are grouped into two classes: class I and II OMT. Each class of OMT has two members. Class I OMT is the major form expressed in healthy plants and seems to be particularly related to lignification, while class II OMT expression is responsive to pathogen attack (Pellegrini et al., 1993; Jaeck et al., 1996).

Monocot OMTs have not been as well characterized as dicot OMTs. However, the organization of maize OMT gene in the genome seems different from the dicot OMTs. As pointed out by Collazo et al. (1992), maize OMT is encoded by a single gene rather than a gene family. Maize OMT mRNA is highly accumulated in roots of the elongating regions, which correlates to the lignification process. Vignols et al. (1995) using *in situ* techniques indicated that maize OMT mRNA also accumulates in leaf midribs. By promoter fusion with GUS, the chimeric gene (OMT promoter-GUS) was introduced in tobacco and maize (Capellades et al., 1996). GUS expression driven by the OMT promoter is observed in xylem and other tissues of tobacco and maize that is undergoing lignification. Interestingly, with that construct, GUS expression also responds to wounding and fungal elicitors.

OMT Down-regulation by Genetic Engineering

Ni et al. (1994) first introduced alfalfa OMT antisense constructs into tobacco and alfalfa plants. Their results indicated that lignin content was reduced but the composition of lignin was not significantly changed. Dwivedi et al. (1994) reported a slight reduction of both lignin content and S/G ratio when an OMT from aspen was used to transform tobacco. However, other authors have reported contradictory results. Several tobacco

showed a strong suppression of OMT activity correlated with a significant reduction of S/G ratio but lignin content was not altered, as compared to the controls (Legrand et al., 1994). Moreover, a new monomeric 5-hydroxylguaiacyl unit was observed in the transgenic tobacco plants, which is similar to the characteristic of *bm3* (Monties et al., 1994). Jan Van Doorselaere et al. (1995) reported similar results when they used a poplar OMT cDNA to manipulate the poplar OMT expression by sense or antisense suppression. To date, there is no report on antisense down-regulation of OMT in monocots.

Most of the studies on OMT down-regulation in transgenic plants have focused on the molecular characterization of transgenic plants and lignin component or content analysis. More recent reports indicated the change of digestibility or pulping characteristics. Vailhe et al. (1996) reported that the stems of the antisense OMT transgenic tobacco plants with a 92% reduction of OMT activity showed a 3.5% improvement in dry matter cellulase degradability by 3.5%. Sewalt et al. (1997) reported in their extreme case that the stem from one of their primary OMT antisense transgenic tobacco had an approximately 50% reduction of OMT activity and improved cell wall cellulase degradability of 48%. Unexpected results were obtained when Lapierre et al. (1999) analyzed OMT down-regulated transgenic poplar plants. They found that the transgenic plants were more resistant to kraft delignification than the control plants during the pulping process when the transgenic plants' lignin composition (S/G ratio) was reduced rather than lignin content.

Plant Transformation

Transformation Methodology

Genetic transformation of higher plants has not only provided an efficient way to study gene structure, function and regulation, but also has allowed expression of unique genes for improved agronomic traits in crop plants. A variety of transformation methods have been developed. Among them, *Agrobacterium*-mediated transformation, microprojectile bombardment, electroporation, protoplast transformation, and silicon carbide whisker-mediated gene transfer are currently employed and have proven useful.

***Agrobacterium*-mediated gene transfer**

Most transgenic plants produced to date were created through the use of the *Agrobacterium* system that is mainly effective with dicots (Gasser and Fraley, 1989; Vasil, 1996). Virulent strains of *Agrobacterium tumefaciens* contain the Ti plasmid which has two sequence regions necessary for gene transfer to plants: the T-DNA region that is transferred to plants and the *vir* region containing genes which are not transferred but are responsible for infection and gene transfer. The mechanism of T-DNA integration into the plant genome is not known, but it involves the interaction of genes from the Ti plasmid (*vir*) and the *Agrobacterium* chromosome (*chv*) (Sheng and Citovsky, 1996). There are two types of transformation vectors used with *Agrobacterium*: cointegrative, and the more commonly used, binary vectors. The production of transgenic plants using *Agrobacterium* includes 3 steps: incubate wounded plant tissues to be transformed with the *Agrobacterium* strain containing the vector to be transferred, screen for the transformed cells, and finally regenerate the transformed cells into plants.

The *Agrobacterium*-mediated method of gene transfer in general has two advantages: (1) it transfers relatively large segments of DNA with little rearrangement; and (2) it integrates a single copy or low-copy number of genes into plant chromosome (Jahne et al., 1995).

Although monocotyledonous plants are not the natural host of *Agrobacterium*, several successful transformations have been made in rice (Chan, 1992), maize (Ishida et al., 1996), wheat (Mooney and Goodwin, 1991) and barley (Tingay et al., 1997). The authors concluded that the starting materials, tissue culture conditions, bacterial strains and vectors had great effect on efficient gene transfer. It is possible that the application of *Agrobacterium*-mediated gene transfer can produce a wider range of transgenic monocots.

Microprojectile bombardment

The development of microprojectile bombardment (biolistics) transformation technology has been significant for plant genetic engineering, because it is equally efficient in the transformation of monocot and dicot species (Sanford et al., 1987; Sanford, 1990). It is now the most routinely used transformation method (Christou, 1995). The principle is that dense microscopic particles (tungsten or gold) are coated with DNA and then accelerated into plant cells, allowing transient gene expression or stable transformation. Several physical and chemical factors, such as particle type, size and density, DNA precipitation method, and particle acceleration speed, have an effect on transformation efficiency, but the critical factor is that the DNA is delivered to cells that can be regenerated (Birch and Bower, 1994). Embryogenic suspension cells, embryogenic callus cultures and primary explants have been used as regenerable tissues for

microprojectile bombardment. Microprojectile bombardment has been used to produce fertile transgenic plants in most major crops (Lazzeri and Shewry, 1993; Christou, 1995; Dunwell, 1995). Microprojectile bombardment has also been successfully utilized to transform plastids (Boynton et al., 1988; Daniell et al., 1990; Daniell and Varma, 1998; Daniell et al., 1998; Hibberd et al., 1998; Kota et al., 1999; Sikdar et al., 1998) and mitochondria (Boynton and Gillham, 1996; Butow et al., 1996).

Tissue electroporation

Transformation via electroporation of intact cells (embryos, seeds and callus) has been attempted by many workers and has provided positive results. However, it is not in general use. The mechanism of transformation by electroporation is not yet understood. It is possible that the electric pulse makes both the cell wall and plasma membrane porous to DNA. There are two major advantages of cell electroporation. (1) Regenerable tissues, such as immature scutella and embryogenic callus, are accessible to transformation; and (2) the procedure does little damage to the treated culture (Lazzeri and Shewry, 1993). Application of this technique to stable transformation has been demonstrated in maize (D' Halluin et al., 1992; Pescitelli and Sukhapinda, 1995), rice (Li et al., 1991) and sugarcane (Arencibia et al., 1995).

Transformation of protoplasts

Another technique is direct uptake of DNA by protoplasts, stimulated by either electroporation or polyethylene glycol (PEG) treatment. This method requires protoplasts. Isolated protoplasts are suspended in a buffer solution containing plasmid DNA and the protoplast plasma membranes are transiently disrupted by a stimulus, allowing DNA uptake. The main disadvantage of this method is that regeneration from protoplasts in

many species, especially cereals, is difficult. The success of obtaining transgenic plants by protoplast transformation has been demonstrated in rice (Tsugawa et al., 1998), maize (Hansen et al., 1997), and barley (Kihara et al., 1998).

Silicon carbide whisker-mediated transformation

This method involves mixing of cells in liquid medium with silicon carbide fibers (0.6 μm average diameter, 10-80 μm length) and plasmid DNA, then vortexing. DNA delivery in this system is presumably due to cell-wall penetration by DNA-coated silicon carbide fibers (Frame et al., 1994; Thompson et al., 1995). The attraction is its simplicity and low requirement of expensive instruments.

Selectable Marker Genes

A selectable marker gene is critical for a plant transformation system. Selectable marker genes encode proteins that can either detoxify, or degrade, or make cells/tissues insensitive to phytotoxic chemicals so that the rare transformed cells can preferentially grow. Selectable marker genes are generally under the control of strong, constitutive promoters. There are two main classes of selectable marker genes used in plant transformation systems: genes for antibiotic resistance and for herbicide resistance. Among these, the bialaphos resistance gene (*bar*) from *Streptomyces hygroscopicus* (Thompson et al., 1987) is the most widely used selectable marker gene for monocot transformation (Vasil, 1994). The *bar* gene encodes the enzyme phosphinothricin acetyltransferase (PAT) which acetylates the free NH_2 group of phosphinothricin (PPT), providing resistance to the PPT herbicides, glufosinate and bialaphos. Glufosinate is the ammonia salt of PPT, and both are chemically synthesized, while bialaphos is a fermented tripeptide containing PPT and two alanine residues (Dennehey et al., 1994).

Transient Expression Assay

Delivery of DNA and its expression in plant cells are essential for a plant transformation system. Several factors, such as promoter strength, target tissues, and proper transformation methods can be involved. These associated parameters can be evaluated by assaying the transient expression of reporter genes 24-48 hr after DNA delivery. The transient assay does not require integration of the plasmid DNA into the plant chromosome. Therefore, it allows quick optimization of transformation parameters.

The β -glucuronidase gene (*gus*), encoded by the *uidA* locus of *E. coli* (Jefferson, 1987) is the most widely used reporter gene in plant transformation. The GUS expression assay can be either visualized by histochemical staining or quantitatively determined by fluorometric or spectrophotometric assays.

Enhancing Transgene Expression

The perspective of plant transformation depends very much on the levels of expression of the introduced genes. Several strategies have been proposed to increase the level of transgene expression (Meyer, 1995). Here, the focus is on promoters, introns, and 3'- untranslated regions (terminators).

Promoters

Generally speaking, a strong promoter leads to a high level of gene transcription. Cauliflower mosaic virus (CaMV) 35S promoter (Oiszwski et al., 1982) has been most useful in dicot species, but the level of gene expression produced by this promoter is much lower in monocots. A number of promoters have been isolated and modified to drive gene expression at a high level in monocots. Among these, Ubi-1 promoter

(Christensen et al., 1992) of maize ubiquitin-1 gene and Act-1 promoter (McElory et al., 1990) of rice actin-1 gene are commonly used in monocot transformation.

Introns

It has been known for a long time that an intron can enhance gene expression. It was found that the presence of the intron does not increase transcription, but improves the processing efficiency at the 3' end of the transcript and nucleocytoplasmic transport. This increases the steady state level of the mRNA and finally increases the protein level (Huang and Gorman 1990; Simpson and Filipowicz 1996). The use of intron sequences in plant expression vectors is almost universal for cereal crop transformation.

3'-untranslated region (terminator)

In eukaryotes, the 3'-untranslated region (including the polyA tail) is essential for mRNA stability and efficient translation (Futterer and Hohn, 1996). Attachment of a 3'-untranslated region from other genes downstream of the specific gene is a common strategy to increase the transgene stability. The 3'-untranslated region from nopaline synthase (NOS) (Kemp et al., 1979) is most often used for such a purpose (Koziel et al., 1996)

Confirmation of Transgenic Plants

There are a number of methods to confirm that plants are transformed. The methods used in this study are described as follows.

PCR assay

The polymerase chain reaction (PCR; Mullis and Faloona 1987; Saiki et al., 1988) has been often used to initially screen regenerated plants for transgenes. Because this technique does not require much DNA from each sample nor high quality DNA, various

protocols have been developed to quickly test plant samples for the transgene (Edwards et al., 1991; Brunel, 1992; Luo et al., 1992; Klimyuk et al., 1993). PCR results are reliable if the conditions of amplification have been optimized and proper controls are used.

Southern blot hybridization analysis

Genomic DNA is isolated and digested with an endonuclease restriction enzyme. The DNA fragments are separated with agarose gel electrophoresis and transferred to a nylon membrane (Southern, 1975). The membrane is then hybridized with a fluorescent or radiolabeled probe and exposed to X-ray film. With the appropriate controls, presence of a hybridizing band in a putative transformed plant provides the strongest evidence of transformation.

Enzyme assay of phosphinothricin acetyltransferase (PAT)

The PAT (encoded by the selectable marker *bar* gene) activity in the transgenic plants can be visualized by autoradiography after the plant extracts incubating with ^{14}C -acetyl coenzyme A followed by elution on the thin-layer chromatography (Spencer et al., 1990). A simpler way is to test for herbicide resistance by spraying or painting the leaf of the transgenic plants with the glufosinate herbicide (Gordon-Kamm et al., 1990). The results are reliable if proper herbicide concentration and controls are used.

Northern blot hybridization analysis

Total RNA or mRNA is extracted from the plant sample and separated by electrophoresis through a denaturing agarose gel. The RNA is then transferred to a nylon membrane. The transgene mRNA can be detected by hybridization with the labeled transgene DNA or RNA probe followed by autoradiography. This analysis provides molecular evidence that the transgene is functionally transcribed.

Transgene Integration, Expression, and Inheritance

Microprojectile-mediated gene transfer has generally resulted in multiple copies of transgene being integrated. Both full-length and rearranged transgenes are frequently detected in variable copy numbers ranging from 1 to more than 20. Transgene rearrangement may involve deletion, ligation, concatenation, and possibly recombination, which could occur before or after integration of the transgene into the host genome (Pawlowski and Somers, 1996; Bower et al., 1996). The multiple copies of the transgene are frequently inherited as a single unit, suggesting that the transgene sequences integrate into the genome at either tightly linked loci or as a single locus rather than randomly distributed in all chromosomes (Pawlowski and Somers, 1996; Takano et al., 1997; Kumpatla et al., 1997). Transgene transmission to the progeny can follow a Mendelian ratio or be distorted (Fromm et al., 1990; Spencer et al., 1992; Walters et al., 1992; Register et al., 1994; Finnegan and McElory, 1994; Kumpatla, 1997).

The expression of the transgene in the primary transgenic plant and its progeny are unpredictable. Transgenes can be inactivated by different mechanisms, such as DNA methylation, and homology-dependent gene silencing (Kilby et al., 1992; Ingelbrecht et al., 1994; Meyer and Heidmann, 1994; Finnegan and McElory, 1994; Matzke, M.A and Matzke, A.J.M., 1995; Stam et al., 1997). Thus, transgene expression has been sometimes considered as an epigenetic trait (Meyer and Heidmann, 1994; Meins and Kunz, 1995; Kumpatla et al., 1997). Selection of highly expressed transgene and stabilization of the introduced trait in the subsequent generations are major challenges for utilization of genetic engineering to improve crop quality.

CHAPTER 3 ANTISENSE VECTOR CONSTRUCTION

Introduction

The objective of the project is to reduce lignin content or modify the lignin component by expressing the antisense version caffeic acid O-methyltransferase (OMT), a gene in the lignin biosynthetic pathway. Before plants can be transformed, a vector must be assembled containing the components necessary for strongly expressing the antisense OMT gene. In addition, an effective selectable marker gene must be incorporated to facilitate recovering transgenic plants. The components for antisense OMT gene expression include the Ubi-1 promoter, first exon and first intron of maize ubiquitin-1 gene (Christensen et al. 1992), a sorghum antisense OMT gene and the nopaline synthase (*nos*) terminator (Jefferson, 1987). The components needed for selectable gene expression are the cauliflower mosaic virus 35S promoter (CaMV 35S; Olszewski et al., 1982), *bar* gene (Thompson et al., 1987), and *nos* terminator. This chapter lists the vector components, their sources and describes how these components were incorporated into a functional unit.

Materials and Methods

The plasmids used to provide the necessary components for the final vectors are summarized in Table 3-1. Dr. Quail (Plant Gene Expression Center, Albany, CA), kindly provided pAHC17 and pAHC20. pBI121 was purchased from Clontech (Palo Alto, CA).

Table 3-1. Plasmids used in the antisense vector construction.

Plasmids	Characteristics	References
pAHC17	Maize Ubi-1 promoter in pUC8	Christensen et al.,1992,1996
pAHC20	Ubi- <i>bar</i> in pUC8	Christensen et al.,1992,1996
pBI121	CaMV 35S-GUS in pUC8	Clontech catalog 1995
pS3	Sorghum OMT in Bluescript	Smith, unpublished data

The construction of the final vectors (see Fig. 3-5) included three steps. (1) The sorghum OMT full-length cDNA, 995 bp 5'-fragment, or 605 bp 3'-fragment were cloned in the antisense direction in pAHC17 downstream of the Ubi-1 promoter in the *Bam*HI site (Fig. 3-1). (2) The Ubi-1 promoter in pAHC20 was replaced with the CaMV 35S promoter from pBI121 to drive the *bar* gene (Fig. 3-2). (3) The OMT antisense gene and the *bar* gene expression units were moved to the same plasmid. All the cloning procedures were done according to standard protocols (Sambrook et al. 1989). The details of those DNA manipulations are described below.

Ubi-OMT Antisense Plasmids

To produce the antisense constructs, the sorghum OMT cDNA had to be cloned in a reverse orientation downstream of the Ubi-1 promoter (Fig. 3-1). The sorghum OMT cDNA full-length (1600 bp), 5'-fragment (995 bp) and 3'-fragment (605 bp) were cleaved by *Eco*RI-*Apa*I, *Eco*RI-*Bam*HI and *Bam*HI-*Apa*I, gel-purified, and made blunt ends by either T4 DNA polymerase (*Eco*RI-*Apa*I and *Bam*HI-*Apa*I fragments) or Klenow fragment (*Eco*RI-*Bam*HI fragment), respectively. pAHC17 was linearized by *Bam*HI and filled in with Klenow polymerase to produce blunt ends. The OMT cDNA fragments were ligated into the blunt-ended pAHC17 by T4 DNA ligase, respectively. The antisense orientation of the insert was determined by restriction digestion. After determination of the antisense orientation by restriction digestion, the junction regions between the Ubi-1 sequence and OMT cDNA antisense of the three constructs were further confirmed by DNA sequencing. A sequence primer in the Ubi-promoter first intron region was designed using Oligo Primer Analysis Software (National Bioscience Inc., MN). The primer contained 17 nucleotides: 5'-TTTTAGCCCTGCCTTCA-3'. The clones were

Fig. 3-1. Diagram of the route for constructing Ubi-OMT full-length antisense construct.

Fig. 3-2. Diagram of the route for constructing CaMV 35S-bar plasmid.

sequenced by the Sequence Core Lab of the ICBR (Interdisciplinary Center for Biotechnology Research) located at the University of Florida.

CaMV 35S-*bar* Plasmid

To construct the CaMV 35S-*bar* plasmid, the Ubi-1 DNA sequence in pAHC20 was replaced with the CaMV 35S promoter from pBI121 (Fig. 3-2). The *HindIII*-*Bam*HI fragment containing the Ubi-1 promoter, first exon and first intron was removed from pAHC20. The *HindIII*-*Bam*HI fragment containing the CaMV 35S promoter in pBI121 was purified from the gel and ligated into the pAHC20 *HindIII*-*Bam*HI site. The resultant plasmid was designated CaMV 35S-*bar*.

Ubi-OMT Antisense/CaMV 35S-*bar* Constructs

The unique *HindIII* site at the 5' end of the Ubi-1 sequence in the antisense constructs was used to insert the chimeric CaMV 35S/*bar* gene (Fig. 3-3). To remove the entire *bar* expression cassette from the CaMV 35S-*bar* plasmid, the plasmid was first digested with *Eco*RI. The *Eco*RI sites were filled in with Klenow and ligated with *HindIII* linkers (5'CAAGCTTG3', New England Labs). Addition of the linker restored the *Eco*RI site. After linker ligation, the DNA was cut with *HindIII* to remove excess linkers and to cut at the 5' end of the cassette. The resulting *HindIII* fragment containing the *bar* expression unit was gel-purified and subcloned into *HindIII*-digested Ubi-OMT antisense constructs, respectively (Fig. 3-3).

Results and Discussion

Ubi-OMT Antisense Plasmids

Different regions of an antisense cDNA may have different effects on the down-regulation of target gene expression (De Lange et al. 1995). Sometimes only the

Fig. 3-3. Procedure for constructing CaMV 35S-bar/Ubi-OMT plasmid.

3'- fragment of the antisense gene has inhibitory effect (Stam et al., 1997). To avoid such problems, sorghum OMT cDNA antisense constructs containing the full-length cDNA (1600 bp), the 995 bp 5'-fragment, and the 605 bp 3'-fragment were assembled. The Ubi-1 promoter of maize, isolated from maize ubiquitin-1 gene, contains 899 bp of promoter sequence, 83 bp of 5' untranslated exon, and 1010 bp of first intron sequence (Christensen et al. 1992). It is a strong monocot promoter and, in addition, it is expressed in all tissues and is stress inducible (Christensen et al. 1996). These features are very useful for the purpose of down-regulating endogenous OMT gene expression. The three OMT antisense fragments were all driven by the maize Ubi-1 promoter and terminated by the *nos* terminator. After determination of the antisense orientation by restriction digestion, the junction regions between the Ubi-1 sequence and OMT cDNA antisense of three constructs were further confirmed by DNA sequencing. The partial sequencing results containing the ligation regions were shown in Fig. 3-4.

CaMV 35S-*bar* Plasmid

CaMV 35S-*bar* plasmid was constructed as an intermediate plasmid for the final vector construction. The *bar* gene, cloned from *Streptomyces hygroscopicus* (Thompson et al., 1987), has often been used as a selectable marker in dicot and monocot transformation systems (De Block et al., 1989; Gordon-Kamm et al., 1990; Gallo-Meagher and Irvine, 1996). To avoid promoter homology that may induce transgene silencing (Meyer 1995), a different promoter was used to drive the *bar* gene. The CaMV 35S promoter is weaker than Ubi-1 promoter in monocots (Cornejo et al., 1993; Gallo-Meagher and Irvine, 1993) but has been successfully used to drive selectable marker

Ubi-1.....AGGATCCCCC TCGAGTTTT.....Full length OMT cDNA, 1600 bp

Ubi-1.....AGGATCGATC CACTTCATGA.....5'- OMT cDNA, 995 bp

Ubi-1.....AGGATCCCCC TCGAGTTTT..... 3'- OMT cDNA, 605 bp

Fig. 3-4. Partial sequencing showing the conjunction regions of Ubi-1 and sorghum OMT antisense cDNA in three different constructs. The underlined sequences resulted from Klenow fill-in of *Bam*HI site on pAHC17.

genes with transgenic plants recovered in rice and maize (Takano et al., 1997; Gordon-Kamm et al., 1990; Zhang et al., 1996).

Ubi-OMT Antisense/35S-*bar* Plasmids

The OMT antisense gene and *bar* gene expression units were cloned on the same plasmid. The final plasmids, containing the two expression units (Ubi-OMT antisense-*nos* and CaMV 35S-*bar-nos*) in the same orientation shown in Fig. 3-5, were used for maize transformation.

Cloning the two genes on the same plasmid has some advantages. Several authors reported coinTEGRATION and coexpression ratios of two transgenes on separate plasmids or on the same plasmid following bombardment-mediated transformation (Klein et al., 1987; Gordon-Kamm et al., 1990; Spencer et al., 1992; Register III et al., 1994). Their results indicated that coexpression ratio could be increased by cloning the two genes together. Moreover, plants with PCR positive for *bar* or herbicide resistance are more likely to carry the OMT antisense gene when they are introduced on the same plasmid. Hence screening is more efficient. This set of constructs can also be used for comparison studies of the most effective target region in OMT for the down-regulation of OMT expression.

Fig. 3-5. Schematic representation of the antisense sorghum OMT vector used for maize transformation. Sorghum antisense OMT can be the full-length, or 5', or 3' of the sorghum OMT cDNA. The arrows on the inner circle indicate the sources of each component.

CHAPTER 4 MAIZE TRANSFORMATION

Introduction

Maize is a major cereal crop and genetic improvement of its agronomic traits by genetic engineering is important. In 1999, nearly one half of the maize grown in the US was transgenic mostly for insect and/or herbicide resistance (Palevitz, 1999). In the past decades, the major efforts were focused on the development of DNA delivery methods. A number of protocols were developed, including protoplast transformation (Fromm et al., 1986; Hansen et al., 1998), electroporation (Dekeyser et al., 1990; D'Halluin et al., 1992), *Agrobacterium*-mediated transformation (Ishida et al., 1996), biolistic particle-mediated (Gordon-Kamm et al., 1990; Walters et al., 1992) and silicon whisker-mediated (Kaeppeler et al., 1992; Frame, et al., 1994) DNA transfer approaches. However, biolistic particle-mediated DNA transfer has been the most commonly reported method. In addition to herbicide, virus and insect resistance, maize has also been used as a biofactory to produce the first commercial recombinant protein 'avidin' in transgenic plants (Hood et al., 1997). Transgene inheritance and expression in maize have also been studied extensively (Fromm et al., 1990; Spencer et al., 1992; Walters et al., 1992; Register III et al., 1994; Zhang et al., 1996).

Tissue culture and regeneration are critical for a transformation system. Maize immature embryos, immature tassels, young leaves, and shoot meristems have been

employed as explant material for tissue culture (Armstrong and Green, 1985; Rhodes et al., 1986; Songstad et al., 1992; Ray and Ghosh, 1990; Lowe et al., 1995). There are two types of embryogenic callus, type-I callus which is compact, and type-II callus which is friable and undifferentiated (Armstrong, 1994). Type-II callus has been generally chosen as bombarded material although type-I and type-II callus and type-II derived suspension cells all have been successfully transformed by direct gene transfer methods. Maize is highly genotype-dependent for type-II callus response and the "Hi-II" line was selected for its high ratio of type-II callus induction (Armstrong et al., 1991). We chose to use "Hi-II" as our model transformation system because of its efficient regeneration.

In the present study, the caffeic acid O-methyltransferase (OMT) gene was selected as the antisense target to modify lignin biosynthesis because OMT is an important enzyme in the lignin biosynthetic pathway. An OMT cDNA, isolated from sorghum, was available for use. Previously, a homologous or heterologous OMT gene was transformed in tobacco and poplar tree to modify lignin components or content (Ni et al., 1994; Atanassova et al., 1995; Doorselaere et al., 1995). To our knowledge, there is no report on monocot transformation using the OMT antisense gene. This chapter reports the successful introduction of heterologous sorghum OMT antisense and *bar* genes into maize "Hi-II" (Armstrong et al., 1991) using the biolistic-mediated method.

Materials and Methods

Plant Material for Callus Initiation

Maize seeds of inbred lines "A" and "B" were obtained from Dr. Marty Sachs, director of Maize Genetics Cooperation Stock Center (University of Illinois at Urbana-Champaign, S-123 Turner Hall, 1102 South Goodwin Ave., Urbana IL 61801-4798). The

"A" line seeds and "B" line seeds were planted in the greenhouse or field, and crossed to obtain hybrid "Hi-II" seeds (Armstrong et al., 1991). The plants produced from these seeds were much stronger plants than the inbred parental lines, and produced high frequency of embryogenic type-II callus.

Callus Initiation

Callus was initiated from both immature embryos and immature tassels of greenhouse grown Hi-II plants. For immature embryo isolation, ears were removed from the plants 9-11 days after pollination and surface sterilized in 10% Clorox plus 0.1% Tween 20 for 20 to 30 min. Immature embryos were isolated using a spatula and forceps under a dissecting microscope. The embryos 1.0-2.0 mm in length were selected and placed onto the initiation medium (Table 4-1) with the embryo axis side down (Armstrong, 1994). Immature tassels were isolated essentially as described by Songstad et al. (1992). Tassels from Hi-II plants were harvested 4-6 weeks after planting in the greenhouse. After the outer leaves were removed, the inner leaves were taken off aseptically in a sterile hood. Immature tassels less than 4 cm were removed, pooled and cut into 1.0-1.5 mm segments. These segments were cultured on the initiation medium (see Table 4-1) and after two weeks of incubation, the fragments were transferred to fresh initiation medium. Once the type-II callus was formed (Fig. 4-1), it was transferred to the maintenance medium (see Table 4-1). Cultures for callus initiation and maintenance were conducted in the dark at 28°C. Type-II callus was selected and transferred to fresh maintenance medium every 14 days.

Table 4-1. Summary of media used in maize tissue culture (all chemicals obtained from Sigma Co.)

Media		Components and Concentrations	Reference
Callus Initiation Medium		N6 salts, L-proline 2.88 g/L, Eriksson's vitamins, thiamine HCl 0.5 mg/L, casamino acids 100 mg/L, 2,4-D 1.0 mg/L, sucrose 2%, silver nitrate 10 μ M	Songstad et al., 1992
Callus Maintenance Medium		The same as initiation medium, except 2,4-D 2.0 mg/L and without AgNO ₃	Armstrong et al., 1994
Selection Medium		The same as maintenance medium except without adding proline and casamino acids. Filter sterilized glufosinate was added to a final concentration of 3 mg/L after autoclaving.	Dennehey et al., 1994
Osmotic Medium		Maintenance medium plus 0.4 M sorbitol.	Vain et al., 1996
Regeneration Medium	R1	MS salts, nicotinic acid 1.3 mg/L, pyridoxine HCl 0.25 mg/L, thiamine HCl 0.25 mg/L, Ca-pantothenate 0.25 mg/L, myo-inositol 100 mg/L, asparagine 1 mM, 2,4-D 0.1 mg/L, ABA 0.1 μ M, sucrose 2%, phytagel 0.2%	Armstrong et al., 1994
	R2	N6 salts, nicotinic acid 0.5 mg/L, pyridoxine HCl 0.5 mg/L, glycine 2.0 mg/L, sucrose 6%, phytagel 0.2%	
	R3	MS salts, nicotinic acid 1.3 mg/L, pyridoxine HCl 0.25 mg/L, thiamine HCl 0.25 mg/L, myo-inositol 100 mg/L, asparagine 1mM, sucrose 2%, phytagel 0.2%

Fig. 4-1. Embryogenic type-II callus initiated from immature tassels.

Bombardment

The plasmids used were *Ubi-bar/Ubi-gus* (pAHC25), *Ubi-gus* (pAHC27) (Christensen and Quail 1996; provided by P. H. Quail, Plant Gene Expression Center, Albany CA), and the three antisense vectors. DNA of these plasmids was purified using the Qiagen kit (Qiagen Inc., Valencia, CA). *Ubi-gus* was used for optimization of bombardment conditions via transient expression of GUS. *Ubi-bar/Ubi-gus* was bombarded alone as a positive control for later screening of stable transformation. For the antisense vector bombardment, cotransformation was performed using 1:1 molar ratio of each of the antisense vectors in combination with pAHC27 (*Ubi-gus*), pAHC27 facilitating analysis of bombardment efficiency. DNA of the above plasmids was precipitated onto 1.0 μm gold particles (Bio-Rad Laboratories, Richmond, CA) using a protocol described by Daines (1990). The precipitation mixture contained 2.1 mg of gold, 25 μl of plasmid DNA (1 $\mu\text{g}/\mu\text{l}$), 220 μl of sterile water, 250 μl of 2.5 M CaCl_2 , and 50 μl of 0.1 M spermidine. After adding the components in the above order, the mixture was vortexed at 4°C for at least 10 min and centrifuged for 5 min. The supernatant was removed and the DNA/gold precipitate was resuspended in 600 μl of absolute ethanol. The ethanol was removed after centrifuging for 1 min and the pellet was resuspended in 36 μl of absolute ethanol by pipetting up and down. About 4-10 μl of the suspension was pipetted onto a macrocarrier for bombardment.

DNA concentration, helium pressure, osmotic treatment of bombarded tissue and numbers of shots per tissue sample were important factors affecting stable transformation, and they were optimized after evaluation with transient GUS assays using *Ubi-gus* (see Fig. 4-2).

Fig. 4-2. GUS histochemical assay 24 hr after bombardment.
A. Non-osmotic treatment, using 6 μ g DNA, 650 psi, one shot.
B. Non-osmotic treatment, 6 μ g DNA/shot, 650 psi, two shots.
C. Osmotic treatment, 6 μ g DNA/shot, 650 psi, two shots.
D. Osmotic treatment, 10 μ g DNA/shot, 1300 psi, two shots.

The optimized bombardment conditions were: 650 psi, 8-10 µg DNA per shot, one or two shots per plate, osmotic treatment (see Table 4-1) of callus 4 hr pre-bombardment and 16 hr post-bombardment. A Petri dish containing callus (ca 1.5-2.0 cm in diameter) was placed in the Biolistic PDS-1000/He device (Bio-Rad) on the third shelf (from the bottom).

Transformant Selection

Bialaphos, glufosinate and Ignite® all can be used for the selection of *bar* gene transformants (Dennehey et al., 1994), because they have phosphinothricin (PPT) as the active component. However, bialaphos and glufosinate are not commercially available. Since selection is crucial for a transformation system and there is a large variation in the reported concentration of selection agent used, kill curves were made for the different selection agents to determine the proper concentration to use. Bialaphos, glufosinate and Ignite® at the rate of 1.0 mg/l, 3.0 mg/l and 5.0 mg/l in the maintenance medium without proline and casamino acids (Dennehey, 1994) were used to compare the efficiency of selection. Two replicates (each containing ca 400 mg type-II callus) were used at each treatment. After two weeks, callus from each plate was weighed and ca 400 mg callus was transferred to the fresh medium. The callus growth rate on each treatment was calculated by the equation: (final weight-initial weight)/initial weight (Dennehey, et al., 1994). The final growth rate was calculated as a percentage of a treatment's growth rate compared to the control (defined as 100% growth). After six weeks of selection, the callus growth rate at 1 mg/l of bialaphos, glufosinate, or Ignite® was 15.8%, 16.2%, and 18.7%; while at 3 mg/l, the growth rate was 2.5%, 3.3%, and 6.3%; at 5 mg/l, the growth rate was 1.5%, 2.5%, and 4.25%, respectively. A significant difference was observed

between 1 mg/l and 3 mg/l of each selective agent ($p=0.033$), but there was no significant difference between 3 mg/l and 5 mg/l ($p=0.21$). Based on the kill curve results and reported data (Zhang et al., 1996; Zhong, et al., 1996), 3 mg/l of glufosinate was used for the selection of transformed callus.

After bombardment, callus remained on the osmotic medium used in the pre-bombardment treatment for 16 hr, transferred to the maintenance medium for 2-6 days (until callus growth resumed), and then transferred to the selection medium (see Table 4-1). Callus was subcultured every 14 days. After 6-8 weeks of selection, resistant callus could be distinguished.

Regeneration of Transformed Callus

Various protocols for maize regeneration were described in the literature. The three-step protocol described by Armstrong (1994) was chosen based on the tests of the four regeneration protocols (Armstrong and Green 1985; Shillito et al., 1989; Armstrong 1994; Tomes et al., 1995) in the present study. The recipes for the three regeneration media are listed in Table 4-1.

The transformed callus was regenerated in the presence of glufosinate. To reduce the number of escapes, glufosinate concentration was increased through the 3 stages from 4 mg/l to 6 mg/l. Resistant callus pieces were broken into small pieces (< 2 mm) and transferred to the R1 medium containing 4 mg/l of glufosinate. After 2 weeks of incubation in the dark at 28°C, the callus was transferred to the R2 medium and grown in the dark for another 2 weeks at 28°C. The first two subcultures stimulated maturation of the type-II callus. The callus was then transferred to R3 medium containing 5 mg/l of glufosinate and moved to a lighted growth chamber (16 hr light : 8 hr dark) at 25°C for

shoot development. After about 2 weeks, the shoots were transferred into Magenta vessels (Sigma) containing R3 medium and 6 mg/l of glufosinate to induce roots. After approximately 2–3 weeks, the plantlets with good root systems were carefully washed with tap water to remove the medium residue and planted in autoclaved, well-watered Metromix-220 (Scotts-Sierra Horticultural Products Company, OH) in 4-inch pots. For the first 2 days after transfer, the plantlets were kept in a high humidity environment for hardening, then the humidity was gradually reduced. After 7-10 days, plantlets were moved to the greenhouse. Plants were covered with two layers of cheesecloth to avoid direct sunshine for 2-3 days and then treated normally as for seed-germinated plants. When plants reached a 6-leaf stage, they were transferred to bigger pots containing soil and Metromix- 220 (2:1). Plants were fertilized with Peter's 20-20-20 (Division of United Industries Corp., MO) weekly and sprayed regularly with Avid (Merck & Co., Inc. NJ) and/or Malathione (Chevron Chemical Company, IL) according to the manufacturer's directions for the control of mites and insects.

Producing the Progeny of Primary Transgenic Plants

Primary transgenic plants were either selfed or crossed with non-transformed regenerated plants to produce seed. Because male flowering and silking were not always synchronous, pollen were collected, dried for ca 2 hr at room temperature, and stored at -70°C. When silks appeared, they were pollinated with the stored frozen pollen.

PCR Analysis

Genomic DNA was extracted using a scaled-down procedure of Dellaporta (Dellaporta et al., 1983). This method uses SDS and potassium acetate to precipitate the impurities. Ten to twenty mg of young leaf tissue was homogenized in an eppendorf tube

with a disposable pellet pestle (Fisher Scientific) in the presence of extraction buffer (Tris-HCl 100 mM, EDTA 50 mM, NaCl 500 mM, pH 8.0, β -mercaptoethanol 10 mM). Remaining steps were the same as Dellaporta's method except that all solutions used were scaled down 1/50.

PCR primers specific for the *bar* gene (sequence obtained from GenBank) were designed using Oligo Primer Analysis Software (National Bioscience Inc., MN). The forward and reverse primers were 5'-GGCGGTCTGCACCATCGT-3' and 5'-GCCAGTTCCTCGTCTGA-3', respectively. The resulting amplified fragment was 419 bp in length.

PCR conditions were as follows: 10 $\times$ PCR buffer (Promega) 2.5 μ l, $MgCl_2$ (50 mM) 1.0 μ l, dNTP (10 mM) 1.0 μ l, *bar* primers (2.5 mM) 0.2 μ l, Taq polymerase (5 u/ μ l) 0.2 μ l, DNA 0.5-1.0 μ g, ddH₂O to a total volume of 25 μ l. PCR was performed as (1) initially 2 min at 94°C, (2) 35-40 cycles for 30 sec at 94°C, 1 min at 55°C, and 1 min 30 sec at 72°C, (3) finally 5 min at 72°C. After amplification, the samples were loaded on 1.5 % agarose gel and the result was visualized by ethidium bromide staining after electrophoresis.

Southern Blot Analysis

Genomic DNA was extracted from 1 g sample of leaf tissue using the Dellaporta protocol with some modification. After isopropanol precipitation, the pellet was resuspended in 700 μ l of TE buffer (50 mM Tris-HCl, 10 mM EDTA, pH 8.0). Ten microliters RNase (10 mg/ml) were added and incubated at 37°C for 30 min. The samples were first extracted with equal volumes of phenol: chloroform (1:1) and then

chloroform: isoamyl alcohol (24:1). The supernatant was removed and DNA precipitated by adding 70 µl of 7.5 M NH₄AC and 700 µl of absolute alcohol.

Ten micrograms of genomic DNA was digested with *Hind*III or *Eco*RI restriction enzyme and separated on a 0.8% agarose gel. For copy number determination, a 1C value (haploid DNA content of maize) of 2.75 pg was used (Arumuganathan and Earle 1991). The plasmid DNA was digested with the same restriction enzymes used for genomic DNA digestion and diluted to the required copy concentration. After DNA was transferred to H-bond N (Amersham) membrane by capillary blotting procedure (Southern, 1975), the membrane blot was UV-crosslinked with a Foto UV 300 DNA Transilluminator (Fotodyne Inc., New Berlin, WI) for 3 min.

DNA probe of *bar* or OMT gene labeled with [³²P] dCTP was synthesized using Prime-A-Gene kit (Promega Co., Madison, WI) and the probe was purified with a Sephadex G-50 chromatography column (Sambrook, 1989). The blot was prehybridized in a solution containing 7% SDS, 0.5 M NaH₂PO₄, 1% BSA and 100 µg/ml fragmented salmon sperm DNA for 4 hr at 65°C. Hybridization was carried out overnight in the same solution plus radiolabeled DNA probe at 65°C. The washing conditions were 2×SSC [20 x SSC stock (g/l): NaCl 175.3 g, Na-citrate 88.2 g, pH 7.0], 0.1% SDS 10 min, 65°C, two times; 0.1×SSC, 0.1% SDS, 65°C, 15 min, two times. The membrane was exposed to X-ray film with intensifying screens at -70°C for 2--5 days.

Northern Blot Analysis

Total RNA was isolated using Trizol reagent (GIBCO, BRL) according to the manufacturer's instruction. Ten micrograms of total RNA were size-fractionated on 1% agarose formaldehyde gels and transferred to H-bond N membrane (Amersham). DNA

probe (*bar* or OMT gene coding region) synthesis and hybridization were performed using the same conditions as Southern blot analysis described above.

GUS Histochemical Staining

To ensure the bombardment conditions were working well, the bombarded callus was tested for GUS transient expression through staining with 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) as described by Jefferson (1987). Callus pieces were incubated with the reagent containing X-Gluc overnight at 37°C in the dark.

Leaf Painting Assay

Regenerated plants and their progeny were screened for herbicide resistance by painting a 2-cm segment of the young and healthy leaf with 1% Ignite® (containing 200 g/l of glufosinate). Results were scored after 5-7 days.

Results

Bombardment, Selection and Regeneration of Putative Transgenic Plants

A total of 54 plates with type-II callus covering the target area were bombarded using the standard bombardment conditions (see Table 4-2). Four plates were shot using pAHC25 (*Ubi-gus/Ubi-bar*) to produce some positive control plants for both *gus* and *bar* genes to facilitate screening. The remaining 50 plates were cobombarded with each of the antisense vectors in combination with pAHC27 (*Ubi-gus*). Several pieces of callus from plates after bombardment were tested for GUS expression. Remaining bombarded calli were then transferred to the selection medium after 2-6 days of recovery. After 6-8 weeks of selection, a total of 68 glufosinate-resistant embryogenic callus pieces were obtained. Fig. 4-3A shows one of the resistant callus pieces. Generally, the resistant

Table 4-2. Summary of the bombarded plasmids and the test results (PCR and herbicide resistance) for the regenerated plants conducted in the transformation experiments.

Experiment	Plasmid DNA bombarded	No. of plates	No. of resistant callus	Plants		
				No. of plants regenerated	No. of PCR positive for <i>bar</i>	No. of herbicide resistant
1	pAHC25	4	2	12	12/12	12/12
2	Full-Length Antisense OMT & pAHC27	25	18	247	125/152	130/221
3	5'- Antisense OMT & pAHC27	12	23	70	37/40	43/70
4	3'-Antisense OMT & pAHC27	13	25	50	Not determined	9/50

Fig. 4-3. Procedure of obtaining transgenic maize plants. (A) A resistant callus showing up after 6 week of glufosinate selection; (B) Resistant calli on the shooting medium (4 mg/L glufosinate); (C) Untransformed control calli on the same shooting medium as in (B); (D) Plantlets on the rooting medium in the Magenta box; (E) Greenhouse grown transgenic maize after pollination.

callus was fast growing while the non-transformed callus became yellow or brown and watery.

Resistant callus was transferred to the regeneration medium containing glufosinate. When shoots developed (Fig. 4-3B), they were transferred into Magenta vessels containing R3 medium and 6 mg/l of glufosinate to produce roots (Fig. 4-3D). Plantlets were transferred to pots containing Metromix-220 when they had a good root system. After a hardening stage, they were moved to the greenhouse. A total of 376 plants were obtained through this process (Fig. 4-3E).

Analysis of Primary Transgenic Plants

PCR analysis

PCR amplification of *bar* gene sequences was used to initially screen transgenic plants. Two hundred and four plants regenerated in the presence of glufosinate were tested by PCR (see Table 4-2). About 85% of these plants were PCR positive. Fig. 4-4 shows representative PCR results.

Herbicide resistance test

Regenerated plants were tested for herbicide resistance to confirm that they were transgenic plants. Since the *bar* confers resistance to glufosinate, if it was expressed in the transgenic plants, the plants would resist glufosinate damage. One percent Ignite® (containing 200 g/l of glufosinate) was painted on a 2-cm leaf segment with a cotton swab. After 5-7 days, leaves of transgenic plants remained green while leaves of control plants became yellow and died (Fig. 4-5). About 55% of the 350 tested plants were herbicide resistant (see Table 4-2), however, some of the PCR positive plants (5% among the PCR-tested plants) were not resistant to the herbicide. This might be due to

Fig. 4-4. PCR analysis of the regenerated plants in the presence of glufosinate. Arrows indicate the expected amplified fragments (418 bp) using *bar* gene primers. M: DNA ϕ x174 marker(*Hae*III); P: Plasmid DNA as positive control; C: Non-transformed plant control. Each lane represented a different plant sample.

Fig. 4-5. Leaf-painting assay. Regenerated plants were screened for glufosinate resistance by painting 2-cm sections of the leaf with 1% Ignite. After one week of herbicide application, transgenic plants showed no response while control and non-transformed plant leaves died. Leaf segments were cut from the plants for photography.

transgene silencing, DNA rearrangement resulting in no gene function, or a low level of *bar* expression so that the transgenic plants would not resist the level of the herbicide tested.

Southern blot analysis

Transformation of maize was further confirmed by Southern blot analysis. Total genomic DNA was isolated from herbicide-resistant plants regenerated from independently bombarded callus. The DNA was digested with *Hind*III, which released the entire expression cassette of *bar* gene (CaMV 35S-*bar-nos*) as a 1.7 kb fragment. After hybridization with the *bar* gene coding sequence, positive bands for the *bar* gene were observed in all the transgenic plants (Fig. 4-6). The hybridization pattern was different from sample to sample, which indicated these samples were from independent transformation events. Some of the samples contained both the 1.7 kb expression unit and higher molecular weight bands, while the other samples contained only larger fragments or the 1.7 kb fragment. The presence of extra bands other than the 1.7 kb fragment suggested the rearrangement of the transgene. The copy number of the *bar* gene (intact and rearranged) varied from 1 to more than 20 copies as compared with the banding intensity of the 1 and 5-copy of the plasmid reconstructions.

Production of Progeny of the Primary Transgenic Plants

Among the 376 regenerated plants, 29 plants died within several days after they were moved to the greenhouse, and less than 10 plants, showing abnormal phenotype such as rosette and dwarf, did not reach maturity. The remaining plants were normal and were tested for herbicide resistance. Only those plants showing herbicide resistance, PCR positive for *bar*, or the brown midrib phenotype were kept to maturity. When these plants

Fig. 4-6. Southern blot analysis of primary transgenic plants. Genomic DNA (10 μ g) was digested with *Hind*III to release the 1.7 kb fragment (arrow position) of the *bar* gene expression unit and hybridized with the *bar* gene probe. Lanes 1 to 4 represent different transgenic plant samples, labeled by plant ID number. Plasmid DNA was also digested with *Hind*III, diluted to represent 1 and 5 copies of the *bar* gene and mixed with the digested genomic DNA from the untransformed plant. λ -DNA (*Hind*III/*Eco*RI) was used as DNA size marker. Bm designations indicate the plants with brown midrib phenotype.

Table 4-3. Summarized data for the regenerated potentially transgenic plants and their progeny.

Regenerated Plants	No. of plants died naturally after move to greenhouse	No. of plants reaching maturity *	No. of plants producing kernels	No. of seeds per ear
376	29	215	198	1 - 3 to more than 100

* Only the PCR positive, or herbicide resistant, or brown midrib plants were kept to maturity.

reached maturity, they were either selfed or cross-pollinated (as male or female parent) with non-transformed control plants. Most of the transgenic plants were fertile and had set seeds (see Table 4-3), with seed numbers varying from 1-3 (5 plants) to more than 100 per ear. Only plants regenerated from one independent callus line did not have seed set at all. Southern blot analysis of those plants indicated that the genomic DNA contained smearing bands of *bar* gene (data not shown).

Analysis of the Progeny of Primary Transgenic Plants

Herbicide test

Five seeds from each of the 40 transgenic plants were planted and herbicide resistance of the seedlings was determined by painting leaves with 1% Ignite. Among the 200 plants tested, 72.8% showed resistance. The negative plants probably resulted from genetic segregation of *bar* gene, as one would expect in progeny of a self-fertilized, hemizygous parent.

Southern blot analysis of the progeny

Southern blot analysis of genomic DNA extracted from progeny of the transgenics was performed to investigate the transgene integration patterns and transgene copy number. For the *bar* gene investigation, genomic DNA was digested with *Hind*III, which released a 1.7 kb (CaMV 35S-*bar-nos*) fragment of *bar* gene expression cassette. After electrophoresis in agarose and transfer to nylon membrane, hybridization was carried out using *bar* coding region as a probe. All herbicide-resistant plants contained bands (Fig. 4-7A and B lanes 4-6), whereas some of the nonresistant progeny (bm10-2, bm4-3, and bm8-3) did not show a hybridization signal (Fig. 4-7B lanes 1-3). This suggested segregation of the *bar* gene. However, plant bm17-2 showed a hybridization band similar

Fig. 4-7. Southern blot analysis of the progeny of transgenic plants. Genomic DNA (10 μ g) was digested with *Hind*III to release the 1.7 kb fragment (arrow position) of the *bar* gene expression cassette and hybridized with the *bar* gene probe. Plasmid DNA was also digested with *Hind*III, diluted to represent 1 copy and 5 copy of the *bar* gene and mixed with the digested genomic DNA from the non-transformed plant. λ -DNA (*Hind*III) and ϕ X174 DNA (*Hae*III) were used as size markers. Bm10-2, bm8-3, and bm4-3 were sensitive to herbicide and showed no hybridization signal. Bm designations indicate the parent had brown midrib phenotype.

to that of other siblings but was sensitive to the herbicide (compare Fig. 4-7A lanes 6-8 with B lane 6), which may suggest the *bar* gene was silenced. Samples from two independent transgenic plants lacked the 1.7 kb expression unit and only contained larger hybridization fragments (see Fig. 4-7A lanes 6-8 and B lane 4). However, these plants were herbicide resistant, and therefore still had a functional *bar* gene. The *bar* gene copy number of these transgenic plants varied from 1 (e.g. family bm17) to more than 20 copies (e.g. family bm8) compared with the plasmid 1 copy and 5 copy reconstructions. In one transformation event, the hybridization profile suggested two integration loci of the transgene as revealed by the segregation of the bands in bm8-6 compared to the other four siblings (compare Fig. 4-7A lanes 1-4 with A lane 5). The above results indicated the *bar* gene was stably transmitted to the progeny.

To determine the integration of the antisense OMT gene in the genome of transgenic plants, Southern blot analysis was done with some of the herbicide-resistant progeny samples. Genomic DNA was digested with *EcoRI* to release a 2.5 kb (from the full-length OMT antisense vector) or a 1.9 kb (from the 5'- OMT antisense vector) fragment. These fragments did not contain the entire expression unit (Ubi-OMT antisense-*nos*) but did contain the sorghum OMT antisense region, ca 600 bp of the Ubi-1 promoter sequence and the *nos* terminator. The maize endogenous OMT was expected as a single band of 5.4 kb (Colloza, 1992). Hybridization was performed using the sorghum OMT gene probe under high stringency conditions. Interestingly, the hybridization results (Fig. 4-8 lanes 16-17) showed that the maize Hi-II control plants had three hybridization bands (ca 9.4 kb, 6.5 kb, and 4.4 kb, respectively) rather than a single band. This might indicate polymorphism for the OMT gene in maize. As indicated in Fig. 4-8,

Fig. 4-8. Southern blot analysis of the progeny of the transgenic plants. Genomic DNA (10 μ g) was digested with *Eco*RI to release the 2.5 kb or 1.9 kb fragment of the partial sorghum OMT antisense expression cassette (full-length or 5'-fragment of sorghum cDNA) and hybridized with sorghum OMT cDNA probe. The partial sorghum OMT antisense expression cassette includes part of the Ubi-1 promoter 1st intron region (ca 600 bp), antisense sorghum OMT (full-length or 5'-fragment of the sorghum OMT cDNA), and *nos* terminator. λ -DNA (*Hind*III) and ϕ X174 (*Hae*III) were used as DNA size marker. Bm designations indicate the parent had brown midrib phenotype.

most of the herbicide-resistant plants contained sequence that hybridized to OMT but differed from the control; some contained the 2.5 kb or 1.9 kb fragment as well as bigger fragments. The copy number of the 2.5 kb or 1.9 kb fragment was 1 to more than 5. The hybridization banding pattern in Fig. 4-8A lane 12 revealed that the antisense OMT in plant bm8-6 segregated as compared with the other siblings (Fig. 4-8 lanes 9-11), which was consistent with the segregation of the *bar* gene. This suggested that the transgene was integrated at more than one locus. Two herbicide-resistant plants (bm17-3 and bm17-4) were similar to the control when hybridized with OMT, which suggested the deletion of the OMT transgene. Some of the transgenic plants lost one or two of the endogenous OMT hybridization bands (e.g. Fig. 4-8 lanes 1-4). OMT banding patterns were different in two ways: (1) progeny from different parents (plant 7 and bm128) having similar *bar* gene hybridization patterns (compare Fig. 4-7 lanes 9-13 with Fig. 4-8 lanes 3-5 and lanes 7-8), and (2) transgenic siblings having similar *bar* gene banding patterns (Fig. 4-8 lanes 3-5, lanes 7-8, and lanes 9-11). These results suggested that the OMT antisense gene has multiple rearrangements.

Transgene expression

Expression of the selectable marker *bar* gene and antisense OMT gene was investigated. Total RNA was extracted from progeny of transgenics regenerated from independent transformation events. Ten micrograms of total RNA were used for each sample. The blot was sequentially hybridized with OMT and *bar* probes. As a control for loading and transfer of RNA, the blot was also hybridized with a 28S rDNA probe. The expression of *bar* varied from plant to plant. The transcripts of the *bar* were not observed in plant 66-5 (Fig. 4-9 lane 2), which was consistent with its lack of herbicide

Fig. 4-9. Northern blot analysis of the progeny of transgenic plants. Total RNA (10 μ g) was loaded for each sample. The membrane was sequentially hybridized with sorghum OMT, *bar* and 28S rDNA probes. The approximate sizes of the transcripts were marked on the right. The 1.7 knt transcript is the endogenous OMT mRNA of maize. The 1.6 knt and 1.0 knt transcripts represent the full-length, and 5'-fragment of the sorghum OMT antisense RNA, respectively.

resistance. OMT antisense transcripts could be detected in only a few samples as compared to the 1.7 knt endogenous OMT mRNA (Fig. 4-9A lanes 1, 4 and 7, and B lanes 1 and 2). In plants bm6-1 and bm6-3 (Fig. 4-9B lanes 1 and 2), the size of the antisense transcripts appeared to remain unspliced, which might be due to the loss of a splicing site resulting from OMT DNA rearrangement (compared with Fig. 4-8 lanes 1 and 2). In some cases, the level of endogenous OMT transcript was lower than the control (Fig. 4-9A lanes 1 and 3), while in other cases, the endogenous OMT mRNA level was higher than the control (Fig. 4-9A lanes 4-8, and B lanes 1 and 2). The reduced endogenous OMT mRNA level suggests the stimulation of RNA turnover by antisense transcripts. No correlation was observed between the antisense OMT transcript level and the endogenous OMT mRNA level, and between *bar* expression level and the antisense OMT expression level.

Discussion

Immature Tassel as Starting Material

Various explants have been used in successful transformation of maize, such as immature embryos, shoot meristem, immature embryo derived type-I or type-II callus or suspension cells initiated from type-II callus. For the maize Hi-II system, we preferred to initiate type-II callus from immature tassels, which has several advantages over immature embryos. (1) It takes 4-6 weeks to harvest immature tassels after first planting, while it takes at least 10 weeks to obtain suitable immature embryos. (2) Surface sterilization is not necessary because the immature tassels are deep inside the leaf whorl and free of contamination. (3) It is easy and quick to manipulate immature tassels, while it is time-consuming and difficult to dissect the tiny immature embryos. (4) Immature tassels are

F1 hybrid tissue and of homogenous genotype because they are pre-meiotic. Type-II callus was initiated directly from the discrete meristematic regions of the immature tassel segments (Songstad, et al. 1992) rather than from the scutella tissue of immature embryos (Armstrong, 1994). Less variation during regeneration was observed compared with the immature embryos derived type-II callus (data not shown).

Optimization of Bombardment Parameters

Gene transfer into plant cells via a microprojectile gun has been successful in transforming many crops including maize, rice, sugarcane, wheat and barley (Gordon-Kamm et al., 1990; Christou et al., 1991; Gallo-Meagher and Irvine 1996; Vasil et al., 1992; Wan and Lemaux 1994). Various protocols have been developed. The details may vary greatly, but the major point is to deliver the DNA-coated particles efficiently into plant cells. A number of factors affect this process. Transient GUS expression assays are very useful to evaluate these factors because the cells expressing GUS can be visualized in 48hr. However, transient expression by cells is not predictive of stable transformation frequencies between different cultures. Sometimes, if the cells were greatly damaged, the stable transformants could not continue to divide and died, although transient expression was very high (Fromm, 1994). Osmotic treatment of callus before and after bombardment is an efficient way to prevent cell damage. Osmotic treatment of callus can increase both transient GUS expression and stable transformation because osmotic treatment is believed to induce plasmolysis of the cells so that fewer cells are disrupted by the penetrating particles (Vain et al., 1993). Another major factor affecting stable transformation is the tissue to be bombarded. Highly embryogenic callus is generally preferred, because it divides vigorously and has a better chance of surviving the stress

during selection and regeneration. Even type-II callus is morphologically heterogenous, and the "pre-embrogenic" morphotype may be more amenable to transformation as this morphotype of type-II callus has "a greater degree of developmental plasticity with respect to cell fate" (p. 729, Welter et al., 1995). The proembroids of type-II calli as bombarded material resulted in more stable transformation events in our experiments.

A Reliable Selection System

Once the bombardment parameters were optimized, the next critical aspect to obtain transgenic plants was to have a reliable selection system because stable transformation is a rare event. Various selectable marker genes, such as *nptII*, *hph*, and *bar* have been used successfully in maize and other monocot species transformation. Of these, the *bar* gene encoding phosphinothricin acetyltransferase (PAT) was most commonly used possibly because (1) phosphinothricin (PPT) is a non-selective herbicide, and (2) the plants can be scored phenotypically by painting or spraying with the herbicide. PPT is an analog of glutamine and it can inhibit glutamine synthetase (GS). PAT detoxifies the herbicide PPT via acetylation so that the transformed cells can survive while untransformed cells are killed. Both glufosinate and bialaphos contain PPT as the active ingredient, but their chemical structure is different. Glufosinate is the ammonium salt of PPT, while bialaphos is the fermented product, which is a tripeptide: PPT with two alanyl groups attached. Bialaphos becomes active only after the two-alanine residues have been cleaved (Tachibana and Kaneko, 1986). Both bialaphos and glufosinate have been used as selective agents to recover transgenic maize (Gordon-Kamm et al., 1990; Zhang et al., 1996). When 3-5 mg/l of bialaphos, or glufosinate, or Ignite (calculated as glufosinate concentration) was used for the kill curve, the callus growth inhibition rate

was more than 90% after six weeks of selection for all of the three selective reagents. Since bialaphos and glufosinate are not commercially available, Ignite® is potentially an alternative for maize transformation.

We were able to obtain 68 resistant callus colonies (some of them might derive from the same transformation event) from 54 bombardments using 3 mg/l of glufosinate and breaking the callus into small pieces. Moreover, about 55% of the regenerated plants were resistant to 1% Ignite. This was achieved by increasing the concentration of glufosinate step by step during regeneration. In some of the transgenic maize work, no selection agent or a low concentration of selective agent was added during regeneration, but the recovery of transgenic plants sometimes failed (Fromm et al., 1990; Pescitelli and Sukhapinda 1995). Perhaps because the resistant callus was chimeric, during regeneration, the non-transformed cells were more competent than the transgenic cells in the absence of selective agent. Bower et al. (1996) reported that increasing selection pressure at the regeneration stage resulted in no escapes with transformed sugarcane. Our results also support this idea. Moreover, to ensure a reliable selection system, it is worthwhile maintain some unbombarded callus with and without selection in parallel with the bombarded callus for comparison.

Transgene Integration, Inheritance and Expression

As summarized by Pawlowski and Somers (1996), microprojectile bombardment-mediated DNA delivery results in transgene integration patterns that generally exhibit the full-length introduced transgene, transgene rearrangements that differ in size from the full-length introduced gene, and variation in copy number. Our data were consistent with such observations. The number of transgene copies varied from 1 to more than 20. In a

portion of analyzed progeny (families 7, bm17 and bm128), these copies seemed to cosegregate as a transgene locus which may indicate that the transgene integrated into the genome at either tightly linked loci or into a single locus. In family bm8, segregation of the hybridization patterns indicated the transgene was integrated at more than one locus.

Transmission of transgenes can be Mendelian or biased (Fromm et al., 1990; Gordon-Kamm et al., 1990; Walter et al., 1992; D'Halluin et al., 1992; Spencer et al., 1992; Omirulleh et al., 1993; Koziel et al., 1996; Register III et al., 1994; Frame et al., 1994; Zhang et al., 1996; Kumpatla et al., 1997). In the present study, the inheritance of the transgene is complicated, as revealed by Southern blot analysis. The selectable *bar* gene was stably transmitted to the progeny. The *bar* gene hybridization pattern in some progeny was the same among siblings (families bm128, 7 and bm17), while in other progeny, the hybridization pattern appeared to segregate (family bm8). The different hybridization pattern of the *bar* gene due to recombination during sexual reproduction was not observed among the progenies studied. However, that was not the case with the OMT antisense gene. The OMT hybridization pattern was different among individual siblings (Fig. 4-8). In two cases (plant bm17-3 and bm17-4) the OMT antisense gene seemed deleted, as hybridization patterns of those plants were the same as the control. OMT DNA rearrangements were observed in both low and high-copy transformation events with higher copy numbers of the OMT antisense gene having the most DNA rearrangements (compare Fig. 4-8 lanes 9-11 with lanes 3-5). The different binding patterns of OMT among transgenic siblings and the disappearance of endogenous OMT bands suggested that recombination between transgene OMT copies, and between transgene OMT and endogenous OMT might occur during meiosis.

We have not focused on the investigation of the transgene transmission, but based on the results of the progenies we analyzed, conclusions can be drawn. (1) The *bar* gene was stably transmitted without detectable rearrangement, and (2) the OMT antisense transgene was not stably transmitted as revealed by the different Southern hybridization patterns among the siblings with multiple rearrangements. Transgene DNA rearrangements include deletion, duplication, and translocation or sometimes even single base pair changes (Pawlowski and Somers 1996; Takano et al., 1997). However, Southern blot analysis cannot determine the exact type of DNA rearrangement. A closer examination of transgene inheritance is necessary to answer whether the transgene transmission follows Mendelian segregations.

Many factors can affect transgene expression, such as the DNA integration site within the genome (position effect), the intactness of the transgene expression cassette, and transgene inactivation. As for the inheritance of transgene expression, it is more complicated (Spencer et al., 1992; Walter et al., 1992; D'Halluin et al., 1992; Zhang et al., 1996; Kumpatla et al., 1997). Progeny of the primary transgenic plants were examined for transgene expression by the herbicide resistance test. Most of the progeny analyzed by leaf-painting assays were herbicide resistant. However, some of them were sensitive, which may mainly be attributed to *bar* segregation as indicated by Southern blot analysis (Plant bm8-3, bm10-2 and bm4-3 in Fig. 4-7). We also observed in the progeny of transgenic plants that while *bar* was transmitted, the plant was sensitive to the herbicide (Plant bm17-2). This suggested that *bar* was silenced.

Northern analysis data indicated that the expression levels of *bar* and the OMT antisense gene varied. No correlation was observed between the *bar* gene and OMT

antisense gene mRNA steady state levels. Furthermore, no correlation was found between the transgene copy number and its expression level. In antisense transgenic plants, several situations were documented (1) endogenous and antisense transcripts disappeared, (2) both existed, and (3) one was more predominant than the other (Van der Krol et al., 1988; Cannon et al., 1990; Stockhaus et al., 1990; De Lange et al., 1996). The true mechanism is not understood yet. In our experiments, we were able to detect antisense transcripts in only a few samples. However, the presence of the antisense transcript did not result in reduction of the endogenous OMT mRNA steady state level. This may be explained as follows. (1) Different localization of the endogenous OMT mRNA and the antisense OMT RNA molecules prevented them from formation of duplex and triggered degradation, or (2) the detected antisense RNA molecules might be transcribed from a different cell type. In some of the samples, antisense transcript was not detected and the endogenous OMT mRNA was reduced, which may suggest the RNA degradation by antisense mechanism. In the case of samples with high endogenous OMT mRNA level and undetectable antisense transcript, it is possible that the antisense transcriptional level was lower than the detectable level or the antisense gene was silenced. Further experiments with run-on assays would be necessary to answer whether gene silencing occurred.

CHAPTER 5 CHARACTERIZATION OF OMT ANTISENSE TRANSGENIC PLANTS

Introduction

Brown midrib mutants of maize *bm3* have been studied extensively because the mutants reduce lignin content and change lignin monomer composition. *Bm3* mutants have a brown midrib phenotype at the 4- to 6-leaf stage. This pigmentation disappears from the leaf midribs as plants grow. However, it can be found in the rind and vascular tissues of the stem cross sections (Cherney et al., 1991; Barriere and Argiller 1993). Grand et al. (1985) first examined the six enzymes in the lignin biosynthetic pathway of the *bm3* mutant and its normal counterpart, and they found that caffeic acid 3-O-methyltransferase (OMT) activity of the whole *bm3* plant was only 10% of the wild type counterpart at 10, 20, and 30 days after germination. The *bm3* mutants have been further characterized as a mutation occurring in the gene encoding caffeic acid O-methyltransferase (OMT). This mutation was caused by a B5 transposon insertion or a deletion in the OMT gene (Vignols et al., 1995; Morrow et al., 1997).

OMT in angiosperms is bifunctional, catalyzing two methylation steps (from caffeic acid to ferulic acid and from hydroxyferulic acid to synapic acid) in lignin biosynthesis, and using S-adenosylmethionine as the methyl group donor. OMT is encoded by a single gene in maize and it is highly expressed in the root elongation region, stem and leaf midrib (Collazo et al., 1992; Vignols et al., 1995; Capellades et al., 1996).

The maize antisense OMT transgenic plants are characterized in this chapter. This is the first report on maize OMT down-regulation by an antisense strategy.

Materials and Methods

OMT Activity Assay

Leaf or stem tissue was ground into a fine powder with a mortar and pestle in the presence of liquid nitrogen and crude protein was extracted as described by Bugos et al. (1991). The leaf or stem powder was suspended in a buffer (4 ml/g tissue) containing 200 mM Tris-HCl (pH 7.2), 14 mM β -mercaptoethanol and vortexed for 1 min. The homogenate was centrifuged at 10,000 x g for 20 min and filtered through one layer of Miracloth (Fisher Scientific, Pittsburgh, PA). All the steps were carried out at 4°C.

The OMT activity assay was a modification based on Bugos et al. (1991), Collendavelloo et al. (1981), Gowri et al. (1991) and Dalkin et al. (1990). The diluted tritiated S-Adenosylmethionine (SAM) was prepared by adding 20 μ l of S-Adenosyl-L-[methyl- 3 H] methionine (specific activity 76.0 Ci/mmol, 1 μ Ci/ μ l, Amersham) to 280 μ l of an unlabeled SAM.HCl solution (100 nmol).

The OMT assay conditions were optimized and the standard OMT assay was established as follows: 50 μ l of plant extract enzyme solution, 10 μ l of caffeic acid (final concentration 1 mM), 137 μ l of 200 mM Tris-HCl pH 8.0 buffer. After incubation at 37°C for 5 min, 3 μ l of S-Adenosylmethionine (0.2 μ Ci/nmol) was added. After 2 hr of incubation at 37°C, 20 μ l of 2 N HCl was added to stop the reaction. Then the product was extracted with a mixture of hexane: ethyl acetate (1:1). One milliliter of the solvent was added to each sample, which was then vortexed for 1 min. The organic phase, recovered after centrifugation for 2 min, was taken into a scintillation vial. Seven

milliliters of Scintilene (Fisher Scientific, Pittsburgh, PA) cocktail were added to each sample and the radioactivity of the sample was measured in a liquid scintillation counter. Each sample was assayed in duplicate and without adding caffeic acid as a control for the assay.

Protein Determination

Protein content of samples for OMT assay was determined by the Bradford (1976) method using the reagent from Bio-Rad with bovine serum albumin (BSA) as the protein standard.

RNA Probe Preparation

Sorghum OMT DNA in Bluescript SK was linearized by *EcoRI* or *ApaI* and recovered from the gel with NA-45 DEAE cellulose membrane (Schleicher & Schuell Company, Keene, NH) after gel electrophoresis following the manufacturer's instruction. Both the OMT sense and antisense probes were made with the *in vitro* transcription kit (Promega, Madison, WI) using ³²P-labeled CTP. The sense strand RNA probe was synthesized using *ApaI*-cut plasmid and T3 RNA polymerase. The *ApaI* fragment was first filled in with Klenow fragment to convert the 3'-overhang to blunt ends. The antisense strand probe was synthesized using *EcoRI*-cut plasmid and T7 RNA polymerase. *In vitro* transcription conditions were performed as recommended by the manufacturer. Following transcription, the DNA template was removed by DNase I treatment. After two phenol and chloroform (1:1) extractions, the RNA probe was purified on a Sephadex G-50 column.

Northern Blot Analysis with cDNA Probe

This was performed as described in the previous chapter. The radioactivity was quantified on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) after hybridization.

Northern Blot Analysis with Riboprobe

Total RNA isolation, size separation and membrane transfer were performed as described in Chapter 4. Prehybridization and hybridization were carried out according to Sambrook et al (1989). The membrane was prehybridized 4 hr at 65°C in 20 ml of a solution containing 6x SSPE [20x stock (g/l): NaCl, 175.3 g, NaH₂PO₄·H₂O, 27.6 g, and EDTA, 7.4 g, adjusting pH to 7.4 with 10 N NaOH], 5x Denhardt's reagent [50x stock (g/l): Ficoll, 10 g, polyvinylpyrrolidone, 10 g, bovine serum albumin, 10 g], 0.5% SDS, 50% formamide and 100 µg/ml (final concentration) denatured salmon sperm DNA. The membrane was then hybridized overnight at 68°C with the radiolabeled RNA probe in the solution as described above except without Denhardt's reagent. The blot washing conditions were as described in Chapter 4.

Histochemical Staining

The control and transgenic plants were harvested five days after anthesis. The seventh and tenth (from the top) internodes of the plants were sliced with a scalpel to 1 mm thick sections. Phloroglucinol-HCl and potassium permanganate-HCl staining were performed as described by Dean (1997).

Statistical Analysis

Analysis of variance was performed to compare the OMT activity of control and transgenic plants according to Lyman Ott (1993) using SAS software version 6.12 (SAS Institute Inc., Cary, NC).

Results

Sampling Stage and Sampling Methods for OMT Assay

In order not to jeopardize obtaining seeds from the transgenic plants, we sampled leaves rather than stems for the OMT activity assay. The following was considered in determining the sampling stage for OMT. The natural brown midrib mutants (*bm3*) showed the brown midrib phenotype at the 4- to 6-leaf stage; OMT mRNA was highly accumulated at the leaf midrib when sampled at the seedling stage (Cherney et al., 1991; Vignols et al., 1995). To determine the distribution of OMT activity in the plant, non-transformed plants at two developmental stages were sampled and OMT activity was measured on the stem, sheath, and leaf. On 2-week plant samples, the second youngest leaf had the highest OMT activity among the four leaves analyzed, and that activity was comparable to the stem and sheath (Fig. 5-1). On 7-week (15-leaf stage) plant samples, the OMT activity was high in the young leaves, extending internodes and leaf sheaths, while it was low in the lower leaves and non-extending young internodes (Fig. 5-2). Because of the highly variable OMT activity within a single plant and at different growth stages, standardization of the sampling was necessary.

To further investigate the correlation of OMT activity and OMT mRNA accumulation, the same plant samples from the 7-week stage were used for northern blot analysis. Ten micrograms of total RNA of each sample fractionated by electrophoresis. After transferring the RNA to a membrane, the blot was sequentially hybridized with the maize OMT cDNA probe and the 28S rDNA probe. As shown in Fig. 5-2, the OMT activity was in agreement with the OMT mRNA accumulation level (correlation coefficient $r = 0.96$).

Fig. 5-1. Distribution of OMT activity from 2-week-old (5-leaf stage) non-transformed seedlings. Three plants were harvested, separated into different parts and pooled for OMT activity assay. The max OMT activity was 0.15 pkat/mg protein.

A.

B.

C.

Fig. 5-2. OMT activity and OMT mRNA level distribution within a maize plant. The maize plant was sampled 7-week after planting in the greenhouse. (A) OMT activity was measured on each plant part and the highest OMT activity (0.76 pkat/mg protein) was considered as 100%. (B) Relative OMT mRNA steady state level was determined by RNA quantification of the northern blot data. (C) Northern blot analysis of the OMT steady state level using the maize OMT cDNA as probe. The blot was also hybridized with 28S rDNA probe to standardize the RNA loading and transfer.

The results also indicated that a single sized transcript homologous to the OMT was present among different plant parts at that sampling stage. In addition, no differential hybridization intensity was observed between sorghum and maize stem tissues (compare Fig. 5-2 lane 10 with lane 12).

OMT Activity of Transgenic Plants

Since both developmental stage and growth conditions influenced OMT activity, it was difficult to utilize the primary transgenic plants for OMT analysis. Our major efforts were focused on the progeny resulting from self-pollination of the primary transgenic plants. Based on the brown midrib phenotype and the potentially independent transformation events, five seeds from each of 40 primary transgenic plants were planted in the greenhouse. Ten control seeds from different plants regenerated from non-transformed callus were planted under the same conditions. Because of the genetic segregation of the transgene, herbicide resistance was first evaluated with the young transgenic plants by painting a leaf with 1% Ignite®. The second youngest leaf was sampled on day 14 and subjected to OMT assay. For the transgenic plants, only the herbicide resistant plants were sampled. A total of 104 transgenic plants were assayed for OMT activity. The OMT activity was highly variable between individual plants. Compared to the mean control OMT activity, 17.3% of the transgenic plants had an OMT activity reduction of greater than 50%; 53.8% of the plants showed a reduction of more than 25%. The extremely down-regulated OMT transgenic plants had about 10% of the control OMT activity. As shown in Fig. 5-3, the OMT activity variation within each transgenic family was as large as between transgenic families, so there were no

Fig. 5-3. OMT activity of two-week-old (5-leaf stage) plants. (A) Control plants (B) Transgenic plants. Five seeds resulting from self-pollination of primary transgenic plants were planted in the greenhouse. The second youngest leaf from control and transgenic plants, 2 weeks after planting, was assayed for OMT activity. The mean of 10 control plants was considered as 100% (0.10 pkat/mg protein). The numbers under the graph denote different progenies. Bm in the family designations indicate the parent had a brown midrib phenotype.

significant differences among the transgenic families. However, the mean OMT activity of the transgenic plants was significantly reduced compared to the control plants ($p = 0.007$). The brown midrib phenotype was not correlated with reduced OMT activity at the 2-week (5-leaf) stage. This could possibly be due to sampling the second youngest leaf while the brown midrib phenotype was observed on the two older lower leaves and suggests the spatially and temporally different expression of the OMT antisense gene.

A number of reports on antisense down-regulation have indicated that only a few samples maintained low enzyme activity when transgenic plants were sampled at a more mature stage (Atanassova et al., 1995; Piquemal et al., 1998). To investigate this problem, we sampled the third youngest leaf when the plants reached the 7-week (15-leaf) stage. A portion of the plants having low OMT activity at the 2-week stage had an increase in activity level similar to the control, and some were even higher than the control. Among the 35 7-week (15-leaf) stage plants analyzed (with down-regulated OMT activity at the 2-week stage), only five plants (bm17-1, bm8-1, bm6-1, 7-3, and bm128-5) had reduced OMT activity ($\leq 50\%$ of the control). Since our major objective was to investigate the potential of forage quality improvement, sampling at a more mature stage (flag leaf stage) may better reflect the effect of OMT down-regulation. Six seeds from each of 10 primary transgenic plants and 12 seeds from regenerated untransformed plants were planted in the greenhouse. The young flag leaf of the herbicide-resistant and control plants was taken for OMT activity assay. The results indicated that OMT activity was less variable within each transgenic family (see Fig. 5-4) and was significantly different among transgenic families ($p < 0.01$). In transgenic families bm8, bm128, bm17, and 7, OMT activity was

Fig. 5-4. OMT activity of flag leaf from transgenic plants. Six seeds resulting from self-pollination of primary transgenic plants were planted in the greenhouse. The young flag leaf from control and transgenic (herbicide resistant) plants, 8 weeks after planting, was assayed for OMT activity. The mean of 10 control plants was considered as 100% (0.32 pkat/mg protein). The plant ID number was indicated under the graph. Bm in the family designation indicates the parent had a brown midrib phenotype. Bm17-4 could be viewed as a control as it lost the OMT antisense gene (see also Fig. 4-8).

Table 5-1. OMT activity of the flag leaf from transgenic and control plants.

Plant Family	Family Mean of OMT Activity (pkat/mg protein)
bm8 (n=4)	0.157 de*
bm 128 (n=2)	0.186 cde
7 (n=4)	0.190 cde
bm 17 (n=5)	0.193 cde
bm 9 (n=2)	0.271 bcd
bm 5 (n=3)	0.277 bc
bm 6 (n=3)	0.288 abc
bm 4 (n=2)	0.399 a
Control (n=10)	0.318 ab

*Means bearing a common letter are not significantly different at 0.05 level of probability using Fisher's least significant difference test.

significantly reduced compared with OMT activity of the control plants as shown in Table 5-1.

Down-regulation of Maize Endogenous OMT mRNA

To further investigate the OMT down-regulation of the endogenous OMT mRNA steady state level, northern blot analysis was performed using an OMT antisense riboprobe with the third leaf of the five plants maintaining reduced OMT activity (15-leaf stage) and with some of the flag leaf samples. As shown in Fig. 5-5A, the third leaf of all five plants showed a significant reduction of the maize OMT mRNA level (1.7 knt) compared to the control. The flag leaf samples having reduced OMT mRNA steady state level were also consistent with their lower OMT activity (comparing Fig. 5-4 and Fig. 5-5B). Plant bm17-4 (Fig. 5-5B) could be viewed as a control because the antisense OMT transgene was lost as evidenced by Southern analysis (see Fig. 4-8). The reduced maize OMT mRNA level may suggest the suppression of endogenous OMT mRNA accumulation by antisense OMT gene expression. The band of 1.3 knt unknown RNA has strand specific binding with OMT antisense probe. That RNA did not hybridize when a cDNA probe or the sense-strand probe was used. Northern analysis also revealed that the reduction of endogenous OMT mRNA level was more pronounced than the reduction of OMT activity compared to the control.

Comparison of the Efficiency of Different Constructs

Regenerated transgenic plants from the three antisense vectors (OMT full-length cDNA, 5'-cDNA and 3'-cDNA) expressed the brown midrib phenotype. However, because of the magnitude of work generated in characterizing the transgenic plants, only the plants bombarded with the OMT full-length and 5'-fragment vectors were

Fig. 5-5. Northern blot analysis with the OMT antisense probe to detect endogenous OMT mRNA steady state level. (A) The third youngest leaf was sampled from 7week old plants with low OMT activity (planted on 07-03-98). (B) The young flag leaf was sampled from 8-week-old plants (planted on 11-20-98). Bm17-4 could be viewed as a control as Southern blot indicated the loss of OMT antisense gene (see Fig. 4-8). The blot was sequentially hybridized with the maize antisense OMT riboprobe and 28s rDNA probe. The 1.7 knt band is the endogenous OMT mRNA. This band is not present in the transgenic plants but present in the control. This suggests the antisense OMT gene expression may promote instability of the endogenous OMT mRNA. The band of 1.3 knt unknown RNA has strand-specific binding with the OMT antisense probe. That RNA did not hybridize when an OMT cDNA probe or the sense riboprobe was used. Bm designations indicate the parent had the brown midrib phenotype.

investigated. In both situations, OMT down-regulation was observed and variation existed among different events as well as individual transgenic plants. The most effective construct could not be determined.

Phenotype of Transgenic Plants

Most transgenic plants were phenotypically normal and were self-fertile. Plants were 0.9--1.8 m in height. Some had pistillate male flowers. Seed set varied from a few seeds per plant to more than 100 seeds, which was consistent with other maize transformation reports. Some mutants didn't reach maturity.

About 10% of the transgenic plants showed the brown-midrib phenotype (Fig. 5-6 Top) at 4-6 leaf stage which is characteristic of *bm3*, a natural maize brown midrib mutant caused by B5 transposon insertion or deletion of OMT gene (Vignols et al., 1995). The brownish color was more obvious on the abaxial side of the leaf than the adaxial side. Unlike the *bm3*, not all the midribs of the leaves turned brown at this stage, only 2 or 3 leaves changed color. The distribution of the brownish color was not homogenous even on a single midrib. When these plants were harvested after maturity, the internodes of some plants also showed discoloration (Fig. 5-6 Bottom). This could be related to spatially and temporarily different expression of the antisense OMT gene. The brown midrib phenotype was also observed in some but not all of the progeny from the primary brown midrib transgenic plants.

Some transgenic plants and their progeny showed strange phenotypes at the 4- to 9-leaf stage (Fig. 5-7), such as variegated leaves, broken and stripped leaves, extremely delayed growth rate, and dying leaf tips of emerging young leaves. These were not

Fig. 5-6. Phenotype of transgenic plants. About 10% of the transgenic plants showed brown midrib leaf at the 4 - 6 leaf stage (top), which is similar to the maize natural brown midrib mutants. Some transgenic plants showed vascular tissue discoloration at the mature stage (bottom). Stem samples of **A**, **C**, and **E** are from a transgenic plant; **B**, **D**, **F** are from an untransformed plant. **A-D** are cross sections and **E-F** are longitudinal sections.

Fig. 5-7. Some unusual phenotypes in the progeny of transgenic plants at the 4- to 9-leaf stage. A. Normal control plant; B. The first emerging young leaf with dead leaf tips but later the rest of the leaf grew normally. C. Two siblings of transgenic plants, the left was normal, the right showed the same phenotype as B. D. The transgenic plant showing stripped and broken leaf. E. The transgenic plant showing variegated leaf. F. The growth rate of the plants in D & F was delayed during the first two months after planting. All the abnormal phenotypes disappeared at a later stage. G. Control and transgenic plants. No distinct phenotype was observed at this stage (The plants were put very close to each other for the photograph).

observed in the regenerated control plant population, or in *bm3* mutants. These phenotypes might be related to the OMT antisense gene's inhibition of some other methyltransferase genes (Ibrahim et al., 1998), which is not uncommon in antisense inhibition experiments (Elomaa, et al., 1996; Fire, et al., 1998).

Histochemical Staining

Two histochemical staining methods were commonly used to roughly detect lignin. One is called phloroglucinol-HCl staining, which can be used to determine lignin deposition sites; the other, potassium permanganate-HCl staining, can be used to determine the change in lignin composition by color difference after the staining. The mechanisms of the staining are not well understood. Free-hand stem sections of the seventh and tenth internode (from top) from transgenic plants with reduced OMT activity or non-transformed plants were stained with phloroglucinol-HCl or potassium permanganate-HCl reagents. For phloroglucinol-HCl staining, the epidermis and vascular tissue of transgenic plants showed less red stain than the control plants (Fig. 5-8A). With potassium permanganate-HCl staining, the reduced intensity of color and the change of brown-red to brown color were also observed in the transgenic plants (Fig. 5-8B). These results suggested that both lignin content and composition have been changed in the transgenic plants with reduced OMT activity.

Discussion

OMT Expression Pattern

Collazo et al. (1992) first indicated that maize OMT mRNA was highly accumulated in the elongation zone of the roots. Lower levels of OMT mRNA were also observed in coleoptiles, mesocotyl, and root tips from 7-day-old plants, leaves

Fig. 5-8. Lignin histochemical staining. The seventh and tenth internode (from the top) from control plants and the OMT-reduced transgenic plants were sliced into 1mm sections, and free-hand sections were subjected to (A) phloroglucinol-HCl staining, and (B) potassium permanganate-HCl staining.

from 3-month-old plants, flowers from 120-day-old plants, and seeds 8, 10, and 12 days after pollination. The OMT mRNA was highly accumulated in the leaf midrib corresponding to the cells undergoing lignification as indicated by *in situ* hybridization (Vignols et al., 1995). GUS expression driven by the maize OMT promoter was observed in the young vascular tissues and was responsive to wounding. Our data further supported the notion that OMT expression is developmentally regulated. OMT mRNA level was high in young leaves, leaf sheath, and elongating internodes; while it was very low in the very young internodes, and old leaves. This OMT mRNA steady state level was correlated well with the OMT activity, which also corresponded to lignification sites. This OMT expression pattern verified standardization of the sampling stage with the same young leaf was necessary to investigate OMT down-regulation.

OMT Down-Regulation by Antisense Strategy

It has been well documented that variations of transgene expression exist among independent transformation events, individual plants from the same transformation event, and progeny of a transgenic plant (Gordon-Kamm et al., 1990; Spencer et al., 1992; Walter et al., 1992; D'Halluin et al., 1992; Gallo-Meagher and Irvine, 1996; Zhang et al., 1996; Kumpatla et al., 1997). We decided to screen progenies from many primary transgenic plants rather than focus on the progenies from several plants. Initially, we chose 40 primary transgenic plants based on the brown midrib and stem discoloration phenotypes of the primary transgenic plants, and the potentially independent transformation events. One hundred and four herbicide resistant plants (2-week-old stage, the second youngest leaf) were analyzed for OMT activity and 17.3% of them showed an OMT reduction of 50% compared to the control mean. Our finding was that OMT activity

was as highly variable within a transgenic family as between transgenic families. The genetic instability and epigenetic variation of the antisense OMT gene expression may explain this. Among these analyzed plants, no correlation was observed between brown midrib phenotype and OMT activity, which may reflect the spatial and temporal expression patterns of the antisense OMT gene. Concerning the "somatic instability" (Kozziel et al., 1996) of transgene expression, we sampled the third youngest leaf from 35 transgenic plants previously having reduced OMT activity at the more mature, 7-week (15-leaf) stage, and found only five plants maintained the depressed OMT activity (about 50% of the control). Northern analysis with the antisense riboprobe showed that the endogenous OMT mRNA level of those five plants was severely reduced. Since our major interest was to investigate the potential of forage quality improvement via down-regulation of maize OMT expression, sampling the plants at more mature stage might better represent the whole process. We replanted seeds from selected plants and sampled the young flag leaves of transgenic and control plants for OMT activity and OMT mRNA analyses. The data showed that both OMT activity and maize OMT mRNA level were reduced in some of those transgenic plants. Interestingly, the more pronounced the reduction of maize OMT mRNA level the lower the OMT activity level, at both 15-leaf and flag-leaf stages.

Antisense induced gene suppression is reported to mainly function at the post-transcriptional level (De Lange et al., 1995; Hamilton et al., 1995; Ardnt and Rank, 1997; Kumur and Carmichael, 1998). Significant reduction of OMT mRNA along with undetectable antisense OMT RNA in leaf tissue in the transgenic plants were in agreement with a number of reports on the action of antisense genes (Crowley et al.,

1985; Kim et al., 1985; Mercola et al., 1988; Van der Krol et al., 1990; Atanassova et al., 1995). These observations may be explained by a rapid turnover of the antisense-endogenous RNA duplex. In only a few transgenic plants, the antisense OMT transcript could be detected (see Fig. 4-9), and the presence of the antisense transcript was not correlated with the reduction of OMT activity or maize endogenous OMT mRNA level, which seemed consistent with other reports (Van der Krol et al., 1988; Cannon et al., 1990; Stockhaus et al., 1990; De Lange et al., 1995). The possible explanation for this could be due to different cellular localization of the endogenous mRNA and the antisense transcript so that the antisense RNA molecules cannot always intervene with the targeted endogenous mRNA (De Lange et al., 1995; Arndt and Rank, 1997).

Generally, only 10-50% of the transgenic plants expressing an antisense gene show suppression of the target gene, and in some cases the antisense gene may have no effect at all (De Lange et al., 1995; Arndt and Rank, 1997). Thus, " 'antisensing' is still a matter of trial and error" (p. 64 in De Lange et al., 1995) until the mechanism is elucidated. Recently, Waterhouse et al. (1998) showed that plants transformed with virus or reporter gene constructs that produced RNAs capable of duplex formation conferred virus immunity or gene silencing much more efficiently than plants with the constructs producing either sense or antisense mRNA alone. This may highlight a new strategy for future down-regulating gene expression in transgenic plants.

Down-regulation of OMT Changed Lignin Profile

The natural brown midrib mutants *bm3* were caused by the deletion of OMT coding region or a B5 transposon insertion in OMT coding region (Vignols, et al., 1995; Morrow et al., 1997). This mutation gave rise to a reduction in lignin content and a

change in lignin composition S/G ratio (Cherney et al., 1991). Therefore, OMT has been considered a good down-regulation target in transgenic tobacco (*Nicotiana tabacum*) and poplar trees (*Populus deltoides*). However, most of the studies of transgenic tobacco and poplar with OMT-down-regulated activity have shown that the lignin level of the transgenic plants was similar to the control, whereas lignin composition S/G ratio was significantly reduced, even where the OMT activity was reduced more than 90% (Dwivedi et al., 1994; Atanassova et al. 1995; Doorsselaere et al., 1995; Vailhe et al., 1996; Zhong et al. 1998; Tsai et al., 1998; Lapierre et al., 1999). Contrary to those reports, Ni et al. (1994) and Sewalt et al. (1997) reported a moderate decrease of lignin content in their OMT down-regulated transgenic tobacco plants. The occurrence of several O-methyltransferases with various substrate specificities and temporal or spatial expression patterns may explain the different results (Matsui et al., 1994; Ye, 1994; Li et al., 1997; Kentaro et al., 1998; Meng and Campbell, 1998; Gauthier et al., 1998; Martz et al., 1998; Lapierre et al., 1999). In the primary transgenic plants and their progeny, we have observed brown midrib phenotype at the 4- to 6-leaf stage, and vascular tissue discoloration at a mature stage. These phenotypes were similar to the natural *bm3* mutants and were not observed in the control plants and progeny lacking the antisense gene. Moreover, results from the lignin histochemical staining of stem sections also indicated that the transgenic plants with down-regulated OMT activity had reduced lignin content and modified lignin composition. Although we did not know how much OMT activity was reduced to cause this change because of different expression in leaf and stem. It has been found recently that there is an alternative methylation pathway to synthesize the lignin monomers (see Fig. 2-1) (Ye et al., 1994; Ye, 1997; Kentaro et al., 1998; Martz et

al., 1998; Zhong et al., 1998). Unlike OMT utilizing caffeic acid and hydroxyferulic acid as substrate, caffeoyl CoA 3-O-methyltransferase (CCoAOMT) catalyzes methylation of caffeoyl and 5-hydroxyferuloyl CoA. Down-regulation of CCoAOMT expression in transgenic tobacco resulted in significantly reduced lignin content and composition S/G ratio, and a similar phenotype of *bm3*. However, down-regulation of CCoAOMT in transgenic maize did not change the phenotype (Dr. Tim Helentjaris, Pioneer Co., personal communication). All above-mentioned experiments suggest that OMT may play a more important role in the monolignol biosynthesis of monocots. Our present understanding of the lignin biosynthetic pathway may be oversimplified, especially when species, tissues and cell specificity are taken into account.

CHAPTER 6 FORAGE QUALITY IMPROVEMENT OF TRANSGENIC PLANTS

Introduction

Lignin is negatively correlated with forage digestibility and has been identified as a major "anti-quality" factor in forage grasses. Natural brown midrib mutants in maize (*Zea mays* L.) and chemically induced mutants in sorghum [*Sorghum bicolor* (L.) Moench] and pearl millet [*Pennisetum glaucum* (L.) R. Br.] resulted in improved digestibility and animal performance because these mutants have reduced lignin content and/or altered lignin composition. There is worldwide interest in forage breeding to introduce the *bm* genes into commercial cultivars (Cherney et al., 1991; Barriere and Argillier, 1993; Barriere et al., 1994). As more genes in the lignin biosynthetic pathway are cloned and their regulatory steps are demonstrated, genetic engineering offers great opportunities of modulating those genes to improve forage quality. There have been several reports on using antisense technology to down-regulate O-methyltransferase (OMT) in tobacco (*Nicotiana tabacum*) and poplar trees (*Populus deltoides*) (Ni et al., 1994; Dwivedi et al., 1994; Atanassova et al., 1995; Doorselaere et al., 1995). Generally, in those cases the S/G ratio of lignin subunit composition was significantly changed but lignin content was not changed. In only one case, lignin content was moderately reduced but the components were not significantly changed (Ni et al., 1994).

The objectives of this section are (1) to examine whether lignin determined by acid detergent lignin (ADL), and cell wall measured by neutral detergent fiber (NDF) changed in the OMT down-regulated transgenic plants; (2) to investigate *in vitro* organic matter digestibility of the transgenic plants.

Materials and Methods

Plant Material

Control and transgenic plants were grown under similar environmental conditions in the same greenhouse. These plants were harvested five days after anthesis and were separated into leaf, sheath and stem (tassels and ear were removed). The plant parts were chopped, dried at 50°C, and ground to pass through a one-millimeter screen in a Wiley Mill. Only the transgenic plants with reduced OMT activity or changed stem lignin staining were selected for forage analysis.

***In Vitro* Dry Matter Digestibility Analysis**

In vitro organic matter digestibility was determined by a revised method of the Tilley and Terry two-stage technique (Tilley and Terry 1963; Moore, 1974), using bovine rumen fluid as inoculum. Plant samples of 0.5 g were incubated in media with inoculum for 48 hr at 39°C under anaerobic conditions, then the samples were further digested by acid pepsin for 48 hr. The undigested residue was recovered by filtration through a crucible containing glass wool, oven-dried, and weighed. The ash weight was determined after the sample was ashed at 500°C in a muffle furnace for 3 hr. Dry matter and organic matter (OM) of the original samples were also determined (OM = oven dry sample weight - ash weight). The OM disappearance was considered to be reflective of digestion. IVOMD was calculated as follows: $IVOMD = [(initial\ OM - (final\ OM - blank\ OM))/initial\ OM]$.

Cell Wall Analyses

Neutral detergent fiber analysis

Neutral detergent fiber (NDF), as an estimate of plant cell wall, was determined by the standard procedure currently used in the University of Florida Agronomy Department Forage Lab (Golding et al., 1985). The composition of the neutral detergent was as follows (per liter): sodium lauryl sulfate 30.0 g, disodium EDTA 18.6 g, sodium borate dehydrate 6.8 g, disodium hydrogen phosphate 4.6 g, 2-ethoxyethanol 10.0 ml. A 0.5 g sample of plant tissue was extracted with neutral detergent for 1 hr at boiling temperature. The residue was recovered by filtration through porcelain crucible containing glass wool and washed with hot water and acetone. The oven-dry and ash weights of the residue were also determined. NDF was calculated:
$$\text{NDF} = (\text{oven dry fiber weight} - \text{fiber ash weight}) / \text{initial oven dry sample weight}.$$

Acid detergent fiber and acid detergent lignin analysis

Acid detergent lignin (ADL) was determined using the method of Goering and Van Soest (1970), which uses two major procedures. The first procedure involves acid detergent extraction of samples. A 0.5 g sample of plant tissue was extracted with acid detergent for 1 hr at the boiling temperature. The components of acid detergent were (per liter): sulfuric acid (A.R.) 0.5 M, and cetyltrimethylammonium bromide 20 g. The residue was recovered with a coarse porosity sintered glass crucible and washed several times with hot water and then acetone. The residue is called acid detergent fiber (ADF), which mainly includes cellulose and lignin. ADF was calculated:
$$\text{ADF} = \text{oven-dry fiber weight} / \text{oven dry sample weight}.$$
 In the second procedure, the ADF residue was further extracted with 72% sulfuric acid for 3 hr. The sample was then washed with hot water until free of

sulfuric acid to give acid detergent lignin (ADL). The dry weight and ash weight was determined. ADL was calculated: $ADL = \text{weight loss upon ashing after } 72\% \text{ H}_2\text{SO}_4 \text{ treatment/oven dry sample.}$

Total Ethanol-Soluble Carbohydrate (TESC) Analysis

Total TESC content of the stem samples was determined using the phenol sulfuric acid assay as described by Dubois et al. (1956). About 0.2 g of sample was extracted with 80% ethanol for 4 hr at ambient temperature. The 80% ethanol extracts were filtered and the solutions were diluted 1:9 with distilled water for analysis.

Statistical Analysis

Statistical analyses including correlation, covariance analysis were performed according to Lyman Ott (1994) using SAS software version 6.12 (SAS Institute Inc., Cary, NC). Plant growing days and dry matter were included as covariables in the model to compare IVOMD, ADL, ADF, and NDF means of transgenic and control plants.

Results

Correlation of Leaf Acid Detergent Lignin (ADL) Content and Flag Leaf OMT Activity

Since OMT activity peaks preceding the completion of lignification, the young flag leaf samples for OMT activity assay were taken at an early stage. It is of interest to determine whether that OMT data could predict lignin content of different plant parts. Correlation analysis was performed to find out whether there was some relationship between flag leaf OMT activity and lignin content of leaf, stem and sheath from plants

Fig. 6-1. Relation between flag leaf OMT activity and ADL content. (▲), and (●) indicate transgenic and control plants, respectively. (A) Leaf, (B) Stem, (C) Sheath.

sampled five days after anthesis. As shown in Fig. 6-1, OMT activity of the young flag leaf was positively related to leaf ADL ($r = 0.62$, $p < 0.01$) and stem ($r = 0.58$, $p < 0.01$), but it was not significantly related sheath lignin content ($r = 0.21$, $p > 0.1$). In general, low OMT activity generally correlated with low ADL content.

***In Vitro* Organic Matter Digestibility (IVOMD), and Cell Wall Parameters of Leaf and Sheath**

In vitro organic matter digestibility (IVOMD), ADL, ADF, and NDF from the leaves and sheath are presented in Tables 6-1 and 6-2, respectively. IVOMD of the transgenic plant leaves averaged 1.9 percentage units higher than leaves of control plants ($p = 0.046$), while IVOMD of the transgenic plant sheath increased 5.2% compared to that of control ($p = 0.002$). Leaves and sheaths of the transgenic plants were 11.8% ($p < 0.001$), and 18.8% ($p = 0.009$) lower in percent lignin than those of control plants, respectively. In addition to lignin, ADF of transgenic leaves and sheaths were reduced 7.1%, and 10.4% ($p < 0.05$), respectively. Moreover, the NDF of transgenic leaves was reduced 7.6%, while that of the transgenic sheaths was reduced 11.4% ($p < 0.05$).

Stem *In Vitro* Organic Dry Matter Digestibility (IVOMD) and Cell Wall Analyses

Stems had higher lignin content and lower NDF content than leaves and sheath (compare Tables 6-1, 6-2 and 6-3). *In vitro* organic matter digestibility of the transgenic plant stems was, on average, 7.4 percentage units greater than that of control stems ($p = 0.016$). Stems of the transgenic plants were approximately 19.8% lower in lignin than

Table 6-1. Leaf *in vitro* organic matter digestibility (IVOMD, % of organic matter), and cell wall analyses (% of dry matter) from transgenic and control plants harvested five days after anthesis.

Plant Sample		IVOMD	ADL	ADF	NDF
bm4-2*		72.8	1.68	32.8	58.5
bm5-4		75.2	1.61	25.3	48.7
bm8-2		75.3	1.66	31.1	58.3
bm8-4		74.2	1.31	32.3	58.2
bm8-5		72.2	1.82	30.6	57.0
bm8-6		77.9	1.61	31.8	58.1
bm9-3		74.6	1.55	31.5	ND
7-1		73.6	1.57	28.5	ND
7-4		74.5	1.76	29.7	52.3
7-5		74.9	1.44	29.6	54.0
7-6		74.4	1.43	32.9	61.6
bm128-2		76.5	1.63	30.1	55.8
bm17-1		76.1	1.60	31.6	ND
bm17-2		75.5	1.38	24.4	ND
bm17-5		75.0	1.58	29.3	53.3
bm17-6		72.5	1.53	30.8	57.2
Control 1		73.4	1.80	31.5	61.6
Control 2		72.1	1.76	30.3	57.8
Control 3		74.7	1.70	30.5	58.6
Control 4		74.4	1.74	32.9	63.6
Control 5		74.4	1.71	32.5	63.0
Control 6		72.2	2.13	31.1	58.0
Control 7		72.2	1.80	35.1	64.3
Control 8		73.0	1.88	34.0	59.6
Control 9		73.5	1.66	33.0	60.3
Control 10		72.9	1.66	33.3	60.2
Mean ± SE	Transgenic	74.7 ± 0.4	1.57 ± 0.03	30.1 ± 0.6	56.1 ± 1.0
	Control	73.3 ± 0.3	1.78 ± 0.04	32.4 ± 0.5	60.7 ± 0.7
P value (n=26)		0.046	0.0003	0.043	0.012 (n=22)

* Bm designations indicate the parent had a brown midrib phenotype.

Table 6-2. Sheath *in vitro* organic matter digestibility (IVOMD, % of organic matter), and cell wall analyses (% of dry matter) from transgenic and control plants harvested five days after anthesis.

Plant Sample		IVOMD	ADL	ADF	NDF
bm4-2*		74.3	1.74	32.5	50.8
bm5-4		76.1	1.59	27.0	48.8
bm8-2		75.7	1.39	28.4	55.0
bm8-4		80.8	1.47	30.0	61.6
bm8-5		79.5	2.22	31.1	57.7
bm8-6		75.1	1.56	34.2	61.6
bm9-3		73.7	1.22	32.4	ND
7-1		79.1	1.50	28.2	ND
7-4		75.0	1.14	30.3	55.3
7-5		78.6	1.11	29.2	60.0
7-6		75.7	1.40	29.8	45.7
bm128-2		75.2	1.30	31.9	58.5
bm17-1		77.5	1.25	34.1	ND
bm17-2		73.1	1.91	34.6	ND
bm17-5		75.7	1.35	30.1	58.3
bm17-6		80.5	1.39	30.6	55.8
Control 1		72.1	1.78	35.7	65.1
Control 2		73.8	2.20	31.1	62.0
Control 3		76.0	1.65	35.8	62.1
Control 4		72.3	1.84	37.3	62.3
Control 5		76.3	1.78	31.3	54.4
Control 6		71.0	1.80	31.5	60.8
Control 7		71.4	1.87	37.4	66.7
Control 8		72.1	1.90	35.3	67.3
Control 9		70.4	1.73	32.7	62.2
Control 10		72.1	1.57	36.6	67.2
Mean ± SE	Transgenic	76.6 ± 0.6	1.47 ± 0.07	30.9 ± 0.6	55.8 ± 1.3
	Control	72.8 ± 0.6	1.81 ± 0.05	34.5 ± 0.8	63.0 ± 1.2
P value (n=26)		0.012	0.010	0.033	0.031(n=22)

* Bm designations indicate the parent had a brown midrib phenotype.

Table 6-3. Stem *in vitro* organic matter digestibility (IVOMD, % organic matter), and cell wall analyses (% of dry matter) from transgenic and control plants harvested five days after anthesis.

Plant Sample		IVOMD	ADL	ADF	NDF
bm4-2*		74.8	2.01	27.8	50.6
bm5-4		78.2	2.03	21.1	39.1
bm8-2		80.3	1.37	25.9	48.3
bm8-4		76.7	2.30	30.5	54.1
bm8-5		74.7	2.57	31.2	53.9
bm8-6		79.9	2.15	27.4	51.4
bm9-3		72.7	2.06	30.4	56.7
7-1		75.6	1.99	24.3	46.2
7-4		76.8	2.10	25.7	47.3
7-5		76.4	1.25	31.8	54.9
7-6		76.3	2.20	30.5	53.2
bm128-2		79.0	1.78	27.6	49.6
bm17-1		71.6	2.49	29.9	53.0
bm17-2		74.6	1.72	29.8	53.7
bm17-5		78.1	2.24	27.1	49.4
bm17-6		81.2	1.36	24.3	43.0
Control 1		69.4	2.27	32.4	55.6
Control 2		75.6	2.05	26.7	47.8
Control 3		68.4	2.81	31.2	53.3
Control 4		68.0	2.41	31.7	54.1
Control 5		72.1	3.20	28.8	49.9
Control 6		67.0	3.31	32.4	55.8
Control 7		74.2	1.89	29.8	53.4
Control 8		72.7	2.32	31.7	54.4
Control 9		72.3	2.17	28.7	50.7
Control 10		74.3	2.25	33.4	57.5
Mean ± SE	Transgenic	76.7 ± 0.7	1.98 ± 0.10	27.8 ± 0.8	50.3 ± 1.2
	Control	71.4 ± 1.0	2.47 ± 0.15	30.7 ± 0.7	53.3 ± 0.9
P value (n=26)		0.0003	0.0033	0.091	0.205

* Bm designations indicate the parent had a brown midrib phenotype.

stem of control plants ($p < 0.001$). Unlike leaf and sheath, ADF and NDF from the transgenic stems were not significantly reduced ($p > 0.05$) compared to the control. Plant bm17-6 has the highest IVOMD (increased 13.7%) and corresponded to its lowest lignin content (reduced 44.9%).

Whole Plant *In Vitro* Organic Matter Digestibility (IVOMD) and Cell Wall Analyses

IVOMD and cell wall parameters of whole plants were calculated by mathematically adjusting the data of the plant parts in proportion to their percentage of the total plant dry matter (Table 6-4 and Table 6-5). The calculated results are presented in Table 6-6. The transgenic plants, on average, had increased IVOMD 5.0% ($p < 0.001$), and reduced lignin 16.5% ($p < 0.001$), ADF 9.3% ($p < 0.01$), and NDF 8.9% ($p = 0.005$) compared to the control plants.

Total Ethanol-Soluble Carbohydrate (TESC) of Stem

Since stems of the transgenic plants had the highest increase in IVOMD, stems from control and transgenic plants with improved IVOMD were subjected to TESC analysis. The results are presented in Table 6-7. The average TESC content increased 29.8% compared to the control. The change in total sugar content was also observed in brown midrib sorghum (Hanna et al., 1981). Since these transgenic plants had significantly reduced ADL, ADF and NDF, it may be possible that carbon flows into non-cell wall components.

Table 6-4. Dry matter of plant parts from transgenic and control plants harvested five days after anthesis.

Sample		Plant Parts (g dry matter)			Total Weight (g dry matter)
		Leaf	Stem	Sheath	
	bm4-2*	13.2	20.9	12.3	46.4
	bm5-4	17.4	25.5	11.6	54.5
	bm8-2	15.6	21.5	12.3	49.4
	bm8-4	16.0	23.2	15.2	54.4
	bm8-5	15.6	22.3	10.7	48.6
	bm8-6	14.8	21.3	10.5	46.6
	bm9-3	14.5	15.7	9.3	39.5
	7-1	12.5	13.4	10.5	36.4
	7-4	17.7	19.7	11.9	49.3
	7-5	17.6	11.8	12.0	41.4
	7-6	14.4	15.9	22.3	52.6
	bm128-2	15.2	17.1	11.3	43.6
	bm17-1	9.6	12.4	7.7	29.7
	bm17-2	10.8	14.1	8.1	33.0
	bm17-5	17.5	23.6	12.5	53.6
	bm17-6	16.0	14.1	10.8	40.9
	Control 1	13.4	13.8	8.1	35.3
	Control 2	9.6	11.0	5.5	26.1
	Control 3	9.0	10.3	7.8	27.1
	Control 4	6.9	8.8	6.0	21.7
	Control 5	18.3	21.2	12.3	51.8
	Control 6	18.2	17.2	11.5	46.9
	Control 7	20.4	23.8	14.3	58.5
	Control 8	21.6	21.2	14.0	56.8
	Control 9	18.2	21.1	12.5	51.8
	Control 10	17.6	21.2	12.9	51.7
Mean $\pm$ SE	Transgenic	14.4 $\pm$ 0.7	17.9 $\pm$ 1.0	11.3 $\pm$ 0.8	43.6 $\pm$ 2.0
	Control	15.8 $\pm$ 1.6	16.5 $\pm$ 1.6	11.1 $\pm$ 1.1	43.4 $\pm$ 4.0

* Bm designations indicate the parent had brown midrib phenotype.

Table 6-5. Percentage of dry matter (% DM) and percentage of organic matter (% OM) of plant parts from transgenic and control plants harvested five days after anthesis.

Plant Sample	Leaf		Stem		Sheath	
	% DM	% OM	% DM	% OM	% DM	% OM
bm4-2*	95.5	91.4	93.6	91.6	95.5	92.4
bm5-4	95.8	91.8	95.5	95.8	96.1	94.7
bm8-2	95.5	91.5	94.1	94.9	95.8	93.9
bm8-4	94.4	91.9	95.4	94.3	93.8	93.8
bm8-5	94.1	90.8	95.6	94.4	93.5	92.7
bm8-6	94.4	91.5	94.4	93.7	93.1	92.7
bm9-3	94.9	91.6	93.2	93.0	94.6	92.1
7-1	95.6	91.6	94.2	93.0	95.7	92.1
7-4	95.9	91.6	94.7	93.7	95.7	92.8
7-5	95.7	90.7	95.3	94.8	95.3	94.1
7-6	95.6	92.3	94.7	92.6	94.3	94.0
bm128-2	95.3	90.8	94.2	93.7	95.4	94.5
bm17-1	95.5	90.4	95.2	93.8	95.8	93.0
bm17-2	94.5	91.4	93.9	92.7	94.6	91.2
bm17-5	95.9	91.4	95.4	95.4	95.9	94.5
bm17-6	95.6	90.7	94.6	93.9	95.2	92.5
Control 1	95.0	90.9	93.0	89.8	94.3	89.8
Control 2	95.3	90.2	94.3	92.4	94.9	90.3
Control 3	94.4	90.6	92.5	90.6	94.0	89.3
Control 4	94.3	90.2	94.5	88.4	94.0	87.9
Control 5	96.2	91.9	94.9	93.2	95.9	91.9
Control 6	96.3	91.2	95.9	93.8	95.7	92.0
Control 7	94.5	90.8	92.7	93.8	94.4	92.0
Control 8	95.2	92.2	93.6	94.1	94.6	93.1
Control 9	94.9	91.2	94.9	94.1	94.9	91.2
Control 10	95.0	91.3	95.6	94.3	94.4	92.0

* Bm designations indicate the parent had brown midrib phenotype.

Table 6-6. Whole plant *in vitro* organic matter digestibility (IVOMD, % of organic matter), and cell wall analyses (% of dry matter) from transgenic and control plants harvested five days after anthesis.

Plant Sample		IVOMD	ADL	ADF	NDF
bm4-2*		74.1	1.85	30.5	52.9
bm5-4		76.8	1.80	23.7	44.2
bm8-2		77.6	1.47	28.2	53.1
bm8-4		77.1	1.77	30.9	57.4
bm8-5		75.0	2.25	31.0	55.7
bm8-6		78.2	1.84	30.3	55.8
bm9-3		73.6	1.68	31.3	ND
7-1		75.9	1.71	26.9	ND
7-4		75.5	1.75	28.2	51.0
7-5		76.4	1.29	30.1	56.0
7-6		75.5	1.65	30.9	52.3
bm128-2		77.1	1.60	29.6	54.1
bm17-1		74.6	1.88	31.5	ND
bm17-2		74.5	1.66	29.2	ND
bm17-5		76.6	1.82	28.5	52.7
bm17-6		77.7	1.43	28.5	51.9
Control 1		71.5	1.98	32.8	60.1
Control 2		74.0	1.98	29.0	54.5
Control 3		72.7	2.11	32.3	57.6
Control 4		71.2	2.04	33.6	59.4
Control 5		73.9	2.34	30.7	55.6
Control 6		70.0	2.48	31.7	57.9
Control 7		72.8	1.85	33.5	60.5
Control 8		72.7	2.05	33.5	59.6
Control 9		72.3	1.88	31.2	56.9
Control 10		73.3	1.88	34.2	60.8
Mean ± SE	Transgenic	76.0 ± 0.4	1.72 ± 0.06	29.3 ± 0.5	53.1 ± 1.0
	Control	72.4 ± 0.4	2.06 ± 0.06	32.3 ± 0.5	58.3 ± 0.7
P value (n=26)		0.0001	0.0004	0.007	0.005(n=22)

* bm designations indicate the parent had a brown midrib phenotype.

Table 6-7. Total ethanol-soluble carbohydrate (TESC) contents of stems from some transgenic and control plants.

Plant Sample		TESC contents (% dry matter)
bm8-2		13.7
bm8-6		15.4
bm5-4		12.1
bm128-2		14.3
bm17-5		14.0
bm17-6		12.2
7-1		13.6
7-4		12.8
Control 1		8.0
Control 2		8.4
Control 3		10.7
Control 7		10.8
Control 8		13.0
Control 10		11.5
Means $\pm$ SE	Transgenic	13.5 $\pm$ 0.4
	Control	10.4 $\pm$ 0.8
P value (n=14)		0.038

* Bm designations indicate the parent had a brown midrib phenotype.

Table 6-8. Leaf ADL/ADF, ADL/NDF, and ADF/NDF from transgenic and control plants harvested five days after anthesis.

Plant Sample		ADL/ADF (%)	ADL/NDF (%)	ADF/NDF (%)
bm4-2*		5.1	2.9	56.1
bm5-4		6.4	3.3	52.0
bm8-2		5.3	2.9	53.3
bm8-4		4.1	2.3	55.5
bm8-5		6.0	3.2	53.7
bm8-6		5.1	2.8	54.7
bm9-3		4.9	ND	ND
7-1		5.5	ND	ND
7-4		5.9	3.4	56.8
7-5		4.9	2.7	54.8
7-6		4.4	2.3	53.4
bm128-2		5.4	2.9	53.9
bm17-1		5.1	ND	ND
bm17-2		5.7	ND	ND
bm17-5		5.4	3.0	55.0
bm17-6		5.0	2.7	53.8
Control 1		5.7	2.9	51.1
Control 2		5.8	3.0	52.4
Control 3		5.6	2.9	52.0
Control 4		5.3	2.7	51.7
Control 5		5.3	2.7	51.6
Control 6		6.9	3.7	53.6
Control 7		5.1	2.8	54.6
Control 8		5.5	3.2	57.0
Control 9		5.0	2.8	54.7
Control 10		5.0	2.8	55.3
Mean ± SE	Transgenic	5.2 ± 0.2	2.9 ± 0.1	54.4 ± 0.4
	Control	5.5 ± 0.2	3.0 ± 0.1	53.4 ± 0.6
P value (n=26)		0.095	0.198 (n=22)	0.059 (n=22)

* Bm designations indicate the parent had a brown midrib phenotype.

Table 6-9. Stem ADL/ADF, ADL/NDF, and ADF/NDF from transgenic and control plants harvested five days after anthesis.

Plant Sample		ADL/ADF (%)	ADL/NDF (%)	ADF/NDF (%)
bm4-2*		7.2	4.0	54.9
bm5-4		9.6	5.2	54.0
bm8-2		5.3	2.8	53.6
bm8-4		7.5	4.3	56.4
bm8-5		8.2	4.8	57.9
bm8-6		7.9	4.2	53.3
bm9-3		6.8	3.6	53.6
7-1		8.2	4.3	52.6
7-4		8.2	4.4	54.3
7-5		3.9	2.3	57.9
7-6		7.2	4.1	57.3
bm128-2		6.5	3.6	55.6
bm17-1		8.3	4.7	56.4
bm17-2		5.8	3.2	55.5
bm17-5		8.3	4.5	54.9
bm17-6		5.6	3.2	56.5
Control 1		7.0	4.1	58.3
Control 2		7.7	4.3	55.9
Control 3		9.0	5.3	58.5
Control 4		7.6	4.5	58.6
Control 5		11.1	6.4	57.7
Control 6		10.2	5.9	58.1
Control 7		6.3	3.5	55.8
Control 8		7.3	4.3	58.3
Control 9		7.6	4.3	56.6
Control 10		6.7	3.9	58.1
Mean ± SE	Transgenic	7.2 ± 0.4	4.0 ± 0.2	55.3 ± 0.4
	Control	8.1 ± 0.5	4.6 ± 0.3	57.6 ± 0.4
P value (n=26)		0.020	0.007	0.030

* Bm designations indicate the parent had a brown midrib phenotype.

Table 6-10. Sheath ADL/ADF, ADL/NDF, and ADF/NDF from transgenic and control plants harvested five days after anthesis.

Plant Sample		ADL/ADF (%)	ADL/NDF (%)	ADF/NDF (%)
bm4-2*		5.4	3.4	64.0
bm5-4		5.9	3.3	55.3
bm8-2		4.9	2.5	51.6
bm8-4		4.9	2.4	48.7
bm8-5		7.1	3.9	53.9
bm8-6		4.6	2.5	55.5
bm9-3		3.8	ND	ND
7-1		5.3	ND	ND
7-4		3.8	2.1	54.8
7-5		3.8	1.9	48.7
7-6		4.7	3.1	65.2
bm128-2		4.1	2.2	54.5
bm17-1		3.7	ND	ND
bm17-2		5.5	ND	ND
bm17-5		4.5	2.3	51.6
bm17-6		4.5	2.5	54.8
Control 1		5.0	2.7	54.8
Control 2		7.1	3.6	50.2
Control 3		4.6	2.7	57.6
Control 4		4.9	3.0	59.9
Control 5		5.7	3.3	57.5
Control 6		5.7	3.0	51.8
Control 7		5.0	2.8	56.1
Control 8		5.4	2.8	52.5
Control 9		5.3	2.8	52.6
Control 10		4.3	2.3	54.5
Mean ± SE	Transgenic	4.8 ± 0.2	2.7 ± 0.2	54.9 ± 1.5
	Control	5.3 ± 0.2	2.9 ± 0.1	54.7 ± 1.0
P value (n=26)		0.094	0.199 (n=22)	0.209 (n=22)

* Bm designations indicate the parent had a brown midrib phenotype.

Table 6-11. Correlation between IVOMD and cell wall analyses of plant parts, and whole plant.

Forage Parameters	r with IVOMD			
	Leaf	Stem	Sheath	Whole Plant
ADL	-0.47 (p = 0.01)	-0.70 (p = 0.0001)	-0.35 (p = 0.088)	-0.69 (p = 0.0002)
ADF	-0.33 (p = 0.10)	-0.66 (p = 0.0003)	-0.60 (p = 0.0015)	-0.66 (p = 0.0004)
NDF	-0.31 (p = 0.16)	-0.58 (p = 0.004)	-0.42 (p = 0.058)	-0.62 (p = 0.0021)
ADL/ADF	-0.21 (p = 0.35)	-0.53 (p = 0.01)	-0.21 (p = 0.36)	-0.36 (p = 0.10)
ADL/NDF	-0.23 (p = 0.25)	-0.41 (p = 0.04)	-0.44 (p = 0.028)	-0.42 (p = 0.049)

Fig. 6-2. Relationship of IVOMD and ADL. (▲) indicates transgenic plants. (●) indicates control plants.

Ratios of ADL/ADF, ADL/NDF, and ADF/NDF in Transgenic and Control Plants

The ratios of ADL/ADF, ADL/NDF, and ADF/NDF were associated with digestibility as pointed out by Van Soest (1967). These calculated ratios are presented in Table 6-8, Table 6-9, and Table 6-10. Leaf and sheath ratios of ADL/ADF, ADL/NDF, and ADF/NDF were not significantly different between transgenic and control plants ($p > 0.05$), while transgenic stem ratios of ADL/ADF, ADL/NDF, and ADF/NDF were significantly decreased compared to the control ($p < 0.03$).

Lignin (ADL), ADF, NDF and *In Vitro* Organic Matter Digestibility Relationship

To further investigate the relationship between cell wall parameters (ADL, ADF, and NDF) and IVOMD, correlation analysis was performed. Table 6-11 presents the Pearson correlation coefficients between IVOMD and cell wall parameters, which reflect the extent of a linear relationship between two data sets. IVOMD was negatively correlated with ADL, and stem IVOMD had strongest negative relationship with stem ADL. Some of the transgenic plants showed higher digestibility even though their ADL levels similar to the controls, which might suggest their lignin chemical composition was changed (see Fig. 6-2). IVOMD was also negatively correlated to ADF and NDF in stem, sheath and whole plant, but not in leaf.

Discussion

Down-regulation of OMT Expression Associated with Lignin Reduction

In the previous chapter, we showed that some transgenic plants had the brown midrib phenotype in early development and stem discoloration at maturity, which are characteristics of the maize natural mutant *bm3*. The histochemical staining also indicated that lignin content was reduced and/or lignin composition was altered. Here we further

showed that OMT activity of the flag leaf was correlated with leaf lignin content five days after anthesis. Grand et al. (1985) suggested that OMT activity could be used as an early marker to screen low lignin lines. However, unlike the structural gene mutation in *bm3*, which is stable during development, the antisense transgenic plants seemed to show unstable OMT down-regulation that may relate to spatially and temporally different expression of the transgene. This observation was consistent with other reports (Atanassova et al., 1995; Piquemal et al., 1998). Sampling at a more mature stage rather than 14-day-old seedling may better reflect the whole process of OMT down-regulation. By standardized sampling of the young flag leaf, we demonstrated that leaves of transgenic plants with reduced OMT activity had lower lignin (ADL) content than the control. For further investigation in the future, comparison of young flag leaf 's OMT activity may offer a way to select low-lignin transgenic plants.

Altered Lignin Improved *In Vitro* Organic Matter Digestibility

Brown midrib mutants in maize, sorghum, and pearl millet all showed improved forage digestibility that was associated with alteration of lignin content and/or composition (Cherney et al., 1991; Barriere et al., 1994). Recently, Vailhe et al. (1996) indicated OMT down-regulated transgenic tobacco improved enzymatic degradability 3.5% without reducing lignin content. Sewalt et al. (1997) reported an extreme improvement of cellulase degradability by 48% in OMT down-regulated transgenic tobacco having moderately reduced lignin. For the purpose of improving tropical grass digestibility, we used maize as a model system. Our data showed that the stem had the greatest improvement of IVOMD (7.4 %), sheath second (5.2 %), and leaf the least (1.9 %), which corresponded to lignin reduction levels of stem -19.8 %, sheath -18.8 %, leaf

-11.8 %. On a whole plant basis, the transgenic plants increased IVOMD averaged 5.0 % with a lignin reduction of 16.5%. The highest IVOMD increase (plant bm17-6) was 8.0 % with a lignin reduction of 30.6%. Some of the transgenic plants (e. g. plant bm8-6, bm5-4) had lignin content levels similar to the controls but increased IVOMD. This might suggest a change of lignin chemical composition. In addition to lignin, ADF and NDF of the transgenic plants were also reduced significantly (see Table 6-4). With stem having the most increased IVOMD, total sugars were also increased. The correlation analysis indicated that IVOMD had a significantly negative association with lignin, ADF and NDF but was positively related to total sugar content. These observations indicated that the IVOMD improvement could not be attributed to only one cell wall component. The plant cell wall is a complex and dynamic structure. The lignin content and/or chemical composition change may also be associated with structural and quantitative change of other cell wall components (such as hemicellulose and cellulose) to compensate the lignin alteration. It requires interdisciplinary cooperation to fully understand the issue. However, when the transgenic plants had lost the OMT antisense gene because of deletion or segregation, no IVOMD improvement was observed in those plants (data not shown). Combining all these together, with the established low lignin *bm* mutants increasing digestibility, our results verified that maize plant digestibility was improved by down-regulation of OMT expression. As OMT is highly conserved in dicots, it is expected to be conserved in monocots (the sequence similarity between sorghum OMT cDNA and maize OMT cDNA is 76.8%). Our results implied that forage quality of tropical grass could be improved by genetic engineering with heterologous OMT antisense gene.

As antisense gene silencing is an epigenetic trait, the inheritance of OMT down-regulation needs to be examined in the successive generations. The other way is to transform some grass species, such as bahiagrass, that can be propagated by vegetative tissue so that non-Mendelian inheritance of transgene expression can be avoided.

CHAPTER 7

SUMMARY AND CONCLUSIONS

Lignin is an aromatic polymer in certain plant cell walls and it has been negatively correlated with forage digestibility. Brown midrib mutants of maize, sorghum and pearl millet have increased digestibility as well as animal intake and performance because of reduced lignin content and/or modified lignin composition. Genetic engineering of the cloned genes in the lignin biosynthetic pathway using antisense technology offers the potential to manipulate lignin biosynthesis and induce the brown midrib phenotype in other grass species.

In the present study, we used maize Hi-II as a model system and the OMT gene as the antisense target to investigate the effectiveness of a sorghum OMT antisense gene on inhibition of the endogenous OMT gene expression, and its effect on forage digestibility. To achieve this, we have constructed a set of sorghum antisense OMT vectors. The vectors contained two expression units, one is the sorghum OMT antisense cDNA (full-length, 5' or 3'-OMT cDNA) driven by maize Ubi-1 promoter, first exon and first intron and terminated by the *nos* terminator. The other is the selectable marker *bar* gene driven by CaMV 35S promoter. The vectors were then introduced into maize via particle bombardment.

From those bombardments (50 shots), three hundred fifty maize plants were regenerated (from 66 resistant calli) in the presence of glufosinate. All the plants were tested by leaf painting with 1% Ignite and 54.5% were herbicide resistant. Among the two

hundred and four plants tested by PCR specific amplification of the *bar* gene, 85% were positive. Southern blot analysis with the primary transgenic plants indicated the *bar* gene was integrated in different patterns with the copy numbers ranging of 1 to more than 20 including both the full-length *bar* gene expression units and the rearranged copies. Southern blot analyses were also performed with the progeny of primary transgenic plants. The data showed that *bar* gene was stably transmitted to the progeny and no rearrangements were observed in the examined plants, whereas the OMT antisense gene was not stably transmitted as revealed by different hybridization patterns among siblings. The inheritance difference between a selectable marker gene and an unselectable gene has also been reported by other groups (Fromm et al., 1990; Spencer et al., 1992; Register III et al., 1994; Zhang et al., 1996).

Expression of the transgenes in progeny was investigated using northern analysis. Results showed that *bar* expression varied among the different transformation events and no correlation was observed between transgene copy number and transgene expression level. OMT antisense gene transcripts were detected in only a few plant samples and no correlation existed between the *bar* gene expression and OMT antisense expression level.

About 10% of the transgenic plants expressed the brown midrib phenotype at the 4- to 6-leaf stage. Some of these plants also had the brownish pigmentation in the vascular tissues of the stems after maturity. These characteristics were similar to the maize natural mutant *bm3*.

OMT activity was highly variable in different plant parts within a single maize control plant. OMT activity was high in the young leaves and extending internodes, while it was low in the old leaves and very young internodes. This OMT activity distribution

was correlated well with the endogenous maize OMT mRNA steady state level. These data demonstrated it was necessary to standardize the sampling stage and sampling method. The second youngest leaf of 2-week old (5-leaf) progeny of the primary transgenic plants was analyzed for OMT activity. The results indicated that OMT activity was significantly down regulated in the transgenic plants compared to the control. However, that activity was as highly variable among family siblings as between families. The down-regulated OMT activity at 2-week stage was not maintained at a more mature stage. This was probably caused by temporally and spatially different expression of the OMT antisense gene. These data indicated that sampling at an early stage was not a reliable indicator for late-stage down regulation of OMT. We replanted selected transgenic and control plants and sampled the young flag leaf for OMT activity assay. The data showed that at this stage OMT was less variable within family siblings but was significantly different among the analyzed families.

The OMT down-regulation was further confirmed by strand-specific northern analysis with an antisense OMT probe. The results indicated the endogenous OMT mRNA steady state level was reduced in the transgenic plants as compared to that of the control. This suggests suppression of the endogenous OMT mRNA accumulation by the antisense OMT RNA.

In vitro organic matter digestibility (IVOMD), neutral detergent fiber (NDF), acid detergent lignin (ADL) and total ethanol-soluble carbohydrate (TESC) analyses were performed with some of the control and transgenic plants. The plants were harvested five days after anthesis and separated into leaf, stem and sheath. Our data showed that stem had most improvement of IVOMD (7.4 %), sheath second (5.2 %), and leaf the last (1.9

%), which corresponded to the lignin reduction level (stem -19.8 %, sheath -18.8 %, leaf -11.8 %) compared to control plants. On a whole plant basis, the transgenic plants increased IVOMD average 5.0 % with lignin reduction of 16.5%. The highest IVOMD increase (plant bm17-6) was 8.0 % with lignin reduction of 30.6%. These data verified that down-regulation of OMT can improve forage digestibility, which offers the potential for the improvement forage quality of other tropical grasses.

REFERENCES

- Akin, D.E., and Rigsby, L.L. (1991). Structure and digestibility of tissues in normal and brown midrib pearl millet (*Pennisetum glaucum*). *Journal of the Science of Food and Agriculture* 56, 523-538.
- Ardnt, G.M., and Rank, G.H. (1997). Colocalization of antisense RNAs and ribozymes with their target mRNAs. *Genome* 40, 785-797
- Arencibia, A., Molina, P.R., De la Riva, G., and Selman-Housein, G. (1995). Production of transgenic sugarcane plants by intact cell electroporation. *Plant Cell Reports* 14, 305-309.
- Armstrong, C.L. (1994). Regeneration of plants from somatic cell cultures applications for *in vitro* genetic manipulation. In Freeling M., and Wallbot, V. (Eds.) *The Maize Handbook*. Springer-Verlag. New York. p 663-677.
- Armstrong, C.L., and Green, C.E. (1985). Establishment and maintenance of friable, embryogenic maize callus and the involvement of L-proline. *Planta* 164, 207-214.
- Armstrong, C.L., Green, C.E., and Phillips, R.L. (1991). Development and availability of germplasm with high type II culture formation response. *Maize Genetics and Cooperation Newsletter* 65, 92-93.
- Arumuganathan, K., and Earle, E.D. (1991). Nuclear DNA content of some important species. *Plant Molecular Biology Reporter* 9, 208-218.
- Atanassova, R., Favet, N., Matz, F., Chabbert, B., Tollier, M.T., Monties, B., Fritig, B., and Legrand, M. (1995). Altered lignin composition in transgenic tobacco expressing O-methyltransferase sequences in sense and antisense orientation. *The Plant Journal* 8, 465-477.
- Barnes, R.F., Muller, L.D., Bauman, L.F., and Colenbrander, V.F. (1971). *In vitro* dry matter disappearance of brown midrib mutants of maize (*Zea mays* L.). *Journal of Animal Science* 33, 881-884.
- Barriere, Y., and Argiller, O. (1993). Brown-midrib genes of maize: A review. *Agronomie* 13, 865-876.

- Barriere, Y., Argiller, O., Chabbert, B., Tollier, M.T. and Monties, B. (1994). Breeding silage maize with brown-midrib genes. Feeding value and biochemical characteristics. *Agronomie* 14, 15-25.
- Barton, F.E., Amos, H.E., Burdick, D., and Wilson, R.L. (1976). Relationship of chemical analysis to in vitro digestibility for selected tropical and temperate grasses. *Journal of Animal Science* 43, 504-512.
- Barro, F., Cannell, M.E., Lazzeri, P.A., and Barcelo, P. (1998). The influence of auxins on transformation of wheat and tritordeum and analysis of transgene integration patterns in transformants. *Theoretical and Applied Genetics* 97, 684-695.
- Birch, R.G., and Bower, R. (1994). Principles of gene transfer using particle bombardment. In Yang, N.S., and Christou, P. (Eds.) *Particle Bombardment Technology for Gene Transfer*. Oxford University Press, New York, p3-37.
- Boynton, J.E., and Gillham, N.W. (1996). Genetics and transformation of mitochondria in the green alga *Chlamydomonas*. *Mitochondrial Biogenesis and Genetics* 264: 279-296. Academic Press, San Diego.
- Boynton, J.E., Gillham, N.W., Harris, E.H., Hosler, J.P., Johnson, A.M., Jones, A.R., Randolph-Anderson, B.L., Robertson, D., Klein, T.M., Shark, K.B., and Sanford, J.C. (1988). Chloroplast transformation in *Chlamydomonas* with high velocity microprojectile. *Science* 240, 1534-1537.
- Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72, 248-254
- Brunel, D. (1992). An alternative, rapid method of plant DNA extraction for PCR analyses. *Nucleic Acids Research* 20, 4676.
- Boudet, A.M., and Grima-Pettenati, J. (1996). Lignin genetic engineering. *Molecular Breeding* 2, 25-39.
- Boudet, A.M., Lapiere, C., and Grima-Pettenati, J. (1995). Biochemistry and molecular biology of lignification. *New Phytologist* 129, 203-236.
- Bower, R., Elliott, A.R., Potier, B.A.M., and Birch, R.G. (1996). High-efficiency, microprojectile-mediated cotransformation of sugarcane, using visible or selectable markers. *Molecular Breeding* 2, 239-249.

- Bugos, R.C., Chang, V.L.C., and Campbell, W.H. (1991). CDNA cloning, sequence analysis and seasonal express lignin-bispecific caffeic acid/5-hydroxyferullic acid methyltransferase of aspen. *Plant Molecular Biology* 17, 1203-1215.
- Butow, R.A., Henke, R.M., Moran, J.V., Belcher, S.M., and Perlman, P.S. (1996). Transformation of *Saccharomyces cerevisiae* mitochondria using the biolistic gun. *Mitochondrial Biogenesis and Genetics* 264 (Part.B)
- Buxton, D.R., and Casler, M.D. (1993) Environmental and genetic effects on cell wall composition and digestibility. *In* Jung, H.G., Buxon, D.R., Hatfield, R.D., and Ralph, J. (Eds.) *Forage Cell Wall Structure and Digestibility*. ASA, Madison, WI, p685-714.
- Campbell, M. M., and Sederoff, R.R. (1996). Variation in lignin content and composition. *Plant Physiology* 110, 3-13.
- Cannon, M., Platz, J., O'Leary M., Sookdeo, C., Cannon, F. (1990). Organ-specific modulation of gene expression in transgenic plants using antisense RNA. *Plant Molecular Biology* 15, 39-47.
- Capellades, M., Torres, M.A., Bastisch, I., Stiefel, V., Vignols, F., Bruce, K.B., Peterson, D., Puigdomenech, P., and Rigau, J. (1996). The maize caffeic acid O-methyltransferase gene promoter is active in transgenic tobacco and maize plant tissues. *Plant Molecular Biology* 31, 307-322.
- Chabbert, B., Tollier, M.T., and Monties, B. (1994). Biological variability in lignification of maize: Expression of the brown midrib bm2 mutation. *Journal of the Science of Food and Agriculture* 64, 455-460.
- Chan, M.T. (1992). Transformation of indica rice mediated by *Agrobacterium tumefaciens*. *Plant Cell Physiology* 33, 577-583.
- Cheng, M., Fry, J.E., Pang, S.Z., Zhou, H.P., Hironaka, C.M. Duncan, D.R., Conner, T.W., and Wan, Y.C. (1997). Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. *Plant Physiology* 115, 971-980.
- Cherney, J.H., Axtell, J.D., Hassen, M.M., and Anliker, K.S. (1988). Forage quality characterization of a chemically induced brown-midrib mutation in pearl millet. *Crop Science* 28, 783-787.
- Cherney, J.H., Cherney, D.J.R., Akin, D.E., and Axtell, J.D. (1991). Potential of brown-midrib, low-lignin mutants for improving forage quality. *Advances in Agronomy* 46, 157-200.

- Chesson, A., and Forsberg, C.W. (1988) Polysaccharide degradation by rumen microorganisms. In Hobson, P.N. (Ed.) The rumen microbial ecosystem. Applied Science Publication, London. p251-284.
- Christensen, A.H., and Quail, P.H. (1996). Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. *Transgenic Research* 5, 213-218.
- Christensen, A.H., Sharrock, R.A., and Quail, P.H. (1992). Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. *Plant Molecular Biology* 18, 675-689.
- Christou, P. (1995). Strategies for variety-independent genetic transformation of important cereals, legumes and wood species utilizing particle bombardment. *Euphytica* 85, 13-27.
- Christou, P., Ford, T.L., and Kofron, M. (1991). Production of transgenic rice plants from agronomically important Indica and Japonica varieties via electric discharge acceleration of exogenous DNA into immature zygotic embryos. *Bio/Technology* 9, 957-962.
- Collazo, P., Montoliu, L., Puigdomenech, P., and Rigau, J. (1992). Structure and expression of the lignin O-methyltransferase gene from *Zea mays* L. *Plant Molecular Biology* 20, 857-867.
- Collendavellou, J., Legrand, M., Geoffroy, P., Barthelemy, J., and Fritig, B. (1981). Purification and properties of the three o-diphenol-O-methyltransferases of tobacco leaves. *Phytochemistry* 20, 611-616.
- Cornejo, M.J., Luth, D., Blackenship, K.M., Anderson, O.D., and Blechl, A.E. (1993). Activity of a maize ubiquitin promoter in transgenic rice. *Plant Molecular Biology* 23, 567-581.
- Crowley, T.E., Nellen, W., Gomer, R.H., and Firtel, R.A. (1985). Phenocopy of Discoidin 1-minus mutants by antisense transformation in *Dictyostelium*. *Cell* 43, 633-641.
- Dalkin, K., Edwards, R., Edington, B., and Dixon, R.A. (1990). Stress responses in alfalfa (*Medicago sativa* L.). *Plant Physiology* 92, 440-446.
- Daniell, H., Datta, R., and Varma, S. (1998). Containment of herbicide resistance through genetic engineering of the chloroplast genome. *Nature Biotechnology* 16, 345-348.

- Daniell, H., and Varma, S. (1998). Chloroplast-transgenic plants: Panacea--No! Gene containment--Yes! *Nature Biotechnology* 16, 602.
- Daniell, H., Vivekananda, J., and Nielsen, B. L. (1990). Transient foreign gene expression in chloroplasts of cultured tobacco cells after biolistic delivery of chloroplast vectors. *Proceedings of the National Academy of Sciences of the United States of America* 87, 88-92.
- Dean, J.F.D. (1997). Lignin analysis. In Dashek, W.V., (Ed.) *Methods in Plant Biochemistry and Molecular Biology*. CRC Press, New York. p199-215.
- De Block, M., De Brouwer, D., and Tenning, P. (1989). Transformation of *Brassica napus* and *Brassica oleracea* using *Agrobacterium tumefaciens* and the expression of the *bar* and *neo* genes in the transgenic plants. *Plant Physiology* 91, 694-701.
- De Lange, P., Van Blokland, R., Kooter, J.M., and Mol, J.N.M. (1995). Suppression of flavonoid flower pigmentation genes in *Petunia hybrida*. In *Gene Silencing in Higher Plants and Related Phenomena in Other Eukaryotes*. Meyer, P. Ed. Springer-Verlag, p57-74.
- Dekeyser, R.A. (1990) Transient gene expression in intact and organized tissues. *The Plant Cell* 2, 591-602.
- De Lange, P., De Boer, G.J., Mol, J.N.M., and Kooter, J.M. (1993). Conditional inhibition of beta-glucuronidase expression by antisense gene fragments in *petunia* protoplasts. *Plant Molecular Biology* 23, 45-55.
- Dellaporta, S.L., Wood, J., and Hicks, J.B. (1983). The plant DNA miniprepation: version II. *Plant Molecular Biology Reporter* 4, 19-21.
- Dennehey, BK., Petersen, W.L., Ford-Santino C., Pajeau, M. and Armstrong, C.L. (1994). Comparison of selective agents for use with the selectable marker gene *bar* in maize transformation. *Plant Cell, Tissue and Organ Culture* 36, 1-7.
- D'halluin, K., Bonne, E., Bossut M., De Beuckeleer, M., and Leemans, J. (1992). Transgenic maize plants by tissue eletroporation. *Plant Cell* 4, 1495-1505.
- Dixon, R.A., Lamb, C.J., Masoud, S., Sewalt, V.J.H., and Paiva, N.L. (1996). Metabolic engineering: prospects for crop improvement through the genetic manipulation of phenylpropanoid biosynthesis and defense responses-a review. *Gene* 179, 61-71.
- Doorselaere, J.V., Baucher, M., Chognot, E., Chabbert, B., Tollier, M.T., Petit-Conil, M., Leple, J.C., Pilate, G., Cornu, D., Monties, B., Montagu, M.V., Inze, D., Boerjan, W., and Jouanin, L. (1995). A novel lignin in poplar trees with a

- reduced caffeic acid/5-hydroxyferulic acid O-methyltransferase activity. The Plant Journal 8, 855-864.
- Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., and Smith, F. (1956). Colorimetric method for determination of sugars and related substances. Analytical Chemistry 28, 350.
- Dumas, B., Doorselaere, J., Van Gielen, J., Legrand, M., Fritig, B. Montagu, M., Van Inze, D. (1992). Nucleotide sequence of a complementary DNA encoding O-methyltransferase from poplar. Plant Physiology 98, 796-797.
- Dunwell, J.M. (1998). Novel food products from genetically modified crop plants: methods and future prospects. International Journal of Food Science & Technology. 33, 205-213.
- Dwivedi, U.N., Campbell, W., Yu, J., Datla, R.S.S., Bugos, R., Chiang, V.L., and Podila, G.K. (1994). Modification of lignin biosynthesis in transgenic *Nicotiana* through expression of an antisense O-methyltransferase gene from *Populus*. Plant Molecular Biology 26, 61-71.
- Edwards, K., Johnstone, C., and Thompson, C. (1991). A rapid and simple method for the preparation of plant genomic DNA for PCR analysis. Nuclear Acids Research 19, 1349.
- Elomaa, P., Helariutta, Y., Kotilainen, M., Terri, C. (1996). Transformation of antisense constructs of the chalcone synthase gene superfamily into *Gerbera hybrida*: different effect on the expression of family members. Molecular Breeding 2, 41-50.
- El-Tekriti, R.A., Lechtenberg, V.L., Bauman, L.F., and Colenbrander, V.F. (1976). Structural composition and in vitro dry matter disappearance of brown midrib corn residue. Crop Science 16, 387-389.
- Finnegan, J., and McElroy, D. (1994). Transgene inactivation: plants fight back! Bio/Technology 12, 883-888.
- Fire, A., Xu, S.Q., Montgomery, M.K., Kostas, S.E., and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391, 806-811.
- Frame, B.R., Drayton, P.R., Bagnall, S.V., Lewnau, C.J., Bullock, Wilson, H.M., Donwell, J.M., Thompson, J.A., and Wang, K. (1994). Production of fertile transgenic maize plants by silicon carbide whisker-mediated transformation. The Plant Journal 6, 941-948.

- Fritz, J.O., Cantrell, R.P., Lechtenberg, J.D., Axtell, J.D., and Hertel, J.M. (1981). Brown midrib mutants in sudangrass and grain sorghum. *Crop Science* 21, 706-709.
- Fritz, J.O., Moore, K.J., and Jaster, E.H. (1990). Digestion kinetics and cell wall composition of brown midrib sorghum×sudangrass morphological components. *Crop Science* 30, 213-219.
- Fromm, M. (1994). Production of transgenic maize plants via microprojectile-mediated gene transfer. *In* Freeling M., and Walbot, V. (Eds.) *The Maize Handbook*. Springer-Verlag, New York. p 677-685.
- Fromm, M.E., Morrish, F., Armstrong, C., Williams, R., Thomas, J., and Klein, T.M. (1990). Inheritance and expression of chimeric genes in the progeny of transgenic maize plants. *Bio/Technology* 8, 833-839.
- Fromm, M., Taylor, L.P., and Walbot, V. (1986). Stable transformation of maize after gene transfer by electroporation. *Nature* 319, 791-793.
- Fukushima, K. Terashima, N. (1990). Heterogeneity in formation of lignin. XIII. Formation of p- hydroxyphenyl lignin in various hardwoods visualized by microautoradiography. *Journal of wood chemistry and technology*. 10, 413-433.
- Futterer, J., and Hohn, T. (1996). Translation in plants. *Plant Molecular Biology* 32, 159-189.
- Gallo-Meagher, M., and Irvine, J.E. (1996). Herbicide resistant transgenic sugarcane plants containing the *bar* gene. *Crop Science* 36, 1367-1373.
- Gallo-Meagher, M., and Irvine, J.E. (1993). Effects of tissue type and promoter strength on transient GUS expression in sugarcane following particle bombardment. *Plant Cell Reports* 11, 567-570.
- Gasser, C.S., and Fraley, R.T. (1989). Genetically engineering plants for crop improvement. *Science* 244, 1293-1298.
- Gauthier, A., Gulick, P.J., Ibrahim, R. (1998). Characterization of two cDNA clones which encoded O-methyltransferases for the methylation of both flavonoid and phenylpropanoid compounds. *Archives of Biochemistry and Biophysics* 351, 243-249.
- Goering, H.K., and Van Soest, P.J. (1970). Forage fiber analyses (apparatus, reagents, procedures and some applications). *Agriculture Handbook* 379. U.S. Government Print Office, Washington D.C.

- Golding, E.J., Carter, M.F., and Moore, J.E. (1985). Modification of the neutral detergent fiber procedure for hays. *Journal of Dairy Science* 68, 2732-2736.
- Gordon-Kamm, W.J., Spencer, T.M., Mangano, M.L., Adams, T.R., Daines, R.J., Start, W.G., O'Brien, J.V., Chambers, S.A., Adams, Jr., W.R., Willetts, N.G., Rice, T.B., Mackey, C.J., Krueger, R.W., Kausch, A.P., and Imaux, P.G. (1990). Transformation of maize cells and regeneration of fertile transgenic plants. *Plant Cell* 2, 603-618.
- Gowri, G., Bugos, R.C., Campbell, W.H., Maxwell, C.A., Dixon, R.A. (1991). Stress responses in alfalfa (*Medicago sativa* L.). X Molecular cloning and expression of S-adenosyl-L-methionine:caffeic acid 3-O-methyltransferase, a key enzyme of lignin biosynthesis. *Plant Physiology* 97, 7-14.
- Grabber, J. H., Ralph, J., Hatfield, R. D., and Quideau, S. (1997). p-Hydroxyphenyl, guaiacyl, and syringyl lignins have similar inhibitory effects on wall degradability. *Journal of Agricultural and Food Chemistry* 45, 2530-2532.
- Grand, C., Parmentier, P., Boudet, A. and Boudet, A.M. (1985). Comparison of lignins and of enzymes involved in lignification in normal and brown midrib (*bmr*) mutant corn seedlings. *Physiologie Vegetale* 23, 905-911.
- Halpin, C., Knight, M.E., Foxon, G.A., Campbell, M.M., Boudet, A.M., Boon, J.J., Chabbert, B., Toller, M.T., and Schuch, W. (1994). Manipulation of lignin quality by down-regulation of cinnamyl alcohol dehydrogenase. *The Plant Journal* 6, 339-350.
- Hamilton, A.J., Fray, R.G., and Grierson, D. (1995). Sense and antisense inactivation of fruit ripening genes in tomato. *In* Gene Silencing in Higher Plants and Related Phenomena in Other Eukaryotes. Meyer, P. Ed. Springer-Verlag, p77-89.
- Hanna, W.W., Monson, W.G. and Gaines, T.P. (1981). IVDMD, total sugars, and lignin measurements on normal and brown midrib (*bmr*) sorghums at various stages of development. *Agronomy Journal* 73, 1050-1052.
- Hansen, G., Shillito, R.D., Chilton, M.D. (1997). T-strand integration in maize protoplasts after codelivery of a T-DNA substrate and virulence genes. *Proceedings of the National Academy of Sciences of the United States of America*. 94, 11726-11730.
- Hayakawa, T., Nanto, K., Kawai, S., Katayama, Y., and Morohoshi, N. (1996). Molecular cloning and tissue-specific expression of that genes that encode caffeic acid O-methyltransferases from *kitakamiensis*. *Plant Science* 113, 157-165.

- Hibberd, J.M., Linley, P.J., Khan, M.S., Gray, J.C. (1998). Transient expression of green fluorescent protein in various plastid types following microprojectile bombardment. *The Plant Journal : for Cell and Molecular Biology* 16, 627-632
- Higuchi, T. (1985). Biosynthesis of lignin. In *Biosynthesis and biodegradation of wood components*. T Higuchi, ed., Academic Press, New York, p141-160.
- Hood, E.E., Witcher, D.R., Maddock, S., Meyer, T., Baszczynski, C., Bailey, M., Flynn, P., Register, J., Marshall, L., Bond, D., Kulisek, E., Kusnadi, A., Evangelista, R., Nikolov, Z., Wooge, C., Mehig, R.J., Hernan, R., Kappel, W.K., Ritland, D., Li, C.P., and Howard, J.A. (1997). Commercial production of avidin from transgenic maize: characterization of transformant, production, processing, extraction and purification. *Molecular Breeding* 3, 291-306.
- Huang, M.T., and Gorman, C.M. (1990). Intervening sequences increase efficiency of RNA 3' processing and accumulation of cytoplasmic RNA. *Nuclear Acids Research* 18, 937-947.
- Ibrahim, R.K., Bruneau, A., and Bantignies B. (1998). Plant O-methyltransferase: molecular analysis, common signature and classification. *Plant Molecular Biology* 36, 1-10.
- Iiyama, K., Lam, T.B.T., Meikle, P.J., Rhodes, D.I., and Stone, B.A. (1993). Cell wall biosynthesis and its regulation. In Jung, H.G., Buxon, D.R., Hatfield, R.D., and Ralph, J. (Eds.) *Forage Cell Wall Structure and Digestibility*. ASA, Madison, WI, p621-683.
- Iiyama, K., Lam, T.B.T., Stone, B.A. (1994). Covalent cross-links in the cell wall. *Plant Physiology*. 104, 315-320.
- Ingelbrecht, I., Van Houdt, H., Van Montagu, M., Depicker, A. (1994). Posttranscriptional silencing of reporter transgenes in tobacco correlates with DNA methylation. *Proceedings of the National Academy of Sciences of the United States of America* 91, 10502-10506.
- Inoue, K., Sewalt, V.J.H., Ballance, G.M., Ni, W.T., Sturzer, C., and Dixon, R.A. (1998). Developmental expression and substrate specificities of alfalfa caffeic acid 3-O-methyltransferase and caffeoyl coenzyme A 3-O-methyltransferase in relation to lignification. *Plant Physiology* 117, 761-770.
- Ishida, Y.J., Saito, H., Ohta, S., Hiei, Y., Komari, T., and Kumashiro, T. (1996). High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. *Nature Biotechnology* 14, 745-751.

- Jaeck, E., Dumas, B., Geoffroy, P., Favet, N., Inze, D., Montagu, M., Van Fritig, B., Legrand, M. (1992). Regulation of enzymes involved in lignin biosynthesis: induction of O-methyltransferase mRNAs during the hypersensitive reaction of tobacco to tobacco mosaic virus. *Molecular Plant-Microbe Interactions* 5, 294-300.
- Jaeck, E., Martz, F., Stiefel, V., Fritig, B., and Lerand, M. (1996). Expression of class I O-methyltransferase in healthy and TMV-infected tobacco. *Molecular Plant-Microbe Interactions* 9, 681-688.
- Jahne, A., Becker, D., and Lorz, H. (1995). Genetic engineering of cereal crop plants: a review. *Euphytica* 85, 35-44.
- Jefferson, R.A. (1987). Assaying chimeric genes in plants: The GUS gene fusion system. *Plant Molecular Biology Reporter* 5, 387-405.
- Johnson, R.R., Dehority, B.A., Parson, J.L., and Scott, H.W. (1962). Discrepancies between grasses and alfalfa when estimating nutritive value from in vitro cellulose digestibility by rumen microorganisms. *Journal of Animal Science* 21, 892-896.
- Jorgenson, L.R. (1931). Brown midrib in maize and its linkage relations. *Journal of the American Society of Agronomy* 23, 549-557.
- Joseleau, J.P., and Ruel, K. (1997). Study of lignification by noninvasive techniques in growing maize internodes. *Plant Physiology* 114, 1123-1133.
- Jung, H.G. (1989). Forage lignins and their effects on fiber digestibility. *Agronomy Journal* 81, 33-38.
- Jung, H.G., and Allen, M.S. (1995). Characteristics of plant cell walls affecting intake and digestibility of forages by ruminants. *Journal of Animal Science* 73, 2774-2790.
- Jung, H.G., and Buxton, D.R. (1994). Forage quality variation among maize inbreds: relation of cell-wall composition and *in-vitro* degradability for internodes. *Journal of the Science of Food and Agriculture* 66, 313-322.
- Jung, H.G., and Deetz, D.A. (1993). Cell wall lignification and degradability. *In* Jung, H.G., Buxon, D.R., Hatfield, R.D., and Ralph, J. (Eds.) *Forage Cell Wall Structure and Digestibility*. ASA, Madison, WI, p315-346.
- Jung, H.G., Mertens, D.R., and Payne, A.J. (1997). Correlation of acid detergent lignin and Klason lignin with digestibility of forage dry matter and neutral detergent fiber. *Journal of Dairy Science* 80, 1622-1628.

- Jung, H.G., Ni, W.T., Chapple, C.C.S., and Meyer, K. (1999). Impact of lignin composition on cell-wall degradability in an *Arabidopsis* mutant. *Journal of the Science of Food and Agriculture* 79, 922-928.
- Kaepper, H.F., Somers, D.A., Rines, H.W., and Cockburn, A.F. (1992). Silicon carbide fiber-mediated stable transformation of plant cells. *Theoretical & Applied Genetics* 84, 560-566.
- Kajita, S., Hishiyam, S., Tomimura, Y., Katayama, Y., and Omori, S. (1997). Structural characterization of modified lignin in transgenic tobacco plants in which the activity of 4-coumarate: coenzyme a ligase is depressed. *Plant Physiology* 114, 871-879.
- Kemp, J.D., Sutton, D.W., Hack, E. (1979). Purification and characterization of nopaline synthase *Agrobacterium tumefaciens*. *Plant Physiology* 63, 58-62.
- Kihara, M., Saeki, K., Ito, K. (1998). Rapid production of fertile transgenic barley (*Hordeum vulgare* L.) by direct gene transfer to primary callus-derived protoplasts. *Plant Cell Reports* 17, 937-940.
- Kilby, N.J., Leyser, H.M.O., Furner, I.J. (1992). Promoter methylation and progressive transgene inactivation in *Arabidopsis*. *Plant Molecular Biology* 20, 103-112.
- Kim, S.K., and Wold, B.J. (1985). Stable reduction of thymidine kinase activity in cells expressing high levels of antisense RNA. *Cell* 42, 129-138.
- Klein, T.M., Wolf, E.D., Wu, R., and Sanford, J.C. (1987). High velocity microprojectiles for delivering nucleic acids into living cells. *Nature* 327, 70-73.
- Klimyuk, V.I., Carroll, B.J., Thomas, C.M., and Jones, J.D.G. (1993). Alkali treatment for rapid preparation of plant material for reliable PCR analysis. *The Plant Journal* 3, 494-494.
- Kota, M., Daniell, H., and Varma, S. (1999). Overexpression of the *Bacillus thuringiensis* (Bt) Cry2Aa2 protein in chloroplasts confers resistance to plants against susceptible and Bt-resistant insects. *Proceedings of the National Academy of Sciences of the United States of America* 96, 1840-1845.
- Kozziel, M.G., Carozzi, N.B., and Desai, N. (1996). Optimizing expression of transgenes with an emphasis on post-transcriptional events. *Plant Molecular Biology* 32, 393-405.
- Kumar, M. and Carmichael, G.G. (1998). Antisense RNA: function and fate of duplex RNA in cells of higher eukaryotes. *Microbiology and Molecular Biology Reviews* 62, 1415-1434.

- Kumapatla, S.P., Teng, W., Buchholz, W.G., and Hall, T.C. (1997). Epigenetic transcriptional silencing and 5-azacytidine-mediated reactivation of a complex transgene in rice. *Plant Physiology* 115, 361-373.
- Lagowski, J.M., Sell, H.M., Huffman, C.F., and Duncan, C.W. (1958) The carbohydrates in alfalfa. I. General composition, identification of a nonreducing sugar and investigation of the pectic substances. *Arch. Biochemistry and Biophysics* 76:306-316.
- Lagrimini, L.M., Gingas, V., Finger, F., Rothstein, S. and Liu, T.T. (1997). Characterization of antisense transformed plants deficient in the tobacco anionic peroxidase. *Plant Physiology* 114, 1187-1196.
- Lapierre, C., Pollet, B., Petit-Conil, M., Toval, G., Romero, J., Pilate, G., Leple, J.C., and Boerjan, W. (1999). Structural alteration of lignins in transgenic poplars with depressed cinnamyl alcohol dehydrogenase or caffeic acid O-methyltransferase activity have an opposite impact on the efficiency of industrial Kraft pulping. *Plant Physiology* 119, 153-163.
- Lazzeri P.A., and Shewry P.R. (1993). Biotechnology of cereals. *Biotechnology & Genetic Engineering Reviews* 13, 79-146.
- Lechtenberg, V.L., Muller, L.D., Bauman, L.F., Rhykerd, C.L. and Barnes, R.F. (1972). Laboratory and *in vitro* evaluation of inbred and F₂ populations of brown midrib mutants of *Zea mays* L. *Agronomy Journal* 64, 657-660.
- Legrand, M., Atanassova, R., Favet, N., Martz, F., Chabbert, B., Tollier, M.T., Monties, B. (1994). Inhibition of O-methyltransferase (OMT) activity in transgenic tobacco plants modified lignin monomeric composition. *International Plant Molecular Biology Meeting*, Amsterdam.
- Li, B. (1991). Introduction of foreign genes into the seed embryo cells of rice by electroinjection and the regeneration of transgenic plants. *Science in China* 34, 923-931.
- Li, L., Popko, J.L., Zhang, X.H., Osakabe, K., Tsai, C.J., Joshi, C.P., Chiang, V.L. (1997). A novel multifunctional O-methyltransferase implicated in a dual methylation pathway associated with lignin biosynthesis in loblolly pine. *Proceedings of the National Academy of Sciences of the United States of America* 94, 5461-5466.
- Lowe, K., Bowen, B., Hoerster, G., Ross, M., Bond, D., Pierce, D., and Gordon-Kamm, B. (1995). Germline transformation of maize following manipulation of chimeric shoot meristems. *Bio/Technology* 13, 677-681.

- Luo, G., Hepburn, A.G., Widholm, J.M. (1992). Preparation of plant DNA for PCR analysis: a fast, general and reliable procedure. *Plant Molecular Biology Reporter* 10, 319-323.
- Lyman Ott, R. (1993). An introduction to statistical methods and data analysis. Duxbury Press, Belmont, California.
- Martz, F., Maury, S., Pincon, G., and Legrand, M. (1998). cDNA cloning, substrate specificity and expression study of tobacco caffeoyl-CoA 3-O-methyltransferase, a lignin biosynthetic enzyme. *Plant Molecular Biology* 36, 427-437.
- Matsui, N., Fukushima, K., Yasuda, S., Terashima, N. (1994). On the behavior of monolignol glucosides in lignin biosynthesis. II. Synthesis of monolignol glucosides labelled with ^3H at the hydroxymethyl group of sidechain and incorporation of the label into magnolia and ginkgo lignin. *Holzforschung* 48, 375-380.
- Matzke, M.A. and Matzke, A.J.M. (1995). How and why do plants inactivate homologous (trans)genes? *Plant physiology* 107, 679-685.
- McElroy, D., Zhang, D., and Wu, R. (1990). Isolation of an efficient actin promoter for use in rice transformation. *Plant Cell* 2, 163-171.
- Meins, F., and Kunz, C. (1995). Gene silencing in transgenic plants: a heuristic autoregulation model. *In* *Gene Silencing in Higher Plants and Related Phenomena in Other Eukaryotes*. Meyer, P. Ed. Springer-Verlag, p105-120.
- Meng, H.B., and Campbell, W.H. (1998). Substrate profiles and expression of caffeoyl coenzyme A and caffeic acid O-methyltransferases in secondary xylem of aspen during seasonal development. *Plant Molecular Biology* 38, 513-520.
- Mercola, D., Westwick, J., Rundell, A.Y.K., Adamson, E.D., and Edwards, S.A. (1988). Analysis of a transformed cell lines using antisense *c-fos* RNA. *Gene* 72, 253-265.
- Meyer, P. (1995) Understanding and controlling transgene expression. *Trends in Biotechnology* 13, 332-337.
- Meyer, P., and Heidmann, I. (1994). Epigenetic variants of a transgenic petunia line show hypermethylation in transgene DNA: an indication for specific recognition of foreign DNA in transgenic plants. *Molecular & General Genetics* 243, 390-399.

- Mol, J.N.M., Van der Krol, A.R., Van Tunen, A.J., Van Blokland, R., De Lange, P., Stuitje, A.R. (1990). Regulation of plant gene expression by antisense RNA. *FEBS Letters* 268, 427-430.
- Monties, B., Tollier, M.T., Chabbert, B., Lapierre, C., Legrand, M., Favet, N., Atanassova, R., Martz, F., and Fritig, B. (1994). Mutant and transgenic plants with similar lignin-structure. *International Plant Molecular Biology Meeting*, Amsterdam.
- Mooney, P.A., and Goodwin, P.B. (1991). *Agrobacterium tumefaciens* gene transfer into wheat tissues. *Plant Cell Tissue and Organ Culture* 25, 209-218.
- Moore, J.E., and Mott, G.O. (1974). Recovery of residual organic matter from *in vitro* digestion of forages. *Journal of Dairy Science*, 1258-1259.
- Morrow, S.L., Mascia, P., Self, K.A., and Altschuler, M. (1997). Molecular characterization of a brown midrib3 deletion mutation in maize. *Molecular Breeding* 3, 351-357.
- Muller, L.D., Barnes, R.F., Bauman, L.F., and Colenbrander, V.F. (1971). Variations in lignin and other structural components of brown midrib mutants of maize. *Crop Science* 11, 413-415.
- Mullis, K.B., and Faloona, F.A. (1987). Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. *Methods in Enzymology* 155, 335-350.
- Ni, W., Paiva, N.A., and Dixon, R. A. (1994). Reduced lignin in transgenic plants containing a caffeic acid O-methyltransferase antisense gene. *Transgenic Research* 3, 120-126.
- Olszewski, N., Hagen, G., and Guilfoyle, T.J. (1982). A transcriptional active, covalently closed minichromosome of cauliflower mosaic virus DNA isolated from infected turnip leaves. *Cell* 29, 395-402.
- Omirlulleh, S., Abraham, M., Golovkin, M., Stefanov, I., Karabaevev, M.K., Mustardy, L., Morocz, S., Dudits, D. (1993). Activity of a chimeric promoter with the doubled CaMV 35S enhancer element in protoplast-derived cells and transgenic plants in maize. *Plant Molecular Biology* 21, 415-428.
- Palevitz, B.A. (1999). Bt or not Bt... Transgenic corn vs. Monarch butterflies. *The Scientist* 13, 12.
- Pawlowski, W.P., and Somers, D.A. (1996). Transgene inheritance in plants genetically engineered by microprojectile bombardment. *Molecular Biotechnology* 6, 17-30.

- Pellegrini, L., Geoffroy, P., Fritig, B., and Legrand, M. (1993). Molecular cloning and expression of a class of ortho-diphenol-O-methyltransferases induced in tobacco (*Nicotiana tabacum* L.) leaves by infection or elicitor treatment. *Plant Physiology* 103, 509-517.
- Pescitelli, S.M., and Sukhapinda, K. (1995). Stable transformation via electroporation into maize type II callus and regeneration of fertile transgenic plants. *Plant Cell Reports* 14, 712-716.
- Piquemal, J., Lapierre, C., Myton, K., O'Connell, A., Schuch W., Grima-Pettenati J., and Boudet, A.M. (1998). Down-regulation of cinnamoyl-CoA reductase induces significant changes of lignin profiles in transgenic tobacco plants. *The Plant Journal* 13, 71-83.
- Poeydomenge, O., Boudet, A.M., Grima-Pettenati, J. (1994). A cDNA encoding S-adenosyl-L-methionine:caffeic acid 3-O-methyltransferase from *Eucalyptus*. *Plant Physiology* 105, 749-750.
- Porter, K.S., Axtell, J.D., Lechtenberg, V.L., and Colenbrander, V.F. (1978). Phenotype, Fiber composition, and in vitro dry matter disappearance of chemically induced brown midrib (*bmr*) mutants of sorghum. *Crop Science* 18, 205-208.
- Provan, G.J., Scobbie, L., and Chesson, A. (1997). Characterization of lignin from CAD and OMT deficient *bm* mutants of maize. *Journal of the Science of Food and Agriculture* 73, 133-142.
- Ray, D.S., and Ghosh, P.D. (1990). Somatic embryogenesis and plant regeneration from cultured leaf explants of *Zea mays*. *Annals of Botany* 66, 497-500.
- Register III, J.C., Peterson, D.J., Bell, P.J., Bullock, W.P., Evans, I.J., Frame, B., Greenland, A.J., Higgs, N.S., Jepson, I., Jiao, S., Lewnau, C.J., Sillick J.M., and Wilson H.M. (1994). Structure and function of selectable and non-selectable transgenes in maize after introduction by particle bombardment. *Plant Molecular Biology* 25, 951-961.
- Rhodes, C.A., Green, C.E., and Phillips, R.L. (1986). Factors affecting tissue culture initiation from maize tassels. *Plant Science* 46, 225-232.
- Robert, L.S., Donaldson, P.A., Ladaique, C., Altosaar, I., Arnison, P.G., and Fabijanski, S.F. (1989). Antisense RNA inhibition of beta-glucuronidase gene expression in transgenic tobacco plants. *Plant Molecular Biology* 13, 399-409.
- Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239, 487-491.

- Sambrook, J., Fritsch, E.F., Maniatis, T. (1989). Molecular cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Sandler, S.J., Stayton, M., Townsend, J.A., Ralston, M.L., Bedbrook, J.R., and Donsmuir, P. (1988). Inhibition of gene expression in transformed plants by antisense RNA. *Plant Molecular Biology* 11, 301-310.
- Sanford, J.C. (1990). Biolistic plant transformation. *Physiology of Plant* 79, 206-209.
- Sanford, J.C., Klein, T.M., Wolf, E.D., and Allen, N. (1987). Delivery of substances into cells and tissues using a particle bombardment process. *Particle Science and Technology* 5, 27-37.
- Sarkanen, K.V., and Ludwig, C.H. (1971). Definition and nomenclature. In K.V. Sarkanen and C.H. Ludwig (Ed.) *Lignins. Occurrence, formation, structure, and reactions*. Wiley-Interscience, New York, p1-18.
- Sewalt, V.J.H., Ni, W.T., Blount, J.W., Jung, H.G., Masoud, S.A., Howles, P.A., Lamb, C. and 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 Physiology* 115, 41-50.
- Sewalt, V.J.H., Weiting, N., Jung, H.G., and Dixon, R.A. (1997). Lignin impact on fiber degradation: increased enzymatic digestibility of genetically engineered tobacco (*Nicotiana glauca*) stems reduced in lignin content. *Journal of Agriculture and Food Chemistry* 45, 1977-1983.
- Sheng, J., and Citovsky, V. (1996). Agrobacterium-plant cell DNA transport: have virulence proteins, will travel. *The Plant Cell* 8, 1699-1710.
- Shillito, R.D., Carswell, G.K., Johnson, C.M., Dimaio, J.J., and Harms, C.T. (1989). Regeneration of fertile plants from protoplasts of elite inbred maize. *Bio/Technology* 7, 581-587.
- Sikdar, S.R., Serino, G., Chaudhuri, S., and Maliga, P. (1998). Plastid transformation in *Arabidopsis thaliana*. *Plant Cell Reports*. 18, 20-24.
- Simpson, G.G., and Filipowicz, W. (1996). Splicing of precursors to mRNA in higher plants: mechanism, regulation, and sub-nuclear organization of the spliceosomal machinery. *Plant Molecular Biology* 32, 1-41.

- Songstad, D.D., Petersen, W.L., and Armstrong, C.L. (1992). Establishment of friable embryogenic (type II) callus from immature tassels of *Zea mays* (Poaceae). *American Journal of Botany* 79, 761-764.
- Southern, E.M. (1975). Detection of specific DNA sequences among fragments separated by gel electrophoresis. *Journal of Molecular Biology* 98, 503-517.
- Spencer, T.M., Gordan-Kamm, W.J., Danies, R.J., Start, W.G. and Lemaux, P.G. (1990). Bialaphos selection of stable transformants from maize cell culture. *Theoretical and Applied Genetics* 79, 625-631.
- Spencer, T.M., O'Brien, J.V., Start, W.G., Adams, T.R., Gordon-Kamm, W.J., and Lemaux, P.G. (1992). Segregation of transgenes in maize. *Plant Molecular Biology* 18, 201-210.
- Stam, M., Mol, J.N.M., and Kooter, J.M. (1997). The silence of genes in transgenic plants. *Annals of Botany* 79, 3-12.
- Stockhaus, J., Hofer, M., Renger, G., Westhoff, P., Wydrzynski, T., and Willmitzer, L. (1990). Anti-sense RNA efficiently inhibits formation of the 10 kd polypeptide of the photosystem II in transgenic plants: analysis of the role of the 10 kd protein. *The EMBO Journal* 9, 3013-3021.
- Tachibana, K., Kaneko, K. (1986). Development of a new herbicide, bialaphos. *Journal of pesticide science* 11, 297-304.
- Taiz, L. and Zeiger E. (1991) Surface protection and secondary defense compounds. In *Plant Physiology*, Brady, E.B. and Donohoe, L. (Eds). Benjamin/Cummings Publishing Company, Inc. p318-343.
- Takano, M., Egawa, H., Ikeda, J.E., and Wakasa, K. (1997). The structures of integration sites in transgenic rice. *The Plant Journal* 11, 353-361.
- Temple, S.J., Knight, T.J., and Unkefer, P. J. (1993). Modulation of glutamine synthetase gene expression in tobacco by the introduction of an alfalfa glutamine synthetase gene in sense and antisense orientation: molecular and biochemical analysis. *Molecular & General Genetics* 236, 315-325.
- Terashima, N., Fukushima, K., He, L.F., Takaba, K. (1993). Comprehensive model of the lignified plant cell wall. In Jung, H.G., Buxon, D.R., Hatfield, R.D., and Ralph, J. (Eds.) *Forage Cell Wall Structure and Digestibility*. ASA, Madison, WI, p247-270.

- Terashima N, Fukushima K, and Takaba K. (1986) Heterogeneity in formation of lignin VIII: an autoradiographic study on the formation of guaiacyl and syringyl lignin in *Magnolia kobus*. *Holzforschung* 42, 347-350.
- Theander, O., and Westerlund, E. (1993). Quantitative analysis of cell wall components. *In* Jung, H.G., Buxon, D.R., Hatfield, R.D., and Ralph, J. (Eds.) *Forage Cell Wall Structure and Digestibility*. ASA, Madison, WI, p83-104.
- Thompson, J.A., Drayton, P.R., Frame, B.R., Wang, K. and Dunwell, J.M. (1995). Maize transformation utilizing silicon carbide whiskers: a review. *Euphytica* 85, 75-80.
- Thompson, C.J., Movva, N.R., Tizard, R., Cramer, R., Davies, J.E., Lauwereys, M., and Botterman, J. (1987). Characterization of the herbicide-resistance gene *bar* from *Streptomyces hygroscopicus*. *The EMBO Journal* 6, 2519-2523.
- Thorsten, E.M.G., Buxton, D.R., and Cherney, J.H. (1992). Apparent inhibition to digestion by lignin in normal and brown midrib stems. *Journal of the Science of Food and Agriculture* 59, 183-188.
- Tilley, J.M.A., and Terry, R.A. (1963). A two-stage technique for the *in vitro* digestion of forage crops. *Journal of British Grassland Society* 18, 104-111.
- Tingay, S., McElory, D., Kalla, R., Fieg, S., Wang, M., and Thornton, S. (1997). *Agrobacterium tumefaciens*-mediated barley transformation. *The Plant Journal* 11, 1369-1376.
- Tomes, D.T., Ross, M.C., Songstad, D.D. (1995). Direct DNA transfer into intact plant cells via microprojectile bombardment. *In* Gamborg, O.L., and Phillips, G.C. (Eds.) *Plant Cell, Tissue and Organ Culture*. Birkhauser Boston, Cambridge. p197-213.
- Tomlin, D.C., Johnson, R.R., and Dehority, B.A. (1964). Relationship of lignification to *in vitro* cellulose digestibility of grasses and legumes. *Journal of Animal Science* 23, 161-165.
- Tsai, C.J., Popko, J.L., Mielke, M.R., Hu, W.J., Podila, G.K., and Chiang, V.L. (1998). Suppression of *O*-methyltransferase gene by homologous sense transgene in quaking aspen causes red-brown wood phenotypes. *Plant Physiol* 117, 101-112.
- Tsugawa, H., Otsuki, Y., and Suzuki, M. (1998) Efficient transformation of rice protoplasts mediated by a synthetic polycationic amino polymer. *Theoretical and applied genetics* 97, 1019- 1026.
- Vailhe, M.A.B., Migne, C., Cornu, A., Maillot, M.P., Grenet, E., and Besle, J.M. (1996). Effect of modification of the *O*-methyltransferase activity on cell wall

- composition, ultrastructure and degradability of transgenic tobacco. *Journal of the Science of Food and Agriculture* 72, 385-391.
- Vain, P., McMullen, M.D., and Finer, J.J. (1993). Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize. *Plant Cell Reports* 12, 84-88.
- Van der Krol, A.R., Lenting, P.E., Veenstra, J., Van der Meer, I.M., Koes, R.E., Gerats, A.G.M., Mol, J.N.M., Stuitje, A.R. (1988). An antisense chalcone synthase gene in transgenic plants inhibits flower pigmentation. *Nature* 333, 866-869.
- Van der Krol, A.R., Mur, L.A., De Lange, P., Mol, J.N.M., and Stuitje, A.R. (1990). Inhibition of flower pigmentation by antisense genes: promoter and minimal sequence requirements for the antisense effect. *Plant Molecular Biology* 14, 457-466.
- Van der Meer, I.M., Stam, M., Van Tunen, A.J., Stuitje, A.R. (1992). Antisense inhibition of flavonoid biosynthesis in *Petunia* anthers results in male sterility. *Plant Cell* 4, 253-262.
- Van Soest, P.J. (1963a). Use of detergents in the analysis of fibrous feeds. I. Preparation of fiber residues of low nitrogen content. *Journal of the Association of Official Analytical Chemists* 48, 825-829.
- Van Soest, P.J. (1963b). Use of detergents in the analysis of fibrous feeds. II. A rapid method for the determination of fiber and lignin. *Journal of the Association of Official Analytical Chemists* 48, 829-835.
- Van Soest, P.J. (1967). Development of a comprehensive system of feed analyses and its application to forages. *Journal of Animal Science* 26, 119-128.
- Van Soest, P.J. (1993). Cell wall matrix interactions and degradation-session synopsis. *In* Jung, H.G., Buxon, D.R., Hatfield, R.D., and Ralph, J. (Eds.) *Forage Cell Wall Structure and Digestibility*. ASA, Madison, WI, p377-395.
- Van Soest, P.J. (1994). Lignin. *In* *Nutritional Ecology of the Ruminant*. Ithaca : Comstock Publication, New York. p177-195.
- Van Soest, P.J., and Wine, R.H. (1967). Use of detergents in the analysis of fibrous feeds. IV. Determination of plant cell-wall constituents. *Journal of the Association of Official Analytical Chemists* 50, 50-55.
- Vasil, I.K. (1994). Molecular improvement of cereals. *Plant Molecular Biology* 25, 925-937.

- Vasil, I.K. (1996). Milestones in crop biotechnology -transgenic cassava and *Agrobacterium*-mediated transformation of maize. *Nature Biotechnology* 14, 702-703.
- Vasil, V., Castillo, A.M., Fromm, M.E., and Vasil, I.K. (1992). Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus. *Bio/Technology* 10, 667-674.
- Vignols, F., Rigau, J., Torres, M.A., Capellades, M., and Puigdomenech, P. (1995). The *brown midrib3 (bm3)* mutation in maize occurs in the gene encoding caffeic acid O-methyltransferase. *The Plant Cell* 7, 407-416.
- Walters, D.A., Vetsch, C.S., Potts, D.E., and Lundaquist, R.C. (1992). Transformation and inheritance of a hygromycin phosphotransferase gene in maize plants. *Plant Molecular Biology* 18, 189-200.
- Wan, Y., and Lemaux, P.G. (1994). Generation of large numbers of independently transformed fertile barley plants. *Plant Physiology* 104, 37-48.
- Waterhouse, P.M., Graham, M.W., and Wang, M.B. (1998). Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. *Proceedings of the National Academy of Sciences of the United States of America* 95, 13959-13964.
- Welter, M.E., Clayton, D.S., Miller, M.A., and Petolino, J.F. (1995). Morphotypes of friable embryogenic maize callus. *Plant Cell Reports* 14, 725-729.
- Whetten, R., and Sederoff, R. (1995). Lignin biosynthesis. *The Plant Cell* 7, 1001-1013.
- Wilman, D., and Rezvani Moghdam, P. (1998). *In vitro* digestibility and neutral detergent fibre and lignin contents of plant parts of nine forage species. *Journal of Agricultural Science, Cambridge* 131, 51-58.
- Ye, Z.H. (1997). Association of caffeoyl coenzyme A 3-O-methyltransferase expression with lignifying tissues in several dicot plants. *Plant Physiology* 115, 1341-1350.
- Ye, Z.H., Kneusel, R.E., Matern, U., Varner, J.E. (1994). An alternative methylation pathway in lignin biosynthesis in *Zinnia*. *The Plant Cell* 6, 1427-1439.
- Ye Z.H., and Varner, J.E. (1995). Differential expression of two O-methyltransferases in lignin biosynthesis in *Zinnia elegans*. *Plant Physiology* 108, 459-467.
- Zhang, S., Warkentin, D., Sun, B., Zhong, H. and Sticklen, M. (1996). Variation in the inheritance of expression among subclones for unselected (*uidA*) and selected

- (*bar*) transgenes in maize (*Zea mays* L.). Theoretical and Applied Genetics 92, 752-761.
- Zhong, R.Q., Morrison, H.M., Negrel, J., and Ye, Z.H. (1998). Dual methylation pathways in lignin biosynthesis. The Plant Cell 10, 2033-2045.
- Zhong, H., Sun, B., Warkentin, D., Zhang, S., Wu, R., Wu, T., and Sticklen, M.B. (1996a). The competence of maize shoot meristems for integrative transformation and inherited expression of transgenes. Plant Physiology 110, 1097-1107.
- Zhong, H., Zhang, S., Warkentin, D., Sun, B., Wu, T., Wu, R., and Sticklen, M.B. (1996b). Analysis of the functional activity of the 1.4-kb 5'-region of the rice *actin1* gene in stable transgenic plants of maize (*Zea mays* L.). Plant Science 116, 73-84.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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