The *brown midrib3* (*bm3*) Mutation in Maize Occurs in the Gene Encoding Caffeic Acid *O*-Methyltransferase

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The *brown midrib* mutations are among the earliest described in maize. Plants containing a *brown midrib* mutation exhibit a reddish brown pigmentation of the leaf midrib starting when there are four to six leaves. These mutations are known to alter lignin composition and digestibility of plants and therefore constitute prime candidates in the breeding of silage maize. Here, we show that two independent *brown midrib3* (*bm3*) mutations have resulted from structural changes in the *COMT* gene, which encodes the enzyme O-methyltransferase (COMT; EC 2.1.1.6), involved in lignin biosynthesis. Our results indicate that the *bm3-1* allele (the reference mutant allele) has arisen from an insertional event producing a *COMT* mRNA altered in both size and amount. By sequencing a *COMT* cDNA clone obtained from *bm3-1* maize, a retrotransposon with homology to the B5 element has been found to be inserted near the junction of the 3′ coding region of the *COMT* gene intron. The second *bm3* allele, *bm3-2*, has resulted from a deletion of part of the *COMT* gene. These alterations of the *COMT* gene were confirmed by DNA gel blot and polymerase chain reaction amplification analyses. These results clearly demonstrate that mutations at the *COMT* gene give a *brown midrib3* phenotype. Thus, the gene genetically recognized as *bm3* is the same as the one coding for COMT.

INTRODUCTION

Lignins are universal components in plants that form cross-links with carbohydrates, such as hemicellulose in the cell wall (for review, see Lewis and Yamamoto, 1990). The resulting amorphous matrix provides the plant with multiple advantages, such as mechanical strength, resistance to pathogens, and waterproof qualities. However, due to their chemical properties, lignins are also responsible for the low agronomic and industrial value of a number of species. Because lignins are strongly linked to other cell wall constituents, they are difficult to extract or to degrade (Sarkanen and Ludwig, 1971). Thus, lignins appear to be an undesirable constituent that increases manufacturers' costs. Once extracted, lignin byproducts produce an adverse impact on the environment. Agronomically, lignin polymers have been shown to lower fiber digestion in ruminants, and it has been proposed that the degree of lignification is inversely proportional to forage crop digestibility (Grand et al., 1985; Cherney et al., 1991). Lignin biosynthesis has been studied extensively both because it is intrinsically interesting scientifically and because it can be used to decrease lignin content in plants of economic interest.

Lignins are biosynthesized through a complex pathway that involves various enzymatic activities and polymerization steps. The first enzymes required for lignin biosynthesis are those from the phenylpropanoid pathway, such as phenylalanine ammonia-lyase and cinnamate 4-hydroxylase (Lewis and

Yamamoto, 1990). Later, a series of hydroxylations and methylations involving various hydroxylases and an *O*-methyltransferase (OMT) provides cinnamic acid derivatives. These compounds are finally converted to respective alcohols by cinnamoyl-coenzymeA reductase and cinnamyl alcohol dehydrogenase (CAD) (Lewis and Yamamoto, 1990). Different approaches designed to produce plants containing low lignin content include backcross selection, chemical mutagenesis (Cherney et al., 1991), and production of antisense plants for various enzymes involved in the lignin pathway (Dwivedi et al., 1993; Halpin et al., 1994; Ni et al., 1994). More logically, special attention has been devoted to plants carrying naturally occurring mutations and exhibiting this property.

The first *brown midrib* mutant was described in maize as early as 1924, in Minnesota (Jorgenson, 1931; Cherney et al., 1991). Plants possessing a *brown midrib* mutation exhibit a reddish brown pigmentation in the leaf midrib, which is initially visible when there are four to six leaves. As the plant grows and the stem lignifies, the reddish brown pigmentation is observed to be associated with vascular bundles and lignified rings in stem cross-sections. Later, the *brown midrib* phenotype gradually disappears from external exposed surfaces of the mature plant, but it is still detectable in internal and lignified tissues. Based on morphologic and genetic descriptions in maize, four mutations (*brown midrib1* [*bm1*], *bm2*, *bm3*, and *bm4*) located on chromosomes 5, 1, 4, and 9, respectively (Neuffer et al., 1968), were shown to produce this phenotype.

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Additional analyses of these mutants indicated that the *brown midrib* mutations could be associated with lower quantity and different quality of lignins (Kuc et al., 1964, 1968; Gentinetta et al., 1990). Moreover, the mutants appeared to be more easily digested by ruminants (Barnes et al., 1971; Lechtenberg et al., 1972). These studies essentially dealt with *bm3* maize plants (and *bm1* to a lesser extent) because the properties of this mutant (cell wall content and composition, agronomic values, and mechanical properties) were shown to be the most appropriate for breeding and feeding purposes (Barnes et al., 1971; Lechtenberg et al., 1972; Barrière and Argillier, 1993; Barrière et al., 1994). For these reasons, the *brown midrib* mutants, and especially *bm3*, have been selected as a forage crop. However, the types of mutations that have occurred in *brown midrib* mutants and their relationship with the lignification process are still not known.

Our group recently characterized the first gene encoding a lignin-related OMT in maize (Collazo et al., 1992). We showed that this gene encodes a bifunctional enzyme able to methylate both caffeic and 5-hydroxyferulic acids to produce ferulic and sinapic acids, respectively. This caffeic acid OMT (COMT; EC 2.1.1.6) protein is encoded by a single gene, although another *OMT*-related gene (44% homologous to the previous one) has been described in maize and is probably involved in the suberin biosynthesis pathway (Held et al., 1993). The lignin-related *COMT* gene is expressed in tissues undergoing lignification; these include elongating roots and vascular bundles in leaves (Collazo et al., 1992). A similar expression has been described for a number of other lignin-related *OMT* cDNAs (Bugos et al., 1991; Gowri et al., 1991; Dumas et al., 1992; Jaeck et al., 1992; Pellegrini et al., 1993). The obvious importance of maize *brown midrib* mutants and the availability of a *COMT* probe from maize allowed us to analyze further the effect of the *brown midrib* mutation on the expression of the *COMT* gene. The experiments described here report the molecular characterization of the lignification-related *bm3* mutation in maize.

RESULTS

COMT Gene Expression Is Modified by the *bm3* Mutation

The maize *COMT* gene is known to be strongly expressed in roots and to a lesser extent in the stem and in leaves (Collazo et al., 1992). To determine whether any of the *brown midrib* mutations modified *COMT* gene expression, different tissues of various *brown midrib* mutants were subjected to RNA gel blot analysis. RNA was first extracted from roots harvested from *bm1*, *bm2*, and *bm3* homozygote mutants maintained in W23 × L317 hybrids and hybridized with the 5′ coding region (XS1, Figure 1A) of the wild-type maize *COMT* gene as a probe. Figure 1B shows that *bm1* and *bm2* mutants exhibited the same expression pattern, as did the wild type. Thus, neither *bm1* nor *bm2* mutations appeared to alter *COMT* mRNA accumulation,
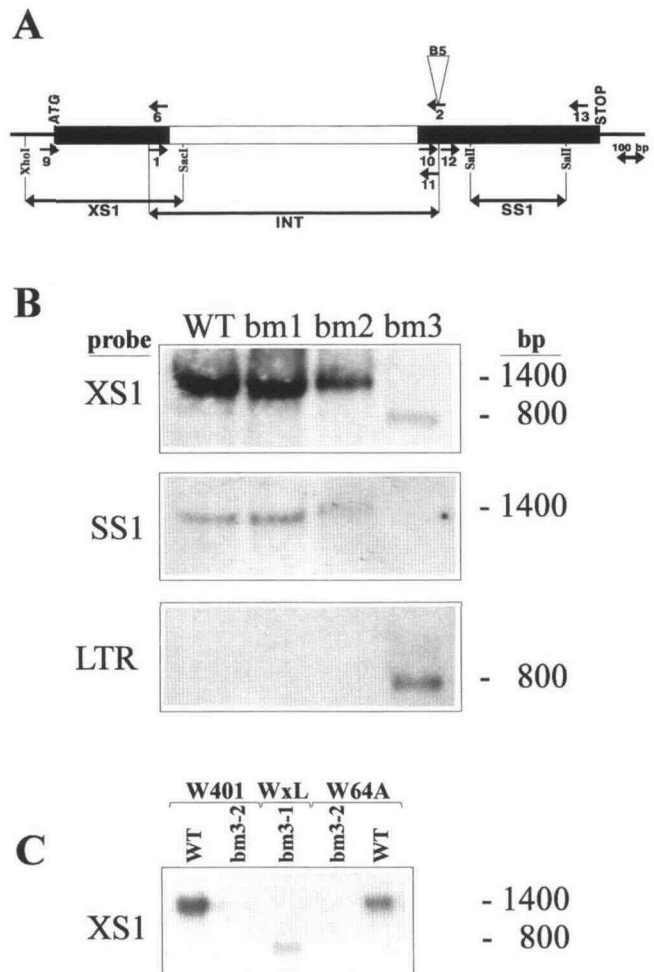
Figure 1. Expression of the Maize *COMT* Gene in Different *brown midrib* Mutants.

(A) Schematic representation of the MG18 *COMT* gene (Collazo et al., 1992). Exons are shown as solid boxes and the single intron as an open box. The positions of the different probes used in hybridization procedures are indicated below the scheme. Oligonucleotides used for PCR experiments are numbered and marked with arrows above and below the gene. The insertion site of the B5 element in the *COMT* is indicated with an open triangle.

(B) *COMT* mRNA accumulation in roots from *brown-midrib* mutants maintained in W23 × L317 hybrids. RNA gel blot analysis was performed from wild-type (WT) or *brown-midrib* homozygote plants for three different loci: *brown-midrib1* (bm1), *brown-midrib2* (bm2), and *brown-midrib3* (bm3). Probes XS1 and SS1 shown in **(A)** or LTR were radiolabeled and used to hybridize to the blot. The length of the hybridizing bands is indicated at right in base pairs.

(C) *COMT* mRNA accumulation in roots from *brown-midrib3* mutants maintained in pure lines. RNA gel blot analysis was performed from *bm3* homozygote plants maintained in W64A and W401 pure lines (bm3-2). WT refers to the W64A and W401 wild-type plants, and *bm3-1* refers to the *brown-midrib3* homozygote from **(B)** maintained in W23 × L317 hybrid (WxL). The length of the hybridizing bands is indicated at right in base pairs.

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CMT cDNA  - ........TCCGCCGCGCCCGTCTGCAAGTGGCTCACCCCCAACGAGGACGGCGTGTCCATGGCCGCCCTCGCGCTCATGAACCAGGACAAGGTCCTCATGGAGAGCTGGTACTATCTCA  480
                   ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
bm3 cDNA  - ........TCCGCCGCGCCCGTCTGCAAGTGGCTCACCCCCAACGAGGACGGCGTGTCCATGGCCGCCCTCGCGCTCATGAACCAGGACAAGGTCCTCATGGAGAGCTGGTACTATCTCA  112
bm3 prot  - ........S  A  A  P  V  C  K  W  L  T  P  N  E  D  G  V  S  M  A  A  L  A  L  M  N  Q  D  K  V  L  M  E  S  W  Y  Y  L
                   :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :
CMT prot  - ........S  A  A  P  V  C  K  W  L  T  P  N  E  D  G  V  S  M  A  A  L  A  L  M  N  Q  D  K  V  L  M  E  S  W  Y  Y  L


CMT cDNA  - AGGACGCGGTGCTGGACGGCGGCATCCCGTTCAACAAGGCGTACGGGATGACGGCGTTC........................................................end  1360
            ::::::::::::::::::::::::::::::::::::::  :  :   : : : :
bm3 cDNA  - AGGACGCGGTGCTGGACGGCGGCATCCCGTTCAACAAGGTGTCAGCCCTCAAGCCAGCAAGGAAGTGCCCAGTGACTCCGTCGCGTCGGATGAGCTATCACGTGCAAGCAGCCAAGAGCA  232
                                                         :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
wxB5 LTR  -                                           TGTCAGCCCTCAAGCCAGCAAGGAAGTGCCCAGTGACTCCGTCGCGTCGGATGAGCTATCACGTGCAAGCAGCCAAGAGCA  81
bm3 prot  - K  D  A  V  L  D  G  G  I  P  F  N  K  V  S  A  L  K  P  A  R  K  C  P  V  T  P  S  R  R  M  S  Y·  H  V  Q  A  A  K  S
             :  :  :  :  :  :  :  :  :  :  :  :  :  :
CMT prot  - K  D  A  V  L  D  G  G  I  P  F  N  K  A  Y  G  M  T  A  F.......


bm3 cDNA  - AGGAACTATGGGCCGTCGTGTCCGCAGCCGAATGGCCGTGTAATGGGTGGAGTATGGGTCAATTAGCTGGGCCGTAAACTTGTAAGCAGTTATTAAGGGAAACACTGGAGGCTGTGAACC  352
            :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
wxB5 LTR  - AGGAACTATGGGCCGTCGTGTCCGCAGCCGAATGGCCGTGTAATGGGTGGAGTATGGGTCAATTAGCTGGGCCGTAAACTTGTAAGCAGTTATTAAGGGAAACACTGGAGGCTGTGAACC  201
bm3 prot  - K  E  L  W  A  V  V  S  A  A  E  W  P  C  N  G  W  S  M  G  Q  L  A  G  P  *


bm3 cDNA  - CTTATGCAAGAAAGAATATTCGCCTAAAAAAAAAAAAAAAA  392
            :::::::::::::::::::::::::::::::  :
wxB5 LTR  - CTTATGCAAGAAAGAATATTCGCCTAACATCTCTCTGTTCCTCTCTCGTTCTCTTCCTCGCCTTCCTCAGCGCCGGGCGCACGTGCTGGTAGCTGACA  299
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Figure 2. Comparison of *COMT* cDNA Sequences Obtained from Wild-Type W64A and the *bm3-1* Mutant of Maize.

The sequence alignment involves *COMT* sequences of the wild-type MC1 (OMT cDNA), *bm3-1* mutant (bm3 cDNA), and the LTR sequence of the B5 element (wxB5 LTR). Nucleotide 520 from which *bm3* cDNA strongly diverges from MC1 *COMT* cDNA is marked with a thin vertical arrow. The position of the *COMT* gene intron in the corresponding genomic clone MG18 is indicated with a solid arrowhead. The "end" at position 1360 indicates the size of the wild-type *COMT* cDNA. A 6-bp sequence that could represent a putative polyadenylation signal in *bm3* cDNA is underlined. The stop codon in the *bm3* COMT sequence is marked with an asterisk. Protein sequence alignment between the wild-type COMT (OMT prot) and *bm3*-derived chimeric protein (bm3 prot) is shown below the nucleotide sequences. The two double-underlined sequences in wxB5 LTR represent the two 6-bp inverted repeat sequences of the LTR of the B5 element. Vertical double dots indicate a sequence identity. The Gen-Bank, EMBL, and DDBJ accession number of the *bm3-1 COMT* cDNA is X80669.

indicating that the *COMT* gene was not affected by these mutations. In the *bm3* mutant, however, the length of the band hybridizing to the XS1 probe was clearly shorter and the intensity of the signal greatly reduced (Figure 1B). This analysis indicated that ~600 nucleotides of the *COMT* mRNA were lacking in this mutant. An identical result was obtained when the same experiment was carried out with RNA extracted from *bm3* mesocotyls or leaves (data not shown). These results suggested that transcription of the *COMT* gene terminated early or that a post-transcriptional regulation event removed a portion of the *COMT* mRNA.

To determine which part of the *COMT* gene was lacking in the *COMT* mRNA, the same blot was hybridized with a probe corresponding to the 3' coding region of the *COMT* gene (SS1, Figure 1A). With such a probe, we observed a similar hybridization pattern for the wild type and *bm1* and *bm2* mutants (Figure 1B). For the *bm3* mutant, a different result was obtained. In this plant, the smaller band previously revealed with the XS1 probe could not be detected, suggesting that at least the SS1-corresponding region in the *COMT* gene was lacking in the *COMT* mRNA (Figure 1B).

All of these analyses were also performed with *bm3* homozygote mutants maintained in pure W64A and W401 maize lines (*bm3-2*, Figure 1C). In *bm3* roots of both W64A and W401 lines, no mRNA corresponding to *COMT* was detected with either the XS1 (Figure 1C) or SS1 (data not shown) probe. The same results were observed when RNA analysis was carried out on other tissues (data not shown). All of these results suggested that at least two events affecting the *COMT* locus may have occurred in *bm3* mutants.

A *bm3* Mutation Produces a Chimeric *COMT* mRNA

To characterize further the *bm3* mutation, we cloned and sequenced the *bm3* cDNA corresponding to the smaller *COMT* mRNA detected in the *bm3* mutant maintained in the W23 × L317 hybrid (so-called *bm3-1* mutant). A part of the *bm3-1 COMT* cDNA nucleotide sequence, shown in Figure 2, appears identical to the wild-type *COMT* cDNA (MC1 cDNA; Collazo et al., 1992), up to nucleotide 519 (Figure 2). Beyond this point, the *bm3-1* cDNA nucleotide sequence strongly diverges from the wild-type *COMT* cDNA and is identical to the long terminal repeat (LTR) of the B5 element, a retrotransposon originally described as interrupting the *waxy* gene in maize (Varagona et al., 1992). The sequence of the B5 LTR in the *COMT* mRNA provides a new stop codon and a putative polyadenylation site producing a shorter mRNA, which is consistent with data previously obtained by RNA gel blot analysis. As a consequence, the presence of the B5 LTR drastically changes the open reading frame of the chimeric transcript (Figure 2). The deduced chimeric protein appears shorter than the COMT

protein (209 amino acids rather than 364) and lacks some of the conserved domains described for all of these proteins (Collazo et al., 1992).

To check for the presence of the LTR of the B5 element in COMT mRNA from the bm3-1 mutant, the region of bm3-1 cDNA corresponding to the LTR was used as a probe to hybridize to the previous RNA gel blot. Figure 1B shows that an mRNA was detected in bm3-1 roots (Figure 1B) that corresponded to the one previously detected with the XS1 probe (Figure 1B). No COMT mRNA was detected with such a probe in either the wild type or plants carrying other mutations. We also checked for the presence of this transcript in bm3-1 mesocotyls and leaves and obtained an identical result (data not shown). These experiments confirmed the presence of the LTR-corresponding portion of the B5 element in bm3-1 COMT mRNA.

Two bm3 Mutations Arise from Retrotransposition and Deletion in the COMT Gene

The data obtained by RNA gel blot analysis and bm3-1 cDNA sequencing indicated that COMT mRNA was modified in the bm3-1 mutant maintained in the W23 × L317 hybrid or was undetectable in the bm3-2 mutant maintained in pure lines. Thus, it was of interest to correlate these data with changes occurring in bm3 alleles by DNA gel blot analysis. Probes were chosen to different regions of the COMT gene and used to compare the structure of both the W23 × L317 hybrid (wild type and bm3-1 mutant) and W401 (wild type and bm3-2 mutant) alleles. The probes from the coding region were the same as those used for RNA gel blot analysis (XS1 and SS1). An INT probe corresponding to the intron (Figure 1A) present in the COMT gene (Collazo et al., 1992) was also used.

Figure 3 demonstrates that bm3 alleles are polymorphic in the COMT locus because different hybridizing bands were observed, depending on the probe used. In the wild type of the W23 × L317 hybrid, the same band (15 kb) was detected using the three probes (Figure 3). In the corresponding bm3-1 mutant, both XS1 and INT probes detected a polymorphic 5.5-kb fragment, whereas SS1 recognized a shorter 3.5-kb fragment. This result indicated that a change occurred in bm3-1 that produced a restriction fragment length polymorphism (RFLP). It also suggested that this modifying event was produced at the junction of the 3' coding region of the intron, which is consistent with data obtained by bm3-1 cDNA sequencing. The result obtained for bm3-1 could be related to B5 element insertion. Using the region of the bm3-1 cDNA corresponding to the LTR as a control probe, a 5.5-kb hybridizing band was detected. It corresponded to the 5.5-kb hybridizing band detected with both XS1 and INT probes (data not shown).

The same experiment was carried out with the bm3-2 mutant and the corresponding W401 alleles from which no COMT transcript was found (Figure 1C). In the W401 wild-type allele, the three COMT-specific probes detected the same 12-kb fragment (Figure 3). In the bm3-2 mutant allele, the hybridization

pattern obtained with both XS1 and INT probes also indicated a polymorphism because a 13-kb fragment was observed. In bm3-2, however, the 3' coding region of the COMT gene appeared to be lacking because the 13-kb band that hybridized with the XS1 and INT probes could not be detected. This result suggested that another mutational event, different from the one detected in bm3-1, had occurred in bm3-2. This second change also affects the COMT gene at the junction of the 3' coding region of the intron. The lack of a hybridizing band when using the SS1 probe indicated that this event might be a deletion of a part of the COMT locus.

The relationship between COMT and bm3 was strengthened by data obtained by mapping the COMT gene on the Pioneer Hi-Bred (Johnston, IA) RFLP map with the MC1 cDNA probe (Collazo et al., 1992). Two populations of maize were analyzed (M.L. Katt, Pioneer Hi-Bred Int., personal communication). In the first population, COMT was placed 8.5 map units proximal to BNL05.46 and 2.1 map units distal to BNL15.45. In the second population, it was located 21.4 map units proximal to BNL05.46 and 8.3 map units distal to umc47. All of these RFLP markers are localized in the same region of maize chromosome 4 in which the bm3 locus has been shown to be mapped (Neuffer et al., 1968; Cherney et al., 1991).
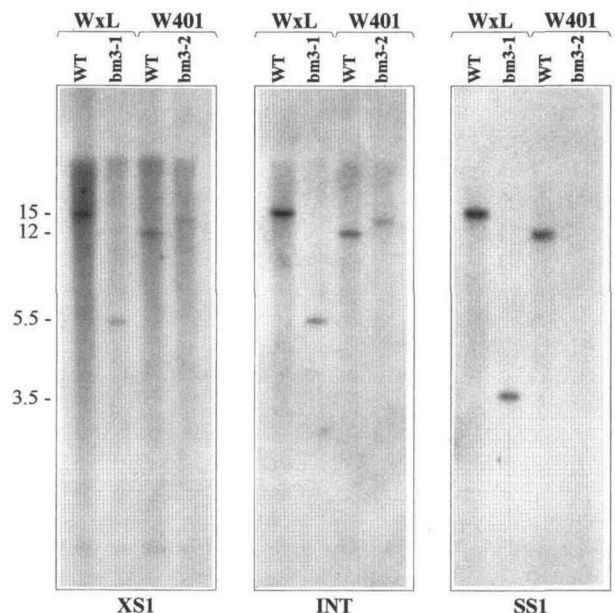
Figure 3. Analysis of Wild-Type and bm3 Maize Genomes by DNA Gel Blot.

Gel blot analysis was performed with 10 μg of genomic HindIII-digested DNA from wild-type (WT) and the corresponding bm3 mutants (bm3-1, bm3-2) of the W23 × L317 hybrid (WxL) and pure W401 (W401) maize lines. The length of the hybridizing bands is indicated at left in kilobases.
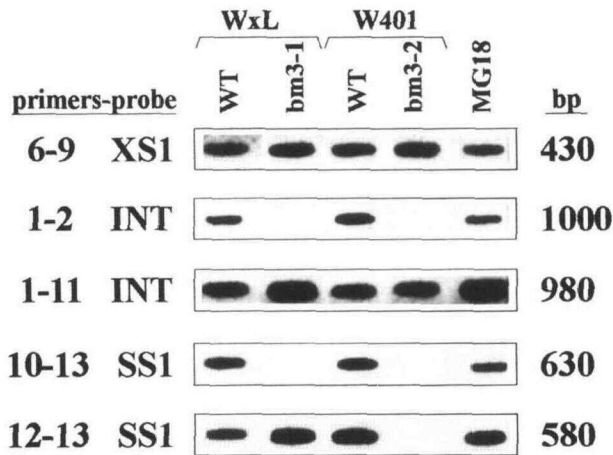
Figure 4. Analysis of Wild-Type and *bm3* Mutant Genomes by PCR.

Different regions of the *COMT* gene were PCR amplified from wild type (WT) and *bm3* mutant (bm3-1, bm3-2) genomic DNA isolated from W23 × L317 hybrid (WxL) and pure W401 (W401) lines. The primers used are described in Figure 1A. Amplifications using the same primers were also performed on genomic clone MG18 (Collazo et al., 1992) as positive controls. PCR products were separated on 1% agarose gels, blotted onto nylon membranes, and hybridized with the probe corresponding to the amplified region of the *COMT* gene (see Figure 1A). Probes and primers are indicated at left, and the length of the bands is given at right in base pairs.

Analysis of *bm3* Alleles by Polymerase Chain Reaction Amplification

The data obtained by DNA gel blot analysis reinforced the presumption that two mutational changes affecting *COMT* occurred in the same region of the gene. To confirm and to characterize further the types of mutations, we used a polymerase chain reaction (PCR) approach involving HindIII-digested DNA from all lines and various *COMT* gene-specific primers.

Using two primers surrounding the *COMT* gene 5′ coding region (primers 6 and 9, Figure 1A), PCR amplification occurred in all alleles (both wild types and mutants), as shown in Figure 4. Two other primers (primers 1 and 2, Figure 1A) surrounding the *COMT* gene intron were then assayed in all lines. Primer 1 corresponded to the inverse sequence of primer 6, and primer 2 was chosen from the site of retrotransposon insertion according to the sequence of *bm3-1 COMT* cDNA. With this primer set, amplification occurred only in the wild-type alleles (Figure 4). No PCR bands were detected in either *bm3-1* or *bm3-2* mutant alleles, indicating that the region corresponding to primer 2 was modified in both *bm3* mutant alleles. When primer 11 (Figure 1A), chosen 12 bp upstream of primer 2, was used along with primer 1, an amplification product of the expected size was derived from all of the alleles. This result indicated that both *bm3-1* and *bm3-2* mutations took place at the same region of the *COMT* gene, within the sequence

corresponding to primer 2 downstream of the intron, which is consistent with the DNA gel blot analysis and the sequence of the *bm3-1* cDNA.

To confirm the insertion of the retrotransposon in the *bm3-1* mutant, amplification of the 3′ coding region was assayed by using primer 10 (complementary to primer 11) and primer 13, chosen 16 bp upstream of the stop codon of the *COMT* gene (Figure 1A). Using such primers, the expected product was obtained only from the wild-type alleles (Figure 4). Because the 3′ coding region of the *COMT* gene could be detected in the *bm3-1* mutant by DNA gel blot analysis (Figure 3), we predicted that the absence of the PCR product from the *bm3-1* allele was the result of the presence of the B5 retrotransposon. This element, which was described as a 6.1-kb DNA fragment (Varagona et al., 1992), might not be amplifiable under our PCR conditions. Alternatively, a HindIII restriction site introduced due to this insertion may have contributed to the lack of amplification between primer 10 and primer 13 and may explain the RFLP detected between *bm3-1* and wild-type alleles.

To characterize further the *bm3-2* mutation, *bm3-2* and corresponding W401 wild-type alleles were first analyzed using a set of primers (primers 10 and 13, Figure 1A) surrounding the *COMT* 3′ coding region. Figure 4 indicates that these primers failed to amplify the expected region. This result reinforced the assumption that the *bm3-2* mutation was produced in the 3′ coding region of the *COMT* gene. However, an identical result was obtained from the *bm3-1* allele when using these oligonucleotides (Figure 4). Therefore, we tested another set of primers (primers 12 and 13, Figure 1A) to understand the difference between the two mutant alleles observed by DNA gel blot analysis (Figure 3). Primer 12 was designed from the 3′ coding region of the *COMT* gene, downstream of the site of retrotransposon insertion according to the sequence of the *bm3-1 COMT* cDNA. Amplification between primer 12 and primer 13 normally produced the expected band from both the wild-type and *bm3-1* alleles, but the band did not occur from the *bm3-2* allele (Figure 4). The lack of a PCR product from the *bm3-2* allele indicated that the region containing primers 12 and 13 had been deleted in the corresponding mutant. This is consistent with the absence of a hybridizing band in the DNA gel blot analysis from the *bm3-2* allele using the SS1 probe (Figure 3).

The Leaf Midrib Is a Location of High *COMT* Gene Expression

The aforementioned data indicated that *COMT* is the gene affected by mutations in *bm3* maize lines. On the other hand, the typical reddish brown pigmentation in *bm3* mutants is associated with the lignified vascular bundles of the leaf midrib (Cherney et al., 1991). Thus, we investigated the cellular distribution of *COMT* mRNA to check whether it is present in the same cells in which the *brown midrib* phenotype is visible. For
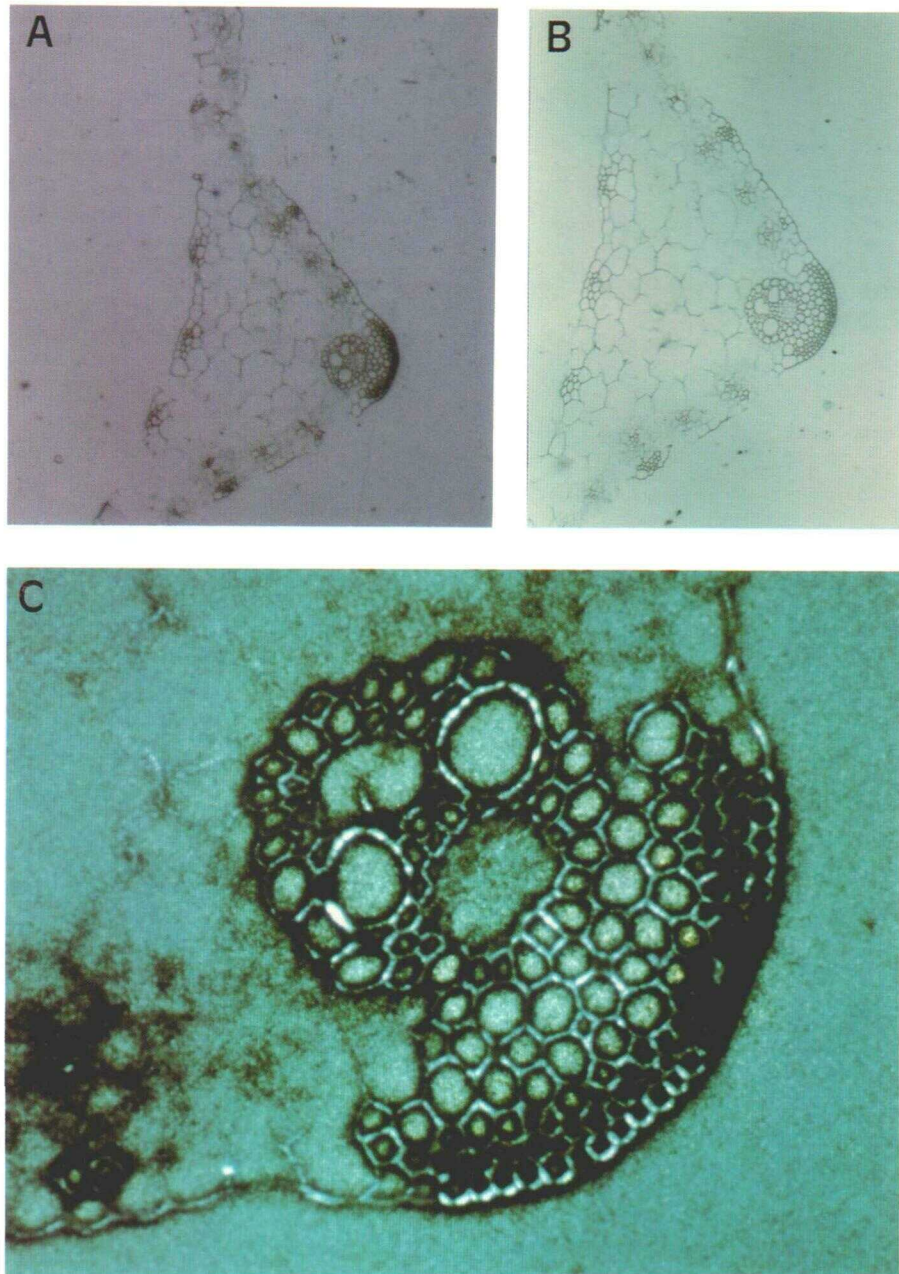
Figure 5. Cellular Expression of the *COMT* Gene in a Wild-Type Maize Leaf.

Bright-field micrographs of 7- to 8-μm-thick tissue sections are shown. In **(A)** and **(C)**, sections were hybridized with ^{35}S-UTP labeled MC1 antisense probes, and in **(B)**, the MC1 sense probe was used. The presence of hybridizing transcripts is indicated by the silver grains that appear as black spots.

(A) Transverse section of a young leaf emerging from the coleoptile.

(B) Same view as shown in **(A)** but obtained after hybridization with the sense probe as negative control.

(C) Detail of the leaf midrib showing the vascular bundles in which *COMT* gene expression occurs.

this purpose, in situ hybridization was carried out with wild-type maize leaves in which *COMT* mRNA is normally detected by RNA gel blot analysis (Collazo et al., 1992). We used the coding region of MC1 cDNA as a probe (Collazo et al., 1992). Figure 5A demonstrates that *COMT* mRNA accumulated to high levels in the leaf midrib. The cellular distribution of the *COMT* mRNA in the leaf midrib corresponded to the cells undergoing lignification located around the differentiated xylem vessels. The *COMT* mRNA was specially abundant in the cells of the sclerenchyma localized under the epidermal layer (Figure 5C).

This result is consistent with the cellular localization of the *brown midrib* phenotype observed in maize (Cherney et al., 1991). In the leaves of *bm3* mutants, subepidermal cells accumulating chimeric *COMT* mRNA produce a reddish brown pigmentation visible from the surface. In stem or in root, the cells expressing *COMT* mRNA are internal (data not shown), which explains why the typical reddish brown pigmentation is not visible.

DISCUSSION

Here, we report the molecular characterization of *bm3* mutations in maize and demonstrate that they were produced in the gene coding for the COMT, a key enzyme in the lignin biosynthetic pathway. Two mutant alleles of *bm3* were found, designated *bm3-1* (reference allele) and *bm3-2*. In the case of the *bm3-1* mutation, a retrotransposon homologous to the B5 family of maize was found to be inserted in the *COMT* gene. This B5 element previously had been described as interrupting one of the *waxy* gene introns (Varagona et al., 1992). The insertion of the B5 element in the *COMT* gene introduced a new stop codon and a polyadenylation signal that produced a shorter *COMT* mRNA. As a consequence, the COMT protein synthesized in the *bm3-1* mutant is considerably shorter. Specifically, it lacks some of the conserved domains of the OMTs that have been described up to now and that are thought essential for their function (Collazo et al., 1992).

Our work also shows that the second *bm3* mutation (*bm3-2*) resulted from a deletion that removed at least a portion of the 3' region of the *COMT* gene. The results obtained by DNA gel blot and PCR analyses indicated that this *bm3-2* mutation was produced in the same region of the *COMT* gene in which the *bm3-1* mutation occurred. As a consequence of both mutations, *COMT* gene expression was dramatically altered. Using several PCR analyses, we did not observe any change in the *COMT* gene promoter that might have suggested a modification of gene regulation. However, without further sequence information, it is not possible to rule out this eventuality. Alternatively, the lower level of chimeric *COMT* transcript detected in the *bm3-1* mutant and the absence of *COMT* mRNA in the *bm3-2* mutant in which the *COMT* gene appeared to be truncated could be the result of post-transcriptional regulation due to the instability of the modified mRNAs.

In both cases, molecular and genetic data demonstrated that *bm3* mutations occur in the *COMT* gene. These data also enabled us to observe the effect of disrupting the *COMT* gene encoding a key enzyme for lignin biosynthesis and to explain the lower and chemically modified lignin content usually observed in these mutants (Kuc et al., 1968; Gaudillere and Monties, 1989; Barrière et al., 1994). Moreover, these data are consistent with the lower OMT activity previously described in such maize lines (Grand et al., 1985; Lapierre et al., 1988). Thus, the availability of the *COMT* probe and its identity to *bm3* may enable its use in RFLP or PCR-derived methods for breeding programs that include *bm3* genes and allow a study of the relationship between the different *bm3* alleles.

Although OMT activity and lignin content are found to be lower in *bm3* mutants, they are still detectable (Grand et al., 1985; Cherney et al., 1991). Considering that lignin-related COMT was shown to be encoded by a single gene in maize (Collazo et al., 1992), we suggest that other methyltransferases exhibiting a lower specificity and efficiency toward COMT substrates may be functional in the lignin biosynthesis pathway. The relative nonspecificity of these enzymes has been demonstrated in plants (Bugos et al., 1991; Edwards and Dixon, 1991; Collazo et al., 1992), and other OMTs normally involved in other pathways, such as flavonoid and isoflavonoid pathways (Vernon and Bohnert, 1992; Held et al., 1993; Maxwell et al., 1993), or disease resistance (Schmitt et al., 1991) could act in an alternative pathway when COMT activity fails. This hypothesis is supported by recent data concerning a caffeoyl-coenzymeA 3-*O*-methyltransferase involved in disease resistance, which has been shown to be effective in the lignification pathway (Ye et al., 1994). The activity of such enzymes might explain the low and modified lignin content typically observed in *bm3* mutants.

Antisense technology with genes involved in the lignin pathway has been used in other species and appears effective in lowering the lignin content (Dwivedi et al., 1994; Halpin et al., 1994; Ni et al., 1994). In tobacco, OMT antisense plants were obtained that exhibited a significant reduction in lignin content (Ni et al., 1994). In the same species, CAD antisense plants were produced with a modified lignin structure that probably would be more accessible to chemical degradation (Halpin et al., 1994). For such purposes, antisense methodology appears useful for species in which no mutants related to the lignification process have been described. However, our results indicated that the characterization of lignin-related mutants might be another useful approach that could be designed for species, including maize.

Maize mutants, such as *bm3*, naturally exhibit the properties of low lignin content and high digestibility (Kuc et al., 1968; Gentinetta et al., 1990; Barrière and Argillier, 1993; Barrière et al., 1994) that are usually the objective of antisense methodology. In some cases, CAD antisense tobacco plants were shown to produce a pigmentation similar to the *bm3* mutant in the leaf midrib (Halpin et al., 1994), indicating that comparable events may occur. Because the *COMT* mRNA appeared highly abundant in the leaf midrib in maize, the characteristic

pigmentation may be explained as the consequence of the accumulation of an unknown phenolic derivative due to blockage of the normal lignin pathway. Studying well-known mutants related to the lignin biosynthesis pathway to identify mutated genes thus appears an alternative to other methods. Analyzing the effects of disrupting such genes of interest enables possible sites and effects of modification of this important pathway to be evaluated.

METHODS

Plant Materials

All of the *brown midrib* mutants analyzed here are homozygous genotypes. Seed of *brown midrib1* (*bm1*), *bm2*, and *bm3* homozygote mutants maintained in W23 × L317 hybrids were provided by E.B. Patterson (Maize Genetics Stock Center, University of Illinois, Urbana, IL). The *bm3* allele from the corresponding mutant (the reference allele) is referred to in this study as *bm3-1*.

Another batch of seed of *bm3* homozygote mutants was a gift of Y. Barrière (Institut National de la Recherche Agronomique, INRA, Lusignan, France). The original seeds were furnished to the INRA in 1972 by L.F. Bauman (Department of Agronomy, Purdue University, Lafayette, IN) under code number 95096-102, without further characterization. Later, the *bm3* mutants were maintained in W64A and W401 pure inbred maize at the INRA of Lusignan. The *bm3* allele from the mutant maintained in the W401 line, which should be given as *bm3-95096-102*, is referred to in this study as *bm3-2*, for ease of presentation.

All plants were grown on filter paper for 7 days and stored at −80°C.

Preparation of OMT Probes

Probes XS1 and SS1 were obtained by digestion of a caffeic acid O-methyltransferase (*COMT*) genomic clone MG18 (Collazo et al., 1992) using XhoI/SacI and SalI restriction enzymes, respectively. Probe INT corresponding to the *COMT* gene intron was obtained by polymerase chain reaction (PCR) amplification of genomic clone MG18 using primers 1 and 11 (Figure 1A). LTR is the region of the *bm3-1* cDNA corresponding to the long terminal repeat of the B5 element. This probe was obtained by PCR amplification of *bm3-1* cDNA subcloned in pBluescript SK+ (Stratagene) using primer 1 (Figure 1A) and pBluescript T7 primer. MC1 probe is the full-length *COMT* cDNA encoding COMT from W64A (Collazo et al., 1992).

RNA Isolation and RNA Blot Analysis

Total RNA was isolated using lithium chloride precipitation and phenol-chloroform extractions according to Verwoerd et al. (1989). Total RNA (10 µg) harvested from 7-day-old roots was separated on a 1.3% agarose gel following standard procedures (Sambrook et al., 1989) and blotted onto a nylon membrane. The hybridization step (16 hr at 65°C) was carried out in 250 mM Na_2HPO_4, 7% SDS, 1 mM EDTA, 100 µg mL⁻¹ salmon sperm DNA containing radiolabeled probe (Feinberg and

Vogelstein, 1984). Washes were performed three times for 20 min each in 20 mM Na_2HPO_4, 1% SDS, 1 mM EDTA at 65°C.

cDNA Library Construction, Screening, and DNA Sequencing

Poly(A)⁺ RNA was isolated from total RNA using the Poly-A-Tract system (Promega). The cDNA library was constructed in λ ZAPII (Stratagene) with 2 µg of poly(A)⁺ RNA from 7-day-old *bm3-1* hybrid roots. The coding XS1 probe was used to screen 300,000 plaques according to standard procedures (Sambrook et al., 1989). Positive clones subcloned in pBluescript SK+ after phagemid in vivo excision were sequenced using the chain termination method (Sambrook et al., 1989) and automatic ALF sequencer (Pharmacia, Sweden). Searches in the data bases (EMBL, GenBank, and DDBJ) and sequence comparisons were performed with the program package, including FASTA 7.2 (Genetics Computer Inc., Madison, WI).

Genomic DNA Isolation and DNA Blot Analysis

Maize genomic DNA was extracted from 7-day-old leaves, using the standard protocol of Dellaporta et al. (1983). Approximately 10 µg of DNA was digested with HindIII and separated on a 0.8% agarose gel. DNA gel blot hybridization was performed as described for total RNA. Washes were performed at 65°C as follows: two washes for 20 min each in 40 mM Na_2HPO_4, 1% SDS, 1 mM EDTA, and one wash for 10 min in 20 mM Na_2HPO_4, 0.5% SDS, 1 mM EDTA.

Polymerase Chain Reaction Amplifications

The location of the primers used for PCR amplifications is shown in Figure 1A. PCR analyses were performed with 2 µg of HindIII-digested genomic DNA for more efficient amplifications, and with 50 ng of non-digested DNA for genomic clone MG18 (Collazo et al., 1992). Primers were chosen from the sequence of clone MG18 as follows (from 5' to 3'): primer 1, ATGAACCAGGACAAGGTCCTCATG; primer 2, CATCCCG-TACGCCTTGTTGAACGG; primer 6, inverse complementary to primer 1; primer 9, TAATCGTAATAGCCATGGGCTCCA; primer 10, GACGGC-GGCATCCCGTTCAAC; primer 11, inverse complementary to primer 10; primer 12, GGCACGGACGCGCGCTTCAAC; primer 13, GCCCAG-GCGTTGGCGTAGATG. PCR amplification consisted of 30 cycles at 94°C for 2 min, 63°C for 2 min, 72°C for 3 min. PCR products were subjected to 1% agarose gel electrophoresis, transferred onto a nylon membrane, and hybridized with the appropriate probe, as described for total RNA.

In Situ Hybridization

In situ hybridization was performed on 7- to 8-µm sections of 7-day-old leaves, using procedures described by Stiefel et al. (1990) and Langdale (1994). Sense and antisense MC1 probes were synthesized with 1 µg of linearized DNA using ³⁵S-UTP. Labeled transcripts were then hydrolyzed by using an alkali treatment. These transcripts were used in a hybridization step as described by Langdale (1994). Photography was carried out using bright-field microscopy and Ektachrome 160 ASA film (Kodak).

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