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Abstract
The midribs of maize brown midrib (bm) mutants exhibit a reddish-brown color associated with reductions in lignin concentration and alterations in lignin composition. Here, we report the mapping, cloning, and functional and biochemical analyses of the bm2 gene. The bm2 gene was mapped to a small region of chromosome 1 that contains a putative methylenetetrahydrofolate reductase (MTHFR) gene, which is down-regulated in bm2 mutant plants. Analyses of multiple Mu-induced bm2-Mu mutant alleles confirmed that this constitutively expressed gene is bm2. Yeast complementation experiments and a previously published biochemical characterization show that the bm2 gene encodes a functional MTHFR. Quantitative RT-PCR analyses demonstrated that the bm2 mutants accumulate substantially reduced levels of bm2 transcript. Alteration of MTHFR function is expected to influence accumulation of the methyl donor S-adenosyl-L-methionine (SAM). Because SAM is consumed by two methyltransferases in the lignin pathway (Ye et al., 1994), the finding that bm2 encodes a functional MTHFR is consistent with its lignin phenotype. Consistent with this functional assignment of bm2, the expression patterns of genes in a variety of SAM-dependent or -related pathways, including lignin biosynthesis, are altered in the bm2 mutant. Biochemical assays confirmed that bm2 mutants accumulate reduced levels of lignin with altered composition compared to wild-type. Hence, this study demonstrates a role for MTHFR in lignin biosynthesis.

Keywords
Zea mays, brown midrib, lignin, methylenetetrahydrofolate, S-adenosyl-L-methionine

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The maize *brown midrib2 (bm2)* gene encodes a methylenetetrahydrofolate reductase that contributes to lignin accumulation

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SUMMARY

The midribs of maize *brown midrib* (*bm*) mutants exhibit a reddish-brown color associated with reductions in lignin concentration and alterations in lignin composition. Here, we report the mapping, cloning, and functional and biochemical analyses of the *bm2* gene. The *bm2* gene was mapped to a small region of chromosome 1 that contains a putative methylenetetrahydrofolate reductase (MTHFR) gene, which is down-regulated in *bm2* mutant plants. Analyses of multiple Mu-induced *bm2-Mu* mutant alleles confirmed that this constitutively expressed gene is *bm2*. Yeast complementation experiments and a previously published biochemical characterization show that the *bm2* gene encodes a functional MTHFR. Quantitative RT-PCR analyses demonstrated that the *bm2* mutants accumulate substantially reduced levels of *bm2* transcript. Alteration of MTHFR function is expected to influence accumulation of the methyl donor S-adenosyl-L-methionine (SAM). Because SAM is consumed by two methyltransferases in the lignin pathway (Ye *et al.*, 1994), the finding that *bm2* encodes a functional MTHFR is consistent with its lignin phenotype. Consistent with this functional assignment of *bm2*, the expression patterns of genes in a variety of SAM-dependent or -related pathways, including lignin biosynthesis, are altered in the *bm2* mutant. Biochemical assays confirmed that *bm2* mutants accumulate reduced levels of lignin with altered composition compared to wild-type. Hence, this study demonstrates a role for MTHFR in lignin biosynthesis.

Keywords: *Zea mays*, *brown midrib*, lignin, methylenetetrahydrofolate, S-adenosyl-L-methionine.

INTRODUCTION

Lignin is a heterogeneous aromatic polymer that is a major component of cell walls. It plays a critical role in the structural integrity of vascular plants (Sarkanen and Ludwig, 1971). In lignified tissues, lignin is heavily cross-linked with cellulose and hemicelluloses to provide protection and strength, and also increases the resistance of biomass to enzymatic digestion by ruminants, bacteria and fungi (Sarkanen and Ludwig, 1971). This high level of resistance to enzymatic digestion has a negative effect on forage quality and animal performance. Reduced lignin content of livestock feed also reduces animal waste (Jung and Vogel, 1986). Therefore, an understanding of the mechanisms that regulate lignin accumulation has the potential to provide important insights that may be used to improve the quality of biomass and forage crops.

Maize (*Zea mays* ssp. *mays* L.) is a widely grown and highly productive food, feed and biofuel crop (Doebley...
et al., 2006; Tang et al., 2010). Brown midrib (bm) mutants are characterized by the reddish-brown color of their leaf midribs (Sattler et al., 2010). The bm phenotype of maize was first reported over 80 years ago (Jorgenson, 1931), and it is now clear that this phenotype is associated with reduced lignin concentrations (Grand et al., 1985; Cherney et al., 1991; Sattler et al., 2010). To date, six bm mutants have been identified. The bm1, bm2, bm3, bm4, bm5 and bm6 loci are located on chromosomes 5, 1, 4, 9, 5 and 2, respectively (Chen et al., 2012; Lawrence et al., 2005; Sattler et al., 2010). The bm1 and bm3 genes encode cinnamyl alcohol dehydrogenase (CAD) (Barrière et al., 2013; Halpin et al., 1998) and caffeic acid O-methyltransferase (COMT) (Vignols et al., 1995), respectively. Both play key roles in lignin biosynthesis. The roles of the other four bm genes in lignin biosynthesis are not clear. Therefore, cloning the remaining bm genes is expected to provide insights into the regulation of lignin biosynthesis.

In this study, a candidate bm2 gene was identified via a map-based approach. Analyses of multiple independent Mutator transposon insertion alleles confirmed that the candidate gene is indeed bm2. A yeast complementation experiment demonstrated that, as predicted based on its sequence, bm2 encodes a functional methyltetrahydrofolate reductase (MTHFR, EC 1.5.1.20). Alteration of MTHFR function is expected to influence accumulation of the methyl donor S-adenosyl-L-methionine (SAM). An RNA-Seq experiment demonstrated that the bm2 mutation significantly affects expression of genes involved in several SAM-related metabolic pathways, including the lignin/phenylpropanoid pathway, ethylene and jasmonate metabolism, and glutathione S-transferase (GST) genes. Collectively, these results demonstrate a role for MTHFR in the lignin biosynthetic pathway.

RESULTS

Characterization of the bm2 mutant phenotype and lignin content in various tissues

The bm2 mutant was originally identified by its brown pigmentation in the leaf midrib (Neuffer et al., 1968), a description that is consistent with our observations of the bm2-ref allele. In the genetic backgrounds used in this study, bm2-ref mutants first exhibit a reddish-brown pigmentation of the leaf midrib beginning at the 6-8-leaf stage, approximately 27 days after planting (Figure 1a). The reddish-brown pigmentation observed on both the adaxial and abaxial surfaces of mutant leaves was not observed in non-mutant siblings (Figure 1a).

In addition to these biochemical assays, bm2 mutant samples from various tissues were stained with phloroglucinol, which detects lignin (Wardrop, 1971). In B73 and non-mutant sibling maize, the midrib, epidermis and tissues around vascular bundles stain red with phloroglucinol, thereby demonstrating lignification of these tissues. However, in the bm2 mutant, these tissues stain only weakly. Although there are no observable alterations in the anatomy of stems and roots associated with the bm2 mutant, significant differences in phloroglucinol staining were detected in these tissues. In B73 and non-mutant sibling stems and roots, strong phloroglucinol staining was detected at xylem vessels and epidermis, whereas bm2 mutant tissues exhibited reduced staining. These phloroglucinol staining results are consistent with previous reports (Vermerris and Boon, 2001; Marita et al., 2003; Sattler et al., 2010) indicating that lignin levels are lower in the bm2 mutant compared to non-mutant controls (Figure 1b).

Mutant bm2 plants have also been reported to accumulate reduced lignin concentrations and levels of G-lignin (Vermerris and Boon, 2001; Marita et al., 2003; Sattler et al., 2010). However, the reports are inconsistent with respect to the effect of the bm2 mutation on concentrations of S-lignin (Vermerris and Boon, 2001; Marita et al., 2003; Saballos et al., 2009; Sattler et al., 2010; Vermerris et al., 2010). In some instances, S-lignin concentrations have been found to
be lower in the bm2 mutant (Vermerris et al., 2010), but have been reported to be unchanged or even elevated in other cases (Vermerris and Boon, 2001; Marita et al., 2003; Barriere et al., 2004; Sattler et al., 2010). Due to this ambiguity, we re-analyzed the lignin in bm2 mutants.

Classically, due to the interest in utilizing stover for feed, biochemical assays of lignin have classically been performed at or around the stage at which maize is harvested for silage (i.e. after anthesis, but before maturity). We were interested to determine whether lignin characteristics changed between this stage and senescence. We therefore determined Klasson lignin and the lignin composition of bm2 mutant and non-mutant sibling (wild-type) stalks collected directly post-anthesis (PA) and post-senescence (PS). Consistent with literature, we found that mutant PA stalks accumulated only approximately 93% of the Klasson lignin of wild-type siblings. Mutant PS samples exhibited similar reductions in lignin concentrations. Both mutant and wild-type PS samples accumulated more Klasson lignin (approximately 112%) than the corresponding PA samples, showing that lignin continues to accumulate in stalks after flowering.

Lignin is composed of at least three hydroxycinnamyl alcohol subunits (the monolignols p-coumaryl, coniferyl and sinapyl alcohol), resulting in hydroxyphenyl (H), guaiacyl (G) and syringyl (S) types of lignin, respectively (Whetten and Sederoff, 1995; Bonawitz and Chapple, 2010). In maize, H-lignin represents only a small fraction (approximately 2%) of total lignin (Barriere et al., 2007). Hence, the monolignol composition of lignin was measured as the μmol of S- or G-lignin per gram of dry stalk tissue. We found that the ratio of S- to G-lignin was elevated in the bm2 mutant compared to wild-type stalks at both stages of development. In agreement with the Klasson lignin data, the absolute amounts of both S- and G-lignin were lower, on average, in mutant samples relative to wild-type and PA samples relative to PS samples (Table 1). These results are consistent with a role for the bm2 gene in lignin biosynthesis.

### Mapping the bm2 gene

The bm2 gene was previously mapped to the long arm of chromosome 1 (www.maizegdb.org) (Neuffer et al., 1968; Lawrence et al., 2005). To map the bm2 gene to a higher resolution, we used a modification of bulked segregant analysis called bulked segregant RNA-Seq (BSR-Seq), which makes use of the quantitative feature of RNA-Seq (Liu et al., 2012). Briefly, RNA-Seq reads are generated from pools of bm2 mutants and non-mutant (wild-type) siblings. Because of the digital nature of next-generation sequencing data, it is possible to perform de novo SNP discovery and quantitatively genotype bulked samples using the same RNA-Seq data.

To generate a bm2 mapping population, a bm2-ref mutant plant was crossed to the non-mutant inbred line B73. A bm2-ref heterozygous individual was self-pollinated to generate an F2 segregating population. From this segregating population, tissue samples from siblings exhibiting the mutant and non-mutant phenotypes were combined into two separate pools and subjected to RNA-Seq (Experimental procedures). RNA-Seq reads were trimmed and aligned to the B73 reference genome (Schnable et al., 2009). In total, 46 289 SNPs were identified and used for the BSR-Seq analysis.

Via the BSR-Seq analysis, the bm2 gene was located to an approximately 2 Mb region of chromosome 1 from 289–291 Mb (Figure 2), which contains 83 genes in the filtered gene set (Table S1). Subsequently, 41 individuals from an F2 mapping population (n = 537) that contained recombination events within this 2 Mb interval were identified using two flanking SNP markers (Experimental procedures). Twelve additional SNP markers (Table S2) located within the 2 Mb interval were used to narrow down the bm2 mapping interval to an approximately 0.5 Mb region that contains 47 genes from the working gene set (Table S3). Only eight of these 47 genes are members of the more confidently defined ‘filtered gene set’ (Table S4). Of the 47 genes, only one (GRMZM2G347056, which is not the member of the filtered gene set) exhibited significant down-regulation in mutant versus wild-type sibling samples as assayed via the RNA-Seq experiments described below (Appendix S1). This bm2 candidate gene encodes a putative methylenetetrahydrofolate reductase (MTHFR).

A blastp (blast.ncbi.nlm.nih.gov/Blast.cgi) search of a variety of plants demonstrated that many dicots contain

### Table 1 Lignin characteristics of wild-type and bm2 mutant siblings at various developmental stages

| Stage | Genotype | Klasson lignin (g g⁻¹ dry weight) | S/G | S (μmol g⁻¹ dry weight) | G (μmol g⁻¹ dry weight) | S + G (μmol g⁻¹ dry weight) |
|-------|----------|-----------------------------------|-----|------------------------|------------------------|----------------------------|
| PA    | Wild-type | 0.162 ± 0.014                     | 1.24 ± 0.06 | 1037 ± 140             | 843 ± 157              | 1880 ± 297                 |
|       | Mutant   | 0.151 ± 0.010                     | 1.74 ± 0.23 | 896 ± 59               | 518 ± 34               | 1414 ± 25                  |
| PS    | Wild-type | 0.182 ± 0.008                     | 1.70 ± 0.18 | 1402 ± 52              | 832 ± 121              | 2233 ± 233                 |
|       | Mutant   | 0.170 ± 0.021                     | 1.97 ± 0.23 | 1054 ± 45              | 539 ± 85               | 1593 ± 130                 |

Each genotype represents the mean of two technical replicates and two biological replicates. One biological replicate is a single whole stalk. PA, post-anthesis (approximately 2 months old); PS, post-senescence (approximately 5 months old).
two apparently full-length MTHFR homologs, while grasses contain only a single apparently full-length MTHFR homolog (Figure S11).

However, maize contains a second protein (AFW67208) encoded by the GRMZM2G034278 gene that exhibits 96% identity to the \( \text{bm2} \)-encoded protein, but with only 52% coverage. We initially hypothesized that this partial MTHFR gene may be part of a Pack-MULE, but it is not included among those identified in the B73 genome (RefGen_v1) by Jiang et al. (2011).

Confirmation that the \( \text{bm2} \) and MTHFR-encoding genes are one and the same

To confirm that the candidate gene is the \( \text{bm2} \) gene, additional \( \text{bm2} \) mutant alleles were identified via a direct \( \text{Mu} \) transposon tagging experiment. A population of plants derived from a cross of plants carrying an active \( \text{Mu} \) transposon system with plants homozygous for the \( \text{bm2-ref} \) allele was screened for \( \text{Mu} \)-induced \( \text{bm2} \) mutant alleles (Experimental procedures). After screening 147 500 individuals, 11 plants were identified that exhibited the characteristic reddish-brown midribs of \( \text{bm2} \) mutants (Figure S2). These individuals were PCR-screened using a \( \text{Mu} \)-specific primer together with \( \text{mthfr} \)-specific primers (Experimental procedures). \( \text{Mu} \) insertions were identified in the MTHFR-encoding gene in each of the 11 identified mutants, including a total of seven unique \( \text{Mu} \) insertion sites (Figure 2 and Figure S1). Although some mutants have identical \( \text{Mu} \) insertion sites (as has been observed previously, Dietrich et al., 2002), the design of our tagging experiment ensured that each mutant arose via an independent event. Homozygous \( \text{bm2-Mu} \) lines were generated for each of the 11 mutants (Experimental procedures), and phloroglucinol staining indicated that these individuals accumulate reduced levels of lignin (Figures S2 and S3), demonstrating that the MTHFR-encoding gene is indeed the \( \text{bm2} \) gene.

The \( \text{bm2} \) gene is expressed constitutively

To test whether the \( \text{bm2} \) gene is specifically expressed in lignified tissues, we used the online tool, qTeller (qteIler.com), to visualize its expression in a variety of organs at multiple stages of development (Figure S12). From this analysis, it is clear that the \( \text{bm2} \) gene is expressed in almost all tissues assayed, including some that are not lignified (e.g. ovules, primordial apices and endosperm).

The accumulation of \( \text{bm2} \) transcripts is dramatically reduced in \( \text{bm2} \) mutants

The RNA-Seq data from the BSR-Seq experiment indicated that the \( \text{bm2} \) gene is down-regulated in midribs of the \( \text{bm2-ref} \) mutant relative to non-mutant sibling controls (Appendix S1). RT-PCR and quantitative RT-PCR performed on multiple biological replicates confirmed this pattern of differential expression in \( \text{bm2-ref} \) and \( \text{bm2-Mu} \) mutants compared to wild-type (B73 and non-mutant siblings). This down-regulation occurs in multiple tissues, including leaf, stem, root and midrib (Figure 3a,b and Figure S1D).

Quantitative RT-PCR analysis of lines homozygous for each of our 11 \( \text{bm2-Mu} \) lines was performed on RNA
alleles contain Mu insertions within the coding region of the bm2 gene.

The RT-PCR products from the bm2-ref allele were sequenced and compared to the B73 reference genome. Seven polymorphisms were identified in the bm2-ref allele relative to the B73 reference genome (Figure S4). One is a synonymous mutation in the coding region; the other six polymorphisms are located in the 3′ UTR (Figure S4). We hypothesized that the reduced accumulation of bm2 mRNA in the bm2-ref mutant may be the result of the 3′ UTR polymorphisms because the 3′ UTR plays a critical role in RNA stability (Gutierrez et al., 1999; Millevi and Vagner, 2010).

To test the stability of the bm2 mRNA from non-mutant (wild-type) and bm2-ref mutant siblings, the corresponding midrib tissues were exposed to the transcription inhibitor actinomycin D (Sawicki and Godman, 1972; Experimental procedures). After a 12 h incubation of the tissues in the presence of actinomycin D, the level of bm2 mRNA in the bm2-ref tissue was reduced, while the level of the non-mutant control remained similar to its original level (Figure 3c). This finding demonstrates that the transcripts from the bm2-ref allele are less stable than non-mutant transcripts and are consistent with our hypothesis that the polymorphisms in the 3′ UTR in the bm2-ref allele reduce the stability of the MTHFR-encoding gene mRNA.

The maize bm2 cDNA complements MET11 knockout yeast

The maize bm2 gene shares 40% amino acid identity and 59% similarity with the yeast MET11 gene, which encodes a functional homolog of the human MTHFR protein (Figure S5). MTHFR catalyzes the conversion of 5,10-methylene-tetrahydrofolate to 5-methyltetrahydrofolate, a co-substrate for homocysteine re-methylation to methionine (Goyette et al., 1994; Vickers et al., 2006). Yeast lacking the endogenous MET11 gene are unable to grow on methionine-free medium (Shan et al., 1999). To test the hypothesis that the maize bm2 gene encodes a functional MTHFR enzyme, an MET11 knockout strain (met11) of yeast was used to test whether expression of the maize bm2 gene rescues met11 yeast. As expected, wild-type yeast (MET11) but not the met11 strain grew in the absence of methionine (Figure 4). Further, consistent with a previous report (Shan et al., 1999), expression of the human MTHFR-encoding gene rescues met11 yeast in the present of galactose, which induces expression of the human MTHFR-encoding gene construct (Figure 4). Significantly, when under the control of a galactose-inducible promoter, the non-mutant bm2 cDNA also rescued met11 yeast in the presence of galactose, but not in the presence of glucose, which inhibits expression of bm2 in this construct (Figure 4). This finding demonstrates that the bm2 gene encodes a functional MTHFR.
We performed two independent RNA-Seq experiments. These experiments differed in terms of the age of plants harvested, the number of replicates analyzed, and the statistical cut-offs used to define differentially expressed genes. The first experiment (#1) included a single replication using 26-day-old midrib tissue. The second experiment (#2) included two biological replications of 38-day-old tissue (Tables S5–S7). In an effort to minimize false discovery, more stringent cut-offs were used in experiment #2 because the former did not include a biological replication.

RNA-Seq experiment #1 identified 369 significantly differentially expressed genes with a >2-fold change between bm2 mutants and their wild-type siblings; of these, 242 were up-regulated and 127 were down-regulated relative to wild-type. RNA-Seq experiment #2 identified 3313 differentially expressed genes (Figures S6–S8 and Appendix S1). Nearly 90% (n = 323) of the 369 differentially expressed genes from RNA-Seq experiment #1 (which lacked a biological replication) were also detected in RNA-Seq experiment #2 (Figure S9). The following functional analysis is based on the results of RNA-Seq experiment #2 that included two biological replicates. A pathway enrichment analysis of the 3313 differentially expressed genes from RNA-Seq experiment #2 revealed statistically significant over-representation of the following functional categories: lignin/phenylpropanoid (Figure S10), hormone ethylene, hormone jasmonate and glutathione S-transferase (GST) gene families, and cytochrome P450s (Table S8).

The p-coumaryl (H), coniferol (G) and sinapyl (S) alcohol subunits of lignin are synthesized by enzymes in the phenylpropanoid pathway, including phenylalanine ammonia lyase (PAL), hydroxycinnamoyl CoA:shikimate hydroxycinnamoyl transferase (HCT), 4-coumarate:CoA ligase (4CL), cinnamyl alcohol dehydrogenase (CAD), cinnamoyl CoA reductase (CCR), caffeoyl CoA 3-O-methyltransferase (CCoAOMT) and caffeic acid 3-O-methyltransferase (COMT) (Lewis and Yamamoto, 1990; Tamasloukht et al., 2011). In RNA-Seq experiment #2, 41 of 109 genes involved in the phenylpropanoid pathway are significantly differentially expressed between the bm2 mutants and wild-type sibling controls (Table 2). The majority of these significantly differentially expressed genes were up-regulated in bm2 mutants as compared to wild-type sibling controls. These included genes that encode members of the PAL, cinnamate-4-hydroxylase (C4H), HCT, CAD and 4CL families. Three CAD-encoding genes (GRMZM2G0990980, GRMZM2G443445 and AC234163.1_FG002), an HCT-encoding gene (GRMZM2G089698) and a 4CL-encoding gene (GRMZM2G048522) were each up-regulated at least 16-fold in the bm2 mutant relative to the wild-type (Table 2). Interestingly, the cloned bm1 gene (GRMZM5G844562) that encodes CAD was down-regulated in the bm2 mutant.

The bm2-encoded MTHFR enzyme is involved in the metabolism of methionine, a precursor of the methyl donor S-adenosyl-L-methionine (SAM). Two SAM-dependent methyltransferases, caffeoyl CoA 3-O-methyltransferase (CCoAOMT) and caffeic acid 3-O-methyltransferase (COMT), participate in the phenylpropanoid pathway. However, among nine genes that are annotated in MapMan (Thimm et al., 2004) as encoding these two methyltransferases, only one (GRMZM2G099363) was significantly down-regulated in the bm2 mutant relative to the wild-type siblings, and this exhibited only a small decrease (0.65-fold). Similarly, the previous cloned COMT-encoding gene bm3 (AC196475.3_FG004) was not significantly differentially expressed in our RNA-Seq experiments with a false discovery rate of ≤5% (Table S9). Two peroxidase genes (AC205413.4_FG001 and GRMZM2G126261) involved in lignin assembly were significantly up-regulated in the bm2 mutant. One of them, AC205413.4_FG001, was up-regulated >16-fold (Appendix S1). Consistent with the role of peroxidases in polymerization of monolignols into lignin (Quiroga et al., 2000), the peroxidase family was enriched among the differentially expressed genes.
DISCUSSION

Plants homozygous for bm2 mutations exhibit reddish-brown pigmentation of their leaf midribs, and also accumulate reduced lignin levels; the lignin that does accumulate has an altered composition as compared to non-mutant maize (Vermerris and Boon, 2001; Marita et al., 2003; Sattler et al., 2010). Here, we describe molecular characterization of the cloned bm2 gene. Analysis of multiple independent Mu-induced alleles of the bm2 gene demonstrated that the putative MTHFR-encoding gene located in this interval that is down-regulated in the bm2 mutant is indeed the bm2 gene. Complementation studies performed in yeast demonstrate that the bm2 gene encodes a functional MTHFR. In addition, a previous study (Roje et al., 1999) showed that expression in MTHFR-deficient yeast strains of a maize cDNA derived from what we now know is the bm2 gene displayed both forward and reverse MTHFR enzyme activities. This observation, in combination with our quantitative RT-PCR analysis of 11 bm2-Mu lines, suggests that the bm2 mutants have, at most, greatly reduced levels of MTHFR activity.

Survival of bm2 mutants

MTHFR plays a critical role in the biosynthesis of methionine (Goyette et al., 1994; Vickers et al., 2006), an essential sulfur-containing amino acid. Apart from its nutritional

Table 2 Differentially expressed genes in the phenylpropanoid pathway in the bm2 mutant as compared to wild-type siblings

| Sort order | Gene            | MapMan annotation | log2 fold change (mutant/wild-type) | P value | Adjusted P value |
|------------|-----------------|-------------------|-------------------------------------|---------|-----------------|
| 1          | GRMZM2G160541   | PAL               | −1                                  | 1.61E-03| 1.73E-02        |
| 2          | GRMZM2G081582   | PAL               | 0.86                                | 2.73E-04| 5.36E-03        |
| 3          | GRMZM2G063817   | PAL               | 1.19                                | 3.05E-03| 2.58E-02        |
| 4          | GRMZM2G334660   | PAL               | 1.77                                | 1.12E-03| 1.36E-02        |
| 5          | GRMZM2G089689   | HCT               | 5.54                                | 5.58E-07| 9.96E-05        |
| 6          | AC210173.4_FG005| F5H               | −2.09                               | 1.59E-04| 3.74E-03        |
| 7          | GRMZM2G099363   | CCoAOMT           | −0.62                               | 7.51E-03| 4.50E-02        |
| 8          | GRMZM5G844562   | CAD (bm1)         | −0.7                                | 7.89E-03| 4.64E-02        |
| 9          | GRMZM2G090980   | CAD               | 4.12                                | 3.78E-06| 3.31E-04        |
| 10         | GRMZM2G443445   | CAD               | 10.27                               | 2.85E-09| 5.78E-06        |
| 11         | AC234163.1_FG002| CAD               | 31.9                                | 8.70E-07| 1.26E-04        |
| 12         | GRMZM2G139874   | C4H               | 1.78                                | 1.18E-04| 3.11E-03        |
| 13         | GRMZM2G147245   | C4H               | 4.22                                | 2.65E-05| 1.14E-03        |
| 14         | GRMZM2G012233   | 4CL               | −0.95                               | 3.89E-03| 2.98E-02        |
| 15         | GRMZM2G049522   | 4CL               | 2.42                                | 1.33E-03| 1.53E-02        |
| 16         | GRMZM2G051005   | No annotation     | −1.79                               | 5.67E-04| 8.77E-03        |
| 17         | GRMZM2G156004   | No annotation     | −1.66                               | 1.37E-05| 7.86E-04        |
| 18         | GRMZM2G158083   | No annotation     | −0.98                               | 1.76E-04| 3.99E-03        |
| 19         | GRMZM2G179703   | No annotation     | −0.97                               | 1.90E-03| 1.92E-02        |
| 20         | GRMZM2G409724   | No annotation     | −0.87                               | 3.41E-03| 2.76E-02        |
| 21         | GRMZM2G017557   | No annotation     | −0.78                               | 4.98E-03| 3.47E-02        |
| 22         | GRMZM2G108714   | No annotation     | −0.77                               | 2.07E-03| 2.02E-02        |
| 23         | GRMZM2G094017   | No annotation     | 0.74                                | 3.16E-04| 5.94E-03        |
| 24         | AC217947.4_FG002| No annotation     | 1.03                                | 2.19E-03| 2.10E-02        |
| 25         | GRMZM2G060210   | No annotation     | 1.11                                | 2.24E-03| 2.13E-02        |
| 26         | GRMZM2G138624   | No annotation     | 1.2                                 | 1.38E-03| 1.55E-02        |
| 27         | GRMZM2G035023   | No annotation     | 1.63                                | 3.43E-04| 6.23E-03        |
| 28         | GRMZM2G147503   | No annotation     | 1.96                                | 2.85E-04| 5.54E-03        |
| 29         | GRMZM2G165192   | No annotation     | 2.05                                | 2.18E-04| 4.61E-03        |
| 30         | GRMZM2G061806   | No annotation     | 2.72                                | 1.09E-04| 2.93E-03        |
| 31         | GRMZM2G408458   | No annotation     | 3.17                                | 2.43E-05| 1.09E-03        |
| 32         | GRMZM2G127418   | No annotation     | 3.94                                | 6.26E-04| 9.32E-03        |
| 33         | GRMZM2G015793   | No annotation     | 4.14                                | 4.52E-06| 3.71E-04        |
| 34         | GRMZM2G124815   | No annotation     | 4.67                                | 5.78E-07| 1.01E-04        |
| 35         | GRMZM2G089698   | No annotation     | 5.54                                | 5.58E-07| 9.96E-05        |
| 36         | GRMZM2G127251   | No annotation     | 6.63                                | 3.08E-06| 2.90E-04        |
| 37         | GRMZM2G362298   | No annotation     | 7.69                                | 2.08E-05| 9.19E-04        |
| 38         | GRMZM2G311036   | No annotation     | 7.84                                | 5.74E-06| 4.25E-04        |
| 39         | GRMZM2G114918   | No annotation     | 19.77                               | 4.43E-05| 1.61E-03        |
| 40         | GRMZM2G023325   | No annotation     | 20.45                               | 7.48E-05| 2.25E-03        |
| 41         | GRMZM2G336824   | No annotation     | 33.71                               | 3.45E-05| 1.37E-03        |
importance and central role in the initiation of mRNA translation, methionine indirectly regulates a variety of important cellular processes, including the biosynthesis of DNA, RNA, protein, lipid and hormones (Amir, 2010). Based on the essential role of this enzyme in yeast, it is predicted that knockout mutants of bm2 would be lethal. However, plants homozygous for the bm2-ref allele or any of the Mu insertion alleles are viable. Because methionine is an essential amino acid, the survival of these bm2 mutants suggests that they accumulate sufficient amounts of methionine. In our RNA-Seq analyses of bm2 mutants, genes associated with protein synthesis are under-represented among the differentially expressed genes, consistent with the hypothesis that protein synthesis is not disrupted in bm2 mutants (Table S8 and Appendix S1). There are at least two possible explanations for the viability of the bm2 mutants. First, the bm2 mutant alleles may be leaky. However, including both the reference and Mu insertion alleles, 12 bm2 mutant alleles have been identified; all are homozygous viable. It is unlikely that all of these are leaky, particularly as each of the 11 Mu-induced alleles contains a transposon insert in the coding region. If the partial bm2 paralog GRMZM2G0343278 (Figure S11) exhibits MTHFR activity, it is possible that in bm2 mutant plants GRMZM2G0343278 may provide sufficient residual MTHFR activity to ensure survival but not normal accumulation of methionine.

Figure 5. Relationship of bm2 to the lignin biosynthetic pathway. Modified biosynthetic pathway of monolignols (Bonawitz and Chapple, 2010; Vanholme et al., 2010). The MTHFR (BM2) enzyme catalyzes the conversion of 5,10-methylene tetrahydrofolate to 5-methyltetrahydrofolate, which serves as a methyl donor to convert homocysteine to methionine. SAMS catalyzes the conversion of methionine to SAM, which is a methyl donor for CCoAOMT and COMT. The number of genes in families with at least one gene differentially expressed between bm2 mutants and wild-type siblings is indicated by the number of rectangles. The number of significantly up- and down-regulated genes in bm2 mutants relative to wild-type siblings are shown in blue and red, respectively. PAL, phenylalanine ammonia lyase; HCT, hydroxycinnamoyl CoA-shikimate hydroxycinnamoyl transferase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate-CoA ligase; C3H, p-coumarate 3-hydroxylase; CAD, cinnamyl alcohol dehydrogenase; CCR, cinnamoyl CoA reductase; CCoAOMT, caffeoyl CoA 3-O-methyltransferase; COMT, caffeic acid 3-O-methyltransferase; F5H, ferulate 5-hydroxylase; MTHFR, methylenetetrahydrofolate reductase; 5,10-methylene THF, 5,10-methylene tetrahydrofolate; 5-methyl THF, 5-methyltetrahydrofolate; THF, tetrahydrofols; MTR, 5-methyltetrahydrofolate homocysteine methyltransferase; SAMS, S-adenosylmethionine synthetase; SAM, S-adenosyl-l-methionine.

Role of the bm2 gene in lignin biosynthesis

Consistent with previous reports (Vermerris and Boon, 2001; Marita et al., 2003; Barriere et al., 2004; Sattler et al., 2010), we found that the bm2 mutation reduces lignin accumulation (Table 1). We also found that lignin continues to accumulate after anthesis in both mutant and non-mutant sibling plants (Table 1).

The bm2-encoded MTHFR generates 5-methyltetrahydrofolate, which is used in the methylation of homocysteine to generate methionine (Goyette et al., 1994; Vickers et al., 2006). Subsequently, S-adenosyl-l-methionine (SAM) may be generated from methionine via the action of S-adenosyl-methionine synthetase. It has previously been shown that knockout of the gene encoding S-adenosylmethionine synthetase suppresses lignin biosynthesis (Shen et al., 2002). Consistent with the fact that MTHFR is involved in the biosynthesis of SAM (Ravanel et al., 1998), a number of SAM-dependent or SAM-related functional sub-groups (i.e. ethylene hormone metabolism, simple phenols and phenylpropanoids) are preferentially affected in the bm2 mutant (Table S8) (Shen et al., 2002). Two of these sub-groups (simple phenols and phenylpropanoids) are related to lignin biosynthesis. Indeed, many of the differentially expressed genes encode enzymes involved in lignin biosynthesis, such as PAL, HCT, ferulate-5-hydroxylase (F5H),...
CCoAOMT, CAD, C4H and 4CL (Table 2). Because SAM is consumed by both CCoAOMT and COMT (Ye et al., 1994), alterations in the accumulation of SAM are expected to reduce the accumulation of both G- and S-lignin (Figure 5). Consistent with this expectation, our data confirm that the bm2 mutant shows reduced accumulation of both G- and S-lignin (Vermeris and Boon, 2001; Marita et al., 2003; Saballos et al., 2009; Sattler et al., 2010; Vermeris et al., 2010).

Experimental Procedures

Genetic stocks

Mutator-derived stocks originally obtained from Don Robertson (Iowa State University, Ames, IA, USA) have been maintained in the Schnable laboratory for many years. Various stocks carrying the brown midrib2 reference allele (bm2-ref) were obtained from the Maize Genetics Cooperation Stock Center (maizecoop.cropsci.uiuc.edu). These include stock numbers 90-896/4-895/2, 93-706-5/705, 93-705-2/706 and 2000-1695-7/1695-4 (Schnable Lab accession numbers Ac3247, Ac3246, Ac3245 and Ac3244, respectively).

The full-length bm2 cDNA was PCR-amplified from each of these stocks using forward primer 5′-GTTATGAGGTTATCGAG AAGATCTCTGGAG-3′ (which includes the start codon) and reverse primer 5′-TCAGATCCTGAAGGCAGCAAACAGG-3′ (which includes the stop codon). The corresponding DNA fragments were then sequenced using the forward primers 5′-ATGGAAGGTTATCGAG GAAGATCTCTGGAG-3′, 5′-GATGCCGTACAAGGCGAGGGG-3′ and 5′-GCCTCTCAAAAAGTGCAAAGTC TTAATGC-3′. Sequence analysis of full-length bm2 cDNAs amplified from these stocks did not detect any polymorphisms among the various bm2 mutant sources, suggesting that these stocks all carry the bm2-ref allele.

Mapping populations

A bm2 mapping population was created by back-crossing homozygous bm2-ref mutant plants (Ac3247) as the male (pollen) parent onto the inbred line B73 (female parent) to generate F1 seeds. Multiple F1 plants were self-pollinated to create F2 seeds that were used in the fine-mapping experiments. The F2 seeds used in the BSR-Seq experiment were derived from a single F1 plant.

Fine-scale mapping

DNA was extracted from leaf tissues sampled from 537 F2 plants derived from a cross between B73 and bm2-ref homozygotes. SNPs identified during the BSR-Seq experiment (see below) and located within the mapping interval were sent to KBiosciences (now LGC Genomics) for KASPar SNP primer design (www.lgcgenomics.com/genotyping/kasp-genotyping-reagents/kasp-assays-kbd-kod/?id=69). The 537 individuals from the F2 mapping population were individually subjected to KASPar-based SNP genotyping (Cuppen, 2007) using the flanking SNP markers bm2-290599838 and bm2-291111263 (Table S2) to identify recombinants. Additional SNPs were used to similarly genotype recombinant plants to more precisely define the bm2 mapping interval.

Isolation of RNA for two independent RNA-Seq experiments

For RNA-Seq experiment #1, F2 seeds from a single heterozygous individual (genotype Bm2/bm2-ref; Maize Genetics Cooperation Stock Center, stock center ID 90-896/4-895-2) (Schnable Lab accession number Ac3247) were grown in a greenhouse under 15 h light/9 h dark (27°C/24°C, 30% humidity). The light intensity was approximately 650–800 µmol m⁻² s⁻¹. Leaf tissue samples were collected from 53 26-day-old phenotypically mutant plants and 53 phenotypically non-mutant plants. A 5.5 cm segment of leaf tissue (measured from the stem) was collected from the second youngest leaf from each individual plant. Corresponding midrib tissue samples were pooled for RNA extraction. RNA was extracted from tissues using RNeasy mini kits (Qiagen, www.qiagen.com) with DNase I treatment according to the manufacturer’s instructions. RNA quality was analyzed using a Bioanalyzer 2100 RNA nanochip (Agilent Technologies, www.agilent.com) according to the manufacturer’s instructions. RNA-Seq libraries were constructed using an Illumina RNA-Seq sample preparation kit according to the manufacturer’s instructions (www.illumina.com). The libraries were sequenced on an Illumina Genome Analyzer II, generating approximately 75 bp reads.

Subsequently, a second RNA-Seq experiment (#2) was performed. F2 seeds from a heterozygous individual (genotype Bm2/bm2-ref) were grown. A 5.5 cm segment of leaf tissue (measured from the stem) was collected from the second youngest leaf of each 38-day-old plant individual. Two biological replicates of mutant and wild-type sibling tissue samples were collected. In each replication, the midribs of leaf samples from 20 mutant and 20 wild-type individuals were pooled separately for RNA extraction. This RNA-Seq experiment was similar to the first RNA-Seq experiment except that: (i) midribs were collected from 38-day-old plants rather than 26-day-old plants, (ii) midribs from 20 individuals were pooled in each sample, and (iii) 101 bp paired-end reads were generated on an Illumina HiSeq2000 instrument.

Trimming and mapping of RNA-Seq reads

Raw reads were subjected to quality checking and trimming to remove low-quality bases using a custom written trimming script (Liu et al., 2012). Trimming parameters were set similarly to the defaults for lucy2 trimming software (Li and Chou, 2004). Trimmed reads were aligned to the B73 reference genome (B73Ref2) using GSNAP (Wu and Nacu, 2010), and uniquely mapped reads (allowing two mismatches every 36 bp, and 3 bp tails per 75 bp) were used for subsequent analyses. The read depth of each gene of the filtered gene set (ZmB73_5b_FGS, see ftp.maizesequence.org/ current/filtered-set/ for files) was computed based on the coordinates of mapped and annotated locations of genes in the B73 reference genome.

Mapping bm2 via BSR-Seq

Reads from the first RNA-Seq experiment were used to map bm2 via BSR-Seq (Liu et al., 2012). After aligning trimmed reads from the bm2 and wild-type sibling pools, SNPs were discovered and quantified at each SNP site in each sample pool (mutant and wild-type). Finally, a Bayesian-based bulked segregant analysis (BSR-Seq), which accounts for biological/technical variation, was used to map the bm2 gene (Liu et al., 2012).

Analysis of RNA-Seq experiment #1

Normalization was performed using a method that corrects for biases introduced by RNA composition and differences in the total numbers of uniquely mapped reads in each sample (Robinson and Oshlack, 2010). Normalized read counts were used to calculate fold changes and statistical significance. Fisher’s exact test was used to test the null hypothesis that the expression level of a given gene is not different between the two samples. Because the
first experiment did not include biological replication, statistically significant variation may be a consequence of either biological or technical variation in gene expression between a pair of samples. Genes identified as candidates for differential expression were further filtered using an absolute log2 fold change >1 and a q value cut-off of 0.00001 to account for multiple testing (Benjamini and Hochberg, 1995). These genes are referred to as ‘significantly differentially expressed’ and were functionally classified using the MapMan functional classification system (Thimm et al., 2004).

**Analysis of the RNA-Seq experiment #2**

Genes with at least one uniquely mapped read across samples and at least two samples with positive read counts were tested for differential expression between the bm2 mutant and wild-type sibling using the R package QuasiSeq (http://cran.r-project.org/web/packages/QuasiSeq). The negative binomial QLSpline method implemented in the QuasiSeq package was used to calculate a P value for each gene (Lund et al., 2012). The 0.75 quantile of reads from each sample was used as the normalization factor (Bullard et al., 2010). A multiple test controlling approach (Nettleton et al., 2006) was used to estimate the number of genes with true null hypotheses among all genes tested, and this estimate was used to convert the P values to q values (Storey, 2002). To obtain approximate control of the false discovery rate at 5%, genes with q values no larger than 0.05 were considered differentially expressed.

**Enrichment analysis of significantly differentially expressed genes in RNA-Seq experiment #2**

Functional annotation of maize genes was downloaded from http://mapman.gabipd.org/. Similar to the PageMan enrichment analysis (Usadel et al., 2006), Fisher’s exact test was used to test the null hypothesis of no enrichment of a certain functional group of genes in the set of differentially expressed genes. To avoid dependency of functional groups across various layers of annotation, which introduces complexity during multiple test control, we performed enrichment analysis for each annotation layer separately.

**Direct transposon (Mu) tagging of bm2**

Additional bm2 alleles were isolated from a forward genetic screen of approximately 147 500 individuals generated by crossing active Mu stocks (genotype of Bm2Bm2; Mu) as females with males homozygous for the bm2-ref allele. The male parent in this cross was derived from a mixture of the four stocks obtained from the Maize Genetics Cooperation Stock Center. A Mutator transposon-specific primer (MuTIR) and bm2 gene-specific primers were used to analyze individuals that exhibited a brown midrib phenotype to identify Mutator insertion alleles in the bm2 gene (bm2-Mu) (Figure S1).

**Measurement of bm2 expression via quantitative RT-PCR**

Quantitative RT-PCR was performed on two biological replicates of 11 homozygous bm2-Mu lines and B73 (described above). Each biological replicate represents a pool of midrib tissue collected from 2 to 4 individual 1-month-old plants. Collection of midrib tissue was performed as described above. RNA was isolated and purified using RNeasy mini kits (Qiagen), before reverse transcription into cDNA using SuperScript™ III reverse transcriptase (Invitrogen, www.lifetech.com) according to the manufacturer’s instructions. qRT-PCR was performed using ABSolute™ qPCR SYBR® Green Mix (Thermo Scientific) on Roche Lightcycler 480 quantitative PCR system (Roche, www.nimblegen.com).

Quantitative RT-PCR data were analyzed using Roche Lightcycler 480 analysis software. The following PCR conditions were used to detect transcript levels: 2 min at 50°C, 10 min at 95°C (pre-denaturation), 40 cycles of 15 sec at 95°C/1 min at 60°C (denaturation/annealing/amplification), with a temperature of 80°C for the melting curve start temperature. Cq values were calculated using the second derivative maximum method with baseline-corrected, 6-carboxy-X-rhodamine (ROX)-normalized parameters (for details, see LightCycler 480 Operator’s Manual accessible at icb.sinica.edu.tw/pubweb/Core%20Facilities/Data/R401-core/LightCycler480%20I_Manual_V1.5.pdf). One plate was analyzed for each group of biological replicates. Each sample was analyzed using bm2 and glyceraldehyde-3-phosphate dehydrogenase gene primers separately. Three technical replicates were analyzed for each reaction, and the mean Cq value of these three replicates was used for further data analysis. Standard curves for quantification were constructed based on four fivefold serial dilutions of B73 DNA with bm2 or glyceraldehyde-3-phosphate dehydrogenase gene primers. Samples were normalized using glyceraldehyde-3-phosphate dehydrogenase gene. The following primers were used for detection of the corresponding mRNAs: bm2 forward primer 5′-ATGATGGTCCGAGAATCA-3′ and reverse primer 5′-GATGTTTCGACCTATATTCTGG-3′; glyceraldehyde-3-phosphate dehydrogenase gene forward primer 5′-GCTTCTCATGATGTTGCT-3′ and reverse primer 5′-CAGGAAGGGAAAGCAAGTG-3′.

**Phloroglucinol staining and light microscopy**

Midrib, stem and root tissue samples were hand-sectioned to a thickness of 200 μm using double-edge razors (Wilkinson Sword, www.wilkinsonword.co.uk), and stored in sterile distilled water for 2 h or less until sample sectioning was completed. Phloroglucinol staining was performed as described previously (Nakano and Mesheetsuka, 1992). Briefly, phloroglucinol (Sigma-Aldrich, www.sigmaaldrich.com) was dissolved in 95% ethanol (Decon Laboratories Inc., www.deconlabs.com) to form a 2% stock solution. Immediately before use, concentrated hydrochloric acid (33% v/v) was mixed with the stock solution to form the phloroglucinol staining solution, which was directly applied to samples. Maize tissue samples were placed on glass slides (Thermo Fisher Scientific, www.thermofisher.com, and stored in sterile distilled water for 2 h or less until sample sectioning was completed. Phloroglucinol staining was performed as described previously (Nakano and Mesheetsuka, 1992). Briefly, phloroglucinol (Sigma-Aldrich, www.sigmaaldrich.com) was dissolved in 95% ethanol (Decon Laboratories Inc., www.deconlabs.com) to form a 2% stock solution. Immediately before use, concentrated hydrochloric acid (33% v/v) was mixed with the stock solution to form the phloroglucinol staining solution, which was directly applied to samples. Maize tissue samples were placed on glass slides (Thermo Fisher Scientific, www.thermofisher.com) and stored in sterile distilled water for 2 h or less until sample sectioning was completed. Phloroglucinol staining was performed as described previously (Nakano and Mesheetsuka, 1992). Briefly, phloroglucinol (Sigma-Aldrich, www.sigmaaldrich.com) was dissolved in 95% ethanol (Decon Laboratories Inc., www.deconlabs.com) to form a 2% stock solution. Immediately before use, concentrated hydrochloric acid (33% v/v) was mixed with the stock solution to form the phloroglucinol staining solution, which was directly applied to samples. Maize tissue samples were placed on glass slides (Thermo Fisher Scientific, www.thermofisher.com). Excess solution was removed from the glass slides using Kimwipes (Kimberly-Clark, www.kimberly-clark.com). Phloroglucinol stain (300 μl) was applied to the samples for 30 sec, with a cover glass (Corning, www.corning.com) on top. Additional phloroglucinol staining solution was added to the sample until it was fully covered by solution. Light images were captured using a Spot RT slider camera (Diagnostic Instruments Inc., www.spotimaging.com) on a Nikon Eclipse E800 microscope (www.nikoninstruments.com) at 20 x magnification, and analyzed using spot version 4.0.6 software (Diagnostic Instruments Inc.).

**Actinomycin D treatment**

A small circular piece of the 2nd youngest leaf (approximately 0.02 g) of various genotypes was obtained using a hole punch. The samples were incubated in water (negative control), actinomycin D (50 μg ml−1) or DMSO (10 μl ml−1, solvent control) for 12 h at room temperature. Solutions were changed every 4 h. The samples were then subjected to RNA purification and RT-PCR.

**Yeast complementation assay**

Saccharomyces cerevisiae strains of the following genotypes were kindly provided by Warren D. Kruger (Division of Population Science, Fox Chase Cancer Center, Philadelphia, PA, USA) (Shan et al., 1999): W303-1A (wild-type, also labeled MET11: Mata,
ade2-1, can1-100, ura3-1, leu2-3, 112, trp1-1, his3-11,15) and XSY3-1A (Mat11 knockout strain, also labeled met11: Mata, ade2-1, can1-100, ura3-1, leu2-3,112, trp1-1, his3-11,15, met11::TRP1). The galactose-inducible human MTHFR expression plasmid pHMTHFR (which contains the human MTHFR-encoding cDNA) inserted in pHMV2.1 from Shan et al. (1999) was also provided by Warren D. Kruger (Shan et al., 1999). To obtain wild-type maize bm2 cDNA, RNA was extracted from wild-type maize (B73), and then subjected to RT-PCR. The full-length (1782 bp) wild-type maize bm2 cDNA was first amplified from B73 cDNA using forward primer 5'-GGTTAGAAGGGTGAT CGAAGAGATCCTGGAG-3' (including the start codon) and reverse primer 5'-TCGAGATCTT GAAGGCAAGAACGAGG-3' (including the stop codon). The fragment was cloned into the galactose-inducible expression plasmid pYES2.1/V5-His-TOPO using a pYES2.1 Topo® TA expression kit (both supplied by Life Technologies, www.lifetechologies.com/en/us/home.html). Plasmids were transformed into yeast using the S.c. EasyComp™ transformation kit (Invitrogen). The cloned fragment was then sequenced using forward primers 5'-ATGAGGTTTATCAGAAGATC-3', 5'-GATGCGATACAAGGCGAGG-3' and 5'-GCGCTTCACAAACTTCTGTC-3', and reverse primer 5'-GTAAGGTCGCAAGGCTTAGC-3'. To test the growth of the transformed yeast for complementation, yeast cells were inoculated on plates containing SD glucose -Met (control) or SD galactose -Met (to induce expression of the insert at the plasmid), which were then incubated at 30°C for 3 days. The fragment was then sequenced using forward primer 5'-GTAAAGGTGCAAAGTCTTAATGC-3' and 5'-GTTATGAAGGTTATCGAGAAGATCCTGGAG-3'. The sequence was confirmed by BLAST analysis (www.ncbi.nlm.nih.gov/blast.cgi) on the maize RefSeqs. Orthologs of maize BM2 were identified using the National Center for Biotechnology Information Protein BLAST algorithm (blast.ncbi.nlm.nih.gov/Blast.cgi) on the maize bm2 protein sequence. All parameters were default except the 'expect' threshold, which was set to 0.00001. The resulting 2099 non-redundant protein sequences were narrowed down to 18 land-plant RefSeqs with greater than 40% identity and coverage of the maize protein sequence. Because these proteins vary in length, a phylogenetic tree was constructed using only the conserved region of MTHFR, which corresponds to residues 167–350 of the BM2 protein sequence. The tree was constructed in Jalview (www.jalview.org) via Blosum62 (www.ncbi.nlm.nih.gov/Class/FieldGuide/BLOSUM62.txt) neighbor joining using a consensus sequence constructed from the 18 RefSeqs.

Expression pattern of bm2

The expression pattern for maize bm2 was visualized using the online tool qTeller (qTeller.com). The RNA-Seq data provided by qTeller (both published and unpublished) comes from a variety of sources throughout the maize community. More information on the origin of the datasets and in-house analysis of the data may be found on the qTeller website.

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and Ann Perera and Zhihong Song (W.M. Keck Metabolomics Research Laboratory, Iowa State University, Ames, IA, USA) for their expertise in GC-MS.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Identification of Mutator insertion alleles in the bm2 locus.

Figure S2. bm2 encodes a putative MTHFR.

Figure S3. Histochemical staining of lignin in tissue sections from wild-type and the bm2-Mu mutant.

Figure S4. Polymorphisms in the bm2-ref allele compared to the Bm2-B73 wild-type allele.

Figure S5. Alignment of deduced amino acid sequences of the Bm2 gene with the sequence of MET11 from Saccharomyces cerevisiae.

Figure S6. Histogram of P values for differential expression tests.

Figure S7. Volcano plot from RNA-Seq.

Figure S8. MA plot from RNA-Seq.

Figure S9. Comparison of fold changes between two RNA-Seq experiments on 368 differentially expressed genes.

Figure S10. Overview of differential expression in the metabolic pathway.

Figure S11. Phylogenetic tree of the maize MTHFR conserved region.

Figure S12. qTeller expression pattern of bm2.

Table S1. Genes in the 2 Mb interval as inferred from BSR-Seq.

Table S2. Fine-mapping primer sequences for the KASPar assay.

Table S3. The 47 genes in the 0.51 MB bm2 interval (working gene set).

Table S4. The eight genes in the 0.51 MB bm2 interval (filtered gene set).

Table S5. Trimming and alignment summary for RNA-Seq experiment #1.

Table S6. Read trimming summary for RNA-Seq experiment #2.

Table S7. Alignment summary for RNA-Seq experiment #2.

Table S8. Overall represented pathways (MapMan).

Table S9. Genes in the phenylpropanoid pathway that did not exhibit significantly differential expression.

Appendix S1. Differential expression between bm2 and the wild-type.

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