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Identification of differentially expressed genes in sorghum (*Sorghum bicolor*) brown midrib mutants

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Sorghum, a species able to produce a high yield of biomass and tolerate both drought and poor soil fertility, is considered to be a potential bioenergy crop candidate. The reduced lignin content characteristic of brown midrib (*bmr*) mutants improves the efficiency of bioethanol conversion from biomass. Suppression subtractive hybridization combined with cDNA microarray profiling was performed to characterize differential gene expression in a set of 13 *bmr* mutants, which accumulate significantly less lignin than the wild-type plant BTx623. Among the 153 differentially expressed genes identified, 43 were upregulated and 110 downregulated in the mutants. A semi-quantitative RT–PCR analysis applied to 12 of these genes largely validated the microarray analysis data. The transcript abundance of genes encoding L-phenylalanine ammonia lyase and cinnamyl alcohol dehydrogenase was less in the mutants than in the wild type, consistent with the expectation that both enzymes are associated with lignin synthesis. However, the gene responsible for the lignin synthesis enzyme cinnamic acid 4-hydroxylase was upregulated in the mutants, indicating that the production of monolignol from L-phenylalanine may involve more than one pathway. The identity of the differentially expressed genes could be useful for breeding sorghum with improved efficiency of bioethanol conversion from lignocellulosic biomass.

Introduction

The development of sources of renewable energy is driven by the depletion of global fossil fuel reserves, a steady increase in the price of oil and gas and the need to reduce net emissions of carbon dioxide into the atmosphere. Plant biomass is a promising source of renewable energy (Schmer et al. 2008). Currently, the majority of biofuel (in particular ethanol) is derived from the fermentation of maize starch or sugarcane juice (Li et al. 2008), so it has been recognized that further increases in the supply of these particular sources of energy will compete with food production for arable land. Thus, the next generation of biofuels should be targeted at plants not only

Abbreviations – ATPC1, ATP synthase gamma chain 1; *bmr* ,brown-midrib; C4H, cinnamic acid 4-hydroxylase; CAD, cinnamyl alcohol dehydrogenase; CCR, cinnamoyl-CoA reductase; COMT, caffeic acid *O*-methyltransferase; EMB2753, embryo-defective 2753; ER, endoplasmic reticulum; EST, expressed sequence tag; GUN4, genomes uncoupled 4; HLH, helix loop helix; HMG, high mobility group protein; PAL, l-phenylalanine ammonia lyase; SSH, suppression subtractive hybridization.

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suitable for producing sufficient biomass for converting to biofuels but also able to grow on marginal land.

Sorghum (*Sorghum bicolor*) has some potential to be developed for this purpose. Being a C4 plant, its photosynthetic efficiency is higher than that of C3 species (Carpita and McCann 2008). Current cultivars are bred for grain, sugar or fodder production, and all types are in principle suitable as a source of bioenergy, either via the hydrolysis and fermentation of starch from grain sorghum, the fermentation of simple sugars from sweet sorghum, or the hydrolysis and fermentation of polysaccharides of forage sorghum. More importantly, sorghum is particularly tolerant of low soil moisture and poor nutrient availability (Corredor et al. 2009). In drought-prone, low fertility soils, it can be cropped successfully for up to 2 years before the fertility of the soil becomes so depleted that there is a need for replenishment.

Cellulose, hemicellulose and lignin are the primary components of the plant cell wall, and so dominate the lignocellulosic biomass (Jung and Ni 1998). The cellulose molecule is formed by the interlinkage of glucose moieties, mostly through glycosidic bonds; the hemicellulose polysaccharide is composed of various pentose and hexose moieties, and *in planta* aggregates the cellulosic fibers into microfibrils. Lignin is a complex polyphenylpropanoid that stiffens the cell wall, and also is associated with resistance to a range of biotic stresses (Del Rio et al. 2007). The current cost of converting lignocellulosic biomass to ethanol is commercially uncompetitive, mainly because the polysaccharides are embedded in a lignin matrix, which impedes the enzymatic hydrolysis process required to generate the necessary monosaccharides. It is the removal of lignin that is the most costly step in the ethanol conversion process (Wyman et al. 2005, Li et al. 2008). Thus, one way to achieve a more cost-effective conversion process would be breed cultivars that accumulate less lignin, while increasing the amount of cellulose present. The brown-midrib (*bmr*) mutants identified in maize, sorghum and millet fit this requirement (Cherney et al. 1991). They are recognized by the brown pigmentation of their leaf midrib and stalk pith, which first becomes apparent at the four- to six-leaf stage. Biochemical analysis has shown that the lignin content in their cell walls and vascular tissues is less than that in the wild type (Barrière et al. 2004). In particular, Porter et al. (1978) demonstrated that the lignin content of the mature stem of the sorghum *bmr* mutant was about half that in the wild type, whereas its leaf lignin content was just one quarter. Similarly, in maize, a 50% higher yield of fermentable sugars was obtainable from the stover of *bmr* mutants than was possible from wild-type stover (Vermerris et al. 2007).

Little is known regarding the location of the various *bmr* mutations within the lignin synthesis pathway, which is both complex and highly regulated. The maize *bm3* mutant has been associated with lesions in the gene encoding caffeic acid *O*-methyltransferase (COMT) (Vignols et al. 1995), whereas the *bm1* mutation affects the expression of cinnamyl alcohol dehydrogenase (CAD) (Halpin et al. 1998). In sorghum, the same genes were affected in, respectively, the *bmr12* and *bmr6* mutants (Bout and Vermerris 2003, Sattler et al. 2009). Here, we describe the identification of differential expression in sorghum *bmr* mutants, by combining suppression subtractive hybridization (SSH) with cDNA microarray analysis. As well as identifying what genes are differentially expressed, the approach also can help resolve questions related to cell-wall metabolism in sorghum. More generally, these data may be applicable to other lignocellulosic bioenergy crops in which there is a need to improve biomass quality.

Materials and methods

Plant materials and growth conditions

The study was based on a comparison between the wild-type sorghum cultivar BTx623 (the variety used to acquire the sorghum genome sequence, see http://www.phytozome.net/sorghum) and 13 ethyl methane sulfonate-mutated *bmr* mutants (*bmr*, *bmr6*, *bmr12*, *bmr29*, *bmr30*, *bmr31*, *bmr32*, *bmr33*, *bmr34*, *bmr35*, *bmr36*, *bmr45* and *bmr49*) all in a genetic background of BTx623. The *bmr* mutant lines have been backcrossed to the wild type for several generations to clean the genetic background (Xin et al. 2009). Grain of all 14 lines were sown in potting composite in a greenhouse held at 29°C and 60% relative humidity, and grown under a 14 h photoperiod up to the five- to seven-leaf stage. The fifth or sixth leaf was snap-frozen in liquid nitrogen and stored at −80°C before being used as a source of RNA. Lignin determinations were made from the leaves of five- and seven-leaf stage seedlings, and from the stem of seven-leaf stage seedlings.

Lignin content determination

The lignin content of *bmr* mutants and BTx623 was determined using an improved acetyl bromide procedure. Plant material was ground into a fine powder in liquid nitrogen and freeze-dried for 48 h. About 0.01 g of powder was rinsed four times with 95% ethanol and twice with distilled water, dried at 60°C and then suspended in 2 ml 25% acetylbromide (v/v in glacial acetic acid). After a 30-min incubation at 70°C, 0.9 ml

2 *M* NaOH was added, followed by 3 ml glacial acetic acid and 0.1 ml 7.5 *M* hydroxylamine hydrochloride. After centrifuging at 4000 *g* for 10 min, the supernatant was diluted 20-fold with glacial acetic acid, and the absorbance determined at 280 nm.

RNA isolation

Total RNA was isolated from frozen leaf tissue using the TRIzol reagent (Invitrogen, Carlsbad, CA). An equimolar mixture of the RNA extracted from the 13 *bmr* mutants was taken forward for mRNA purification and reverse transcription. An Oligotex mRNA Midi kit (Qiagen, Valencia, CA) was employed to acquire the mRNA from a 1-mg aliquot of total RNA.

Suppression subtractive hybridization

A PCR-based cDNA subtraction was carried out using a PCR-Select cDNA Subtraction kit (Clontech, Mountain View, CA). Both a forward (BTx623 as the driver and *bmr* as the tester) and a reverse (*bmr* as the driver and BTx623 as the tester) subtraction library was created from 2 µg mRNA. The tester cDNA was digested with *Rsa* I and ligated to adaptors 1 and 2R, and two rounds of hybridization and amplification were performed. The resulting amplicon was cloned into the pJET1.2/blunt vector (Fermentas, Glen Burnie, MD) and introduced into *Escherichia coli* DH5α cells (Invitrogen), which were then cultured overnight in liquid LB medium. A 2-µl aliquot of the bacterial culture was taken as the template for a PCR based on the primer pair Nested 1 and 2R, provided in the PCR-select cDNA subtraction kit. Insert sizes were checked by agarose gel electrophoresis, resulting in a set of 6600 recombinant clones carrying inserts in the size range 100–500 bp.

Preparation of the cDNA microarray

The surplus amplicon of the 6600 recombinant clones was precipitated by the addition of two volumes of ethanol, chilling at −80°C for 1 h and centrifuging at 17 200 *g* for 10 min. After washing with 70% ethanol, the DNA pellet was dissolved in 3× saline-sodium citrate buffer. The cDNA clones were arrayed in duplicate on two amino-silane-coated slides (Corning Inc., Acton, MA) with each clone spotted three times on each slide. The slides were then rehydrated with hot vapor and held at 80°C overnight to immobilize the cDNA.

Probe labeling and hybridization

cDNA was reverse-transcribed from 100 µg total RNA from each of wild-type BTx623 and the *bmr* mix using an Array 350 hybridization kit (Genisphere, Hatfield, PA). A 100-pg aliquot of two controls (spikes 1 and 3) were added to the total RNA of each sample to allow normalization. During reverse transcription, the BTx623 cDNA was labeled with Cy5 and the *bmr* mix with Cy3 and then the two probes were mixed in equimolar amounts. The combined probe was applied to the array and incubated at 42°C overnight. The subsequent washing regime followed the manufacturer's recommendations.

Microarray scanning and data analysis

The slides were analyzed using a ScanArray Express scanner (Perkin-Elmer, San Jose, CA). A linear normalization based on the signal intensities of the internal controls (spikes 1 and 3) spotted on the slide was performed with the aid of GenePix Pro v4.0 software (Axon Instrument, Union City, CA). Pre-processing of the normalized microarray data was performed according to Park et al. (2006). Two independent hybridizations were performed. cDNAs showing an intensity ratio ≥2 were considered to be differentially expressed and taken forward for sequencing on an ABI 3730 DNA sequencer (Applied Biosystems, Foster City, CA). After the removal of vector sequence, the sequence set was used as a BLAST query against the GenBank database (http://www.ncbi.nlm.nih.gov).

Semi-quantitative RT–PCR analysis

A 2-µg aliquot of DNase-treated total RNA extracted from the leaf of BTx623 and each of the *bmr* mutants was reverse-transcribed using an iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA). The subsequent PCR comprised an initial denaturation of 95°C/4 min, followed by 25–28 cycles of 95°C/30 s, 53°C/30 s, 72°C/40 s and a final extension of 72°C/5 min. Cycle number was optimized for each target gene, and at least three technical replicates per target gene were analyzed. The resulting amplicons were visualized in EtBr-stained 1% agarose gels following electrophoresis.

Results

Lignin content of the *bmr* mutants

As 5 of the 13 mutants grew very poorly, lignin determination was restricted to 8 of them. Each mutant

accumulated significantly less lignin than the wild type did, although the extent of the reduction varied from mutant to mutant, and also was dependent on the developmental stage of the seedlings (Fig. 1). The *bmr34* mutant contained the least lignin in the leaf at the five-leaf stage (26.8% less than the wild type), whereas that of the other mutants reduced about 11–17% compared with the wild-type BTx623 except *bmr6* (Table 1). In seven-leaf stage seedlings, the leaf lignin content in *bmr36* and *bmr32* was, respectively 37.5 and 28.6% lower than the wild type, and the stem lignin content was reduced , respectively, 27.6 and 29.1%. The lignin content in the other mutants was reduced 9–16% at this stage except *bmr35* (Table 1).

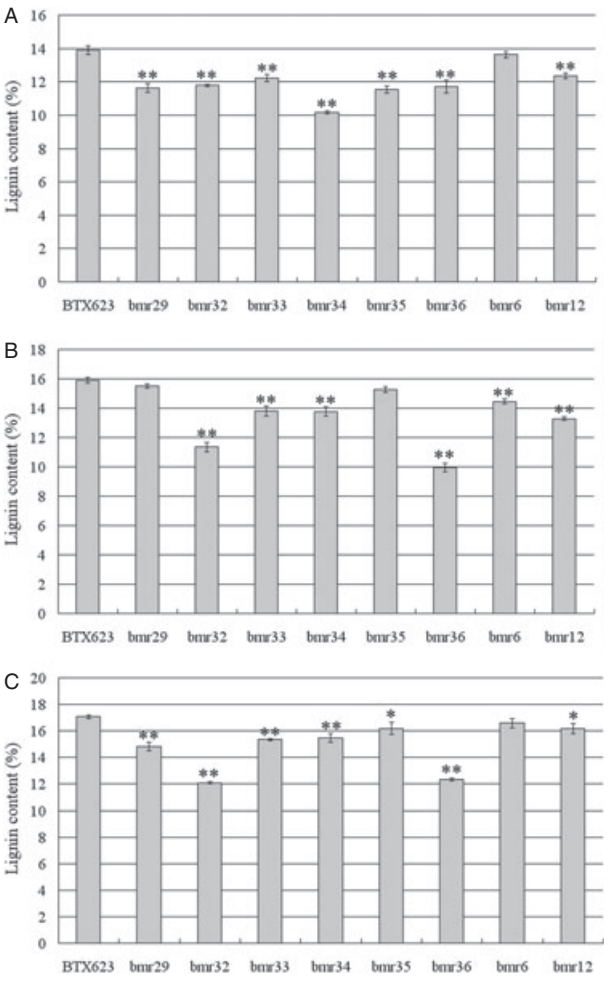

Fig. 1. Lignin content of eight *bmr* mutants and wild-type BTx623. (A) Lignin content in the leaf of five-leaf stage seedlings; (B) lignin content in the leaf of seven-leaf stage seedlings; (C) lignin content in the stem of seven-leaf stage seedlings. All data given in the form mean ± SD. * and ** represent significant differences determined by ANOVA at $P < 0.05$ and $P < 0.01$, respectively.

Identification of differentially expressed genes

Insert size in the 6600 recombinant clones selected from the forward and reverse subtraction libraries lay in the range of 100–500 bp (Fig. 2). The cDNA microarray analysis revealed that among these, 356 (5.4%) experienced differential expression (based on a criterion of a minimum of a twofold difference between the wild-type and the *bmr* mutant bulk. Sequencing of these clones produced 210 non-redundant sequences, of which 153 showed significant homology to genes of known function; 43 of these were upregulated in the *bmr* mutants and the remainder were downregulated. At the protein level, it was possible to classify the gene products of the 153 genes into 11 functional groups, namely metabolism, photosynthesis, genetic information processing, stress response, protein fate, signal transduction, transport, lignin synthesis, cell processes and mobility, development and regulation, and others (Tables 2 and 3, Fig. 3).

The most abundant group was metabolism, which included 28 downregulated and 19 upregulated genes. Among the 17 differentially expressed genes associated with photosynthesis, 16 were downregulated in the *bmr* mutants (Tables 2 and 3). These included three genes associated with carbon fixation, four with photosystem I, four with photosystem II and one each with antenna pigment and electron transfer. The single gene upregulated in the mutants encoded a component of the light-harvesting complex. Nine genes associated with development and regulation were downregulated in the mutants, which included those encoding cytochrome P450 78A9 (CYP78A9), ATP synthase gamma chain 1 (ATPC1), histone-binding protein RBBP4, genomes uncoupled 4 (GUN4) protein, RelA/SpoT domain containing protein, seed maturation protein PM23, embryo-defective 2753 (EMB2753) and two senescence-associated proteins (Tables 2 and 3).

Most of those associated with lignin synthesis and protein fate were downregulated in the mutants. In particular, these included genes encoding the three enzymes L-phenylalanine ammonia lyase (PAL), cinnamyl alcohol dehydrogenase (CAD7) and nicotianamine aminotransferase A; however, the gene encoding cinnamic acid 4-hydroxylase (C4H) was upregulated (Tables 2 and 3). Eleven genes involved in protein fate were downregulated in the mutants, which included those encoding DNAJ-related Chaperone protein, DNAJ heat-shock N-terminal domain-containing protein, HSP91, HSP101, ubiquitin-protein ligase, ubiquitin-specific peptidase 54, C13 endopeptidase NP1 precursor, peptidase M48 family protein, aspartic proteinase, ATP-dependent Clp protease adaptor protein ClpS family protein and

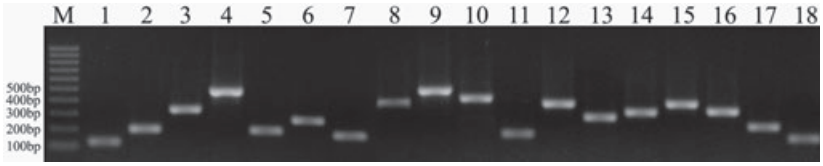

Fig. 2. PCR analysis of clones developed from SSH libraries. M: DNA size marker. Lanes 1–18 illustrate the insert present in each of 18 clones.

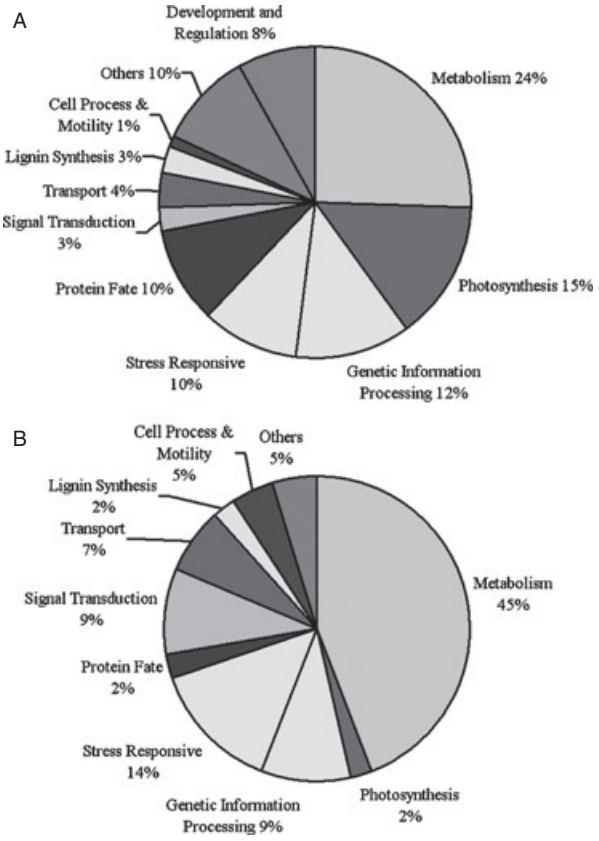

Fig. 3. Functional grouping of the differentially expressed genes in the sorghum *bmr* mutants. (A) Downregulated genes; (B) upregulated genes.

an ORMDL family protein. Only one gene-encoding prolyl endopeptidase was found to be upregulated in the mutants (Tables 2 and 3).

For the genetic information processing category, a total of 17 genes were obtained and 13 of them showed downregulated expression in the *bmr* mutants, which included those encoding 5′–3′ exoribonuclease (XRN3), basal transcription factor complex subunit-related protein, translation initiation factor SUI1, translation initiation factor-5, 5.8*S* ribosomal RNA rhizome 2 (RHIZ2), 40*S* ribosomal protein S7 (RPS7A), 40*S* ribosomal protein S8 (RPS8B), two peptidylprolyl isomerase and high mobility group (HMG) protein. The four upregulated genes involved in genetic information processing encoding RAP2, diphosphonucleotide phosphatase, Scarecrow-like protein and a bHLH domain containing protein, respectively (Tables 2 and 3). Seventeen proteins responsive to stress were obtained, of which 11 genes showed repressed expression whereas 6 showed enhanced expression in the *bmr* mutants (Tables 2 and 3). For the signal transduction and transport category, seven differentially expressed genes were obtained for each of the two categories (Tables 2 and 3).

Expression pattern of candidate genes

Semi-quantitative RT–PCR was applied to 12 of the putatively differentially expressed genes (Fig. 4). Seven of these, namely *BSSS*, *CYP78A9*, *GlyT*, *PAL*, *HMG*, *SUI1* and *SDR*, were downregulated in the *bmr* mutants, whereas the other five (*C4H*, *CytAD*, *GlyH*, *bHLH* and *RAP2*) were upregulated. Among the former group, the RT–PCR analysis demonstrated that *CYP78A9*, *PAL*, *HMG* and *SDR* were repressed in most of the mutants, although the extent of the repression in some of the mutants was only slight. *BSSS*, *GlyT* and *SUI1* were downregulated in between five and seven of the mutants, with their transcription level remaining indistinguishable from wild type in the remainder (Fig. 4). Among the five upregulated genes, the transcription of *C4H*, *bHLH* and *RAP2* was substantially enhanced in at least five of the mutants, whereas *GlyH* was upregulated in only

Table 1. Relative reduction in lignin content of the eight *bmr* mutants compared with wild-type BTx623 (%).

	bmr29(%)	*bmr32* (%)	*bmr33* (%)	*bmr34* (%)	*bmr35* (%)	*bmr36* (%)	*bmr6* (%)	*bmr12* (%)
Leaf of 5-leaf stage	16.3	15.3	12.1	26.8	17.0	15.8	1.9	11.1
Leaf of 7-leaf stage	2.5	28.6	13.1	13.4	4.0	37.5	9.1	16.4
Stalk of 7-leaf stage	13.1	29.1	10.1	9.4	5.1	27.6	2.8	5.2

Table 2. Downregulated genes in the *bmr* mutants.

Clone	Homology	Log 2 of signal ratio	E-value
Metabolism			
P2-B09	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	−2.607	1.00E−131
P1-H12	Formamidase	−2.246	2.00E−88
P4-C10	Nine-*cis*-epoxycarotenoid dioxygenase 4 (NCED4)	−2.069	3.00E−69
P2-G09	Lipoxygenase 2 (LOX2)	−2.002	1.00E−69
P3-B10	4-Alpha-glucanotransferase (GluT)	−1.957	3.00E−167
P2-E10	Alanine-glyoxylate aminotransferase (AGT)	−1.954	5.00E−32
P1-A06	Carbonic anhydrase	−1.823	1.60E−36
P2-C06	Glyceraldehyde-3-phosphate dehydrogenase B subunit	−1.786	8.00E-66
P4-D11	Cysteine protease	−1.479	0
P4-H10	Glyceraldehyde-3-phosphate dehydrogenase (GADPH)	−1.418	0
P3-A10	Uridylyltransferase-related	−1.360	2.00E−73
P3-B05	GDS2631 record; anaerobic carbohydrate metabolism	−1.288	0
P2-F03	1-Aminocyclopropane-1-carboxylate synthase	−1.277	3.00E−22
P4-B02	Pyridoxine biosynthesis 1 (PDX1)	−1.272	3.00E−53
P3-F09	Adenosylmethionine decarboxylase family protein	−1.235	6.00E−77
P3-C01	Ferrochelatase	−1.210	6.00E−93
P3-C10	Albino or pale green mutant 1 (APG1)	−1.141	1.00E−35
P3-G02	Nicotinamidase 1 (NIC1)	−1.098	1.00E−15
P3-G07	Lipoxygenase	−1.090	1.00E−14
P3-E04	Cysteine-type peptidase	−1.085	8.00E−26
P3-H08	Short-chain dehydrogenase/reductase family protein (SDR)	−1.078	4.00E−52
P4-A03	Glycosyl transferase family 17 protein (GlyT)	−1.074	7.00E−55
P3-H09	6-phosphogluconate dehydrogenase2	−1.074	1.00E−119
P4-B11	Glutathione transferase 20	−1.074	9.00E−33
P4-D07	Aldo/keto reductase family protein	−1.035	5.00E−56
P3-B08	CP12 domain-containing protein 1 (CP12-1)	−1.032	8.00E−26
P3-E12	Fructose-bisphosphate aldolase	−1.020	2.00E−38
P1-C03	Carbonic anhydrase	−0.985	2.00E−28
Photosynthesis			
P1-G05	Photosystem II subunit O-2 (PSBO-2)	−2.150	6.00E−93
P1-C08	23 kDa polypeptide of photosystem II	−1.973	1.00E−49
P4-D08	Pyruvate orthophosphate dikinase (PPDK)	−1.758	1.00E−136
P2-D04	Chlorophyll *a*/*b* binding protein	−1.754	4.00E−37
P2-B01	Photosynthetic electron transfer C (PETC)	−1.682	5.00E−37
P1-E07	Photosystem I light harvesting complex gene 4 (LHCA4)	−1.628	1.00E−31
P1-B12	Photosystem I reaction center subunit VI	−1.516	8.00E−07
P4-F3	PGR5-Like B	−1.478	2.00E−129
P1-C01	Photosystem II type II chlorophyll *a*/*b* binding protein	−1.420	3.00E−82
P1-F02	Phosphoenolpyruvate carboxylase	−1.374	1.00E−11
P1-G09	Photosystem I N subunit 1	−1.344	1.00E−43
P1-H09	Chlorophyll *a*/*b*-binding apoprotein CP24 precursor	−1.275	6.00E−57
P2-E03	Photosystem II light harvesting complex gene 1.5	−1.169	1.00E−32
P1-D02	Phosphoenolpyruvate involved in C4 photosynthesis	−1.166	6.00E−52
P4-A10	Pyruvate orthophosphate dikinase 1	−1.008	9.00E−54
P1-F12	Plastocyanin	−0.997	7.00E−30
Genetic information processing			
P1-F08	Peptidylprolyl isomerase	−2.867	4.00E−137
P1-A11	HMG	−2.256	1.70E−35
P2-F02	Eukaryotic translation initiation factor-5	−1.766	1.00E−58
P1-F01	5.8*S* ribosomal RNA rhizome 2 (RHIZ2)	−1.748	1.00E−55
P1-B02	5′−3′ Exoribonuclease (XRN3)	−1.710	1.00E−22
P3-A6	Basal transcription factor complex subunit-related	−1.605	3.00E−90
P3-F7	Peptidyl-prolyl *cis*−trans isomerase	−1.227	1.00E−52
P3-D8	Variegated 1 (VAR1)	−1.204	2.00E−28
P4-B4	40*S* ribosomal protein S8 (RPS8B)	−1.118	3.00E−122
P4-D6	40*S* ribosomal protein S7 (RPS7A)	−1.103	0

Table 2. Continued

Clone	Homology	Log 2 of signal ratio	E-value
P3-B7	Thioredoxin family protein	−1.097	2.00E−28
P3-D4	Embryo-defective 2184 (EMB2184)	−1.062	1.00E−17
P4-A11	Eukaryotic translation initiation factor SUI1	−1.034	7.00E−169
Stress responsive			
P1-H07	Protochlorophyllide reductase A	−2.607	3.00E−86
P2-F06	Peroxisomal biogenesis factor 11 family protein	−2.231	0
P2-C04	Bundle sheath strand-specific gene 1 (BSSS)	−1.796	4.00E−64
P4-E3	Low expression of osmotically responsive genes 1 (LOS1)	−1.609	5.00E−135
P4-H2	Low expression of osmotically responsive genes 2 (LOS2)	−1.437	5.00E−145
P3-D3	Plant basic secretory protein (BSP)	−1.332	1.00E−24
P3-E8	Light regulated Lir1 family protein, contains InterPro domain	−1.330	7.00E−27
P2-H01	Metallothionein 2A (MT2A)	−1.290	1.00E−58
P4-C2	Light regulated Lir1 family protein	−1.288	1.00E−37
P3-A5	Calmodulin-related protein	−1.079	4.00E−19
P4-D3	NADPH HC toxin reductase-like protein	−1.030	5.00E−67
Protein fate			
P2-D01	Chaperone protein DNAJ-related	−1.853	1.00E−69
P4-F8	DNAJ heat-shock protein	−1.798	8.00E−102
P1-A04	Ubiquitin-protein ligase	−1.645	3.00E−122
P2-B08	Heat-shock protein 101	−1.215	7.00E−14
P4-C5	Heat-shock protein 91	−1.635	0
P4-B3	C13 endopeptidase NP1 precursor	−1.534	3.00E−106
P3-D12	ATP-dependent Clp protease adaptor protein ClpS	−1.465	3.00E−101
P3-E11	Ubiquitin-specific peptidase 54	−1.362	7.00E−128
P4-A12	Aspartic proteinase nepenthesin I-like	−1.280	3.00E−95
P3-A2	Peptidase M48 family protein	−1.137	3.00E−37
P3-H11	ORMDL family protein	−1.078	3.00E−101
Signal transduction			
P2-F09	Adenylate kinase	−1.999	5.00E−38
P1-D09	Tyrosine-specific protein phosphatase	−1.840	1.00E−96
P1-G06	MAPK1	−1.638	4.00E−117
Transport			
P2-A08	H^+-transporting two-sector ATPase	−1.830	4.00E−86
P4-E12	Na^+/H^+ antiporter NhaC	−1.543	1.00E−120
P4-H8	Acclimation of photosynthesis to environment 2 (APE2)	−1.488	6.00E−134
P3-E10	Secretion-associated RAS super family 2	−1.010	2.00E−17
Lignin synthesis			
P4-C7	Cinnamyl-alcohol dehydrogenase 7 (CAD7)	−1.571	8.00E−38
P4-C1	Phenylalanine ammonia-lyase (PAL)	−1.115	2.00E−38
P3-H5	Nicotianamine aminotransferase A	−1.036	2.00E−23
Cell process and motility			
P4-G4	Myosin heavy chain	−1.578	2.00E−175
Development and regulation			
P4-H6	Senescence-associated protein	−2.339	4.00E−46
P2-B04	RelA/SpoT domain containing protein	−1.693	6.00E−84
P4-E6	Seed maturation protein PM23	−1.648	7.00E−89
P3-E3	Senescence-associated protein	−1.471	2.00E−115
P3-H10	EMB2753	−1.298	4.00E−47
P2-E04	Cytochrome P450 78A9 (CYP78A9)	−1.271	1.00E−53
P1-F04	Histone-binding protein RBBP4	−1.728	7.00E−43
P3-G10	ATP synthase gamma chain 1 (ATPC1)	−1.618	1.00E−110
P4-D9	GUN4	−1.298	0
Others			
P2-G12	Esterase/lipase/thioesterase family protein	−1.724	7.00E−129
P2-A11	VirB2-interacting protein 2 (BTI2)	−1.620	3.00E−118
P4-B1	Putative thiol-disulphide oxidoreductase DCC	−1.259	2.00E−65
P4-A9	Pentatricopeptide repeat-containing protein (PPR)	−1.230	4.00E−35

Table 2. Continued

Clone	Homology	Log 2 of signal ratio	E-value
P3-D9	Thioredoxin M1	−1.025	1.00E−47
P3-G9	MTN3	−1.002	4.00E−57
P3-G1	InterPro domain containing protein	−1.236	1.00E−27
P2-G05	Hypothetical protein, contains InterPro domain	−1.024	2.00E−88
P3-F4	Membrane protein	−1.290	5.00E−99
P3-B4	PB1 domain-containing protein	−1.110	3.00E−37
P3-A11	Peroxisomal membrane protein-related	−1.039	3.00E−42

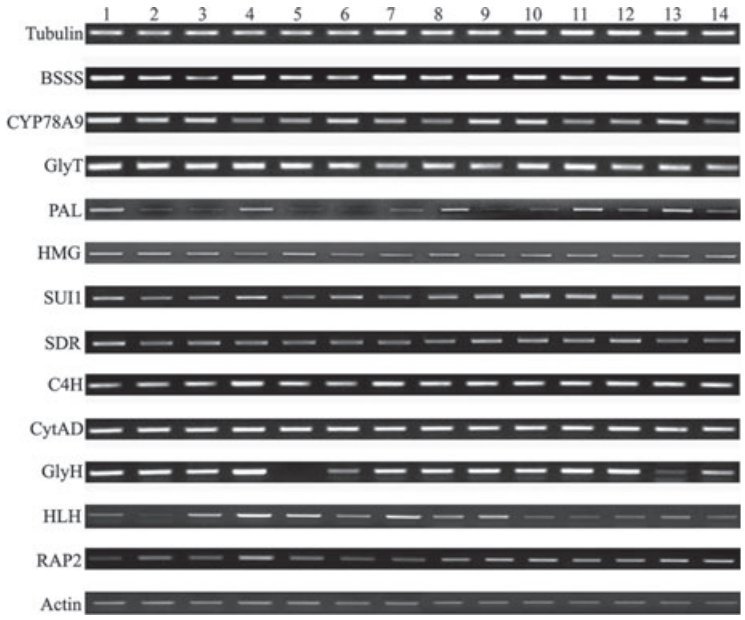

Fig. 4. RT−PCR profiles of a set of genes differentially expressed in the 13 *bmr* mutants compared with wild-type BTx623. Lane 1: BTx623, lane 2: *bmr*, lane 3: *bmr29*, lane 4: *bmr30*, lane 5: *bmr31*, lane 6: *bmr32*, lane 7: *bmr33*, lane 8: *bmr34*, lane 9: *bmr35*, lane 10: *bmr36*, lane 11: *bmr45*, lane 12: *bmr49*, lane 13: *bmr6*, lane 14: *bmr12*. BSSS: bundle sheath strand-specific gene 1; CYP78A9, cytochrome P450 78A9; GlyT, glycosyl transferase family 17 protein; HMG, high mobility group protein; SUI1, eukaryotic translation initiation factor SUI1; SDR, short-chain dehydrogenase/reductase family protein; CytAD, cytosolic aldehyde dehydrogenase; GlyH, glycosyl hydrolase family 17 protein; HLH, bHLH domain-containing protein; RAP2, ras family small GTP-binding protein. Tubulin and Actin represent internal loading controls.

three mutants and clearly downregulated in four others. *CytAD* transcript abundance was marginally raised in five of the mutants. Overall, the RT−PCR outcomes were reasonably consistent with those obtained from the microarray analysis.

Discussion

Brown midrib mutants are of potential interest in the context of improving bioethanol conversion efficiency, because the lignin content of their vegetative tissue is lower than that of wild-type tissue. Our current understanding of the molecular basis of the mutant phenotype is, however, rather limited. In particular, it will be of relevance to know which of the genes involved in cell-wall metabolism (and their regulation) are affected in the mutants, as this will guide any molecular-based strategy aimed at the genetic improvement of bioenergy crops. In a study of certain maize *bm* mutants, SSH was combined with microarray analysis to identify 53 differentially expressed genes in *bm3* and 32 in all of *bm1*, *bm2* and *bm3* (Shi et al. 2006), whereas Guillaumie et al. (2007) showed that, among a set of 144 genes associated with the synthesis of phenylpropanoid and related compounds, 69 were differentially expressed in the young stems of at least one of the four *bm* mutants *bm1*–*bm4*. As yet in sorghum, apart from the documented effects on the transcription of the genes encoding COMT and CAD in *bmr12* and *bmr6* (Bout and Vermerris 2003, Sattler et al. 2009), there is a lack of knowledge of the influence of any of the *bmr* mutations on the transcriptome.

Table 3. Upregulated genes in the *bmr* mutants.

Clone	Homology	Log 2 of signal ratio	E−value
Metabolism			
P4-G6	Cytosolic aldehyde dehydrogenase (CytAD)	1.075	2.00E−60
P1-G04	Acyl-coenzyme A oxidase 2	1.082	4.3
P1-A02	Fructose-bisphosphate aldolase	1.096	7.30E−03
P4-F9	Polyamine oxidase 1	1.109	4.00E−73
P4-E9	Alkaline alpha galactosidase 1	1.125	4.00E−35
P1-E08	NADP-dependent malic enzyme	1.128	8.8
P4-F2	Acid phosphatase (class B) family protein	1.137	2.00E−33
P1-H05	Alkaline alpha galactosidase 1	1.229	3.00E−50
P1-B10	Glutathione *S*-transferase	1.241	2.00E−24
P2-F04	Carbonic anhydrase	1.376	3.50E−05
P4-H12	Nitrate reductase 2 (NIA2)	1.390	3.00E−14
P4-G8	Catalase-3	1.417	8.00E−32
P4-C9	Lipoxygenase	1.475	1.00E−35
P4-E7	Inositol-3-phosphate synthase isozyme 2	1.710	7.00E−35
P4-E4	Acid phosphatase	1.804	9.00E−54
P4-E1	Acid phosphatase class B family protein	2.106	3.00E−42
P1-C10	Low phytic acid 1	2.264	8.00E−24
P2-H09	Beta-amylase	2.284	3.00E−23
P2-A01	Glycosyl hydrolase family 17 protein (GlyH)	2.361	5.00E−25
Photosynthesis			
P4-A2	Photosystem I light-harvesting complex gene 6 (LHCA6)	0.960	2.00E−33
Genetic information processing			
P2-G04	Ras family small GTP-binding protein (RAP2)	0.950	2.00E−35
P2-H02	Diphosphonucleotide phosphatase	1.090	1.20E−04
P2-H12	Scarecrow-like protein	2.209	2.30E−02
P4-E11	bHLH domain containing protein	2.683	2.00E−72
Stress responsive			
P4-C3	In2-1 protein	1.045	3.00E−37
P2-C03	Early responsive to dehydration 8	1.304	1.00E−33
P1-D01	Low molecular mass early light-induced protein	1.411	2.00E−20
P1-F06	Secretory acid phosphatase	1.669	1.50E−06
P1-D08	Osr40c1 protein	1.988	2.90E−09
P1-A12	Early light-induced protein precursor	1.992	1.90E−16
Protein fate			
P2-A06	Prolyl endopeptidase	1.659	4.40E−07
Signal transduction			
P2-G11	ADP-ribosylation factor	1.148	2.80E−03
P4-G11	PAP27	1.276	3.00E−41
P3-F10	PAP10	1.763	2.00E−26
P1-D06	Purine and other phosphorylases	2.020	2.20E−06
Transport			
P2-D07	Legumain-like protease precursor	1.042	3.40E−09
P4-G1	NOD26-like major intrinsic protein (NIP2-2)	1.156	3.00E−36
P4-G3	Mannitol transporter, putative	1.177	1.00E−35
Lignin synthesis			
P1-D04	C4H	1.768	0.037
Cell process and motility			
P4-F6	TUA6	1.573	1.00E−30
P2-H08	Loricrin-like	2.191	3.00E−45
Others			
P3-D2	Harpin-induced 1 domain containing protein	1.163	4.00E−57
P3-E7	Contains InterPro domain	1.259	2.00E−36

Here, we have combined SSH and microarray technology in an attempt to identify a set of differentially expressed genes in the *bmr* mutants. In the conventional SSH method adopted to uncover differentially expressed genes, the contrast is made between a single mutant and the wild type; however, this approach typically results in a large number of candidate sequences, which greatly complicates the process of identifying the key gene(s) responsible for the phenotype. For this reason, we based the SSH libraries on a bulk template formed from 13 independent mutants, reasoning that although this risked concealing some of the genes underlying the phenotype of a subgroup of the mutants, it would help to discover those genes involved in lignin synthesis and expression regulation that might have similar expression patterns in different *bmr* mutants because all the *bmr* mutants showed reduced lignin content. The RT–PCR analysis applied to the individual *bmr* mutant templates indicated that the genes involved in lignin synthesis showed quite similar expression patterns in the various *bmr* mutants, which proved the efficiency of this strategy.

The 153 genes identified by the microarray analysis as being differentially expressed between the *bmr* mutants and the wild type fell into 11 functional groups. A comparison between maize and sorghum confirms a level of consistency in the functional classification of differentially expressed genes. In both species, most of the genes affected in the brown midrib mutant belong to the categories metabolism, photosynthesis, lignin synthesis, signal transduction and regulation (Shi et al. 2006, Guillaumie et al. 2007). However, there is less consistency in the identity of the individual genes, perhaps reflecting species differences and/or variation in experimental design. Nevertheless, a regular outcome of such expression studies is that metabolism is the most prominent functional group. The synthetic pathway leading to lignin production is rather complex, and includes a large number of precursors and intermediates. Thus, it may not be so surprising to find that any modification (whether quantitative or qualitative) of a single pathway component results in a major alteration in the output of other components in the pathway, or even thanks to cross-talk, of components in other metabolism pathways. An attractive example demonstrated that the reduction in the lignin content and an alteration in its composition induced by the downregulation of the genes encoding either COMT or CAD in switchgrass (*Panicum virgatum*) raised the rate of sugar released and the production of ethanol, but at the same time had hardly any impact on cell-wall polysaccharide content or structure (Fu et al. 2011a, 2011b).

Among the 17 differentially expressed genes involved in photosynthesis, 16 were downregulated in the *bmr* mutants. The inhibition of photosynthesis must inevitably result in a reduction in biomass, as indeed was shown by the phenotype of some of the *bmr* mutants. Whether a negative effect on photosynthesis was causally correlated with the observed reduction in lignin content in the *bmr* mutants has not been established, but it may be relevant that in tobacco, plants engineered to give reduced levels of both CCR and CAD, the resulting perturbation of the lignin synthesis pathway has been shown to have a measurable impact on other metabolic pathways including photorespiration (Dauwe et al. 2007). Fortunately, it appears that hybrids between the wild type and at least some of the *bmr* lines do not suffer any yield reduction, even though their lignin content is low and the digestibility of their biomass is high (Sattler et al. 2010).

Given the reduced lignin content of the tissue of *bmr* plants, it is hardly surprising that three of the candidates emerging from the analysis were associated with lignin synthesis; a similar outcome was reached with respect to the maize *bm1*, *bm2* and *bm3* mutants (Shi et al. 2006, Guillaumie et al. 2007). The RT–PCR-based analysis of *PAL* expression showed that it was repressed in at least 9 of the 13 *bmr* mutants. PAL catalyzes the de-amination of L-phenylalanine to form cinnamic acid, and represents the first enzyme in the phenylpropanoid pathway. In *bm1*, as in the *bmr* mutants, *PAL* was downregulated; in contrast, it was substantially upregulated in young *bm2* and *bm4* plants, even though the lignin content of mature *bm2* and *bm4* plants is lower than in their wild-type equivalent. C4H is the second enzyme in this same pathway, and combines with PAL and possibly other phenylpropanoid pathway enzymes to create a metabolic channel through which intermediates can be processed without any diffusion into the cytosol (Achnine et al. 2004). In tobacco, PAL and C4H both co-localize to the endoplasmic reticulum (ER) membrane, and it has been proposed that one role of C4H is to anchor the channeling complex to the ER (Achnine et al. 2004). Unlike *PAL*, the transcription of *C4H* was upregulated in most of the sorghum *bmr* mutants. Transgenic reduction in C4H and PAL activity in tobacco both reduces lignin content and changes its composition (Sewalt et al. 1997). PAL is encoded by a multigene family in most of the species investigated by Fukasawa-Akada et al. (1996). The different transcription profiles of the genes encoding PAL and C4H between the *bm* and *bmr* mutants, together with their unconformity to lignin content, suggest that the synthesis of monolignol from L-phenylalanine is achieved via more than one route, so that different sets of genes are probably responsible for the reduced lignin content of the *bm* and *bmr* mutants.

A further downregulated lignin synthesis gene in the *bmr* mutants was *CAD*, the reduced transcription of which was also observed in maize *bm1* and sorghum *bmr6* (Halpin et al. 1998, Sattler et al. 2009). CAD, a member of the alcohol dehydrogenase superfamily, catalyzes the conversion of hydroxycinnamoyl aldehydes into monolignols, prior to their incorporation into the lignin polymer. Except for *ZmCAD2*, four *CAD* genes in *bm1* and two in *bm2* were also downregulated compared with their wild-type transcription levels (Guillaumie et al. 2007). Reduced CAD activity induced a phenotype similar to brown midrib in transgenic tobacco and poplar (Halpin et al. 1994, Baucher et al. 1996), whereas the transgenic downregulation of *CAD* is associated with a reduced lignin content and/or changed lignin composition in a range of plant species, resulting in improved sugar release and increased digestibility and pulping efficiency (Halpin et al. 1994, Baucher et al. 1996, 1999, Lapierre et al. 1999, Chen et al. 2003, Fu et al. 2011b). The implication is therefore that a reduction in CAD activity on its own is sufficient to induce the brown midrib phenotype. If this is correct, then *CAD* would represent a good candidate for manipulating the lignin content of cellulosic plant biomass.

Much progress has been made in understanding the transcriptional regulation of lignin synthesis over the last two decades. Bioinformatics-based analysis of the promoters of lignin synthesis genes has identified the AC element (ACCT/AAA/CC) as a frequent motif, perhaps serving as a shared *cis* regulatory element driving the coordinated regulation of lignin synthesis (Zhong and Ye 2009). The AC element sequences resemble the maize MYB protein P-binding site (CCT/AACC) (Grotewold et al. 1994) as well as those of a number of MYB proteins encoded by *Arabidopsis thaliana*, pine and eucalyptus. These transcription factors have emerged as strong candidates for the regulation of lignin synthesis, as their over-expression has been associated with the induction of various lignin synthesis genes, resulting in the ectopic deposition of lignin or secondary wall thickening (Goicoechea et al. 2005, Zhou et al. 2009). In addition to these AC-binding MYBs, some other MYBs have also proven able to activate lignin synthesis genes and cause ectopic lignin deposition when over-expressed (Zhong et al. 2008). None of the differentially expressed sequences in the *bmr* mutants proved to encode an MYB, but the transcription of a bHLH transcription factor was upregulated in several of them. The heterologous expression of this gene in *A. thaliana* substantially reduced stem lignin content (data not shown), which suggested that this transcription factor functions as a repressor in the lignin synthesis pathway. The reduction of lignin content in plants achieved via the transgenic downregulation of lignin synthesis genes is often accompanied by abnormal growth and development (Li et al. 2008), but the heterologous expression of the bHLH transcription factor had only a marginal negative effect on biomass accumulation (data not shown). Thus, along with *CAD*, this transcription factor may represent an interesting candidate for the improvement of biomass productivity.

Conclusions

A number of genes that were differentially expressed in the *bmr* mutants were identified by comparing the pooled cDNAs of 13 independent *bmr* mutants with that of the wild type. This set of genes include several strong candidates underlying the *bmr* mutations by in silico mapping through comparing the differentially expressed ESTs-enriched genome region with the chromosome location of *bmr* as a result of the availability of sorghum genome sequences and may provide molecular leads relevant for breeding sorghum cultivars well suited to bioethanol production.

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