Agrotechnology
ISSN: 2168-9881

Tanmay et al., Agrotechnol 2016, 5:2
DOI: 10.4172/2168-9881.1000146

Research Article Open Access

Gene Expression Studies in Lignin Synthesis Pathway of Sorghum [*Sorghum Bicolor*] (L. Moench)

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Abstract

Gene expression play significant role in lignin synthesis pathway in sorghum. Expression level of brown-midrib sorghum was studied in brown midrib sorghum *bmr* 6 and *bmr* 12 mutant of Atlas, Kansas collier, Early hagari Sart, Rox Orange. Gene expression levels for *bmr* 6, CAD 4, SBCAD2, *bmr* 12, COMT3 COMT were compared for wild sorghum genotypes with their *bmr* 6 and *bmr* 12 counterparts. *bmr* 6 has negative non-significant correlation with lignin content (-0.075).

Keywords: Brown-midrib sorghum; Gene expression; Lignocellulosic; Kansas collier

Introduction

Gene expression is the process by which genetic information is converted into protein or a functional product. This process uses an intermediate molecule, m-RNA, which is transcribed from DNA and then used as a template to translate the message into a protein product. Studies of gene expression provide a window into how an organism's genetic makeup enables it to function and respond to its environment. Real-Time PCR can be used to quantify gene expression by two methods: relative and absolute quantification. The relative quantification method compares the gene expression of one sample to that of another sample: drug-treated samples to an untreated control, for example, using a reference gene for normalization. Absolute quantification is based on a standard curve, which is prepared from samples of known template concentration. The concentration of any unknown sample can then be determined by simple interpolation of its PCR signal (Cq) into this standard curve [1-6]. As RT-q PCR performance is affected by the RNA integrity, Fleige and Pfaffl recommend an RNA quality score (RIN or RQI) higher than five as good total RNA quality and higher than eight as perfect total RNA for downstream applications [4-7]. A study on the impact of RNA quality on the expression stability of reference genes indicated that it is inappropriate to compare degraded and intact samples, necessitating sample quality control prior to RT-q PCR measurements [8]. (Vermeulen *et al*., submitted for publication) data indicate that RNA quality has a profound impact on the results, in terms of the significance of differential expression, variability of reference genes and classification performance of a multi-gene signature. In addition or as an alternative to the use of capillary gel electrophoresis methods that assess the integrity of the ribosomal RNA molecules as discussed in [4,8] PCR based tests are also frequently used to determine mRNA integrity. In one such a test, the ratio between the 5' and 3' end of a universally expressed gene is measured upon anchored oligo-dT cDNA synthesis, reflecting integrity of that particular poly-adenylated transcript [7] finally, another PCR based assay is often used in clinical diagnostics to determine sample purity. By comparing the Cq value of a known concentration of a spiked DNA or RNA molecule in both a negative water control and in the sample of unknown quality, enzymatic inhibition can be determined [7] (Figure 1).

***Bmr* 6:** The *bmr* 6 mutation in sorghum encodes cinnamyl alcohol dehydrogenase 2 (CAD2). In the final step of monolignol biosynthesis, CAD catalyzes the reduction of cinnamyl aldehydes (Coniferyl, Coumaryl and Sinapyl aldehyde) to their corresponding cinnamyl alcohols, using NADPH as a cofactor, prior to their incorporation into the lignin polymer (Figure 2). ZmCAD2 is an ortholog to both the sorghum bmr6 and rice Gh2, mutations in either gene resulted in reduced CAD activity and altered lignin composition similar to the bm1 phenotype.

The bmr 6 allelic group consists of *bmr* 6-3, *bmr*6-4, *bmr*6-20, *bmr*6-22, *bmr*6-23, *bmr*6-24, *bmr*6-27, *bmr*6-28, *bmr*6-39, *bmr*6-40 and *bmr*6-41 including *bmr* 6-refference allele. Alleles *bmr* 6-39 and *bmr* 6-40 both are resulted due to G to C 3699bp and *bmr* 6-41 consist of C-to-T transition at 3619bp and G-to-T transversion at 3620bp [9-11]. Although CAD2 protein was absent from *bmr* 6 tissues, CAD activity was still detectable in the tissues, though activity was reduced

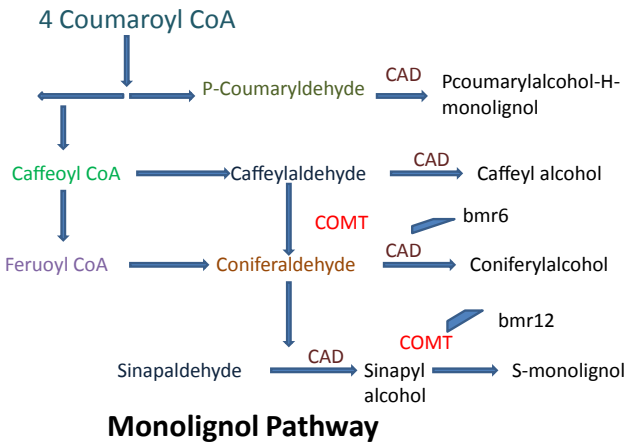

Figure 1: Monolingnol Pathway.

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Received November 24, 2015; **Accepted** July 06, 2016; **Published** July 13, 2016

Citation: Tanmay K, Umakanth AV, Madhu P, Bhat V (2016) Gene Expression Studies in Lignin Synthesis Pathway of Sorghum [*Sorghum Bicolor*] (L. Moench). Agrotechnol 5: 146. doi: 10.4172/2168-9881.1000146

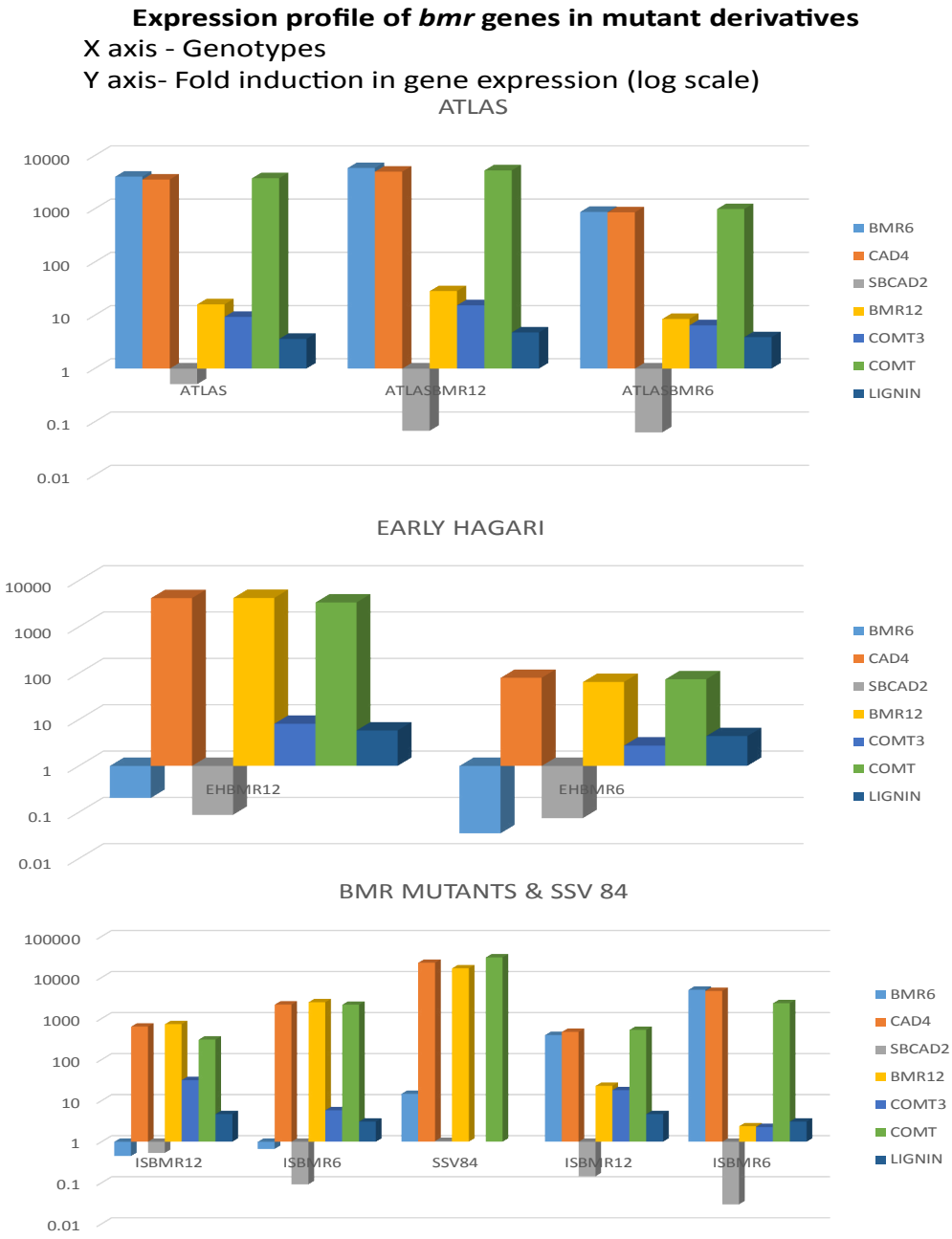

Figure 2: Expression Profile of *Bmr* Genes In Mutant Derivatives.

to 15-50% of wild type activity. This indicate that there are other CAD proteins present in sorghum that can utilize cinnamyl substrates, but the brown midrib phenotype reveals that bmr6 encode the main CAD protein in the monolignol biosynthetic pathway in sorghum (Figure 3).

Mutations in brown mid-rib lines

***Bmr* 12:** Sorghum bmr12 locus encodes orthologous caffeic O-methyl transferase (COMT). Caffeic O methyl transferase (COMT) is members of an evolutionary conserved O-methyl transferase family, whose function in lignin biosynthesis has been documented in both monocots and dicots. The lignin monomeric compositions of bmr 12 plants has shown that syringyl-lignin was greatly reduced, while p-hydroxyphenyl and guaiacyl-lignin were slightly reduced.

About ten distinct alleles of sorghum *bmr* 12 have been isolated and the mutated sites identified i.e *bmr* 12-ref, *bmr* 12-7, *bmr* 12-15, *bmr* 12-18, *bmr* 12-25, *bmr* 12-26, *bmr* 12-30, *bmr* 12-34, *bmr* 12-35 and *bmr* 12-820 [1,2,9]. The mutations in the *bmr* 12and *bmr* 18 alleles are located 27 (C-to-T 486) and 80nt (G-to-A4 36) upstream of the exon-exon junction respectively, whereas the *bmr* 26 mutation is (G-to-A2292) 388nt downstream of this boundary [2]. The *bmr* 25 consist of same mutation as that of *bmr* 18 Nonsense mutations are responsible for four of the characterized alleles *i.e.*, *bmr* 12-30 consist of G-to-A transistion at 2364nt leading to Gly225Asp, bmr12-34 is due to C-to-T at 518 and 2139nt causing Ala71Val and Pro 150 Leu respectively, *bmr* 12-34 and *bmr* 12-820 contain two mutations in *bmr*12, which are identical, *bmr*12-35 is due to G-to-A transistion at

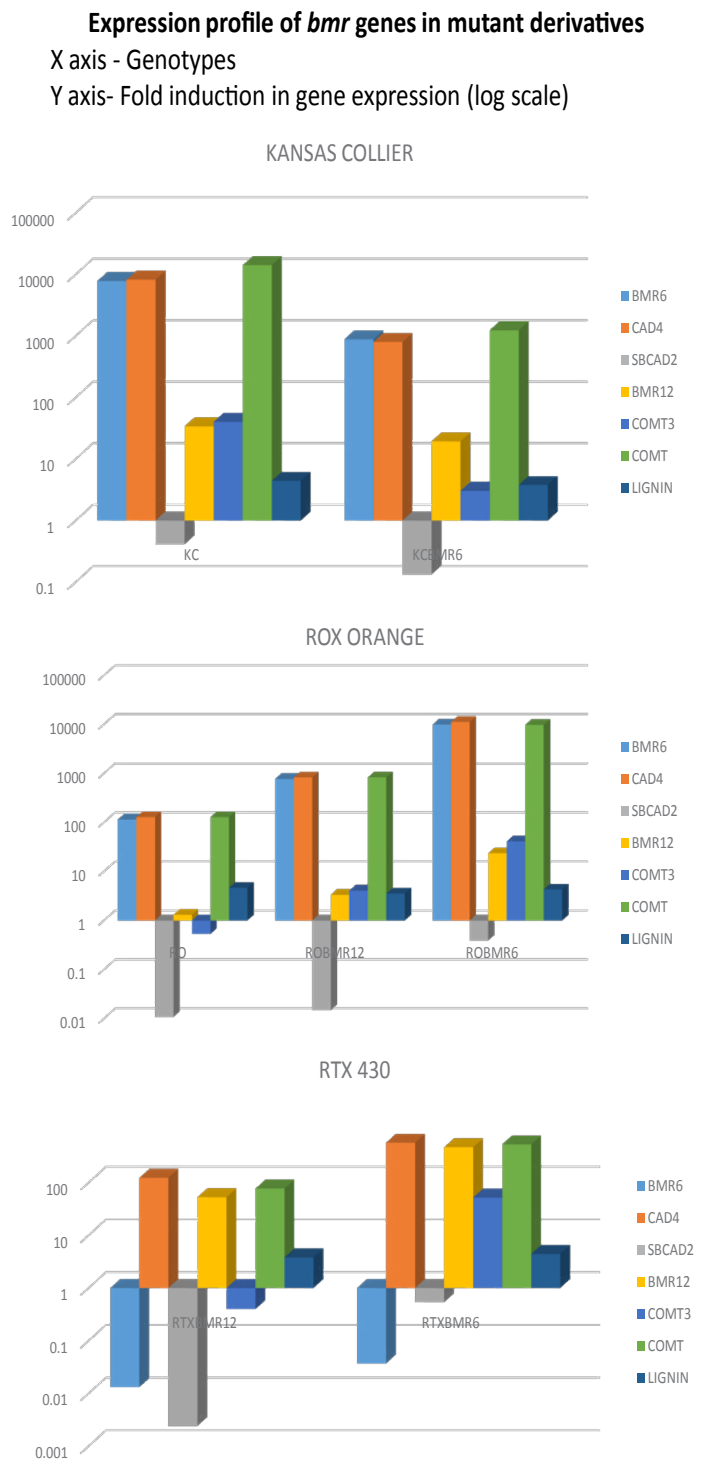

Figure 3: Expression profile of *bmr* genes in mutant derivatives.

2663 leading to Gly325Ser [9,10]. These four nonsense mutations are all presumably null alleles, because the premature stop codons would truncate the polypeptide prior to the SAM binding site of the enzyme.

Lignin is one of the most important biomolecules in vascular plants and is uniquely involved in the structure support, water transport, and other functions [3,5]. Lignin biosynthesis has been subject to intensive study during the past two decades, mainly driven by the significant needs in forage and biofuel industries [12].

Material and Methods

Sorghum DNA isolation

Breaking the cell wall and cell lysis: Plant tissue very well

ground up, caution taken to prevent DNA from degrading during the procedure. The cells are lysed in the presence of chaotropic agent CTAB.

1. 9 ml of warm (65° C) CTAB extraction buffer added to 1g freeze dried, ground tissue (young leaves) in a 30 ml centrifuge tube.
2. Incubated for 3h at 65° C in a water bath with occasional mixing.

Separate DNA from other cell components: Chloroform helps bind up the complex proteins and polysaccharides. Chloroform is denser than water solutions and thus after spinning this solution. Chloroform and water will separate into two distinct phases. The lower phase will be chloroform. This is the phase that proteins and polysaccharides find most chemically attractive. The upper aqueous phase phase will contain DNA. Iso-propanol is used to precipitate DNA present in aqueous phase. Precipitation with iso-propanol has the advantage that the volume of liquid to be centrifuged is smaller. However, iso-propanol is less volatile than ethanol and it is more difficult to remove the last traces; moreover, solutes such as sucrose or sodium chloride are more easily co-precipitated with DNA when iso-propanol is used, especially at -70° C.

1. Tubes were removed from water bath, wait for 4-5 min and added 10ml chloroform/iso-amyl alcohol (24:1). Mixed gently for several times.
2. Centrifuged at 6000 rpm for 10 min at RT.
3. Transfer aqueous phase to a clean 30 ml (or 15ml) tube. 6 ml chloroform/isoamyl alcohol (24:1) added and mixed gently several times.
4. Centrifuged at 6000 rpm for 10 min at RT.
5. Transferred aqueous phase to clean 30 ml (or 15 ml) tube. 6 ml (2/3 volume) isopropanol added, mixed gently by inversion several times. Removing DNA by hook minimizes the contamination by salt precipitations, DNA washed to remove impurities.
6. Precipitated DNA removed with glass hook, or centrifuge at 6000 rpm for 10 min and pellets DNA.
7. Place hook with DNA (or DNA pellet) in a 5 ml plastic tube containing 2 ml of washing buffer 1(76 % ethanol, 0.2 M sodium acetate). Leave DNA on hook in tube for at least 20 min.
8. Rinse DNA on hook briefly in 1-2 ml of washing buffer 2 (76% ethanol, 10Mm ammonium acetate) and air dry at 37° C.
9. Transfer DNA to 1.5 ml micro-centrifuge tube containing 0.4 ml of TE buffer and place at 4° C overnight to disperse DNA. Next day, treat with RNase for 3 h at 37° C.

Phenol extraction and ethanol precipitation of DNA:

1. Equal volume of phenol/chloroform/isoamyl alcohol added (24:24:1) to the DNA solution in a 1.5 ml micro centrifuge tube.
2. Vortex vigorously 10 sec and micro centrifuge for 10 min at maximum speed.
3. The top (aqueous) phase removed carefully containing the DNA using a pipette and transfer to a new tube. If a white precipitate is present at the aqueous organic interface, re-extract the organic phase and pool aqueous phases.
4. An equal volume of chloroform /isoamyl alcohol (24:1) added to the DNA solution in a micro centrifuge tube.
5. Vortex vigorously 10 sec and micro centrifuge for 10min maximum speed.
6. 1/10 volume of 3 M sodium acetate Ph 5.2 added. Mix by vortexing briefly or by flicking the tube several times with a finger.
7. Add 2 to 2.5 volume (calculated after salt addition) of ice-cold 100% ethanol. Mix by vortexing and place in crushed dry ice for 5min or longer.
8. Spin 10 min at high speed and remove supernatant.
9. 1 ml of RT 70% ethanol added. Inverted the tube several times and Micro-centrifuged as in step 6.
10. The supernatant removed, dry the pellet and dissolve in appropriate volume of water or TE buffer, Ph 8.0.

RNA isolation: It is essential to use correct amount of starting material to obtain optimal RNA Yield and purity. A maximum of 100 mg plant material or 1x107 cells can generally be processed.

1. β-Mercaptoethanol (β-ME) added to Buffer RLT or Buffer RLC before use. 10 µl β-ME per 1 ml Buffer RLT or Buffer RLC added. Buffer RLT or Buffer RLC containing β-ME can be stored at room temperature (15-25° C) for up to 1 month.
2. Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96-100%) as indicated on the bottle to obtain a working solution.
3. 100 mg of plant material weighed. Weighing tissue is the most accurate way to determine the amount.
4. Immediately placed the weighed tissue in liquid nitrogen, and grounded thoroughly with a mortar and pestle. Tissue powder decanted and liquid nitrogen into an RNase-free, liquid-nitrogen cooled, 2 ml micro centrifuge tube. The liquid nitrogen allowed to evaporate, but do not allow the tissue to thaw. Proceed immediately to step3.
5. RNA in plant tissues is not protected until the tissues are flash-frozen in liquid nitrogen. Frozen tissues not allowed to threw during handling.

Discussion

Total RNA was isolated from wild type and *bmr* 6 and *bmr* 12 leaves and q RT-PCR was used to measure *bmr* 6 and *bmr* 12 leaves and q RT-PCR was used to measure *bmr* 6 and *bmr* 12 RNA expressions. Expression levels were determined using the ΔCT method and *bmr* 6, 12 gene expression was relativized against wild trait level. The presence of lignin reduces the quality of lignocellulosic biomass for biofuels (Figure 4). The reduced lignin content characteristic of brown midrib (*bmr*) mutants improves the efficiency of bioethanol conversion from biomass. bmr 6 gene encode cinnamyl alcohol dehydrogenase the final step of monolignol pathway. bmr 6 has overall negative correlation with lignin content for all the genotype studied (Table 1).

Atlas, Rox orange, Kansas collier, Tx 430, Early Hagaris sart, was studied for gene expression. Gene expression levels for *bmr*6, CAD 4,

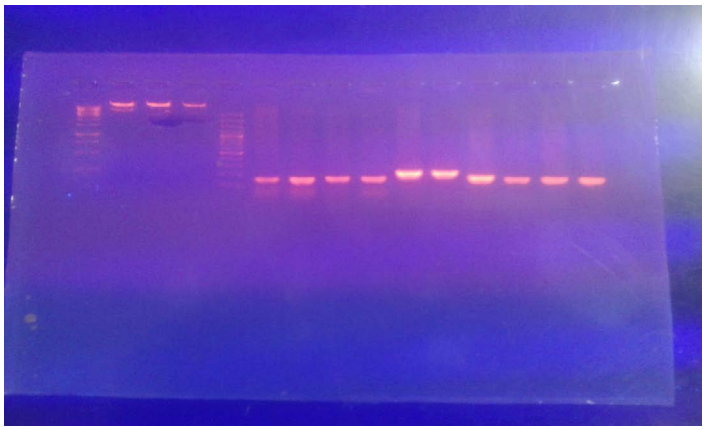

Figure 4: PCR Product at 52° C Showing Bands of Different Brown Mid rib Genotypes Atlas (234), Kansas Collier(67), Early Hagari Sart(89), Rox Orange(10,11) Tx 430 (12,13), Tx 631 (14,15).

	Wild trait	Bmr 6	Bmr 12
Atlas	√	√	√
Rox Orange	√	√	√
Kansas collier	√	√	√
Early Hagari		√	√
RTx 430		√	√
IS 1		√	√
IS 2		√	√
SSV 84	Sweet sorghum variety		

Table 1: Sorghum genotypes for gene expression studies.

	ATLAS	ATLASBMR12	ATLASBMR6	EHBMR12	EHBMR6	KC	KCBMR6	RO	ROBMR12	ROBMR6	Correlation with lignin content
BMR12	16.111289	28.640802	8.5741877	4211.1542	64.893407	34.059846	19.427118	1.3013419	3.3403517	23.425371	-0.169
BMR6	4039.6092	5873.4807	879.17101	0.2030631	0.0349152	7858.2917	885.28612	114.56321	770.68633	9877.9777	0.170
SbC3H4	0.0587202	1.7776854	0.0308198	0.0356489	0.9592641	0.2624292	0.0448111	0.0100268	0.0064343	0.0211969	-0.184
SbC3HF	3147.5204	4067.7069	709.17605	2957.167	116.97043	8364.1313	497.99933	130.68955	471.13608	8306.3561	-0.064
SbCAD4	3565.7751	5007.9346	861.07793	4182.0657	79.893155	8192	797.86453	128	820.29555	11113.303	-0.093
SbCCR1	43.713288	75.061437	17.267652	16	3.8370565	103.96831	7.8353624	1.4339552	10.556063	112.20553	-0.228
SbCOMT	3769.0886	5293.4772	995.99867	3373.4288	74.028044	14164.578	1243.3356	128	826.00116	9741.9847	-0.192
SbCOMT3	9.3826796	15.454981	6.4980192	8.1116758	2.7510836	40.224428	3.0525184	0.528509	4.0840485	41.069629	-0.149
SbHCT	1024	2368.8974	342.50945	2740.0756	37.530718	3125.7789	436.54906	51.625073	310.83389	2574.3634	-0.287
SbCAD2	0.5069797	0.0674518	0.0629347	0.0877778	0.0743254	0.4117955	0.1330463	0.0107464	0.0147822	0.3895823	-0.195
Lignin content	3.61	4.78	3.9	5.83	4.4	4.42	3.78	4.63	3.54	4.27	

Table 2: Correlation of *bmr* mutants with lignin content.

SBCAD2, *bmr*12, COMT3 COMT were compared for wild sorghum genotypes with their *bmr* 6 and *bmr* 12 counterparts. *bmr* 6 mutation in sorghum encodes cinnamyl alcohol dehydrogenase 2 (CAD2). bmr 6 has negative non-significant correlation with lignin content (-0.075) (Table 2).

Atlas

Gene expression level were compared with lignin content in atlas (WT), atlas *bmr* 6 and bmr 12. It was found that expression level not much differs in gene *bmr*6, COMT and CAD 4. SBCAD2 gene activity suppressed. Lignin content of Atlas *bmr* 6 (3.9), *bmr* 12(4.78) and Atlas (WT) (3.61) was measured.

Kansas collier

Bmr 6 and CAD 4 were equally expressed in Kansas collier (WT) and bmr 6 genotypes. Lignin content for Kansas collier (WT) (4.42) and Kansas collier *bmr* 6 (3.78) was measured. Gene expression level was less in *bmr* 6 mutant for CAD 4, *bmr* 6 and COMT genes than wild versison.

Rox orange

Bmr 6, COMT and CAD 4 were equally expressed for wild and mutant genotypes in Rox orange (WT). Maximum expression for *bmr*6, COMT and CAD was observed in Rox Orange *bmr* 6 mutant. Lignin content for Rox orange (WT) (4.63), *bmr* 6(4.27) and *bmr* 12 (3.54) was measured.

Early hagari

Gene SBCAD 2 and *bmr* 6 supressed in Early Hagari. Lignin content in Early Hagari *bmr* 6 (4.4) and *bmr* 12 (5.83) was measured. *Bmr* 6, COMT and CAD 4 were equally expressed *bmr* 6 and bmr 12 mutants.

RTx 430

Gene *bmr* 6 is negatively correlated with expression of CAD 4, *bmr* 12 and COMT3.

Conclusion

Brown midrib mutants *bmr* 6 and *bmr* 12 are having low lignin content could be used introgression breeding program. Expression level of *bmr* 6 gene was negatively correlated with lignin content in all *bmr* 6 mutants. Sorghum cultivars with reduced lignin can have a better way to increase second generation cellulosic ethanol production as compared with other crop residues and also improve process economics targeting higher conversion efficiency. Reduced lignin content will be highly beneficial for improving biomass conversion yield.

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