# **RESEARCH ARTICLE**



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# Functional characterization of cinnamyl alcohol dehydrogenase and caffeic acid *O*-methyltransferase in *Brachypodium distachyon*

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# Abstract

**Background:** Lignin is a significant barrier in the conversion of plant biomass to bioethanol. Cinnamyl alcohol dehydrogenase (CAD) and caffeic acid *O*-methyltransferase (COMT) catalyze key steps in the pathway of lignin monomer biosynthesis. Brown midrib mutants in *Zea mays* and *Sorghum bicolor* with impaired CAD or COMT activity have attracted considerable agronomic interest for their altered lignin composition and improved digestibility. Here, we identified and functionally characterized candidate genes encoding CAD and COMT enzymes in the grass model species *Brachypodium distachyon* with the aim of improving crops for efficient biofuel production.

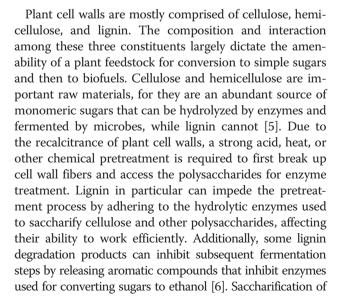
**Results:** We developed transgenic plants overexpressing artificial microRNA designed to silence *BdCAD1* or *BdCOMT4*. Both transgenes caused altered flowering time and increased stem count and weight. Downregulation of *BdCAD1* caused a leaf brown midrib phenotype, the first time this phenotype has been observed in a C<sub>3</sub> plant. While acetyl bromide soluble lignin measurements were equivalent in *BdCAD1* downregulated and control plants, histochemical staining and thioacidolysis indicated a decrease in lignin syringyl units and reduced syringyl/guaiacyl ratio in the transgenic plants. *BdCOMT4* downregulated plants exhibited a reduction in total lignin content and decreased Maule staining of syringyl units in stem. Ethanol yield by microbial fermentation was enhanced in *amiR-cad1-8* plants.

**Conclusion:** These results have elucidated two key genes in the lignin biosynthetic pathway in *B. distachyon* that, when perturbed, may result in greater stem biomass yield and bioconversion efficiency.

### Background

Plant biomass offers a sustainable, low-carbon-emitting source of biofuel feedstock to potentially alleviate both environmental and economic disadvantages of fossil fuel usage [1]. Fossil fuel combustion has resulted in elevated atmospheric  $CO_2$  levels that continue to rise, threatening air quality, wildlife habitat, and human health [2,3]. A viable, cost-effective alternative is to replace and/or blend gasoline and diesel fuels with biofuel. The efficiency of existing approaches to generating biofuels can be improved through research into plant feedstock attributes, namely biomass yield and recalcitrance to conversion [4].

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*Medicago sativa* expressing antisense transcripts designed to silence lignin biosynthesis genes showed an inverse relationship between lignin content and sugar yield, revealing that lignin is indeed a significant obstacle to obtaining high yields of cell wall sugars [7]. Consequently, there has been strong interest in the engineering of bioenergy crops, namely grasses, that are more amenable to feedstock conversion, notably by the manipulation of lignin biosynthesis, in order to improve efficiency of the pretreatment process and obtain maximum fuel yield [8].

Lignin is a complex phenolic polymer, and despite its recalcitrant properties, it is important in providing structural support, hydrophobicity, and protection against pathogens [9]. It is comprised of *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units derived from the monolignol precursors *p*-coumaryl, coniferyl, and sinapyl alcohols, respectively. A series of ten enzymes is involved in the phenylpropanoid pathway for monolignol biosynthesis [9]. The eudicot *Arabidopsis thaliana* has served as a valuable tool to understand lignin biosynthesis [9]. However, there are major plant cell wall structural differences between eudicots and grasses including the type and abundance of lignocellulosic components, pectins, and proteins and the linkages between them [10,11].

Cinnamyl-alcohol dehydrogenase (CAD) functions in one of the final steps of monolignol biosynthesis that catalyzes the reduction of cinnamyl aldehyde to cinnamyl alcohol prior to polymerization into the lignin polymer. The highly conserved Rossmann fold NAD(P)H/NAD(P)+ binding domain found in CAD monolignol-synthesizing proteins indicates the use of NADPH as a cofactor in the reduction reaction [12]. CAD tends to exist in multi-gene families with one gene being primarily responsible for lignin biosynthesis. The effect of CAD downregulation has been studied using a transgenic approach in several eudicot species including Nicotiana tabacum [13-16], M. sativa [17], Populus sp. [18-20] and Eucalyptus camaldulensis [21]. Reports of lignin modification by downregulation of CAD in grasses are limited to Panicum virgatum [22,23], Zea mays [24], and Festuca arundinacea [25]. In general, the transgenic downregulation of CAD does not affect total lignin content; instead, the inhibition of monolignol biosynthesis leads to changes in lignin composition, such as incorporation of accumulated aldehyde precursors or novel units into the lignin polymer, changes that make biomass more digestible [26].

Caffeic acid *O*-methyltransferase (COMT) is an *O*methyltransferase that tends to be broad in substrate affinity and can potentially act in various branches of the phenylpropanoid pathway. The highly conserved *S*adenosyl methionine (SAM) binding domain in COMT proteins indicates the use of SAM as the methyl group donor to the hydroxyl group of a methyl acceptor molecule [27]. COMT is involved in the methylation of caffeic acid to ferulic acid, which is then hydroxylated at position five by ferulate-5-hydroxylase. The subsequent methylation by COMT at this position yields sinapic acid. Similarly, COMT catalyzes the 3-O-methylation of caffeal- and coniferal- aldehyde/alcohol precursors to G and S lignin [28,29]. In Brachypodium distachyon, COMT has high affinity for a variety of substrates including flavonoid compounds, with the greatest activity with caffeic acid and caffealdehyde [30]. Transgenic downregulation of COMT has been reported in eudicots including M. sativa [31], N. tabacum [32,33], and Populus sp. [18,34,35]. Reports of COMT-downregulated transgenic grasses are more limited, including P. virgatum [36], Z. mays [37], F. arundinacea [38] and Lolium perenne [39]. Perturbation of the COMT enzyme often results in the inhibition of S lignin formation and consequently the accumulation of 5-OH coniferyl alcohol that could not be synthesized into S lignin is instead converted into a G lignin monomer, resulting in incorporation of a novel 5-hydroxyguaiacyl lignin unit into the lignin polymer [33,40]. Accordingly, COMT transgenics with altered lignin composition tend to be more digestible [26].

In the early 1920's, brown midrib (bmr) mutants were identified in Z. mays as displaying a brownish-red to tan pigmentation of the leaf midrib associated with reduced lignin content or altered lignin composition [41]. A similar discoloration was observed in lignified tissues in the stalk. Changes in development are also apparent, as exemplified by variation in flowering time [42]. While the bmr mutants tend to have improved digestibility, various unfavorable traits including decreased grain and stem yield, increased lodging, and increased disease susceptibility sometimes develop [40,43,44]. Mutants have been isolated in the  $C_4$  grasses Z. mays, S. bicolor, and Pennisetum glaucum. Four bmr loci in Z. mays, bm1, bm2, bm3 and bm4, represent spontaneous mutants that were first isolated almost a century ago; an additional locus, bm5, was identified later [40]. In S. bicolor, four brown midrib loci, bmr2, bmr6, bmr12, and bmr19, were isolated from mutagenized populations [45,46]. Three brown midrib mutants have been isolated in P. glaucum, although they have not been as well characterized [47]. In Z. mays and S. bicolor, the bmr phenotype is associated with orthologous loci that encompass CAD and COMT genes. The bm1 mutation affects expression of ZmCAD2 and five alleles of the orthologous bmr6 have been characterized in S. bicolor [48-51]. The bm3 locus in Z. mays and bmr12 in S. bicolor correspond to orthologous COMT genes [52,53]. The bmr mutants have the potential to act as viable bioenergy crops, as the visual phenotype seems to be an effective marker for impaired lignin biosynthesis associated with improved digestibility. A more complete understanding of the genes responsible for the phenotype will help provide novel breeding strategies and expand the resources of conversion-efficient plants.

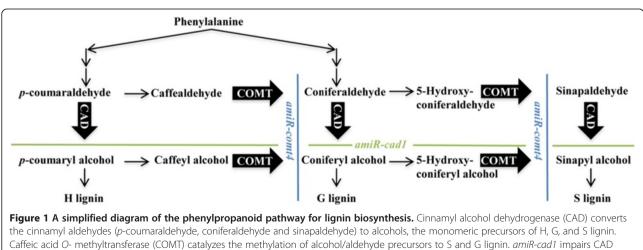
Grasses are a key source of grain and forage and have recently gained in importance as feedstocks for the biofuel industry. Large perennial bioenergy crops such as P. virgatum and Miscanthus sp. have a relatively long generation time, complex genomes, and tall stature that make these species difficult research subjects. In order to better understand grasses, the small annual grass, B. distachyon, was used here as a research model from which we can translate results to the improvement of crops for efficient biofuel production [54]. It has many attributes of a model system including a small diploid and sequenced genome, rapid generation time, short stature, and it is easily transformable [54]. In this study, we used artificial microRNAs to disrupt the function of candidate *CAD* or *COMT* genes, with the objective of characterizing their role in lignin biosynthesis in B. distachyon.

### Results

# Characterization and phylogenetic analysis of *BdCAD* and *BdCOMT* gene families

Given their importance to monolignol synthesis in other plants, *CAD* and *COMT* genes with a potential role in secondary cell wall lignification were identified and perturbed in order to evaluate their role in *B. distachyon* (Figure 1). Candidate genes were selected based on sequence homology to previously characterized genes. Seven putative BdCADs were identified by BLAST search of the *B. distachyon* genome with the *Oryza sativa* CAD protein family [55]. The BdCAD family consists of Bradi3g06480 (BdCAD1), Bradi3g17920 (BdCAD2), Bradi3g22980 (BdCAD3), Bradi4g29770 (BdCAD4), Bradi4g29780 (BdCAD5), Bradi5g04130 (BdCAD6), and Bradi5g21550 (BdCAD7); numbered sequentially as they appear in the genome. Multiple amino acid sequence alignment was

performed with the seven candidate B. distachyon CAD family members along with S. bicolor (Sbbmr6), Saccharum officinarum (SoCAD), Z. mays (Zmbm1), P. virgatum (PviCAD), O. sativa (OsCAD2), F. arundinacea (FaCAD), and Triticum aestivum (TaCAD) CAD proteins. Alignment results indicate a high degree of similarity in conserved domains and binding residues characteristic of alcohol dehydrogenases, especially in BdCAD1 (Figure 2). All seven CAD family members in B. distachyon contain the Zn-1 binding domain motif  $GHE(X)_2G(X)_5G(X)_2V$  and the conserved Zn-1 catalytic residues C47, H69, and C163. The sequence of the Zn-1 binding motif is most highly conserved in BdCAD1, with 99.3% homology to the motif in the other aforementioned grass species CAD proteins. A glycine-rich repeat  $GXG(X)_2G$ , involved in NADP(H) cosubstratebinding, is conserved amongst all CAD proteins. The consensus sequence GD(X)<sub>9,10</sub>C(X)<sub>2</sub>C(X)<sub>2</sub>C(X)<sub>7</sub>C for binding the Zn-2 metal ion is preserved in the BdCADs. Additionally, twelve amino acids have been identified as substratebinding residues in the bona fide CADs of various plant species [56]. Of the seven CADs in B. distachyon, BdCAD1 contains ten of these twelve conserved residues, while the other family members are more variable at these positions. Interestingly, only BdCAD1 contains both active substratebinding residues, W119 and F298, which determine specificity for aromatic alcohols, and the conserved S212 residue that determines NADP(H) binding at that position, as seen in OsCAD2 in rice [55]. Pairwise sequence alignments with the BdCAD1 protein revealed high percent identity to F. arundinacea (89.7%), T. aestivum (89.4%), O. sativa (89.2%), P. virgatum (87.5%), Z. mays (88.3%), S. officinarum (86.9%), and S. bicolor (86.9%). Based on amino acid sequence, it appears that BdCAD1 (Bradi3g06480) contains the conserved functional and structural features of a medium chain dehydrogenase/reductase specific to enzymes involved in lignin biosynthesis in secondary cell walls.



activity; amiR-comt4 impairs COMT activity.

	10	20	30	40	50	60	70	80
Sbbmit						MOSLAS	ERKVVGWAAR	ATGHLSPYTY
SoCAD							ERKVVGWAAR	
Zmbm1							ERKVVGWAAR	
PviCAD2							ERTVVGWAARD	
OsCAD2							EKTVTGWAARD	
FaCAD							SEKTITGWAARD	
TaCAD						· · · MGSVDA	SETTVTGWAARD	ATGHLSPYRY
BdCAD1						MG S   A S	EERTVTGWAARD	ADGHLSPYTY
BdCAD3							ESGNCHAWAAK	
BdCAD6							TQTVSGWAAT	
BdCAD2							TKKAVGLAAR	
BdCAD7 BdCAD4					···MAPIPIII		PRKVLGLAAH	
BdCAD4	MSHHLHLRGSVPPLSFR	GOL DARESPON	DWUFASSS	SNVAVAAASS			GKAALGWAAR	
BUCKUS	Monnenerosveresrk	OOL DART SPOT	r whr Assos	3449444433	ANTENFEMINA	A VINANEAU EN	I I AALVHAARI	ASSALSFIDI
	90 100	110	120	130	140	150	160	170
Sbbmit	TLRNTGPEDVVVKVLYC	GICHTDIHQAH	NHLGASKY	PMVPGHEVVG	EVVEVGPEVS -	KYGVGDVVG	GVIVGCCRECS	PCKANVEQYC
SoCAD	TLRSTGPEDVVVKVLYC	GICHTDIHQAP	(NHLGASKY)	PMVPGHEVVG	EVVEVGPEVT.	KYGVGDVVG	VGVIVGCCRECH	PCKANVEQYC
Zmbm1	TLRNTGPEDVVVKVLYC							
PviCAD2	TVRKTGPEDVVVKVLYC							
OsCAD2	TLRKTGPEDVVVKVLYC							
FaCAD	NLRRTGAEDVVLKVLYC TLRKTGPEDVVLKVKYC							
TaCAD BdCAD1	TLRKTGPEDVLVKVLYC							
BdCAD3	NRTVQSGDISLRITHC							
BdCAD6	KRRENGVDDVTIKVEYC							
BdCAD2	TRRSTGDDDVAIKILYC							
BdCAD7	SRRNTGDDDVAIKVLYC							
BdCAD4	SRRVQKDDDVTIKVLFC							
BdCAD5	SRRAQKDDDVTIKVLYC	GICHTDLYIIK	(NEWGNAMY	PVVPGHEILG	VVTDVGSGVT-	KFKAGETVG	VG YF VG SCRSCE	SCGNGYENYC
	180 190	200	210	220	230	240	250	260
Sbbmi®	NKKIWSYNDVY - TDGR							
SoCAD	NKKIWSYNDVY - TDGR							
Zmbm1	NKKIWSYNDVY TDGR							
PviCAD2	NKRIWSYNDVY TDGR							
OsCAD2	NKRIWSYNDVY TDGR	PTQGGFASAM	VDQKFVVK	PAGLAPEQA	APLL <mark>C</mark> AGLTVY	S - PLKHFGL	S PGLRGG	L <mark>GLGGVG</mark> HMG
FaCAD	NKKI <mark>W</mark> SYNDVY - · TDGK							
TaCAD	NKKIWSYNDVY - TDGK							
BdCAD1	NKRIWSYNDVY TDGR							
BdCAD3 BdCAD6	SKFVFTFNGVD TDGT DKVTLTYNGVF WDGS							
BdCAD2	PGMIF TYNSTD RDGT							
BdCAD7	SKVVF SYNSLD RDGT							
BdCAD4	PTLVLTSNGVD - · YDGA	TTQGGFSDVVV	VNQDYVVR	VPDTLPPDGA	APLL <mark>C</mark> AGVTVY	S . PMMQYGL	A POKHLON	VGLGGLGHMA
BdCAD5	YGMVLTSNGIDAEHGGA	VTQGGFSDVIV	VNEDYVVR	VPDGLPLDKA	APLL <mark>C</mark> AGVTVY	S - PMMRFGL	NA PGKHLG	V <mark>GLGGLG</mark> HVA
	270 280	290	30	0 31	0 320	33	0 340	350
Sbbmit	VKVAKAMGHHVTVISSS							
SoCAD	VKVAKAMGHHVTVISSS							
Zmbm1	VKVAKAMGHHVTVISSS							
PviCAD2								
OsCAD2	VKVAKSMGHHVTVISSS							
FaCAD	VKVAKSMGHHVTVISSS							
TaCAD	VKVAKSMGHHVTVISSS							
BdCAD1	VKVAKSMGHHVTVISSS							
BdCAD3 BdCAD6	VKFGKSFGLKVTVFSTS VKFGKAFGLRVTVISTS							
BdCAD2	VKFGKAFGMKVTVISSS							
BdCAD7	VKFARAFGAKVTVISTS							
BdCAD4	VKFGKAFGMKVTVISSS	LRKREEALDRL	GADDFLVS	SDAEQMKAAA	GAMDGIIDTVS	AG · · HP I VP	LELLRPMGQM	VCG APSEPLQ
BdCAD5	VKFGKAFGMKVTVIS <mark>T</mark> S	PGKREEAIEKL	GADDFLVS	RDPGQMQAAF	GTMDGILDTVS	AW · · HPISPI	FALMKPMGQM	FVGGPTKPLE
	360	370 3	80	390	400	410	420 4	30
Sbbmrð	FVSPMVMLGRKAITGSF		OFCVDKOL	SOLEVVENO			VAGENVEED	AADAPSN
SoCAD	FVSPMVMLGRKAITGSF							
Zmbm1	FVSPMVMLGRKAITGSF							
PviCAD2								
OsCAD2	F I S P MVML G R KA I T G S F							
FaCAD	FVSPMVMLGRKTITGSF							
TaCAD	FVSPMVMLGRKTITGSF							
BdCAD1	F VS PMVML GRKS I TG SF							
BdCAD3 BdCAD <sup>6</sup>	I H P A T L N L G A R T L A G S V L P S F P L I F G K R T V S G S M							
BdCAD <sup>©</sup> BdCAD2	VPAFALVAKNKTLAGSC							
BdCAD7	VPAF ALVOGGKTLAGSF							
BdCAD4								
BdCAD5	LPAVAIVPGGKGIAGNC	GGMRDCQAML	DFAGKHGI	TAEVEVIKMD	VVNTALERLQK	NDVRYRFVI	VAGSLGTTA	
Figure	2 Sequence alignment of	the CAD family	of Brachung	dium distach	on and other c	Amino	acid sequence or	mparison of
-		•						
	<i>distachyon</i> CAD proteins w							
(SoCAD)	), Zea mays (Zmbm1), Panice	<i>um virgatum (</i> Pvi	CAD2), Oryza	<i>sativa</i> (OsCAD2	), Festuca arundir	<i>nacea</i> (FaCAD),	and Triticum aest	ivum (TaCAD).
The Zn-	1 binding domain motif is l	abeled in pink ar	nd Zn-1 catalv	rtic residues are	labeled in vellow	w. The NADP(H	) cosubstrate-bind	ding domain
	labeled in red and the Zn-2							9
	1) binding residue is labeled			g.ccm. Jubbildi	- Sinaniy icsidde			ciere specific
INAL PLH								

NADP(H) binding residue is labeled in purple.

Four COMTs were identified in B. distachyon, Bradilg 14870 (BdCOMT1), Bradi2g02380 (BdCOMT2), Bradi2g 02390 (BdCOMT3), and Bradi3g16530 (BdCOMT4). Multiple amino acid sequence alignment was then performed with the four derived B. distachyon COMT family members and with known COMTs in T. aestivum (TaCM), F. arundinacea (FaCOMT), S. bicolor (Sbbmr12), S. offici narum (SoCOMT), Z. mays (Zmbm3), and P. virgatum (PviCOMT). Sequence comparison with the functionally characterized COMT proteins from various plants showed a high degree of similarity at the amino acid level (Figure 3). The S-adenosyl-L-methionine (SAM) binding domain, LVDVGGGxG, a signature of O-methyltransferases, is conserved in BdCOMT1, BdCOMT3, and BdCOMT4; however, one residue is inconsistent at position 193 in BdCOMT2, where isoleucine replaces valine in the sequence motif. Catalytic residues E310, E342, and R343 are conserved in all B. distachyon COMT family members and all other characterized COMT lignin proteins. Interestingly, catalytic residue H281 found in *P. virgatum*, *F. arundinacea*, *S. bicolor*, and *Z. mays*, is conserved only in BdCOMT1 and BdCOMT4. Of the four BdCOMT family members, only BdCOMT4 has all substrate-binding and positioning residues M130, N131, L136, A162, H166, F176, M180, H183, I319, M320, N324 conserved in the known COMTs specific to the synthesis of secondary cell wall lignin. Pairwise sequence alignments with BdCOMT4 revealed high percent identity to *F. arundinacea* (89.2%), *T. aestivum* (87.4%), *O. sativa* (83.4%), *P. virgatum* (83.4%), *S. officinarum* (80.2%), *Z. mays* (80.0%), and *S. bicolor* (78.8%). The prevalence of common signatures in the amino acid sequence supports the idea that BdCOMT4 is a COMT ortholog functioning as a SAM-dependent *O*-methyltransferase in *B. disatchyon*.

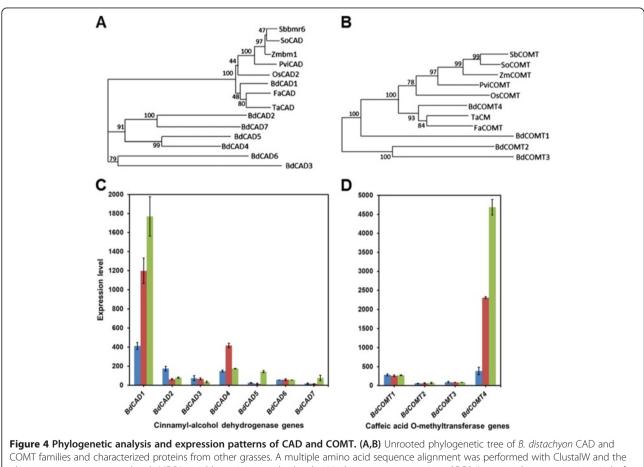
Evidence for the role of BdCAD1 and BdCOMT4 as functional lignin biosynthesis proteins was further enhanced by phylogenetic analysis. An unrooted neighbor-joining tree with 1000 bootstrap permutations was generated from the

10     20     20     40     60     70     80     90       FacOMT     MGST     AADABASADERGENMIALDIVESSILLETLAAAC     OKTLIFFACVAALD-SAANP     FAPDWUDRULLL       SacOMT     MGST     AADABASADERGENMIALDIVESSILLETLAAAC     OKTLIFFACVAALD-SSANP     FAPDWUDRULLL       SacOMT     MGST     AADAMASADERGENMIALDIASSILPMILKALELOLLETLAAAC     OKTLIFFACVAALD-SSANP     FAPDWUDRULLL       SacOMT     MGST     AEDVAAVADERGENMIALDIASSILPMILKALELOLLEVLORAA     OKTLIFFECVAALD-SSANP     FAPDWUDRULLL       SacOMT     MGST     AEDVAAVADERGENMIALDIASSILPMILKALELOLLEVLORAA     OKTLIFFECVAALD-VARLPTIFF     AADAWD RULLLL       MGGOMT     MGST     ATDVAAAADEEGACWALGLASSILPMILKALELOLLEVLORAA     OKTLIFFECVAALD-VARLPTIFFECVAALD-SARPO     DAADMUDRULLL       MGGOMT     MGST     ATDVAAADEEGACWALGLASSILPMILKALELOLLEVLORAA     REAFLIPSOVAELO-SARPO     DAADMUDRULLL       MGGOMT     MGST     ATDVAAADEEGACWALGLASSILPMILKALELOLEVLORAA     REAFLIPSOVAELO-SARPO     DAADMUDRULLL       MGGOMT     MGST     ADDVAADEGACWALGLASSILPMILKALELOLEVLORAA     REAFLIPSOVAELO-SARPONDURULL     AEGMUNRULL       MGGOMT     MGST     ADDVAADEGACWANDELONDURULL     ADDV								
isodut   MoST   AADMAASDEEACUFALCLASSSILPMTLKNAIELOLLEVILOKAA   SKSLTPTEVAAKUP-SANNP-EAPDMVSNUERLE     isodut   MoST   AEDVAANDEEACUFALCLASSSILPMTLKNAIELOLLEVILOKAA   SKSLTPTEVAAKUP-SANNP-EAPDMVSNUERLE     isodut   MOST   AEDVAANDEEACUFALCLASSSILPMTLKNAIELOLLEVILOKAA   SKSLTPTEVAAKUP-SANNP-EAPDMVSNUERLE     isodut   MOST   AEDVAANDEEACUFALCLASSSILPMTLKNAIELOLLEVILOKAA   SKSLTPTEVAAKUP-SANNP-EAPDMVSNUERLE     isodut   MOST   AEDVAANDEEACUFALCLASSSILPMTLKNAIELOLLEVILOKAA   SKSLTPTEVAAKUP-SANNP-AADMVSNUERLE     isodut   MOST   AEDVAANDEEACUFALCLASSSILPMTLKNAIELOLLEVILOKAA   SKSLTPTEVAOKUP-SANNP-AADMVSNUERLE     isodut   MOST   AEDVAANDEEACUFALCLASSSILPMTLKNAIELOLLEVILOKAA   SKRTPTAANDEOKAALLANDEKA   SKRTPTAANDEOKAALLANDEKA     isodut   MOSARTEMVYFGAAGABOEEACUFALCLASSSILPMTLKNAIELOLLEVILAGAA   SKRTPTAANDEOKAALLANDEKA   MAEEACOMANDECAKUPT   SKRTPTAANDEOKAALLANDEKA     isodut   MOSARTEMVYFGAAGABOEKAKUPT   MAEEACOMANDECKUPT   MAEEACOMANDECKUPT   SKRTPTAANDEOKAALLANDEKA   MESMYFLKAANDEOKAALLANDEKA   MESMYFLKAANDEKA   MAMADEEACUFAKUPT   SKRTPTAANDEKAKUPT   SKRTPAANDEKAKUPT   SKRTPAANDEKAKUPT   SKRTPAANDEKAKUPT   SKRTPAANDEKAKUPT   SKRTPAANDEKAKUPT   SKRTPAANDEKAKUPT   SKRTPAANDEKAKUPT   SKRTPAANDEKAKUPT		10	20	30 4	o 50	eo	70 80	eo
BGODIT   MOST A ADMAATA DE RACMT ALOLASS SILPMIT KINA LEUGLEU LOVAS OKSL TPAEVAAKLP - SSSMT - AAPDWORMLELL     SGODIT   MOST A ED VAAVADE CA KMY AMOLASS SILPMIT KINA LEUGLEU LOVAS OKAL APE EVVANKUPAT TPF - AAADWORMLELL     SGODIT   MOST A ED VAAVADE CA KMY AMOLASS SILPMIT KINA LEUGLEU LOVAS	TaCM	MG S	AAGADEDACMYALQ	LVSSSILPMTLKN	AIELGLLETLMAAG	GKFLTP/	AEVAAKLP - SAANP	EAPDMVDRMLRLL
Stocht   MOST	FaCOMT	MGST AADM	AASADEEACMFALQ	LASSSILPMTLKN	AIELGLLEILVAAG	GKSLTP	TEVAAKLP - SAANP	EAPDMVDRMLRLL
signal   Mostr AEDVAAVADEEACMYAMOLASS ELPMITIKNALELOILEULGAGAGO	BdCOMT4	MGST · · · · AADM	AATADEEACMFALQ	LASSSILPMTLKN	AIELGLLDTLVQAS	GKSLTP	AEVAAKLP - SSSNP	AAPDMVDRMLRLL
<pre>mediation with the set of th</pre>	SECOMT							
Pricedu     MostAtlance     Atlance     Atlance     Event of the service     Atlance     A								
OCOUNT   MOST	ZmCOMT							
BGGONT   MSSARTEWVYPGAAGAODEEACUYALCILASSILPHTLKNIELOHLEIUVAGA   KTLSPSQVAERLG-AKPPP.DAPAMLDRWLERU     BGGONT   MAEEEACUYALCILASSULPHTLKNIELOHLEIUVAGA   YoKTMSPEEVTAKLP-AKANP.SAASUVDRHLRVL     10								
BGGONT								
BECONT3								
110   120   120   140   150   150   170   180   190     Tack   CRTEEGKDGRLSRRYGAAPVCKYLTPREDGVSMALALMNORVL MESWYYLKDAVLOG.01FPNKAYDMSAFEHGTDPRINKYNEOHKHSI   15000000000000000000000000000000000000								
Tem   CRT EEGK DG BLS RRY DAAPV CKYL TP HE DQ YSMALAL UND KYL UHE SWYL KDAL DG - 01 FP NK YG WSAFE FY DT DP FN NVY NÉ OKKHS I J FGCWT     TagGWT   CAVEE DG NG LS RSY DAAPV CKYL TP HE DQ YSMALAL NUD KYL UHE SWYL KDAL DG - 01 FP NK YG WSAFE FY DT DP FN NVY NE OKKHS I J SCOWT     GAWE A. DG VYE RRY SAAPV CKWL TP HE DQ YSMALAL NUD KYL UHE SWYL KDAL DG - 01 FP NK YG WSAFE FY DT DP FN NVY NE OKKHS I J SCOWT   CGME D. KDG KYE RRY SAAPV CKWL TP HE DQ YSMALAL NUD KYL UHE SWYL KDAL DG - 01 FP NK YG WTAFE FY DT DP FN NVY NE OKKHS I J SCOWT     SCOWT   CGME D. KDG KYE RRY SAAPV CKWL TP HE DQ YSMALAL LUN DQ KL UHE SWYL KDAL DG - 01 FP NK YG WTAFE FY DT DP FN NVY NE OUKKHS Y WCOWT   CGME D. KDG KYE RRY SAAPV CKWL TP HE DQ YSMALAL LUN DQ KL UHE SWYL KDAL DG - 01 FP NK YG WTAFE FY DT DP FN NVY NE OUKKHS Y WCOWT     GAWE D. KDG KYE RRY SAAPV CKWL TP HE DQ YSMALAL LUN DQ KL UHE SWYL KDAL DG - 01 FP NK YG WTAFE FY DT DP FN NVY NE OUKKHS Y WCOWT   CGME D. KDG KYE RRY SAAPV CKWL TP HE DQ YSMALAL LUN DQ KL UHE SWYL KDAL DG - 01 FP NK YG WTAFE FY DT DP FN NVY NE OUKKHS Y WCOWT     GAWE D. KDG KYE RRY SAAPV CKWL TP HE DQ YSMAP AL LL QD KY ME SWYL KDAL DG - 01 FP NK YG WTAFE FY DT DP FN NVY NE OUKKHS Y WCOWT   CGME D. KDG KYE RRY SAAPV CKWL TP HE DQ YSMAP AL LL QD KY ME SWYL KDAL DG - 01 FP NK YG WTAFE FY DT DP FN NVY NE OUKKHS Y WCOWT     GAWE D. KDG KYE RRY SAAPV CKWL TP HE DQ YSMAP AL LL QD KY MA FE FY DT DP FN NVY NE OUKKHS Y WCOWT   CGME DE AND YG KWY KY TP HE DQ YSMAP AL LUN DQ KY MA FE YG DT DP FN NVY NE OWKHS Y WCOWT   CGME DE AND YG KWY KY HE HE DQ YSMAP AL LUN DQ KY MA FE YG TT DP FN NVY NE ALKNY HE HE KY HE T DY TY TA WA KY HE HE KY HE T DY TY TA WA KY HE T HE T T W T T T T T T T T T T T T T T T T	BdCOMT3		- MAEEEAGCMHALM	LASSVVQPMAVRT	AIELGLLEILVAGA	G · · · · YGKTMSP	EEVTAKLP . T . SNP	EAASMVDRLLRVL
Tem   CRTEEGENDGALS REVGAAPVCKYL TP NEDOVSALAL UNDKYL WE SWYL KDAL DO - 01 FP NK YOWSAFE FV 01 DP FN NYL NEOVKH SI TAGOUT     TagoUT   CVVEEGENDGLS REVGAAPVCKYL TP NEOVSALALAL UNDKYL WE SWYL KDAL DO - 01 FP NK YOWSAFE FV 01 DP FN NYL NEOVKH SI SCOUT   COMED + KOCKYERRYSAAPVCKYL TP NEOVSALALAL UNDKYL WE SWYL KDAL DO - 01 FP NK YOWSAFE FV 01 DP FN NYL NEOVKH SI SCOUT   COMED + KOCKYERRYSAAPVCKYL TP NEOVSALAL UNDKYL WE SWYL KDAL DO - 01 FP NK YOWTAFE FV 01 DP FN NYL NEOVKH SI SCOUT   COMED + KOCKYERRYSAAPVCKYL TP NEOVSALAL UNDKYL WE SWYL KDAL DO - 01 FP NK YOWTAFE FV 01 DP FN NYL NEOVKH SI SCOUT   COMED + ROKYERRYSAAPVCKYL TP NEOVSALAL UNDKYL WE SWYL KDAL DO - 01 FP NK YOWTAFE FV 01 DP FN NYL NEOVKH SI SCOUT   COMED + ROKYERRYSAAPVCKYL TP NEOVSALAL UNDKYL WE SWYL KDAL DO - 01 FP NK YOWTAFE FV 01 DP FN NYL NEOVKH SI SCOUT   COMED + ROKYERRYSAAPVCKYL TP NEOVSMAL AL UNDKYL WE SWYL KDAL DO - 01 FP NK YOWTAFE FV 01 DP FN NYL NEOVKH SI SCOUT   COMED + ROKYERRYSAAPVCKYL TP NEOVSMALAL UNDKYL WE SWYL KDAL DO - 01 FP NK YOWTAFE FV 01 DP FN NYL NEOVKH SI SCOUT   COMED + ROKYERRYSAPVCKYL TP NEOVSMALAL UNDKYL WE SWYL KDAL DO - 01 FP NK YOWTAFE FV 01 DP FN NYL NEOVKH SI SCOUT   SCOUT SVERANDAPVCKYL TP NEOVSMALAL UNDKYL WE SWYL KDAL DO - 01 FP NK YOWTAFE FV 01 DP FN NYL NEOVKH SI SCOUT   SCOUT SVERANDAPVCKYL TP NEOVSMALAL UNDKYL WE SWYL KDAL DO - 01 FP NK YOWTAFE FV 01 DP FN NYL NEOVKH SI SCOUT   SCOUT SVERANDAPVCKYL TP NEOVSMALAL UNDKYL WE SWYL KDAL DO - 01 FP NK YOWTAFE FV 01 DP FN NYL NEOVKH SI SCOUT   SCOUT SVERANDAPVCKYL TP NEOVSMALAL UNDKYL WE SWYL KDAL DO - 01 FP NK YOWTAFE FV 01 DP NK NEOVKH SI SCOUT   SCOUT SVERANDAPVCKYL TP NEOVSMALAL UNDKYL WE SWYL KDAL DO - 01 FN KKYD NA SAD TF NK SCOUT   SCOUT SVERANDAPVCKYL TP NEOVSMALAL UNDKYL WE SWYL		110	120	130 140	150	160 1	180	190
FACONT   CLVEEEKKORLSREYGAAPVCKWLTPNEDOSYMAALALMNDDKVL MESWYYLKDAVLDO-0 IPFKKA YOMAFEYNOTDPRINNEV NEDUKKHS I BCONTA     SECONTA   CAWEEGKORKLSREYGAAPVCKWLTPNEDOSYMAALALMNDDKVL MESWYYLKDAVLDO-0 IPFKKA YOMAFEYNOTDPRINNEV NEDUKKHS I COMED - KOGKYERRYSAAPVCKWLTPNEDOSYMAALALMNDDKVL MESWYYLKDAVLDO-0 IPFKKA YOMTAFEYNOTDPRINNEV NEDUKKHS Y COMED - KOGKYERRYSAAPVCKWLTPNEDOSYMAALALMNDDKVL MESWYYLKDAVLDO-0 IPFKKA YOMTAFEYNOTDPRINNEV NEDUKKHS Y COVEE AKOGSLSRRYDAPVCKWLTPNEDOSYMAAFLA DDKV MESWYLKDAVLDO-0 IPFKKA YOMTAFEYNOTDPRINNEV NEDUKKHS Y COVEE AKOGSLSRRYDAPVCKWLTPNEDOSYMAAFLA DDKV MESWYLKDAVLEO - OPFTKA OMTAFEYNOTDPRINNEV NEDUKKHS Y COVEE AKOGSLSRRYDAPVCKWLTPNEDOSYMAAFLA DDKV MESWYLKDAVLEO - OPFTKA OMTAFEYNOTDPRINNEV NEDUKKHS Y COVEE AKOGSLSRRYDAPVCKWLTPNEDOSYMAAFLA DDKV MESWYLKDAVLEO - OPFTKA OMTAFEYNOTDPRINNEV NEDUKKHS Y COVEE AKOGSLSRRYDAPVCKWLTPNEDOSYMAAFLA ADKV MKATWYNKDAVLEO - OPFTKA OMTAFEYNOTDPRINNEV NEDUKKHS Y COVEE AKOGSLSRRYDAPVCKWLTPNEDOSYMAAFLA ADKV MKATWYNKDAVLEO - OPFTKA OMTAFEYNOTDPRINNEV NEDUKKHS Y COVEE AKOGSLSRRYDAPVCKWLTPNEDOSYMAFLA ANAPYA KONTPLENTYN NEDUKKAN Y COVEE AKOGSLSRRYDAPVCKWLTPNEDOSYMAFLA ANAPYA KONTPLENTYNKDAVLEO - OPTTKA OMTAFEYNOTDPRINNEV NEDUKKAN Y COVEE AKOGSLSRRYDAPVCKWLTPNEDOSYMPHTAFIN Y COV					1	1		1
BECONT GAVE BEGENG KLS RRYAAAPVC KWL TP NED OVS MAAL AL WOD KVL ME SWYYL KDAVLOD • 0 IF PF KKA YG MTAF EYH OT DPR NRY NED KKH SV SCONT GCMED • KOG KY ERRYSAAPVC KWL TP NED OVS MAAL LL WOD KVL ME SWYYL KDAVLOD • 0 IF PF KKA YG MTAF EYH OT DPR NRY NED KKH SV SCONT GCMED • ROG KY ERRYSAAPVC KWL TP NED OVS MAAL LL WOD KVL ME SWYYL KDAVLOD • 0 IF PF KKA YG MTAF EYH OT DPR NRY NED KKH SV PVCONT GCMED • ROG KY ERRYSAAPVC KWL TP NED OVS MAAL LL WOD KVL ME SWYYL KDAVLOD • 0 IF PF KKA YG MTAF EYH OT DPR NRY NED KKH SV PVCONT GCMED • ROG KY SRYAAPVC KWL TP NED OVS MAAL LL WOD KVL ME SWYYL KDAVLOD • 0 IF PF KKA YG MTAF EYH OT DPR NRY NED KKH SV PVCONT GEVEBE QG EG LL ARRYPARYC KWL TP NED OVS MAAL LL WOD KVL ME SWYYL KDAVLOD • 0 IF PF KKA YG MTAF EYH OT DAR NRY NED KKH SV BGCONT GEVEBE QG EG LL ARRYPARYC KWL TP NED OVS MAAL LL WOD KVL ME SWYYL KDAVLOD • 0 IF PF KKA YG MTAF EYH OT DAR NRY NED KKH SV BGCONT GEVEBA CO SL S RRYPARYC KWL TP NED OVS MAAL LL WOD KVL ME SWYL KDAVLOD • 0 IF PF KKA YG MTAF EYH YN FD AKN NRY NED KKH SV BGCONT GVVEE AKO SL S RRYPARYC KWL TP NED OVS MAAF LL AD DKV ME SWYL KDAVLOD • 0 IF PF KKA YG MTAF EYH NED KVN NED KKH SV BGCONT GVVEE AKO SL S RRYPARYC KWL TP NED OVS MAAF LL AD DKV ME SWYL KDAVLOD • 0 IF PF KKA YG MTAF EYH NED KVN NED KKH SV BGCONT GVVEE AKO SL S RRYPARYC KWL TP NED OVS MAAF LL AD DKV MAT WY NKDAVLED • 0 OF PF KKA YG MTAF EYH NED KVN NED KKH SV BGCONT FYKOFFO · UT V VOOG YG ATVAAI ANTYPK IK INFD IPHYI SEAPPFFO • VTHYG OD MF KYP • SD AI LMKWI LH WUSD CH AT LLKKN BGCONT FYT OF FO · ST LV DVOOG YG ATVAAI ANTYPK IK INFD IPHYI SEAPPFFO • VTHYG OD MF KYP • SD AI LMKWI LH WUSD AH AT LLKKN BGCONT FYT OF FO · ST LV DVOOG YG AT LHAI TS HFP I HS (NYFD IPHYI SEAPPFFO • V HVG OD MF KYP • SD AI LMKWI LH WSD AH AT LLKKN BGCONT 0 KYV VY CU LV VY SC LHAN YG KYWY VY KLH PH HS (NYFD IPHYI SEAPPFFO • V HVG OD MF KYP • AD AI LMKWI LH WSD AH AT LLKKN BGCONT 0 KVI VY CU LV VY SC LHAN YG KYWY VY SC HHAN HYP KYM FYF • SD AI LMKWI LH WSD AH AT LLKKN BGCONT 0 KVI VY CU LV VY SC								
Secont COMED & CONKYERRYSAAPVOKULTPNEDOGYSMALAL MODKYL MESWYYL KOAVL DO & O IPFNKA YOMTAFEY & DTPR NRY NEGUKH SY COMED & ROGRYERRYSAAPVOKULTPNEDOGYSMALAL MODKYL MESWYYL KOAVL DO & O IPFNKA YOMTAFEY & DTPR NRY NEGUKH SY COMED & ROGRYERRYSAAPVOKULTPNEDOGYSMALAL MODKYL MESWYYL KOAVL DO & O IPFNKA YOMTAFEY & DTPR NRY NEGUKH SY COMED & ROGRYERRYSAAPVOKULTPNEDOGYSMALAL MODKYL MESWYYL KOAVL DO & O IPFNKA YOMTAFEY & DTPR NRY NEGUKH SY COMED & ROGRYERRYSAAPVOKULTPNEDOGYSMALAL MODKYL MESWYYL KOAVL DO & O IPFNKA YOMTAFEY & DTPR NRY NEGUKH SY COMED & ROGRYERRYSAAPVOKULTPNEDOGYSMALAL MODKYL MESWYYL KOAVL DO & O IPFNKA YOMTAFEY & DTPR NRY NEGUKH SY COVERAKDOSLSRRYOPAPYOKULTPNEDOGYSMAFAL A ODKYL MESWYYL KOAVL DO & O IPFNKA YOMTAFEY & GTDAR NRY NEGUKH SY COVERAKDOSLSRRYOPAPYOKULTPNEDOGYSMAFAL A ODKYL MESWYHLKOAVL DO & O IPFNKA YOMTAFEY & GTDAR NRY NEGUKH SY COVERAKDOSLSRRYOPAPYOKULTPNEDOGYSMAFAL A ODKYL MESWYHLKOAVL DO & O IPFNKA YOMTAFEY & GTDAR NRY NEGUKH SY COVERAKDOSLSRRYOPAPYOKULTPNEDOGYSMAFAL A ODKYL MESWYHLKOAVL DO & O IPFNKA YOMTAFEY & GTDAR NRY NEGUKH SY COVERAKDOSLSRRYOPAPYOKULTPNEDOGYSMAFAL A ODKYL MESWYHLKOAVL DO & O IPFNKA YOMTAFEY & GTDAR NRY NEGUKH SY COVERAKDOSLSRRYOPAPYOKULTPNEDOGYSMAFAL A ODKYL MESWYHLKOAVL DO & O IPFNKA YOMTAFEY & GTDAR NRY NEGUKH SY COVERAKDOSLSRRYOPAPYOKUKUTPNEDOGYSMAFAL COVERAKDOSLSRRYOPAPYOKUKUTPNEDOGYSMAFAL COVERAKDOSLSRRYOPAPYOKUKUTPNEDOGYSMAFAL COVERAKDOSLSRRYOPAPYOKUKUTPNEDOGYSMAFAL COVERAKDOSLSRRYOPAPYOKUKUTPNEDOGYSMAFAL COVERAKDOSLSRRYOPAPYOKUKUTPNEDOGYSMAFAL COVERAKDOSLSRRYOPAPYOKUKUTPNEDOGYSMAFAL COVERAKDOSLSRRYOPAPYOKUKUTPNEDOGYSMAFAL COVERAKDOSLSRRYOPAPYOKUKUTPNEDOGYSMAFAL COVERAKDOSLSRRYOPAPYOKUKUTPNEDOGYSMAFAL COVERAKDOSUSSTRYNAAPYOKUKUTPNEDOGYSMAFAL COVERAKDOSUSSTRYNAAPYOKUKUTPNEDOGYSMAFAL COVERAKDOSUSSTRYNAAPYOKUKUTPNEDOGYSMAFAL COVERAKDOSUSSTRYNAAPYOKUKUTPNEDOGYSMAFAL COVERAKDOSUSSTRYNAAPYOKUKUTPNEDOGYSMAFAL COVERAKDOSUSSTRYNAAPYOKUKUTPNEDYNAA COVERAKDOSUSSTRYNAAPYOKUKUTPNEDYNAA COVERAKDOSUSSTRYNAAPYOKUKUTPNEDYNEDYN CONT O VSTLUVUVOGOVATUAA NANPPEN COVERAKDOSUSSTRYNAAPYOKU								
SCONT COMED - KOGKYERRYSAAPVOKWLTPNEDOSYSMALTLM NDXKU MESWYYLKDAVLDG. 6 IPFNK YOMTAFEY GTDPR NRY NGOVKH SY WCONT COMED - ROORYERRYSAAPVOKWLTPNEDOSYSMALALLM NDXKU MESWYYLKDAVLDG. 6 IPFNK YOMTAFEY GTDPR NRY NGOVKH SY WCONT COMED - ROORYERRYSAAPVOKWLTPNEDOSYSMALALLM NDXKU MESWYYLKDAVLDG. 6 IPFNK YOMTAFEY GTDPR NRY NGOVKH SY WCONT CEWEEGOGOLLARRYOPAVCKWLTPNEDOSYSMALALLM NDXKU MESWYYLKDAVLDG. 6 IPFNK YOMTAFEY GTDPR NRY NGOVKH SY BCONT CEWEEGOGOLLARRYOPAVCKWLTPNEDOSYSMAPLALL DXX MESWYYLKDAVLDG. 6 IPFNK YOMTAFEY GTDPR NRY NGOVKH SY BCONT CEWEEGOGOLLARRYOPAVCKWLTPNEDOSYSMAPLALL DXX MESWYYLKDAVLG. 6 OPFNK YOMTAFEY GTDPR NRY NGOVKH SY BCONT CEWEEGOGOLLSRRYOPAVCKWLTPNEDOSYSMAPLALL DXX MESWYYLKDAVLG. 6 OPFNK YOMTAFEY GTDPR NRY NGAVKH SY 20 20 20 20 20 20 20 30 30 20 ESYKOFEO LOT LVXXGOVGATVALTANYTIKONY SEAPPFPG. VTNYGOMTKEW Y GDDTR NNY NGAVLH SS BCONT SYNGFEO LOT LVXXGOVGATVALTANYTIKON NFDLPHVISEAPPFPG. VTNYGOMTKEWP SODALLMKWLH WSDOHGATLLKKO FYTOFEO LOT LVXXGOVGATVALTANYTIKON NFDLPHVISEAPPFPG. VTNYGOMTKEWP. SODALLMKWLH WSDOHGATLLKKO FYTOFEO LOT LVXXGOVGATVALTANYTIKON NFDLPHVISEAPPFPG. VTNYGOMTKEWP. SODALLMKWLH WSDOHGATLLKKO BCONT FYTOFEO LOT LVXXGOVGATVALTANYTIKON NFDLPHVISEAPPFPG. VTNYGOMTKEWP. SODALLMKWLH WSDOHGATLLKKO FYTOFEO LOT LVXXGOVGATVALTANYTIKON NFDLPHVISEAPPFPG. VSNYGODALLMKWLH WSDOHGATLLKKO BCONT FYTOFEO VSTLVVXGOVGATVANYSNYHPH NGINFDLPHVISEAPPFPG. VSNYGODALLMKWLH WSDOHGATLLKKO BCONT CYVLVECLVVXGOVGATLAHITSNYPHISONFDLPHVISEAPPFPG. VSNYGODALLMKWLH WSDOHGATLLKKO BCONT SYNYKYCVUVGOVGATLAHITSNYPHINGINYDLPHVISEAPPFPG. VSNYGODALLMKWLH WSDOHGATLLKKO BCONT SYNYKYCVUVGOVGATLAHITSNYPHINGINYDLPHVISEAPPFPG. VSNYGODALLMKWLH WSDOHGATLLKKO BCONT SYNYKYCVUVGOVGATLAHITSNYPHINGINYDLPHVISEAPPFPG. VSNYGODALLMKWLH WSDOHGATLLKKO BCONT SYNYVYCVUVGOVGATLAHITSNYPHINGINYDLPHVISEAPPFPG. VSNYGODALLMKWLH WSDOHGATLKKKO BCONT SYNYVYCVUVGUVGAVATHAHITSNYPHINGINYDLPHVISEAPPFPG. VSNYGODALLKWULH WSDOHGATLKKKO BCONT SYNYVYCVUVGUVGATLAHITSNYPHINGINYDLPHVISEAPPFPG. VSNYGODALLKWULH WSDOHGATLKKKO BCONT SYNYVYCVUVGUVGATLAHITSNYPHI								
Dimont   COME 0: ROORYSERYSAAPVCKWLTPNEDOSYMAALAL MODKYL WESWYYLKDAVLE0.0 IF FNK.YOWTAFEYLGTDSR NRY NEGUKHESY     Dividout   Comes 6x000rySERYAAAPVCKWLTPNEDOSYMAALAL MODKYL WESWYYLKDAVLE0.0 IF FNK.YOWTAFEYLGTDSR NRY NEGUKHESY     Bidcouti   CEVEEGAOCKLSRRYSARAAPVCKWLTPNEDOSYMAALAL MODKYL WESWYYLKDAVLE0.0 IF FNK.YOWTAFEYLGTDAR NRY NEGUKHESY     Bidcouti   CEVEEGAOCSLARRYSPAPVCKWLTPNEDOSYMAAFAL AODKYL WESWYLKDAVLE0.0 IF FNK.YOWTAFEYLGTDAR NRY NEGUKHESY     Bidcouti   CEVEEGAOCSLARRYSPAPVCKWLTPNEDOSYMAAFAL AODKYL WESWYLKDAVLE0.0 COPFTKALOUSWEYLGADTR NRY NEGUKHESY     Bidcouti   CUVEEAKDOSLSRRYSPAPVCKWLTPNEDOSYMAAFAL AODKYL WESWYLKDAVLE0.0 COPFTKALOUSWEYLGADTR NRY NEGUKHESY     Bidcouti   CUVEEAKDOSLSRRYSPAPVCKWLTPNEDOSYMAAFAL AODKYL WESWYLKDAVLE0.0 COPFTKALOUSWEYLGADTR NRY NEGUKHESY     Bidcouti   CUVEEAKDOSLSRRYSPAPVCKWLTPNEDOSYMAAFAL AODKYL WESWYLKDAVLE0.0 COPFTKALOUSWEYLGADTR NRY NEGUKHESY     Bidcouti   CUVEEAKDOSLSRRYSPAPKCKWLTPNEDOSYMAAFALLANDYKL WESWYLKDAVLE0.0 COPFTKALOUSWEYLKANDYKEN NEGUKHESY     Bidcouti   CUVEEAKDOSLSRRYSPAPKCKWLTPNEDOSYMAAFALLANDYKL WESWYLKANDYKEALLEGROGAFNKAFOLKANDYKKANDYKKANDYKKANDYKL     Bidcouti   CUVEEAKDOSLSRRYSPAPKCKWLTPNEDOSYMAAFALLANDYKL WESWYLKANDYKEALLEGROGAFNKAFOLKANDYKKAND								
PUCOUNT   COMEEGACDGRYSRYAAAPVCKWLTPREDOVSMAALALWNODKVL MESWYLKDAVLEG-OIPFNKAYGWTAFEYHGTDPRENKYGNKHSY     COCONT   CEWEEGACGLISRRYAAAPVCKWLTPREDOVSMAALALWNODKVL MESWYLKDAVLEG-OIPFNKAYGWTAFEYHGTDPRENKYGNEAHKSY     BECONTI   CEWEEGACGLISRRYAAAPVCKWLTPREDOVSMAAFALA ODKVY MESWYLKDAVLEG-OIPFNKAYGWTAFEYHGTDARFNAY NEAHKHST     BECONTI   CEWEEGACGLISRRYGPAPVCKWLTPREDOVSMAAFALA ODKVY MATWPYMKOAVLEG-OIPFNKAYGWTEFYGADARFRWYNEAHTHSG     BECONTI   CYVEEAKDOSLSRRYGPAPVCKWLTPREDOVSMAAFALA ODKVY MATWPYMKOAVLEG-OIPFNKAYGWTEFYGAVDARFRWYNEAHTHSG     VECAKDOSLSRRYGPAPVCKWLTPREDOVSMAAFALA ODKVY MATWPYMKOAVLEG-OIPFNKAYGWTEFYGAVDARFRWYNEAHTHSG   CVVEEAKDOSLSRRYGPAPVCKWLTPREDOVSMAAFALA ODKVY MATWPYMKOAVLEG-OIPFNKAYGWTEFYGAVDARFRWYNEAHTHSG     SCONT   210   220   230   240   260   270   280   280     TaCM   ESYNGTEG-LGTLVDVOGGGAATVAALAAHYPTIKGINFDLPHVISEAPPFPG-VTHVOGDMFKEVP-SODALLMKWILHDWSDAHGATLLKNG   50000T   EFYTGFEG-LGTLVDVOGGGAATVAALAHYPTIKGINFDLPHVISEAPPFPG-VTHVOGDMFKEVP-SODALLMKWILHDWSDAHGATLLKNG   50000T   5000T   5000T   5000T   5000T   5000T   5000T   5000T <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td>								
Occount   CEMEEGADGKLSRRYAAAPVCKWLTPNEDGVSMAALALMNODKY, MESWYLKDAVLDG-GLPFNKAYOMTAFEY, GTDARF NRVF NEDMKNHSY BKODMT     BKODMT   CEVEEGGCELLARRYGPAPVCKWLTPNEDGVSMAAFALAAGDKY HATWPYMKDAVLEG-GDPFTKALGMSWFEYAGADTRFNRVHEAMTHHSG CVVEEAKDGSLSRRYGPAPVCKWLTPNEDGVSMAAFALAAGDKY HATWPYMKDAVLEG-GDPFTKALGMSWFEYAGADTRFNRVHEAMTHHSG CVVEEAKDGSLSRRYGPAPVCKWLTPNEDGVSMAAFALAAGDKY HATWPYMKDAVLEG-GDPFTKALGMSWFEYAGADTRFNRVHEAMTHHSG CVVEEAKDGSLSRRYGPAPVCKWLTPNEDGVSMAAFALAAGDKY HATWPYMKCALLEGRGGATKAFGTTWFHACOTTRFNNL     210   220   230   240   200   270   280   290     TacM FacOMT   ESYKGFEG-LGTLVDVGGOVGATVAATAAHYPTIKGINFDLPHVISEAPPFPGVTHVGGDMFGKYP-SOAALLMKWILH DWSDHCATLLKKC ELYHOFGG-LGTLVDVGGOVGATVAATAAHYPTIKGINFDLPHVISEAPPFPGVTHVGGDMFGKYP-SOAALLMKWILH DWSDHCATLLKKC ELYHOFGG-LGTLVDVGGOVGATVAATAAHYPTIKGINFDLPHVISEAPPFPGVTHVGGDMFGKYP-AGOALLMKWILH DWSDHCATLLKKC EGYKGFEG-LGTLVDVGGOVGATVAATVASINFDLPHVISEAPPFPGVTHVGGDMFGKYP-AGOALLMKWILH DWSDHCATLLKKC EGYGGTT   EYYGFEG-VSTLVDVGGOVGATVAATVASINFDLPHVISEAPPFPGVKHVGGDMFGKYP-AGOALLMKWILH DWSDHCATLLKKC EGYGGTT   EYYGFEG-VSTLVDVGGOVGATVAATVSNYFHFHISGNFDLPHVISEAPPFPGVKHVGGDMFGKYP-AGOALLMKWILH DWSDHCATLLKKC EGYGGTT   EYYGFEG-VSTLVDVGGOVGATVAATVSNYFHFHISGNFDLPHVISEAPPFPGVKHVGGDMFGKYP-AGOALLMKWILH DWSDHCATLLKKC EYYGFEG-VSTLVDVGGOVGATHAATSNYFHISGNFDLPHVISEAPPFPGVKHVGGDMFGKYP-AGOALLMKWILH DWSDHCATLLKKC EYYGFEG-VSTLVDVGGOVGATHAATSNYFHISGNFDLPHVISEAPPFPGVKHVGGDMFGKYP-AGOALLMKWILH DWSDHCAALLKKC EYYGFEG-VSTLVDVGGOVGATHAATSNYFHISGNFDLPHVISEAPPFPGVKHVGGDMFGKYP-AGOALLMKWILH DWSDHCAALLKKC EYYGFEG-VSTLVDVGGOVGATHAATSNYFHISGNFDLPHVISEAPPFPGVKHVGGDMFGKYP-AGOALLMKWILH DWSDHCAALLKKC EYYGFEGA-VSTLVDVGGOVGATHAATSNYFHISGNFDLPHVISEAPPFPGVKHVGGDMFGKYP-AGOALLMKWILH DWSDHCAALLKKC EYYGFEGA-VSTLVDVGGOVGATHAATSNYFHISG								
Becontine   CEVEEGAGESLLARRYOPAPVCKWLTPNEDOOVSMOPLALL   DDKVMESWYHLKOVVLDG-GLPFNKAHGIIJEEY GKAARDRVK HAKNAKT     Becontine   CVVEEAKDOSLSRRYOPAPVCKWLTPNEDOOVSMAPFCLLADRVFTETWCYMKEALLEGRGGAFNKAFGTWFEHGAVDATES-GOPFTKALGONSWEEY GKAADTRNNLENKKHKOV     Picontine   270   270   270   270   270   270   270     Picontine   ESYNGFEGAL   DTVDEGAKOGOVGATVAAITANYPTIKO INFOLOPHVISEAPGTPG- VTHVGODMFGKVP-SODAILMKWILD OVSDGHGATLLKNC     Bicontine   ESYNGFEGAL   DTVDGOVGATVAAITANYPTIKO INFOLOPHVISEAPGTPG- VTHVGODMFGKVP-SODAILMKWILD OVSDGHGATLLKNC     Bicontine   ESYNGFEGAL   DTVDVGOVGATVAAITANYPTIKO INFOLOPHVISEAPGTPG- VTHVGODMFGKVP-SODAILMKWILD OVSDGHGATLLKNC     Bicontine   ESYNGFEGAL   DTVDVGOVGATVAAITANYPTIKO INFOLOPHVISEAPGTPG- VTHVGODMFGKVP-SODAILMKWILD OVSDGHGATLLKNC     Bicontine   ESYNGFEGAL   DTVDVGOVGATVAAITANYPTIKO INFOLOPHVISEAPFTPG- VTHVGODMFGKVP-SODAILMKWILD OVSDGHGATLLKNC     Socontine   ESYNGFEGAL   DTVDVGOVGATVAAITANYPTIKO INFOLOPHVISEAPFTPG- VTHVGODMFGKVP-SODAILMKWILD OVSDGHGATLLKNC     Socontine   ESYNGFEGAL   DTVDVGOVGATVAAITANYSRHPHIKISSANFEDEPVLSEAPFTPG- VTHVGODMFGKVP-SODAILMKWILD OVSDHGATLLKNC     Socontine   Socontine   Socontine   DVSDHGATLLKNC     Bicontine   Socontine   Socontine   Socontine   Socontine     Socontine								
Bucontz   CVVEEAK00SLSRRY0PAPVCKWLTPNED0VSWAAFALAADDKVHMATWPYWKDAVLE0.OPFTK LLOMSWFEYAGADTR FNRM YHEAUTHHS0     Bucontz   210   220   230   240   200   270   200   200     TSCM   ESYK0FE0.L0TLVDV000C0ATVAAITAHYPTIK0INFDLPHVISEAPPFP0.VTHV00OMFGKVP.SADAILMKWILH0WSDHCATLLKNC     Bucont   EYYK0FE0.L0TLVDV000C0ATVAAITAHYPTIK0INFDLPHVISEAPPFP0.VTHV00OMFGKVP.SADAILMKWILH0WSDHCATLLKNC     Bucont   EYYK0FE0.L0TLVDV000C0ATVAAITAHYPTIK0INFDLPHVISEAPPFP0.VTHV00OMFGKVP.SDAAILMKWILH0WSDAHCATLLKNC     Bucont   EYYK0FE0.L0TLVDV000C0ATLHAITSHHHIKOINFDLPHVISEAPPFP0.VTHV00OMFKSVP.ADDAILMKWILH0WSDAHCATLLKNC     Sucont   EYYK0FE0.VSTLVDV000C0ATLHAITSHHHIKOINFDLPHVISEAPPFP0.VGNVGNKFSVP.ADDAILMKWILH0WSDAHCATLLKNC     Zmcont   EYYK0FE0.VSTLVDV000C0ATLHAITSHHHIKOINFDLPHVISEAPPFP0.VGNVGNKFSVP.ADDAILMKWILH0WSDAHCATLLKNC     Zmcont   EYYK0FE0.VSTLVDV00C0ATLHAITSHHHIKOINFDLPHVISEAPPFP0.VGNVGNKFSVP.ADDAILMKWILH0WSDAHCATLLKNC     Zmcont   EYYK0FE0.VSTLVDV00C0ATLHAITSRHPHIKOINFDLPHVISEAPPFP0.VGNVGNKFSVP.ADDAILMKWILH0WSDAHCATLLKNC     Bucont   EYYK0FE0.VSTLVDV00C0ATHAITSRHPHIKOINFDLPHVISEAPPFP0.VGNVGNKFSVP.ADDAILMKWILH0WSDAHCATLLKNC     Bucont   EYYK0FE0.VSTLVDV00C0ATHAITSRHPHIKOINFDLPHVISEAPPFP0.VGNVGNKFSVP.ADDAILMKWILH0WSDAHCATLLKNC     Bucont   EYYK0FE0.VSTLVDV00C0ATHAITSRHPHIKOINFDLPHVISEAPPFP0.VGNVGNKFSVP.ADDAILMKWILH0WSDAHCATLLKNC								
BdcOM3   CVVEEAKD0SLSRRY0PAPVCKWLTPNED0VSMAPFCLLAQDRVFTETWCYMKEAILE0R0GAFNK4F0TTWFEHA0VDTRFNNLFNALKAHSV     210   220   20   20   20   20   20   20   20   20   20   20   200								
210220230240250260270280290TGMESYKGFE6-LGTLVDVGGGVGATVAALTAHYPTIKGINFDLPHVISEAPDFF6-VTHVGGDMFGKVP-SADALLMKWILHOWSDEHCATLLKNCB4C0MT4DLYPGFG0-LGTLVDVGGVGATVAALAPPAIKGVNFDLPHVISEAPDFF6-VTHVGGDMFGKVP-SADALLMKWILHOWSDEHCATLLKNCB4C0MT4DLYPGFG0-LGTLVDVGGVGATVAALAPPAIKGVNFDLPHVISEAPDFF6-VTHVGGDMFGKVP-SADALLMKWILHOWSDEHCATLLKNCB4C0MT4DLYPGFG0-LGTLVDVGGVGATVAALAPPAIKGVNFDLPHVISEAPDFF6-VTHVGGDMFGKVP-SADALLMKWILHOWSDEHCATLLKNCB4C0MT4EFYTGFDESVSTLVDVGG0GATTHAITSHPAIKGINFDLPHVISEAPPFF6-VGHVGGDMFKVP-SODALLMKWILHOWSDAHCATLLKNCB4C0MT4EFYTGFDE-VSTLVDVGG0GGATLHAITSRHPHISGVNFDLPHVISEAPPFF6-VGHVGGDMFKVP-SGOALLMKWILHOWSDAHCATLLKNCB4C0MT4EFYTGFDE-VSTLVDVGG0GGATHAATSKYPFIGB4C0MT4EFYTGFDE-VSTLVDVGG0GGATHAATSKYPFIGB4C0MT4EFYTGFDE-VSTLVDVGG0GGATHAATSKYPFIGB4C0MT4EFYTGFDE-VSTLVDVGG0GGATHAATSKYPFISGVNFDLPHVISEAPPFF6-VFHVG0DMFASVPFRGGDALLMKWILHOWSDAHCATLLKNCB4C0MT4EFYTGFDE-VSTLVDVGG0GGATHAATSKYPFISGVNFDLPHVISEAPPFF6-VFHVG0DMFASVPFRGGDALLMKWILLOWSDAHCATLLKNCB4C0MT4EFYTGFDE-VSTLVDVGG0GGATHAATSKYPFISGVNFDLPHVISEAPPFF6-VFHVG0DMFASVPFRGGDALLMKWILLOWSDAHCATLLKNCB4C0MT4EFYTGFDE-VSTLVDVGG0GGATHAATSKYPFISGVNFDLPHVISEAPPFF6-VFHVG0DMFASVPFRGGDALLMKWILLOWSDAHCATLLKNCB4C0MT5EFYTGFDE-VSTLVDVGG0GGATHAATSKYPFISGVNFDLPHVISEAPPFF6-VFHVG0DMFASVPFRGGDALLMKWILLOWSDAHCATLLKNCB4C0MT5EFYTGFDE-VSTLVDVGG0GGATHAATSKYPFISGVNFDLPHVISEAPPFF6-VFHVG0DMFASVPFRGGDALLMKWILLOWSDAHCATLLKNCB4C0MT5EFYTGFDE-VSTLVDVGG0GGATHAATSKYPFISGVNFDLPHVISEAPPFF6-VFHVG0DMFASVPFRGGDALLMKWILLOWSDAHCATLLKNCB4C0MT5EFYTGFDE-VSTLVDVGG0GGATHAATSKYPFISGVNFDLPHVISE								
Tight Esyköfes - Lött V0VGGGVGATVAAITAHYPTIKÖINFDLPHVISEAPPFPG - VTHVGGDMFGVP - SADAILMKWILHDWSDEHCATLLKNC Fight ElytgfGs - Lött V0VGGGVGATVAAIAAHYPTIKÖINFDLPHVISEAPPFPG - VTHVGGDMFGVP - SADAILMKWILHDWSDEHCATLLKNC ElytgfGs - Lött V0VGGGVGATVAAIAAHYPTIKÖINFDLPHVISEAPPFPG - VTHVGGDMFGVP - SADAILMKWILHDWSDAHCATLLKNC Scont EfytgfGes - Lött V0VGGGVGATLHAITSHHPAIKGINFDLPHVISEAPPFPG - VGHVGGDMFKSVP - AODAILMKWILHDWSDAHCATLLKNC Scont EfytgfGs - VST V0VGGGVGATLHAITSHHPAIKGINFDLPHVISEAPPFPG - VGHVGGDMFKSVP - AODAILMKWILHDWSDAHCATLLKNC Zmount DFYtGFGs - VST V0VGGGVGATLHAITSHHPAIKGINFDLPHVISEAPPFPG - VGHVGGDMFKSVP - AODAILMKWILHDWSDAHCATLLKNC BCONT DFYtGFGS - VST V0VGGGVGATLHAITSHPHISGVNFDLPHVISEAPPFPG - VGHVGGDMFASVP - AODAILMKWILHDWSDAHCATLLKNC BCONT DFYtGFGS - VST V0VGGGVGATLHAITSHPHISGVNFDLPHVISEAPPFPG - VGHVGGDMFASVP - AODAILMKWILHDWSDAHCATLLKNC BCONT DLYtGFGA - ST V0VGGGVGATLHAITSHYPGIRGVNFDLPHVISEAPPFPG - VGHVGGDMFASVP - AODAILMKWILHDWSDAHCATLLKNC BCONT DLYtGFGA - ST V0VGGGVGATLHAITSHYPFISGVNFDLPHVISEAPPFPG - VGHVGGDMFASVP - SODAILMKWILHDWSDAHCATLLKNC BCONT DLYtGFGA - ST V0VGGGVGATHAITSHYPFISGVNFDLPHVISEAPPFPG - VGHVGGDMFFKVP - SODAILMKWILHDWSDAHCATLLKNC BCONT DLYtGFGA - ST V0VGGGVGATHAITSHYPFISGVNFDLPHVISEAPPFPG - VGHVGGDMFFKVP - SODAILMKWILHDWSDAHCATLLKNC BCONT ELYTGFD - VKT V0VGGGVGAT HAITSHYPFISGVNFDLPHVISEAPPFPG - VGHVGGDMFFKVP - SODAILMKWILHDWSDAHCATLLKNC BCONT CLVVVFCILPVNPEATPKAGGVFHVDM MLAHNPGGRERYEFFEFALARGAGFTGVKSTYIYANAWAIEFTK BCONT G - KVVVVFCILPVNPEATPKAGGVFHVDM MLAHNPGGRERYEFFEFALARGAGFTGVKSTYIYANAWAIEFTK BCONT G - KVVVVFCULPVNFEATPKAGGVFHVDM MLAHNPGGRERYEFFEFALARGAGFTGVKSTYIYANAWAIEFTK BCONT G - KVIVVFCULPVNFEATPKAGGVFHVDM MLAHNPGGRERYEFFEFALARGAGFTGVKSTY	buoolario	OVVELARDOJEJR			a b k v l i l i w o i w k l		of the chaovor h	
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BicoMT4 ShCOMT EFYTGFDESVSTLVDVGGCVGATVGATVARHPAIKGINFDLPHVISEGIPFPGVCHVGGDMFGXVP.SCDATLMKWILHOWSDAHGATLLKNG ShCOMT EFYTGFDESVSTLVDVGGCVGATLHAITSHHBHIRGINFDLPHVISEAPPFPGVCHVGGDMFKSVP.AGDATLMKWILHOWSDAHGATLLKNG ZMCOMT DFYTGFEG.VSTLVDVGGCVGATLHAITSRHPHISGVNFDLPHVISEAPPFPGVCHVGGDMFKSVP.AGDATLMKWILHOWSDAHGATLLKNG PWCOMT EFYTGFDE.VSTLVDVGGCVGATLHAITSRHPHISGVNFDLPHVISEAPPFPGVCHVGGDMFKSVP.AGDATLMKWILHOWSDAHGATLLKNG PWCOMT EFYTGFDE.VSTLVDVGGCVGATLHAITSRHPHISGVNFDLPHVISEAPPFPGVCHVGGDMFKSVP.AGDATLMKWILHOWSDAHGATLLKNG BCGMT2 EFYTGFDA.XSTVVDVGGCVGATLHAITSRYPGINGVNFDLPHVISEAPPFPGVCHVGGDMFKSVP.AGDATLMKWILHOWSDAHGATLLKNG BCGMT3 ELYTGLDA.XSTVVDVGGCVGATLHAITSRYPGINGVNFDLPHVISEAPPFPGVCHVGGDMFKSVP.AGDATLMKWILHOWSDAHGATLLKNG BCGMT4 BCGMT4 BCGMT4 GCVVVLVCILPVVPGATVAAVSRPHIRGINVDLPHVISEAPPFPGVCHVGGDMFKSVP.SODATLMKWILHOWSDAHGATLLKNG BCGMT3 ELYTGLDA.VKTLVDVGGCVGATTRAITSRYPGINGVNFDLPHVISEAPPFPGVCHVGGDMFKSVP.SODATLMKWILHOWSDAHGATLLKNG BCGMT3 ELYTGLDA.VKTLVDVGGCVGATTRAITSRYPGINGVNFDLPHVISEAPPFPGVCHVGGDMFKSVP.SODATLMKWILHOWSDAHGATLLKNG BCGMT3 ELYTGLDA.VKTLVDVGGCVGATTRAITSRYPGINGVFHVDMVLAHNPGGRERYEHFFALXGGFAAMKTTYIYNAWAIEFTK BCGMT4 GCVVVLVCILPVNPEATPKAQGVFHVDMVLAHNPGGRERYEHFFALXGGFAAGFSGFKATYIYNAWAIEFTK BCGMT4 GCVVVVCILPVTTAAVKAQGVFHVDMVLAHNPGGRERYEHFFFALAKGAGFSGFKATYIYNAWAIEFTK BCGMT4 GCVVVVCILPVTTAAVKAQGVFHVDMVLAHNPGGRERYEHFFFALAKGAGFSGFKATYIYNAWAIEFTK BCGMT4 GCVVVVCILPVTTAAVKAQGVFHVDMVLAHNPGGKERYEHFFFALAKGAGFSGFKATYIYNAWAIEFTK BCGMT4 GCVVVVCILPVTTAAVKAQGVFHVDMVLAHNPGGKERYEHFEFELAKGAGFSGFKATYIYNAWAIEFTK BCGMT4 GCVVVVCILPVNPEATPKAQGVFHVDMVLAHNPGGKERYEHFEFELAKGAGFSGFKATYIYNAWAIEFTK BCGMT4 GCVVVVCILPVNPEATPKAQGVFHVDMVLAHNPGGKERYEHFEFELAKGAGFSGFKATYIYNAWAIEFTK BCGMT5 GCMT4 GCVVVVCILPVNPEATPKAQGVFHVDMVLAHNPGGKERYEHFEFELAKGAGFSGFKATYIYNAWAIEFTK BCGMT5 GCMT4 GCVVVVCILPVNPEATPKAQGVFHVDMVLAHNPGGKERYEHFEFELAKGAGFSGFKATYIYNAWAIEFTK BCGMT5 GCMT4 GCMT7 GCVVVVCILPVNPEATPKAQGVFHVDMVLAHNPGGKERYEHFEFELAKGAGFSGFKATYIYNAWAIEFTK BCGMT5 GCMT6 GCVVVVCILPVNPEATPKAQGVFHVDMVLAHNPGGKERYEHFEFEF	TaCM	ESYKGFEG - LGTL	VDVGGGVGATVAAI	TAHYPTIKGINFD	LPHVISEAPPFPG -	- VTHVGGDMFQKV	P-SADAILMKWILH	WSDEHCATLLKNC
SECONT EFYTGFDESVST VDVGGG GATLHAITSHHSHIRGINFDLPHVISEAPFFPG. VQHVGGDMFKSVP.AGDAILMKWILHOWSDAHGATLLKNG SACONT EFYTGFEG.VST VDVGGG GATLHAITSHHPGIKGINFDLPHVISEAPFFPG. VQHVGGDMFKSVP.AGDAILMKWILHOWSDAHGATLLKNG DFYTGFEG.VST VDVGGG GATLHAITSHPHGIKGINFDLPHVISEAPFFPG. VQHVGGDMFKSVP.AGDAILMKWILHOWSDAHGATLLKNG DFYTGFEG.VST VDVGGG GATLHAITSRPHGIRGVNFDLPHVISEAPFFPG. VCHVGGDMFKSVP.AGDAILMKWILHOWSDAHGATLLKNG DFYTGFEG.VST VDVGGG GATLHAITSRPHGIRGVNFDLPHVISEAPFFPG. VCHVGGDMFKSVP.AGDAILMKWILHOWSDAHGATLLKNG DLYTGFDA.STVVDVGGG GATLHAITSRPHGIRGVNFDLPHVISEAPFFPG. VCHVGGDMFKVP.AGDAILMKWILHOWSDAHGATLLKNG BGCOMT EFYTGFDD.VKT VDVGGG GATLHAITSRPHFISGVNFDLPHVISEAPFFPG. VCHVGGDMFKVP.SODAILMKWILHOWSDAHGATLLKNG BGCOMT EFYTGFDD.VKT VDVGGG GATLHAITSRPFIKGINYDLPHVISEAPFFPG. VCHVGGDMFKVP.SODAILMKWILHOWSDAHGATLLKNG BGCOMT EFYTGFDG.ISVVVGGG GATLHAITSRPFIKGINYDLPHVIADAPAYPGGRVGHVGGNMFEKVPSGO DAILMKWILHOWSDAHGATLLKNG BGCOMT SUTTGFDG.ISVVVGGG GATLHAITSRPFIKGINFDLPHVVSEAPAFPGGRVGHVGGNMFEKVPSG DAILMKWILHOWSDAHGATLLKNG BGCOMT 310 320 330 340 350 370 TAGM 6.KVVVVC CILPVNPEATPKAQGVFHVDM MLAHNPGGRERYEREFEALAKGAGFFAMKTTYIYANAWAIEFTK BGCOMT 6.KVVVVC CILPVNPEATPKAQGVFHVDM MLAHNPGGRERYEREFEALAKGAGFFAMKTTYIYANAWAIEFTK SGCOMT 6.KVVVVC CULPVTTDAVKAQGVFHVDM MLAHNPGGRERYEREFEELARGAGFFGVKSTYIYANAWAIEFTK SGCOMT 6.KVIVVC CULPVTTEAVFAQGVFHVDM MLAHNPGGRERYEREFEELARGAGFSGFKATYIYANAWAIEFTK SGCOMT 6.KVIVVC CULPVTTEAVFAQGVFHVDM MLAHNPGGKERYEREFEFLDLAKAGFSGFKATYIYANAWAIEFTK SGCOMT 6.KVIVVC CULPVTTEAVFAQGVFHVDM MLAHNPGGKERYEREFELALAKGAGFSGFKATYIYANAWAIEFTK SGCOMT 6.KVIVVC CULPVTEATPKAQGVFHVDM MLAHNPGGKERYEREFELALAKGAGFSGFKATYIYANAWAIEFTK SGCOMT 6.KVIVVC CULPVNFEATPKAQGVFHVDM MLAHNPGGKERYEREFELALKGAGFSGFKATYIYANAWAIEFTK SGCOMT 6.KVIVVC CULPVNFEATPKAQGVFHVDM MLAHNPGKERYEREFELALKGAGFSGFKATYIYANAWAIEFTK SGCOMT 6.KVIVVC CULPVNFEATPKAQGVFHVDM MLAHNPGKERYEREFELALKGAGFFAVRATYIYANAWAIEFTK SGCOMT 6.KVIVVC CULPVNFEATPKAQGVFHVDM MLAHNPGKERYEREFELALKGAGFFAVRATYIYANAWAIEFTK SGCOMT 6.KVIVVC CULPVNFEATPKAQGVFHVDM MLAHNPGKERYEREFELALKGAGFFAVRATYIYANAWAIEFTK SGCOMT 6.KVI								
SCONT DFYTGFEG-VSTLVDVGGOVGATLHAITSHHPQIKGINFDLPHVISEAPPFPGVQHVGGDMFKSVP-AGDAILMKWILHDWSDAHCATLLKNC DFYTGFEG-VSTLVDVGGOVGATLHAITSRHPHISGVNFDLPHVISEAPPFPGVPHVGGDMFKSVP-AGDAILMKWILHDWSDAHCATLLKNC DFYTGFEG-VSTLVDVGGOVGATLHAITSRHPHISGVNFDLPHVISEAPPFPGVPHVGGDMFASVP-AGDAILMKWILHDWSDAHCATLLKNC DFYTGFEG-VSTLVDVGGOVGATLHAITSRYPHISGVNFDLPHVISEAPPFPGVPHVGGDMFASVPRGGDAILMKWILHDWSDAHCATLLKNC DFYTGFDA-XSTLVDVGGOVGATLHAITSRYPHISGVNFDLPHVISEAPPFPGVPHVGGDMFASVPRGGDAILMKWILHDWSDAHCATLLKNC DFYTGFDA-XSTLVDVGGOVGATLHAITSRYPHISGVNFDLPHVISEAPPFPGVPHVGGDMFASVPRGGDAILMKWILHDWSDAHCATLLKNC BGCOMT BGCOMT DLYTGFDA-XSTLVDVGGOVGATHAITSRYPFIKGINYDLPHVISEAPPFPGVPHVGGDMFASVPRGGDAILMKWILHDWSDAHCATLLKNC HTT DVGGOVGATTHAITSRYPSIKGINFDLPHVVAEAPAYPGGRVGHVGGDMFEKVPSGDAILMKWILHDWSDAHCATLLKNC 310 320 330 340 350 370 TaCM OKVVVVCCILPVNPEATPKAGGVFHVDMIMLAHNPGGRERYERFEFALAKGAGFTAMKTTYIYANAWAIEFTK SCOMT OKVVVVCCILPVNPEATPKAGGVFHVDMIMLAHNPGGRERYERFEFALAKGAGFTGVKATYIYANAWAIEFTK SCOMT OKVVVVCCULPVNTEAVPKAGGVFHVDMIMLAHNPGGRERYERFEFALAKGAGFTGVKATYIYANAWAIEFTK SCOMT OKVVVVCCVLPVNTEAVPKAGGVFHVDMIMLAHNPGGRERYERFEFALAKGAGFTGVKATYIYANAWAIEFTK SCOMT OKVVVVCCVLPVNTEAVPKAGGVFHVDMIMLAHNPGGRERYERFEFALAKGAGFSGFKATYIYANAWAIEFTK SCOMT OKVVVVCCVLPVNTEAVPKAGGVFHVDMIMLAHNPGGRERYERFEFALAKGAGFTGVKATYIYANAWAIEFTK SCOMT OKVVVVCCVLPVNTEAVPKAGGVFHVDMIMLAHNPGGRERYERFEFALAKGAGFSGFKATYIYANAWAIEFTK SCOMT OKVVVVCCVLPVNTEAVPKAGGVFHVDMIMLAHNPGGRERYERFEFALAKGAGFSGFKATYIYANAWAIEFTK SCOMT OKVVVVCCVLPPSDATAREGOVFHVDMIMLAHNPGGRERYERFEFELAKGAGFTGVKATYIYANAWAIEFTK SCOMT OKVVVVCCVLPPSDATAREGOVFHVDMIMLAHNPGGKERYERFEFELAKGAGFTGVKATYIYANAWAIEFTK SCOMT OKVVVVCCVLPESSDATAREGOVFHVDMIMLAHNPGGKERYERFEFELAKGAGFTGVKATYIYANAWAIEFTK SCOMT OKVVVVCCVLPPSDATAREGOVFHVDMIMLAHNPGGKERYERFEFELAKGAGFTGVKATYIYANAWAIEFTK SCOMT OKVVVVCCVLPPSDATAREGOVFHVDMIMLAHNPGGKERYERFEFELAKGAGFTGVKATYIYANAWAIEFTK SCOMT OKVVVVCCVLPVDETSDAVSLLAYSPGGKERYERFEFELAKGAGFTGVKATYIYANAWAIEFTK SCOMT OKVVVVCCVLPVDETGAGUISVDVSLLAYSPGGKERYERFEFELAKGAGFTGVKATYIYANAWAIEFTK SCOMT SKVINCCCIPVNEAF								
Zmcomt PuicoutDFYTGFEG-VST VDV0G0VGATLHAITSRHPHISGVNFDLPHVISEAPPFPG-VRHVGGDMFASVP-AGDAILMKWILHDWSDAHCATLLKNC EFYAGFEG-VGTLDDVGGGVGATLHAITSRYPGIRGVFDLPHVISEAPPFPG-VCHVGGDMFASVP-AGDAILMKWILHDWSDAHCATLLKNC DLYGFDD-ASTVDVGGGVGATVAAVVSRHPHRGINYDLPHVISEAPPFPG-VCHVGGDMFASVP-AGDAILMKWILHDWSDAHCAAILKNC BdCOMT1BdCOMT1 EFYTGFDD-VKTLVDVGGGVGATVAAVVSRHPHRGINYDLPHVISEAPPFPG-VCHVGGDMFASVP-SGDAILMKWILHDWSDAHCAAILKNC ELYKGFDD-VKTLVDVGGGVGATVAAVSSRHPHRGINYDLPHVISEAPPFPG-VCHVGGDMFASVP-SGDAILMKWILHDWSDAHCAAILKNC BdCOMT2BdCOMT3 ELYKGFDD-VKTLVDVGGGVGATVAAVSSRHPHRGINYDLPHVISEAPPFPG-VCHVGGDMFASVP-SGDAILMKWILHDWTDDHCMMLLRNC BdCOMT3BdCOMT3 ELYKGFEG-ISVLVDVAGGVGATHAITSRYPSIKGINFDLPHVVAEAPAYPGGRVGHVGGDMFEKVPSGOAILMKWILNCFSDKACATLLKNC310320330340350370TaCM FaCOMT6-KVVLVECILPVNPEATPKAGGVFHVDM NLAHNPGGRERYERFEEALAKGAGFTGVKATYIVANAWAIEFTK SCOMT6-KVVLVECILPVNPEATPKAGGVFHVDM NLAHNPGGRERYERFEEALAKGAGFTGVKATYIVANAWAIEFTK SCOMTSCOMT SCOMT6-KVVLVECULPVNTEAVPKAGGVFHVDM NLAHNPGGRERYERFEFBLAKGAGFTGVKATYIVANAWAIEFTK SCOMT 6-KVVLVECULPVNTEAVPKAGGVFHVDM NLAHNPGGRERYERFEFBLAKGAGFSKATYIVANAWAIEFTK SCOMT 6-KVVLVECULPVNTEAVPKAGGVFHVDM NLAHNPGGRERYERFEFBLAKGAGFSKATYIVANAWAIEFTK SCOMT 6-KVIVVECULPVNTEAVPKAGGVFHVDM NLAHNPGGRERYERFEFBLAKGAGFSKATYIVANAWAIEFTK SCOMT 6-KVIVVECULPVNTEAVPKAGGVFHVDM NLAHNPGGRERYERFEFBLAKGAGFSKATYIVANAWAIEFTK BCOMT3BdCOMT1 6-KVIVVECULPVNTEAVPKAGGVFHVDM NLAHNPGKERYERFEFELAKGAGFSKATYIVANAWAIEFTK BCOMT3BdCOMT3 6-KVIVVECULPVNTEAVPKAGGVFHVDM NLAHNPGKERYERFEFELAKGAGFSKATYIVANAWAIEFTK BdCOMT3FJGURGSSLAMGGUGGAVFHVDM NLAHNPGKERYERFEFELAKGAGFSKATYIVANAWAIEFTK BGCOMT3BdCOMT1 6-KVIVVECULPVNTEAVPKAGGVFHVDM NLAHNPGKERYERFEFELAKGAGFAVATYIVANAWAIEFTK BGCOMT3 <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td>								
PWICOMT   EFYAGFEG.VGT LVDVGGGVGATLHAITSRYPGIRGVNFDLPHVISEAPPFPG.VEHVGGDMFKAVP.AGDAILMKWILHDWSDAHGAAILKNC     BdCOMT   LYTGFDA.ASTVVDVGGGVGATVAAVVSRPHIRINGUPHVISEAPPFPG.VEHVGGDMFKKVP.AGDAILMKWILHDWSDAHGAAILKNC     BdCOMT2   ELYTGLDC.ISTLVDVGGGVGATIAAISKYPHIRSVPFIFVISSAPTCPG.VCHVGGDMFKKVP.SGDAILMKWILHDWSDAHGAAILKNC     BdCOMT2   ELYTGLDC.ISTLVDVGGGVGATIAAISKYPTIKGINYDLPHVISSAPTCPG.VCHVGGDMFKKVP.SGDAILMKWILHDWSDAHGALLKNC     BdCOMT3   ELYTGLDC.ISTLVDVGGGVGATTHAITSKYPTIKGINYDLPHVISAPAYPGGRVGHVGGDMFEKVPSG.DAILMKWILHDWSDAHGALLKNC     BdCOMT3   SLUKFGE.ISVLVDVGGVGATTHAITSKYPTIKGINYDLPHVVAEAPAYPGGRVGHVGGDMFEKVPSG.DAIFMKWILNGFSDKDCATLLKNC     BdCOMT3   SLUKFEG.ISVLVDVGGVGATTHAITSKYPTIKGINFDLPHVVAEAPAYPGGRVGHVGGDMFEKVPSG.DAIFMKWILNGFSDKDCATLLKNC     BdCOMT3   GKVVLVCCILPVNPEATPKAGGVFHVDM MLAHNPGGRERYEREFEALAKGAGFTGVKATYIYANAWAIEFTK     SCOMT   GKVVLVCCILPVNPEATPKAGGVFHVDM MLAHNPGGRERYEREFEALAKGAGFTGVKATYIYANAWAIEFTK     SCOMT   GKVIVVCCULPVTTDAVPKAGGVFHVDM MLAHNPGGKERYEREFERLAKGAGFSGFKATYIYANAWAIEFTK     SCOMT   GKVIVVCCULPVNTEAVPKAGGVFHVDM MLAHNPGGKERYEREFERELAKGAGFTGFKATYIYANAWAIEFTK     SGCOMT   GKVIVVCCULPVNTEAVPKAGGVFHVDM MLAHNPGGKERYEREFERELAKGAGFTGFKATYIYANAWAIEFTK     SGCOMT   GKVIVVCCULPVNTEAVPKAGGVFHVDM MLAHNPGGKERYEREFERELAKGAGFTGFKATYIYANAWAIEFTK     SGCOMT   GKVIVVCCULPVNTEAVPKAGGVFHVDM MLAHNPGGKERYEREFERELAKGAGFTGFKATYIYANAWAIEFTK     SGCOMT   GKVIVVCCULPVNTEATPKAGGVFHVDM								
OscOMT   DLYTGFDA.AST VVDVGGGVGATVAAVVSRHPHIRGINYDLPHVISEAPPFPG.VEHVGGDMFASVPRGGDAILMKWILLDWIDDFARLLKNC     BdCOMT1   EFYTGFDD.VKT VVDVGGGVGATIRAIISKYPHISGINYDLPHVISAAPTCPG.VQHIGGDMFAKVP.SGDAILMKWILLDWIDDFDHCMMLLRNC     BdCOMT3   ELYKGFED.SVLVDVGGGVGATIRAIISKYPHISGINYDLPHVISAAPTCPG.VQHVGGDMFEKVPSGAAILMKWILLDWIDDFDHCMMLLRNC     BdCOMT3   ELYKGFED.SVLVDVGGGVGATIRAIISKYPHISGINYDLPHVIAAPAYPGGRVQHVGGDMFEKVPSGAAILMKWILLDWIDDFDHCMMLLRNC     BdCOMT3   ELYKGFED.SVLVDVGGGVGATTHAITSKYPSIKGINFDLPHVVAEAPAYPGGRVQHVGGDMFEKVPSG.AAILMKWILLDWIDDFSDKDCATLLKNC     310   320   330   340   350   370     TaCM   G.KVVLVECILPVNPEATPKAQGVFHVDM MLAHNPGGRERYEREFEALARGAGFTGVKATYIYANAWAIEFTK     BdCOMT3   G.KVVLVECILPVNPEATPKAQGVFHVDM MLAHNPGGRERYEREFEALARGAGFTGVKATYIYANAWAIEFTK     SbCOMT   G.KVVVVECUPVTTDAVPKAQGVFHVDM MLAHNPGGRERYEREFEAKAAGFSGFKATYIYANAWAIEFTK     SbCOMT   G.KVIVVECUPVTTDAVPKAQGVFHVDM MLAHNPGGRERYEREFEAKAAGFSGFKATYIYANAWAIEFTK     SbCOMT   G.KVIVVECUPVTEATPKAQGVFHVDM MLAHNPGGKERYEREFEELARGAGFTGFKATYIYANAWAIEFTK     SbCOMT   G.KVIVVECUPVTEATPKAQGVFHVDM MLAHNPGGKERYEREFEELAKGAGFTGFKATYIYANAWAIEFTK     SbCOMT   G.KVIVVECUPVTEATPKAQGVFHVDM MLAHNPGGKERYEREFEELAKGAGFTGFKATYIYANAWAIEFTK     SbCOMT   G.KVIVVECUPVTEATPKAQGVFHVDM MLAHNPGGKERYEREFEELAKGAGFTGFKATYIYANAWAIEFTK     SbCOMT   G.KVIVVECUPVTEATPKAQGVFHVDM MLAHNPGGKERYEREFEELAKGAGFTGFKATYI								
BdcOMT1   EFYTGFDD.VKT_VDVGGGVGATIRALISKYPHISGVNFDLPHVISSAPTCPG.VGHIGGDMFKKVP.SGDAILMKWILHDWTDDHCMMLLRNC     BdcOMT3   ELYTGLDD.ISTLIDVGGGGATHAVTSKYPTIKGINYDLPHVIADAPAYPGGRVGHVGGNMFEKVPSGADAILMKWILHDWTDDHCMMLLRNC     BdcOMT3   310   320   340   350   340   370     TaCM   6.KVVLVCCILPVNPEATPKAGGVFHVDM NLAHHPGGRERYEREFEALAKGAGFAGMKTYIYANAWAIEFTK   6.KVVLVCCILPVNPEATPKAGGVFHVDM NLAHHPGGRERYEREFEALAKGAGFAGWKTYIYANAWAIEFTK     BdcOMT4   6.KVVLVCCILPVNPEATPKAGGVFHVDM NLAHHPGGRERYEREFEALAKGAGFSGFKATYIYANAWAIEFTK     BdcOMT4   6.KVVLVCCILPVNPEATPKAGGVFHVDM NLAHHPGGRERYEREFEALAKGAGFSGFKATYIYANAWAIEFTK     BdcOMT4   6.KVVLVCCILPVNPEATPKAGGVFHVDM NLAHHPGGRERYEREFEALAKGAGFSGFKATYIYANAWAIEFTK     BdcOMT4   6.KVVLVCCILPVNPEATPKAGGVFHVDM NLAHHPGGRERYEREFEALAKGAGFSGFKATYIYANAWAIEFTK     SdcOMT   6.KVIVVCCVLPVTTDAVFKAGGVFHVDM NLAHHPGGKERYEREFERLAKGAGFSGFKATYIYANAWAIEFTK     SdcOMT   6.KVIVVCCVLPVTTAVFKAGGVFHVDM NLAHHPGGKERYEREFERLAKGAGFTGFKATYIYANAWAIEFTK     SdcOMT   6.KVIVVCCVLPVTEAVFKAGGVFHVDM NLAHHPGGKERYEREFERLAKGAGFTGFKATYIYANAWAIEFTK     SdcOMT   6.KVIVVCCVLPVTEAVFKAGGVFHVDM NLAHHPGGKERYERFERELAKGAGFTGFKATYIYANAWAIEFTK     SdcOMT   6.KVIVVCCVLPVTEAVFKAGGVFHVDM NLAHPGGKERYERFEREFERE     SdcOMT   6.KVIVVCCVLPVTEAVFKAGGVFHVDM NLAHPGGKERYERFERELAKGAGFTGFKATYIYANAWAIEFTK     SdcOMT   6.KVIVVCCVLPVTEAVFKAGGVFHVDM NLA								
BdCOMT2   ELYTELD6.IGT_LDVGGG_CATIHAVTSKYPTIKGINYDLPHVIADAPAYPGGRVGHVGGNMFEKVPSGADAILMKWILNCFRDEECATLLKNC     BdCOMT3   BLUKGFEG.ISVLVVAGGVGATHAITSKYPSIKGINFDLPHVVAEAPAYPGGRVGHVGGNMFEKVPSG.DAILMKWILNCFSDKDCATLLKNC     310   320   330   340   360   370     TacM   G.KVVLVCILPVNPEATPKAQGVFHVOM MLAHNPGGRERYEREFEALARGAGFAGVKATYIYANAWAIEFTK   G.KVVLVCILPVNPEATPKAQGVFHVOM MLAHNPGGRERYEREFEALARGAGFTGVKATYIYANAWAIEFTK     BdCOMT4   G.KVVLVCILPVNPEATPKAQGVFHVOM MLAHNPGGRERYEREFEELARGAGFTGVKATYIYANAWAIEFTK     SdCOMT   G.KVVLVCILPVNPEATPKAQGVFHVOM MLAHNPGGRERYEREFEELARGAGFTGVKATYIYANAWAIEFTK     SdCOMT   G.KVVLVCULPVNTEAVPKAQGVFHVOM MLAHNPGGRERYEREFEELARGAGFTGVKATYIYANAWAIEFTK     SdCOMT   G.KVIVVCULPVNTEAVPKAQGVFHVOM MLAHNPGGRERYEREFERDLAKGAGFSGFKATYIYANAWAIEFTK     SdCOMT   G.KVIVVCULPVNTEAVPKAQGVFHVOM MLAHNPGGKERYEREFERLAKGAGFSGFKATYIYANAWAIEFTK     SdCOMT   G.KVIVVCULPVNTEAVPKAQGVFHVOM MLAHNPGGKERYEREFERLAKGAGFSGFKATYIYANAWAIEFTK     SdCOMT   G.KVIVVCULPVNTEAVPKAQGVFHVOM MLAHNPGGKERYEREFERLAKGAGFGFKATYIYANAWAIEFTK     SdCOMT   G.KVIVVCULPVNTEAVPKAQGVFHVOM MLAHNPGGKERYEREFERLAKGAGFAGVKATYIYANWAIEFTK     SdCOMT   G.KVIVVCULPVNTEAVPKAQGVFHVOM MLAHNPGGKERYERFERELAKGAGFGAAVKATYIYANWAIEFTK     SdCOMT   G.KVIVVCULPVNTEAVPKAQGVFHVOM MLAHNPGGKERYERFEREFERLAKGAGFGAAVKATYIYANWAIEFTK     SdCOMT   G.KVIVVCULPVNTEAVPKAQGVFHVOM MLAHNPG								
BICOMT3   ELYKGFEG. ISVLVDVAGOVGATTHAITSRYPSIKGINFDLPHVVAEAPAYPGGRVQHVGGDMFEKVPSG.DAIFMKWILNGFSDKDCATLLKNC     310   320   330   340   350   360   370     TacM   G. KVVLVE CILPVNPEATPKAQGVFHVDM NLAHNPGGRERYEREFEALAKGAGFAAMKTTYIYANAWAIEFTK     BdCOMT3   G. KVVLVE CILPVNPEATPKAQGVFHVDM NLAHNPGGRERYEREFEALARGAGFTGVKATYIYANAWAIEFTK     BdCOMT4   G. KVVLVE CILPVNPEATPKAQGVFHVDM NLAHNPGGRERYEREFEALARGAGFTGVKATYIYANAWAIEFTK     BdCOMT5   G. KVVLVE CULPVNEAKPSSGVFHVDM NLAHNPGGRERYEREFEALARGAGFTGVKATYIYANAWAIEFTK     SbCOMT   G. KVIVVE CULPVNTEAVPKAQGVFHVDM NLAHNPGGRERYEREFEALARGAGFSGFKATYIYANAWAIEFTK     SbCOMT   G. KVIVVE CULPVNTEAVPKAQGVFHVDM NLAHNPGGRERYEREFEAKAAGFSGFKATYIYANAWAIEFTK     SbCOMT   G. KVIVVE CULPVNTEAVPKAQGVFHVDM NLAHNPGGKERYEREFEELAKGAGFTGFKATYIYANAWAIEFTK     SbCOMT   G. KVIVVE CULPVNTEATPKAQGVFHVDM NLAHNPGGKERYEREFEELAKGAGFTGFKATYIYANAWAIEFTK     SbCOMT   G. KVIVVE CULPVNTEATPKAQGVFHVDM NLAHNPGGKERYEREFEELAKGAGFTGFKATYIYANAWAIEFTK     BdCOMT3   G. KVIVVE CULPVNPEATPKAQGVFHVDM NLAHNPGGKERYEREFEELAKGAGFTGFKATYIYANAWAIEFTK <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td>								
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Tack   6 - KVVLVECILPVNPEATPKAQQVFHVDM INLAHNPGGRERYEREFEALAKGAGFAAMKTTYIYANAWAIEFTK     FacOMT   6 - KVVLVECILPVNPEAKPSSQVFHVDM INLAHNPGGRERYEREFEALARGAGFTGVKATYIYANAWAIEFTK     BdCOMT4   6 - KVVLVECILPVNPEATPKAQQVFHVDM INLAHNPGGRERYEREFEALARGAGFTGVKATYIYANAWAIEFTK     ScOMT   6 - KVVLVECILPVNPEATPKAQQVFHVDM INLAHNPGGRERYEREFEALARGAGFTGVKATYIYANAWAIEFTK     ScOMT   6 - KVIVECULPVTDAVFKAQQVFHVDM INLAHNPGGRERYEREFEDLAKGAGFSGFKATYIYANAWAIEFTK     ScOMT   6 - KVIVECULPVTTAVFKAQQVFHVDM INLAHNPGGRERYEREFEDLAKGAGFSGFKATYIYANAWAIEFTK     ScOMT   6 - KVIVECULPVTTAAPFKAQQVFHVDM INLAHNPGGRERYEREFEDLAKGAGFSGFKATYIYANAWAIEFTK     ScOMT   6 - KVIVECULPVTTAAPFKAQQVFHVDM INLAHNPGGKERYEREFELAKGAGFTGFKATYIYANAWAIEFTK     ScOMT   6 - KVIVECULPSSDATAREQOVFHVDM INLAHNPGGKERYEREFETELAKGAGFTGFKATYIYANAWAIEFTK     SdCOMT   6 - KVIVECULPSSDATAREQOVFHVDM INLAHNPGGKERYEREFERELAKGAGFTGFKATYIYANAWAIEFTK     BdCOMT3   6 - KVIVECULPSSDATAREQOVFHVDM INLAHNPGGKERYEREFERELAKGAGFTGFKATYIYANAWAIEFTK     BdCOMT3   6 -	B4COMT3	ELYKGFEG - ISVL	VDVAGGVGATTHAI	TSRYPSIKGINFD	LPHVVAEAPAYPGG	RVQHVGGDMFEKVI	PSG - DAIFMKWILN	CFSDKDCATLLKNC
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FacOMT 6 - KVVLVE CILPVNPEAKPSSGGVFHVOM NLAHNPGGRERY EREFEALARGAGFTGVKSTYIYANAWAIEFTK BdCOMT4 6 - KVVIVE CILPVNPEATPKAGGVFHVOM NLAHNPGGRERY EREFELARGAGFTGVKATYIYANAWAIEFTK SdCOMT 6 - KVIVE CVLPVTTDAVPKAGGVFHVOM NLAHNPGGRERY EREFELARGAGFSGFKATYIYANAWAIEFTK 7 - KVIVE CVLPVTTAVPKAGGVFHVOM NLAHNPGGRERY EREFHDLAKGAGFSGFKATYIYANAWAIEFTK 7 - KVIVE CVLPVTEATPKAGGVFHVOM NLAHNPGGRERY EREFELAKGAGFSGFKATYIYANAWAIEFTK 7 - KVIVE CVLPVTEATPKAGGVFHVOM NLAHNPGGKERY EREFELAKGAGFSGFKATYIYANAWAIEFTK 7 - KVIVE CVLPVTEATPKAGGVFHVOM NLAHNPGGKERY EREFERELAKGAGFSGFKATYIYANAWAIEFTK 8 - KVIVE CVLPVESDATAREGOVFHVOM NLAHNPGGKERY EREFERELAKGAGFSGFKATYIYANAWAIEFTK 8 - KVIVVE CVLPESSDATAREGOVFHVOM NLAHNPGGKERY EREFERELAKGAGFAGFKATYIYANAWAIEFTK 8 - KVIVVE CVLPESSDATAREGOVFHVOM NLAHNPGGKERY EREFERELAKGAGFAGVKATYIYANAWAIEFTK 8 - KVINVECILPVNPEATPRARMAFED DM NLTY PGGKERY EREFEVELAKGAGFAGVKATYIYANAWAIEFTK 8 - KVINVECILPVNPETPSAGGLID DMSLLAYS PGGKERYL RELEKLAKGAGFAGVKATYIYANFWAIEYTK 8 - KVINLECIMPVNPETHGAGGLISVOVSLLAYS PGGKERYL RELEKLAKGGGAGVGGGY 8 - KVINLECIMPVNEGTGGY 8 - KVINLEGIMGGY 8 - KVINLEGIMGGY	TaCM	G - KVVLVECILPV	NPEATPKAQGVFHV	DMINLAHNPGGRE	RYEREFEALAKGAG	FAAMKTTYIYANAN	NAIEFTK	
BdcOMT4   G+KVVIVE CILPVNPEATPKAQGVFHVOM INLAHNPGGKERYEREFELARGAGFTGVKATYIYANAWAIEFTK     ScOMT   G-KVVIVE CVLPVTTDAVPKAQGVFHVOM INLAHNPGGRERYEREFRDLAKGAGFSGFKATYIYANAWAIEFTK     ScOMT   G-KVIVVE CVLPVTTEATPKAQGVFHVOM INLAHNPGGRERYEREFRDLAKGAGFSGFKATYIYANAWAIEFTK     OxCOMT   G-KVIVVE CVLPVTTEATPKAQGVFHVOM INLAHNPGGRERYEREFRELAKGAGFSGFKATYIYANAWAIEFTK     OxCOMT   G-KVIVVE CVLPVTTEATPKAQGVFHVOM INLAHNPGGKERYEREFRELAKGAGFSGFKATYIYANAWAIEFTK     OxCOMT   G-KVIVVE CVLPVTTEATPKAQGVFHVOM INLAHNPGGKERYEREFRELAKGAGFTGFKATYIYANAWAIEFTK     BdCOMT1   G-KVIVVE CVLPESSDATAREGGVFHVOM INLAHNPGGKERYEREFRELAKGAGFTGFKATYIYANAWAIEFTK     BdCOMT2   G-KVIVVE CVLPESSDATAREGGVFHVOM INLAHNPGGKERYEREFRELAKGAGFTGFKATYIYANAWAIEFTK     BdCOMT3   G-KVIVVE CVLPESSDATAREGGVFHVOM INLAHNPGGKERYEREFRELAKGAGFAAVKATYIYANAWAIEFTK     BdCOMT4   G-KVIVVE CVLPESSDATAREGGVFHVOM INLAHNPGGKERYEREFRELAKGAGFAAVKATYIYANWAIEFTK     BdCOMT2   G-KVIVVE CVLPESSDATAREGOVFHVOM INLAHNPGGKERYEREFRELAKGAGFAAVKATYIYANWAIEFTK     BdCOMT3   G-KVIVECILPVNPEATPRARMAFED MILAHNPGGKERYEREFERVEREFRELAKGAGFAAVKATYIYANWAIEFTK     BdCOMT4   G-KVIVECILPVNPETPHAQGIIS VDVSLAY PGGKERYEREFERELAKGAGFAAVKATYIYANWAIEFTK         BdCOMT5   G-KVINECILPVNPETPHAQGIIS VDVSLAY PGGKERYEREFERELAKGAGFAAVKATYIYANWAIEFTK         BdCOMT3   G-KVINECILPVNPETPHAQGUIS VDVSLAY PGGKERYEREFERELAKGAGFAAVKATYIYANGKAIEYTK         Figure 3 Seq								
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ZmcOMT   G - KVIVVE CVL PVNTEATPKAQGVFHVOM INLAHNPGGKERYEREFRELAKGAGFSGFKATYIYANAWAIEFIK     PVICONT   G - KVIAVECIL PVNPEATPKAQGVFHVOM INLAHNPGGKERYEREFRELAKGAGFTGFKATYIYANAWAIEFIK     OsCOMT   G - KVIAVECIL PVNPEATPKAQGVFHVOM INLAHNPGGKERYEREFRELAKGAGFTGFKATYIYANAWAIEFIK     BdCOMT1   G - KVIAVECIL PVNPEATPRARMAFED DM INLTY PGGKERYEREFRELAKGAGFTGFKATYIYANAWAIEFIK     BdCOMT2   G - KVINVECIL PVNPEATPRARMAFED DM INLTY PGGKERYEREFRELAKGAGFAAVKATYIYANSWAIEYTK     BdCOMT3   G - KVINVECIL PVNPEATPRARMAFED DM INLTY PGGKERYEREFEVLAKGAGFAAVKATYIYANSWAIEYTK     BdCOMT3   G - KVINVECIL PVNPEATPRARMAFED DM INLTY PGGKERYEREFEVLAKGAGFAAVKATYIYANSWAIEYTK     BdCOMT3   G - KVINVECIL PVNPETPHOAQOLI SVDVSLLAYS PGGKERYEREFEKLAKGAGFAAVKATYIYANSWAIEYTK     Figure 3 Sequence alignment of the COMT family of Brachypodium distachyon and other species. Amino acid sequence comparison of the four COMT proteins in <i>B. distachyon</i> with functionally characterized COMT proteins in <i>Triticum aestivum</i> (TaCM), <i>Festuca arundinacea</i> (FaCOMT), Sorghum bicolor (Sbbmr12), Zea mays (Zmbm3), Panicum virgatum (PViCOMT), and Oryza sativa (OsCOMT). The conserved motif for the	SECOMT	GGKVIVVECVLPV	TTDAVPKAQGVFHV	DMIMLAHNPGGRE	RYEREFROLAKAAG	FSGFKATYIYANAN	NAIEFIK	
Pwicomt   6 - KVIAVE CILPVNPEATPKAQOVFHYDMINLAHNPOOKERYEREFEELAKGAGFTOFKATYIYANAWAIEFTK     Occomt   6 - KVVVVCULPESSDATAREGOVFHYDMINLAHNPOOKERYEREFRELARGAGFTOFKATYIYANAWAIEFTK     Bdcomts   6 - KVIVVECULPESSDATAREGOVFHYDMINLAHNPOOKERYEREFRELARGAGFTOFKATYIYANAWAIEFTK     Bdcomts   6 - KVIVVECULPESSDATAREGOVFHYDMINLAHNPOOKERYEREFRELARGAGFTOFKATYIYANAWAIEFTK     Bdcomts   6 - KVIVVECULPVNPETPRARMAFEDDMINLTY POOKERYEREFRELARGAGFAOVKATYIYANAWAIEFTK     Bdcomts   6 - KVINVECILPVNPETPRARMAFEDDMINLTY POOKERYEREFRELARGAGFAOVKATYIYANAWAIETK     Bdcomts   6 - KVINVECILPVNPETPRARMAFEDDMINLTY POOKERYEREFRELARGAGFAOVKATYIYANAWAIETK     Bdcomts   6 - KVINVECILPVNPETPRARMAFEDDMINLTY POOKERYEREFRELARGAGFAOVKATYIYANFWAIEYTK     Bdcomts   6 - KVINVECILPVNPETPRARMAFEDDMINLTY POOKERYERFEREFRELARGAGFAOVKATYIYANFWAIEYTK     Figure 3 Sequence alignment of the COMT family of Brachypodium distachyon and other species. Amino acid sequence comparison of the four COMT proteins in <i>B. distachyon</i> with functionally characterized COMT proteins in <i>Triticum aestivum</i> (TaCM), <i>Festuca arundinacea</i> (FaCOMT), Sorghum bicolor (Sbbmr12), Zea mays (Zmbm3), Panicum virgatum (PviCOMT), and Oryza sativa (OsCOMT). The conserved motif for the	SOCOMT	G . KVIIVECVLPV	NTEAVPKAQGVFHV	DMIMLAHNPGGRE	RYEREFHDLAKGAG	FSGFKATYIYANAN	NAIEFIK	
Oscomt 6 · KVVVVE CVLPESSDATAREGGVFHVOM MLAH PROCKERY EREFRELARAAGFTGFKATYIYANAWAIEFTK Bdcomt 0 · KLIIIESILPVNPEATPRARMAFED MMLTYT PROKERY KREFEVLAKGARFASVRTTYIYANAWAIEYTK 6 · KVIVECILPVNPETPSARGLIG DMSLLAYSPOCKERY KRELEKLAKGAGFAAVKATYIYANFWAIEYTK 6 · KVINLECIMPVNPEPTHGAGGLISVDVSLLAYSPOCKERY KRELEKLAKGAGFADVKATYIYANFWAIEYTK Figure 3 Sequence alignment of the COMT family of Brachypodium distachyon and other species. Amino acid sequence comparison of the four COMT proteins in <i>B. distachyon</i> with functionally characterized COMT proteins in <i>Triticum aestivum</i> (TaCM), <i>Festuca arundinacea</i> (FaCOMT), <i>Sorghum bicolor</i> (Sbbmr12), <i>Zea mays</i> (Zmbm3), <i>Panicum virgatum</i> (PviCOMT), and <i>Oryza sativa</i> (OsCOMT). The conserved motif for the	ZmCOMT	G . KVIVVECVLPV	NTEATPKAQGVFHV	DMIMLAHNPGGKE	RYEREFRELAKGAG	FSGFKATYIYANAN	NAIEFIK	
Bdcomta 6-KLIIIE SILPVNPEATPRARMAFED DMINLTY TPOCKERY KREFEVLAKGARFASVRTTYIYANSWAIEYTK Bdcomta 6-KVINVECILPVNPETPSARGLIG DMSLLAYS POCKERY LRELEKLAKGAGFAAVKATYIYANFWAIEYTK Figure 3 Sequence alignment of the COMT family of Brachypodium distachyon and other species. Amino acid sequence comparison of the four COMT proteins in <i>B. distachyon</i> with functionally characterized COMT proteins in <i>Triticum aestivum</i> (TaCM), <i>Festuca arundinacea</i> (FaCOMT), <i>Sorghum bicolor</i> (Sbbmr12), <i>Zea mays</i> (Zmbm3), <i>Panicum virgatum</i> (PviCOMT), and <i>Oryza sativa</i> (OsCOMT). The conserved motif for the	PviCOMT							
BIGOMT2 G-KVINVECILPVNPDETPSARGLIQ DMSLLAYSPOCKERYLRELEKLAKGAGFAAVKATYIYANFWAIEYTK BIGOMT3 G-KVINLECIMPVNPEPTHGAGGLISVOVSLLAYSPOCKERYLRELEKLAKGAGFADVKATYIYANFWAIEYTK Figure 3 Sequence alignment of the COMT family of <i>Brachypodium distachyon</i> and other species. Amino acid sequence comparison of the four COMT proteins in <i>B. distachyon</i> with functionally characterized COMT proteins in <i>Triticum aestivum</i> (TaCM), <i>Festuca arundinacea</i> (FaCOMT), <i>Sorghum bicolor</i> (Sbbmr12), <i>Zea mays</i> (Zmbm3), <i>Panicum virgatum</i> (PviCOMT), and <i>Oryza sativa</i> (OsCOMT). The conserved motif for the								
<b>BIGOMT3 G: KVINLECIMPVNPEPTHOAGOLISYDVSLLAYSPOCKERYLRELEKLAKGAGFADVKATYIYADFWAIEYTK</b> <b>Figure 3 Sequence alignment of the COMT family of Brachypodium distachyon and other species.</b> Amino acid sequence comparison of the four COMT proteins in <i>B. distachyon</i> with functionally characterized COMT proteins in <i>Triticum aestivum</i> (TaCM), <i>Festuca arundinacea</i> (FaCOMT), <i>Sorghum bicolor</i> (Sbbmr12), <i>Zea mays</i> (Zmbm3), <i>Panicum virgatum</i> (PviCOMT), and <i>Oryza sativa</i> (OsCOMT). The conserved motif for the								
<b>Figure 3 Sequence alignment of the COMT family of </b> <i>Brachypodium distachyon</i> and other species. Amino acid sequence comparison of the four COMT proteins in <i>B. distachyon</i> with functionally characterized COMT proteins in <i>Triticum aestivum</i> (TaCM), <i>Festuca arundinacea</i> (FaCOMT), <i>Sorghum bicolor</i> (Sbbmr12), <i>Zea mays</i> (Zmbm3), <i>Panicum virgatum</i> (PviCOMT), and <i>Oryza sativa</i> (OsCOMT). The conserved motif for the								
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the four COMT proteins in <i>B. distachyon</i> with functionally characterized COMT proteins in <i>Triticum aestivum</i> (TaCM), <i>Festuca arundinacea</i> (FaCOMT), <i>Sorghum bicolor</i> (Sbbmr12), <i>Zea mays</i> (Zmbm3), <i>Panicum virgatum</i> (PviCOMT), and <i>Oryza sativa</i> (OsCOMT). The conserved motif for the	Einung 2	Convonco alianno	nt of the CONT for	mily of Brachurs	dium distachus	d ather marine ^	mino acid coqueres	comparison of
(FaCOMT), Sorghum bicolor (Sbbmr12), Zea mays (Zmbm3), Panicum virgatum (PviCOMT), and Oryza sativa (OsCOMT). The conserved motif for the	-				•	•		
	the four C	COMT proteins in B. a	<i>distachyon</i> with fund	tionally characteriz	zed COMT proteins i	n <i>Triticum aestivum</i>	(TaCM), Festuca aru	ndinacea
		Sorahum hicolor (Cl	$\frac{1}{2}$ $\frac{1}{2}$ $\frac{1}{2}$ $\frac{1}{2}$ $\frac{1}{2}$ $\frac{1}{2}$ $\frac{1}{2}$	7mhm3) Danicum	viraatum (DuiCOMT)	and Oniza cativa (	OCOMT) The cons	arved motif for the
SAM binding domain is labeled in pink, catalytic residues are labeled in green, and active site substrate-binding residues are labeled in blue.								
	SAM bindi							

deduced multiple amino acid sequence alignments of the aforementioned CAD and COMT proteins. The phenogram established a single clade in which the characterized CAD proteins known to be involved in monolignol biosynthesis in monocots were clustered with one protein from our candidate B. distachyon family, BdCAD1 (Figure 4A). Within that clade, BdCAD1 clustered with F. arundinacea, T. aestivum and O. sativa proteins. A second, closely related group consisted of CADs in P. virgatum, Z. mays, S. bicolor, and S. officinarum. The remaining seven CAD family members in B. distachyon did not fall into any such existing clades. A similar trend was observed in the phylogenetic analysis of the COMT sequences isolated from the previously mentioned species. A common clade formed amongst the known COMT lignin proteins and included one COMT from the B. distachyon family, BdCOMT4 (Figure 4B). Within the clade, BdCOMT4 clustered with F. arundinacea and T. aestivum proteins, while a closely related group consisted of P. virgatum, Z. mays, S. bicolor, and S. officinarum proteins. In contrast, the other three BdCOMTs did not show a similar relationship to known COMT enzymes. The relationships among the CAD and COMT sequences specifically portray *BdCAD1* and *BdCOMT4* as unique from any other candidates in the corresponding multi-gene families in *B. distachyon*.

# *BdCAD1* and *BdCOMT4* are highly expressed in developing stem

Anatomical expression data were also considered in evaluating the multi-gene *BdCAD* and *BdCOMT* families for a role in lignification. We used microarray data to analyze gene expression of each member of the family in developing stems, roots, and leaves. Lignin biosynthesis genes are expected to be highly expressed in stems, where secondary cell walls are prevalent and lignification occurs, while remaining at relatively low levels in roots and especially leaves. Of the seven CAD genes identified in *B. distachyon*, *BdCAD1* expression was greatest in stem tissue, exhibiting ten-fold higher transcript level than any of the other seven *BdCAD* genes (Figure 4C). Expression of *BdCOMT4* was also greatest in the stem and was eighteen-fold greater than the expression of the



phenogram was constructed with MEGA neighbor-joining method with 1000 bootstrap permutations. **(C,D)** Anatomical gene expression in leaf (blue), root (red), and stem (green) of *CAD* and *COMT* families in *B. distachyon*.

other three *BdCOMT* genes (Figure 4D). As might be expected, both *BdCAD1* and *BdCOMT4* were expressed at a slightly lower level in roots, where lignin is also present, and at a significantly lower level in leaves, where there is not much lignin at all.

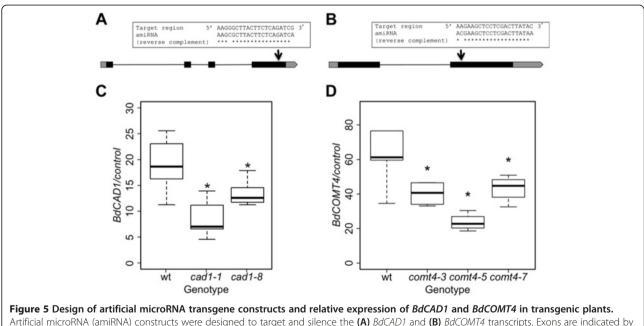
### BdCAD1 and BdCOMT4 transgenic plants

In order to functionally characterize BdCAD1 and BdCOMT4, reduction-of-function mutants were developed and assayed for changes in secondary cell wall composition. Highly specific artificial microRNA (amiRNA) constructs were designed to target and silence BdCAD1 or BdCOMT4 (Figure 5A, B). The WMD Version 3 webbased tool was used to design amiRNA sequences for the transgenes. The BdCAD1 transcript was targeted by a 21mer sequence at nucleotides 950-970 in the fourth exon (Figure 5A). The BdCOMT4 transcript was targeted by a 21-mer sequence at nucleotides 556-576 in the second exon (Figure 5B). Two transformation events targeting BdCAD1 (amiR-cad1-1 and amiR-cad1-8) and three transformation events targeting BdCOMT4 (amiR-comt4-3, amiR-comt4-5, and amiR-comt4-7) were selected for further characterization in the  $T_2$  generation. Transgenic *B*. distachyon containing the empty binary vector pOL001 were used as control in all experiments. Anatomical expression data revealed that BdCAD1 and BdCOMT4 were highly expressed in stems; therefore, we analyzed the expression levels of the target genes in stems of the selected transgenic lines for both *BdCAD1* and *BdCOMT4* in order to confirm silencing. Quantitative real-time PCR of the *BdCAD1* or *BdCOMT4* transcript was performed to investigate the artificial microRNA induced suppression of *CAD* or *COMT*. Relative expression of *BdCAD1* was significantly reduced in *amiR-cad1-1* by 55% and *amiR-cad1-8* by 31% compared to empty vector control (Figure 5C). The expression level of *BdCOMT4* was significantly decreased by 40, 64, and 34% in lines *amiR-comt4-3, amiRcomt4-5,* and *amiR-comt4-7* respectively, compared to the empty vector control (Figure 5D).

The velocity of CAD in crude protein extracts was determined for aboveground tissue of empty vector control and *amiR-cad1* transgenic plants as the inflorescent first emerged from the flag leaf (Additional file 1: Figure S1). Sinapaldehyde was used as a substrate to evaluate total CAD activity that includes seven other putative CAD enzymes (Additional file 1: Figure S1). Although not statistically significant, total CAD activity was reduced by 6% in *amiR-cad1-1* and 17% in *amiR-cad1-8* plants relative to empty vector control.

# Effects of downregulation of *BdCAD1* and *BdCOMT4* on development

Transgenic plants were assayed for changes in growth and development typical of lignin deficiency including time to flower, tiller number, and stem weight. Both *CAD*-downregulated events showed a significant delay



Artificial microRNA (amiRNA) constructs were designed to target and silence the (A) *BdCAD1* and (B) *BdCOMT4* transcripts. Exons are indicated by solid black boxes, untranslated regions by grey boxes, and intron by thin, black lines. The genes are drawn to scale. Arrows indicate the artificial microRNA target regions. RNA was prepared from stems of developmentally equivalent transgenic and empty vector control plants and subjected to quantitative real-time PCR to assay for target gene expression. Relative expression of (C) *BdCAD1* and (D) *BdCOMT4* with control housekeeping gene Bradi5g25870. The boxes show interquartile range, the whiskers show the outer quartile edge, and the black line represents the median of each distribution. Open circles represent outliers, when present. \* Denotes significance at the 5% level.

in inflorescence emergence, flowering on average ten days later than empty vector control plants (Figure 6A, B). On the other hand, amiR-comt4-3 and amiR-comt4-7 plants flowered significantly earlier than the empty vector control, but this difference was not observed in amiR-comt4-5 (Figure 6E, F). The amiR-cad1-1 and amiR-cad1-8 events showed a significant, near two-fold increase in tiller number (Figure 6C). The tiller count among the amiR-comt4 mutants was significantly increased relative to the empty vector control for amiRcomt4-3 and amiR-comt4-5 (Figure 6G). Total stem weight was measured at senescence following the removal of seeds and leaves. Events amiR-cad1-1 and amiR-cad1-8 showed a significant increase in stem biomass (Figure 6D), while all amiR-comt4 events were similar to the empty vector control (Figure 6H).

#### Transgenic plants have lignin-associated phenotypes

The typical brown midrib phenotype was observed in all *CAD*-downregulated plants, but not in empty vector control or *COMT*-downregulated plants (Figure 7A-C). The leaf midrib appeared a brownish tan color in leaves of *amiR-cad1-1* and *amiR-cad1-8* plants and discoloration was consistently observed in successive leaves, while the control leaves remained green until senescence. Leaf midribs in *amiR-comt4-3, amiR-comt4-5,* and *amiR-comt4-7* appeared similar in color to the control.

The effect of changes in BdCAD1 and BdCOMT4 transcript abundance on lignification was evaluated by histochemical analysis. Since CAD is involved in the last step in the production of the precursors to S, G, and H lignin, we expected BdCAD1-deficient plants to be altered in lignin (Figure 1). Lignin amount and localization was observed with Wiesner staining of hand-cut stem cross sections from the first internode of developmentally equivalent transgenic and empty vector control plants. The Wiesner reaction stains lignin in a concentration-indicative manner, whereby lignified tissue stains a dark red color and less lignified tissue an orange-yellow color. Empty vector control stem stained the dark red color for lignin (Figure 8A). There was a noticeable difference in staining in both amiRcad1 and amiR-comt4 stem sections relative to the control. The amiR-cad1 stems stained a visibly lighter yellow color in regions that appeared red in the control, most notably in the sclerenchyma fibers, epidermal cells, and vascular bundle sheath (Figure 8B). The amiR-comt4 stems stained an orange color relative to the red in the control, although this was mainly restricted to sclerenchyma fibers (Figure 8C).

Since *COMT* is important in the production of the sinapyl alcohol precursor for S lignin, we expected *BdCOMT4*-downregulated plants would exhibit a more drastic change, specifically in S lignin, more so than in total G and H lignin content (Figure 1). The Maule reagent was used to observe S lignin amount and localization in stems;

the reagent stain S lignin a dark red-purple color. A shift to yellow-brown is indicative of a decrease in S lignin. Empty vector control stem sections treated with the Maule reagent stained a red-purple color (Figure 8D). Examination of the *amiR-cad1* stems revealed a reduction in red staining and a slight shift to yellow-orange color, notably in the epidermal cells and sclerenchyma fibers (Figure 8E). There was a striking decrease in staining of the *amiRcomt4* stem sections, in which the majority of tissue stained pale brown in color, with slight purple coloration seen in the outer regions of the pith (Figure 8F). The dramatic shift in color was reflective of a severe loss of S lignin in *amiRcomt4* stems compared to the empty vector control.

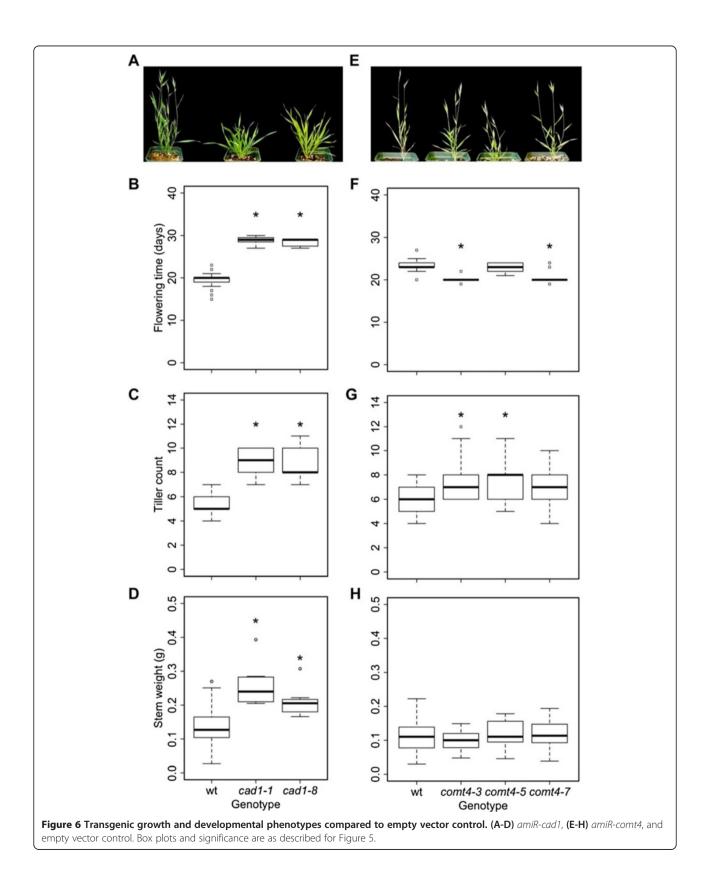
# Effect of *BdCAD1* or *BdCOMT4*downregulation on lignin composition

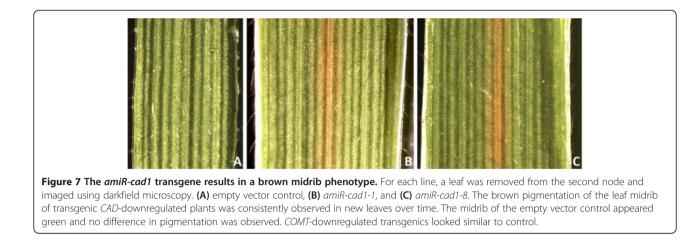
To determine total lignin polymer content, plant material was subjected to hydrolysis by acetyl bromide (AcBr) and fluorescent products, AcBr soluble lignin, were quantified by spectrometry. There was no significant difference in AcBr lignin content in *CAD*-downregulated plants as compared to the empty vector control (Figure 9A). On the other hand, AcBr lignin was significantly reduced by an average of 31.5% in *amiR-comt4-3* and *amiR-comt4-7* plants and 24% (P = 0.08) in *amiR-comt4-5* relative to the control.

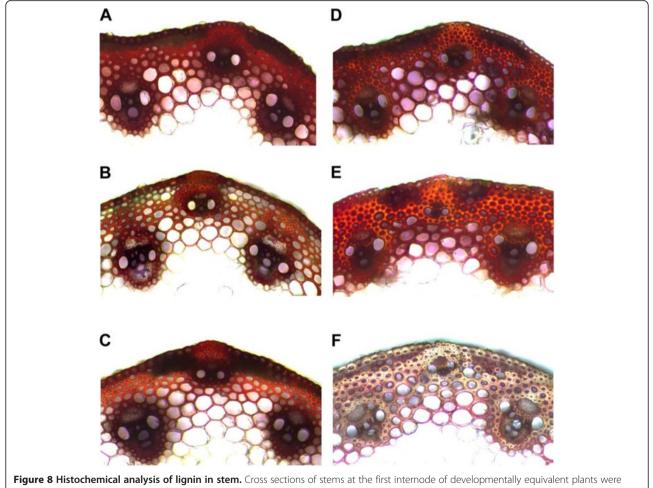
Lignin composition was evaluated by thioacidolysis, which cleaves  $\beta$ -O-4 ether bonds within the lignin polymer to reveal the monomer components. The recovered monomeric degradation products, namely S, G, and H units, were quantified by gas chromatography mass spectrometry. Empty vector control samples consisted of approximately 55% S, 40% G, and 5% H lignin units. Monomer levels were altered in amiR-cad1 and amiR-comt4 plants (Table 1). Downregulation of BdCAD1 was associated with a significant decrease in S units and a slight yet not statistically significant increase in G units, resulting in a reduced S/G ratio. Although H units were relatively scarce, they were increased in amiR-cad1 plants. The amiR-cad1-8 plants showed the most dramatic phenotype, in which the amount of S units and the S/G ratio were 16% and 22% lower than control. Although not statistically significant, the BdCOMT4-downregulated plants exhibited a 10% decrease in S units and a 17% reduction in G units, along with an increase in the S/G ratio relative to empty vector control plants. No consistent difference in H lignin was observed in amiR-comt4 plants.

### Biological conversion efficiency in transgenic plants

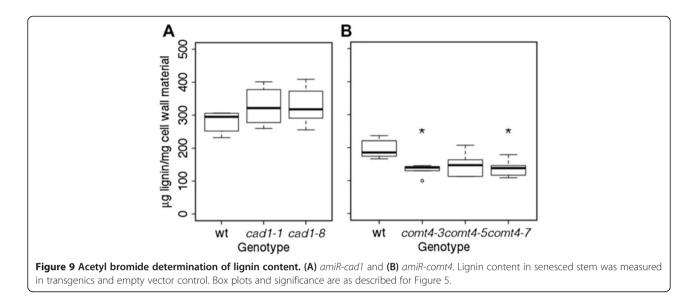
In order to evaluate the impact of lignin pathway modification on biofuel feedstock quality, we measured the potential of the transgenic plants to produce ethanol following inoculation with the cell-wall-degrading, ethanogenic bacterium *C. phytofermentans*.







**Figure 8 Histochemical analysis of lignin in stem.** Cross sections of stems at the first internode of developmentally equivalent plants were dissected when the flag leaf was 4 cm below the spike. (A) to (C) Wiesner staining of empty vector control (A), *amiR-cad1-1* (B), *amiR-comt4-5* (C). Wiesner reagents stain lignified tissue in a concentration-indicative manner; heavily lignified tissue stains dark red, while areas with less lignin stain orange-yellow. (D) to (F) Maule reagent staining of empty vector control (D), *amiR-cad1-1* (E), and *amiR-comt4-5*. The Maule reagent stains S lignin; a shift from red to brown-yellow is representative of a decrease in S lignin.



A slight increase in average ethanol concentration was detected for both the *amiR-cad1* and *amiR-comt4* lines (Figure 10). Fermentation of the *CAD*-downregulated plants resulted in ethanol yields that were increased by 9% in *amiR-cad1-1* and 17% (p = 0.01) in *amiR-cad1-8* lines relative to empty vector control plants. Digestion was slightly improved by *COMT*-downregulation, as ethanol yield was increased by 4% in *amiR-comt4-3* (p = 0.76), 10% in *amiR-comt4-5* (p = 0.10), and 8% in *amiR-comt-4-7* lines (p = 0.24).

#### Discussion

In this study, we used a candidate gene approach to identify the *CAD* and *COMT* genes involved in monolignol biosynthesis in *B. distachyon*. While well studied as key enzymes in the lignin pathway that influence forage quality, understanding of these enzymes is now of increased interest due to an apparently similar effect on biofuel feedstock quality [8]. To date, there have only been a few

Table 1 Lignin composition in CAD1- and COMT4downregulated transgenic plants

Plant line <sup>1</sup>	S (µmol/g) <sup>2,3</sup>	G (µmol/g) <sup>2,3</sup>	Η (μmol/g) <sup>2,3</sup>	S/G <sup>2,3</sup>
Control 1	89.7 ± 2.3	69.5 ± 1.4	8.9 ± 0.1	1.29
amiR-cad1-1	87.7 ± 1.8	75.8 ± 1.8	10.0 ± 0.1	1.16
amiR-cad1-8	75.2 ± 2.0	75.0 ± 1.7	9.4 ± 0.2	1.00
Control 2	97.2 ± 5.6	65.7 ± 3.6	$9.5 \pm 0.5$	1.48
amiR-comt4-3	86.6 ± 3.8	54.2 ± 1.4	8.9 ± 0.2	1.60
amiR-comt4-5	92.4 ± 1.9	$60.0 \pm 0.9$	10.8 ± 0.2	1.54
amiR-comt4-7	88.5 ± 2.7	55.8 ± 1.3	9.1 ± 0.2	1.58

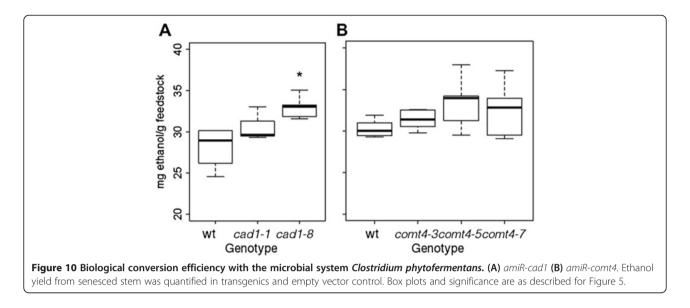
<sup>1</sup>Senesced stem tissue of *amiRcad1* and *amiRcomt4* were grown in different experiments with separate empty vector controls.

<sup>2</sup>Values are means  $\pm$  SD (n = 3).

<sup>3</sup>Syringyl (S), guaiacyl (G), p-hydroxphenyl (H) units.

reports of *CAD* or *COMT* downregulation in transgenic monocots [36-39]. These species, such as *F. arundinacae* and *L. perenne* have proven to be challenging research subjects. We report here the effectiveness of artificial microRNA silencing in a species considerably more amenable to research, *B. distachyon*, from which gained knowledge can be contributed towards the optimization of bioenergy grasses.

In monocots such as Z. mays, S. bicolor and O. sativa, CAD tends to exist as a multi-gene family with one CAD primarily involved in monolignol biosynthesis [48,49,55]. In contrast, in the eudicot A. thaliana, the last step of monolignol biosynthesis is controlled synergistically by two genes, AtCAD-C and AtCAD-D [57]. Based on amino acid sequence and gene expression pattern, we identified BdCAD1 as a likely candidate for having a role in lignin biosynthesis. Albeit with significantly lower efficiency than BdCAD1, Bradi4g29770 can also use coniferyl aldehyde and coniferyl alcohol as substrates and may also have a role in monolignol biosynthesis [58]. Unlike the other BdCADs, BdCAD1 contains all sequence features characteristic of a zinc-dependent alcohol dehydrogenase and more specifically, appears to be a member of the medium-chain dehydrogenase/reductase superfamily. High similarity in sequence motifs and substrate binding position suggests that BdCAD1 shares the same function as the bona fide CADs [56]. Consistent with multi-gene CAD families in Z. mays, S. bicolor and O. sativa, one particular protein in B. distachyon shared a significantly higher degree of homology with other known CADs than any other family member [48,49,55]. Gene expression analysis revealed that BdCAD1 was the most highly expressed member of the gene family and that transcript abundance was particularly high in stem and root, where secondary cell walls are prevalent. Lignin-



associated *CAD* expression was similarly high in stem and root tissues in *P. virgatum*, *F. arundinacae*, *O. sativa*, and *S. bicolor* [22,38,55].

The downregulation of *BdCAD1* caused phenotypes characteristic of lignin deficiency without reducing plant biomass. The delay in flowering time of the amiR-cad1 plants is consistent with the phenotypes in five S. bicolor bmr mutants including CAD impaired bmr6 [42,59]. The variation in flowering time observed in lignin mutants from S. bicolor and B. distachyon reinforces the possibility of an evolutionarily conserved mechanism between cell wall biosynthesis and production of flowers [60]. Because lignin has a significant role in xylem function, it is possible that changes in lignin may alter development by perturbing water transport. However, this rationale seems unlikely considering that, while amiR-cad1 plants were developmentally delayed, mature transgenic plants were significantly larger than empty vector control plants (Figure 6D). An increase in aboveground stem biomass, even coupled with delayed flowering, is a favorable trait for a perennial energy crop, considering crop rotation will not be part of cultivation. While mutations in CAD can sometimes lead to pleiotropic effects of dwarfing, lodging, and a decrease in grain and/or biomass yield, these effects are mostly background-dependent [40]. Further understanding of gene-by-gene interactions that result in these deleterious effects will increase the efficiency of cultivar development.

We present here the brown midrib leaf phenotype for the first time in a  $C_3$  grass [40]. Genetic redundancy may explain why the phenotype has not been observed in mutant polyploid species such as *T. aestivum* and *F. arundinacae*; however, CAD mutants of diploid *O. sativa* do not exhibit a leaf brown midrib [25,55,61,62]. It has been suggested that the brown midrib phenotype may

present itself differently in various species. In O. sativa, a mutation in GOLD HULL AND INTERNODE 2, which encodes a CAD enzyme, caused a red-brown pigment in the hull, internode, and basal leaf sheath while the leaf midrib did not show the same discoloration [63]. Similarly, recent research in B. distachyon indicated that CAD mutants displayed the red-brown pigmentation in various tissues including nodes and flowers, but not in the leaf midrib [64]. We did not observe color differences in tissues other than leaf midrib. In other species, including Populus sp. and N. tabacum, transverse stem cross-sections of transgenic CAD-downregulated plants exhibited unusually red xylem [13,16,20]. On the other hand, no visible mutant phenotype was observed in CAD-RNAi Z. mays plants [13]. Here, the BdCAD1downregulated plants phenotypically resembled Z. mays, S. bicolor, and P. glaucum leaf brown-midrib mutants. The brown-midrib phenotype may occur only when CAD activity is decreased beneath a certain threshold [65]. For example, in four lines of antisense-CAD transgenic tobacco with residual CAD activity ranging from 8-56%, the extent of CAD downregulation was correlated with the presence and pattern of reddish-brown xylem [16]. We measured relatively small changes in CAD activity, but at a developmental stage that had not yet exhibited the brown-midrib phenotype. Nonetheless, the amiR-cad1-8 plants which were most reduced in CAD activity were significantly more digestible when the plant had completely senesced. It is possible at a subsequent developmental stage characterized by greater lignin biosynthesis that diminished CAD activity would be more evident.

The quality of lignin was altered in *amiR-cad1* plants, as indicated by a significant decrease in S units observed by thioacidolysis and in agreement with histochemical

staining by the Maule reagent. Previous research in CADdownregulated N. tabacum and CAD mutant B. distachyon showed that the most dramatic change in lignin composition in plants was a severe decrease in S lignin [16,64]. Some CAD gene knockouts produce functional lignin through increased incorporation of cinnamyl aldehyde subunits into the lignin polymer [43]. Previous reports for Z. mays, N. tabacum, and Populus sp. demonstrated an incorporation of aldehydes into the lignin polymer, in which increased coniferyl aldehyde caused an increase in intensity of the Wiesner stain [13,20]. On the contrary, the Wiesner stain in amiR-cad1 lines was less intense than control plants. A likely explanation is that the inhibition of S lignin synthesis still caused an accumulation of aldehydes, but specifically sinapyl aldehydes, which are not detected by Wiesner staining. This is consistent with decreased Wiesner staining and incorporation of 8-O-4coupled sinapyl aldehyde in B. distachyon CAD mutant plants [64].

Consistent with reports in *N. tabacum, M. sativa, E. camaldulensis, Populus sp., F. arundinacae,* and *Z. mays, amiR-cad1* plants were unchanged in the amount of acetyl bromide soluble lignin polymer, but thioacidolysis indicated changes in lignin monomer composition [13,16-18, 20,21,24,38]. The lignin in *CAD* downregulated plants was generally more reactive. This has been illustrated by improved pulping properties in *Populus sp.,* forage digestibility in *N. tabacum* and *F. arundinacae,* saccharification in *P. virgatum* and *B. distachyon,* and digestibility in *Z. mays* [13,16-18,20,22,23,25,64]. Along the same lines, the modified lignin in *amiR-cad1-8* improved biological conversion efficiency by a statistically significant 17%.

One protein among the four COMTs identified in B. distachyon, BdCOMT4 (Bradi3g16530), contained all of the signature features of a plant O-methyltransferase. Plant O-methyltransferases tend to have broad substrate specificity, and all nine substrate binding and positioning residues in BdCOMT4 are common to COMT proteins in other species. Similar to L. perenne, F. arundinacae, P. tremuloides, and M. sativa COMT genes [38,66-68], BdCOMT4 was the most highly expressed COMT in stem, root, and leaf tissues than any of the three other B. distachyon family members. Similar to observations made in P. tremuloides, BdCOMT4 expression was relatively low in leaves compared to stems [68]. Phylogeny, amino acid sequence, and the abundance of transcript in lignified tissues concurrently support that BdCOMT4 is an O-methyltransferase involved in monolignol biosynthesis in B. distachyon.

The downregulation of *COMT* resulted in changes in various phenotypic traits. Mutants tended to flower earlier than the empty vector control, as seen for the lignin mutants *bm1* in *Z. mays* and *bmr7* in *S. bicolor* [42,59]. In general, the brown midrib phenotype is not common in

COMT-downregulated transgenic plants, with the only reports of a reddish-brown coloration of the leaf and internodes being in Z. mays [37,69]. Although the cause of discoloration in plants with impaired CAD activity is often attributed to the incorporation of aldehydes into the lignin polymer, there is no obvious correlation between the phenotype and the activities of other enzymes of the monolignol biosynthesis pathway. Previous biochemical analysis has indicated that the brown coloration is not a result of accumulated carotenoids, anthocyanins, flavones, tannins, or flavonols, but could possibly be due to incorporation of other phenolic compounds into the lignin polymer [40]. Accumulation of novel 5-OH-G units has been observed in COMT-downregulated transgenic M. sativa and Z. mays, although a visual phenotype associated with this phenomenon has not been defined [33,37,69,70].

In our transgenic *B. distachyon*, the perturbation of the COMT enzyme had a deleterious effect on the total quantity of lignin produced in the plant. Similarly, down-regulated *COMT* mutants in *Z. mays*, *F. arundinacae*, *L. perenne*, *S. bicolor*, and *Saccharum spp*. were also reduced in total lignin [38,39,69,71]. Staining with the Maule reagent revealed an obvious difference between control and *amiR-comt4* transverse stem cross sections. We measured an increase in ethanol yield of up to 10% in *amiR-comt4* lines, which is consistent with the characterization of *COMT* mutants in other species [33,37,38,69,71-73].

# Conclusion

One of the more costly steps of producing liquid fuels from biomass on the biochemical platform is the pretreatment required to reduce biomass recalcitrance for the enzymatic and fermentation steps. As a result, there has been increasing effort to identify the factors behind biomass recalcitrance. In this study, modification of CAD and COMT expression induced changes in cell wall composition that improved amenability to conversion. A significant 17% increase in ethanol yield from plant biomass, as observed here by CAD downregulation, would increase industrial efficiency of processing such feedstock. Genetic modification of lignin biosynthesis may provide a means of improvement of biofuel crop conversion efficiency by reducing biomass pretreatment costs, thereby improving the bioethanol production process overall.

# Methods

### **Phylogenetic analysis**

Candidate CAD and COMT in *B. distachyon* were identified by amino acid homology with known proteins in other plant systems by BLAST search of the Phytozome v8.0 and NCBI databases [74,75]. Multiple amino acid sequences were aligned using ClustalW and analyzed with the associated editing program JalView 2.0 [76]. A neighbor-joining tree with bootstrap 1000 was constructed with MEGA [77]. Sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers: Sbbmr6 (BAF42789.1), Zmbm1 (ACG45271.1), PviCAD2 (ADO01602.1), OsCAD2 (NP\_001046132.1), FaCAD (AAK97809.1), SoCAD (O82056.1), TaCAD (ADI59734.1), SbCOMT (AAO43609.1), Zmbm3 (NP\_001106047.1), PviCOMT (ADX98508.1), TaCM (ABP63535.1).

#### Microarray expression profiling

Expression patterns of the multi-gene CAD and COMT families were observed using microarray expression profiling of leaf, root, and stem tissue of B. rachypodium distachyon. Plants were in a growth chamber at 20°C with 20 h light:4 h dark cycles at a fluence rate of 220 µmol of photons<sup>-2</sup>·s<sup>-1</sup> and relative humidity of 67–69. Additionally, for plate-grown plants, seeds were de-hulled and then imbibed in water for two hours with shaking. Then, seeds were treated with 70% ethanol for 20 seconds, rinsed with sterile water, then soaked in 1.3% sodium hypochlorite for 4 minutes at room temperature while shaking. Seeds were subsequently rinsed three times with sterile water and stored in the dark at 4°C for a minimum of 2 days in a sterile Petri dish with filter paper. Seedlings were grown for seven days on 0.5X Murashige and Skoog (MS) medium containing 0.7% bactoagar adjusted to a pH of 5.8 with KOH.

Approximately 30 days following germination, total leaf and stem were collected as the inflorescence emerged from the flag leaf. Leaves were collected from the stems with a curved-tip probe. Nodes and internodes from the second leaf junction to the internode below the inflorescence were placed in a tube cooled with liquid nitrogen. Seven-day-old whole seedlings were flash frozen in liquid nitrogen and then the roots were snapped off into a sterile culture tube. The six time points were collected over the course of one day at ZT2, 6, 10, 14, 18, and 22. Three plants were dissected for each time point and in triplicate for each tissue type. Samples were stored in liquid nitrogen or at -80°C until RNA extraction. Tissue was ground with mortars and pestles in liquid nitrogen. RNA was extracted using the Qiagen (Valencia, CA) Plant RNaeasy Kit according to the manufacturer's instructions. Labeled sense strand cDNA probes were synthesized using the Ambion WT expression kit.

Transcript abundance of three biological replicates of three-week-old leaves and stems, and seven day old roots was measured using the Affymetrix *B. distachyon* BradiAR1b520742 whole genome tiling array. The array contains ~6.5 M unique 25-mer oligonucleotide features, both the forward and reverse strand sequence. The complete genome sequence is tiled with an average of 30 bases between each array feature; 1.6 million features

correspond to exons and introns and 4.9 million features between gene models. Version 1.0 genome annotation includes a total of 25,532 protein coding genes and 2,542 non-coding genes [74]. Approximately ~95% (~26,670) of the genes have at least five corresponding exon array features and from those a summary value was calculated for each gene model. The average number of array features corresponding to the CAD and COMT families is 38, ranging from 10 to 111. Probeset values were calculated using gcRMA. The Affymetrix BradiAR1b520742 GeneChip data (.CEL files) have been deposited at PLEXdb [Accession no: BD3].

#### Generation of transgenic plants

The WMD Version 3 web-based tool (http://wmd3. weigelworld.org) was used to design highly specific artificial microRNA (amiRNA) constructs to target the BdCAD1 (Bradi3g06480) and BdCOMT4 (Bradi3g16 530) transcripts. This program selects a 21-mer sequence in the target gene from which an amiRNA can be produced. The gene aliases for BdCAD1 and BdCOMT4 were searched in the WMD-3 B. distachyon 1.0 genome database for the amiRNA sequence which would most likely hybridize to the target mRNA without affecting the rest of the genome. The native BdCAD1 transcript was targeted by the amiRNA sequence AA GCGCTTACTTCTCAGATCA, corresponding to part of the fourth exon, with hybridization energy of the target site in the target gene of -38.50 kcal/mol. The native BdCOMT4 transcript was targeted by the amiRNA sequence, ACGAAGCTCCTCGACTTATAA, correspon ding to the second exon, with hybridization energy of -39.85 kcal/mol.

Modified polymerase chain reactions were performed as described in the protocols section of the WMD3 website to engineer the amiRNA sequences into the endogenous O. sativa microRNA precursor osa-MIR528 in the PNW55 vector [78]. All PCRs were performed with Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswitch, MA) according to the manufacturer's instructions. AmiRNA precursors were then cloned into the Gateway compatible vector pENTR/D-TOPO (Invitrogen, Grand Island, NY). Following sequence confirmation, entry clones were recombined into the destination vector pOL001 using the LR Clonase II Plus enzyme (Invitrogen, Grand Island, NY). This vector confers hygromycin resistance with the HptII gene and will express the amiRNAs under the constitutive ubiquitin promoter UBQ from Z. mays. Electroporation was used to transform the amiRNAs into Agrobacterium tumefaciens strain AGL1.

Transgenes were integrated into the *B. distachyon* accession Bd21-3 genome by *Agrobacterium*-mediated transformation of embryogenic calli following the protocol

described by Vogel and Hill [79]. Regenerated plants from *BdCAD1* transformation events *amiR-cad1-1* and *amiR-cad1-8* along with regenerated *BdCOMT4* plants from transformation events *amiR-comt4-3*, *amiR-comt4-5*, and *amiR-comt4-7* were selected for characterization in the  $T_2$  generation. Control plants were obtained by transformation with the empty binary vector pOL001. Plants were grown in a growth chamber at 20°C with 20 h light: 4 h dark cycles at a fluence rate of 220 µmol of photons m<sup>-2</sup>·s<sup>-1</sup> and relative humidity of 67–69.

Transgenic and empty vector control plants were genotyped by PCR of leaf genomic DNA. Leaf tissue was frozen in liquid nitrogen and pulverized with 6.35 mm stainless steel beads (BioSpec, Bartlesville, OK) in a Retsch Mixer Mill MM400. Pulverized tissue was treated with 600 µl DNA Extraction Buffer (100 mM NaCl, 50 mM Tris, 25 mM EDTA, 1% SDS, 10 mM 2-mercaptoethanol) at 65°C for 10 minutes. Samples were then placed on ice, mixed with 250 µl 5 M potassium acetate, and incubated for 20 minutes. The solution was centrifuged in a tabletop centrifuge at 12,000 rpm for 10 minutes. The supernatant was transferred to tubes containing 600 µl 100% isopropanol, mixed, and centrifuged to pellet the DNA. The supernatant was discarded and the pellet rinsed with 300 µl 70% ethanol. The pellet was resuspended in 225  $\mu$ l T<sub>10</sub>E<sub>5</sub> (10 mM Tris, 5 mM EDTA), mixed with 25 µl 3 M sodium acetate, 500 µl 100% ethanol and centrifuged in a tabletop centrifuge at 10,000 rpm for 7 minutes. Supernatant was discarded and the pellet was rinsed with 70% ethanol and centrifuged an additional 7 minutes. The pellet was allowed to air dry and was then resuspended in 30 µl  $T_{10}E_1$  (10 mMTris, 1 mM EDTA). All putative transgenic and empty vector control plants were tested for the presence of the HptII transgene by PCR of leaf genomic DNA with primer forward: 5' AGAATCTCGTGCTTT CAGCTTCGA 3' and primer reverse: 5' TCAAGACC AATGCGGAGCATATAC 3'. Only confirmed positive transformants were analyzed in subsequent experiments.

### Molecular characterization of transgenic plants

Transgenic and empty vector control plants were subjected to quantitative real-time PCR to assay for *BdCAD1* and *BdCOMT4* gene expression. Whole stem tissue was sampled as the inflorescence first emerged from the flag leaf, frozen in liquid nitrogen, and pulverized with 6.35 mm stainless steel beads (BioSpec, Bartlesville, OK) using Retsch Mixer Mill MM400. Total RNA was extracted from pulverized tissue using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions; RNA purification was analyzed with the NanoDrop1000 (ThermoScientific, Waltham, MA). RNA samples were reverse-transcribed into cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Grand Island, NY). Primers for gene-specific real-time PCR were selected using the QuantPrime design tool [80]. Reactions were completed using a QuantiFast SYBR Green PCR Kit (Qiagen, Valencia, CA) in an Eppendorf Mastercycler ep realplex2. To assay for BdCAD1 expression, cDNA from amiR-CAD1 and empty-vector plants was amplified with BdCAD1-specific primers (forward: 5'AGGATAGAATG GGCAGCATCGC 3'; reverse: 5' ATCTTCAGGGCCT GTCTTCCTGAG 3'). To assay for BdCOMT4 expression, cDNA from amiR-COMT4 and empty vector plant was amplified with BdCOMT4-specific primers (forward: 5' TGGAGAGCTGGTACTACCTGAAG 3'; reverse: 5' CGACATCCCGTATGCCTTGTTG 3'). Expression values were normalized with the real-time PCR signal for the housekeeping gene Bradi5g25870 with its gene specific primers (forward: 5'- TCAGCAGGGTGCTAATTCAGT TC 3'; reverse: 5' CGACAGAGTTTAGCGGTCTTAGC 3'). The selected housekeeping gene Bradi5g25870 exhibits moderate expression levels and extremely low variance across numerous B. distachyon array experiments. All qRT-PCR reactions were performed in triplicates.

#### **Enzymatic assays**

CAD activity was determined by generally following previously published procedures [50,81-83]. Approximately 150 mg of frozen plant tissue that had been ground as previously described and stored at -80°C was taken up in 700 µL buffer (100 mMTris-Cl, pH 7.5, 5 mM DTT, 5% ethylene glycol) and then sonicated to disrupt cell walls using a sonicator equipped with a micro-tip (Branson Digital Sonifier 450, Branson Ultrasonic Corp., Danbury CT). Sonicated extracts were centrifuged at 4°C for 10 min. at 14,000 RPM and the crude protein extract was placed into new 1.5 mL tubes and kept on ice. Each plant sample was extracted twice and enzyme activity was tested by monitoring absorbance changes on a microplate reader (BioTek Synergy HT, BioTek Instruments, Winooski, VT) at A<sub>340</sub>. All reactions were carried out in a volume of 200 µL and consisted of 100 mM MES at pH 6.5, 200  $\mu$ M NADPH, 100 mMsinapyl aldehyde, and 10 µL of the crude protein extract. Absorbance changes were monitored for three minutes after addition of crude protein extract. Enzyme velocities were determined by fitting a line using linear least squares to the absorbance data; the slope of the line was used in velocity determinations. Protein concentrations were determined using the Pierce 660 nm protein assay (Pierce Biotechnology, Rockford, IL) and served to normalize the calculated velocity for each extract.

### Histochemical staining of lignin

Cross sections of stems were manually dissected from the first internode of developmentally equivalent transgenic and control plants. The Wiesner staining method [84] was used to visualize total lignin content and localization in the stem. Sections were stained with 1% phloroglucinol for 2 minutes followed by a wash in 50% HCl and were mounted onto microscope slides for observation. The Maule reagent [85] was used to observe S lignin content and localization in stems. Sections were treated with 1% KMnO<sub>4</sub> for 5 minutes and rinsed with water. Sections were then treated with 10% HCl for 2 minutes, rinsed with water, and mounted on microscope slides in 1% NH<sub>4</sub>OH. Images were captured using a Nikon Eclipse E200MV R microscope with a 3 PixeLINK 3 MP camera.

#### Lignin content and composition analysis

Senesced stem material was dried thoroughly following wash steps with 70% ethanol at 65°C for 1 hour, and following ethanol removal was rinsed with acetic acid and allowed to air dry. Stem was pulvarized into powder with metal beads in a Retsch Mixer Mill MM400. Acetyl-bromide-soluble lignin was quantified using the procedure described by Foster et al. [86]. Triplicate samples of 1.5 mg powdered stem material were used for AcBr lignin analysis. Lignin composition was evaluated by thioacidolysis and S, G, and H unit quantification was conducted as described by Foster et al. [86].

### **Digestibility analysis**

Biological conversion efficiency was measured using the microbial system developed for *Clostridium phytofermentans*, which converts plant biomass to ethanol, following the protocol as described by Lee et al. [72]. Triplicate 20 mg samples, dried and prepared as described above, were incubated with *C. phytofermentans* in 96-well plates and supernatant ethanol concentration was measured with high-performance liquid chromatography (HPLC) with RI detection [87].

#### Statistical analysis

For each measurement, 4 to 17 independent plants were sampled. Analysis of variance and Dunnett's contrasts were performed in R v2.15.0.

# Additional file

**Additional file 1: Figure S1.** CAD enzyme activity in empty vector control and *amiR-cad1* transgenic plants. Activity of CAD was measured in aboveground tissue using sinapaldehyde as a substrate. Box plots and significance are as described for Figure 5.

#### **Competing interests**

The authors declared that they have no competing interests.

#### Authors' contributions

GMT and SPH designed research; GMT, DAM, SJL, and AJS performed research; HDP, TCM, GS and SPH contributed new reagents/analytic tools;

SPH and HDP analyzed data; and GMT and SPH wrote the paper. All authors read and approved the final manuscript.

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#### References

- 1. Somerville C: Biofuels. Curr Biology 2007, 17(4):R115–R119.
- Archer D, Eby M, Brovkin V, Ridgwell A, Cao L, Mikolajewicz U, Caldeira K, Matsumoto K, Munhoven G, Montenegro A, et al: Atmospheric lifetime of fossil fuel carbon dioxide. Annu Rev Earth Pl Sc 2009, 37:117–134.
- Luthi D, Le Floch M, Bereiter B, Blunier T, Barnola J-M, Siegenthaler U, Raynaud D, Jouzel J, Fischer H, Kawamura K, *et al*: High-resolution carbon dioxide concentration record 650,000-800,000 years before present. *Nature* 2008, 453(7193):379–382.
- Lynd L, Laser M, Brandsby D, Dale B, Davison B, Hamilton R, Himmel M, Keller M, McMillan J, Sheehan J, et al: How biotech can transform biofuels. Nat Biotechnol 2008, 26:169–172.
- Carroll A, Somerville C: Cellulosic biofuels. Annu Rev Plant Biol 2009, 60(1):165–182.
- Keating JD, Panganiban C, Mansfield SD: Tolerance and adaptation of ethanologenic yeasts to lignocellulosic inhibitory compounds. *Biotechnol Bioeng* 2006, 93(6):1196–1206.
- Chen F, Dixon R: Lignin modification improves fermentable sugar yields for biofuel production. Nat Biotechnol 2007, 25:759–761.
- Vanholme P, Morreel K, Ralph J, Boerjan W: Lignin engineering. Curr Opin Plant Biol 2008, 11:278.
- Bonawitz ND, Chapple C: The genetics of lignin biosynthesis: connecting genotype to phenotype. Ann Rev Genet 2010, 44:337–363.
- Vogel J: Unique aspects of the grass cell wall. Curr Opin Plant Biol 2008, 11(3):301–307.
- Handakumbura PP, Hazen SP: Transcriptional regulation of grass secondary cell wall biosynthesis: playing catch-up with Arabidopsis thaliana. Front Plant Sci 2012, 3.
- 12. Rossmann MG, Moras D, Olsen KW: Chemical and biological evolution of a nucleotide-binding protein. *Nature* 1974, **250**(5463):194–199.
- Halpin C, Knight ME, Foxon GA, Campbell MM, Boudet AM, Boon JJ, Chabbert B, Tollier M-T, Schuch W: Manipulation of lignin quality by downregulation of Cinnamyl alcohol dehydrogenase. *Plant J* 1994, 6(3):339–350.
- Bernard Vailhé MA, Besle JM, Maillot MP, Cornu A, Halpin C, Knight M: Effect of down-regulation of Cinnamyl alcohol dehydrogenase on cell wall composition and on degradability of tobacco stems. J Sci Food Agric 1998, 76(4):505–514.
- O'Connell A, Holt K, Piquemal J, Grima-Pettenati J, Boudet A, Pollet B, Lapierre C, Petit-Conil M, Schuch W, Halpin C: Improved paper pulp from plants with suppressed Cinnamoyl-CoA reductase or Cinnamyl alcohol dehydrogenase. *Transgenic Res* 2002, 11(5):495–503.
- Yahiaoui N, Marque C, Myton KE, Negrel J, Boudet AM: Impact of different levels of Cinnamyl alcohol dehydrogenase down-regulation on lignins of transgenic tobacco plants. *Planta* 1997, 204(1):8–15.
- 17. Baucher M, Bernard-Vailhe M, Chabbert B, Besle J, Opsomer C, Van Montagu M, Botterman J: Down-regulation of Cinnamyl alcohol dehydrogenase in

transgenic alfalfa (*Medicago sativa* L.) and the effect on lignin composition and digestibility. *Plant Mol Biol* 1999, **39**(3):437–447.

- Lapierre C, Pollet B, Petit-Conil M, Toval G, Romero J, Pilate G, Leple J, Boerjan W, Ferret V, De Nadai V, *et al*: Structural alterations of lignins in transgenic poplars with depressed cinnamyl alcohol dehydrogenase or caffeic acid O-methyltransferase activity have an opposite impact on the efficiency of industrial kraft pulping. *Plant Physiol* 1999, **119**(1):153–164.
- Lapierre C, Pilate G, Pollet B, Mila I, Leple J-C, Jouanin L, Kim H, Ralph J: Signatures of cinnamyl alcohol dehydrogenase deficiency in poplar lignins. *Phytochemistry* 2004, 65(3):313–321.
- Baucher M, Chabbert B, Pilate G, Van Doorsselaere J, Tollier MT, Petit-Conil M, Cornu D, Monties B, Van Montagu M, Inze D, et al: Red xylem and higher lignin extractability by down-regulating a Cinnamyl alcohol dehydrogenase in poplar. *Plant Physiol* 1996, 112(4):1479–1490.
- Valério L, Carter D, Rodrigues JC, Tournier V, Gominho J, Marque C, Boudet A-M, Maunders M, Pereira H, Teulières C: Down regulation of Cinnamyl alcohol dehydrogenase, a lignification enzyme, in *Eucalyptus camaldulensis*. *Mol Breed* 2003, **12**(2):157–167.
- Fu C, Xiao X, Xi Y, Ge Y, Chen F, Bouton J, Dixon R, Wang Z-Y: Downregulation of Cinnamyl alcohol dehydrogenase (CAD) leads to improved saccharification efficiency in switchgrass. *BioEnergy Res* 2011, 4:153–164.
- Saathoff AJ, Sarath G, Chow EK, Dien BS, Tobias CM: Downregulation of Cinnamyl-alcohol dehydrogenase in switchgrass by RNA silencing results in enhanced glucose release after cellulase treatment. *PLoS One* 2011, 6(1):e16416.
- Fornale S, Capellades M, Encina A, Wang K, Irar S, Lapierre C, Ruel K, Joseleau J-P, Berenguer J, Puigdomenech P, et al: Altered lignin biosynthesis improves cellulosic bioethanol production in transgenic maize plants down-regulated for Cinnamyl alcohol dehydrogenase. Mol Plant 2011, 5(4):817–830.
- Chen L, Auh CK, Dowling P, Bell J, Chen F, Hopkins A, Dixon RA, Wang ZY: Improved forage digestibility of tall fescue (*Festuca arundinacea*) by transgenic down-regulation of cinnamyl alcohol dehydrogenase. *Plant Biotechnol J* 2003, 1(6):437–449.
- 26. Li X, Weng J-K, Chapple C: Improvement of biomass through lignin modification. *Plant J* 2008, **54**(4):569–581.
- Joshi CP, Chiang VL: Conserved sequence motifs in plant S-adenosyl-Lmethionine-dependent methyltransferases. *Plant Mol Biol* 1998, 37(4):663–674.
- 28. Whetten R, Sederoff R: Lignin biosynthesis. Plant Cell 1995, 7(7):1001-1013.
- 29. Humphreys JM, Chapple C: Rewriting the lignin roadmap. *Curr Opin Plant Biol* 2002, **5**(3):224–229.
- Wu X, Wu J, Luo Y, Bragg J, Anderson O, Vogel J, Gu YQ: Phylogenetic, molecular, and biochemical characterization of caffeic acid omethyltransferase gene family in *Brachypodium distachyon*. Int J Plant Genomics 2013, 2013:12.
- Guo D, Chen F, Wheeler J, Winder J, Selman S, Peterson M, Dixon RA: Improvement of in-rumen digestibility of alfalfa forage by genetic manipulation of lignin O-methyltransferases. *Transgenic Res* 2001, 10(5):457–464.
- Ni W, Paiva NL, Dixon RA: Reduced lignin in transgenic plants containing a Caffeic acid O-methyltransferase antisense gene. *Transgenic Res* 1994, 3(2):120–126.
- Guo D, Chen F, Inoue K, Blount JW, Dixon RA: Downregulation of Caffeic acid 3-O-methyltransferase and Caffeoyl CoA 3-O-methyltransferase in transgenic alfalfa: impacts on lignin structure and implications for the biosynthesis of G and S lignin. *Plant Cell* 2001, 13(1):73–88.
- Van Doorsselaere J, Baucher M, Chognot E, Chabbert B, Tollier M-T, Petit-Conil M, Leplé J-C, Pilate G, Cornu D, Monties B, et al: A novel lignin in poplar trees with a reduced Caffeic acid/5-hydroxyferulic acid Omethyltransferase activity. Plant J 1995, 8(6):855–864.
- Jouanin L, Goujon T, de Nadai V, Martin M-T, Mila I, Vallet C, Pollet B, Yoshinaga A, Chabbert B, Petit-Conil M, et al: Lignification in transgenic poplars with extremely reduced Caffeic acid O-methyltransferase activity. Plant Physiol 2000, 123(4):1363–1374.
- Fu C, Mielenz JR, Xiao X, Ge Y, Hamilton CY, Rodriguez M, Chen F, Foston M, Ragauskas A, Bouton J, et al: Genetic manipulation of lignin reduces recalcitrance and improves ethanol production from switchgrass. Proc Natl Acad Sci USA 2011, 108:3803–3803.

- Pichon M, Deswartes C, Gerentes D, Guillaumie S, Lapierre C, Toppan A, Barrière Y, Goffner D: Variation in lignin and cell wall digestibility in Caffeic acid O-methyltransferase down-regulated maize half-sib progenies in field experiments. *Mol Breed* 2006, 18(3):253–261.
- Chen L, Auh C-K, Dowling P, Bell J, Lehmann D, Wang Z-Y: Transgenic down-regulation of Caffeic acid O-methyltransferase (COMT) led to improved digestibility in tall fescue (*Festuca arundinacea*). Funct Plant Biol 2004, 31(3):235–245.
- Tu Y, Rochfort S, Liu Z, Ran Y, Griffith M, Badenhorst P, Louie GV, Bowman ME, Smith KF, Noel JP, et al: Functional analyses of caffeic acid Omethyltransferase and cinnamoyl-CoA-reductase genes from perennial ryegrass (Lolium perenne). Plant Cell 2010, 22(10):3357–3373.
- Sattler S, Funnell-Harris D, Pedersen J: *Brown midrib* mutations and their importance to the utilization of maize, sorghum, and pearl millet lignocellulosic tissues. *Plant Sci* 2010, 178:229–238.
- 41. Jorgensen JR: Brown midrib in maize and its linkage relationships. J Am Soc Agron 1931, 23:549–557.
- 42. Vermerris W, McIntyre LM: Time to flowering in brown midrib mutants of maize: an alternative approach to the analysis of developmental traits. *Heredity* 1999, **83**:171–178.
- Barriere Y, Ralph J, Mechin V, Guillaumie S, Grabber JH: Genetic and molecular basis of grass cell wall biosynthesis and degradability. II. Lessons from brown-midrib mutants. C R Biol 2004, 327:847.
- 44. Pedersen JF, Vogel KP, Funnell DL: Impact of reduced lignin on plant fitness. Crop Sci 2005, 45(3):812–819.
- Porter KS, Axtell JD, Lechtenberg VL, Colenbrander VF: Phenotype, fiber composition, and in vitro dry matter disappearance of chemically induced brown midrib (bmr) mutants of sorghum. Crop Sci 1978, 18(2):205–208.
- Saballos A, Vermerris W, Rivera L, Ejeta G: Allelic association, chemical characterization and saccharification properties of *brown midrib* mutants of sorghum (*Sorghum bicolor* (L.) Moench). *BioEnerg Res* 2008, 1(3):193–204.
- Cherney JH, Axtell JD, Hassen MM, Anliker KS: Forage quality characterization of a chemically induced brown-midrib mutant In pearl millet. Crop Sci 1988, 28(5):783–787.
- Halpin C, Holt K, Chojecki J, Oliver D, Chabbert B, Monties B, Edwards K, Barakate A, Foxon GA: Brown-midrib maize (bm1) - a mutation affecting the cinnamyl alcohol dehydrogenase gene. *Plant J* 1998, 14:545–553.
- Saballos A, Ejeta G, Sanchez E, Kang C, Vermerris W: A genomewide analysis of the cinnamyl alcohol dehydrogenase family in sorghum [Sorghum bicolor (L.) Moench] identifies SbCAD2 as the brown midrib6 gene. Genetics 2009, 181(2):783–795.
- Sattler SE, Saathoff AJ, Haas EJ, Palmer NA, Funnell-Harris DL, Sarath G, Pedersen JF: A nonsense mutation in a Cinnamyl alcohol dehydrogenase gene Is responsible for the sorghum *brownmidrib6* phenotype. *Plant Physiol* 2009, 150(2):584–595.
- Gorthy S, Mayandi K, Faldu D, Dalal M: Molecular characterization of allelic variation in spontaneous brown midrib mutants of sorghum (Sorghum bicolor (L.) Moench). Mol Breed 2013, 31(4):795–803.
- Vignols F, Rigau J, Torres M, Capellades M, Puigdomenech P: The brown midrib3 (bm3) mutation in maize occurs in the gene encoding caffeic acid O-methyltransferase. Plant Cell 1995, 7(4):407–416.
- Bout S, Vermerris W: A candidate-gene approach to clone the sorghum Brown midrib gene encoding Caffeic acid O-methyltransferase. MGG 2003, 269(2):205–214.
- Brkljacic J, Grotewold E, Scholl R, Mockler T, Garvin DF, Vain P, Brutnell T, Sibout R, Bevan M, Budak H, *et al*: Brachypodium as a model for the grasses: today and the future. *Plant Physiol* 2011, 157(1):3–13.
- 55. Tobias C, Chow E: Structure of the Cinnamyl-alcohol dehydrogenase gene family in rice and promoter activity of a member associated with lignification. *Planta* 2005, **220**(5):678–688.
- Youn B, Camacho R, Moinuddin SGA, Lee C, Davin LB, Lewis NG, Kang C: Crystal structures and catalytic mechanism of the Arabidopsis cinnamyl alcohol dehydrogenases AtCAD5 and AtCAD4. Org Biomol Chem 2006, 4(9):1687–1697.
- Sibout R, Eudes A, Mouille G, Pollet B, Lapierre C, Jouanin L, Seguin A: Cinnamyl alcohol dehydrogenase-C and -D are the primary genes involved in lignin biosynthesis in the floral stem of Arabidopsis. *Plant Cell* 2005, 17(7):2059–2076.

- Bukh C, Nord-Larsen PH, Rasmussen SK: Phylogeny and structure of the cinnamyl alcohol dehydrogenase gene family in *Brachypodium distachyon. J Exp Bot* 2012, 63(17):6223–6236.
- Vermerris W, Thompson KJ, McIntyre LM: The maize Brown midrib1 locus affects cell wall composition and plant development in a dosedependent manner. *Heredity* 2002, 88(6):450–457.
- 60. Vermerris W, Thompson K, McIntyre L, Axtell J: Evidence for an evolutionarily conserved interaction between cell wall biosynthesis and flowering in maize and sorghum. *BMC Evol Biol* 2002, **2**(1):2.
- Ma Q-H: The expression of caffeic acid 3-O-methyltransferase in two wheat genotypes differing in lodging resistance. *J Exp Bot* 2009, 60(9):2763–2771.
- Hirano K, Aya K, Kondo M, Okuno A, Morinaka Y, Matsuoka M: OsCAD2 is the major CAD gene responsible for monolignol biosynthesis in rice culm. Plant Cell Rep 2012, 31(1):91–101.
- Zhang K, Qian Q, Huang Z, Wang Y, Li M, Hong L, Zeng D, Gu M, Chu C, Cheng Z: GOLD HULL AND INTERNODE2 encodes a primarily multifunctional cinnamyl-alcohol dehydrogenase in rice. *Plant Physiol* 2006, 140(3):972–983.
- 64. d'Yvoire MB, Bouchabke-Coussa O, Voorend W, Antelme S, Cézard L, Legée F, Lebris P, Legay S, Whitehead C, McQueen-Mason SJ, et al: Disrupting the cinnamyl alcohol dehydrogenase 1 gene (BdCAD1) leads to altered lignification and improved saccharification in Brachypodium distachyon. Plant J 2012, 73:496–508.
- Anterola AM, Lewis NG: Trends in lignin modification: a comprehensive analysis of the effects of genetic manipulations/mutations on lignification and vascular integrity. *Phytochemistry* 2002, 61(3):221–294.
- Inoue K, Sewalt VJH, Murray Ballance G, Ni W, Sturzer C, Dixon RA: Developmental expression and substrate specificities of slfalfa Caffeic acid 3-O-methyltransferase and Caffeoyl coenzyme A 3-Omethyltransferase in relation to lignification. *Plant Physiol* 1998, 117(3):761–770.
- McAlister FM, Jenkins CLD, Watson JM: Sequence and expression of a stem-abundant caffeic acid O-methyltransferase cDNA from perennial ryegrass (Lolium perenne). Funct Plant Biol 1998, 25(2):225–235.
- Bugos RC, Chiang VLC, Campbell WH: cDNA cloning, sequence analysis and seasonal expression of lignin-bispecific caffeic acid/5-hydroxyferulic acid O-methyltransferase of aspen. *Plant Mol Biology* 1991, 17(6):1203–1215.
- Piquemal J, Chamayou S, Nadaud I, Beckert M, Barriere Y, Mila I, Lapierre C, Rigau J, Puigdomenech P, Jauneau A, et al: Down-regulation of Caffeic acid O-methyltransferase in maize revisited using a transgenic approach. *Plant Physiol* 2002, 130(4):1675–1685.
- 70. Lapierre CT, Tollier MT, Monties B: A new type of constitutive unit in lignins from the corn bm3 mutant. *CR Acad Sci* 1988, **307**:723–728.
- Jung JH, Vermerris W, Gallo M, Fedenko JR, Erickson JE, Altpeter F: RNA interference suppression of lignin biosynthesis increases fermentable sugar yields for biofuel production from field-grown sugarcane. *Plant Biotech J* 2013, 11(6):709-16.
- Lee SJ, Warnick TA, Pattathil S, Alvelo-Maurosa JG, Serapiglia MJ, McCormick H, Brown V, Young NF, Schnell DJ, Smart LB, et al: Biological conversion assay using *Clostridium phytofermentans* to estimate plant feedstock quality. *Biotechnol Biofuels* 2012, 5(1):5.
- Dien B, Sarath G, Pedersen J, Sattler S, Chen H, Funnell-Harris D, Nichols N, Cotta M: Improved sugar conversion and ethanol yield for forage sorghum (Sorghum bicolor L. Moench) lines with reduced lignin contents. *BioEnerg Res* 2009, 2(3):153–164.
- 74. Initiative TIB: Genome sequencing and analysis of the model grass *Brachypodium distachyon*. *Nature* 2010, **463**(7282):763–768.
- Altschul S, Madden T, Schaffer A, Zhang J, Zhang Z, Miller W, Lipman D: Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997, 25(17):3389–3402.
- Thompson JD, Higgins DG, Gibson TJ: CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl Acids Res 1994, 22(22):4673–4680.
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S: MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 2011, 28(10):2731–2739.

- Warthmann N, Chen H, Ossowski S, Weigel D, Hervre P: Highly specific gene silencing by artificial miRNAs in rice. PLoS One 2008, 3(3):e1829.
- Vogel J, Hill T: High-efficiency Agrobacterium-mediated transformation of Brachypodium distachyon inbred line Bd21-3. Plant Cell Rep 2008, 27(3):471–478.
- Arvidsson S, Kwasniewski M, Riano-Pachon D, Mueller-Roeber B: QuantPrime - a flexible tool for reliable high-throughput primer design for quantitative PCR. *BMC Bioinforma* 2008, 9(1):465.
- Hawkins SW, Boudet AM: Purification and characterization of cinnamyl alcohol dehydrogenase isoforms from the periderm of *Eucalyptus gunnii* hook. *Plant Physiol* 1994, 104(1):75–84.
- Mansell RL, Gross GG, Stöckigt J, Franke H, Zenk MH: Purification and properties of cinnamyl alcohol dehydrogenase from higher plants involved in lignin biosynthesis. *Phytochemistry* 1974, 13(11):2427–2435.
- Saathoff A, Tobias C, Sattler S, Haas E, Twigg P, Sarath G: Switchgrass contains two cinnamyl alcohol dehydrogenases involved in lignin formation. *BioEnergy Res* 2011, 4(2):120–133.
- Wiesner J: Note über das verhalten des phloroglucins und einiger verwandter körper zur verholzten zellmembran. Sitzungsberichte der kaiserlichen akademie der wissenschaften. Math Nat Classe 1978, 77:60–66.
- Maule C: Das verhalten verholzter membranen gegen kaliumpermanganat, eine holzreaktion neuer art. Beiträge zur wissenschaftlichen Botanik 1901, 4:166–185.
- Foster CE, Martin TM, Pauly M: Comprehensive compositional analysis of plant cell walls (lignocellulosic biomass) Part I: lignin. J Vis Exp 2010, 37:e1745.
- Lee SJ, Warnick TA, Leschine SB, Hazen SP: A high-throughput biological conversion assay for determining lignocellulosic quality. *Methods Mol Biol* 2013, 918:341–349.

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