

# SUPPLEMENTARY MATERIAL

## **Misregulation of *p-Coumaroyl-CoA:Monolignol Transferase* in *Brachypodium distachyon***

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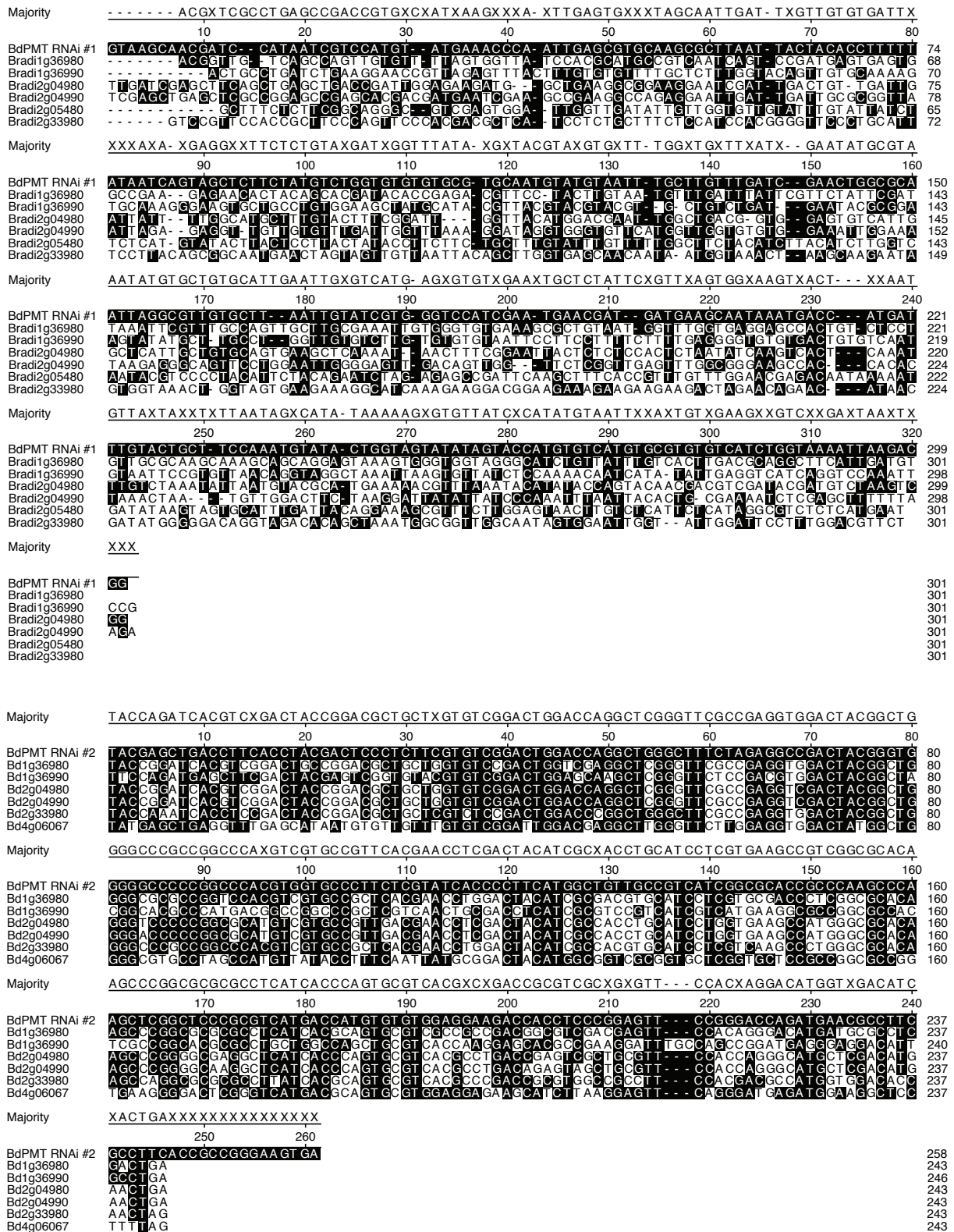
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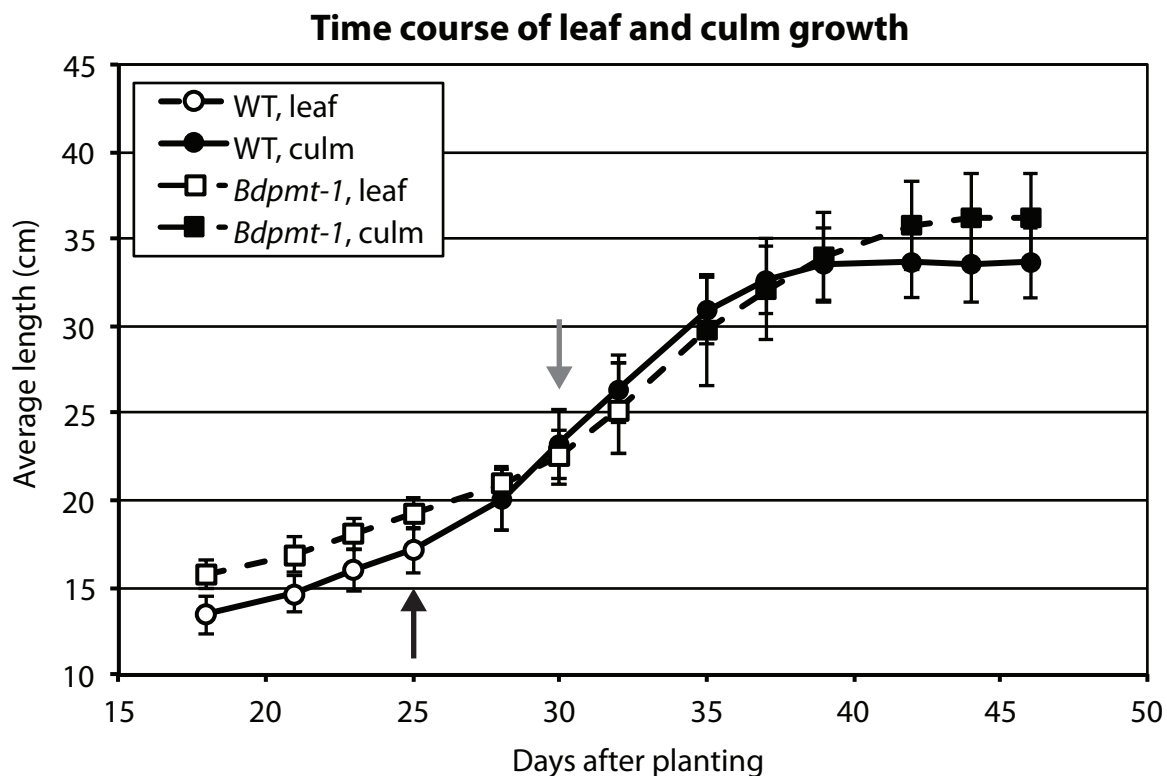
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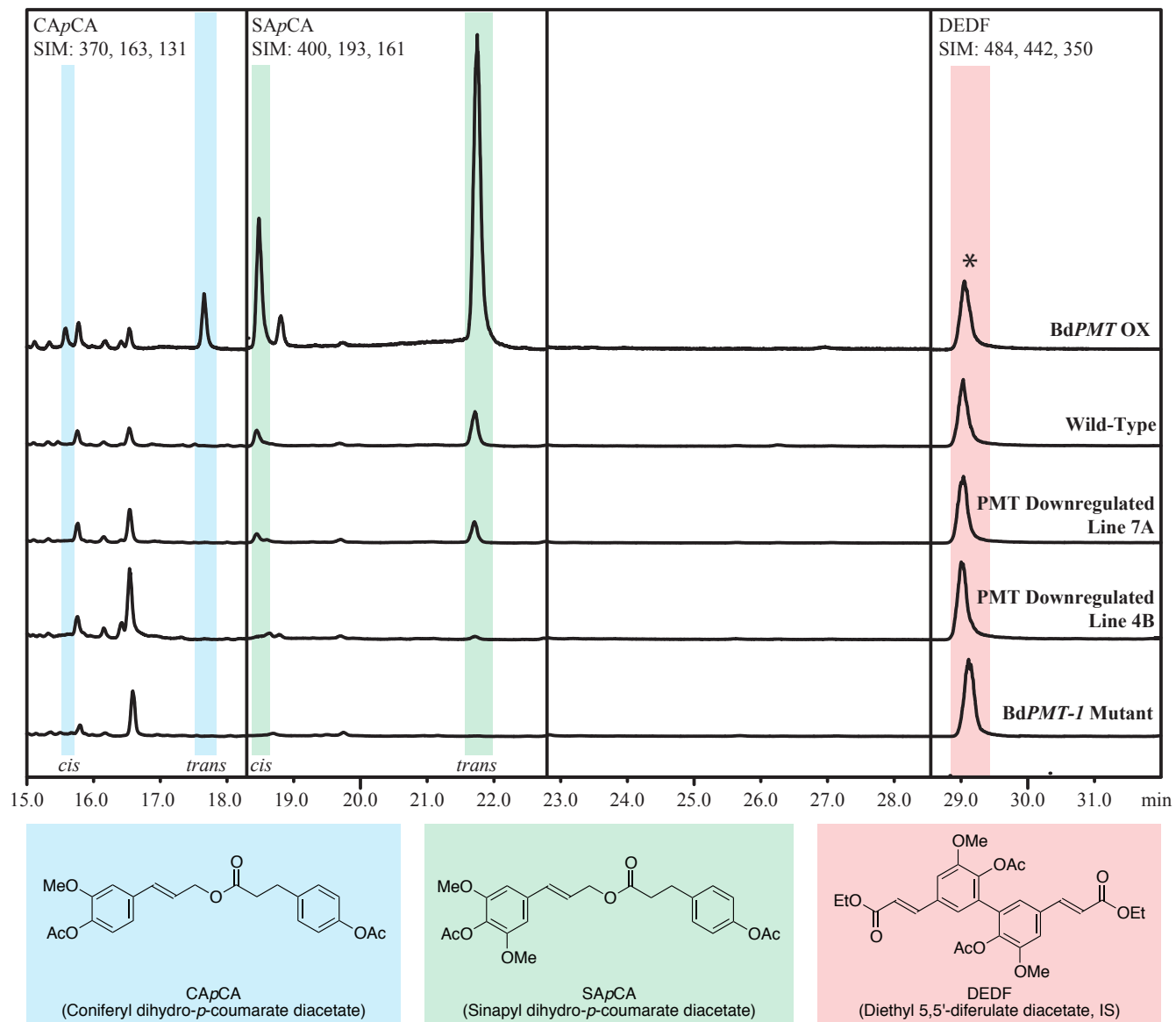
Address for correspondence: John Sedbrook, School of Biological Sciences, Illinois State University, Normal, IL 61790, phone 309-438-3374, fax 309-438-3722, email [jcsedbr@ilstu.edu](mailto:jcsedbr@ilstu.edu)



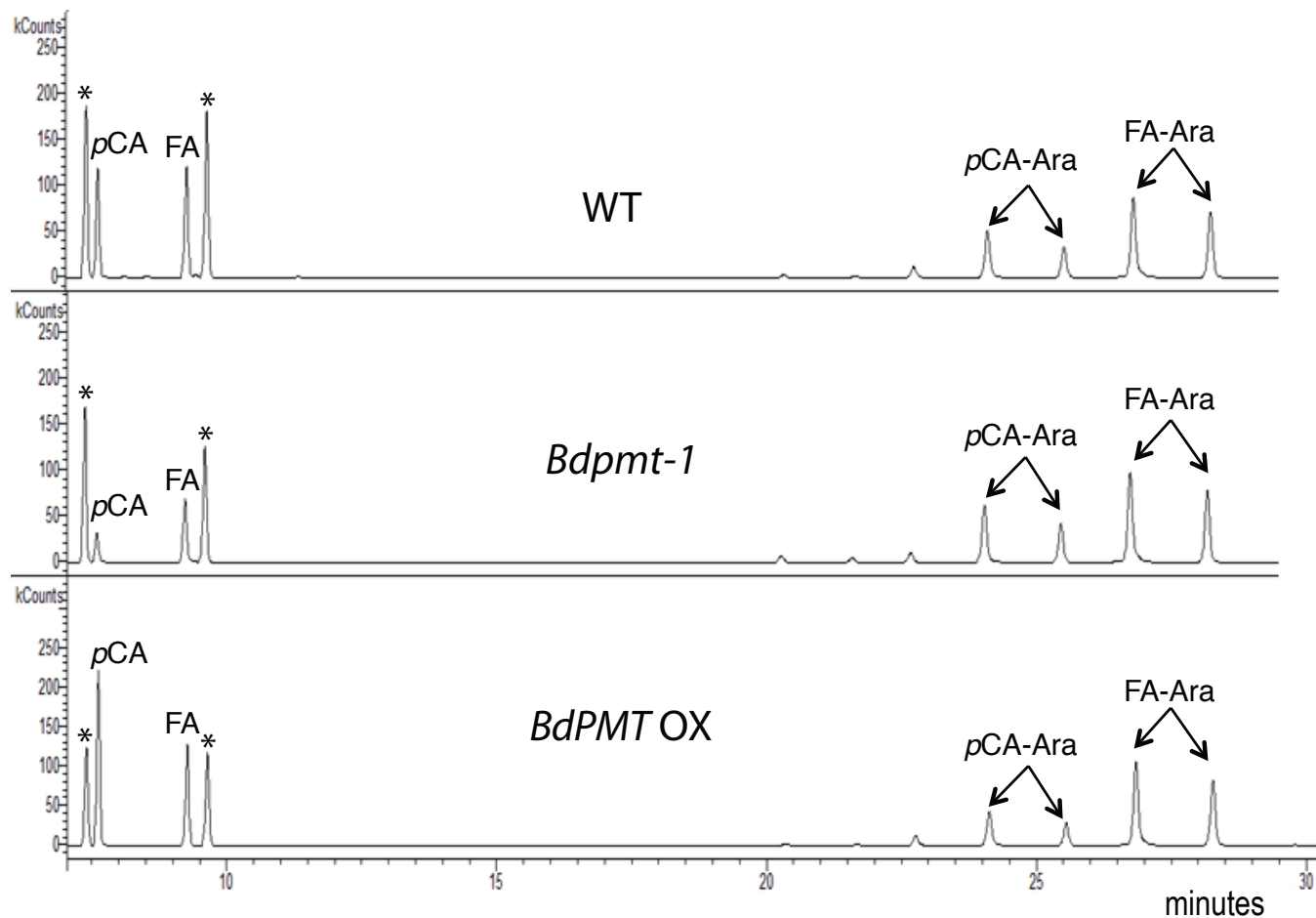
**Figure S1.** Clustal W alignments of the nucleotide sequences comprising *BdPMT* RNAi constructs #1 (top alignment) and #2 (bottom alignment) with the coding sequences of closely related Brachypodium *BdPMT*-like genes. Nucleotides in black boxes are identical to the corresponding *BdPMT* sequences.



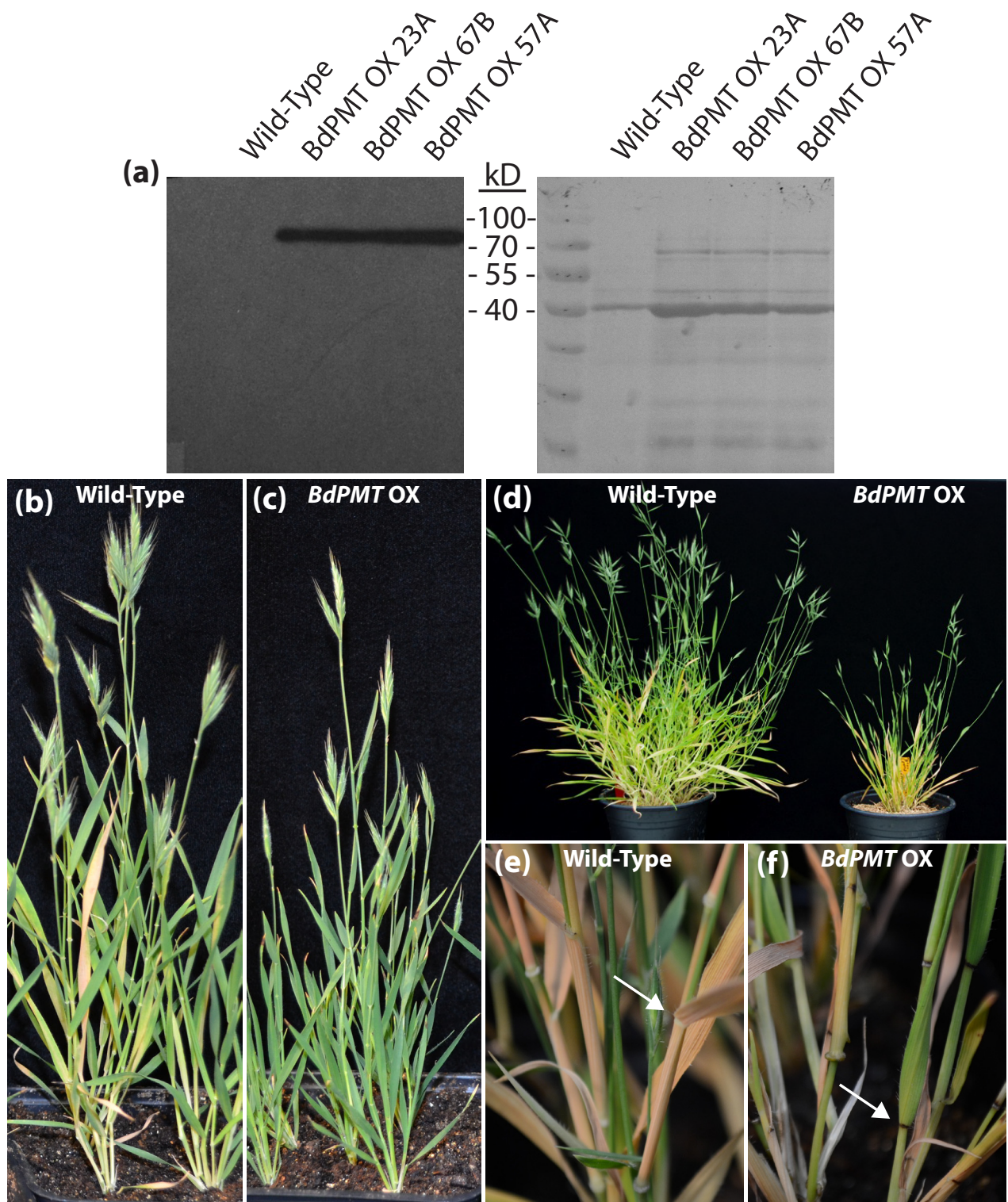
**Figure S2.** Time course growth measurements of *Bdpmt-1* (squares) and WT (circles) plants in soil. Arrows delineate the time points at which 50% of the spikelets could be seen emerging from the leaf whorl (black arrow for WT, grey arrow for *Bdpmt-1*). Open symbols represent average lengths of the longest leaves, whereas closed symbols represent average culm height. Vertical bars are standard deviations. Note that, with this particular experiment, *Bdpmt-1* culms emerged later from the leaf whorl than did WT culms. However, this is not always the case (sometimes average culm emergence occurs at the same time or earlier). Note that *Bdpmt-1* culms grow to be about the same heights as WT, which is usually the case for WT and *Bdpmt-1* and RNAi mutant plants grown side by side in our controlled growth chambers (20 h/day light at 200-250  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , 22 °C). n=51.



**Figure S3.** Partial GC-SIM traces of the diacetylated monolignol conjugates generated by DFRC treatment of extractive-free senesced stems from (top to bottom) *BdPMT* overexpressed (*BdPMT* OX), wild-type (WT), *BdPMT* downregulated line 7A (*PMT* RNAi line 7A), *BdPMT* downregulated line 4B (*PMT* RNAi line 4B), and *BdPMT-1* mutant. \* = internal standard peaks.



**Figure S4.** Partial GC-MS traces of phenolics recovered by mild acidolysis of extractive-free senesced stems from wild-type (WT) and *BdPMT* misregulated lines (*Bdpmt-1* mutant and *BdPMT* overexpressor *BdPMT OX*). *p*-Coumaric acid (*p*CA), ferulic acid (FA), *p*-coumaroylated arabinose (*p*CA-Ara, 2 anomers) and feruloylated arabinose (FA-Ara, 2 anomers) are analyzed as their TMS derivatives. \* = internal standard peaks.



**Figure S5.** Western blot analysis of *BdPMT OX* expression, and growth phenotypes of *BdPMT OX* plants. (a) Western blot analysis of *EYFP:BdPMT* transgenic plant extracts (three independent lines *BdPMT OX* 23A, 67B, and 57A) probed with an anti-GFP antibody, which detected a band of the expected 80 kiloDalton (kD) size (blot image on left; image on right is the same blot protein-stained with amido black). (b-f) Images of WT (b, d, e) and *BdPMT OX* (c, d, f) plant phenotypes. (b, c) In growth chambers with tightly controlled conditions, WT (b) and *BdPMT OX* (c) plants grew to similar sizes. (d) Under greenhouse growth conditions, which were variable, *BdPMT OX* plants grew smaller than WT. (e, f) Leaf collars (delineated by arrows) of *BdPMT OX* plants (f) typically became blackened and the leaf blades grew alongside the culms, whereas WT leaf collars (e) never blackened and WT leaves grew at about 60 degree angles from the culms (these phenotypes were observed in both growth chamber and greenhouse conditions).

**Table S1.** Classes of genes predicted by PlaNet to be co-expressed with Bradi2g36910.1 (*BdPMT*).  
<http://aranet.mpimp-golm.mpg.de/index.html>

Mapman termID	Term description	Terms in NVN	Terms on microarray	p-value
11.10.1	lipid metabolism.glycolipid synthesis.MGDG synthase	1	3	0.01
13.1.6.3.1	amino acid metabolism.synthesis.aromatic aa.phenylalanine.arogenate dehydratase / prephenate dehydrat	1	6	0.03
16	secondary metabolism	7	350	0.01
16.2	secondary metabolism.phenylpropanoids	6	115	0
16.2.1	secondary metabolism.phenylpropanoids.lignin biosynthesis	4	39	0
16.2.1.1	secondary metabolism.phenylpropanoids.lignin biosynthesis.PAL	2	8	0
16.2.1.10	secondary metabolism.phenylpropanoids.lignin biosynthesis.CAD	1	7	0.03
16.2.1.3	secondary metabolism.phenylpropanoids.lignin biosynthesis.4CL	1	16	0.05
17	hormone metabolism	7	395	0.02
17.5	hormone metabolism.ethylene	4	117	0.01
17.5.3	hormone metabolism.ethylene.induced-regulated-responsive-activated	2	16	0.01
17.7.1.5	hormone metabolism.jasmonate.synthesis-degradation.12-Oxo-PDA-reductase	1	11	0.04
20.2.3	stress.abiotic.drought/salt	2	46	0.04
26.21	misc.protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	2	37	0
27.3	RNA.regulation of transcription	22	2052	0.01
27.3.13	RNA.regulation of transcription.CCAAT box binding factor family, DR1	1	2	0.02
27.3.14	RNA.regulation of transcription.CCAAT box binding factor family, HAP2	1	5	0.04
27.3.22	RNA.regulation of transcription.HB,Homeobox transcription factor family	3	75	0.04
27.3.25	RNA.regulation of transcription.MYB domain transcription factor family	3	114	0.04
27.3.4	RNA.regulation of transcription.ARF, Auxin Response Factor family	2	21	0.01
27.3.5	RNA.regulation of transcription.ARR	2	17	0.01
3.2.1	minor CHO metabolism.trehalose.TPS	1	8	0.04
31	cell	9	651	0.01
31.1	cell.organisation	8	352	0
33	development	8	484	0.02
33.99	development.unspecified	7	409	0.02
34	transport	15	1010	0
34.13	transport.peptides and oligopeptides	7	108	0
34.3	transport.amino acids	3	85	0.01
34.6	transport.sulphate	2	10	0

List of genes predicted to co-express with Bradi2g36910 (*BdPMT*). Highlighted in yellow are known and putative lignin biosynthesis genes.

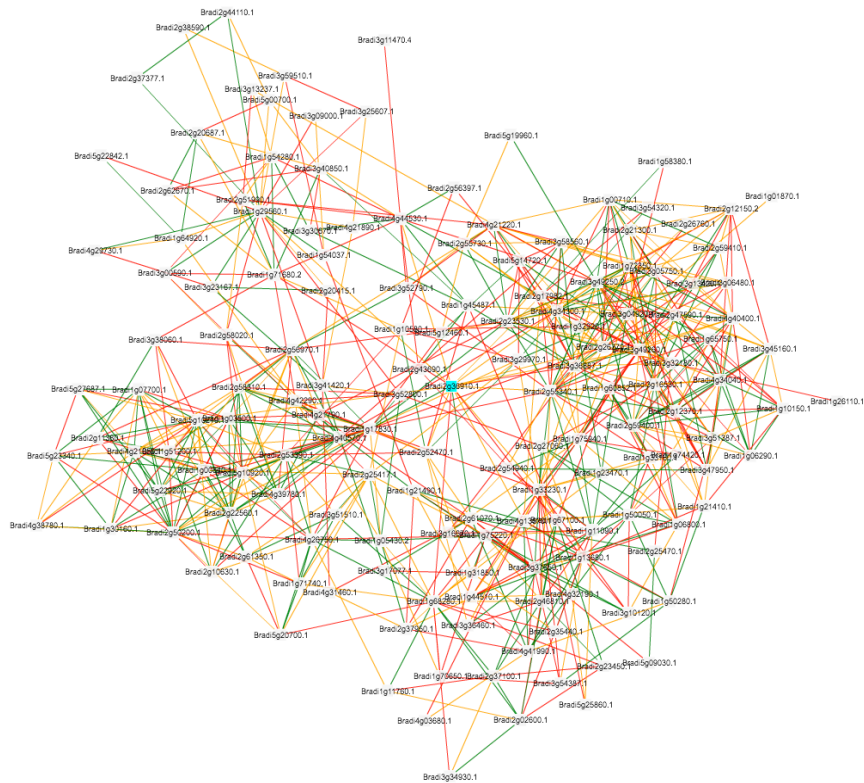
Gene ID	Description	
Bradi4g38780.1	gibberellin 20 oxidase 2, putative, expressed	
Bradi2g37377.1	MYB family transcription factor, putative, expressed	
Bradi5g20700.1	WRKY68, expressed	
Bradi2g55340.1	transmembrane amino acid transporter protein, putative, expressed	
Bradi1g75220.1	Citrate transporter protein, putative, expressed	
Bradi3g59510.1	protein kinase domain containing protein, expressed	
Bradi2g26760.1	annexin, putative, expressed	
Bradi1g58380.1	ERD4 protein, putative, expressed	
Bradi3g37650.1	12_oxophytodienoate reductase, putative, expressed	
Bradi3g13237.1	transferase family protein, putative, expressed	
Bradi1g01870.1	pirin, putative, expressed	
Bradi1g31850.1	cell cycle control protein, putative, expressed	
Bradi3g11470.4	ras_related protein, putative, expressed	
Bradi1g45487.1	annexin, putative, expressed	
Bradi1g21490.1	zinc_finger protein, putative, expressed	
Bradi3g16670.1	peptide transporter PTR2, putative, expressed	
Bradi3g25607.1	expressed protein	
Bradi4g32190.1	EF hand family protein, putative, expressed	
Bradi4g44530.1	peroxidase precursor, putative, expressed	
Bradi2g55730.1	cytochrome P450, putative, expressed	
Bradi2g59400.1	exostosin family domain containing protein, expressed	
Bradi2g11380.1	polygalacturonase, putative, expressed	
Bradi3g30670.1	dehydration response related protein, putative, expressed	
Bradi3g54320.1	expressed protein	
Bradi2g10630.1	expressed protein	
Bradi4g21050.1	ANTH domain containing protein, expressed	
Bradi3g00590.1	auxin_induced protein 5NG4, putative, expressed	
Bradi1g07700.1	subtilisin_like protease, putative, expressed	
Bradi1g32920.1	nodulin MtN3 family protein, putative, expressed	
Bradi3g51387.1	aquaporin protein, putative, expressed	
Bradi2g59410.1	exostosin family domain containing protein, expressed	
Bradi1g50050.1	lung seven transmembrane domain containing protein, putative, expressed	
Bradi3g47950.1	expressed protein	
Bradi3g54387.1	monogalactosyldiacylglycerol synthase, putative, expressed	
Bradi3g32180.1	expressed protein	
Bradi2g23450.1	membrane associated DUF588 domain containing protein, putative, expressed	
Bradi2g61350.1	RNA recognition motif containing protein, putative, expressed	
Bradi2g62670.1	senescence_induced receptor_like serine/threonine_protein kinase precursor, putative, expressed	
Bradi2g27060.1	golgi transport complex protein_related, putative, expressed	
Bradi3g36887.1	cinnamoyl_CoA reductase, putative, expressed	CCR1
Bradi3g29970.1	hydrolase, alpha/beta fold family domain containing protein, expressed	
Bradi4g31460.1	HD domain containing protein, putative, expressed	
Bradi5g12460.1	uncharacterized Cys_rich domain containing protein, putative, expressed	
Bradi1g64920.1	pathogenesis_related Bet v I family protein, putative, expressed	
Bradi1g70650.1	natural resistance_associated macrophage protein, putative, expressed	
Bradi3g05750.1	AMP_binding domain containing protein, expressed	4CL

<b>Bradi2g21300.1</b>	<b>cytochrome P450, putative, expressed</b>	<b>C3H</b>
Bradi1g74420.1	sulfate transporter, putative, expressed	
Bradi1g33230.1	expressed protein	
Bradi2g02600.1	PB1 domain containing protein, expressed	
Bradi1g51200.1	sulfate transporter, putative, expressed	
Bradi1g10580.1	LTPL118 _ Protease inhibitor/seed storage/LTP family protein precursor, expressed	
Bradi5g25860.1	OsRR6 type_A response regulator, expressed	
Bradi1g06800.1	tubulin/FtsZ domain containing protein, putative, expressed	
Bradi2g17982.1	myb_like DNA_binding domain containing protein, putative, expressed	
Bradi5g00700.1	ethylene receptor, putative, expressed	
Bradi5g09030.1	prephenate dehydratase domain containing protein, expressed	
Bradi3g38060.1	gibberellin receptor GID1L2, putative, expressed	
<b>Bradi3g16530.1</b>	<b>O_methyltransferase, putative, expressed</b>	<b>COMT</b>
Bradi1g71680.2	LTPL69 _ Protease inhibitor/seed storage/LTP family protein precursor, expressed	
Bradi5g19960.1	RING_H2 finger protein ATL5G, putative, expressed	
Bradi2g46810.1	expressed protein	
Bradi2g58020.1	two_component response regulator, putative, expressed	
Bradi1g54280.1	expressed protein	
Bradi4g21790.1	peptide transporter PTR2, putative, expressed	
Bradi1g68280.1	actin_depolymerizing factor, putative, expressed	
Bradi4g26750.1	expressed protein	
Bradi2g61070.1	NADPH quinone oxidoreductase, putative, expressed	
Bradi2g38590.1	CGMC_GSK.6 _ CGMC includes CDA, MAPK, GSK3, and CLKC kinases, expressed	
Bradi1g65750.1	glycosyltransferase family 43 protein, putative, expressed	
Bradi2g26770.1	annexin, putative, expressed	
Bradi3g41420.1	zinc finger DHHC domain_containing protein, putative, expressed	
Bradi4g41990.1	harpin_induced protein 1 domain containing protein, expressed	
Bradi3g34930.1	histone_like transcription factor and archaeal histone, putative, expressed	
Bradi1g29560.1	pollen signalling protein with adenyl cyclase activity, putative, expressed	
Bradi1g54037.1	DUF630/DUF632 domains containing protein, putative, expressed	
Bradi3g13420.1	esterase, putative, expressed	
Bradi2g12150.2	S_adenosylmethionine synthetase, putative, expressed	
Bradi1g75940.1	protein phosphatase 2C, putative, expressed	
Bradi4g21220.1	auxin_induced protein 5NG4, putative, expressed	
Bradi4g42290.1	expressed protein	
Bradi1g00840.1	avr9/Cf_9 rapidly elicited protein, putative, expressed	
Bradi1g26110.1	expressed protein	
<b>Bradi5g14720.1</b>	<b>transferase family protein, putative, expressed</b>	<b>HCT</b>
Bradi1g33160.1	auxin response factor 18, putative, expressed	
Bradi1g06290.1	fasciclin domain containing protein, expressed	
Bradi2g25470.1	alpha/beta hydrolase fold, putative, expressed	
Bradi4g13670.1	patatin, putative, expressed	
<b>Bradi3g49260.1</b>	<b>phenylalanine ammonia_lyase, putative, expressed</b>	<b>PAL</b>
Bradi5g27687.1	peroxidase precursor, putative, expressed	
Bradi3g51510.1	dof zinc finger domain containing protein, putative, expressed	
Bradi2g22560.1	DUF640 domain containing protein, putative, expressed	
Bradi2g44110.1	No annotation	
Bradi4g40570.1	amino acid transporter, putative, expressed	
Bradi1g71740.1	ethylene_responsive transcription factor, putative, expressed	
Bradi1g00710.1	expressed protein	
Bradi3g45160.1	harpin_induced protein 1 domain containing protein, expressed	
Bradi2g20687.1	serine/threonine_protein kinase receptor precursor, putative, expressed	
Bradi2g37950.1	tetraspanin family protein, putative, expressed	
Bradi3g23167.1	expressed protein	
Bradi4g21890.1	NB_ARC domain containing protein, expressed	
Bradi1g13680.1	nuclear transcription factor Y subunit, putative, expressed	
Bradi1g60852.1	expressed protein	
Bradi3g52790.1	basic helix_loop_helix, putative, expressed	
Bradi5g22842.1	NBS_LRR disease resistance protein, putative, expressed	
Bradi2g12370.1	GDSL_like lipase/acylhydrolase, putative, expressed	
Bradi2g47590.1	MYB family transcription factor, putative, expressed	
Bradi1g11760.1	zinc finger family protein, putative, expressed	
Bradi5g10920.1	peptide transporter PTR2, putative, expressed	
Bradi2g54940.1	homeodomain protein, putative, expressed	
Bradi1g30160.1	receptor protein kinase CLAVATA1 precursor, putative, expressed	
Bradi1g21410.1	expressed protein	
Bradi2g56970.1	amino acid transporter, putative, expressed	
Bradi3g10120.1	expressed protein	
Bradi2g43690.1	oxidoreductase, aldo/keto reductase family protein, putative, expressed	
Bradi5g22920.1	ethylene_responsive protein related, putative, expressed	
Bradi4g29730.1	trehalose synthase, putative, expressed	
Bradi4g34300.1	membrane protein, putative, expressed	
Bradi1g50280.1	expressed protein	
Bradi2g20415.1	MATH domain containing protein, expressed	
Bradi1g44510.1	B12D protein, putative, expressed	
Bradi3g17077.1	expressed protein	
Bradi1g03500.1	peptide transporter PTR2, putative, expressed	
Bradi1g11090.1	GRAS family transcription factor domain containing protein, expressed	
<b>Bradi3g06480.1</b>	<b>dehydrogenase, putative, expressed</b>	<b>CAD</b>
Bradi4g03680.1	heparanase_like protein precursor, putative, expressed	
Bradi2g36910.1	transferase family protein, putative, expressed	
Bradi2g52470.1	ICE_like protease p20 domain containing protein, putative, expressed	
Bradi4g34040.1	CHIT13 _ Chitinase family protein precursor, expressed	
Bradi2g50200.1	RING_H2 finger protein, putative, expressed	



Bradi2g37100.1	ankyrin repeat domain_containing protein 28, putative, expressed	
Bradi2g25417.1	peptide transporter PTR2, putative, expressed	
Bradi3g09000.1	spotted leaf 11, putative, expressed	
Bradi4g39780.1	peptide transporter PTR3_A, putative, expressed	
Bradi1g72350.1	glycosyl transferase, putative, expressed	
Bradi1g17830.1	potassium transporter, putative, expressed	
Bradi3g40850.1	ras_related protein, putative, expressed	
<b>Bradi3g49250.2</b>	<b>phenylalanine ammonia_lyase, putative, expressed</b>	<b>PAL</b>
Bradi1g05430.2	BHLH transcription factor, putative, expressed	
Bradi2g58310.1	No annotation	
Bradi3g04920.1	auxin response factor 6, putative, expressed	
Bradi2g23530.1	homeodomain protein, putative, expressed	
Bradi1g67100.1	OsWLM2 _ LIM domain protein, putative actin_binding protein and transcription factor, expressed	
Bradi2g56397.1	proton_dependent oligopeptide transport, putative, expressed	
Bradi3g52800.1	bg55, putative, expressed	
Bradi2g35440.1	lipase, putative, expressed	
Bradi5g23340.1	transcription factor, putative, expressed	
Bradi1g23470.1	ZOS7_06 _ C2H2 zinc finger protein, expressed	
Bradi2g51990.1	bHelix_loop_helix transcription factor, putative, expressed	
Bradi5g10210.1	OsSub41 _ Putative Subtilisin homologue, expressed	
Bradi4g40400.1	plant_specific domain TIGR01627 family protein, expressed	
Bradi2g53390.1	homeobox domain containing protein, expressed	
Bradi1g10150.1	tubulin/FtsZ domain containing protein, putative, expressed	
Bradi3g36460.1	RAD23 DNA repair protein, putative, expressed	
Bradi3g58560.1	plastocyanin_like domain containing protein, putative, expressed	

The node vicinity network below contains the query gene (light blue) and all genes two steps away from the query gene.



**Table S2.** Impact of misregulating the *BdPMT* gene on *BdPMT* transcript levels and on the amount of *p*-coumaric acid (*p*CA) and ferulic acid (FA) released by mild alkaline hydrolysis.

Genotype	<i>BdPMT</i> transcript level (n, SD) <sup>a</sup>	Mild alkaline hydrolysis <sup>b</sup>	
		<i>p</i> CA(mg/g)	FA (mg/g)
WT	ND	7.34 ± 0.30	4.06 ± 0.10
<i>Bdpmt-1</i>	ND	1.63 ± 0.09**	3.03 ± 0.41*
WT	1 (4, 0.06)	6.40 ± 0.26	8.62 ± 0.41
RNAi#2 4B	0.08 (3, 0.04)**	3.14 ± 0.18**	8.31 ± 0.58
RNAi#2 7A	0.25 (6, 0.07)**	5.04 ± 0.29**	8.64 ± 0.65
WT	1 (5, 0.00)	5.55 ± 0.13	6.24 ± 0.30
RNAi#1 1A	0.42 (15, 0.03)**	4.77 ± 0.38*	6.40 ± 0.47
RNAi#1 8A	0.47 (12, 0.03)*	5.39 ± 0.54	6.04 ± 0.14
WT	1 (3, 0.01)	7.45 ± 0.83	5.42 ± 0.48
Empty vector	ND	7.56 ± 0.41	5.09 ± 0.44
PMT OX 23A	34.82 (7, 6.72)**	16.16 ± 1.07**	7.14 ± 0.69*
PMT OX 57A	55.78 (12, 15.06)**	17.33 ± 0.61**	7.12 ± 0.17*
PMT OX 67B	ND	16.37 ± 1.14*	6.38 ± 0.52

ND: not determined. Asterisks denote statistically significant differences (Student's test) compared to the corresponding WT plants grown alongside (\*:  $p < 0.02$ ; \*\*:  $p < 0.0001$ ).

<sup>a</sup>Quantitative real-time PCR analyses were run on the top 2 internodes collected when the inflorescences were fully elongated but before seed fill had occurred. Transcript level values for misregulated plants are expressed relative to the corresponding WT value normalized to one. In parentheses are the number of individually analyzed plants, *n*, and the standard deviation of the ratio of gene expression. <sup>b</sup>Mild alkaline hydrolyses were run on extract-free senesced stems. Data are mean values ± SD.  $n \geq 3$ .

**Table S3.** Amount of DFRC-released *p*-coumaric acid (*p*CA) and amounts of *p*CA, ferulic acid (FA), *p*-coumaroylated arabinose (*p*CA-Ara) and feruloylated arabinose (FA-Ara) released by mild acidolysis of extract-free senesced stems.

Genotype	DFRC	Mild acidolysis			
	<i>p</i> CA (mg/g)	<i>p</i> CA (mg/g)	FA (mg/g)	<i>p</i> CA-Ara (mg/g)	FA-Ara (mg/g)
WT	2.63 ± 0.60	0.64 ± 0.07	0.49 ± 0.06	2.94 ± 0.49	6.06 ± 0.74
<i>Bdpmt-1</i>	0.01 ± 0.02*	0.18 ± 0.03*	0.47 ± 0.09	3.21 ± 0.56	5.93 ± 0.57
WT	1.20 ± 0.28	0.53 ± 0.09	0.53 ± 0.10	4.37 ± 0.34	8.35 ± 0.92
RNAi#2 4B	0.10 ± 0.03*	0.25 ± 0.04*	0.54 ± 0.11	4.91 ± 0.50	8.52 ± 0.67
RNAi#2 7A	0.60 ± 0.20*	ND	ND	ND	ND
WT	2.31 ± 0.96	0.87 ± 0.05	0.90 ± 0.06	4.85 ± 0.44	8.53 ± 0.51
PMT OX	7.98 ± 2.71*	1.84 ± 0.13*	1.15 ± 0.07	4.43 ± 0.40	11.57 ± 1.01*

Values are means ± SD from individually analyzed plants (n≥3). Asterisks denote statistically significant differences (Student's test) compared to the corresponding wild-type (WT) values at p<0.001, except for RNAi#2 7A line where p<0.006. ND= not determined. Note that for the DFRC treatment, *p*CA is derived exclusively from lignin. For the Mild Acidolysis treatment, FA, *p*CA-Ara, and FA-Ara are derived exclusively from arabinoxylan, whereas *p*CA is derived from both lignin and arabinoxylan.

**Table S4.** Primer sequences and qRT-PCR amplification efficiencies.

Primer name	Purpose	Amplicon Length	Sequence (5' - 3')
Bradi2g36910_RNAi#1_F	Cloning	301 bp	GGGGGGTACCGGATCCGTAAGCAACGATCCATAATCG
Bradi2g36910_RNAi#1_R			GGGGACTAGTGGCGCGCCCCGTCTTAATTTTACCAGATG
Bradi2g36910_RNAi#2_F		258 bp	GGGGGGTACCGGATCCTACGAGCTGACCTTCACCT
Bradi2g36910_RNAi#2_R			GGGGACTAGTGGCGCGCCTCACTTCCCGGCGGTGAAG
Bradi2g36910_ORFBamF		1350 bp	GAGAGGATCCGATGGAGAAGAAGTTCACGGTG
Bradi2g36910_ORFXhoR			GAGAACTAGTCTCGAGTCACTTCCCGGCGGTGAAGGCCA- AGGCCG
pStarling_F1	Screening	470 bp	CTTCATACGCTATTTATTTGCTTGGTA
pStarling_R1			TGACTTACCATGGATGATGTGTTTCTA
pStarling_F2		532 bp	TCTGAATGGTCACAAGTCACATACAAT
pStarling_R2			GTGTTCTAAGCTAGCCTGGTAATAATC
PMT_qRTPCR_3F	RT-qPCR	175 bp	GGCGAGGCACCTTGATGGG
PMT_qRTPCR_3R2R			CCCGTCGGCGATGGTGTG
BdUBC18for		196 bp	ATCCCATGGAGGCACCTCAGGTC
BdUBC18rev			CGGTCATTGTCTTGCGGACGTTG
EF1a_Forward		150 bp	CATGCCCTCCTTGCTTTCACCTT
EF1a_Reverse			AGGGTTGTAGCCGACCTTCTTGAG

Gene	Locus	Amplification efficiency $\pm$ SD
BdPMT	Bradi2g36910	2.05 $\pm$ 0.19
UBC18	Bradi4g00660	1.92 $\pm$ 0.09
EF1 $\alpha$	Bradi1g06860	2.05 $\pm$ 0.01