Protein Arginine Methyltransferase Expression Affects Ectomycorrhizal Symbiosis and the Regulation of Hormone Signaling Pathways

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The genomes of all eukaryotic organisms, from small unicellular yeasts to humans, include members of the protein arginine methyltransferase (PRMT) family. These enzymes affect gene transcription, cellular signaling, and function through the post-translational methylation of arginine residues. Mis-regulation of PRMTs results in serious developmental defects, disease, or death, illustrating the importance of these enzymes to cellular processes. Plant genomes encode almost the full complement of PRMTs found in other higher organisms, plus an additional PRMT found uniquely in plants, PRMT10. Here, we investigate the role of these highly conserved PRMTs in a process that is unique to perennial plants—the development of symbiosis with ectomycorrhizal fungi. We show that PRMT expression and arginine methylation is altered in the roots of the model tree *Eucalyptus grandis* by the presence of its ectomycorrhizal fungal symbiont *Pisolithus albus*. Further, using transgenic modifications, we demonstrate that *E. grandis*-encoded PRMT1 and PRMT10 have important but opposing effects in promoting this symbiosis. In particular, the plant-specific EgPRMT10 has a potential role in the expression of plant hormone pathways during the colonization process and its overexpression reduces fungal colonization success.

Keywords: arginine methylation, *Eucalyptus grandis*, hormone regulation, mycorrhizal fungi, *Pisolithus albus*, posttranslational modification

Ectomycorrhizal (ECM) fungi associate with the roots of most tree species in temperate and subtropical forests worldwide,

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improving plant access to nutrition and protecting them from biotic and abiotic stressors in exchange for photosynthates (Smith and Read 2008). The establishment of a successful mutualistic relationship between plant and fungus requires a carefully balanced exchange of signaling molecules and appropriate transcriptomic alteration on the part of both partners. Several recent studies have considered the transcriptional changes in host roots throughout the colonization process (Plett et al. 2015a and b; Sebastiana et al. 2014; Tarkka et al. 2013). Typically, plant roots will launch a general defense response upon perception of the fungus, although less pronounced than with a pathogen attack. At later stages of the interaction, this response will shift to include hormone signaling and changes to cell-wall architecture and nutrient shuttling to accommodate the establishing symbiotic partnership. Proper regulation of hormone signaling within plant tissues is crucial to the interaction (Daguerre et al. 2016; Martin et al. 2001); however, the underlying mechanisms and signaling pathways to produce these necessary transcriptional changes are not well-understood.

One mechanism by which both transcription and protein function can be altered is via protein posttranslational modifications (PTMs), which can include phosphorylation, acetylation, glycosylation, ubiquitination, or methylation. PTMs of histones, transcription factors, or DNA binding proteins can directly alter transcription of specific genes or gene clusters (DesJarlais and Tummino 2016) or PTMs can change the function of a given protein through changes to its structure, stability, or localization. Posttranslational modifications such as phosphorylation are known to affect the outcome of some beneficial plant-microbe interactions (Antolín-Llovera et al. 2012). One emerging PTM of interest, protein methylation at arginine residues, is catalyzed by protein arginine methyltransferases (PRMTs) using *S*-adenosyl-L-methionine (SAM) as a methyl donor (Ahmad and Cao 2012; Bedford and Clarke 2009; Blanc and Richard 2017; Peng and Wong 2017). PRMTs are well-conserved in all eukaryotic organisms and have been implicated in altered transcription, RNA processing, transport and translation, signal transduction, DNA repair, chromatin structure, cell-cycle regulation, and cellular differentiation (Bedford and Clarke 2009; Blanc and Richard 2017; Raposo and Piller 2018). Common targets of PRMTs are histones (Liu et al. 2010), although many nonhistone methylated proteins have also been identified as PRMT substrates (Lee and Stallcup 2009; Wei et al. 2014).

Nine PRMT homologs have been identified in the *Arabidopsis thaliana* genome (Ahmad and Cao 2012). PRMT homologs have also been identified in other annual plants, such as rice (Ahmad et al. 2011) and soybean (Liew et al. 2013). While most of these

PRMTs are conserved and present in other higher eukaryotic organisms, plants uniquely have evolved an additional type I PRMT—PRMT10—which has proposed roles in histone modification and flowering time in *Arabidopsis* (Cheng et al. 2011; Niu et al. 2007). Type I PRMTs produce mono-methylated arginine as an intermediate and asymmetrically dimethylated arginines as the final product and, in *Arabidopsis*, have been implicated in transcription and RNA processing (Ahmad et al. 2011; Yan et al. 2007), ribosomal biogenesis (Hang et al. 2014), and flowering time (Liew et al. 2013; Niu et al. 2007, 2008). Type II PRMTs produce symmetrically dimethylated arginine as the final product and, in plants, have known roles in precursor mRNA splicing (Deng et al. 2010, 2016), flowering time (Deng et al. 2016; Wang et al. 2007), salt stress tolerance (Zhang et al. 2011), primary root length (Pei et al. 2007), root stem cell maintenance (Li et al. 2016), and circadian rhythms (Sanchez et al. 2010). While less is known about the role of PRMTs in perennial plants, previous research on *E. grandis* (Plett et al. 2017) has demonstrated an important role for type I PRMTs in the development of roots. *E. grandis* roots with transgenically downregulated type I PRMT genes had significantly shorter roots and fewer lateral roots formed (Plett et al. 2017). Repression of PRMT1 activity using chemical inhibitors resulted in a broad transcriptomic shift in the roots (Plett et al. 2017), highlighting the importance of PRMTs in cellular signaling and transcription regulation networks.

Here, we consider the effect of protein methylation by two type I PRMTs, EgPRMT1 and EgPRMT10, on the colonization outcomes and transcriptomic regulation of the interaction between *E. grandis* and *Pisolithus albus*, an ECM fungal symbiont. PRMT1 is considered to be the most active PRMT with as much as 85% of arginine methylation in mouse cells attributed to it (Ahmad and Cao 2012). PRMT10 is of interest as it has uniquely evolved within plant genomes. While the role of PRMTs in mutualistic plant-fungal associations has not been studied, there has been some work in pathogenic interactions. There are a number of examples during pathogenesis whereby aberrant histone methylation in the plant causes reduced immunity due to lower transcription of defense-related genes (Zhu et al. 2016). The majority of what is known concerning protein methylation and its impact on the outcome of pathogenic interactions, however, focuses on lysine methylation rather than arginine methylation. One of the few studies considering asymmetric arginine dimethylation during pathogenesis focused on the activity of a type I PRMT from a fungal pathogen and found that it bolstered fungal virulence (Wang et al. 2012).

Here, we show that EgPRMT1 and EgPRMT10 have important but opposing effects in the promotion of mutualistic symbiosis between *E. grandis* and its ECM partner *P. albus*. Elevated expression of *EgPRMT1* in the roots of *E. grandis* results in a significant improvement in the number of lateral roots colonized by *P. albus*, while increased expression of *EgPRMT10* significantly decreases the colonization success of the fungus. Gene expression analysis demonstrates that EgPRMT10 has an effect on the expression of key hormone-responsive genes in various hormone signaling pathways. These results suggest that PRMT10 may have evolved control over the plant-specific hormone pathways and, concurrently, mycorrhizal symbiosis.

RESULTS

PRMT genes are differentially regulated in roots undergoing colonization by ECM fungi.

The roots of *E. grandis* seedlings were placed in contact with the ECM fungus *Pisolithus albus* SI-12 (Plett et al. 2015a) and

expression of *E. grandis* PRMT genes was monitored over a two-week colonization timecourse. Within 24 h of contact with the host plant, *P. albus* hyphae began growing around active root tips, increasing through 48 h to 1 week of contact. Between one and two weeks of the colonization process, the hyphae condense around the root to form a thick mantle and establish the mycorrhizal root tip (Fig. 1A and B). Expression of the seven PRMT genes encoded by *E. grandis* varied widely across this colonization timeline relative to axenic controls (Fig. 1C). *EgPRMT1* is significantly repressed at nearly all timepoints, although this repression is quite small (expressed at about 65 to 75% of control levels). *EgPRMT10*, meanwhile, was universally overexpressed except for the 48-h timepoint. At the 48-h timepoint, *EgPRMT3* and *EgPRMT4* were also significantly overexpressed, while *EgPRMT5* expression was significantly reduced. *EgPRMT7* was also significantly up-regulated during the later timepoints of the colonization process.

Colonization by *P. albus* alters protein methylation in *E. grandis*.

Total protein was extracted from *E. grandis* control roots (grown without fungal contact), roots in indirect contact with *P. albus* for 48 h (i.e., presymbiosis, an early symbiosis establishment timepoint), and fully colonized roots (2 weeks after contact, a symbiosis maintenance timepoint). The resulting proteins were analyzed by mass spectrometry to identify proteins with methylated arginine residues (Table 1). In some cases, proteins that were methylated in control roots were not found to be methylated in roots exposed to *P. albus* and vice versa. For example, at 48 h in ATP synthase subunit beta proteins, R159 is methylated only in axenic controls, while in the elongation factor 1-alpha protein, R166 is nonmethylated in axenic controls but gains a methyl group upon indirect fungal contact. Also, in many cases, proteins were methylated in both control and test roots, but one or more specific arginines methylated within the protein sequence changed (e.g., HSP80 is methylated at R329 and R375 in axenic root samples but at R98 during the 48-h presymbiosis stage with *P. albus*). Thus, the presence of *P. albus* causes alterations not only in the transcription of PRMTs but in the methylation pattern on different proteins expressed in the plant roots. It should be noted that mass spectrometry cannot differentiate between asymmetric and symmetric arginine dimethylation; thus, this table represents activity of both type I and II PRMTs.

Chemical inhibition of PRMT activity and transgenic variation of PRMT gene expression alters colonization of *E. grandis* roots by *P. albus*.

To determine the effect of PRMT activity on *E. grandis* root colonization by *P. albus*, we used a combination of chemical inhibitors of PRMT activity and transgenic roots differentially expressing EgPRMT1 and EgPRMT10. All inhibitors of PRMT activity (adenosine dialdehyde [AdOx, a general methylation inhibitor], arginine methyltransferase inhibitor 1 [AMI-1], and 2,3-dimethoxynitrostyrene [DMNS, a PRMT1 inhibitor]) applied to *E. grandis* roots in contact with *P. albus* caused the percentage of mycorrhizal root tips to significantly decline to approximately half the number found on the control treated plants (Fig. 2A). As PRMT inhibitors can equally affect the plant roots and the colonizing fungus, these experiments were complemented with the expression of *E. grandis* PRMTs modified transgenically. Similar to the inhibitor experiments, transgenic repression of *EgPRMT1* resulted in a significant reduction in the percentage of lateral roots colonized by *P. albus*. Elevated expression of *EgPRMT1* resulted in an increase in the number of roots colonized by *P. albus* (Fig. 2B). Interestingly, *35S::EgPRMT10* roots showed lower levels of

colonization and *EgPRMT10* RNA interference (RNAi) roots had elevated percentages of mycorrhizal root tips, as compared with controls (Fig. 2B). While this may seem to contradict the results from the inhibitor experiments, AdOx and AMI-1 are general inhibitors, and the resulting repression of other PRMTs

may be masking the effect of *EgPRMT10* repression. While previous reports on *E. grandis* have demonstrated that transgenic knockdown of either *EgPRMT1* or *EgPRMT10* results in shorter roots with fewer lateral roots (Plett et al. 2017), contact with *P. albus* caused both *EgPRMT1* RNAi and *EgPRMT10*

Fig. 1. Change in expression of protein arginine methyltransferase (PRMT) genes in *Eucalyptus grandis* roots during colonization by the ectomycorrhizal fungus *Pisolithus albus*. **A**, *E. grandis* undergoing colonization by *P. albus* (2 weeks after contact). **B**, Closeup of mature mycorrhizal root tips (2 weeks after contact). **C**, The relative expression ($\log_2$) of PRMT genes encoded by *E. grandis* in roots undergoing colonization, as compared with axenically growing roots. Data are presented over the timecourse of colonization: 24 h (white), 48 h (dark gray), 1 week (black), and 2 weeks (light gray) after contact and is the average of three biological replicates $\pm$ standard error; an asterisk (*) = significant expression change as compared with axenically grown roots ($P < 0.05$).

RNAi roots to grow and branch such that, at harvest, they did not contain significantly fewer lateral roots than PRMT over-expressors or controls (based on students *t* test, $P > 0.05$ in all cases). Data from all transgenic roots are presented as an average (Fig. 2B); however, results from individual roots and lateral rooting data can be found in Supplementary Figure S3.

***EgPRMT1* or *EgPRMT10* affect the expression of different functional gene categories in *P. albus*-colonized roots.**

RNA sequencing analysis of transformed mycorrhized roots (35S::*EgPRMT1*, *EgPRMT1* RNAi, 35S::*EgPRMT10*, and *EgPRMT10* RNAi) revealed a set of 2,089 genes that exhibited differential expression when compared with mycorrhized wild-type controls (more than fivefold change in gene expression;

$P < 0.001$) (Fig. 3). Many of these genes encode kinases, disease resistance proteins (including toll interleukin 1 receptor-nucleotide binding site-leucine-rich repeat and NB-ARC), cytochrome P450s, various enzymes (e.g., hydrolases, peptidases), DNA-binding proteins, or transcription factors.

This list of genes was further filtered to produce two gene groupings: those regulated in an opposite manner between 35S::*EgPRMT1* and *EgPRMT1* RNAi transgenic roots (Group A, 143 genes total [Supplementary Table S2]) and those regulated in an opposite manner between 35S::*EgPRMT10* and *EgPRMT10* RNAi transgenic roots (Group B, 467 genes total [Supplementary Table S3]). Genes without at least a fivefold change in expression in at least one of the considered conditions were discarded. There is some overlap between the two

Table 1. Identification of arginine methylation sites from total protein extracted from 48-h ($n = 3$) or 2-week axenic controls ($n = 3$), 48-h indirect fungal contact ($n = 3$), or 2-week mycorrhized ($n = 2$) *Eucalyptus grandis* roots^a

Protein name	Gene no.	48-Hour control		After 48 h of indirect contact		2-Week control		After 2-week mycorrhization	
		Number	Mean %	Number	Mean %	Number	Mean %	Number	Mean %
187-kDa microtubule-associated protein	Eucgr.E02482	<u>R981</u>	100 ± 0	ND	N/A	NM	N/A	R90 R889	35 ± 21 100 ± 0
30S ribosomal protein S3	Eucgr.J02481	<u>R26</u>	100 ± 0	ND	N/A	NM	N/A	ND	N/A
5-methyltetrahydropteroyltrimethylglutamate-homocysteine methyltransferase	Eucgr.A02126	<u>R447*</u> <u>R571*</u>	100 ± 0 46 ± 15	<u>R705</u> <u>R714*</u>	52 ± 9 60 ± 14	<u>R705</u>	33 ± 57	ND	N/A
ATP synthase subunit beta	Eucgr.G02224	<u>R159*</u> <u>R176</u>	63 ± 32 33 ± 28	<u>R176</u>	56 ± 38	NM	N/A	NM	N/A
Catalase	Eucgr.F01776	NM	N/A	R102 R146	53 ± 11 45 ± 18	ND	N/A	NM	N/A
Cell division control protein	Eucgr.H03858	ND	N/A	R315	100 ± 0	ND	N/A	NM	N/A
Cell division cycle-associated 7-like protein	Eucgr.H00548	ND	N/A	<u>R50</u>	100 ± 0	ND	N/A	ND	N/A
E3 ubiquitin transferase	Eucgr.J00838	ND	N/A	<u>R990</u>	100 ± 0	ND	N/A	NM	N/A
E3 ubiquitin-protein ligase	Eucgr.E00402	<u>R20</u>	51 ± 8	ND	N/A	NM	N/A	ND	N/A
Elongation factor 1-alpha	Eucgr.J01121	R411	75 ± 25	<u>R166*</u> <u>R411</u>	46 ± 8 60 ± 15	NM	N/A	<u>R254</u>	100 ± 0
Elongation factor 2	Eucgr.F01462	<u>R592</u> <u>R774</u>	48 ± 13 83 ± 15	<u>R592</u> <u>R774</u>	65 ± 31 70 ± 26	ND	N/A	NM	N/A
Fructose-bisphosphate aldolase	Eucgr.A01538	<u>R53</u>	100 ± 0	NM	N/A	ND	N/A	ND	N/A
Fructose-bisphosphate aldolase	Eucgr.K02073	R249	66 ± 30	NM	N/A	ND	N/A	ND	N/A
Glyceraldehyde-3-phosphate dehydrogenase	Eucgr.B00144	<u>R169</u> <u>R293</u>	100 ± 0 61 ± 13	NM	N/A	NM	N/A	R16 R236	33 ± 57 33 ± 57
Heat shock 70-kDa protein-like	Eucgr.J00025 Eucgr.F03980	<u>R145</u> R225* R608*	100 ± 0 46 ± 6 85 ± 13	R145	100 ± 0	<u>R145</u>	100 ± 0	R647	100 ± 0
Heat shock 70-kDa protein	Eucgr.E01024	R149	100 ± 0	NM	N/A	R546 R568	33 ± 57 100 ± 0	R256	100 ± 0
Histone H3	Eucgr.D00584	R43	78 ± 20	<u>R64</u>	58 ± 8	R84	33 ± 57	ND	N/A
Histone H4	Eucgr.J02062	ND	N/A	<u>R56</u> <u>R93</u>	48 ± 3 51 ± 8	NM	N/A	NM	N/A
HSP80	Eucgr.F03673	<u>R329*</u> <u>R375*</u>	62 ± 9 59 ± 14	R98*	70 ± 26	ND	N/A	NM	N/A
Nucleoside triphosphate hydrolase superfamily protein	Eucgr.D01797	ND	N/A	<u>R303</u>	100 ± 0	ND	N/A	NM	N/A
Phosphoenolpyruvate carboxylase	Eucgr.F01229	ND	N/A	<u>R574</u>	61 ± 10	ND	N/A	ND	N/A
Phosphoglycerate kinase	Eucgr.F04463	<u>R99</u>	100 ± 0	NM	N/A	ND	N/A	ND	N/A
Proteasome activator complex subunit	Eucgr.F02001	ND	N/A	ND	N/A	ND	N/A	R1062	100 ± 0
Survival motor neuron interacting protein	Eucgr.A00251	ND	N/A	ND	N/A	ND	N/A	<u>R67</u> <u>R69</u>	100 ± 0
TIR-NBS-LRR type disease resistance protein/TMV resistance protein ^b	Eucgr.J01296	ND	N/A	<u>R730</u>	100 ± 0	ND	N/A	ND	N/A
Transmembrane protein	Eucgr.F04115	ND	N/A	ND	N/A	ND	N/A	R273	65 ± 21
Tubulin alpha	Eucgr.G01186	ND	N/A	R79	100 ± 0	ND	N/A	ND	N/A
Tubulin beta	Eucgr.K00264	ND	N/A	<u>R77</u> <u>R262</u> <u>R276</u>	63 ± 16 54 ± 12 62 ± 62	ND	N/A	ND	N/A

^a As specific proteins were detected multiple times in each sample, the number of methylated residues as well as the mean percentage detected as methylated is given (± standard deviation) to indicate the consistency of the methyl modification across all proteins detected. Underlined arginine residues are monomethylated, while bolded arginine residues are di-methylated. An asterisk (*) indicates methylated residues that are unique to that given sample condition as the residue was detected and confirmed as not being methylated in other sample conditions. ND indicates that the protein was not detected by mass spectrometry. NM indicates that a portion of the protein was detected, but no methylation was detected. N/A indicates not applicable.

^b TIR-NBS-LRR = toll interleukin 1 receptor-nucleotide binding site-leucine-rich repeat; TMV = tobacco mosaic virus.

groups (Fig. 3). Group A (EgPRMT1-affected) does not contain as many genes as Group B, nor does it contain unique gene families that are not represented in some form in Group B. EgPRMT10 affects the expression of a number of genes found in hormone biogenesis and signaling pathways not similarly found in EgPRMT1 transformants. Table 2 lists some of the most notable genes in these hormone pathways.

PFAM enrichment analysis of the genes included in Groups A and B gives an extensive list of enriched PFAM families in the areas of biological processes (Supplementary Tables S4, S5, S6, and S7). Those PFAM families that are within the top ten of each dataset (based on Z-score) that also pertain to defense or symbiosis responses or hormone signaling are listed in Table 3. Both Groups A and B show enrichment in defense responses to a fungus, the flavonoid and terpenoid pathways, and gibberellin (GA) biosynthetic pathways. Group B genes (EgPRMT10-affected) are also enriched in genes pertaining to responses to general hormones and jasmonic acid (JA). To ensure that these were meaningful results, we performed the same PFAM enrichment analysis on genes upregulated in all four conditions (446 genes) or downregulated in all four conditions (537 genes) and found none of the same enriched PFAM domains, except for one instance of 'response to hormone' where genes were upregulated in all four conditions.

EgPRMT10 expression is correlated to the expression of key genes in plant hormone pathways.

As the transcriptomic data indicates that EgPRMT10 may affect plant hormone pathways, we treated plant roots with double-stranded interfering RNA (dsiRNA) designed to specifically reduce the expression of *EgPRMT10* and considered the effect on the expression of genes involved in hormone response. dsiRNA was shown to enter plant cells, within 2 h, through application of a fluorescently labeled control (Fig. 4A) and to persist in the roots for the duration of the experiment. Application of the test EgPRMT10 dsiRNA caused a significant reduction in the expression of *EgPRMT10* within 4 h of exposure, as compared with a scrambled control dsiRNA treatment (Fig. 4B). After 8 h, however, expression levels of *EgPRMT10* began to return to normal or to be overexpressed compared with controls, possibly due to the depletion of the dsiRNA construct or to plant feedback. Quantitative (q)PCR analysis demonstrated that a number of hormone-responsive genes were affected by the reduction in *EgPRMT10* expression, including genes involved in JA and GA pathways complementing the transcriptomic datasets (Fig. 4C). These genes showed a strong, significant positive correlation between their relative expression levels and that of *EgPRMT10*. Thus, the alteration in *EgPRMT10* expression simultaneously affected gene expression in multiple hormone pathways.

DISCUSSION

E. grandis is an economically important tree in forestry and bioenergy sectors and a useful model organism, given its fully sequenced genome (Myberg et al. 2014). In natural settings, its key ECM associates are *P. albus* and the closely related *P. microcarpus*. While many aspects of the signaling and transcriptomic regulation involved in the formation of ECM symbiosis between these partners are known (Duplessis et al. 2005; Plett et al. 2015a), the role of posttranslational modifications, particularly methylation, has not been investigated. We demonstrate, here, a novel role of host PRMTs in balancing the symbiotic interaction between *E. grandis* and the mycorrhizal fungus *P. albus*. Expression of EgPRMT1 facilitates mycorrhizal colonization, while expression of EgPRMT10 is inhibitory to colonization, thus EgPRMT1 and EgPRMT10

appear to affect pathways that work antagonistically in the colonization process. qPCR analysis of the expression of *EgPRMT* genes throughout the colonization process shows that *EgPRMT1* is generally repressed, as compared with control roots, and *EgPRMT10* is overexpressed. Thus, based on our results, the plant host appears to regulate the expression of these PRMTs and any downstream pathways in a way that is antagonistic to fungal colonization. While defenses raised by a plant host against a mycorrhizal fungus are generally much milder than those raised against a pathogen, they are believed to serve as a mechanism to protect the plant from over-colonization by the fungus (Plett and Martin 2018; Plett et al. 2014b). The regulation of these PRMTs may serve as a protection for the plant as with other defense-related gene pathways. Other *E. grandis* PRMTs are also differentially expressed over the timecourse of colonization and their more specific involvement in the mycorrhization process remains a question for future research.

In addition to demonstrating differential transcription of PRMT genes throughout the colonization process, we used analysis of protein methylation by mass spectrometry to demonstrate that the protein targets of *E. grandis* PRMTs are altered by the presence of *P. albus*. While we have not detected all methylated proteins from the tissues analyzed, it is interesting to note within those proteins found here that there were a

Fig. 2. Effect of protein arginine methyltransferase (PRMT) inhibition or transgenic misexpression on the percentage of *Eucalyptus grandis* lateral roots colonized by *Pisolithus albus*. **A**, Percent colonization of *E. grandis* roots after 2 weeks of contact with *P. albus* SI-12 when treated with water (control; $n = 9$), AdOx ($n = 12$), AMI-1 ($n = 11$), 1% dimethyl sulfoxide (DMSO control) ($n = 10$) or 2,3-dimethoxynitrostyrene (DMNS) ($n = 17$) $\pm$ standard error (SE); **B**, Percent colonization of *Rhizobium rhizogenes*-transformed control ($n = 9$) and test (35S::EgPRMT1 ($n = 7$); EgPRMT1 RNAi ($n = 3$); 35S::EgPRMT10 ($n = 6$); EgPRMT10 RNAi ($n = 6$)) roots after 2 weeks of contact with *P. albus* SI-12 $\pm$ SE; an asterisk (*) = significant change in the percentage of lateral roots colonized as compared with the control treatment ($P < 0.05$).

Fig. 3. Heat-map representation of differentially expressed genes in mycorrhized *Eucalyptus grandis* roots mis-expressing either EgPRMT1 or EgPRMT10. Log₂-transformed heat map of the 2,089 significantly regulated genes ($P < 0.001$; excepting those that are similarly up- or down-regulated in all four cases) in 35S::EgPRMT1, EgPRMT1 RNAi, 35S::EgPRMT10, EgPRMT10 RNAi mycorrhized roots as compared with mycorrhized controls. Data are the average of three biological replicates. Genes used in the assembly of Group A and B gene lists are indicated.

number of examples whereby arginine methylation changed depending upon whether the root was grown axenically or if it was exposed to *P. albus*. In many of these cases, exposure to *P. albus* results in additional methylation at arginine residues, highlighting the specific activity of PRMTs in the plant response to the fungus. In other cases, we show that the original methylation seen in axenically grown roots is no longer detected. For example, the heat shock 70-kDa protein-like protein has two arginine residues, R225 and R608, which are only methylated under axenic conditions. Until recently, the presence of an arginine demethylase protein was highly debated, but it is now commonly accepted that a Jumonji-domain containing protein (JMJD6) might have a role in the demethylation of arginines (Chang et al. 2007; Poulard et al. 2016). Alternatively, protein turnover may describe the effects seen. As old protein is recycled and new protein is transcribed, a new pattern of posttranslational modifications can be applied. It will be interesting to determine in future investigations how these differences in methylation sites affect protein function with relation to plant-microbe interactions.

Previous work on the effect of type I PRMTs on root development in *E. grandis* showed that repression of either EgPRMT1 or EgPRMT10 resulted in the same phenotype, i.e., a reduction in root growth and lateral rooting (Plett et al. 2017). This raised the question of whether *E. grandis* type I PRMTs acted redundantly, in concert, or affected different parts of the same pathways. Our results show that, while EgPRMT1 and EgPRMT10 may alter some pathways in common, they also have distinct roles and pathways that they control as evidenced by the different effect of their expression on mycorrhization. Additionally, these results demonstrate that the mycorrhization phenotype observed is unlikely to be caused entirely by the root-growth phenotypes previously reported. The process of mycorrhizal root-tip formation is highly dependent on the induction of lateral roots and, thus, the short-root phenotype of the RNAi knockdown roots would appear to be inhibitory to colonization. ECM fungi, however, are known to stimulate lateral root formation (Burgess et al. 1996; Felten et al. 2009) and, in our experiments, contact with *P. albus* caused both EgPRMT1 RNAi and EgPRMT10 RNAi roots to grow and branch such that, at harvest, they were similar in appearance to controls. Transcriptomic analysis of ECM-colonized roots differentially expressing EgPRMT1 or EgPRMT10 (both 35S:: and RNAi) was conducted to indicate potential pathways that were being affected to cause the observed phenotype. The total of 2,089 genes significantly ($P < 0.001$) differentially expressed across these transgenic roots highlights the large impact that PRMTs have on cellular processes. In *E. grandis* roots differentially expressing EgPRMT10, we observed significant regulation of genes associated with plant hormone synthesis and regulation, including genes associated with the ethylene (ET), JA, and auxin pathways. Appropriate balance of hormone signaling within plant roots is important for the successful establishment of ECM symbiosis. ET and JA production are antagonists to ECM colonization of host tissues (Plett et al. 2014a and b), while auxin improves colonization through modification of root growth and architecture (Vayssières et al. 2015). Overall, enrichment analysis shows a significant over-representation of genes with PFAM domains associated with hormone stimuli in the Group B transcriptomic dataset. This indicates that EgPRMT10 may have a potentially important effect on proteins in or upstream of multiple plant hormone signaling pathways. Alternatively, differential expression of EgPRMT10 could also affect other physiological pathways in the root that indirectly result in a shift in hormone signaling pathways. To address this possibility, experiments using dsRNA to reduce the expression of

EgPRMT10 on a shorter time scale demonstrated that the expression of *EgPRMT10* was significantly correlated to the expression of several genes within hormone-responsive pathways. The high level of correlation to several genes in multiple hormone pathways may be indicative of the rapid cross talk between hormone pathways or that *EgPRMT10* targets a master regulator of these pathways. For example, in *Arabidopsis*, the SWI/SNF chromatin remodeling complex is known to affect the expression of all genes tested in this study (Sarnowska et al. 2016). As PRMT10 has evolved only in plant lineages, there is very little indication of its role or activity in the literature. However, one study has shown that it is able to methylate histones and has a role in flowering time through controlling the expression of FLOWERING LOCUS C in *Arabidopsis* (Niu et al. 2007). Like the establishment of ECM symbiosis, flowering time in plants is a process that is highly dependent on hormonal and epigenetic regulation (Campos-Rivero et al. 2017; Conti 2017)). It remains to be seen whether the impact of *EgPRMT10* activity on hormone signaling is direct, through modification to histones or proteins in hormone pathways, or indirect, via the methylation of a transcriptional regulator.

Overall, we here present a framework, identifying unique and opposing effects of *E. grandis* PRMT1 and 10 homologs in mutualistic symbiosis. Additionally, we suggest a potential role for *EgPRMT10* in the regulation of plant-based hormones. This work highlights the evolutionary importance of posttranslational modifications like arginine methylation in biotic interactions and paves the way for future work on the specific mechanisms of control.

MATERIALS AND METHODS

Mycorrhization timecourse and PRMT expression.

E. grandis seedlings were grown from sterile seed on 1% agar media for 1 month followed by growth on one-half MMN (modified Melin-Norkrans media, 1 g of glucose per liter) on a sterile cellophane membrane for an additional month. Two-week old colonies of *P. albus* (strain SI-12) (Plett et al. 2015a), grown on one-half MMN media were placed on top of the plant roots and were left for 2 weeks (16 h of light and 8 h dark cycle, 25°C). Control seedlings were placed on one-half MMN

without any fungus. For the mycorrhization qPCR timecourse, three biological replicates of both test and control seedlings were harvested at 24 h, 48 h, 1 week, and 2 weeks after contact. These samples were immediately frozen in liquid nitrogen upon harvest and RNA was extracted using Qiagen RNeasy plant mini kits as per manufacturer instructions. RNA was used as a template for cDNA synthesis using the iScript cDNA synthesis kit (BioRad) and the SensiFAST SYBR No-ROX kit (Bioline) was used for qPCR analysis on a Corbett rotor-gene 6000 RT-PCR cycler. Log₂-fold expression change from axenic roots was calculated, using three biological replicates for *EgPRMT1* (*Eucgr.C03665*), *EgPRMT10* (*Eucgr.C01117*), *EgPRMT3* (*Eucgr.G03214*), *EgPRMT4* (*Eucgr.B01318*), *EgPRMT5* (*Eucgr.D02618*), *EgPRMT6* (*Eucgr.J00342*), and *EgPRMT7* (*Eucgr.D02075*). qPCR gene expression results were normalized using *Eucgr.B03031* and *Eucgr.B02502* as control genes (Cassan-Wang et al. 2012). Primer sequences can be found in Supplementary Table S1).

Protein extraction and analysis.

To produce protein samples, *E. grandis* seedlings were grown as above. The seedlings were either put in indirect contact with *P. albus* SI-12 for 48 h (accomplished using a sterile cellophane membrane between the plant and fungal colony to allow the passage of nutrients and small molecules but not roots or hyphae), were put in direct contact with SI-12 for 2 weeks, or were left in axenic culture (harvested at 48-h and 2-week timepoints). All cultures were grown on one-half MMN. Roots from three to five seedlings from each of these three treatments were pooled to generate sufficient tissue for protein extraction and were treated as one biological replicate. Results presented are from three biological replicates (i.e., three independent sets of pooled samples) for all conditions. After extraction, proteins were separated on a sodium dodecyl sulfate-polyacrylamide gel and were analyzed by mass spectrometry according to the methods of Plett et al. (2017).

Mycorrhization of inhibitor-treated roots.

E. grandis seedlings were grown as above and were placed in contact with a 2-week-old colony of SI-12. They were treated with water, 5 µM AdOx, 50 µM AMI-1 (Cheng et al. 2004), 1%

Table 2. Selected *Eucalyptus grandis* genes pertaining to hormone synthesis or signaling from those that are significantly differentially regulated in mycorrhized roots with modified *EgPRMT10* expression as compared with wild-type mycorrhized roots (Group B genes)^a

Gene ID	Arabidopsis homolog	Annotation	Role ^b	Average fold change	
				35S::EgPRMT10 roots	EgPRMT10RNAi roots
Hormone synthesis pathways					
Eucgr.C03886.1	AT2G19590.1	ACC oxidase 1	ET biosynthesis	0.13	2.20
Eucgr.K00739.1	AT1G05010.1	ET-forming enzyme	ET biosynthesis	0.20	7.76
Eucgr.K00740.1	AT1G05010.1	ET-forming enzyme	ET biosynthesis	0.02	12.46
Eucgr.K00746.1	AT1G05010.1	ET-forming enzyme	ET biosynthesis	0.12	9.85
Eucgr.F00649.1	AT5G55250.1	IAA carboxylmethyltransferase 1	Auxin conversion	0.11	1.13
Eucgr.H02641.1	AT4G15550.1	Indole-3-acetate β-D-glucosyltransferase	Auxin conversion	0.52	5.13
Eucgr.F02495.1	AT1G67080.1	ABA-deficient 4	ABA biosynthesis	0.57	14.54
Hormone responsive transcription factors/complexes					
Eucgr.K03266.1	AT3G23240.1	ET response factor 1	ET signaling	0.12	5.18
Eucgr.B03050.1	AT1G68840.2	Related to ABI3/VP1 2	ABA signaling	0.02	2.79
Eucgr.B03545.1	AT5G13220.1	Jasmonate-zim-domain protein 10	JA signaling	0.08	11.99
Eucgr.G01954.1	AT5G13220.1	Jasmonate-zim-domain protein 10	JA signaling	0.12	1.64
Expression regulated by hormones					
Eucgr.D00773.1	AT5G54490.1	Pinoid-binding protein 1	Upregulated by auxin [*]	0.65	6.84
Eucgr.K00884.1	AT5G54490.1	Pinoid-binding protein 1	Upregulated by auxin [*]	0.10	2.12
Eucgr.F00588.1	AT1G75750.2	GAST1 protein homolog 1	GA/ABA regulated ^{**}	0.88	8.62
Eucgr.I02349.1	AT4G09600.1	GAST1 protein homolog 3	GA/ABA regulated ^{**}	0.03	1.53

^a ACC = 1-aminocyclopropane-1-carboxylate; ET = ethylene; IAA = indole 3-acetic acid; ABA = abscisic acid; JA = jasmonic acid; GA = gibberellin.

^b Role in hormone signaling. One asterisk (*), Benjamins et al. 2003; two (**), Shi and Olszewski 1995.

dimethyl sulfoxide (DMSO), or 30 μ M 2,3-dimethoxynitrostyrene (DMNS) in 1% DMSO (Dillon et al. 2012) for the inhibition trials ($n = 9$ to 17 plants per treatment). Inhibitor treatment was delivered, via aerosol (to avoid over-wetting the fungus in one place) to the root system, using a sterilized atomizer. Approximately 1 ml of liquid was added each time, and treatments were administered six times over the 2-week fungal contact period at regular intervals. At the end of the 2 weeks, percent fungal colonization on each root system was scored (number of colonized lateral roots/total lateral roots $\times$ 100%). Determination of whether a root tip is colonized or not is assessed visually based on morphological changes in the shape and color of the root tip.

Production and mycorrhization of roots mis-expressing *EgPRMT1* or *EgPRMT10*.

EgPRMT1 (*Eucgr.C03665*) and *EgPRMT10* (*Eucgr.C01117*) 35S:: and RNAi constructs were cloned from cDNA synthesized, using iScript (BioRad), from total RNA extracted from *E. grandis* roots using the RNeasy plant mini RNA extraction kit according to manufacturer instructions (Qiagen). The amplified fragments were gel-purified and ligated into pDONR222 and were sequence verified. Positive inserts were then transferred to pH2GW7 (35S::) or to pH7GWIWG2(II) (RNAi) vectors using Gateway gene cloning (Life Technologies) and were transformed into *Rhizobium rhizogenes* isolate K599. *E. grandis* seedlings were grown from seed to 1 month old on 1% agar media. The root system of the plant was cut off and the resulting wound was dipped in freshly grown *R. rhizogenes* expressing the plasmid of choice or wild-type *R. rhizogenes* as a control. Dipped plants were embedded in Murashige Skoog (MS) media and were left for 1 week, placed upside down in a growth cabinet with a constant temperature of 25°C and a 16-h photoperiod. Plants were then transferred to fresh MS media supplemented with 150 μ g of Timentin per milliliter and were grown upright under the same conditions (Macrae and van Staden 1993). Transformed roots typically emerged within one or two weeks, were allowed to grow for another month, and were then transferred to one-half MMN media, covered with a

sterile cellophane membrane. Successful transformation of the roots was confirmed by verifying alteration to the expression of the transgene using the RNA sequencing (Supplementary Fig. S1). We also used our RNA-seq dataset to determine the expression of genes previously reported as positively (+) or negatively (–) coregulated with *PRMT1* and *PRMT10* (based on *E. grandis* publicly available transcriptomic datasets available at the Joint Genome Institute Phytozome vs. 12 database and based on *Arabidopsis thaliana* transcriptomic datasets publicly available on the BAR Expression Angler database). The logic behind this test was that genes positively coregulated by *PRMT1* or *PRMT10* should also be increased in expression in their respective 35S::*PRMT* transgenic roots versus control and more repressed in *PRMT* RNAi transgenic roots versus control (and vice versa for negatively coregulated genes). Given the fact that these two lines of evidence were complementary to each other, we concluded that our transgenic roots were i) properly transformed and ii) that *PRMT* activity was being affected in the expected manner as determined by the expression patterns of previously described coregulated genes.

For mycorrhization trials, 2-week old colonies of *P. albus* (strain SI-12) grown on one-half MMN media were placed on top of the plant roots and were left for 2 weeks (16 h light and 8 h dark cycle; 25°C) before percent mycorrhization was scored.

Transcriptomic analysis.

Three independent transformed (35S::*EgPRMT1*, 35S::*EgPRMT10*, *EgPRMT1* RNAi, *EgPRMT10* RNAi) or control (wild-type *R. rhizogenes*) mycorrhized root systems were harvested at the 2-week timepoint, were frozen immediately in liquid nitrogen, and RNA was extracted using Qiagen RNeasy plant mini kits per manufacturer instructions. Transcriptional analyses of all tissues were performed using RNA-seq via conventional poly-A library preparation for Illumina sequencing. Library construction and 100-bp paired-end read sequencing was performed by the Western Sydney University Next Generation Sequencing Facility. The samples were indexed and run on a high-output lane of an Illumina Hi-Seq 2000 flow-cell. Raw reads were trimmed for quality and were aligned to the primary transcripts of the *E. grandis* genome taken from the Phytozome database (accessed November 2015 [Myburg et al. 2014]), using CLC Genomics Workbench 7. For mapping, the minimum length fraction was 0.9, the minimum similarity fraction 0.8, and the maximum number of hits for a read was set to 10. The unique and total mapped reads number for each transcript were determined, and then, unique reads were normalized to reads per kilobase of exon model per million mapped reads (RPKM). Variation in the expression of the transgene between transformed mycorrhized root systems was checked to ensure sufficient correlation of the data generated. Based on RPKM, the standard error in *EgPRMT1* or *EgPRMT10* expression was between 2.6 and 6.7% for transgenic roots, only slightly higher than or equivalent to 1.4 to 2.6% for wild-type roots. Genes listed as significantly regulated (based on a student's *t* test with false discovery rate (FDR) Benjamini-Hochberg multiple testing correction applied to the data) were differentially regulated a minimum of five times as compared with wild-type control roots in at least one of the four sets of transformants. Group A and B gene lists were generated from this larger group of genes by considering only genes oppositely regulated in 35S:: and RNAi pairs (*EgPRMT1* for Group A and *EgPRMT10* for Group B) and having a differential expression of at least five times from control roots in the 35S:: or RNAi roots or both.

PFAM enrichment analysis of PFAM domains associated with Group A and B genes was done using the online program

Table 3. Over-represented PFAM families that pertain to defense, symbiotic responses, or hormone signaling from the Group A and B datasets that are within the top ten most enriched families (based on Z-score) within the dataset

PFAM family	Z-score	P value
Group A (<i>EgPRMT1</i> responsive)		
Upregulated in 35S:: <i>EgPRMT1</i>		
Defense response to fungus	21.43	3.24E-14
Gibberellin biosynthetic process	20.85	3.05E-07
Diterpenoid biosynthetic process	18.91	3.11E-09
Response to fungus	18.21	1.24E-13
Gibberellin metabolic process	18.02	1.22E-06
Flavonoid biosynthetic process	18.02	1.22E-06
Downregulated in 35S:: <i>EgPRMT1</i>		
Response to chitin	15.6	3.52E-07
Diterpenoid metabolic process	14.25	8.99E-08
Response to oxidative stress	13.11	1.16E-13
Terpenoid metabolic process	12.67	2.95E-07
Group B (<i>EgPRMT10</i> responsive)		
Upregulated in 35S:: <i>EgPRMT10</i>		
Diterpenoid biosynthetic process	17.72	3.37E-10
Gibberellin biosynthetic process	16.29	1.35E-06
Flavonoid metabolic process	15.27	2.16E-07
Downregulated in 35S:: <i>EgPRMT10</i>		
Defense response to fungus	22.27	4.85E-22
Response to fungus	17.93	8.27E-19
Response to jasmonic acid	17.4	2.56E-13
Response to hormone	17.23	1.06E-31

Fig. 4. Uptake of *EgPRMT10* targeting double-stranded interfering RNA (dsiRNA) into plant cells and its effect on gene expression. **A**, Merged bright field and fluorescent confocal microscopy images of the control *Eucalyptus grandis* root tip or an *E. grandis* root tip after 2 h of exposure to Cy3-labeled dsiRNA. Scale bar = 100 μ m. **B**, Relative expression ($\log_2$) of *EgPRMT10* in *EgPRMT10* dsiRNA treated roots as compared with scrambled control dsiRNA treated roots after 4 and 8 h of exposure. $n = 5 \pm$ standard error; an asterisk (*) = significant change ($P < 0.05$). **C**, Correlations between the relative expression of *EgPRMT10* in dsiRNA treated root tissues [$\log_2$ (fold change), x axis] and the expression of hormone responsive genes [$\log_2$ (fold change), y axis] for individual replicates as compared with scrambled control dsiRNA treated roots. Genes chosen are indicative of auxin, jasmonic acid (JA), cytokinin (CK), gibberellin (GA), or abscisic acid (ABA) responses. Light gray diamonds represent 8-h timepoint replicates, while dark gray diamonds represent 4-h timepoint replicates. The gene considered, Pearson's correlation coefficient (r), and significance of the correlation are indicated on each plot.

dcGO Enrichment (accessed December 2017 [Fang and Gough 2013]). PFAM families were assessed using the full ontology function and FDR < 001.

dsiRNA treatment of *E. grandis* roots and hormone analysis.

Two-month-old *E. grandis* seedlings, grown as above, were treated with solutions of custom-synthesized dsiRNA (Integrated DNA Technologies). Roots were treated with dsiRNA designed to reduce expression of EgPRMT10 (complementing GATATGAGTATCTTGACCAAACCAT sequence in gene transcript), a scrambled control sequence, or a Cy 3 fluorescently labeled cell-entry control. All dsiRNA was diluted to a concentration of 2 nM in liquid one-half MMN and roots were immersed in these solutions for 4 or 8 h. At each harvest timepoint, roots were removed from the solution, were rinsed in fresh one-half MMN, were blotted dry, and were snap-frozen in liquid nitrogen. Fluorescently labeled dsiRNA-treated roots were harvested into 4% paraformaldehyde in 1× PBS (phosphate buffered saline) for microscopy. Roots for microscopy were rinsed three times in 1× PBS and were observed using a confocal microscope (Leica TCS SP5). The presence of the dsiRNA inside the root cells was confirmed by Cy 3 fluorescence emission at 570 nm, as compared with nontreated control roots. RNA was extracted from five to six biological replicates of roots (over two independent experiments) treated with either test or scrambled dsiRNA, and qPCR analysis was conducted. RNA was used as a template for cDNA synthesis using the SensiFAST cDNA synthesis kit (Bioline) and the SensiFAST SYBR No-ROX kit (Bioline) was used for qPCR analysis on a BioRad CFX96 Touch RT-PCR cycloer. Log₂-fold expression change from control roots treated with scrambled dsiRNA was calculated for EgPRMT10 (*Eucgr.C01117*), and the closest *E. grandis* homologs to the known *Arabidopsis* hormone responsive genes: GA3ox1 (*Eucgr.F02568*), Myc2 (*Eucgr.E00277*), VSP2 (*Eucgr.J02927*), ARR16 (*Eucgr.G03141*), ARR6 (*Eucgr.B02571*), PIN3 (*Eucgr.B02902*), PIN2 (*Eucgr.C00078*), and ABI3 (*Eucgr.H00815*). qPCR gene-expression results were normalized using *Eucgr.C00350* and *Eucgr.K02046* as control genes.

Phylogenetic tree construction.

To confirm that PRMT10 homologs are only present in plant genomes a phylogenetic tree was constructed (Supplementary Fig. S2). PRMT protein sequences from human (*Homo sapiens*) (Venter et al. 2001), mouse (*Mus musculus*) (Dietrich et al. 1996), cow (*Bos Taurus*) (Fries et al. 1993), *Xenopus laevis* (Roe et al. 1985), zebrafish (*Danio rerio*) (Woods et al. 2000), fruit fly (*Drosophila melanogaster*) (Adams et al. 2000), yeast (*Saccharomyces cerevisiae*) (Mewes et al. 1997), *Arabidopsis thaliana* (Lamesch et al. 2012), rice (*Oryza sativa*) (Ouyang et al. 2007), and eucalyptus (*E. grandis*) (Myberg et al. 2014) were retrieved using the PANTHER protein classifications PTHR11006 and PTHR10738. A phylogenetic tree was constructed using the online tool Phylogeny.fr (Dereeper et al. 2008). The sequences were aligned using MUSCLE (default settings) and the phylogenetic tree was reconstructed using the maximum likelihood method (PhyML program v3.1/3.0 aLRT). All the PRMT-like protein sequences were downloaded from the Phytozome database (Phytozome v10.3, accessed 27/02/2018) for plant sequences and the Uniprot database (accessed February 27, 2018) for all other sequences.

Statistical methods.

Three or more independent biological replicates were performed for each test outlined. A biological replicate is represented by one seedling root system except in the protein

extraction experiments, in which each biological replicate consisted of three to five pooled root systems to generate sufficient sample for each of three extractions. For all transgenic experiments, a minimum of three independent transgenic root systems were used. A student's two-tailed independent *t* test was calculated to determine the statistical significance of the differences observed (*P* < 0.05) unless otherwise noted. Correlations between EgPRMT10 and test gene expression in the dsiRNA treatment experiment were determined by calculating the Pearson's correlation coefficient (*r*) and the correlation was deemed as significant for *P* < 0.05.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

BAR Expression Angler database:
<http://bar.utoronto.ca/ExpressionAngler/>
 dcGO (domain-centric Gene Ontology) Enrichment database:
<http://supfam.org/SUPERFAMILY/cgi-bin/dcenrichment.cgi>
 Hawkesbury Institute for the Environment data collection site:
<http://hie-pub.westernsydney.edu.au/47b96970-1b57-11e8-a446-525400daae48>
 Joint Genome Institute Phytozome 12 database: <https://phytozome.jgi.doe.gov>
 Uniprot database: www.uniprot.org

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