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Expression profiling and down-regulation of three histone lysine methyltransferase genes (*PvATXR3h*, *PvASHH2h*, and *PvTRX1h*) in the common bean

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Abstract.

Histone methylation is a conserved epigenetic mechanism in eukaryotes. Most of the histone lysine methyltransferases (HKMTases) conferring such modifications are proteins with a conserved SET domain responsible for enzymatic activity. Genetic studies in *Arabidopsis thaliana* have revealed that proteins from the Trithorax group (TrxG) are critical in activating transcription by methylating lysine 4 and lysine 36 of histone H3. Two TrxG proteins, ATXR3 and ATX1 (also called SET DOMAIN GROUP 2 and 27, respectively) are necessary for global genome-wide H3K4me3 deposition in Arabidopsis, whilst ASHH2 (also called SDG8) is a multi-functional enzyme with H3K4 and H3K36 methylation activity. Using phylogenetic analysis, we have identified the common bean (*Phaseolus vulgaris* L.) gene orthologs to Arabidopsis *ATXR3, ASHH2,* and *ATX1* genes, which we have designated *PvATXR3h*, *PvASHH2h,* and *PvTRX1h*, respectively. Analysis of these genes with qRT-PCR reveals that all three are broadly expressed during plant and nodule development. Through a reverse genetics approach, we created common bean composite plants to knock-down *PvATXR3h, PvTRX1h,* and *PvASHH2h* expression. From analysis of the transgenic root phenotype, we conclude that transgenic root growth and development in the common bean was hindered by *PvASHH2h* gene downregulation.

Keywords: Common bean, epigenetics, histone-methyltransferases, phylogeny, root growth.
Abbreviations: HKMTases_histone lysine methyltransferases; PTMs_post-translational modifications; TrxG_Trithorax group; SDG_SET DOMAIN GROUP; ATX1_ARABIDOPSIS HOMOLOG OF TRITHORAX 1; ATXR3_ARABIDOPSIS TRITHORAX-RELATED 3; ASHH2_ABSENT, SMALL, OR HOMEOTIC DISCS 1 HOMOLOG 2; RNAi_RNA interference; GFP_green fluorescent protein; RFP_red fluorescent protein; ML_Maximum Likelihood tree; NJ_Neighbor-Joining tree; SET_(Su[var]3-9, Enhancer of Zeste, Trithorax) domain

Introduction

Chromatin is a highly ordered structure comprised of DNA, histones, and other proteins. Post-translational modifications of histone proteins and DNA are two epigenetic mechanisms that modify gene expression. The information conveyed by these alterations is not encoded by the nucleotide sequence, but is heritable and can be transmitted, once established, to daughter cells and likely from generation to generation (Probst et al., 2009; Muramoto et al., 2010). The functional consequences of post-transcriptional modification of histones can be direct, causing structural changes to chromatin, or indirect, acting through the recruitment of effector proteins. The type of histone modification has a characteristic effect on transcription and is associated with either transcriptionally active or repressed chromatin (Berger, 2007). For example, methylation of lysine 9 of histone H**3** (H3K9), lysine 27 (H3K27), and lysine 20 of histone H4 (H4K20) is generally associated with silenced chromatin regions, while methylation of H3K4, H3K36, and H3K79 is associated with active genes (Berger, 2007; Zhang and Ma, 2012). The major subclass of lysine-specific histone methyltransferases (HMTases), the 'writers' of lysine methylation, are Su[var]3-9, Enhancer of Zeste, Trithorax (SET)-domain-containing enzymes, which catalyze mono- (me1), di- (me2), or tri-methylation (me3) of distinctive Lys residues on histone H3 or H4 (Hennig and Derkacheva, 2009). The types of histone methylation marks that have been primarily examined as representatives of plant epigenetic modification systems are: mono-, di-, or tri-methylated histone H3K4 (H3K4me1, H3K4me2, and H3K4me3), tri-methylated histone H3K27 (H3K27me3), tri-methylated histone H3K36 (H3K36me3), and di-methylated histone H3K9 (H3K9me2; reviewed in Liu et al., 2010). Of particular interest, in the model plant *Arabidopsis thaliana*, is the ARABIDOPSIS TRITHORAX-RELATED 3 (ATXR3, or SET DOMAIN GROUP 2) histone-methyltransferase, considered the major Arabidopsis H3K4 tri-methyltransferase. It is broadly expressed during development and highly conserved in plants (Guo et al., 2010). In *atxr3* mutants there is global reduction of H3K4me3, a slightly reduced level of H3K4me2, and an enhanced level of H3K4me1 compared to wild-type (WT) plants, while levels of H3K36me1, H3K36me3, and H3K27me3 are unaffected (Berr et al., 2010; Guo et al., 2010). Loss of function of another *Arabidopsis* SET-domain gene, "*ABSENT, SMALL, OR HOMEOTIC DISCS 1 HOMOLOG 2" (ASHH2* or *SDG8*), which has pleiotropic effects in the Arabidopsis plant, results in reduced di-

methylation of histone H3K36 compared to WT plants. Reduced di-methylation was particularly apparent in chromatin associated with the *FLOWERING LOCUS C* (*FLC*) (Zhao et al., 2005). Arabidopsis plants with mutant *ASHH2* also have less H3K4 tri-methylation in *FLC*-associated chromatin (Kim et al., 2005). This indicates that ASHH2, considered a major H3K36-specific histone lysine methyltransferase (HKMT; Xu et al., 2008), is a multi-functional enzyme with H3K4 and H3K36 methylation activity. A third major HMTase in Arabidopsis is the ARABIDOPSIS TRITHORAX 1 (ATX1), a regulator of flower development (Alvarez-Venegas et al., 2003), that is also involved in the timing of root development, stem-cell-niche maintenance, and cell patterning during primary and lateral root development (Napsucialy-Mendivil et al., 2014). However, SET-domain containing proteins belonging to the Trx-G modify only a fraction of the nucleosomes that are known to be modified in Arabidopsis (Alvarez-Venegas and Avramova, 2005), which implies the involvement of several or many histone H3K4 methyltransferases. Histone modifications and their functions have been extensively studied in yeast and mammals (Kouzarides, 2007), and to a lesser extent in Arabidopsis (Alvarez-Venegas, 2010). We have chosen to extend these studies to the analysis of HKMT and histone modifications in the common bean (*Phaseolus vulgaris* L.*)*, about which little is known. This endeavor was particularly appealing as the genome of the common bean line G19833, an Andean *P. vulgaris* genotype (Phytozome v9.1, http://www.phytozome.net), has recently been sequenced. This has allowed us to identify the *P. vulgaris* gene orthologs to the Arabidopsis *ATXR3*, *ASHH2,* and *ATX1* genes by phylogenetic analysis. These are *Phvul.005G112000* (designated *PvATXR3h*), *Phvul.009G130100* (designated *PvASHH2h*), and *Phvul008G018500* (designated *PvTRX1*), respectively. Based on what is known at present, these genes are considered the major HKMTs in plants. Thus, their expression patterns in plant organs and root nodules as well as their role in root growth and development are of great interest. Grain legumes are recalcitrant to *in vitro* regeneration and *P. vulgaris* is particularly recalcitrant to *in vitro* induction of somatic embryogenesis and regeneration. However, *P. vulgaris* composite plants, with WT shoots and transgenic hairy roots derived from *Agrobacterium rhizogenes*-mediated genetic transformation, have been successfully developed (Estrada-Navarrete et al., 2007) and were employed in this study. We acquired transgenic roots from composite plants induced by *A. rhizogenes* strain NCPPB2659 (also known as K599; Costantino et al., 1981; Combard et al., 1987) in a reverse genetic approach. The method has been described previously (Estrada-Navarrete et al., 2007; Blanco et al., 2009; Sánchez-López et al., 2011; Barraza et al., 2013). Since genetic transformation by *A. rhizogenes* is restricted to roots and nodules in the composite plants, our experiments were limited to these organs. In this study, we evaluated the effects of gene downregulation by RNAi of *PvATXR3h, PvASHH2h,* and *PvTRX1h* in transgenic hairy root growth of common bean plants. Downregulation of *PvASHH2h* (*PvASHH2h*-RNAi) arrested hairy root growth and we suggest that *PvASHH2h* is crucial for root growth and development. Down-regulation of *PvATXR3h* and *PvTRX1h* (*PvATXR3h*-RNAi and *PvTRX1h*-RNAi, respectively) had no apparent effect on the transgenic roots, which were phenotypically similar to those of the NCPPB2659 (K599) transformed plants. The results are consistent with an important and distinct role for *PvASHH2h* in regulation of gene expression and root development and function. This information should increase our understanding of post-transcriptional modifications in legumes. The combined information derived from studies of this kind should eventually be applicable to increased crop productivity and lead to better understanding of plant-microorganisms interactions. The development of techniques for transformation of the whole legume plant should further enhance our knowledge base.

Results

Phylogeny of SET-domain containing proteins in the common bean

Our first goal was to characterize SET-domain containing proteins from common bean with respect to their number, type, and biochemistry. We used sequence similarity, multiple alignments, and tree reconstruction methods based on the eudicot *A. thaliana* genome as the model plant for comparison. Using the SET-domain sequences from *A. thaliana* as a probe, we have, to date, identified 32 genes encoding SET-domain-containing proteins in *P. vulgaris* (Fig. 1; Phytozome v9.1, http://www.phytozome.net), that belong to classes I–V of plant SET-domain-containing proteins (according to Ng et al., 2007; Thorstensen et al., 2011). As can be seen in the Maximum Likelihood (ML) and Neighbor-Joining (NJ) trees in Figure 1 and Supplementary Figure S1, the *P. vulgaris* SET-domain proteins were grouped with their *Arabidopsis* counterparts within classes I–V (Ng et al., 2007; Thorstensen et al., 2011). The clustering pattern is supported by the architecture of the proteins, as recognized by the SMART database (Letunic et al., 2004; Schultz et al., 2000). Based on the phylogenetic analysis and the known catalytic activities of some of the *Arabidopsis* proteins, the position of the *P. vulgaris* proteins with respect to class and group allows us to infer their catalytic activities (Supplementary Table S1).

Major histone lysine methyltransferases in plants

Three *P. vulgaris* genes (*Phvul.005G112000, Phvul.009G130100*, and *Phvul.008G018500*; GenBank accession numbers XP_007149940, XP_007137477, and XP_007139309, respectively*)*, were identified as orthologs to the genes in Arabidopsis that have been considered to be the major histone lysine methyltransferases: *ATXR3* (Guo et al., 2010), *ASHH2* (Xu et al., 2008), and *ATX1* (Alvarez-Venegas et al., 2003). We designated these common bean genes *PvATXR3h* (for "*P. vulgaris ATXR3 homolog"*), *PvASHH2h* (for "*P. vulgaris ASHH2 homolog"*), and *PvTRX1h* (for "*P. vulgaris ATX1 homolog"*), respectively. As shown in the ML and NJ trees of Figure 1 and Supplementary Figure S1, the clustering of these putative pairs had high support: ATXR3 with PvATXR3h had 100% bootstrap and 1.0 branch support values, ASHH2 with PvASHH2h had 93% bootstrap and 0.962 branch support values, and ATX1 with PvTRX1h had 99% bootstrap and 0.817 branch support values. Based on these values, we conclude that the *P. vulgaris* genes are orthologs of their *Arabidopsis* counterparts. The phylogenetic relationship between the proteins is supported by their domain composition and distribution (Supplementary Fig S2), in addition to the relatedness of their SET domains. Finally, ASHH2 and PvASHH2h proteins have a 44% amino acid sequence identity, ATXR3 and PvATXR3h have a 54% sequence identity, and ATX1 and PvTRX1h have a 57% sequence identity (Supplementary Table S2).

Expression patterns of PvATXR3h, PvASHH2h, and PvTRX1h during development in P. vulgaris

The next endeavor was to characterize the expression of the *P. vulgaris* genes in the WT cultivars by quantitative RT-PCR (qRT-PCR). Differential expression of genes with respect to developmental stage and location may provide clues to their biological functions. With this in mind, we analyzed the transcriptional levels of *PvATXR3h, PvASHH2h,* and *PvTRX1h* in several tissues and organs, in two cultivars of *P. vulgaris,* and at various times after germination. Since we were particularly interested in the role of these genes in root and nodule development, the effect of inoculation with *Rhizobium* was also examined. The results for non-inoculated plants are shown in Figure 2 and the results for inoculated plants are shown in Figure 3.

Non-inoculated plants

In plants that had not been inoculated with *R. etli*, *PvATXR3h* and *PvASHH2h* exhibited similar expression patterns in seedlings, plantlets, and leaves of the BAT477 cultivar at all developmental stages measured (and for both reference genes, Supplementary Fig S3), with the exception of a striking decrease in expression at seven days post germination (dpg). The decrease in expression in leaves at seven dpg was also apparent in expression of *PvTRX1h*, which was much more variable in leaves and seedlings than were the other two genes. In the roots of this cultivar, there was a gradual decline in expression of *PvATXR3h* and *PvASHH2h* with age of the roots, but there was a large increase in expression of *PvTRX1h* in roots at seven dpg.

In the DOR364 cultivar, expression levels of *PvATXR3h* and *PvASHH2h* were quite stable in seedlings, plantlets, and leaves at the ages studied, with the exception of decrease in expression at seven dpg, which was much more striking for *PvASHH2h* than for *PvATXR3h*. Expression levels of *PvTRX1h* in shoot tissue declined after germination but, in contrast to the other two genes, there was a large increase at seven dpg. A similar pattern of *PvATXR3h* and *PvASHH2h* expression was apparent in the roots: expression was similar at all ages, except for a marked decrease at seven dpg. Expression of *PvTRX1h* also declined, compared to seedlings, but occurred earlier than for the other two genes, at three dpg and had declined further by seven dpg.

Inoculated plants

Figure 3 shows the analysis of the transcription levels of the *PvATXR3h, PvASHH2h,* and *PvTRX1h* genes in roots and nodules of inoculated plants. Transcript accumulations of *PvATXR3h* and *PvASHH2h* had similar patterns in both cultivars (and for both reference genes, Supplementary Fig S4), declining at seven days post inoculation (dpi) and then increasing thereafter. *PvASHH2h* expression was extremely low at seven dpi. The expression levels of *PvATXR3h* and *PvASHH2h* in mature nodules (at 14 and 21 dpi) of the BAT477 cultivar were much higher when compared to other organs and to the *PvTRX1h* gene. The accumulation of these transcripts at stages of root and nodule development corresponding to cessation of cell division and expansion growth (Islas-Flores et al., 2011), suggests that *PvATXR3h* and *PvASHH2h* transcripts accumulated during nodule cell expansion. However, in the DOR364 cultivar, *PvASHH2h* levels were similar in germinated seeds and mature nodules (when normalized to *PvActin11* and *PvEF1α*; Supplementary Table S4 b). In addition, the expression levels of *PvTRX1h* in roots and nodules of inoculated plants were two-fold lower than in germinated seeds, lower when compared to the other two genes, and did not increase during nodule cell expansion.

Silencing of the PvATXR3h, PvASHH2h, and PvTRX1h genes in Agrobacterium rhizogenes-derived composite plant roots display dissimilar phenotypes

We next asked if *PvATXR3h, PvASHH2h,* or *PvTRX1h* are important in the development of *P. vulgaris* roots or nodules. This question was approached by repressing gene expression in hairy roots. Composite plants were produced with *Agrobacterium rhizogenes* strain NCPPB 2659 (also known as K599). *A. rhizogenes* K599 infection results in neoplastic, plagiotropic transformed ‘hairy’ roots that have distorted geotropic behavior and a high degree of branching (Odegaard et al., 1997; Estrada-Navarrete et al., 2006). The transgenic hairy roots develop from individually infected plant cells that have integrated a root inducing (Ri) plasmid-derived T-DNA into the plant genome (Collier et al., 2005). The genes were silenced with RNA interference (RNAi), often referred to as gene knock-down, by introducing *PvATXR3h*-RNAi, *PvASHH2h*-RNAi, and *PvTRX1h*-RNAi silencing vectors into *A. rhizogenes* before infection. Figure 4a and b shows the hairy-root phenotype after infection with *A. rhizogenes* with no vector. Figure 4c-l shows the phenotypes after infection with *A. rhizogenes* K599 containing silencing vectors for one of the three common bean genes of interest or with the control vector, pK7NEG. Transgenic roots emerged from the tumor at the wounded sites in all cases, except when the PvASHH2h-RNAi silencing vector was present. This was true for both cultivars, which had similar phenotypes (Fig. 4). Specifically, in three separate experiments, there were 73 plants of the DOR364 cultivar and 68 of the BAT477 that were infected with *A. rhizogenes* K599 that carried the PvASHH2h-RNAi silencing vector; none developed transgenic hairy roots (Table 1; Fig. 4). Two weeks after infection, most plants that did not carry the *PvASHH2h*-RNAi silencing vector had abundant hairy roots (Fig. 5). At this time, the primary root was removed from these plants, and the plants were transplanted and inoculated with *R. tropici* strain CIAT899 GFP (*R. tropici*-GFP; Estrada-Navarrete et al., 2007). As shown in Figure 5, the composite plants, with abundant hairy roots, were able to form nodule primordia that developed into determinate nodules harboring the *R. tropici-GFP*, as evidenced by green fluorescence. We also detected red fluorescence in the transgenic hairy roots from the RFP reporter included in the vector, but not in the *A. rhizogenes* K599 transformed control roots that did not carry the vector (data not shown). We next wished to demonstrate that the *PvASHH2h*-RNAi silencing vector was functional. We measured red fluorescence in stems at the site of infection after inoculation with *A. rhizogenes K599* containing the *PvASHH2h*-RNAi silencing binary vector. As shown in the confocal images (Fig. 5q-r), we detected red fluorescence in stems three weeks after inoculation, in agreement with reports stating that Gateway-compatible vectors with fluorescent tags (driving its expression under the pAtUBQ10 promoter) remains elevated for more than two weeks after transient transformation (Grefen et al., 2010). Fluorescence of the RFP reporter gene in *PvASHH2h*-RNAi-transformed stems suggest two possibilities: transient expression resulting from the continued presence of the *Agrobacterium* in the tissue (persisting in the tissue but repeatedly transferring T-DNA from *A. rhizogenes* K599 *PvASHH2h*-RNAi into plant tissue through time; if so, the pAtUbq10 promoter driving the expression of the RFP

Table 1. Transformation of *Phaseolus vulgaris* wild-type cultivars with *Agrobacterium rhizogenes* strain K599 containing specific *PvATXR3h*-RNAi, *PvASHH2h*-RNAi, and *PvTRX1h*-RNAi silencing vectors. Numbers of hairy roots were taken 14 days after infection with *A. rhizogenes*.

Treatment	DOR364 + K599	BAT477 + K599	DOR364 + pK7NEG -DsRed	BAT477 + pK7NEG-DsRed	DOR364 + *PvATXR3h*-RNAi	BAT477 + *PvATXR3h*-RNAi	DOR364 + *PvASHH2h* -RNAi	BAT477 + *PvASHH2h* -RNAi	DOR364 + *PvTRX1h* -RNAi	BAT477 + *PvTRX1h*-RNAi
Inoculated plants	21	21	26	19	43	48	73	68	21	18
% Survival	100	100	100	89.5	52	55.6	78	83	68	76
Plants with hairy roots (%)	15 (71.5%)	18 (85.7%)	21 (80.8%)	17 (100%)	14 (77.7%)	15 (68%)	0 (0%)	0 (0%)	16 (76.2%)	14 (77.7%)
Hairy roots per plant (X)	4.25	6	4.62	10.8	2.5	2.65	0	0	5.8	6.5

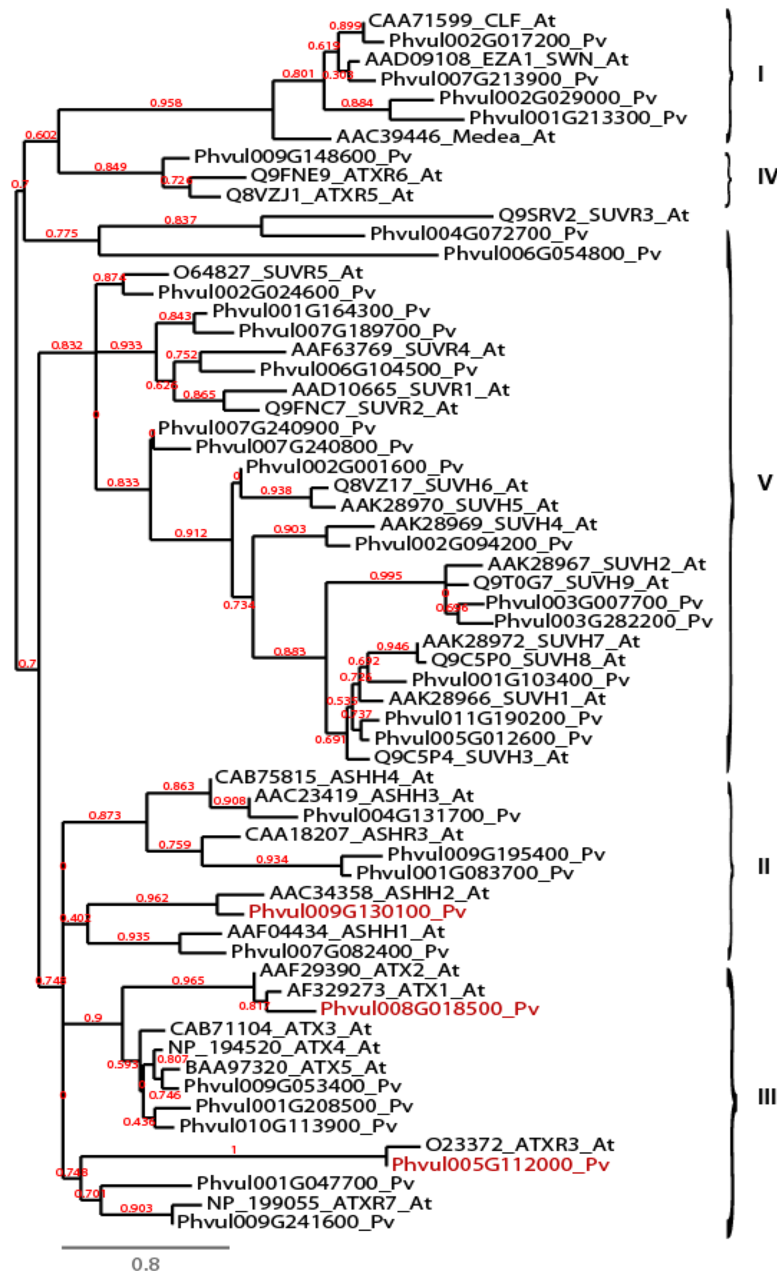

Fig 1. Maximum Likelihood phylogeny of SET-domain containing proteins in *A. thaliana* and *P. vulgaris*. SET-domain sequences with the highest BLAST scores were used to generate the Maximum Likelihood phylogenetic tree. The distinct SET-domain classes are indicated by numbers. The branch length is proportional to the number of substitutions per site. Numbers in red correspond to the branch support values. (At, *A. thaliana*; Pv, *P. vulgaris*.).

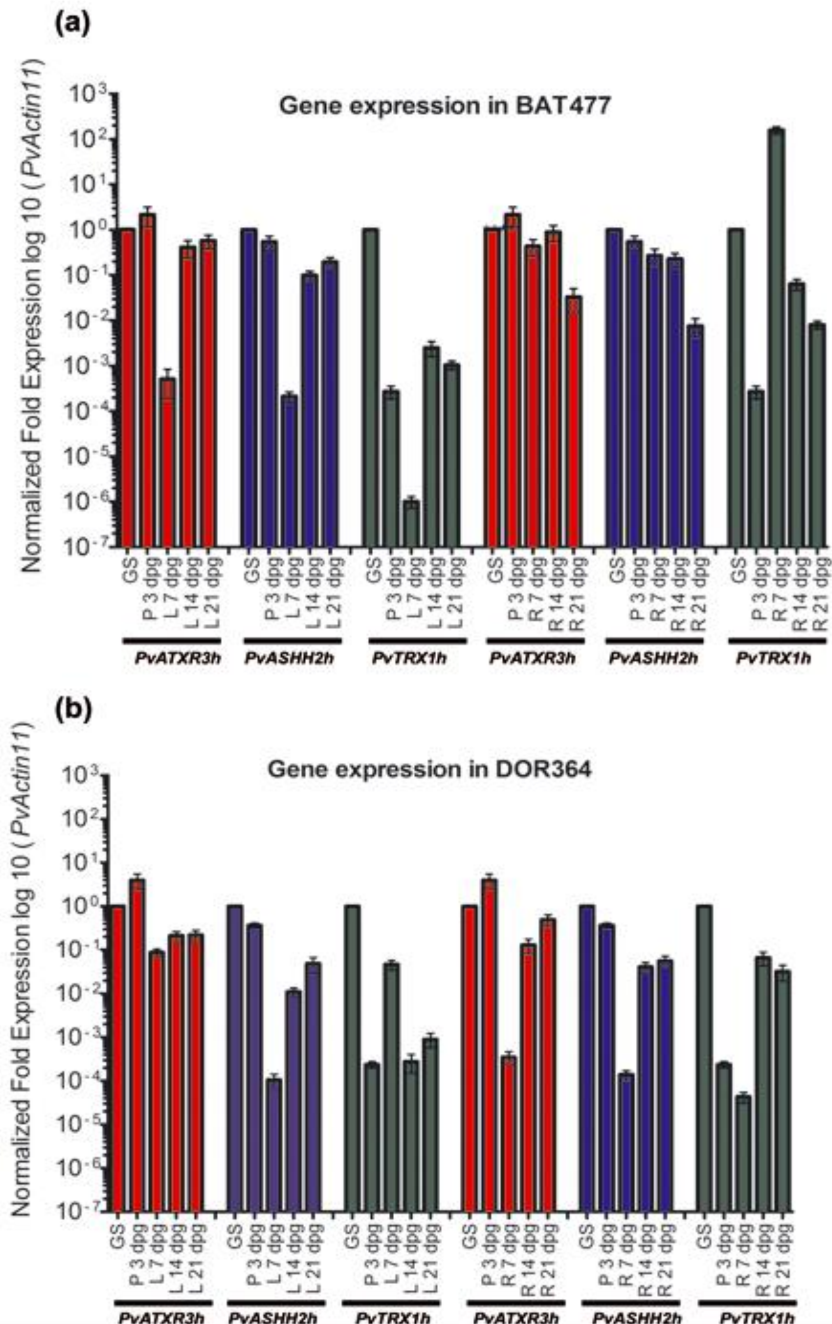

Fig 2. Transcript abundance of *PvATXR3h*, *PvASHH2h,* and *PvTRX1h* genes as determined by qRT-PCR in WT *P. vulgaris* plants that had not been inoculated with *R. etli.* Expression values were normalized to those of the *PvActin11* reference gene. (a) The BAT477 cultivar. (b) The DOR364 cultivar. (GS, germinated seedlings; P, plantlets; L, leaves; R, roots; dpg, days post germination; data represent mean ± SD; ****, $P<0.0001$ as determined with an unpaired two-tailed Student's *t*-test.).

protein was still active in *A. rhizogenes*), or incorporation of the T-DNA into the plant genome. In any event, Figure 6 shows that downregulation of the *PvASHH2h* was achieved, and we conclude that this downregulation was directly associated with the non-hairy-root phenotype. Furthermore, we rule out the possibility that root formation was not affected simply by turning on the RNAi machinery, since the phenotype was restricted to transformed plants that contained the *PvASHH2h*-RNAi silencing vector.

Root growth and development in P. vulgaris is hindered by down-regulation of PvASHH2h expression

We also wished to confirm downregulation of *PvATXR3h*, *PvASHH2h,* and *PvTRX1h* gene expression. This was done by measuring the transcript levels in transgenic roots by qRT-PCR. For the plants transformed with the *A. rhizogenes* K599 *PvASHH2h*-RNAi silencing vector that did not develop transgenic hairy roots, we excised the inoculation zone in the stems and analyzed the expression patterns. As shown on Figure 6, *PvATXR3h* was downregulated about 3-fold (Fig.

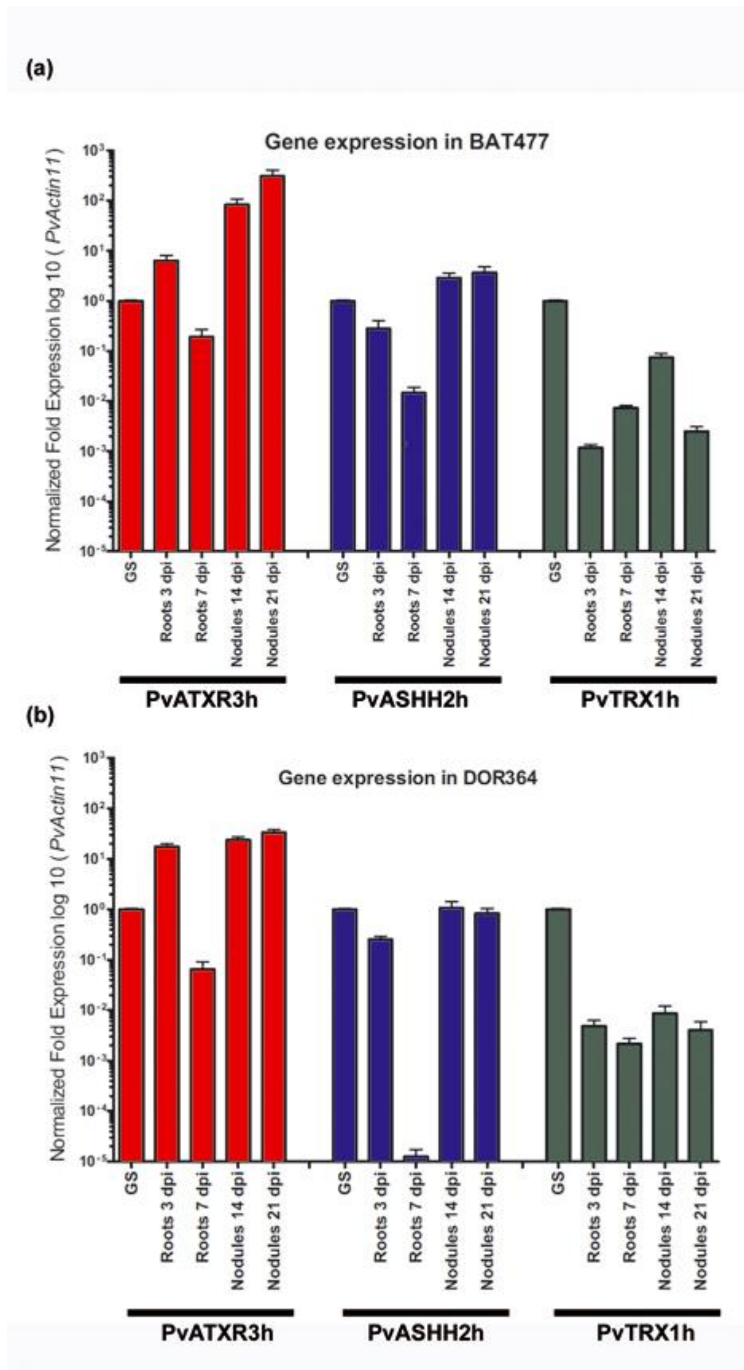

Fig 3. Transcript abundance of *PvATXR3h*, *PvASHH2h,* and *PvTRX1h* genes as determined by qRT-PCR in wild-type *P. vulgaris* plants inoculated with *R. etli.* (a) The BAT477 cultivar. (b) The DOR364 cultivar. (GS, germinated seedlings; P, plantlets; L, leaves; R, roots; dpi, days post inoculation; data represent mean ± SD; ****, $P<0.0001$ as determined with an unpaired two-tailed Student's *t*-test.).

6a), *PvASHH2h* about 2.8-fold (Fig. 6b), and *PvTRX1h* about 1.7-fold (Fig. 6c) when compared to control hairy roots from *A. rhizogenes* K599 and to transgenic hairy roots from *A. rhizogenes* K599 pK7NEG (and when normalized to both reference genes, *PvEF1α* and *PvActin11).* This indicates that the phenotype seen (absence of root development) can be attributed to downregulation of *PvASHH2h* expression and that hairy root development is hindered specifically by the down-regulation of *PvASHH2h.* On the contrary, *PvATXR3h*-RNAi and *PvTRX1h*-RNAi transgenic hairy roots formed nodule primordia that developed into determinate nodules.

Discussion

In this study, we used phylogenetic analysis and protein architecture to identify the common bean orthologs of the three major histone lysine methyl transferases described so far in plants, specifically, the Arabidopsis *ATXR3*, *ASHH2,* and *ATX1* genes. The BLAST searches performed against the common bean genome identified only one gene homolog that clustered in the same clade for each one of the Arabidopsis genes. These were *Phvul005G112000* as the homolog of *ATXR3*, *Phvul009G130100* as the homolog of *ASHH2*, and *Phvul008G018500* as the homolog *ATX1* (Fig. 1 and

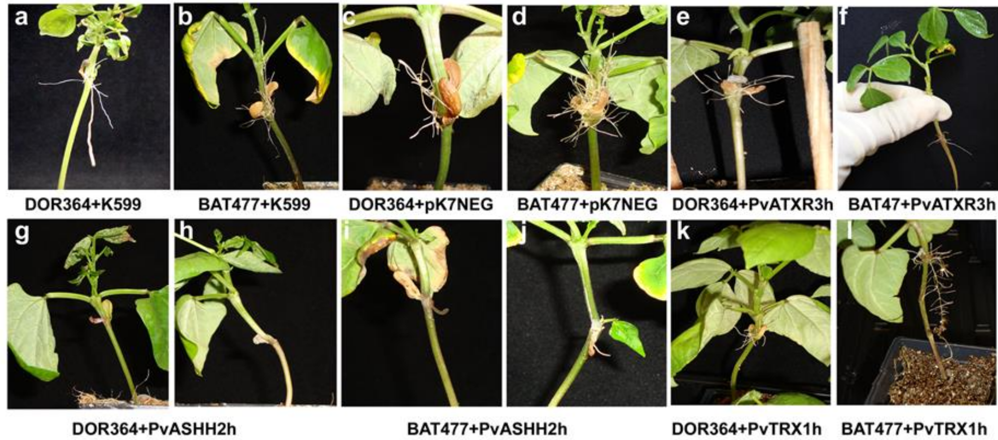

Fig 4. Images showing hairy root phenotypes of composite common bean plants of cultivar DOR364 and BAT477. **(a-b)** Seedlings transformed with *Agrobacterium rhizogenes* K599. (**c-d)** Roots from plants transformed with *A. rhizogenes* K599 that harbored the empty vector pK7NEG as control. (**e-f)** Roots from plants transformed with *A. rhizogenes* K599 that harbored the PvATXR3h-RNAi silencing vector. (**g-j)** Roots from plants transformed with *A. rhizogenes* K599 that harbored the PvASHH2h-RNAi silencing vector. (**k-l**) Roots from plants transformed with *A. rhizogenes* K599 that harbored the PvTRX1h-RNAi silencing vector.

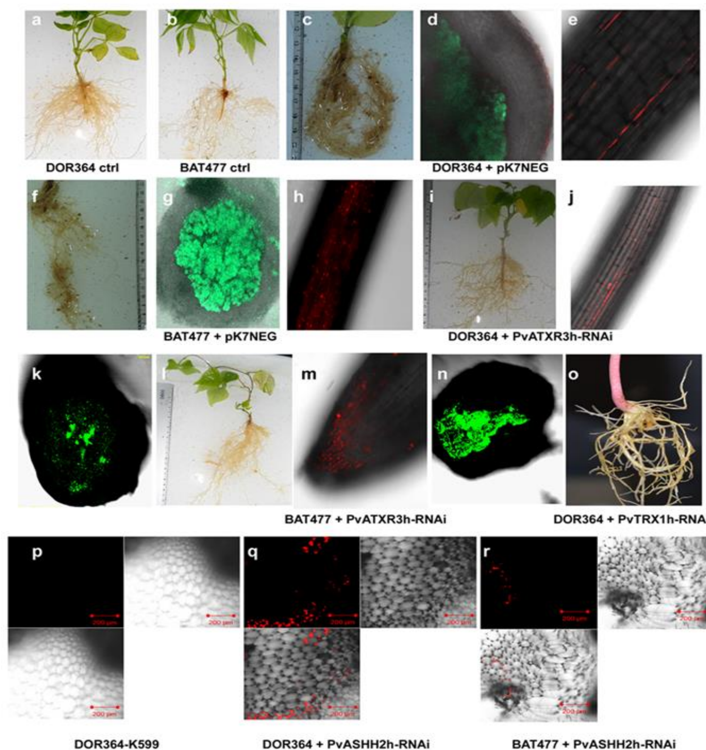

Fig 5. Images of phenotypes of composite common bean plants of cultivars DOR364 or BAT477. **(a-b)** Seedlings of cultivars DOR364 and BAT477 transformed by *Agrobacterium rhizogenes* K599. (**c-e)** Roots and nodules from plants of cultivar DOR364 transformed with *A. rhizogenes* that harbored the empty vector pK7NEG as control: (**c**) bright-field image of hairy roots inoculated with *R. tropici*-GFP, (**d**) green fluorescent protein (GFP) expression in nodules inoculated with *R. tropici*-GFP, (**e**) red fluorescent protein (RFP) expression in hairy roots transformed with *A. rhizogenes*. **(f-h)** Roots and nodules from plants of cultivar BAT477 transformed with *A. rhizogenes* that harbored the empty vector pK7NEG as control: (**f**) bright-field image of hairy roots inoculated with *R. tropici*-GFP, (**g**) green fluorescent protein (GFP) expression in nodules inoculated with *R. tropici*-GFP, (**h**) red fluorescent protein (RFP) expression in hairy roots transformed with *A. rhizogenes*. **(i-k)** Plants and tissues from cultivar DOR364 transformed with *A. rhizogenes* that harbored the *PvATXR3h*-RNAi silencing vector. **(l-n)** Plants and tissues from cultivar BAT477 transformed with *A. rhizogenes* that harbored the *PvATXR3h*-RNAi silencing vector. **(o)** Bright-field image of hairy roots from cultivar DOR364 transformed with *A. rhizogenes* that harbored the *PvTRX1h*-RNAi silencing vector. **(p)** Confocal image of stems from plants of cultivar DOR364 plants transformed with *A. rhizogenes* (the images on the top merge the bottom image with the DIC image). **(q)** Confocal image of stems from plants of cultivar DOR364 transformed with *A. rhizogenes* that harbored the *PvASHH2h*-RNAi silencing vector (the images on the top merge the bottom image with the DIC image). **(r)** Confocal images of stems from plants of cultivar BAT477 transformed with *A. rhizogenes* that harbored the *PvASHH2h*-RNAi silencing vector (the images on the top merge the bottom image with the DIC image). Scale bar = 200µm.

Supplementary Figure S1). We designated these as *PvATRX3h*, *PvASHH2h,* and *PvTRX1h,* respectively. Each of these common bean proteins have all the domains present and in the same arrangement as their Arabidopsis homologs (Supplementary Fig S2 and Supplementary Table S2). We examined the expression level of *PvATRX3h*, *PvASHH2h,* and *PvTRX1h* with respect to plant location and developmental stage. The three genes were broadly expressed in the common bean, namely, we detected expression in seedlings, plantlets, leaves, roots, and nodules of two different cultivars, at several times after germination. Plants of cultivar BAT477 that were not inoculated with *R. etli* had the lowest expression of the three genes in leaves at seven dpg (Fig. 2). On the contrary, expression of *PvATXR3h* and *PvASHH2h* in roots had very small changes when compared to seedlings, whereas expression of the *PvTRX1h* gene showed the highest levels in roots at 7dpg. However, some of the differences in gene expression seem to depend on the cultivar. That is, expression of *PvATRX3h*, *PvASHH2h,* and *PvTRX1h* in roots of the DOR364 cultivar had the lowest levels at 7dpg, but all in the BAT477 cultivar, *PvATRX3h* and *PvASHH2h* in roots did not show this decrease (Fig. 2). When the common bean plants were inoculated with *R. etli*, there were noticeable differences in gene expression compared to the non-inoculated plants (Fig. 3). We observed upregulation of *PvATXR3h* and to a lesser extent *PvASHH2h* in mature nodules, and downregulation of *PvTRX1h* in roots and nodules of inoculated plants, at all times tested. The transcript accumulation of *PvATXR3h* in mature nodules suggests an important role for this gene after nodule meristem initiation and *Rhizobium* infection. These time points match the developmental stages in which determinate nodule meristem cells cease to divide and cells continue to grow by cell expansion (Islas-Flores et al., 2011). Thus, *PvATXR3h* transcript accumulation could be involved in nodule growth during nodule cell expansion. However, even though *ATX1*, the Arabidopsis ortholog to *PvTRX1h,* is essential for proper root apical meristem organization and cell patterning during primary and lateral root development in *Arabidopsis* (Napsucialy-Mendivil et al., 2014), the downregulation of *PvTRX1h* during nodule growth and cell expansion in the common bean indicates that other HMTases are involved in root and nodule activity than *PvTRX1h* alone. Next, we assessed the possible involvement of *PvATRX3h*, *PvASHH2h,* and *PvTRX1h* in root development by gene silencing with RNAi. Composite plants were generated by infection with *A. rhizogenes* K599, which causes hairy roots. We introduced silencing vectors for the genes of interest into *A. rhizogenes* K599 and determined that transgenic hairy roots emerged from the wounded sites in the cultivars tested with all vectors except the *PvASHH2h*-RNAi silencing vector (Figs. 4 and 5). Furthermore, when the hairy roots were inoculated with *R. tropici*, the transformed plants formed nodule primordia that developed into determinate nodules harboring the *R. tropic*i. It appears, then, that *PvASHH2h,* but not the other genes, is essential for root -and probably nodule- development. We confirmed the effectiveness of *PvASHH2h* silencing by showing that *PvASHH2h* was, indeed, down-regulated approximately 2.8 fold in stems at the site of inoculation and tumor formation, apparently enough downregulation to prevent transgenic root growth and development. These values were similar to the downregulation of *PvATXR3h* and *PvTRX1h* when these genes were silenced, although silencing them to this degree had no apparent effect on hairy root or nodule formation. The results strongly suggest that the genes tested here are involved in multiple signal pathways with different target genes, both in *Arabidopsis* and in *P. vulgaris.*

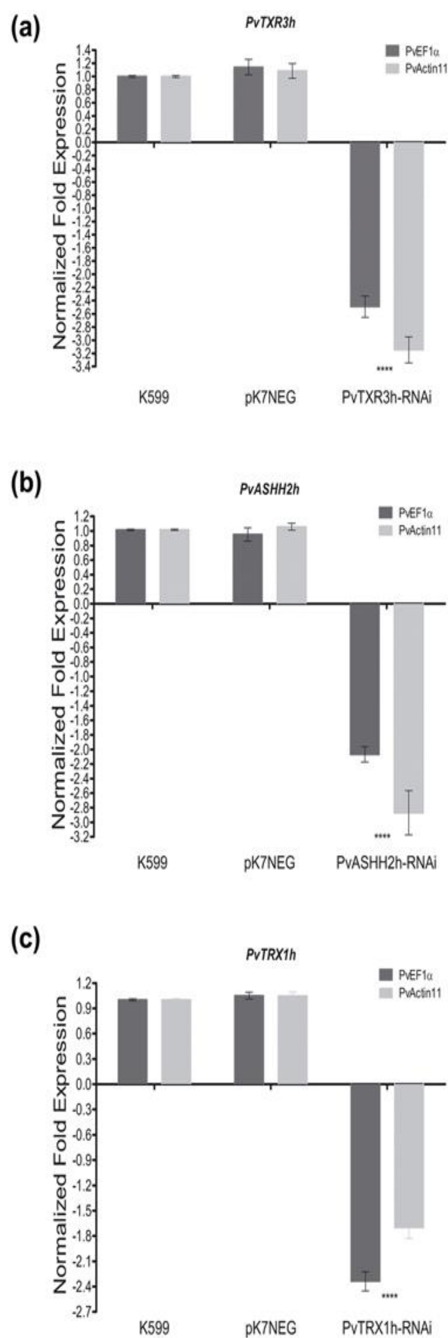

Fig 6. Transcript abundance of *PvATXR3h*, *PvASHH2h,* or *PvTRX1h* in composite plants of cultivar BAT477 as determined by qRT-PCR. (a) Expression of the *PvATXR3h* gene in hairy roots transformed with *A. rizhogenes*, *A. rizhogenes* containing the pK7NEG control vector, or *A. rizhogenes* containing the *PvATXR3h*-RNAi silencing vector. (b) Expression of the *PvASHH2h* in stems of plants transformed with *A. rizhogenes*, *A. rizhogenes* containing the pK7NEG control vector, or *A. rizhogenes* containing the *PvASHH2h*-RNAi silencing vector. (c) Expression of the *PvTRX1h* gene in hairy roots from plants transformed with *A. rizhogenes*, *A. rizhogenes* containing the pK7NEG control vector, or *A. rizhogenes* containing the *PvTRX1h*-RNAi silencing vector. Expression values were normalized to those of the *PvActin11* and *PvEF1α* reference genes, as shown. (Data represent mean ± SD; ****, $P<0.0001$ as determined with an unpaired two-tailed Student's *t*-test).

However, *PvASHH2h* is an ortholog of the permissive chromatin-modifying enzyme ASHH2. The fact that it is necessary for hairy root growth introduces the possibility that hairy root formation is regulated by a universal regulator.Previous studies have demonstrated that the *Arabidopsis thaliana ASHH2* plays important roles in cell proliferation, control of organ size, and fertility. Loss of function of this gene reveals that it has pleiotropic phenotypic effects (Xu et al., 2008). During seedling development, *ASHH2* expression can be observed within the seed and emerging root, in young seedlings, at the shoot apical meristem region, in cotyledons, in the vasculature, and more variably in the primary root (Cazzonelli et al., 2010). Similarly, the *Arabidopsis ATXR3* gene is ubiquitously expressed and the loss of *atxr3* leads to pleiotropic phenotypic effects, including shorter roots, smaller rosettes, shorter stems, and impaired gametophyte development (Yao et al., 2013). More recently, Napsucialy-Mendivil and co-workers (2014) have shown that, in addition to its known role in flower development (Alvarez-Venegas et al., 2003), ATX1 is important in root development. Specifically, ATX1 is essential for proper root apical meristem organization and activity, cell production, the timing of root development, stem-cell-niche maintenance, and cell patterning during primary and lateral root development (Napsucialy-Mendivil et al., 2014). However, regardless of such important roles of members of the TrxG proteins in plant growth and development, the mechanisms of their function in root and nodule development remains to be explored and will be essential for understanding epigenetic regulation in legumes, specifically in the response to developmental and environmental stimuli and to *Rhizobium*. New approaches of this kind and the development of new technologies should enhance our knowledge of post-translational modifications and facilitate the understanding of their roles in root and nodule development and in plant-microorganism interactions. This information should also be relevant to increasing crop productivity and disease management.

Materials and Methods

Plant material and growth conditions

Wild-type *P. vulgaris* cultivars BAT 477 and DOR 364 were used in this study (Davis et al., 1988). Both cultivars were acquired from the germplasm bank at the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP)-Celaya, México. Seeds were surface sterilized with 2% sodium hypochlorite, rinsed five times, placed in sterile plates, covered with tin foil, and germinated under sterile conditions for two days at 28°C in a Percival growth chamber (Percival Scientific, Perry, IA). The seedlings were transferred to pots containing vermiculite and placed in a greenhouse, at which time half of the plants were inoculated with 1 mL of a *Rhizobium etli* strain CE3 cell suspension. All plants were watered every other day with B&D solution described in Broughton and Dilworth (1971), with or without nitrogen (non-inoculated and inoculated plants with *R. etli*, respectively). Leaves and roots of non-inoculated plants were collected at 7, 14, or 21 days post germination (dpg). Roots of inoculated plants were collected at 3 and 7 days post-inoculation and nodules were collected at 14 and 21 days post-inoculation. All samples were frozen in liquid nitrogen and stored at -80°C.

Plasmid construction

The pRED-PvATXR3h-RNAi (PvATXR3h-RNAi) and pRED-PvASHH2h-RNAi (PvASHH2h-RNAi) vectors, which drove the expression of the *PvATXR3h* and *PvASHH2h* inverted repeats, respectively, were under the control of the CaMV35S promoter. They were created as follows: a 730 bp PCR fragment of the *PvATXR3h* cDNA (*Phvul.005G112000*; GenBank accession number XP_007149940) was amplified by using gene-specific forward (PvXR3RiF: 5´atggttgagggacgaggtccga-3') and reverse (PvXR3RiR: 5´ccacgctaaaacaggcagcagc-3') primers. Likewise, a 388 bp fragment of the *PvASHH2h* cDNA (*Phvul.009G130100*; GenBank accession number XP_007137477) was amplified with a gene-specific forward (PvH2RiF: 5'-cacaggaaagctgtcctgccca-3') and reverse (PvH2RiR: 5'-tgcagtgacccaacgctctggaa-3') primer. The PCR products were cloned separately into the pCR8/GW/TOPO TA Cloning vector (Invitrogen, Carlsbad, CA, U.S.A.), to generate the entry vectors and then sequenced. After that, the DNA fragments were recombined from the entry vector to the MultiSite Gateway pK7GWIWG2(II)-RedRoot destination vector (http://gateway.psb.ugent.be/) by using the Gateway LR Clonase II Enzyme mix (Invitrogen, Carlsbad, CA, U.S.A.), to generate the final silencing vectors (pRED-PvATXR3h-RNAi [*PvATXR3h*-RNAi] and pRED-PvASHH2h-RNAi [*PvASHH2h*-RNAi]). Construction of the *PvTRX1h*-RNAi vector that drove the expression of an antisense sequence from the *PvTRX1h* gene (*Phvul.008G018500*; GenBank accession number XP_007139309) under control of the ectopic CaMV35S promoter, was created as follows: a 602bp PCR fragment of the *PvTRX1h* cDNA was amplified by using gene-specific forward and reverse primers (PvRX1RiF 5'-tctagagcaaagcatccacataaagg-3'; PvRX1RiR 5'- ggatcccgaaac-aatgggaagaatcag-3'; underlined sequences correspond to artificially introduced *XbaI* and *BamHI* restriction sites, respectively). Next, the pFGC5941 binary vector was digested with *XbaI* and *BamHI* and the backbone was purified and ligated to the *PvTRX1h* PCR product with the T4 DNA ligase at the *XbaI* and *BamHI* sites of the pFGC5941 plasmid to generate the PvTRX1h-RNAi vector. The control expression vector, pK7NEG with the red fluorescent protein from *Discoma sp.* (DsRed) was generated by digesting the pK7GWIWG2(II)-RedRoot vector with the *Sal*I restriction enzyme and the purified fragment was self-ligated with the T4 DNA ligase.

Bacterial strains and growth conditions

Competent cells of *Agrobacterium rhizogenes* strain NCPPB 2659, also known as K599, were prepared according to McCormac et al. (1988). After preparation, 40 µL of competent cells were mixed with 1 µg of plasmid DNA (*PvATXR3h*-RNAi, *PvASHH2h*-RNAi, *PvTRX1h*-RNAi, or pK7NEG) and electroporated in a 0.2-cm cuvette using a Bio-Rad Gene Pulser (25 µF, 200 ohms, 2.0 Kv). Cells were diluted with Luria Broth (LB) media, incubated for two hours at 28°C in an orbital incubator, plated on solid LB media supplemented with 100 µg/mL streptomycin and 100 µg/mL spectinomycin, and grown at 28°C for 36 h. *A. rhizogenes* K599 with *PvATXR3h*-RNAi, *PvTRX1h*-RNAi, *PvASHH2h*-RNAi, or pK7NEG were used as described by Estrada-Navarrete et al. (2007) to generate composite common bean plants. *Rhizobium etli* strain CE3 was grown at 28°C for 48 h to 72 h on peptone-yeast (PY) liquid media containing 20 µg/mL nalidixic acid, 100 mg/mL streptomycin, and 7 mM

$CaCl_2$, until the bacterial culture reached an OD_{600} of 0.5 to 0.6 (Cárdenas et al., 2006). After that, roots from the different plants were inoculated with 1 mL of the *Rhizobium* suspension and watered every other day with the B&D nitrogen-free solution. *Rhizobium tropici* strain CIAT 899 with the reporter gene *green fluorescent protein (GFP)* or with the reporter gene *GUS* were grown at 28°C on PY liquid media supplemented with 7 mM $CaCl_2$ (containing 25 µg/mL of rifampicin and 20 µg/mL of tetracycline for the GFP strain, and 20 µg/mL nalidixic acid and 50 µg/mL of kanamycin for the GUS strain) until the culture reached an OD_{600} of 0.4 to 0.6. Then, the bacterial culture was centrifuged, the pellet washed in 10 mM $MgSO_4$, and the cells resuspended in 10 mM $MgSO_4$. Next, the *A. rhizogenes*-transformed roots were inoculated with 4 mL of *R. tropici* solution and watered every other day with the B&D nitrogen-free solution.

Generation of transgenic hairy roots

A. rhizogenes K599 containing the *PvATXR3h*-RNAi, *PvTRX1h* -RNAi, or *PvASHH2h*-RNAi silencing vectors, or the pK7NEG control vector were used to transform both *P. vulgaris* cultivars. Composite common bean plants were generated as described by Estrada-Navarrete et al (2007). When the transgenic roots were fully grown (12-14 days after infection with *A. rhizogenes*), the primary root was removed by cutting the stem below the transgenic roots and the composite common bean plants were transferred to new pots containing sterile vermiculite. The transgenic roots were inoculated with *R. tropici* strain CIAT 899. Transgenic roots and nodule structures were analyzed and collected as indicated.

cDNA synthesis and qRT-PCR analysis

TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.) was used to isolate total RNA from all the samples. For qRT-PCR analysis, RNA was treated with DNaseI (Invitrogen, Carlsbad, CA, U.S.A.) to remove genomic DNA. The absence of DNA was confirmed by performing PCR (40 cycles, similar to the real-time PCR program) on the DNaseI-treated RNA using Taq-DNA polymerase. One microgram of total RNA was reverse transcribed in 20 µL reaction mix with 200 U of SuperScript II-Reverse Transcriptase (Invitrogen, Carlsbad, CA, U.S.A.) and oligo(dT) primer. A StepOne® Real-time PCR system (Applied Biosystems, Carlsbad, CA, U.S.A.) was used for real-time PCR quantifications. qRT-PCR was performed according to the Maxima® SYBR Green/ROX qPCR Master Mix (2x) protocol (Thermo Scientific, Waltham, MA, U.S.A.). A "no DNA" template control was used in each analysis. Primers used were as follows: for *PvATXR3h*, PvATXR3hF 5'-gcaagaaaaggtgcagaaggaggaagttgagga-3', and PvATXR3hR 5'-acaatctccccgttctcaatctcgcccct-3'; for *PvTRX1h*, PvTRX1hF 5'-agaggaaaattgggagcactgagctggagagac-3', and PvTRX1hR 5'-cacacttaaaattcccagcagcaccagaattcc-3'; and for *PvASHH2h*, PvASHH2hF 5'- ggtgaggggtcttgcgatgctttgggtt-tgg-3', and PvASHH2hR 5'-gggaacttcgatgcccccataagaaacccc-caaac-3'.

The results presented are from three independent biological replicates from different composite common bean plants. Statistical significance was determined with an unpaired two-tailed Student's *t*-test. Each biological replicate was tested in triplicate and data were normalized to the *Actin11* (*PvActin11*) reference gene (*PvActin11F* 5'-tgcatacgttggtgatgagg-3', and *PvActin11R* 5'-agccttggggttaagaggag-3' [Borges et al., 2012]); and to the *elongation factor 1-α* (*PvEF1α*) reference gene (PvEF1aF 5'-ggtcattggtcatgtcgactctgg-3', and PvEF1aR 5'-gcacccaggcatacttgaatgacc-3') (Livak and Schmittgen, 2001; Barraza et al., 2013).

Confocal microscope settings

All imaging was done using a Zeiss LSM 510 inverted confocal microscope with EC Plan-Neofluar 20X/0.50 ph2 M27 and EC Plan-Neofluar 10X/0.30 ph1 objectives; plant tissues were temporarily under water during the imaging. Auto-fluorescence was eliminated by adjusting the detection gain, amplifier offset, and amplifier gain parameters with negative, un-transformed control plants. To image GFP and DsRED, we used an HFT 458/561/633 dichroic mirror. We used a 565 (NFT565) dichroic secondary filter to split the emission in conjunction with a 561-nm laser line and a 575- to 615-nm (BP575-615) band-pass filter for DsRED, and the 488-nm laser line and a 500- to 550-nm (BP500-550) band-pass filter for GFP. The 561-nm laser was attenuated to 13% and the 488-nm laser was attenuated to 5%. For imaging of DsRED, we used a 561-nm laser line together with an HFT 458/561/633 dichroic mirror and a band-pass filter of 575-615 nm. For GFP, we used a 488-nm laser line and a 500- to 550-nm (BP500-550) band-pass filter.

SET-domain sequence search

SET-domain containing sequences from *Arabidopsis thaliana* were initially searched at the National Center for Biotechnology Information (NCBI) database. With these proteins, new BLAST searches were conducted to collect *P. vulgaris* sequences from the Phytozome database (Phytozome v9.1, http://www.phytozome.net). After performing the similarity searches, all non-redundant hits were compiled and each of these sequences was examined to confirm the presence of the SET-domain by searching the CCD Database (NCBI; Marchler-Bauer et al., 2005) as well as the SMART database (Letunic et al., 2004; Schultz et al., 2000).

Multiple sequence alignments and phylogenetic analyses of SET-domain sequences.

The ClustalW2 software (http://www.ebi.ac.uk/Tools/msa/clustalw2/) was employed to generate multiple sequence alignments of the SET-domain sequences. Once created, the ClustalW2 alignments were used to generate Neighbor Joining (NJ) phylogenetic trees with the MEGA4 program (Tamura et al., 2007). Multiple sequence alignment of proteins to generate the Maximum Likelihood (ML) phylogenetic trees was performed with the MUSCLE software (Edgar, 2004). ML phylogeny of SET-domain sequences was performed with the "Phylogeny Pipeline" at http://phylogeny.lirmm.fr/ (Dereeper et al., 2008). Multiple sequence alignment of proteins was performed with the MUSCLE software (Edgar, 2004), curation of the alignment was done with the GBLOCKS program (Castresana, 2000), Maximum Likelihood trees with approximate Likelihood Ratio Test for branches (PhyML+ aLRT) was performed with the PhyML3.0 program (Guindon and Gascuel, 2003; Anisimova and Gascuel, 2006), and trees were drawn with TREEDYN software (Chevenet et al., 2006). NJ phylogeny was conducted with the MEGA4 program (Tamura et al., 2007). The evolutionary history was inferred using the NJ method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 5000 replicates was taken to represent the

evolutionary history of the analyzed taxa (Felsenstein, 1985). Branches corresponding to partitions reproduced in fewer than 50% bootstrap replicates were collapsed.

Conclusion

In this study, transgenic roots (composite plants) induced by *Agrobacterium rhizogenes* strain NCPPB2659 (also known as K599) were used, in a reverse genetic approach, to investigate the role of *PvATXR3h*, *PvASHH2h* and *PvTRX1h* in the common bean. Considering that genetic transformation in common bean composite plants is restricted to roots and nodules, we evaluated the effects of gene down-regulation by RNAi in transgenic root growth. Our findings suggest that down-regulation of *PvASHH2h* exhibits transgenic root growth arrest in common bean composite plants. Therefore, *PvASHH2h* is crucial for transgenic root growth and development. However, down-regulation of *PvATXR3h* and *PvTRX1h* gave rise to apparent normal transgenic roots, even though downregulation of *PvATXR3h* affects the number of transgenic hairy roots per plant. Accordingly, our study highlights a distinct role of PvASHH2h in regulation of genome function and root activity.

Acknowledgements

We thank Dr. Federico Sánchez for kindly providing the *Agrobacterium rhizogenes* K599 strain, *Rhizobium tropici* CIAT899 GFP, and *R. tropici* CIAT 899 GUS strains. Thanks to Dr. Luis Cárdenas for giving us the *R. etli* strain CE3. This work was supported by "CONACYT", grant CB-2011/167693 to RA-V.

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