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ORIGINAL ARTICLE

Analysis of tomato plasma membrane H$^+$-ATPase gene family suggests a mycorrhiza-mediated regulatory mechanism conserved in diverse plant species

Junli Liu[1] · Jianjian Liu[1] · Aiqun Chen[1] · Minjie Ji[2] · Jiadong Chen[2] · Xiaofeng Yang[2] · Mian Gu[2] · Hongye Qu[1] · Guohua Xu[1,2]

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Abstract In plants, the plasma membrane H$^+$-ATPase (HA) is considered to play a crucial role in regulating plant growth and respoding to environment stresses. Multiple paralogous genes encoding different isozymes of HA have been identified and characterized in several model plants, while limited information of the HA gene family is available to date for tomato. Here, we describe the molecular and expression features of eight HA-encoding genes (*SlHA1-8*) from tomato. All these genes are interrupted by multiple introns with conserved positions. *SlHA1*, *2*, and *4* were widely expressed in all tissues, while *SlHA5*, *6*, and *7* were almost only expressed in flowers. *SlHA8*, the transcripts of which were barely detectable under normal or nutrient-/salt-stress growth conditions, was strongly activated in arbuscular mycorrhizal (AM) fungal-colonized roots. Extreme lack of *SlHA8* expression in M161, a mutant defective to AM fungal colonization, provided genetic evidence towards the dependence of its expression on AM symbiosis. A 1521-bp *SlHA8* promoter could direct the GUS reporter expression specifically in colonized cells of transgenic tobacco, soybean, and rice mycorrhizal roots. Promoter deletion assay revealed a 223-bp promoter fragment of *SlHA8* containing a variant of AM-specific *cis*-element MYCS (vMYCS) sufficient to confer the AM-induced activity. Targeted deletion of this motif in the corresponding promoter region causes complete abolishment of GUS staining in mycorrhizal roots. Together, these results lend cogent evidence towards the evolutionary conservation of a potential regulatory mechanism mediating the activation of AM-responsive HA genes in diverse mycorrhizal plant species.

Keywords Tomato · H$^+$-ATPase · *SlHA8* · GUS reporter · Arbuscular mycorrhizal symbiosis

Junli Liu and Jianjian Liu contributed equally to this work.

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✉ Aiqun Chen
chenaq8@njau.edu.cn

[1] State Key Laboratory of Crop Genetics and Germplasm Enhancement, College of Resources and Environmental Sciences, Nanjing Agricultural University, Nanjing 210095, China

[2] MOA Key Laboratory of Plant Nutrition and Fertilization in Lower-Middle Reaches of the Yangtze River, Nanjing Agricultural University, Nanjing 210095, China

Introduction

Plasma membrane H$^+$-ATPase, a member of the superfamily of P-type ATPases, which are characterized by the formation of phosphorylated intermediates during catalysis, is a ubiquitous enzyme existent in all cell types of plants (Kanczewska et al. 2005; Okumura et al. 2012). The H$^+$-ATPase catalyzes ATP hydrolysis coupled with proton pumping out of cells, thereby establishing electrochemical gradient of H$^+$ across plasma membrane, which is required for a number of substance transport processes dependent on proton motive force (PMF) and ATP-released energy (Sondergaard et al. 2004; Duby and Boutry 2009). Recent physiological studies have demonstrated that the activity of PM H$^+$-ATPase was highly correlative with numerous physiological processes essential for plant growth, such as nutrient uptake, ion homeostasis, intracellular pH regulation, stomatal controlling, and cellular expansion (Alsterfjord et al. 2004; Haruta et al. 2010, 2012; Hayashi et al. 2014).

In all plant species examined so far, plasma membrane H$^+$-ATPase is encoded by a multigene family (hereafter referred to

as HA family) with members having distinct but partially overlapping expression profiles and physiological roles (Gaxiola et al. 2007; Janicka-Russak et al. 2012). For example, *Arabidopsis* has 11 (*AHA1-11*) members encoding different PM H^+-ATPase isoforms (Baxter et al. 2003). Expression of *AHA1* and *2* in a variety of tissues (Haruta et al. 2010), *AHA3* in vasculature and reproductive tissues (Robertson et al. 2004), *AHA6*, *8*, and *9* in floral organs (Arango et al. 2003), *AHA7* in root hairs (Lan et al. 2013), and *AHA10* in endothelium of developing seed coat (Baxter et al. 2005) provided strong evidence towards the evolution of their specialized roles in a tissue-specific and development-dependent manner (Arango et al. 2003; Lefebvre et al. 2005). Several studies in different plant species have also shown differential regulation of HA members in response to various abiotic (nutrient deficiencies, high salinity) and biotic (pathogen infection) stresses, as well as arbuscular mycorrhizal (AM) fungal colonization (Schaller and Oecking 1999; Krajinski et al. 2002; Sibole et al. 2005; Shen et al. 2006; Janicka-Russak and Kobus 2007; Liu et al. 2009; Sperandioa et al. 2011; Zeng et al. 2012). Their roles in eliciting accentuated transport of solutes and metabolites in specific cells have been postulated (Rosewarne et al. 2007).

AM symbiosis, formed between AM fungi in rhizospheres and roots of most land plants, is the most widespread mutualistic symbiotic association in nature (Smith and Smith 2011; Bitterlich et al. 2014). One of the major benefits for host plants is the increased uptake of water and nutrients through the symbiotic uptake pathway (Smith et al. 2004; Balestrini et al. 2005; Maeda et al. 2006; Yang et al. 2012; Chen et al. 2014). Multiple AM-induced transporters responsible for translocating nutrients, such as P and N, across the intraradical symbiotic interface have been isolated (Glassop et al. 2005; Nagy et al. 2005; Javot et al. 2007; Guether et al. 2009; Kobae et al. 2010; Yang et al. 2012; Breuillin-Sessoms et al. 2015), and their function were proposed to be dependent on the activity of H^+-ATPase located on the periarbuscular membrane (Karandashov and Bucher 2005). Recently, the AM-responsive HA genes, which were considered to be essential for energizing and regulating the secondary transport systems at the symbiotic interfaces, have been identified in several plant species. Nine HA genes (*PMA1-9*) have been annotated for tobacco (*N. plumbaginifolia*), and two (*PMA2* and *4*) of them were found to be induced in cortical cells containing arbuscules of mycorrhizal roots (Gianinazzi-Pearson et al. 2000; Oufattole et al. 2000). In tomato, three full-length complementary DNAs (cDNAs) and four short genomic sequences (*c.* 200 bp) encoding putative HA isoforms (LHA1-7) have been reported (Ewing and Bennett 1994). On the basis of in situ hybridization, two members, *LHA1* and *4*, were reported to be AM-responsive (Rosewarne et al. 2007). Both the tobacco and tomato HA genes showing induction in AM fungal-colonized cells were also expressed in a variety of tissues (Gévaudant et al. 2007; Bobik et al. 2010). Notably, *MtHA1* from *Medicago* and *OsA8* (also named as *Os-HA1*) from rice are the only two known HA genes whose expression is exclusively confined to specific root cells containing AM fungal structures (Krajinski et al. 2014; Wang et al. 2014). The AM-specific *MtHA1* and *OsHA8* fall into a phylogenetic clade distinct from that to which tobacco and tomato HA genes showing AM-induced expression belong (Rosewarne et al. 2007). Therefore, it would be interesting to investigate the presence of some other AM-induced/specific HA genes in tomato or other solanaceous species.

In this study, we report a comprehensive genome-wide inventory of the tomato HA gene family, including exon/intron structure, phylogenetic evolution, and expression regulation in different tissues and in response to AM symbiosis. A newly identified HA member, *SlHA8*, was revealed to be strongly and specifically expressed in AM fungal-colonized roots. The promoter activity of *SlHA8* in response to AM symbiosis was further investigated in the mycorrhizal roots of tobacco, soybean, and rice plants. Promoter deletion/truncation assay was also employed to screen the putative *cis*-acting element(s) responsible for conferring the symbiosis-responsive expression of *SlHA8* in mycorrhizal roots.

Materials and methods

Plant material and cultivation conditions

Micro-Tom (*Solanum lycopersicum* cv.), a miniature tomato cultivar, and M161, a tomato mutant showing resistance to AM fungal infection and colonization by significantly repressing spore germination and appressorium formation (David-Schwartz et al. 2001), were used in this study. The tomato seeds were surface-sterilized and cultivated in a growth chamber with a photoperiod of 14-h light (30 °C) and 10-h dark (20 °C). After being germinated and grown on MS medium for 2 weeks, the seedlings were then transplanted to sterilized quartz-sand for a 2-week culture irrigated with full-strength nutrient solution containing the following: 1 mM NH_4^+-N, 4 mM NO_3^--N, 2 mM K^+, 1 mM Pi, 0.75 mM Ca^{2+}, 0.5 mM Mg^{2+}, 0.25 mM Cl^-, 0.5 mM SO_4^{2-}, 20 μM Fe^{2+}, 9 μM Mn^{2+}, 46 μM BO_3^{3-}, 8 μM Zn^{2+}, 3 μM Cu^{2+}, and 0.03 μM MoO_4^{2-} (Chen et al. 2014). The plants were then transferred to pot culture for continuing growing for either collecting the roots, young leaves (newly expanded leaves), flowers, and fruit samples or conducting the following treatments.

For AM fungi colonization, three plantlets were transplanted to a 3-dm^3 pot filled with sterilized sand. A sand-based inoculum containing *Rhizophagus irregularis* was used for inoculation. Each plant was inoculated with 2 g of inoculum (150–200 spores) that was placed in the sterilized sand around the plant roots. The irrigating nutrient solution

contained 50 μM Pi, in addition to the other essential nutrients (see above). After inoculation for 1 month, the plants were harvested and the roots and young leaves were sampled for subsequent RNA isolation. For K-, Mg-, and Pi-deficient treatments, the concentrations of K^+, Mg^{2+}, and Pi in the irrigating solution were decreased to 50, 25, and 50 μM, respectively. For salt-stress treatment, the plants were irrigated with full-strength nutrient solution but additional 200 mM NaCl. All the treatments comprised three replicates. After being treated for 2 weeks, the plant tissues were harvested for the subsequent RNA isolation.

Identification of PM H^+-ATPase genes in tomato and potato genomes

Members of the plasma membrane H^+-ATPase (HA) family in tomato genome were identified using the TBLASTN algorithms. In order to find out all the potential HA genes in tomato, the *Arabidopsis* HA genes were employed as queries for blast searches against the tomato genomic sequence database at Solanaceae Genomics Network (www.sgn.cornell.edu). Sequences with a query over 50 % and e-value less than −10 were taken as the putative HA candidates. The obtained sequences were submitted to NCBI (http://www.ncbi.nlm.nih.gov/) and TIGR (Plant Transcript Assembly Report, http://blast.jcvi.org/euk-blast/plantta_blast.cgi) for EST blast searches. Eventually, a total of eight distinct genomic sequences encoding putative different isoforms of plasma membrane H^+-ATPase were identified and named as *SlHA1-8*, respectively.

For identification of the potential HA homologues from potato genome, the potato genomic sequence database (http://solgenomics.net/organism/Solanum_tuberosum/genome) was also extensively searched using tomato HA genes as queries. Accordingly, eight distinct sequences were identified as the putative potato HA genes. These genes were therefore named as *StHA1* to *8*, based on the phylogenetic homology with their tomato orthologs.

Phylogenetic and gene structure analysis

Multiple sequence alignments were performed using the program ClustalX (v1.8) with default gap penalties. An unrooted phylogenetic tree was constructed using the deduced amino acid sequences of HA genes by neighbor-joining algorithm wrapped in MEGA 5.1 software (www.megasoftware.net). Exon/intron structure analysis of the tomato HA genes was performed by comparing the messenger RNA (mRNA) sequences with their genomic DNA sequences.

RNA extraction

Total RNA was isolated from 100 mg of various tomato tissues, including roots, stems, leaves, flowers, and fruits (green and ripe). The RNA from fruit samples was isolated using a CTAB-sour phenol extraction method as described previously (Chang et al. 1994). The total RNA from other tissue samples was all isolated using the guanidine thiocyanate extraction method with TRIzol reagent (Invitrogen).

cDNA preparation and quantitative real-time RT-PCR analysis

For cDNA preparation, approximately 2 μg of DNase-treated total RNA from each sample was used to synthesize first-strand cDNA in a 20-μl reaction solution using a reverse transcription kit (TaKaRa), and the synthesized cDNAs were used as templates in the following real-time RT-PCR reactions.

Real-time qRT-PCR analysis was conducted to quantify the relative transcription levels of tomato HA genes in different tissues or in roots and leaves in response to different treatments. The reaction was run on an Applied Biosystems (ABI) Plus Real-Time PCR System using the SYBR premix ExTaq kit (TaKaRa). The reactions were conducted in a final volume of 20 μl containing 10 μl of SYBR Green premix (2×) (TaKaRa), 0.2 μM of each gene-specific primers, and 1 μl of cDNA template. The PCR procedure consisted of an initial incubation at 95 °C for 15 s, followed by 40 cycles of 95 °C for 5 s, 68 °C for 30 s, and an additional final cycle of dissociation curves to guarantee a specific amplification. Relative quantification of the transcription level for each target gene was normalized to the transcripts of the tomato constitutive *Actin* gene (Chen et al. 2014). The specificity of primer sets for the qRT-PCR (Table S3) was further confirmed by sequencing after the PCR reaction.

Detection of mycorrhizal fungal colonization

The examination of mycorrhizal fungal colonization was performed as described previously (Chen et al. 2007). Root segments were treated with 10 % (*w*/*v*) KOH solution at 90 °C for 1–2 h and then acidified with 1 % (*v*/*v*) HCl solution for 10 min. The root segments were then stained with 0.3 % (*w*/*v*) trypan blue solution at 90 °C for 3 h. After being rinsed in 50 % glycerol to remove excess stain, the root segments were examined for fungal colonization under a microscope by using the magnified line intersect method (Trouvelot et al. 1986; Herrera-Medina et al. 2007).

Construction of binary vector and transformation of tobacco and soybean plants

A series of promoter fragments with different length (1521, 778, 639, 510, 383, 223, 119 bp) of *SlHA8* immediately upstream of the translation start ATG were PCR amplified to introduce Hind III and BamH I restriction sites at the end of the 5′ and 3′ regions. After digestion with the two restriction enzymes, the amplified

fragments were cloned into binary vector pBI121 to replace the CaMV35S promoter in front of the GUS reporter gene. The constructs were named as *pSlHA8*$_{-x}$ (*x* denotes the length of corresponding promoter fragments). *Agrobacterium*-mediated transformation of tobacco plants was performed using the leaf-disk infection method (Horsch et al. 1985). The transformation of rice plants was carried out as described by Upadhyaya et al. (2000). The *pSlHA8*$_{-1521}$ construct was also introduced into *A. rhizogenes* strain K599 and transformed into soybean hairy roots as described previously (Guo et al. 2011).

Histochemical GUS assays

Histochemical staining of the transgenic roots for GUS activity was performed as described previously (Karandashov et al. 2004). For visualization of mycorrhizal fungal structures, the Magenta-GUS-stained root segments were counterstained for 1–2 h at 90 °C with 0.3 % (*w*/*v*) trypan blue. The co-localization of Magenta-GUS and trypan blue stains in mycorrhizal roots was indicated by purple staining. The stained root segments were then rinsed in 50 % glycerol to remove excess stain before being photographed using a stereomicroscope with a color CCD camera (Olympus, Japan).

Results

Genome-wide identification of tomato plasma membrane H^+-ATPase genes

To identify and characterize all the potential HA genes in tomato, the coding sequence (CDS) of the three accessioned tomato HA genes *LHA1*, *LHA2*, and *LHA4*, and 11 homologues from *Arabidopsis* HA family (*AHA1–11*) was employed for BLAST search against tomato genomic sequence database (http://solgenomics.net/). This resulted in the identification of eight nonallelic genomic sequences on five (3, 6, 7, 8, and 12) of the 12 tomato chromosomes as the putative tomato HA genes (Fig. S1). These putative genes, including the previously accessioned three tomato HA members, were (re)named as *SlHA1* to 8. Except closely located *SlHA5* and *6* on chromosome 7, none of the other members were found to be located in clusters. A synteny search of ±500-kb genomic regions encompassing each tomato HA gene permitted to identify four significant homologous blocks embodying five SlHA genes (Fig. S1).

Molecular analysis of the deduced polypeptides showed that all the putative proteins of the tomato HA genes contain 924–966 amino acids with their molecular weights predicted to be between 100.60 and 106.08 kDa (Table S1), similar to that of the known HA isozymes from other plant species. Sequence alignment analysis revealed high levels of identities (71.2–98.3 % at the amino acid level) between these HA paralogues (Fig. S2, Table S2). A comparison between the mRNA and genomic sequences revealed the interruption of tomato HA genes by multiple and position-conserved introns. *SlHA1*, *2*, and *8* contain 20 introns in their coding regions, whereas the other five members are characterized by having only 10–13 introns (Fig. 1).

A phylogenetic tree was constructed to estimate the phylogenetic relationships among the tomato HA genes and other plant homologues. As observed, the phylogenetic tree clusters the HA genes into five major subfamilies (I–V) (Fig. 2), and members from tomato were represented in four of them. In subfamily I, *SlHA1* and *2* were separated into two independent dicot clusters with members from potato, tobacco, grape, and *Medicago*. In subfamily II, *SlHA4* groups together with two members form other two solanaceous species, potato (*StHA4*) and tobacco (*PMA4*), and a member from rice (*OsA7*). In subfamily IV, the three SlHA members, *SlHA5*, *6*, and *7*, clustered closely with orthologs from two other solanaceous species potato and tobacco. On the other hand, *SlHA3* and *8* in subfamilies IV and V, respectively, formed clades with other members also from Brassicaceae, Leguminosae, Vitaceae, and Gramineae (Fig. 2).

Tissue-specific expression profiles of the tomato HA family genes

To better learn the possible function of each of the tomato HA genes, the transcriptional levels of *SlHA1-8* were quantitatively examined in various tissues, including roots, young leaves, flowers, and fruits at green and ripe stages (Fig. 3). Transcripts of all the members, with a notable exception of *SlHA8*, could be detected in a certain tissue(s) with distinct and partially overlapping expression profiles. Although *SlHA1*, *2*, and *4* showed ubiquitous expression in all the tissues examined, relatively the transcript abundance of *SlHA1* was significantly lower compared with those of *SlHA2* and *4*. *SlHA2* showed accentuated expression in leaves and flowers, but to much lesser extent in roots and fruits. *SlHA1* had a similar, but significant, lower expression tendency in all the tissues examined as compared to *SlHA2*. In addition to lower transcript abundance in fruits, *SlHA4* was observed to be expressed relatively highly and constitutively in roots, leaves, and flowers. Although the expression of *SlHA3* could be detected in all the tissues with relatively higher levels in flowers, its levels were much lower compared with those of *SlHA1*, *2*, and *4*. On the contrary, the expression of other three paralogues, *SlHA5*, *6*, and *7*, showed more distinct tissue-specific patterns with their transcripts almost only detectable in flowers (Fig. 3).

SlHA8 is specifically responsive to arbuscular mycorrhizal fungal colonization

Several HA genes in different plant species, including the tomato *SlHA1* and *4*, have been shown to be regulated by

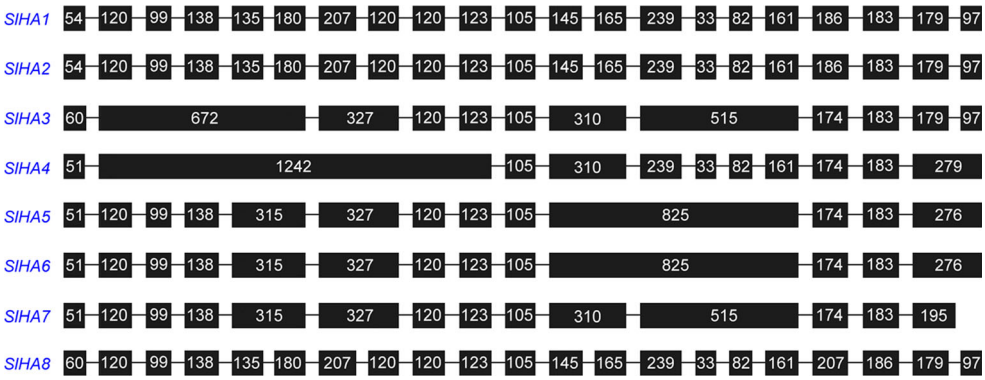

Fig. 1 Exon/intron structures of the tomato HA genes. *Black boxes* represent exons within coding regions, and the *lines* connecting them denote introns. *Numbers in boxes* denotes the sizes (bp) of exons

AM fungal colonization. To determine whether the presence of other HA members in tomato is also responsive to AM symbiosis, the relative transcript levels of each tomato HA gene were further evaluated in roots and leaves in response to AM fungal colonization. It was shown that *SlHA8*, whose transcripts were undetectable in all the tissues under normal growth condition, was significantly induced in the roots colonized by AM fungi (Fig. 4). Besides, the *SlHA2* transcripts were also found to be notably increased in colonized roots of mycorrhizal plants. No significant upregulation of the *SlHA1* and *SlHA4* transcripts were observed in the colonized roots as compared to those in the roots of nonmycorrhizal plants (Fig. 4).

To define whether the activation of *SlHA8* in mycorrhizal roots was dependent on AM colonization, we further examined the *SlHA8* expression in wild type and M161, a tomato mutant with a defect in AM fungal infection and colonization (Fig. 5a, b). It was shown that the transcripts of *SlHA8* in M161 were only 1/28 as compared with those in the mycorrhizal roots of wild-type plants (Fig. 5c), which is relatively consistent with the intensity of mycorrhizal colonization (M%) and the arbuscule abundance (A%) in the root system of M161 mutants (M% 3.3 ± 0.8 and A% 1.3 ± 0.4, respectively) and wild-type plants (M% 37.8 ± 4.5 and A% 17.6 ± 2.1, respectively). Additionally, we also investigated whether the AM-induced *SlHA8* were also responsive to other environmental factors, such as nutrient and salinity stresses, and the expression of *SlHA8* was further determined in roots in response to K, Mg, and Pi deficiency, as well as high salinity. As observed, the AM-induced *SlHA8* showed no response

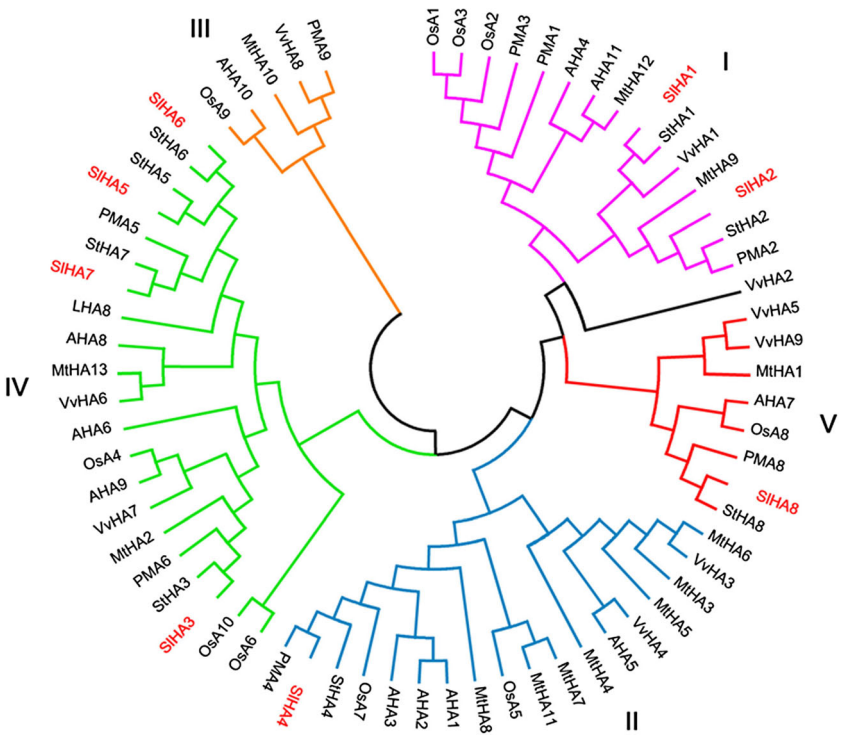

Fig. 2 Phylogenetic analysis of tomato HA family genes and the other homologues. The unrooted phylogenetic tree was constructed using the neighbor-joining method within MEGA 5.2 program. The roman numerals *I–V* represent the five groups of the HA gene family, respectively. HA proteins and corresponding plant species are as follows: tomato, SlHA1–8; potato, StHA1–8; tobacco, PMA1–6 and PMA8; *Arabidopsis*, AHA1–11; rice, OsA1–10; *Medicago*, MtHA1–13; grape, VvHA1–9

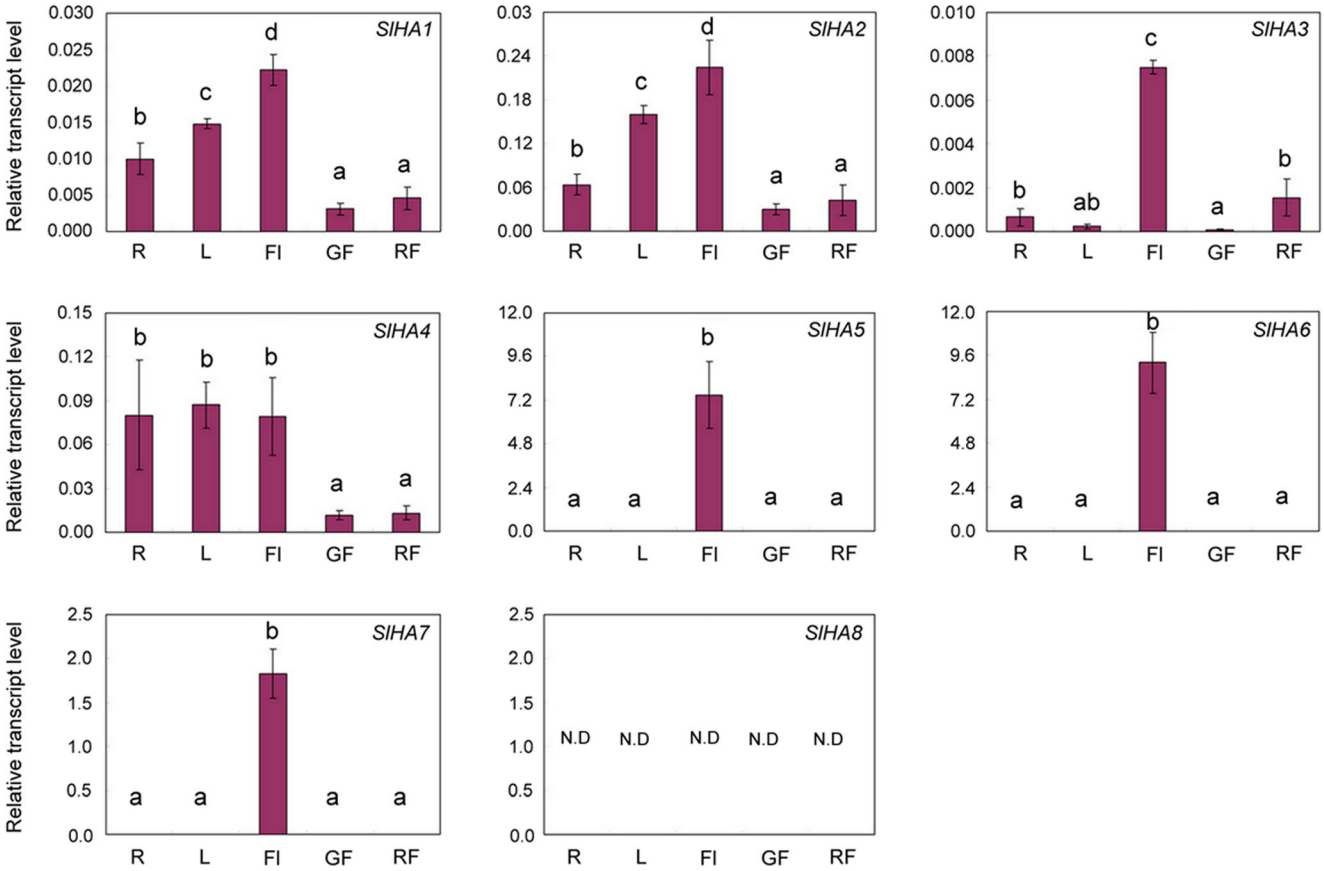

Fig. 3 Tissue-specific expression analysis of tomato HA genes. The RNA were prepared from different tissues, including roots (*R*), newly expanded young leaves (*L*), flowers (*Fl*), and fruits at green (*GF*) and ripe (*RF*) stages. The relative transcript levels of each of the tomato HA genes were indicated as percentage of the constitutive *Actin* expression activity. Values are the means of three biological replicates with SDs. In each individual graph, *different letters* indicate a significant difference ($P < 0.05$). *N.D* indicates that the expression were not detectable

to all these nutrient/salinity stresses (Fig. 5d). These results suggested that *SlHA8* might be a mycorrhiza-specific gene, and its expression is highly correlated with mycorrhizal colonization.

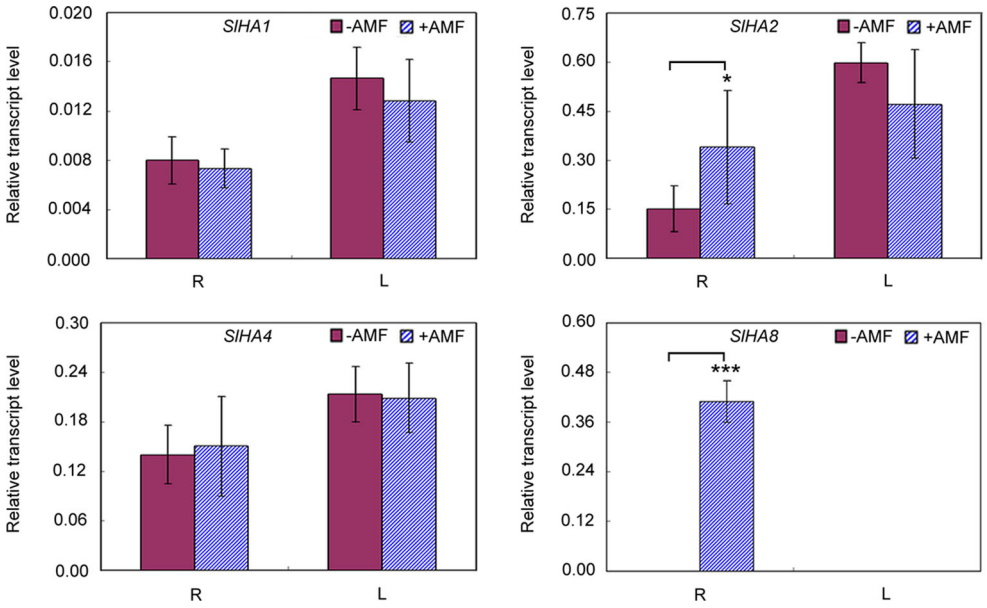

Fig. 4 Transcriptional analysis of the tomato HA genes in response to AM fungal (*Rhizophagus irregularis*) colonization. Real-time qRT-PCR was performed to determine the relative transcript levels of the tomato HA genes in roots (*R*) and newly expanded young leaves (*L*) of mycorrhizal plants (+*M*) and nonmycorrhizal plants (−*M*). The relative transcript levels of each of the tomato HA genes were indicated as percentage of the constitutive *Actin* expression activity. Values are the means of three biological replicates with SDs. $^{*}P < 0.05$; $^{***}P < 0.001$

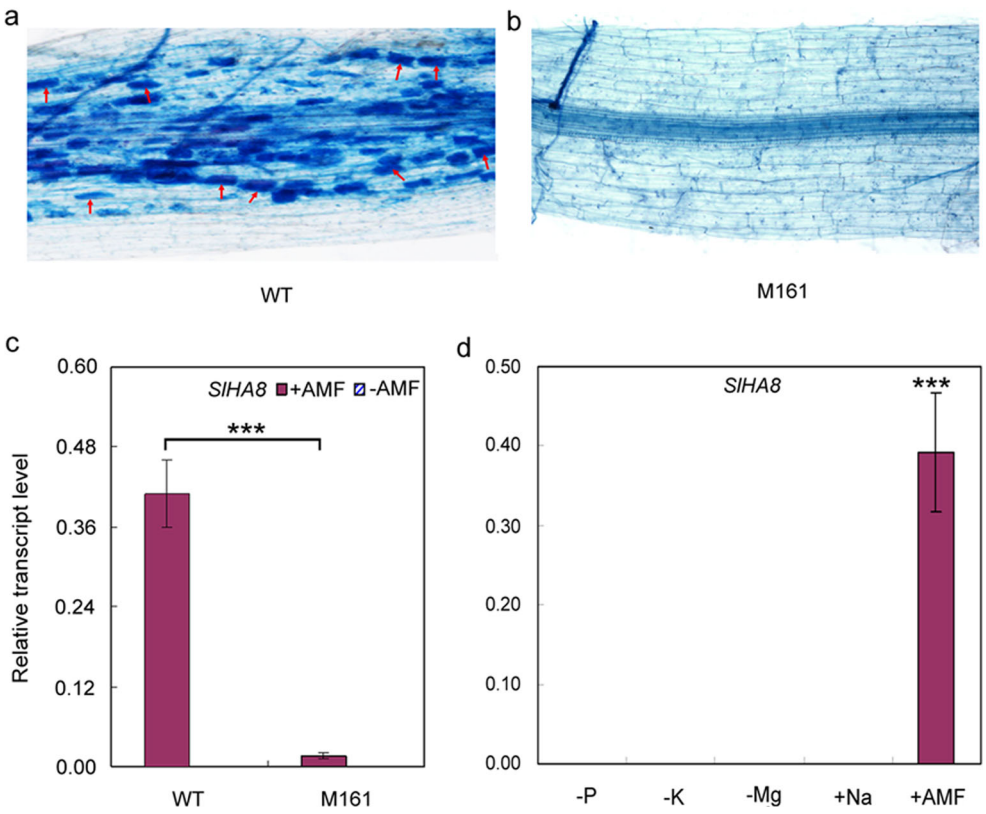

Fig. 5 Expression analysis of *SlHA8* in mycorrhizal roots of wild type (**a**) and M161 mutant (**b**) plants. *Red arrows* represent arbuscules in the cortical cells. Real-time qRT-PCR was performed to determine the relative transcript levels of *SlHA8* in mycorrhizal roots and leaves of wild-type plants and M161 mutant (**c**), as well as in wild-type plants in response to Pi, K^+, and Mg^{2+} deficiency and high NaCl (+Na) irrigation (**d**). The relative transcript levels of *SlHA8* were indicated as percentage of the constitutive *Actin* expression activity. Values are the means of three biological replicates with SDs. ***$P < 0.001$

The *SlHA8* promoter could drive AM-specific expression in mycorrhizal roots of transgenic tobacco, soybean, and rice plants

To obtain a more distinct view of the *SlHA8* expression in mycorrhizal roots, a 1521-bp promoter fragment of *SlHA8* was fused to the β-glucuronidase (GUS) reporter gene and introduced into tobacco plants via *A. tumefaciens*-mediated transformation. The transgenic plants were subsequently inoculated with *R. irregularis*. Four weeks after inoculation, the transcripts of *NtPT4*, the AM-specific phosphate transporter gene (Chen et al. 2011), and *NtHA8*, the ortholog of *SlHA8*, were strongly detected in tobacco mycorrhizal roots (Fig. S3). It was observed that the GUS activity could be detected only in AM fungal-colonized roots of transgenic tobacco plants (Fig. 6a) and no GUS staining could be detected in nonmycorrhizal transgenic roots (Fig. 6b). Co-localization of GUS expression and AM fungal structure by overlay of Magenta-GUS with trypan blue staining revealed that GUS activity driven by the *SlHA8* promoter was confined to distinct cells containing arbuscules (Fig. 6c). No GUS staining could be observed in vesicle-containing cells or in noncolonized cells (Fig. 6d).

To test whether the tomato *SlHA8* promoter could also direct the GUS reporter expression in other eudicot or monocot mycorrhizal plants, we further introduced the *pSlHA8*$_{-1521}$*::GUS* chimeric gene into soybean hairy roots and rice plants. As observed, the GUS staining could also be detected specifically in colonized cells of soybean and rice mycorrhizal roots (Fig. 6e, f). These results indicate that the *SlHA8* promoter fragment contains a *cis*-acting element(s) that is required for conferring AM-induced expression of HA genes in diverse mycorrhizal plants.

A variant of the MYCS *cis*-element was required to confer the AM-specific activity of *SlHA8*

To gain further insights into the regulatory mechanisms underlying the AM-activated expression of *SlHA8*, a series of promoter truncations fusing the GUS reporter were generated and introduced into transgenic tobacco plants (Fig. 7). Histochemical staining revealed that a 223-bp promoter fragment was still sufficient to confer the AM-specific GUS activity in transgenic tobacco roots (Fig. 7, S4). No known AM-responsive *cis*-element, except a variant of MYCS (vMYCS) (−124 to −113) elements (Chen et al. 2011; Favre et al. 2014), was found in the 223-bp promoter region (−223 to −1) of *SlHA8* (Fig. 7). A further deletion of *pSlHA8* down to −119 (*pSlHA8*$_{-119}$) that contains only partial vMYCS motif resulted in complete absence of GUS staining in transgenic mycorrhizal roots. Another construct with targeted deletion of the vMYCS motif from *pSlHA8*$_{-223}$ was generated for further investigating the role of vMYCS in AM-activated response. Histochemical staining for GUS activity revealed that deletion of the vMYCS (*pSlHA8*$_{-223\_Del\text{-}vMYCS}$) caused almost complete abolition of GUS staining in transgenic mycorrhizal

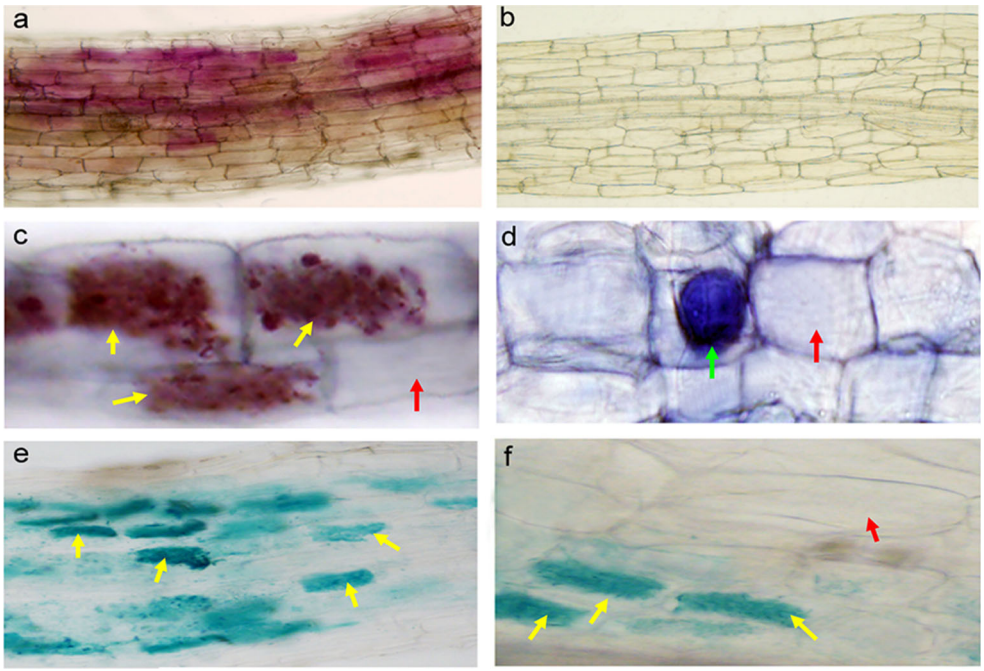

Fig. 6 Histochemical staining for the promoter activity of *SlHA8* using GUS reporter gene. **a**, **b** GUS staining driven by *SlHA8* promoter in tobacco mycorrhizal roots (**a**) and non-mycorrhizal roots (**b**). **c** Co-localization of GUS activity (indicated by the *purple color*, from the overlay of the Magenta-GUS and trypan blue stains) in the tobacco cortical cells harboring arbuscules. **d** No GUS staining could be detected in noncolonized cells or in vesicle-containing cells. **e**, **f** GUS (blue-GUS) staining directed by *SlHA8* promoter in soybean (**e**) and rice (**f**) mycorrhizal roots. *Yellow arrows* indicate arbuscules, *green arrows* indicate vesicles, and *red arrows* indicate noncolonized cells

roots. These results highlight the functional role of vMYCS for the AM-specific response of *SlHA8* in tomato.

Discussion

Evolutionary history of the HA gene family in tomato

In this study, through extensive searches of available database, a total of eight HA paralogues were identified in tomato genome. Phylogenetic analysis of these HA genes as well as the other homologues corroborated the earlier suggestion that plant HA genes could be classified into five subfamilies (I–V) (Arango et al. 2003).

The clustering of *SlHA1* and *2* in subfamily I along with members from grape and *Medicago*, but not Solanaceae only, suggested the divergence of the two paralogues occurring before Solanaceae split with Leguminosae and Vitaceae. Whereas the grouping of *SlHA5*, *6*, and *7*, in an independent solanaceous subclade in subfamily IV (Fig. 2) suggested the

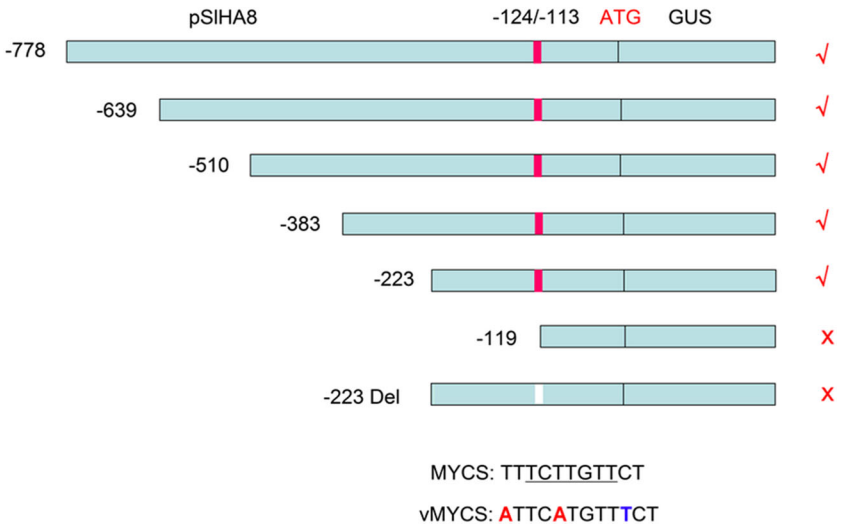

Fig. 7 *SlHA8* promoter truncation constructs and their activity in mycorrhizal tobacco roots. *-233 Del* denotes the deletion of the vMYCS motif from the promoter fragment *pSlHA8*$_{-233}$. *Tick symbols* (√) indicate that AM-induced GUS staining could be detected in mycorrhizal roots harboring corresponding *SlHA8* promoter truncation/deletion constructs, whereas *cross symbols* (×) indicate no visible GUS staining in corresponding mycorrhizal roots. *Red squares* represent the variant of MYCS element (vMYCS). The sequence (*TCTTGTT*) underlined in the MYCS element denotes its conserved core motif (Chen et al. 2011; Lota et al. 2013). The deviations from the consensus sequence in vMYCS motif are indicated by *red* and *blue bold letters*

likely divergence of the three paralogues as a relatively recent event that would have occurred after Solanaceae split with other dicot families. Based on phylogenetic distribution and chromosomal localization, it could be assumed that *SlHA5* and *6* are the two closest members that would have derived from the most recent tandem duplication occurring before the speciation of tomato and potato lineages. Their closeness could gain further creditability form their identical exon/intron structure (Fig. 1), as well as high sequence identity (Table S1). Therefore, it might be not surprising that the two genes would have a similar tissue-specific expression (Fig. 3) and/or biological functions. The distribution of *SlHA3*, *4*, and *8* in three different subfamilies that cluster together with members from both dicots and monocots suggested that these three HA members could be evolutionarily older than the other five paralogues.

Transcriptional regulation of tomato HA genes in response to AM symbiosis

In the present study, differential but partial overlapping expression of the tomato HA family genes was revealed (Fig. 3). Several HA genes in subfamilies I and II have been documented to be regulated by AM symbiosis. In tomato, *SlHA1* and *4*, each representing one of the two subfamilies, I and II, were previously shown to be AM-responsive (Rosewarne et al. 2007). In another earlier study and also in our present study, expression analysis of the SlHA genes, however, showed that the transcripts of *SlHA1* and *4* were not remarkably increased in mycorrhizal roots (Ferrol et al. 2002) (Fig. 4). Such discrepancy could be reasonably explained from in situ study demonstrating that the high inducibility of *SlHA1* and *4* in arbuscular-containing cortical cells was accompanied by an observable decrease of their transcripts in epidermal cells (Rosewarne et al. 2007). Similar alteration in expression distribution in response to AM symbiosis could also be observed from the study of LjSultr1;2, a sulfate transporter showing both S-starvation- and AM-induced responses (Giovannetti et al. 2014). Another study reported relative higher levels of *SlHA2* transcripts in mycorrhizal roots (Ferrol et al. 2002). Consistently, our study also revealed a substantial increase of *SlHA2* transcripts in mycorrhizal roots (Fig. 4). However, an earlier study based on in situ hybridization revealed that *SlHA2* was neither expressed in arbuscule-containing cortical cells nor had any change in expression in mycorrhizal roots as arbuscules developed (Rosewarne et al. 2007). Such discrepancies in *SlHA2* expression obtained by different studies could possibly be attributed to different analytical methods, sampling stages, and use of different interspecies or AM fungi.

Contrary to most of the known AM-regulated HA genes exhibiting ubiquitous expression and inducible responses to various environmental factors, *SlHA8*, the newly identified tomato HA gene, and two other orthologous genes, *OsA8/OsHA1* from rice and *MtHA1* from *Medicago*, belonging to subfamily V, showed an AM-specific expression profile (Figs. 4 and 5) (Krajinski et al. 2014; Wang et al. 2014). Arbuscules that developed from highly differentiated fungal hyphae in colonized cells are commonly considered to be the major sites for nutrients and signal exchange between host plants and AM fungi (Pumplin and Harrison 2009; Gutjahr and Parniske 2013). Multiple genes involved in transporting nutrients, mainly P and N, and signal molecules, such as strigolactones, have been shown to be specifically and/or strongly expressed in arbuscule-containing cells and play a key role in modulating AM symbiosis (Maeda et al. 2006; Chen et al. 2007; Javot et al. 2007; Kobae et al. 2010; Kretzschmar et al. 2012; Yang et al. 2012). The inactivation of *SlHA8* in nonmycorrhizal plants under normal growth condition, or nutrient/salt-stress conditions, but strong induction in arbuscule-containing cells of mycorrhizal roots well suggested its specialized role in energizing the secondary membrane transport processes at intraradical symbiotic interface and modulating AM symbiosis. Such hypothesis gained well support from the recent studies on its two orthologs, *MtHA1* and *OsA8* (also named as Os-HA1) from *Medicago* and rice (Krajinski et al. 2014; Wang et al. 2014). Mutation of the two AM-induced orthologs resulted in significant reduction in Pi uptake and impaired arbuscule development in the two mycorrhizal plant species.

Evolutionary conservation in regulatory mechanisms mediating the AM-activated HA expression in diverse plant species

Based on phylogenetic localization, it is tempting to contemplate the likely responsiveness of the members in subfamily V to AM symbiosis. Surprisingly, *SlHA8* with its two solanaceous orthologs, *StHA8* and *PMA8*, preferably group together with *OsA8* and *Arabidopsis AHA7*, but not *MtHA1* (Fig. 2). Since *Arabidopsis* is a nonmycorrhizal plant that is unable to form symbiosis with AM fungi, it could be presumed that functional divergence related to these orthologs would have occurred after the split of *Arabidopsis* from a common ancestor shared by other mycorrhizal plants (Santi and Schmidt 2009).

The similar expression patterns and relatively close phylogenetic relationships among *MtHA1*, *OsA8*, and *SlHA8* prompted us to investigate the possibility regarding the conservation in the regulatory pathway controlling AM-specific HA induction in different mycorrhizal plants. Previous studies on dissecting promoter activity of several AM-activated Pi transporter (PT) genes have evidenced that the promoters of potato *StPT3* and *Medicago MtPT4* were able to direct the AM-specific GUS expression in several dicot species, while the promoter of rice *OsPT11* showed no activity in driving GUS expression in the corresponding eudicots (Karandashov et al.

2004). The absence of a conserved MYCS element, which was proven to be a mandatory requirement for conferring the AM-induced expression of dicot PT genes, in the promoter region of *OsPT11*, led to the suggestion that the regulatory pathway associated with the AM-activated response of PT genes might be divergent between eudicots and monocots (Karandashov and Bucher 2005; Chen et al. 2011; Lota et al. 2013; Favre et al. 2014). The presence of GUS staining in not only tobacco and soybean but also rice mycorrhizal roots driven by *SlHA8* promoter (Fig. 6f, g) thus gave a novel proposal that a conserved regulatory mechanism involved in the induction of AM-specific HA genes might be shared by diverse eudicots and monocots.

Promoter deletion/truncation assay is a common and useful tool to identify *cis*-elements responsible for tissue-specific or development-dependent expression patterns. Tomato and tobacco are two closely related solanaceous species, and it has been repeatedly evidenced by our previous studies that tobacco, as its high transformation efficiency, could be used efficiently, instead of other solanaceous species, in conducting promoter activity analysis (Chen et al. 2011; Liao et al. 2015). Promoter deletion analysis in transgenic tobacco roots in this study revealed a 100-bp functional promoter region of *SlHA8* (−223 to −119) which might contain *cis*-element(s) essential for conferring the AM-specific response (Fig. 7). A variant of the AM-specific MYCS (vMYCS) motif present in the short promoter region led to the hypothesis that the vMYCS motif might be the suitable candidate responsible for the regulation of AM-specific HA genes in plants. The function of vMYCS in AM-induced expression was further evidenced by its deletion from the $pSlHA8_{-223}$ promoter fragment. Targeted deletion of this motif resulted in almost complete absence of GUS staining in mycorrhizal roots, indicating that vMYCS is indeed required for the activation of AM-specific HA expression. Since it has been well recognized that the function of HA is tightly coupled with the activation of a number of secondary transporters located in the plasma membrane, including the Pi transporters, it thus gave us another hint that the AM-specific HA genes and the AM-induced Pht1 transporter genes might be co-regulated by a conserved regulatory mechanism involving the MYCS (vMYCS) component.

In conclusion, in the present work, through genome-wide searches of available database, we identified a total of eight HA genes in tomato. Further analysis of these genes led to the identification of a member, *SlHA8*, strongly responsive to AM symbiosis. The AM-specific expression of the GUS reporter driven by *SlHA8* promoter in tobacco, soybean, and rice plants and the requirement of a variant of the AM-responsive MYCS element to confer its AM-mediated expression provided strong evidence towards the potentially highly conserved nature of a regulatory mechanism involved in the regulation of AM-specific HA and other AM-induced genes in diverse mycorrhizal plant species.

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Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare.

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