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Note

Possible contribution of TED6 and TED7, secondary cell wall-related membrane proteins, to evolution of tracheary element in angiosperm lineage

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Abstract The Tracheary Element Differentiation-Related6 (TED6) and TED7 membrane proteins function in the differentiation of xylem vessel elements, the cellular units for water conduction in angiosperm plants. Functional analysis of TED6 and TED7 had suggested that these proteins directly bind to a subunit of the secondary cell wall (SCW)-related cellulose synthase complex, to promote SCW formation in xylem vessel elements. However, whether TED6 and TED7 function in SCW formation of xylem vessel elements only, or function broadly in other cell types has remained unclear. To clarify this, we conducted detailed expression analysis of *TED6* and *TED7* genes in *Arabidopsis thaliana*. This showed that *TED6* and *TED7* are expressed in differentiating vessel elements of all organs examined here, including roots, leaves, and inflorescence stems. We detected no *TED6* and *TED7* promoter activity in other types of cells with SCW thickening, such as fiber cells and anther endothecium, indicating that TED6 and TED7 have specific roles in SCW formation of vessel elements. Homology searches identified TED6/7-like proteins only in the angiosperm lineage. These data suggest that development of TED6/7 proteins could have coincided with the emergence of the angiosperm lineage, and that TED6/7 may have made key contributions to the evolution of water-conducting cells from tracheids to vessels.

Key words: Angiosperm, secondary cell wall, TED6, TED7, xylem vessel element.

Development of water-conducting cells greatly advanced the ability of plants to colonize the land during early plant evolution. Land plants have several kinds of water-conducting systems, such as hydroids, tracheids, and vessels, which all consist of dead cells (Sperry, 2003). Tracheids and/or vessels, which conduct water in vascular plants, are composed of cellular units called tracheary elements. Hydroid cells are found in certain species of bryophytes, which are non-vascular plants, and show large variation in terms of cell size and cell wall modification (Ligrone et al. 2000). Some moss hydroids possess thickened cell walls, but they do not form secondary cell wall (SCW) (Ligrone et al. 2002). By contrast to hydroid cells, tracheary elements deposit lignified and patterned SCW (Myburg and Sederoff 2001; Sperry, 2003). In tracheids, the tracheary elements are drastically elongated, single cells with tapered or pointed ends. Gaps of SCW, called pits, form in the walls of the tracheary elements, allowing the tracheid cells to conduct water.

Xylem vessel elements, another type of tracheary element, are constituents of xylem vessels and occur mainly in angiosperm plants. These vessel elements are shorter and wider than tracheid cells, and have specific perforated ends that align end-to-end to form connective pipes. These features confer greatly improved hydraulic efficiency to vessel pipes through their increased diameter and length (ca. 500 μm and $>$10 m, respectively) compared with single-celled tracheids (Myburg and Sederoff 2001; Sperry, 2003). Molecular phylogenetic analysis and anatomical analysis of tracheids in early fossil tracheophytes showed a single origin of tracheids (Qiu et al. 1998; Kenrick 2000), although large variation in vessel elements suggested that evolution of vessel elements (i.e. the conversion of tapered/pointed ends to a perforated plate) likely had polyphyletic origins (Myburg and Sederoff 2001). Based on developmental and comparative works, the SCW

Abbreviations: GUS, β-glucuronidase; SCW, secondary cell wall; TED6, Tracheary Element Differentiation-Related6; TED7, Tracheary Element Differentiation-Related7.

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properties has been considered as one of the big gaps between tracheids and vessel elements (Friedman and Cook 2000; Kishi et al. 1979).

The molecular mechanisms of differentiation of water-conducting systems have been studied using model plant systems such as *Zinnia elegans* (Zinnia), *Arabidopsis thaliana* (Arabidopsis), *Populus trichocarpa*, and *Physcomitrella patens*, resulting in successful identification of many regulatory factors for cellular differentiation (Demura and Fukuda 2007; Demura et al. 2002; Devillard and Walter 2014; Escamez and Tuominen 2014; Nakano et al. 2015; Ohtani et al. 2011; Schuetz et al. 2013; Turner et al. 2007; Xu et al. 2014). Two membrane protein-coding genes, *Tracheary Element Differentiation-Related6* (*TED6*) and *TED7* were originally found to be upregulated during vessel element in cultured Zinnia cells (Demura et al. 2002; Endo et al. 2009). TED6 and TED7 share the transmembrane domain and C-terminal domain, although Pro-rich N-terminal region is only found in TED7. Artificial knock-down of *TED6* and *TED7* affected the SCW formation during vessel element differentiation in Zinnia and Arabidopsis, clearly indicating that these proteins have critical roles in SCW formation. Importantly, TED6 can directly interact with the IRREGULAR XYLEM3 (IRX3)/CELLULOSE SYNTHASE A7 (CESA7) protein, a subunit of the SCW-related cellulose synthase (CesA) complex. These results indicate that TED6 and TED7 regulate SCW formation through interaction with the CesA complex during vessel element differentiation (Endo et al. 2009). However, several critical issues related to molecular functions of TED6/7 remain unclear, in particular, whether TED6 and TED7 function in SCW formation of xylem vessel elements only or other cell types broadly. Recent advances in the analysis of transcriptional regulatory networks for SCW formation revealed that many SCW-related factors commonly participate in the differentiation of both vessel elements and xylem fibers, which are SCW-forming supporting cells found in the xylem of hardwood plants (Nakano et al. 2015).

Here, we performed detailed expression analysis of *TED6* and *TED7* using Arabidopsis plants harboring chimeric reporter genes containing 1- and 0.5-kb upstream promoter sequence of *TED6* and *TED7*, respectively, fused with the *β*-glucuronidase (*GUS*) reporter gene (Endo et al. 2009). Previous work showed that *TED6* and *TED7* are specifically expressed in the differentiating vessel elements in roots of young Arabidopsis seedlings (Endo et al. 2009). For further expression analysis, homozygous reporter lines were established and aseptically grown on Murashige-Skoog medium for 14 days, and then transferred to soil. The 7-d-old and 40-d-old plants were sampled, fixed with 90% (w/v) acetone, and subjected to GUS assay according to Pyo et al. (2004). The sample incubation in the GUS substrate solution was performed for 24 h, except in the case of inflorescence stem (for 48 h), at 37°C, and after a rinse with phosphate buffer, the samples were mounted with a few drops of 8 : 1 : 2 (w/v/v) chloral hydrate, glycerin, and water. Observations were made under a microscope equipped with Nomarski optics and a digital camera (BX51 and DP72; Olympus, Tokyo, Japan). In the 7-d-old seedlings, strong GUS signals were detected in developing xylem vessel elements of hypocotyls, leaves, and roots (Figure 1A–F). As described in Endo et al. (2009), the GUS signals were apparent both in protoxylem and metaxylem-types of vessel elements, but not in the differentiated vessel cells, indicating that *TED6* and *TED7* are expressed in differentiating vessel elements to regulate SCW formation of vessel elements (Endo et al. 2009).

In Arabidopsis, SCW formation occurs not only in xylem vessel elements, but also in several non–vascular cells, such as fiber cells in inflorescence stem, endothecium cells in anther, and valve cells in the silique (Dardick and Callahan 2014; Nakano et al. 2015; Wilson et al. 2011). To check if TED6 and TED7 are involved in SCW formation of anther and silique cells, the promoter activities of *TED6* and *TED7* were tested in the reporter lines at the reproductive stages. The GUS activities were also apparent in vessel elements in the young reproductive organs, but not detected in the anther endothecium cells or the valve cells of the silique (Figure 1G–J). In addition, cross sections of inflorescence stems revealed that *TED6* and *TED7* are expressed in the differentiating vessel elements but not in the differentiating interfascicular fibers (Figure 1K–L). *TED7* promoter activity appeared to be relatively weak in the middle part of inflorescence stems compared with the top part (Figure 1M, N). This observation was supported by semi-quantitative RT-PCR analysis of *TED6* and *TED7* expression in inflorescence stems (Figure 1O). Thus, TED7 appears to preferentially function in vessel element differentiation at the early stages of inflorescence stem development. These findings suggest that TED6 and TED7 are involved only in SCW formation during vessel element differentiation, but not in the differentiation of other types of cells with SCW thickening.

Based on the xylem vessel element-specific expression of *TED6* and *TED7*, we conducted in silico searches for TED6/7 homologs in a wide range of plant species from angiosperm, gymnosperm, fern, moss, and algae, to obtain information on the contribution of TED6/7 to the evolution of plant water-conducting cells. We conducted BLAST and PSI-BLAST searches of multiple public databases, including NCBI (http://www.ncbi.nlm.nih.gov), Phytozome version 9 and 10.2 (http://phytozome.jgi.doe.gov), and the Dendrome database (http://dendrome.ucdavis.edu/resources/blast), using the amino acid sequences of Arabidopsis TED6 and TED7 proteins

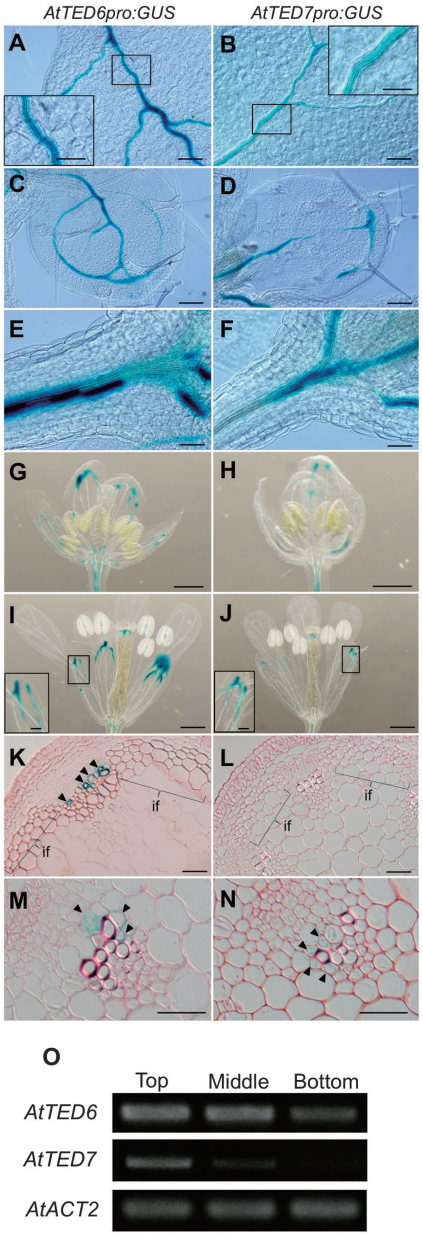

Figure 1. Expression of *TED6* and *TED7* in Arabidopsis plants. (A–F) GUS signals detected in 7-d-old Arabidopsis seedlings carrying *AtTED6pro:GUS* (A, C, E) and *AtTED7pro:GUS* (B, D, F). The promoter activities were found in differentiating xylem vessels in cotyledons (A, B), young leaves (C, D), and hypocotyls (E, F). (G–N) GUS signals detected in 40-d-old Arabidopsis plants carrying *AtTED6pro:GUS* (G, I, K, M) and *AtTED7pro:GUS* (H, J, L, N). GUS expression patterns in developing flowers (G, H), opened flowers (I, J) and inflorescence stems (K–N). The promoter activities were found in the vasculature region of floral organs (G–J). In the middle (K, L) and top (M, N) parts of inflorescence stems, the GUS signals were apparent in xylem vessels (arrowheads) but not in fiber cells (indicated by "if" in K and L). (O) Semi-quantitative RT-PCR analysis of *AtTED6* and *AtTED7*. Total RNA was isolated from the top, middle, and bottom regions of inflorescence stems of 5-week-old Arabidopsis wild-type plants, and subjected to semi-quantitative RT-PCR analysis using the primer sets described in Endo et al. (2009). *AtACT2* was examined as an internal control. PCR was performed for 31 cycles (*AtTED6* and *AtTED7*) and for 33 cycles (*ACT2*). Bar=100 μm (A–D), 50 μm (E, F, K, L), 200 μm (G, H), 500 μm (I, J) and 20 μm (M, N). Insets represent magnified images; scale bar=50 μm (A, B), and 100 μm (I, J).

as queries. Manually curated alignment of candidate sequences based on the transmembrane domain and the C-terminal domain conserved between TED6 and TED7 yielded proteins with high sequence similarity to TED6/7 (Figure 2; Supplemental Table 1; Supplemental Figure 1). It is notable that genes homologous to *TED6/7* occur in all the examined angiosperm species, but not in the examined gymnosperm species (*Picea abies, Picea glauca*, and *Pinus taeda*), fern (*Selaginella moellendorffii*), moss (*Physcomitrella patens*), and green algae (*Chlamydomonas reinhardtii*, and *Volvox carteri*) (Figure 2; Supplemental Table 1), indicating that the *TED6/7* developed exclusively within the angiosperm lineage, as implied in the report from the Amborella Genome Project (2013). Figure 2 also shows that the TED7-type protein structure, i.e. a protein containing a Pro-rich N-terminal region, could be the original form of the TED6/7-related proteins. Additionally, we observed multiplexing of the conserved C-terminal domain in several species, as well as length variation in the Pro-rich N-terminal region.

Our results also identified a *TED7* homolog in *Amborella trichopoda*, a woody plant phylogenetically located at the basal part of the flowering plant lineage (Bailey and Swamy 1948; Qiu et al. 1999). The distinct feature of this plant is its lack of xylem vessels; xylem tissues of *A. trichopoda* are composed of tracheid cells (Bailey and Swamy 1948; Feild et al., 2000). Feild et al. (2000) demonstrated that *A. trichopoda* has a typical tracheid-bearing system, based on morphological and functional analysis of *A. trichopoda* water-conducting systems. However, they also showed that pit membranes are sometimes absent in *A. trichopoda* tracheids, and further hypothesized the capacity to hydrolyze pit membranes in *A. trichopoda* (Feild et al. 2000). Comparative analysis of the *A. trichopoda* genome revealed many angiosperm lineage-specific gene families possibly involved in cell wall biosynthesis and/or modification (Amborella Genome Project 2013); therefore, acquisition and diversification of cell wall-related proteins, including TED6/7 and other cell wall-related enzymes, could be one of the major events leading to the transition from tracheids to vessel elements.

The differentiation of the SCW-forming cells is regulated by specific NAC transcription factors, including VASCULAR-RELATED NAC-DOMAIN (VND), SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN1, and NAC SECONDARY WALL THICKENING PROMOTING FACTOR (NST) of Arabidopsis (Kubo et al. 2005; Mitsuda et al. 2005, 2007, 2008; Zhong et al. 2006). This NAC protein family is called the VNS family based on evolutionary conservation among land plant species (Nakano et al. 2015; Ohtani et al. 2011; Xu et al. 2014). Functional analysis of *Physcomitrella patens* VNS proteins showed

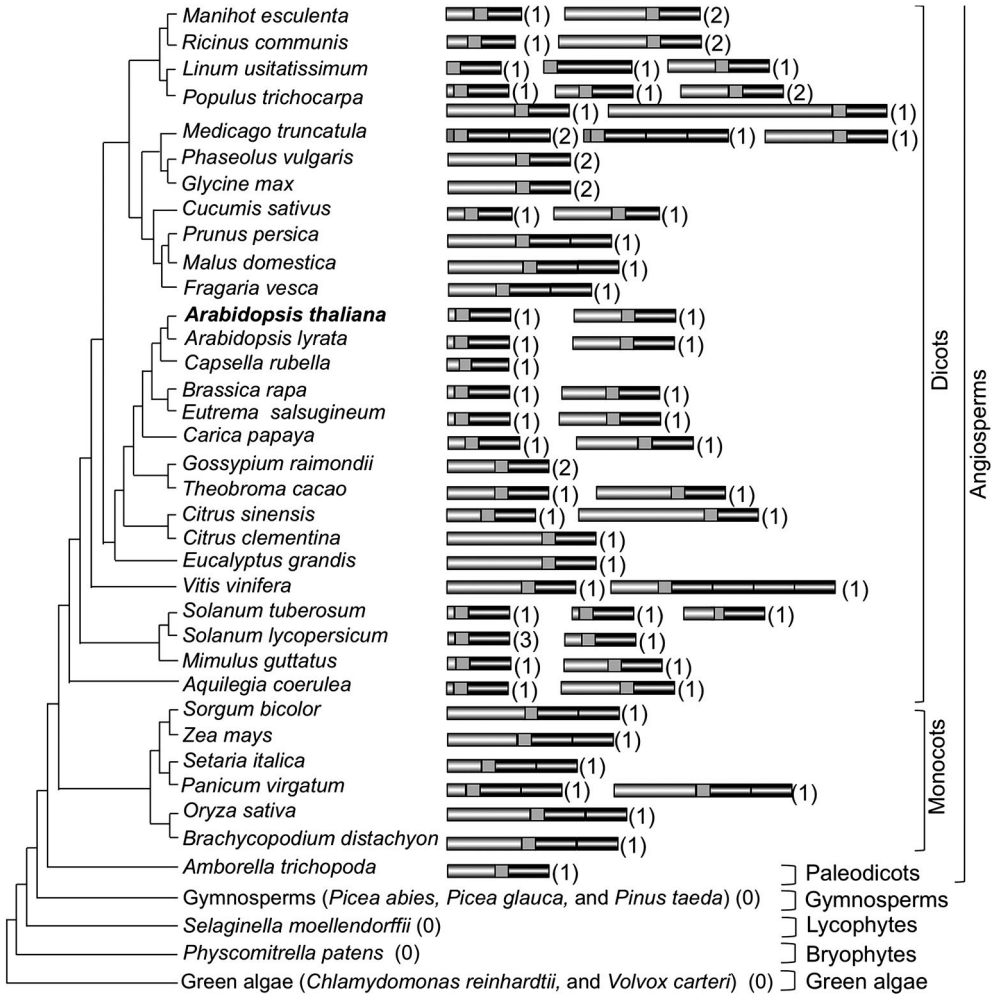

Figure 2. TED6 and TED7 homologs found in the angiosperm lineage. Schematic drawings of homologous proteins to TED6/7 found in the examined 39 plant species. Phylogenetic information is obtained from Phytozome (http://phytozome.jgi.doe.gov). Boxes indicate putative structures of TED6/7 homolog proteins; green box, Pro-rich N-terminal domain specific to TED7; grey box, N-terminus without Pro-rich domain; yellow box, transmembrane domain; blue box, conserved C-domain (Supplemental Figure 1). Numbers in brackets represent the number of TED6/7 homologs in each species.

that VNS-based transcriptional regulation for the differentiation of water-conducting cells had been already established in the last common ancestor of moss and vascular plants (Xu et al. 2014), strongly suggesting that evolution of water-conducting systems largely depended on diversification of events downstream of VNS regulation, such as SCW biosynthesis. This view is consistent with the idea that the diversification of cell wall-related genes would contribute to the vessel evolution described above. Development of vessel elements from tracheids is recognized as one of the key innovations during land plant evolution (Broadribb and Feild, 2010). Further functional analysis of TED6 and TED7 in SCW formation will give us additional clues as to the molecular basis of tracheary element evolution.

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