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A NAC Domain Transcription Factor, XVP, Regulates Vascular patterning, Stem Cell Proliferation and Xylem Differentiation in Arabidopsis

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A NAC Domain Transcription Factor, XVP, Regulates Vascular patterning,
Stem Cell Proliferation and Xylem Differentiation in *Arabidopsis*

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B.S., The University of Utah, [2011]

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at The

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APPOVAL PAGE

Master of Science Thesis

A NAC Domain Transcription Factor, XVP, Regulates Vascular patterning, Stem Cell
Proliferation and Xylem Differentiation in *Arabidopsis*

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Abstract

Vascular stem cells proliferate and differentiate into vascular tissues, which transport water and nutrients throughout the plant and form the majority of terrestrial biomass. Vascular stem cell maintenance is regulated by small peptide Tracheary Element Differentiation Inhibitory Factor (TDIF), but the mechanism is still elusive. We report the identification of a novel NAC domain transcription factor *XVP* in regulating xylem differentiation and vascular patterning, two well-known functions of TDIF signal. Overexpression of *XVP* resulted in the disruption of vascular patterning, precocious xylem differentiation and reduced cell numbers in vascular bundles. In accordance with its function in vascular meristem, *XVP* was expressed in the vasculatures of leaf, root, and reproductive tissues. In the stem, the expression of *XVP* was specifically detected in phloem and cambium/developing xylem cells. The *XVP* showed activation activity in yeast. Investigation of subcellular localization of *XVP* in *N.benthamiana* indicated that *XVP* is localized on plasma membrane, but may be relocated to nucleus with C-terminal region being truncated. The *XVP* activated the TDIF coding gene *CLE44*. On the other hand, overexpression of the *CLE41/44* inhibited *XVP* expression, indicating that *XVP* participates in a positive feedback regulation of TDIF signaling. The expression of *VND6*, a master regulator of xylem differentiation, is activated by *XVP*. The precocious xylem differentiation is recovered in the double mutant *xvp-d vnd6* compared to *xvp-d*. This result indicated that *XVP* is epistatic to *VND6*. This study demonstrates that *XVP* is a key regulator of stem cell proliferation, xylem differentiation and vascular patterning through small peptide TDIF signaling.

Introduction

Vascular tissue provides physical strength, and transports water, nutrients and other growth substances vital for plant growth. Plant vascular tissues are composed of xylem, phloem and cambial cells. The proliferation of stem cells in the vascular meristem produces progeny cells, which either maintain their stem cell property or differentiate into xylem toward the center and phloem toward the periphery of plant stems (Miyashima et al. 2013). During cambial differentiation process, xylem fibers and tracheary elements (TEs), including vessels and tracheids, develop secondary cell walls (SCW) (Ohashi-Ito and Fukuda 2010; Schuetz, Smith, and Ellis 2013). The development of TEs and xylary fibers undergoes a programmed cell death (PCD) process. The SCW contains the majority of lignocellulosic biomass that serves as a renewable resource for biofuel production (Carroll and Somerville 2009; Pauly and Keegstra 2010; Carpita 2012).

The current paradigm of stem cell maintenance is derived from studies in apical meristems; shoot apical meristem (SAM) and root apical meristem (RAM). In the SAM and RAM, stem cell populations are tightly maintained by balancing cell proliferation and differentiation through signaling transduction or transcriptional regulation with adjacent niche cells (Fletcher et al. 1999; Sarkar et al. 2007; Schoof et al. 2000). A key mechanism of stem cell maintenance involves the interaction between small mobile peptides and their receptors, Leucine-Rich Repeat Receptor-Like Kinase (LRR-RLK). In the SAM, dodecapeptide CLV3 diffuses from the dividing stem cells into the underlying layer of niche cells, where it interacts with CLV1, a member of LRR-RLK, or a receptor complex composed of CLV2 and CORYNE, a membrane localized Serine/Threonine kinase (Fletcher et al. 1999; Jeong, Trotochaud, and Clark 1999; Muller, Bleckmann, and Simon 2008; Ogawa et al. 2008). The receptor-ligand interaction transduces signals that repress the

expression of *WUSCHEL* (*WUS*), a homeobox transcription factor that specifies niche cells and maintains the stem cell population through cell-to-cell movement (Mayer et al. 1998; Schoof et al. 2000; Yadav et al. 2011a). A similar regulatory mechanism in the SAM was also identified in the RAM. This signaling pathway also comprises a small peptide ligand, CLE40 and an LRR-RLK protein ACR4 (ARABIDOPSIS CRINKLY4) (De Smet et al. 2008) and WOX5 (WUSCHEL RELATED HOMEBOX5) acts in the quiescent center to maintain the stem cell pool while the CLE40-ACR4 ligand-receptor complex restricts its function and triggers distal stem cell differentiation (Meyer et al. 2015). The paradigm of plant stem cell maintenance involves a feedback loop that comprises a small peptide CLE, LRR-RLKs and a WOX transcription factor.

Although substantial progress has been made in understanding stem cell functions in apical meristems, our knowledge about the maintenance of vascular stem cells is still very limited (Sablowski 2011). In a similar way to the SAM and RAM, the interaction between CLEs and LRR-RLKs controls proliferation of vascular cambium in vascular tissue. In *Arabidopsis*, *CLE41* and *CLE44* encode the dodeca-peptide Tracheary Element Differentiation Inhibition Factor (TDIF), which activity was originally identified from a *Zinnia* cell culture system. Consistent with the finding in *Zinnia*, overexpressing *CLE41/44* in *Arabidopsis* inhibited the differentiation of TEs and resulted in the discontinuous xylem strand formation (Ito et al. 2006a). Furthermore, TDIF promoted the division of vascular stem cells in the hypocotyl (Hirakawa et al. 2008; Whitford et al. 2008). The receptor of TDIF is a LRR-RLK, named as TDR or PXY. Mutation of *PXY* resulted in an aberrant organization of xylem and phloem in the stem (Fisher and Turner 2007). In the absence of TDR/PXY, plants became insensitive to high levels of TDIF. Therefore, the TDIF-TDR signaling has three functions: to act as a positive signal for vascular stem cell proliferation, to act as a negative signal for xylem differentiation and to act as a positional signal for vascular

organization. Similar to CLV3-CLV1 regulation in the SAM, *CLE41/44* and *TDR/PXY* are expressed in different cell types. *CLE41* and *CLE44* are produced in the phloem and diffuse into vascular cambial cells where *TDR/PXY* is expressed (Etchells and Turner 2010). The TDIF-TDR signal induces the expression of *WOX4* in *Arabidopsis*. *WOX14* may function redundantly with *WOX4* in TDIF-TDR signaling (Hirakawa, Kondo, and Fukuda 2010; Ji et al. 2010; Suer et al. 2011). This positive regulation is opposite to what the CLV3-CLV1 complex does to *WUS*. Further investigation suggested that *WOX4* and *WOX14* have no function in the differentiation of tracheary elements in response to TDIF (Etchells et al. 2013; Suer et al. 2011). Three other LRR-RLKs, *PXC1* (*PXY-CORRELATED1*), *MOL1* (*MORE LATERAL GROWTH1*) and *RUL1* (*REDUCED IN LATERAL GROWTH1*) were shown to be involved in regulating cambium activity (Agusti et al. 2011a; Wang et al. 2013).

Recently, a new molecular mechanism was revealed that Brassinosteroid (BR) signaling is involved in TDIF-TDR signaling in repressing xylem differentiation (Kondo et al. 2014). *BIN2* (*BRASSINOSTEROID-INSENSITIVE 2*), a member GSK3 (*GLYCOGEN SYNTHASE KINASE 3*) protein, was identified as an interacting partner of *TDR/PXY* in a yeast two-hybrid screening. TDIF association with *TDR/PXY* at the plasma membrane activates *BIN2* in turn suppresses the expression of *BES1* (*BRI-EMS-SUPPRESSOR1*). *BES1* is one of the *BIN2* downstream TFs that positively regulate xylem cell differentiation. Overall, TDIF binding to its receptor *TDR/PXY* disassociates *BIN2* from the complex, suppresses the function of *BES1*, and subsequently inhibits xylem formation (Kondo et al. 2014; Li and Nam 2002; Yin et al. 2002). Therefore, the TDIF-TDR signaling participates in regulating vascular cell division orientation, cambial cell proliferation and xylem differentiation. In spite of the importance of the

vascular meristem to plant development and wood formation, the regulation of the TDIF-TDR signal on vascular patterning and xylem differentiation is still largely unknown.

Xylem differentiation and secondary cell wall development are under transcriptional regulation. Transcription factors in the transcriptional network often have orthologs in tree species suggesting conserved functions. The NAC domain transcription factors VND6 (VASCULAR-RELATED NAC-DOMAIN6) and VND7 play essential roles in xylem vessel differentiation in both *Arabidopsis* and *Populus* (Kubo et al. 2005; Yamaguchi et al. 2010b). In *Arabidopsis*, VND6 promotes the differentiation of metaxylem and VND7 induces the developmental fate of protoxylem. Transcriptional profiling of transgenic plants ectopically expressing either *VND6* or *VND7* gene revealed that a wide array of genes is differentially expressed (Yamaguchi et al. 2010a; Yamaguchi et al. 2010b). Both *VND6* and *VND7* activate the expression of a broad range of genes involved in programmed cell death (PCD), such as *XCPI* (*XYLEM-SPECIFIC PAPAIN-LIKE CYSTEINE PEPTIDASE*) (Funk et al. 2002). Other VND family members, *VND1* to *VND5*, function redundantly with *VND6* and *VND7* in vessel development (Zhou, Zhong, and Ye 2014).

Another group of NAC transcription factors, NST1 (NAC SECONDARY WALL THICKENING PROMOTING FACTOR 1), NST2 and NST3/SND1 (SECONDARY WALL ASSOCIATED NAC DOMAIN PROTEIN1), function as master regulators for the differentiation of interfascicular and xylary fibers, and secondary cell wall deposition (Demura and Ye 2010; Zhong, Demura, and Ye 2006; Zhong et al. 2008). The *nst1 nst3* double mutant disturbs SCW formation in the vascular and interfascicular fibers, meanwhile, ectopic expression of *NST1* and *NST3* leads thicker SCW deposition (Mitsuda et al. 2007; Zhong, Demura, and Ye 2006). These NAC domain master regulators activate the entire transcriptional network through their immediate downstream MYB transcription factors, MYB46 and MYB83 (Ko, Kim, and Han 2009; Zhong,

Richardson, and Ye 2007). Numerous other transcription factors were identified to function downstream of the NAC and MYB domain master regulators. The transcriptional cascade ensures the biosynthesis of cellulose, hemicellulose and lignin in xylem vessels and fibers. It is still unclear whether the transcriptional cascade has a role in balancing vascular stem cell proliferation with xylem differentiation.

Here, I report the identification of the novel transcription factor, XVP (precocious xylem differentiation and altered vascular patterning), to function in TDIF signaling and to control xylem differentiation and vascular organization in *Arabidopsis*. The gain-of-function *xvp-d* mutant showed a phenotype of disorganized vascular tissues, prematurely differentiated xylem cells and reduced cell numbers per vascular bundle. The expression of the *XVP* gene was highly upregulated in the *xvp-d* mutant. Promoter reporter analyses suggest that *XVP* is specifically expressed in the phloem and cambium/developing xylem cells. Further analysis showed that expression of *XVP* is regulated by TDIF, and *CLE44* expression is positively regulated by XVP. The functional characterization of XVP will enhance our understanding of TDIF-TDR signaling and will shed light on the regulation of vascular stem cell maintenance.

Result

Identification of the *xvp-d* mutant with defects in vascular development

Forward genetics is a powerful tool to identify specific gene function in plant development. Following the forward genetic screening of activation tagged mutant population, we isolated a dominant mutant that showed precocious xylem differentiation and altered vascular patterning, and we named the mutant as *xvp-d*.

To better understand the *xvp-d* phenotypes, we characterized the *xvp-d* mutant with detailed histochemical analysis. Stem cross sections from the control and mutant plants were observed after toluidine blue, phloroglucinol, and aniline blue staining. In wild type plants, cambium cells (black arrowheads) were located between phloem (phl) and xylem cells (xyl) (Figure 1A). The periclinal division of the cambium cells and subsequent differentiation formed well-separated and well-organized vascular tissues in wild type plants. In the *xvp-d* mutant plants, xylem and phloem cells were intercalated, and the division orientation of the cambium cell is disturbed (Figure 1B to 1D). The intercalated mutant phenotype was especially evident through the aniline blue staining, which stains sieve cells of phloem to a bright blue-green color under the UV light (Figure 1I and 1J). Using phloroglucinol staining, we could detect prematurely differentiated xylem cells that are small and lignified in the *xvp-d* stem sections (Figure 1E to 1G). Although the heterozygous plants (*xvp-d/+*) also showed some characteristic abnormalities, homozygous plants (*xvp-d*) had much stronger phenotypes (Figure 1).

The *Arabidopsis* hypocotyl has an anatomic organization that is comparable to the stem of tree species, and undergoes significant secondary growth. Because xylem development was affected in the stem tissues of *xvp-d*, we reasoned that hypocotyl development may have also been affected. As shown in Figure 2, the mutant plants showed a much severely disorganized vascular

organization in hypocotyls compared to the stem tissues. We found that the phloem tissues were interspersed with xylem cells in the heterozygous plants, and the disorganized tissue developed almost to the center of the hypocotyls in the homozygous plants (Figure 2C and 2F). Furthermore, the division of cambium cells in the mutant plants was no longer in the periclinal orientation as that of the wild type (Figure 2A and 2B, black arrowheads). The phenotypes of *xvp-d* resemble those observed in mutant lines of vascular stem cell maintenance regulators, such as the *tdr/pxy* null mutants and transgenic plants with disrupted TDIF signaling (Fisher and Turner 2007). These results indicated that *XVP* may function directly or indirectly as a negative regulator of TDIF signaling in vascular patterning and xylem differentiation.

The vascular structure and organization are well-defined in root tissues. To determine whether root vascular development was affected in the *xvp-d* mutants, we further analyzed the root growth and root vascular organization. We found that the root growth was normal in *xvp-d* mutant seedlings. Both root length and lateral root numbers were comparable to that of wild type (Figure 3A to 3C). We also found that the root vasculature was normally developed in the *xvp-d* mutants. The xylem, phloem, and cambium cells showed no defects in development in the stele region (Figure 3D and 3E). The metaxylem and protoxylem cells were also well developed and well organized as observed in the cleared root tissues in the *xvp-d* mutants (Figure 3F and 3G). Unlike the vascular tissues in stem and hypocotyl, the leaf venation development also showed no significant defects in the *xvp-d* mutant compared to those of wild type (Figure 4).

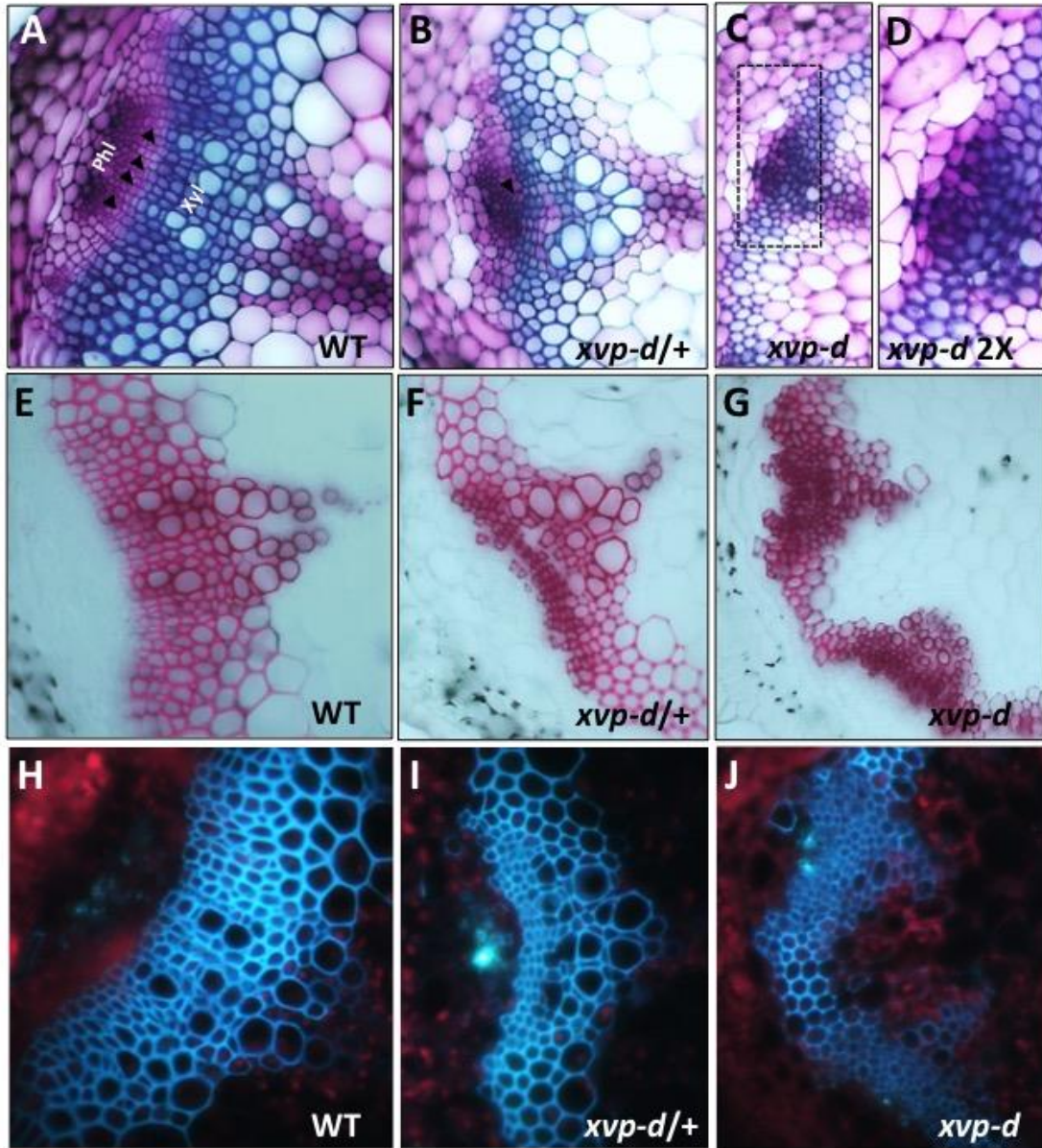


Figure 1. Xylem differentiation and vascular patterning was affected in the *xvp-d* mutants. (A) to (D) Toluidine blue staining of stem sections of wild-type (A), *xvp-d* heterozygous plants (B) and *xvp-d* homozygous plants (C) and (D).

(E) to (G) Phloroglucinol staining of stem sections of wild-type (E), *xvp-d* heterozygous plants (F) and *xvp-d* homozygous plants (G).

(H) to (J) Aniline blue staining of stem sections of wild-type (H), *xvp-d* heterozygous plants (I) and *xvp-d* homozygous plants (J).

▲ Black arrowheads indicated cambium cells.

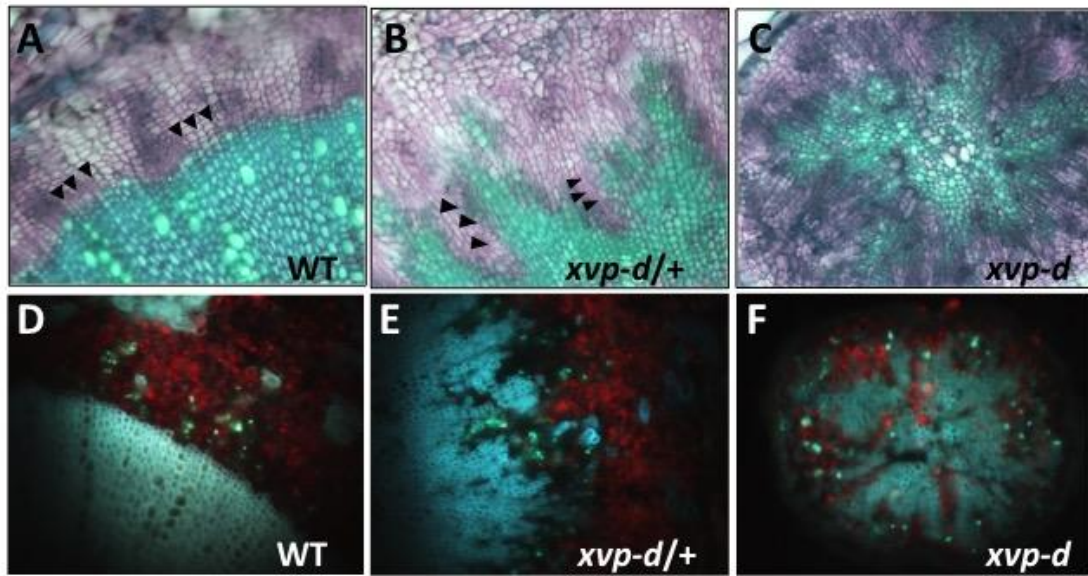


Figure 2. Xylem and phloem cells are interspersed in *xvp-d* hypocotyls. (A) to (C) Toluidine blue staining in wild-type (A), *xvp-d* heterozygous plants (B) and *xvp-d* homozygous plants (C). (D) to (F) Aniline blue staining in wild-type (D), *xvp-d* heterozygous plants (E) and *xvp-d* homozygous plants (F). ▲ Black arrowheads indicated cambium cells.

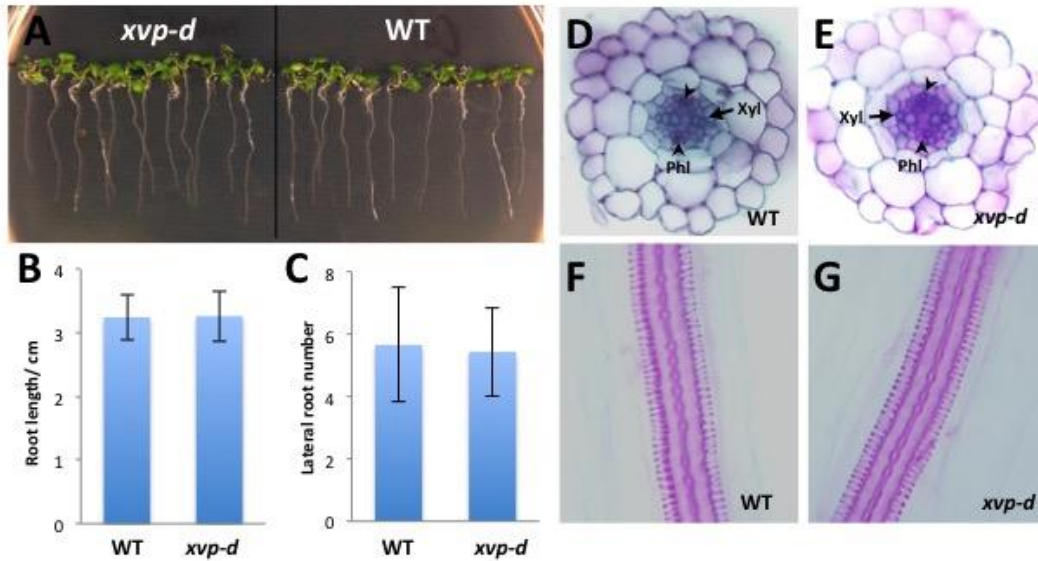


Figure 3. The development of root and lateral root in the *xvp-d* mutants.
 (A) The growth of 7-day old seedlings of wild type and *xvp-d* mutant on 1/2MS plate.
 (B) Measurement of root length in the wild type and *xvp-d* mutant seedlings.
 (C) Lateral root number in wild type and the *xvp-d* mutant seedlings.
 (D) and (E) Cross sections of root showing vascular tissues organization in wild type (D) and the *xvp-d* mutants (E).
 (F) and (G) xylem development and organization in root.
 Error bar indicated mean \pm SE (n=10) in each genotype.

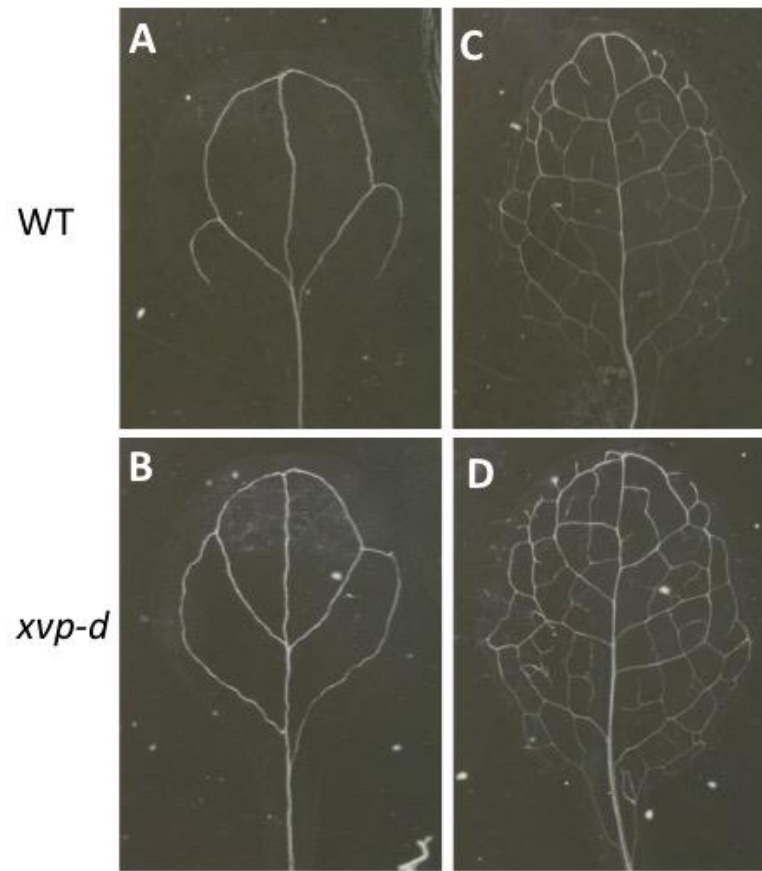


Figure 4. The venation development in cotyledons and leaves
 (A) and (B) Venation of the cotyledons in wild type (A) and *xvp-d* mutant.
 (C) and (D) Venation of the leaves in wild type (C) and *xvp-d* mutant.

The *xvp-d* mutant showed a dwarf phenotype

In the vegetative growth stage, mutant plants developed smaller rosette leaves in three weeks old plant compared to wild type. Also, leave edges were curved down irregularly as compared to wild type. Overall, homozygous, *xvp-d*, developed much smaller and narrower leaves than heterozygotes. *xvp-d/+* (Figure 5A and 5B).

The inflorescent stem of six weeks old mutant plants was shorter than the wild type. According to our measurement, the main stems of *xvp-d/+* were 25% shorter than the wild type, and *xvp-d* were 75% shorter than the wild type (Figure 5C and 5D). However, the number of branches in the mutant was not different from wild type (Figure 5E). Also, the development of flower and siliques were not significantly affected in *xvp-d* mutant (Figure 5C). These results indicated that the dwarf plant phenotype in *xvp-d* mutant might be caused by abnormal vascular development.

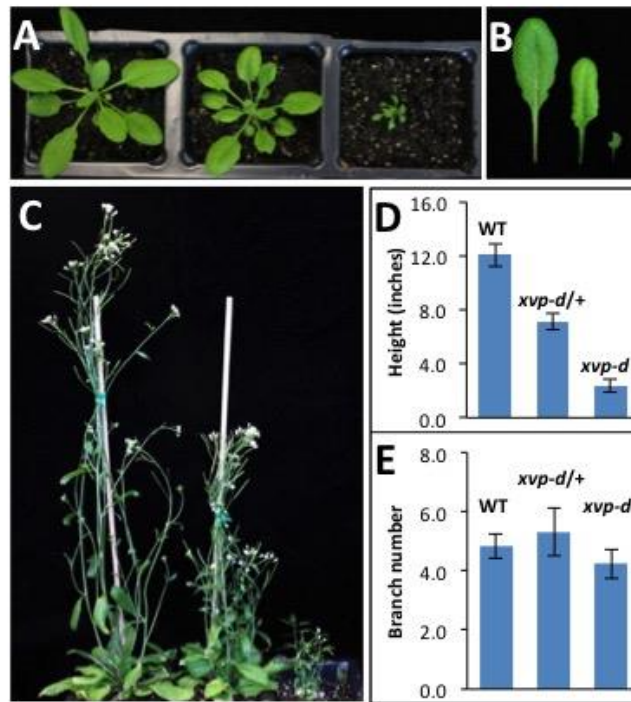


Figure 5. Plant growth phenotypes of the *xvp-d* mutant

(A) Morphology of three-week old plants of WT (left), Het (middle) and Hom (right).

(B) Representative fully expanded the sixth leaves of WT (left), Het (middle) and Hom (right).

(C) Morphology six-week old plants. From left to right, the plants are WT (left), Het (middle) and Hom (right).

(D) Plant height measurement for main stem height in each genotype.

(E) Number of branches in six-weeks old plants in each genotype.

Error bar indicated mean \pm SE (n=10) in each genotype.

The *xvp-d* phenotype is caused by overexpression of *NAC003* gene

The *xvp-d* mutant was identified from an activation tagged population and showed semi-dominant phenotypes, indicating that the mutant phenotype may be resulted from activation tagging of a regulator of vascular development. To identify the gene(s) that was responsible for the *xvp-d* phenotype, we determined the T-DNA insertion locus in the mutant genome using thermal asymmetric interlaced PCR (TAIL-PCR) and subsequent sequencing of the amplified DNA fragment (Liu et al. 1995). We identified a single T-DNA insertion that is located 3.6 kb upstream of *At1G02220* (*NAC003*) and 969 bp downstream of *At1G02230* (*NAC004*) (Figure 6A). To determine whether the T-DNA insertion was correlated with the mutant phenotype, we genotyped a segregating T3 population (103 plants) using a PCR based analysis and identified 25 plants of wild type, 52 plants of *xvp-d*/+ and 26 plants of *xvp-d*. Thus, the single T-DNA insertion was identified by 3:1 ratio of segregation (Figure 6B).

Using qRT-PCR (Quantitative real-time PCR) analyses, we determined the expression of genes that located in a range of 10 kb from the T-DNA insertion site. A strong activation of the *NAC003* expression was detected in the mutant lines, with about 100 times overexpression in the heterozygous plants and 220 times in the homozygous plants (Figure 6C). The expressions of *NAC004* and *NAC005* were also activated compared to that of the wild type, but to a much lower level than *NAC003* gene (Figure 6D and 6E). There is a predicted pseudogene (*At2g02228*) located 385bp from the right border of the T-DNA insertion, but we did not detect its expression in both wild type and the *xvp-d* mutant plants. Because the *NAC003* gene was the most overexpressed among the three activated genes in *xvp-d* mutant, we first focused our functional analysis on *NAC003*.

We performed both overexpression (*OX*) and knockdown (*KD*) transgenic experiments to determine the function of *NAC003* gene. First, we overexpressed *NAC003* in the wild type background driven by the Cauliflower Mosaic Virus (CaMV) 35S promoter. The plant growth of 10 out of the 36 primary transgenic plants was affected. We choose two of the *OX* transgenic lines (*OX5* and *OX11*) for further characterization (Figure 7A). The transgenic plants showed precocious xylem differentiation and intercalated phloem and xylem, similar to the *xvp-d* mutant (Figure 7B). In consistent with the observed vascular development defects, the expression of *NAC003* was highly increased as determined by qRT-PCR analysis (Figure 7C and 7D). The transgenic plants were dwarf and had small curved leaves like the *xvp-d* mutants (Figure 7A). Secondly, we knocked down the expression of *NAC003* in the *xvp-d* mutant background using a microRNA construct (Schwab et al. 2006). The designed microRNA was specific to the target and silenced the *NAC003* transcripts post transcriptionally. Because the homozygous plants were compromised in plant growth, we used heterozygous mutant plants for the transformation. We successfully identified positive transgenic plants in the *xvp-d* homozygotes background in the T2 generation. As shown in Figure 7, the phenotype of stem was two independent microRNA knockdown transgenic plants (*miRKD2* and *miRKD6*) was reversed to wild type (Figure 7E to 7H). The overexpression and knockdown experiments indicated that *NAC003* was responsible for the *xvp-d* mutant phenotype. Therefore, we renamed the *NAC003* gene as *XVP*.

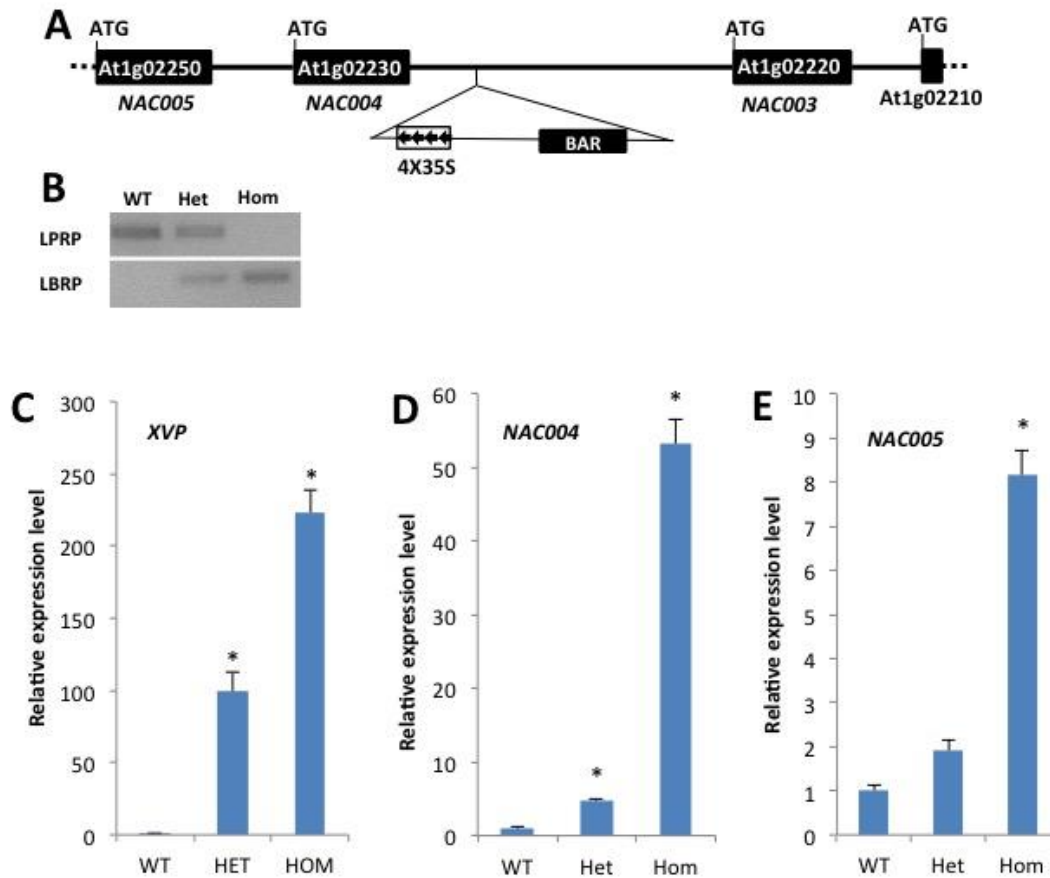


Figure 6. Identification of the *XVP* gene

(A) Schematic diagram showing the genomic region with T-DNA insertion. Start codon was shown to indicate the transcription orientation for each gene. The *CaMV* 35S tetrad enhancer was marked as 4X35S that located at the right border of the T-DNA. The blocks denote genes and lines denote intergenic regions.

(B) Gel electrophoresis results showing gel patterns of different genotypes.

(C) qRT-PCR analysis confirmed activation of *XVP* expression in the heterozygotes (Het) and homozygotes (Hom) compared to the wild type (WT) plants

(D) and (E) qRT-PCR characterization of the expression of *NAC004* (D) and *NAC005* (E) in the heterozygotes (Het) and homozygotes (Hom) compared to the wild type (WT) plants

Error bar indicated mean \pm SE (n=3) of transcription levels.

Asterisks indicated significant differences ($P < 0.05$) from WT.

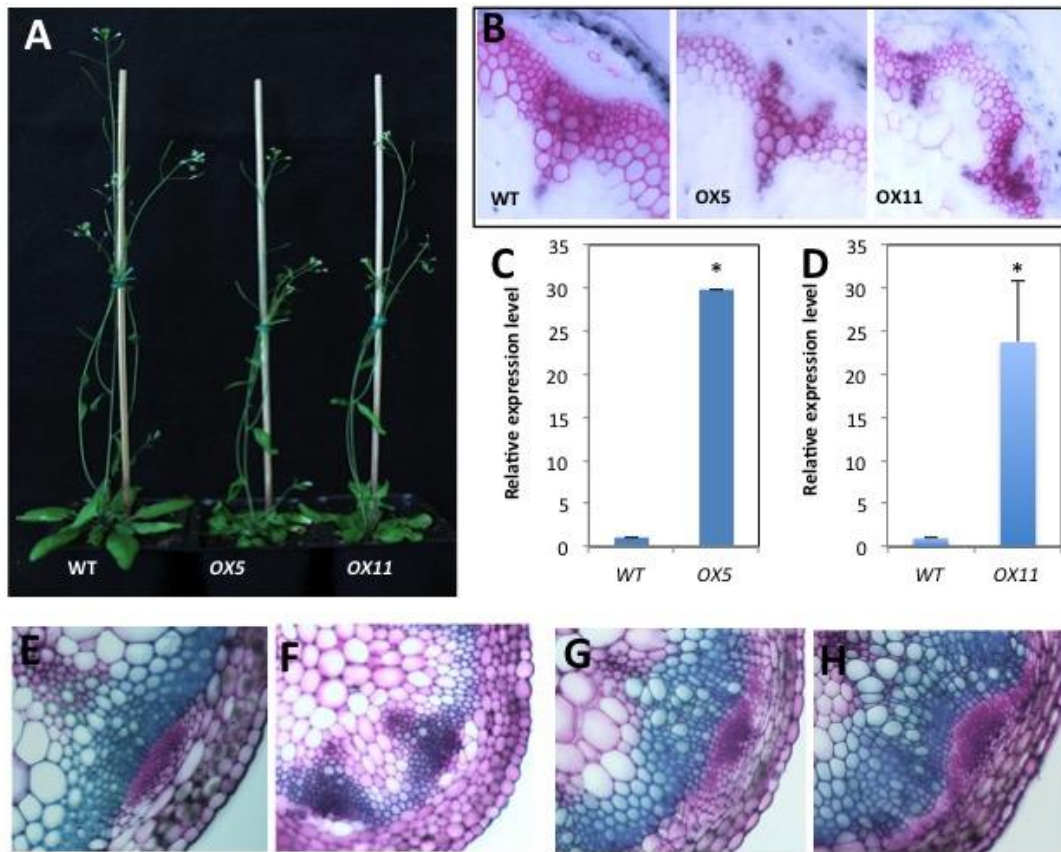


Figure 7. Molecular characterization of the *XVP* overexpression (OX) and *XVP* knockdown (KD) lines

(A) Morphological phenotypes of two *XVP* overexpression lines OX5 and OX11.

(B) Characterization of stem cross section of *XVP* OX5 and *XVP* OX 11 with phloroglucinol staining.

(C) and (D) Confirmation of *XVP* overexpression in transgenic lines OX5 and OX11.

(E) to (H) Toluidine blue staining of the stem cross sections of wild type (E), *xvp-d* (F), and two *XVP* knockdown (KD) transgenic lines (G) and (H).

Error bar indicated mean \pm SE (n=3) of transcription levels.

Asterisks indicated significant differences (P<0.05) from WT.

The *XVP* gene is predominantly expressed in phloem and cambium/developing xylem

To investigate the expression pattern of *XVP* gene, we first performed a qRT-PCR experiment using samples prepared from different tissues of the wild type plants. As shown in Figure 8, the *XVP* gene was highly expressed in roots, stems and leaves, but the expression was lower in flowers and siliques (Figure 8A). The expression of *NAC004* and *NAC005* showed similar expression pattern, but with much lower expression in the stem tissues compared to *XVP* (Figure 8B).

To understand whether *XVP* was expressed in developing vascular tissues, we cloned the promoter of *XVP* and fused it with the GUS (β -glucuronidase) reporter. The reporter construct *ProXVP::GUS* was transformed into wild type *Arabidopsis* plants. Six lines were selected for GUS staining and all of the lines showed similar staining patterns, with various staining intensity. GUS staining was observed in the developing vasculature in the cotyledon and young leaves (Figure 8C and 8D). In the root tissues, GUS staining was detected in the stele tissues, but no staining was observed at the root tips of the both main and lateral roots (Figure 8E). In flowers, GUS signal is specifically observed in vascular bundles of the anthers (Figure 8F and 8G).

We also performed GUS staining from the basal part of matured stems (five weeks old plants). The GUS signal was mainly observed in phloem cells of the vascular bundle and cambium/developing xylem cells (Figure 9A and 9C). To determine the expression location of GUS staining, we counter-stained stem tissue with Ruthenium red, which stains pectin of cell wall as red, thus we could easily distinguish all cell type in stem tissue. Considering the cell shapes and relative locations of the staining signals, we could confirm the GUS activity should be in phloem and cambium/developing xylem (Figure 9B and 9D).

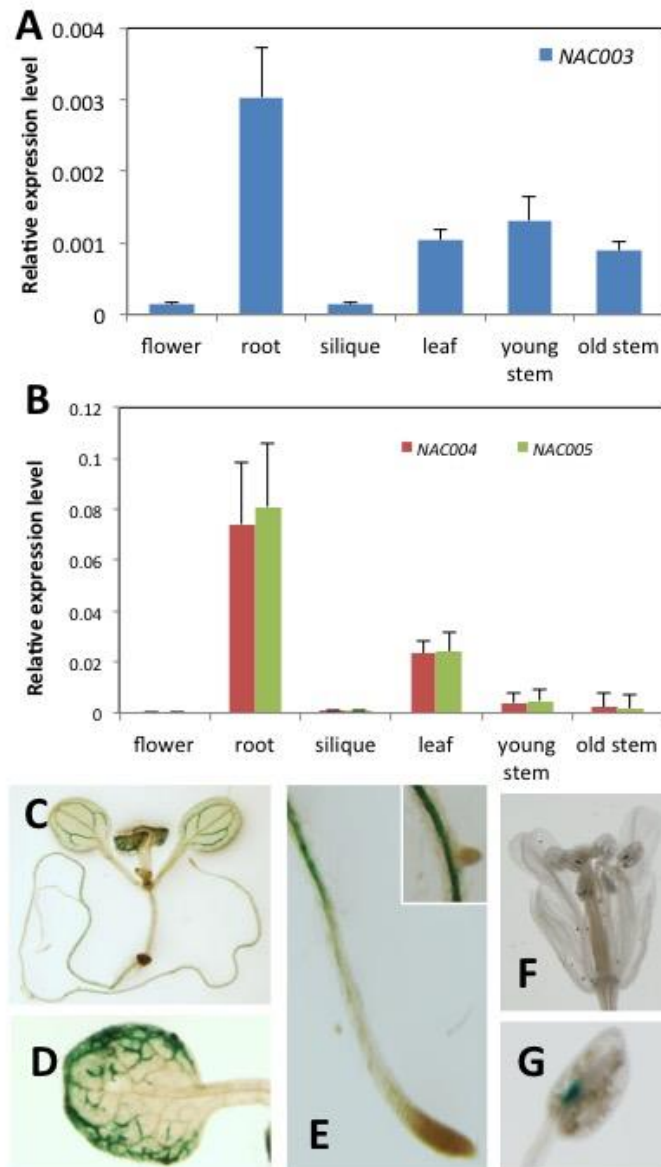


Figure 8. The expression pattern of *XVP* gene

(A) qRT-PCR analysis of *XVP* (*NAC003*) expression in different tissues of the wild type plants.

(B) qRT-PCR analysis of *NAC004* and *NAC005* expression in different tissues of the wild type plants.

(C) to (G) Expression of *XVP* in vasculature as determined by GUS staining of the 12 days old seedling (C), leaf (D), root (E), flower (F) and anther (G).

(A) and (B) Expression of the Ubiquitin5 gene was used for normalization.

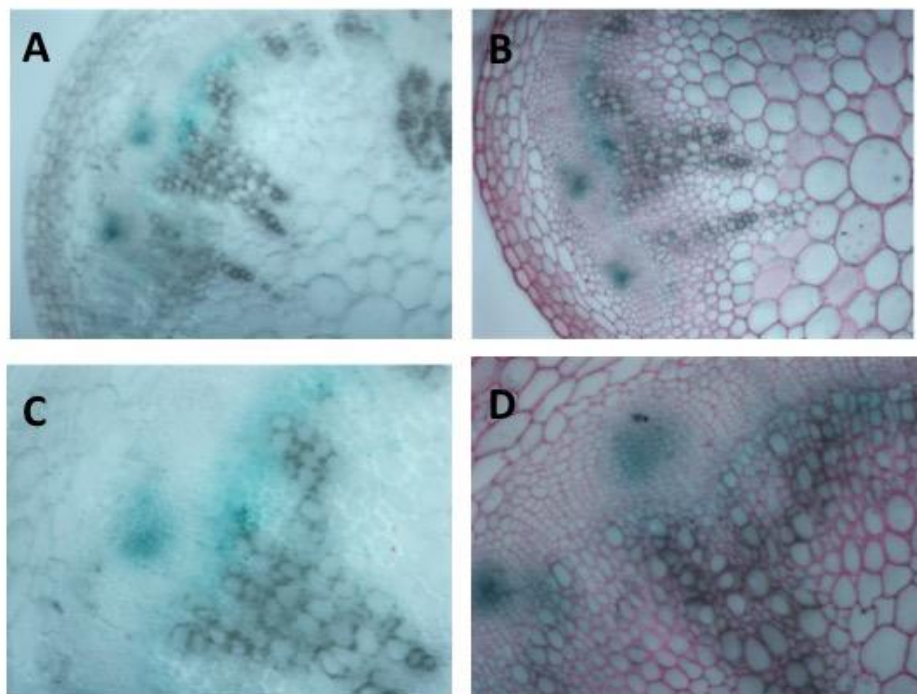


Figure 9. The expression pattern of *XVP* gene in basal stem
(A) and (C) GUS staining image of two stem cross sections.
(B) and (D) GUS staining image of two stem cross sections counter-stained with
Ruthenium red.

XVP has transactivation activity

XVP is a member of the Group II (or NAC-a) NAC domain transcription factors, and no functions have been assigned to the genes in this clade (Ooka et al. 2003). Although most of the NAC domain transcription factors function as activators, some of the members are transcriptional repressors (Yamaguchi et al., 2010a). Sequence alignment of XVP with its close homologous proteins indicated that the full length XVP can be divided into to three subdomains, a NAC domain, a trans-activation region (TAR) and a c-terminus unknown function sequence (UK). To determine the transcriptional activity of XVP and elucidate which region was essential for its activity, we generated a series of truncation of XVP and fused them to the GAL4 DNA binding domain. The resulting constructs were transformed into yeast strain *AH109* to test the activation activities (Figure 10). The full length XVP protein showed activation activity comparable to the positive control, SND, a known NAC domain transcriptional activator (Wang et al. 2011; Zhong, Demura, and Ye 2006). The TAR domain was confirmed to have the activation activity, and the NAC domain had no activation activity as expected (Figure 10). The UK domain did not interfere with the activation activity of TAR domain (Figure 10).

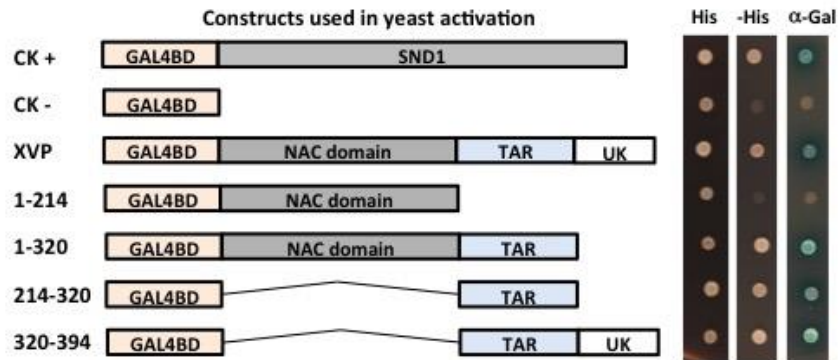


Figure 10. Transactivation activity of XVP

CK+ (Positive control): GAL4BD + Full-length open reading frame of SND1

CK- (Negative control): GAL4BD

XVP: GAL4BD + Full-length open reading frame of XVP

1-214: GAL4BD + NAC domain of XVP

1-320: GAL4BD + NAC domain + trans-activation region (TAR)

214-320: GAL4BD + trans-activation region (TAR)

320-394: GAL4BD + trans-activation region (TAR) with unknown functions (UK)

The subcellular localization of XVP

Through the transactivation assay in yeast, we detected activation activity of XVP. In general NAC domain transcription factors localize in nucleus due to the presence of a nuclear localization sequence (NLS). To investigate the subcellular localization of XVP, we prepared constructs with GFP fusion to the N-terminus of XVP protein or its truncated form as specified in the activation assay (Figure 10 and 11). Two different promoters, a *CaMV 35S* promoter (*35S::GFP-XVP*) and the XVP endogenous promoter (*XVPpro::GFP-XVP*) were used for the subcellular localization experiments (Figure 11A). Constructs were infiltrated individually into *N. benthamina* leaves, and GFP signals were checked under confocal microscope three days after inoculation. Surprisingly, GFP signals of *35S::GFP-XVP* were observed on the plasma membrane (Figure 11B and 11C). The XVP native promoter (*XVPpro::GFP-XVP*) construct also showed similar localization on the plasma membrane (Figure 11D and 11E). Interestingly, the other two constructs, *35S::GFP-NAC* and *35S::GFP-NAC+TAR*, showed signals in nucleus (Figure 11F to 11I). These results indicated that the UK domain of the XVP protein is responsible for plasma membrane localization, and the full length XVP may have the potential to be re-located into the nucleus.

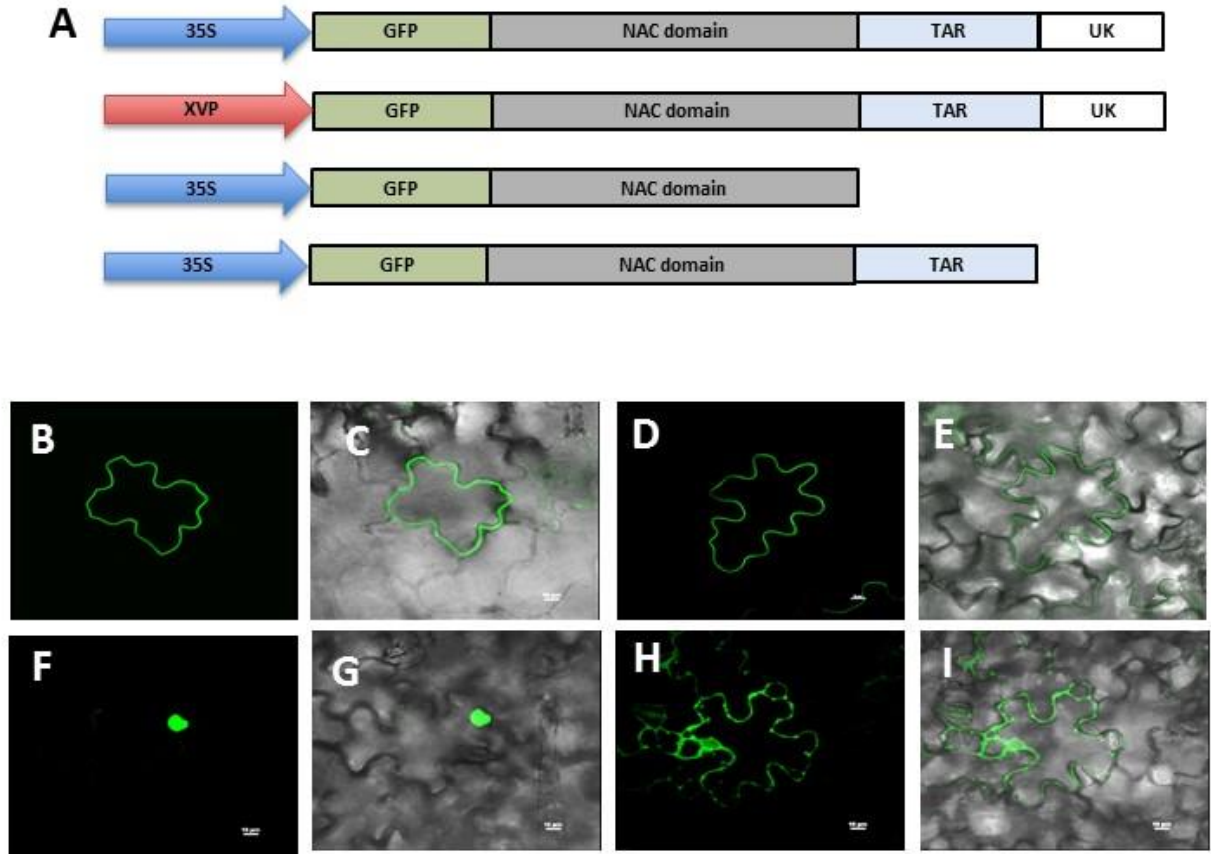


Figure 11. XVP localization as determine in *N.benthamina* .

(A) Diagram showing different constructs used for GFP fusion (domain information in Figure10).

(B) to (I) Fluorescent microscopic images of *35S::GFP-XVP* (B) and (C), and *XVPpro::GFP-XVP* (D) and (E), *35S::GFP-NAC* (F) and (G) and *35S::GFP-NAC+TAR* (H) and (I).

Images merged with bright field showing relative position of the GFP signals in leaf epidermal cells (C, E, G and I).

Investigation of the XVP function in vascular development using transcriptome analysis

To understand the functional mechanism of XVP in vascular development, we performed a transcriptome analysis using the Arabidopsis Gene1.1 ST Array Strips. There were over 500 genes either up- or down-regulated greater than 2 times in *xvp-d* mutants compared to the wild type. A number of the changed genes function in regulating xylem differentiation and stem cell proliferation. Among them, *VND6* was up-regulated more than 6 times in the *xvp-d* mutant (Figure 12A). Another xylem related NAC domain transcription factor, *XND1*, was also up regulated. The function of *XND1* in xylem differentiation includes promoting tracheary element (TE) elongation, and inhibiting both programmed cell death and secondary wall development (Zhao et al. 2008). We have also found that *AtXYLP9*, encoding a xylogen like protein, was up regulated in the *xvp-d* mutant plants. Xylogens are extracellular small peptides that promote xylem differentiation in plants (Schuetz, Smith, and Ellis 2013). Interestingly, the expression of secondary wall master regulators, *SND1*, *MYB46* and *MYB83*, were all down regulated in the *xvp-d* mutant (Figure 13). These results confirmed that the *xvp-d* mutant had precocious xylem differentiation, rather than secondary wall over-accumulation.

We also found that the expression of *CLE44* was up-regulated in the *xvp-d* mutant, which was confirmed by qRT-PCR (Figure 12B). The *CLE44* gene encodes the TDIF peptide that regulates vascular stem cell maintenance (Etchells and Turner, 2010; Hirakawa et al., 2010). The expression of *WOX4* and *WOX14* were not significantly affected in the *xvp-d* mutant indicating that XVP function is independent of WOX proteins. The microarray analysis and qRT-PCR results confirmed that XVP functions in regulation of genes known to affect xylem differentiation and vascular stem cell maintenance.

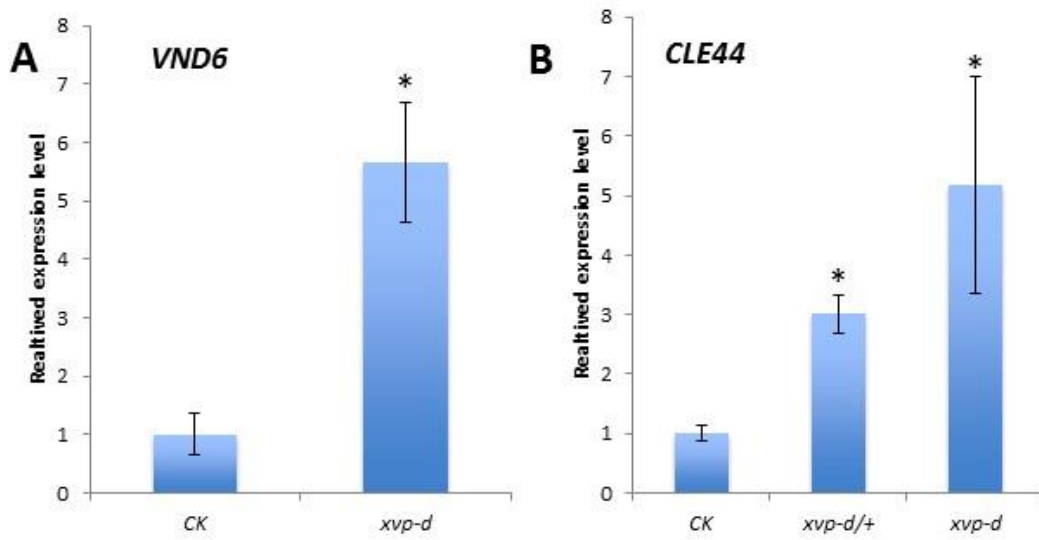


Figure 12. qRT-PCR analysis shows up-regulation of *VND6* and *CLE44* in *xvp-d* mutant.

(A) In *xvp-d*, expression of *VND6* was induced.

(B) In *xvp-d*, expression of *CLE44* was induced.

Error bar indicated mean \pm SE (n=3).

Asterisks indicated significant differences (P<0.05) from WT.

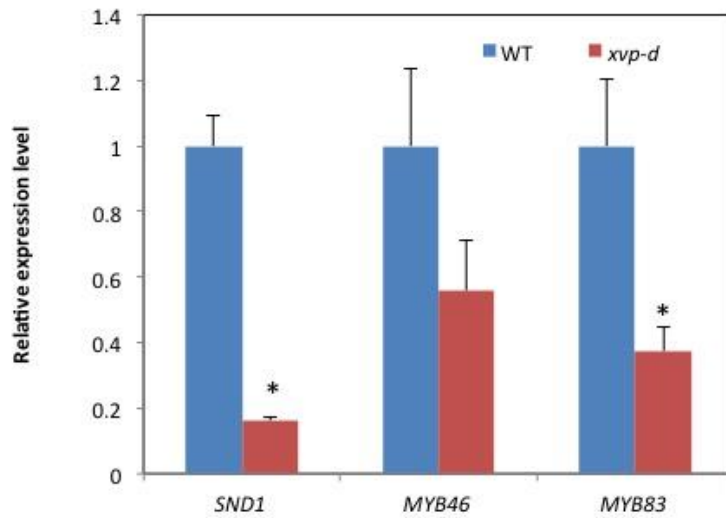


Figure 13. qRT-PCR analysis of the master regulators of secondary cell wall development

In *xvp-d*, the expression levels of three master regulators of secondary cell wall development were repressed compared to wild type.

Error bar indicated mean \pm SE (n=3).

Asterisks indicated significant differences ($P < 0.05$) from WT.

The expression of *XVP* gene is regulated by TDIF

The activation of *CLE44* in the *xvp-d* mutant suggested that *XVP* might be involved in small peptide TDIF signaling. To investigate the functional mechanism of *XVP*, we performed expression analysis using transgenic plants overexpressing TDIF coding gene *CLE41* and *CLE44*, respectively. The *35S::CLE41* line was reported previously in controlling vascular development (Hirakawa, Kondo, and Fukuda 2010; Ito et al. 2006b; Kondo et al. 2015). TDIF is synthesized in phloem, diffuses into cambium and binds to its receptor, PXY that is a member of LRR-RLKs. TDIF signal activates the expression of *WOX4* and *WOX14* in cambium to promote cambial cell proliferation (Etchells and Turner 2010; Etchells et al. 2013; Hirakawa et al. 2008; Ji et al. 2010).

We obtained a *CLE41 OX* lines (*35S::CLE41*) and checked *XVP* gene expression. In the *CLE41 OX* line, expression of *XVP* was suppressed (Figure 14). In addition, we overexpressed *CLE44* in the wild type plants. The phenotypes of *CLE44* overexpression (*CLE44 OX*) were characterized using histochemical analysis. Compared to wild type, *CLE44 OX* lines showed disrupted cell orientation in vascular bundles similar to *xvp-d* (Figure 15A to 15D). Using qRT-PCR, overexpression of *CLE44* was confirmed in *CLE44 OX* lines (Figure 15E). Meanwhile, the expression of *XVP* was repressed in the *CLE44 OX* lines (Figure 15F). Taken together, these experiments provided convincing evidence that TDIF negatively regulates the expression of *XVP*, which in turn negatively regulate stem cell proliferation. This result indicated that *XVP* is involved in the *CLE44*-PXY signaling pathway in regulating vascular tissue orientation in vascular bundles.

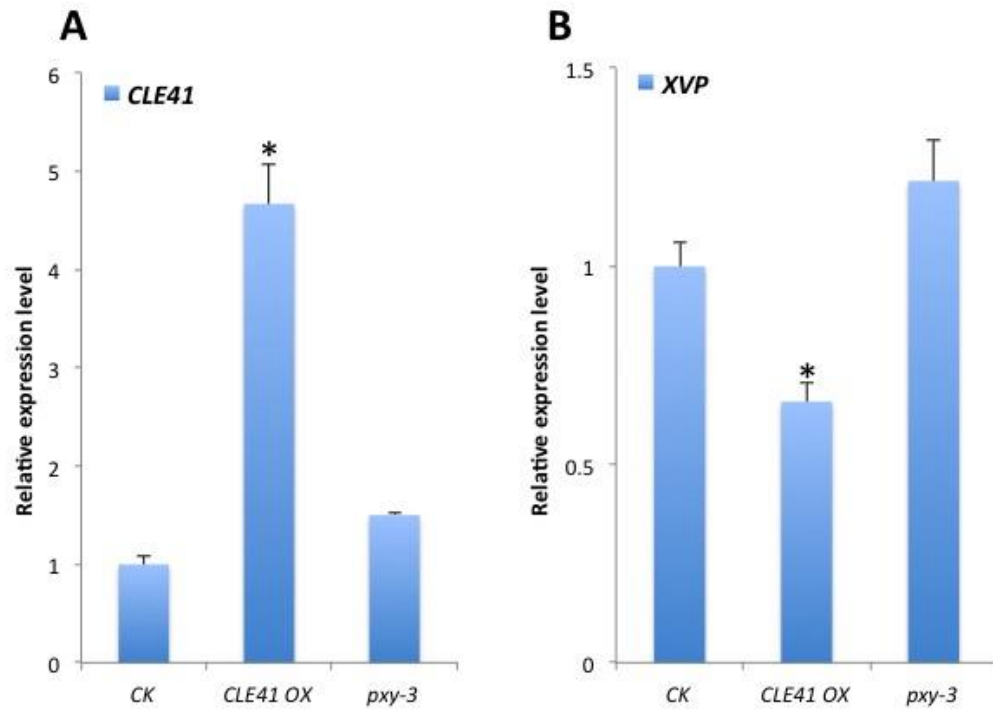


Figure 14. Investigation of gene expression in *CLE41* overexpression lines
 (A) qRT-PCR analysis showing *CLE41* expression level in *CLE41 OX* and *pxy*.
 (B) qRT-PCR analysis showing *XVP* expression level in *CLE41 OX* and *pxy*.
 Error bar indicated mean \pm SE (n=3).
 Asterisks indicated significant differences (P<0.05) from WT.

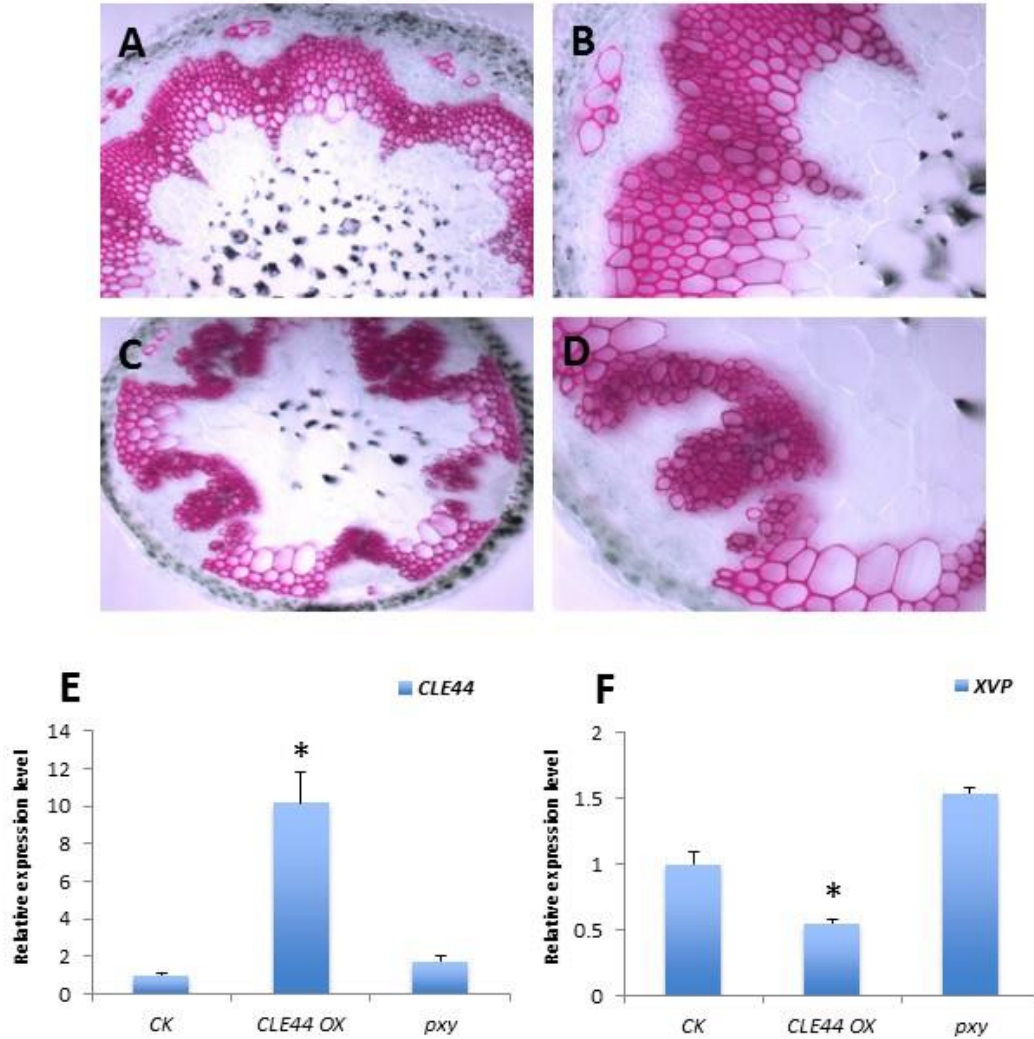


Figure 15. Overexpression of *CLE44* disrupted cell division orientation and negatively regulated *XVP* expression.

(A) and (B) Transverse stem cross section for wild type with phloroglucinol staining.
 (C) and (D) Transverse stem cross section for *CLE44 OX* lines with phloroglucinol staining.

(E) qRT-PCR analysis showing *CLE44* expression level in *CLE44 OX* and *pxy*.

(F) qRT-PCR analysis showing *XVP* expression level in *CLE44 OX* and *pxy*.

Error bar indicated mean \pm SE (n=3).

Asterisks indicated significant differences ($P < 0.05$) from WT.

XVP regulates xylem differentiation through VND6

As mentioned above, the expression of *VND6* was up-regulated more than six times in the *xvp-d* following transcriptome analysis (Figure 12A). *VND6* is a positive regulator of metaxylem formation (Yamaguchi et al. 2010a). To investigate if activation of *VND6* is required for the precocious xylem differentiation in *xvp-d* plant, we generated a double mutant, *xvp-d vnd6*, by crossing *vnd6* mutant into the *xvp-d* background. The growth of *xvp-d vnd6* plants was similar to *xvp-d* (Figure 16A). To characterize the vascular development, we performed histochemical analysis (Figure 16B to 16I). In *vnd6* mutant plant, we did not observe any obvious abnormality in metaxylem formation due to functional redundancy with *VND7* (Yamaguchi et al. 2008; Zhou, Zhong, and Ye 2014)(Figure 16C and 16G). In contrast, the precocious xylem formation in the *xvp-d vnd6* mutant was repressed compared to *xvp-d* plants (Figure 16E and 16I). These results indicate that the *VND6* function is necessary for the precocious xylem differentiation in *xvp-d* plants.

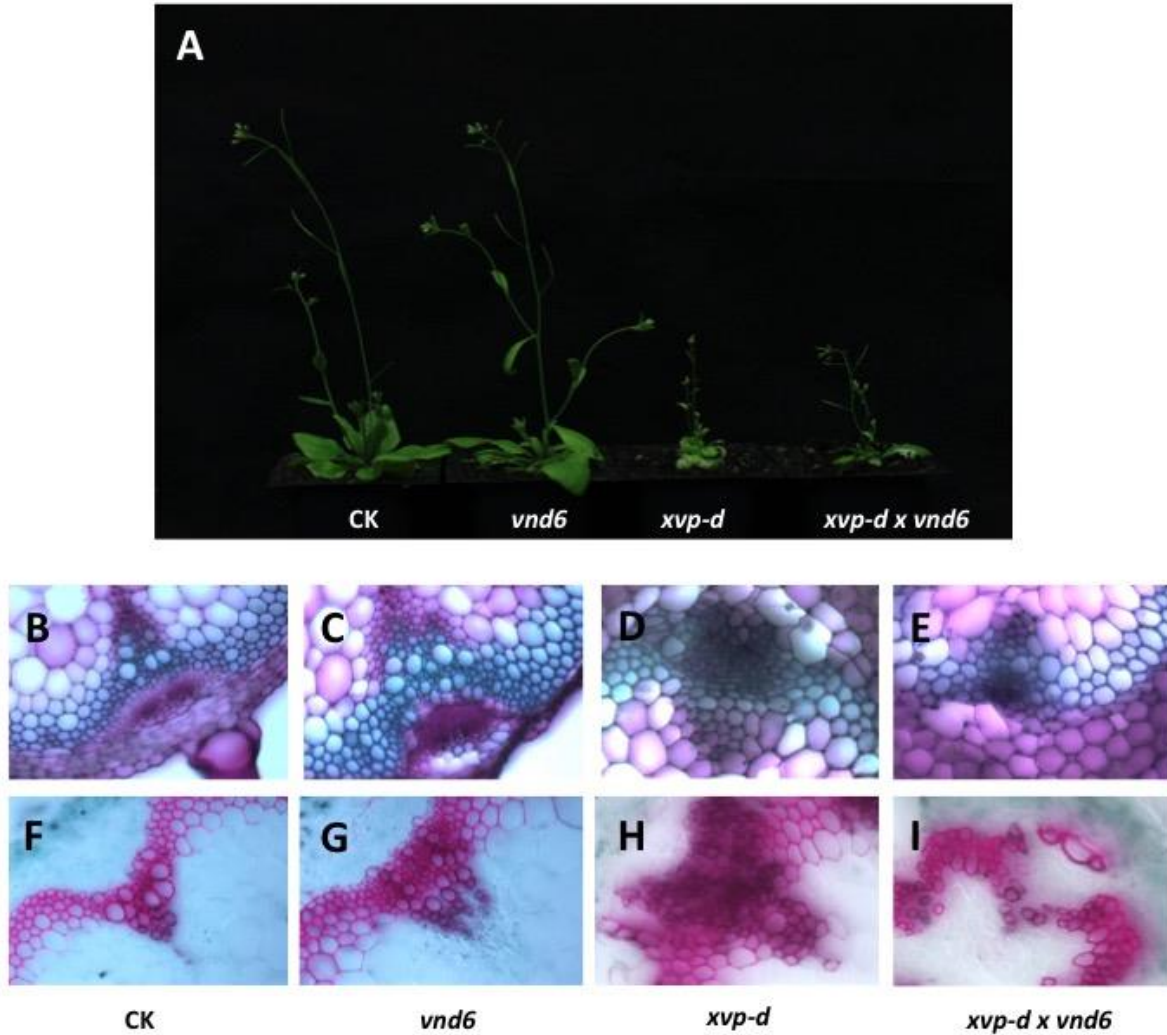


Figure 16. Double mutant of *xvp-d vnd6* showed suppressed precocious metaxylem development.

(A) Plant morphology phenotypes in each genotype

(B) to (E) Transverse stem cross section with toluidine blue staining.

(F) to (I) Transverse stem cross section with phloroglucinol staining.

(B) and (F) wild type/ (C) and (G) *vnd6*/ (D) and (H) *xvp-d*/ (E) and (I) *xvp-d vnd6*

The *XVP* regulates cambium activity

To investigate whether overexpression of *XVP* suppressed cambial activity, we counted cell numbers in the vascular bundle in plant of the genotypes; wild type, *xvp-d/+* and *xvp-d*. The number of cambium cells in vascular bundle was fewer in *xvp-d* mutants compared to wild type. Also, reduced number of cells in the phloem and xylem was observed (Figure 17). Thus, overexpression of *XVP* caused fewer cells for all cell types in vascular bundles due to compromised cambial activity.

XVP may function as a negative regulator for cambial proliferation. Thus, we expected that knockdown of *XVP* may lead to higher cambial proliferation and result in thicker cambium cell layers. We therefore analyzed the phenotypes of a *nac003* T-DNA insertion mutant (Flag_346F11). However, *nac003* line showed no difference in cell numbers compared to the wild type plant.

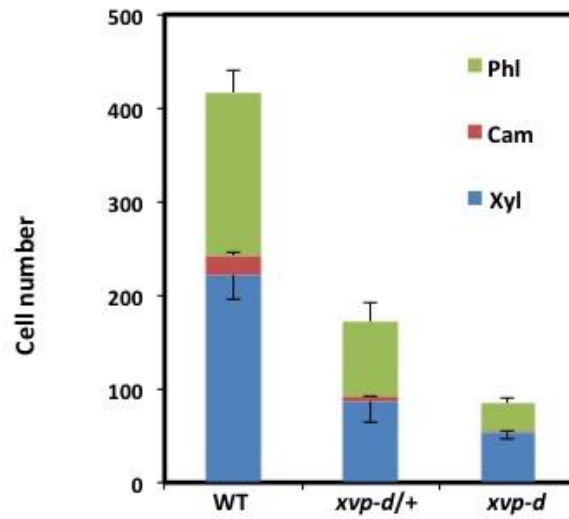


Figure 17. The *xvp-d* mutants have fewer cells per vascular bundle. Number of cambium (Red), Phloem (Green) and Xylem (Blue) cell in each genotypes. Error bar indicated mean \pm SE (n=10) of vascular bundles.

The XVP function redundantly with other NAC domain transcription factors

XVP is a member of the NAC domain transcription factor gene family. Phylogenetic analysis indicated that *NAC048* showed the highest homology with XVP (Figure 18). To investigate the function of *NAC048*, we overexpressed this gene using the *CaMV* 35S promoter. The overexpression of *NAC048* (*NAC048 OX*) was confirmed using qRT-PCR in two transgenic lines (Figure 19G). Histochemical analysis indicated that these two *NAC048 OX* lines showed similar phenotypes as those of *xvp-d* mutant, such as disrupted vascular cell orientation and precocious xylem differentiation in vascular bundles (Figure 19A to 19F). Expression analyses confirmed that both *CLE44* and *VND6* were up-regulated in *NAC048 OX* lines (Figure 19H and 19I). These results indicated that *NAC048* may have similar functions as XVP.

NAC004 and *NAC005* are also closely related with *NAC003/XVP* based on phylogenetic analysis, and may also have similar functions. To test this possibility, these two genes were also overexpressed in the wild-type background. Some small plants were identified in both transgenic studies. Detailed analyses are still needed to make a conclusion on their functions.

```

NAC003      METPVGLRFCPTDEEIVVDYLWPKNSDRDTSVDRFINTVPVCRLDPWELPCQSRIKLKD 60
NAC048      MENPVGLRFRPTDKEIVVDYLRPKNSDRDTSVDRVISTVTIRSFDPWELPCQSRIKLKD 60
NAC004      MMNPVGFRFRPNDEEIVDHYLRPKNLSDTSHVDEVISTVDICSFEPWDLPSKSMIKSRD 60
NAC005      MANPVGFRFRPTDGEIVDIYLRPKNLESNTSHVDEVISTVDICSFDPWDLPSHSRMKTRD 60
      * .***:** * . * *** ** ** : :*****. . ** : :***:** : * : *

NAC003      VAWCFFRPKENKYGRGDQQMRKTKSGFWKSTGRPKPIMR---NRQQIGEKKILMFYTSKE 117
NAC048      ESWCFFSPKENKYGRGDQQIRKTKSGYWKITGKPKPILR---NRQEIGEKKVLMFYMSKE 117
NAC004      GVWYFFSVKEMKYNRGDQQRRRTNSGFWKKTGKTMTVMRKRGNREKIGEKRVLVFKNRDG 120
Nac005      QVWYFFGRKENKYGKGRQIRKTKSGFWKKTGVTMDIMRKTGDREKIGEKRVLVFKNHGG 120
      * * * * * .***:** * * :***:** * * . : * :***:** : * *

NAC003      ---SKSDWVIHEYHG-FSHNQMMMTYTLCKVMFNGGMREKSSSSPSSSGVSGIEQSRDS 173
NAC048      LGGSKSDWVMHEYHA-FSPTQMMMTYTICKVMFKGDVREISSSSASYG--SEIEQSR-DS 173
NAC004      ---SKTDWVMHEYHATSLFPNQMMMTYTVCKVEFKGEETEISSSSSTGSE----IEQIH--S 171
NAC005      ---SKSDWAMHEYHATFSSPNQIMTYTLCKVKFKGERREFS-VATGSG----IKHTH--S 170
      **:**. :***. : :***:** * * * * . ** : * : *

NAC003      LIPQLVNNSEGSSLHREDPSQFGDVLQEAPIEDAKLTEELVKWLMNDEDDAQIEDAIP-- 231
NAC048      LIPLLVNDSE-----EEAQIEDAIP-- 193
NAC004      LIP-LVNSSG---GSEGSSFHSQELQNSS-----QSGVFANVQGESQIDDATTPI 217
NAC005      LIP-PTNNSGVLVSVETEGSLFHSQESQNPS-----QFSGFLDVALD--RDFCNIL 218
      *** . * . * *

NAC003      IEEWETWLN-DIDDAKEKSIMFMHDNRSDYRPPNSLTGVFSDDVSSDDNDSDLLTPKTNS 290
NAC048      IEEWETWLTDDGVDEQVNHIMNMKDDRNHRPQKPLTGVLIDDSSDDDDSDLLSPTTNS 253
NAC004      EEWKTWLNNDGDEQRN--IMFMQDHRSDYTPLKSLTGVSDD-SSDDNDSDLISPKTNS 274
NAC005      SDDFKGFFND-DEQSK--IVSMQDDRNHTPQKPLTGVSDDH-STDGSDSDPISATTIS 274
      : : : : . * : * : * . : : * : * . : * . : * . : * . : * . : *

NAC003      IQTSSTCDSFGSSNHRIDQIKDLQESPTSTINLVSLTQEVSQALITSIDTAEKKNPYDD 350
NAC048      IENSSTCDSFGSSDQ-----INLVSLTQEVSQALITSIDTPEKIKSPYDD 298
NAC004      IGTSTCASFASN--HQIDQTQHSPTSTVQLVSLTQEVSQGPG----- 316
NAC005      IQTLSTCPSFGSSNP-LYQITDLQESPNS-IKLVSLAQEVSKTPGTGID-----ND 323
      * . *** ** .** : :***:** : * : *

NAC003      AQGTEIGEHLGQETIKKKRAGFFHRMIQKFKVKKIHLCSISRT 394
NAC048      AQGTGAGGQKLGQETREKKRAGFFHRMIQIFVKKIHQCISISRT 342
NAC004      -QVTVIREHKLGEESVKKKRASFVYRMIHRLVKKIHCYSISRT 359
NAC005      AQGTEIGEHLGQETIKNKRAGFFHRMIQKFKVKKIHLRT----- 362
      * * :***:** :***:** :***:** :***:** :***:**

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Figure 18. Sequence alignment of XVP and its homologous proteins. Using CLUSTAL 2.1 multiple sequence alignment tool, NAC048 showed highly homology with XVP. NAC004 and NAC005 also showed homology with XVP.

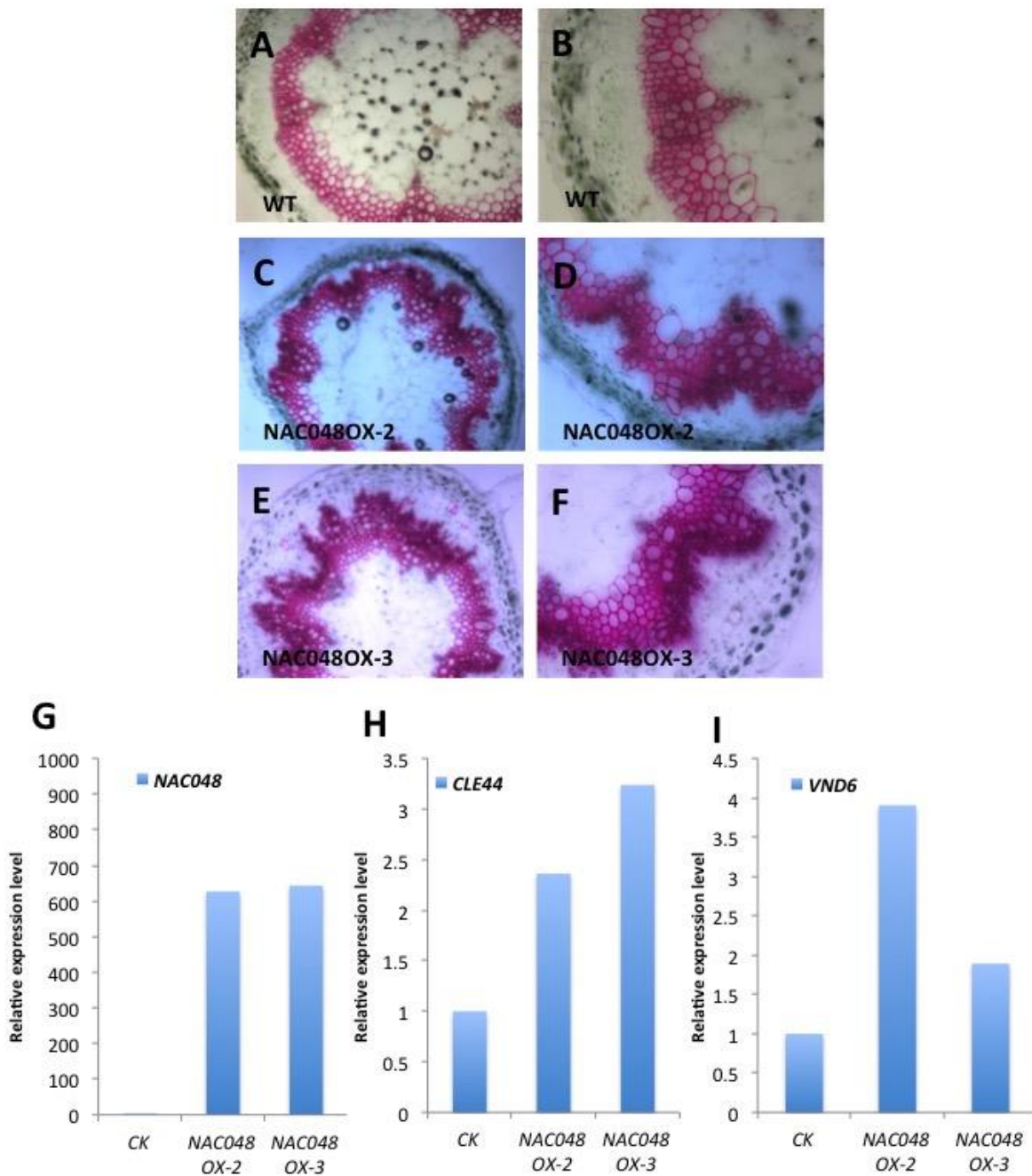


Figure 19. Functional analysis of *NAC048*.

(A) and (B) Phloroglucinol staining of transverse stem sections of wild type plants.

(C) to (F) Phloroglucinol staining of transverse stem sections of *NAC048 OX-2* and *NAC048 OX-3* lines

(G) qRT-PCR showing *NAC048* expression level in *NAC048 OX* lines

(H) qRT-PCR showing *CLE44* expression level in *NAC048 OX* lines

(I) qRT-PCR showing *VND6* expression level in *NAC048 OX* lines

Discussion

The functional mechanism of XVP in small peptide TDIF signaling

Based on previous studies and the results presented herein, we proposed a model for XVP function in TDIF-TDR signaling (Figure 20). In this model, XVP positively regulates xylem differentiation, negatively controls stem cell proliferation and acts as a position signal for vascular patterning. The TDIF-TDR signal exerts its functions through negatively regulating XVP. To be specific, the TDIF-TDR signal represses the expression of XVP, as a result inhibiting xylem differentiation, promoting stem cell proliferation and maintaining normal vascular patterning. On the other hand, XVP positively regulates the expression of *CLE44* that encoding TDIF. In detail, overexpression of XVP in the *xvp-d* mutants or in the *35S::XVP* and *35S::NAC048* transgenic lines resulted in disorganized vasculature, which was similar to the transgenic lines overexpressing *CLE41* or *CLE44* (Figure 7, 15, 16 and 20). For both cases, the phenotypes can be explained by the disruption of TDIF signaling polarity, which is initiated from phloem expressed TDIF and transmitted to the procambium/cambium localized TDR/PXY (Figure 20). The feedback regulation is essential to stabilize the TDIF signaling pathway, and is similar to the mechanism identified in apical meristems (Mayer et al. 1998; Yadav et al. 2011b). In order to confirm the feedback regulation involved in *CLE44*/PXY-XVP, we created double mutant, *xvp-d pxy*. We expect the double mutant show rescued phenotype from *xvp-d* due to abolishment of activated TDIF/PXY signaling (Figure 20). This approach could give more confident data to interpret the polarized signal and relay XVP function in TDIF-PXY mediated signaling pathway.

Stem cell maintenance in the vascular cambium

Plant stem cells reside in three different meristems, i.e. shoot apical meristem (SAM), root apical meristem (RAM) and vascular meristem. As in animals, stem cells in plants produce progenies that either become new stem cells or differentiate into specialized tissues. The balance between these two cell fates needs to be maintained through a robust regulatory network. The current paradigm of plant stem cell maintenance involves a CLE peptide, LRR-RLK receptor (s), and downstream homeobox WOX proteins (Aichinger et al. 2012; Elo et al. 2009; Heidstra and Sabatini 2014). Similar to the apical meristems, small signal peptide TDIF plays a critical role in stem cell maintenance in the vascular meristem. The TDIF signaling pathway also involves a LRR-RLK, TDR/PXY, which is localized in procambium/cambium (Hirakawa, Kondo, and Fukuda 2010; Wang et al. 2013b). Despite the similarities, vascular meristem is also significantly different from the apical meristems in several ways. First, the vascular meristem differentiates into only two different cell types, phloem and xylem, while SAM and RAM produce cell lineages that eventually differentiate into all of the above and underground organs. This makes the vascular meristem a simplified model to study the regulation of tissue specification and differentiation. Secondly, the polarity of vascular stem cell division and tissue differentiation is also simpler due to its simpler organization. In the stem and hypocotyls, phloem differentiates on the peripheral side and xylem differentiates on the central side. The root vascular organization is in two poles and well defined. Thirdly, the homeobox WOX proteins and BIN2 are downstream factors have been suggested in stem cell maintenance in the apical meristems. In vascular bundles, TDIF activates TDR through its binding, and activated TDIF-TDR induces to expression of *WOX4/14* and *BIN2* (Etchells et al. 2013; Hirakawa, Kondo, and Fukuda 2010; Ji et al. 2010; Kondo et al. 2014; Kondo et al. 2015;

Miyashima et al. 2013; Ohashi-Ito and Fukuda 2010). Expression of *WOX4/14* and *BIN2* signaling promotes cambium proliferation and inhibits xylem differentiation.

The novel transcription factor *XVP* regulated cambium activity and xylem differentiation through TDIF-TDR signaling pathway. In our research data, *xvp-d* has less cambial cells because overexpressed *XVP* promotes precocious xylem differentiation and suppressed cambium proliferation (Figure 1 and 20). Interestingly, we found that *CKX1/5* (*CYTOKININ OXIDASE 1/5*) were up-regulated in the *xvp-d* mutant (Figure 21). In general, *CKX1/5* are enzymes that reduce the level of functional cytokinin and repress cell division (De Rybel et al. 2014; Ohashi-Ito and Fukuda 2010). Therefore, *XVP* may involve in cytokinin signaling pathway to induce expression of *CKX1/5* which results in reduced cambium proliferation due to lower cytokinin level. For this reason, we expect that knockout of *XVP* lead to increase cambial proliferation activity. However knockout mutant for *XVP* did not show expanded cambium cell layers. According to sequence alignment of *XVP* and its homologues such as *NAC004*, *NAC005* and *NAC048*, we assumed that *XVP* functions redundantly with its homologues (Figure 18 and 19). In order to overcome gene redundancy, we created double mutant, *nac003 nac048* by crossing *nac048* mutant into the *nac003* background. Also, we try to generate quadruple mutant, *nac003 nac004 nac005 nac048*, which does not express most homologue genes of *XVP*. First, we made triple mutant, *nac003 nac004 nac005* using CRISPR-Cas9 system. We designed two sgRNAs to knockout 20 nucleotides in *NAC004* and *NAC005* (Feng et al. 2014; Zhang et al. 2015). Next, we made sgRNA(*NAC004*)-CRSIPR-Cas9-sgRNA(*NAC005*) construct and transformed into *nac003* mutant. Therefore, we generated triple mutant, *nac003 nac004 nac005*. Next, we could generate quadruple mutant by crossing *nac048* mutant into triple mutant background, *nac003 nac004 nac005*. We expect to

observe expanded cambium cell layers either *nac003 nac048* or *nac003 nac004 nac005 nac048* mutant.

Xylem differentiation is a specific feature of the vascular meristem

The NAC domain transcription factors, such as VND6 and VND7, function in regulating xylem differentiation (Ohashi-Ito and Fukuda 2010; Yamaguchi et al. 2010b), while other NAC transcription factors, such as SND1, function as master regulators in regulating secondary cell wall development (Zhong, Demura, and Ye 2006; Zhong, Richardson, and Ye 2007). How these master NAC domain transcription factors were regulated by their upstream signals is largely unknown. In the *xvp-d* mutant plants, the overall secondary cell wall development was compromised with less numbers of secondary thickened fibers cells in the mutant. In accordance with these observations, the expressions of *SND1*, *MYB46* and *MYB83* were down regulated in the *xvp-d* mutant (Figure 14). We also found that *XVP* overexpression activated the expression of *VND6*, which function in regulating metaxylem differentiation (Figure 13). In particular, double mutant, *xvp-d vnd6*, showed disrupted metaxylem formation (Figure 17). These results supported that precocious xylem differentiation in *xvp-d* caused by induction of *VND6*. As we shown above, *XVP* showed activation activity, which could be explained the up-regulation of *VND6* in the *xvp-d* mutant, while the repression on *SND1*, *MYB46* and *MYB83* may be resulted from indirect effects.

Expression pattern and subcellular localization of XVP

Previous study reported *CLE41/44* are expressed in phloem and TDR/PXY is localized in cambium (Etchells and Turner 2010). Ectopic expression of *CLE44* with 35S promoter resulted in disruption of cell division plane and cellular orientation due to losing cellular polarization in

vascular bundle (Etchells and Turner 2010; Fisher and Turner 2007). Analysis of the expression pattern using *XVPpro::GUS* construct, *XVP* expressed in phloem and *XVP* could activate the expression of *CLE44* (Figure 9 and 12). Based on these data, *XVP* could regulate *CLE44* by directly or indirectly in the phloem. In the cambium, on the other hand, activated TDIF-TDR transmitted signal to phloem, resulted in suppressed expression of *XVP*. In the *XVPpro::GUS* analysis, GUS signal was also observed in cambium/developing xylem (Figure 9). In previous study, expression of *VND6* was detected in developing xylem region (Zhou, Zhong, and Ye 2014). Taken together, *XVP* may promote xylem differentiation through activation of *VND6* expression directly/ indirectly.

In general, NAC domain transcription factors localize in nucleus and regulate transcription. In the subcellular localization study, we confirmed that full length of *XVP* was localized on plasma membrane. However, *XVP* could be localized into nucleus without its C-terminal region. Some NAC domain transcription factor family such as NTL (NAC with transmembrane motif1-like), localize on plasma membrane due to presenting a transmembrane region (TM) in C-terminal region. NTL subfamily transcription factors are relocated into nucleus after removing TM by proteolytic processing (Liang et al. 2015). Though *XVP* showed similar subcellular localization pattern to NTL subfamily, *XVP* does not have a TM region. However, the C-terminus region of *XVP* may interact with other membrane proteins such as membrane integral receptors. To test this possibility, we conducted yeast-two-hybrid (Y2H) assay to check physical interaction between *XVP* and several other membrane-localized proteins. PXY is an integral membrane protein, and membrane localization of PXY was reported (Kondo et al. 2014). In our Y2H result, we did not detect interaction between *XVP* and PXY. We also checked interaction between *XVP* and BIN2. The *XVP* did not interact to BIN2 (Kondo et al. 2014). Similarity, we did not detect interaction

between XVP and PXC1 and RUL1, two membrane localized receptor like proteins function in xylem development (Agusti et al. 2011b; Wang et al. 2013b). Although XVP did not interact to receptors (PXY, PXC1 and RUL1) or BIN2 in our analysis, XVP is associated plasma membrane. Our hypothesis is that XVP could be dissociated from plasma membrane and translocated to nucleus under certain cellular signaling stimulus.

In this research, we identify the novel regulatory factors, XVP, function in cambial activity and xylem differentiation in the TDIF-TDR signaling pathway. Identification of new genes function in vascular stem cell maintenance and tissue differentiation will certainly advance our understanding cambial activity and vascular development. Knowledge gained from this research may help design better strategy in feedstock improvement for renewable biofuel production.

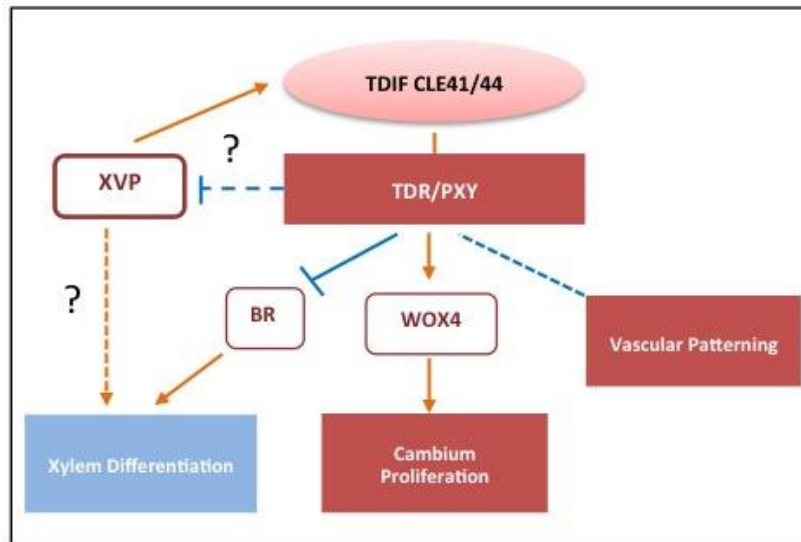


Figure 20. The functional mechanism of *XVP* in TDIF-TDR regulated vascular development.

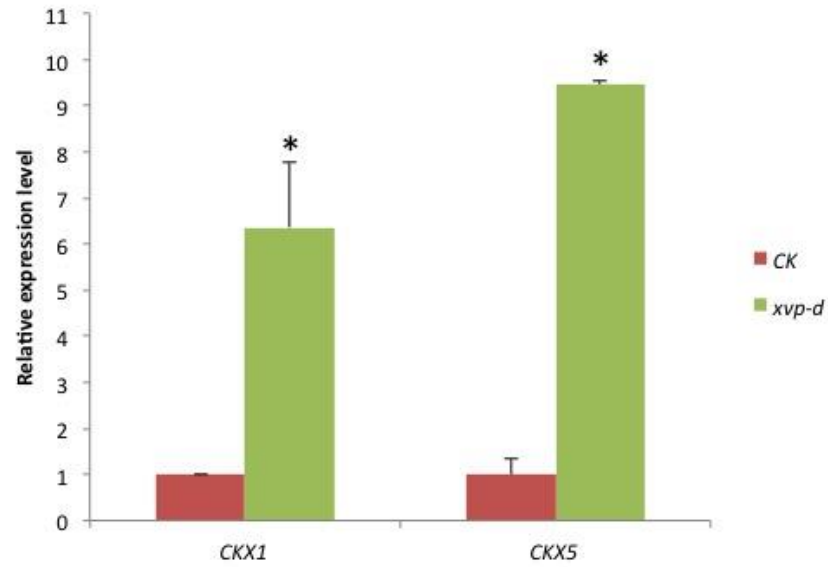


Figure 21. qRT-PCR analysis shows the expression of *CKX1* and *CKX5* in *xvp-d*. The qRT-PCR result indicated that both *CKX1* and *CKX5* were induced by *XVP*. Error bar indicated mean \pm SE (n=3) of transcription levels. Asterisks indicated significant differences ($P<0.05$) from WT.

Materials and Methods

Plant Materials and Growth Conditions

The *Arabidopsis thaliana* ecotype Columbia-0 was used as wild type in this research. The *xvp-d* mutant was identified from a large-scale mutant screening using UV microscopy. *Arabidopsis* growth and treatment were performed following the protocol described previously. Plants were grown in growth chambers with the following settings: photoperiod, 16 h day/8 h night; temperature, 22 °C day/ 20 °C night; relative humidity 70-80%; and light intensity 150 $\mu\text{mol m}^{-2}\text{s}^{-1}$.

Phenotypic Characterization

The activation-tagged mutant population was created by transformation of wild type *Arabidopsis* plants with activation-tagging vector pSKI015 as described. Identification of mutants with defects in vascular development was performed using stem cross section and UV microscopy. In brief, cross sections (100 μm) of the stem were cut just above the rosette leaves with a Leica RM 2255 microtome. Stem sections were put on glass slides and observed using a Nikon Microphot-FX microscope. Micrographs were taken with an INFINITY3 color camera with consistent settings. Mutants identified from the UV microscopy screening were further characterized in detail using growth phenotype analysis and cell wall related histochemical staining. In phloroglucinol staining, we prepared phloroglucinol-HCl Staining solution (2% phloroglucinol in 95% EtOH) and applied to stem section for two minutes. In toluidine blue staining, we applied toluidine blue staining solution (0.4% toluidine blue in 0.1M NaAC pH=4.00) to stem tissue for two minutes, and wash residual staining solution with water. In ruthenium red staining, we applied ruthenium red staining solution (0.04% of Ruthenium red in water) to stem

tissue, and wash residual staining solution with water. After staining stem tissue with appropriate staining solution, we observed stained stem tissues under Nikon Microphot-FX microscope and took a pictures using INFINITY3 color camera.

TAIL-PCR

Gene responsible for the *xvp-d* mutant phenotype was cloned using the Thermal Asymmetric Interlaced PCR (TAIL-PCR) method as described (Liu et al., 1995). The T-DNA specific primers and degenerated primers used in this research were provided in Table 1. Amplified DNA fragments were cut from the agarose gel, purified using a DNA purification kit (Qiagen) and subsequently sent for sequencing. The precise insertion site of the T-DNA containing the activation tag was determined according to the sequencing results. Further genotyping assay and genetic analyses confirmed the activation tag is responsible for the mutant phenotype.

Gene cloning and Constructs

We developed a Gateway compatible artificial microRNA vector based on a previously reported method (Schwab et al., 2006). In brief, the miR319a sequence (409 bp) was cloned using primer miR319Fw and miR319Re (Table 1), and inserted into the TOPO-D vector (Invitrogen). The resulting vector named TOPO319 contains the functional mir319a precursor including the stem loop sequence. To knockdown the expression of *XVP* artificial microRNAs targeting *XVP* alone or these three genes were designed using a web microRNA designer (<http://wmd3.weigelworld.org/cgi-bin/webapp.cgi>). The designed artificial microRNAs were engineer into miR319 precursor in TOPO319 vector using a PCR based mutagenesis method. PCR primers used to engineer the artificial microRNA are listed in Table 1.

To overexpress *XVP*, the full-length coding sequence of the *XVP* was cloned using high-fidelity polymerase and inserted into the TOPO-D vector. Primers *XVP* Fw and *XVP* Re were provided in Table 1. All constructs were confirmed by sequencing, and sub-cloned to destination vector pK2GW7 by LR reaction. Also, we made overexpression of *CLE44* and *NAC048* following same method to overexpression of *XVP*.

To make the *XVP_{Pro}::GUS* reporter line, a 1.5 kb DNA fragment in front of the *XVP* start codon was amplified using primers Pro*XVP* Fw and Pro*XVP* Re (Table 1), and inserted to the TOPO-D vector. After sequencing, the vector was ligated into the pBGWFS7 vector by LR reaction. All constructs in binary vectors were transformed into *Agrobacterium* strain *AGL1* or *GV3101* for plant transformation.

GUS Staining

Staining of the GUS reporter lines were performed as described (Wang et al., 2010). To stain the stem tissues, cross section was cut at 100-120 μ m and submerged into the staining buffer (2mM X-Gluc (5-bromo-4-chloro-3-indolyl glucuronide), 50mM M Na₂HPO₄, pH 7.0, 5 mM potassium ferricyanide/ferrocyanide, and 0.06% Triton X-100). Samples were infiltrated under vacuum for 10 min and then incubated at 37 °C overnight. Staining buffer was replaced with 70% ethanol to clear the tissue. Observations of the stained tissues were made using an Olympus SZX stereoscope and/or light Nikon Microphot-FX microscope.

XVP localization in *N.benthamiana*

Full length and the truncated versions of *XVP* were cloned using primer containing respective restriction enzyme sites (Table 1), and were inserted into TOPO-D vector. After sequencing confirmation, individual *XVP* fragments were sub-cloned to the destination vector pK7WGF2 (N' GFP fusion) by LR reaction. In order to make *XVPpro::GFP-XVP* construct, we replaced 35S promoter in construct of *XVP* (pK7WGF2) to *XVP* promoter by using traditional enzyme digestion and ligation. Using same constructs, we transformed all constructs into *Agrobacteria* (*AGL-1*), and we infiltrated transformed *Agrobacteria* into *N.benthamiana* leaves. After three days, we observed GFP signal under confocal microscope

Plant Transformation

Floral dipping method was used for *Arabidopsis* transformation (Clough and Bent, 1998). Seeds collected from the transformed *Arabidopsis* plants were plated on half strength MS (Murashig and Skoog) medium supplied with appropriate antibiotics. Resistant plants survived on selective plate were transferred to soil for further analysis.

Gene Expression Analysis

Plant tissues were collected from stems of 35-day old plants or whole seedlings, flash frozen in liquid nitrogen and stored in a -80 °C freezer. Total RNAs were isolated using a RNA isolation kit (Qiagen). RNA samples were treated with RNase-free DNase (Qiagen) to eliminate contamination from genomic DNA. Three micrograms of total RNA was reverse transcribed using the Superscript III RT kit (Invitrogen) in a 20 µL reaction system. The cDNA was diluted 50 times and used as the templates for RT-PCR or qRT-PCR as previously described (Wang et al., 2010).

Briefly, the PCR amplification was performed on an ABI 7900HT real-time PCR machine using SYBR green q-PCR master mix. For each reaction, 1 μ L diluted cDNA sample was used in a total 10 μ L reaction system. The PCR reaction program was set according to the manufacturer's protocol. Primers used for real-time RT PCR were listed in Table 1.

Microarray Analysis

Microarray analysis was performed to compare the transcriptomes of the wild type and the *xvp-d* mutant. Because the *xvp-d* homozygous plants are extremely dwarf, we used Col-0 and *xvp-d/+* heterozygous line. For both genotypes, plants were grown for five weeks under long day conditions. Three biological replicates were used for microarray analysis. For each replicate, 3-6 primary inflorescence stems were used with cauline leaves and side branches removed. Total RNAs were isolated from the collected materials using a RNA isolation kit (Qiagen). The Affymetrix Gene Atlas System was used to perform the microarray analysis. GeneAtlas® WT Expression Kit was used for RNA treatment, cDNA synthesis, fragmentation and labeling, and hybridization following the manufacture's manual. Background correction, quantile normalization, and gene expression analysis were performed using the Expression Console and Transcriptome Analysis Console from Affymetrix. Gene level differential expression filter was set at Fold Change (linear) < -2 or Fold Change (linear) > 2 , and ANOVA p-value (Condition pair) < 0.05 . The microarray experiment was solely used as a screen for candidate genes that changed significantly in the *xvp-d* mutant, and selected genes were then validated independently with real-time PCR experiments.

Yeast Activation Assay

The full-length coding sequences of the control gene *SND1*, the full length or truncated *XVP* were amplified and fused with GAL4-BD domain in vector pGBKT7 (Clontech). The resulting plasmids were transformed individually into yeast strain *AH109* (Clontech), and plated on selection SD medium to identify the positive transformants. Activation analysis was carried out by dropping 2 µl of yeast culture that harboring corresponding constructs onto solid SD medium with or without histidine, or supplied with X- α -Gal to check for activation of corresponding reporter genes. Yeast growth and color development were observed and documented after two days cultivation in a 30 °C incubator.

Table 1. Primers used in thesis research.

XVP FW	CACCGaattcATGGAAACTC	NAC004 Q-Fw	GTCGTTTCACAGCCAAGAATTAC
XVP RE	gtcgacAGTTCTTGAGATGGAA	NAC004 Q-Re	CATTCTCTCAATCGGTGTAG
XVP FW (EcoRI)	CACCGAATTCATGGAAACTC	NAC005 Q-Fw	ATGATGACGATGAGCAGAGTAAG
XVP RE (Sall)	GTCGACAGTTCTTGAGATGGAA	NAC005 Q-Re	CACTGAAGACACCGGTCAAT
XVP NAC domain 214 Re	gtcgacTTATACCAATTCCTCGGTCAGTTAGC	XND1 Q-Fw	GAAGGGAGGCAATGGTACTT
XVP NAC&TAR 320 Re	gtcgacTTATGTTGAGGTAGGAGATTCTGTC	XND1 Q-Re	GTGTGTGGAGCTTGTGTAGAT
XVP TAR Fw	CACCGaattcATGAAATGGCTGATGAATGATGAGGAT	CKX1 Q-Fw	CCTGGACAGACTACCTTCATTT
XVP TAR Re	gtcgacAAATGGCTGATGAATGATGAGGAT	CKX1 Q-Re	GCTGGTAGACGTTGTTGATTTG
XVP UK FW (EcoRI)	CACCGATTCAAAGACCTGCAAGAATCTCCT	CKX5 Q-Fw	ccgactccgaaatcgtgatcagg
XVP UK RE (Sall)	GTCGACTCAAGTTCTTGAGATGGAAGAACAT	CKX5 Q-Re	gtgtaccggctcgagaaagcca
XVP genotyping Rp	TCCCCACAAAAGATGTCAGGTTC	CLE44 Q-FW	TTTGGACCACTTGGAACTTC
XVP genotyping Lp	TGTGATAGTGCCCAATCTGCC	CLE44 Q-RE	ACGCAGTGGCACTTCTTCTT
pSKI LB1	ATACGACGGATCGTAATTTGTC	VND6 Q-Fw	tccgggaatgcaagttgatgtt
pSKI LB2	TAATAACGCTGCGGACATCTAC	VND6 Q-Re	ccggttgctctattggctcgtgc
pSKI LB3	TTGACCATCATACTATTGCTG	WOX4 Q-FW	TCACGACCACTGGTGTCTTT
DG1	WGCNAGTNAGWANAAG	WOX4 Q-Re	CCCAGCTCCTACATGTCCTC
DG2	AWGCANGNCWGANATA	SALK_026128 LP	CCCCACACAAAAACCATAATG
DG3	NGTCGASWGANAWGAA	SALK_026128 RP	AAAAATCGAGAAGCTTGAGGG
DG4	WGTGNAGWANCANAGA	CS874140 LP	CGCCTGTCTAATTGACAGCTC
DG5	STTGNTASTNCTNTGC	CS874140 RP	AGGACGACACTCGGTGATATG
DG6	NTCGASTWTSGWGTT	CS836364 LP	GTGGAAGAGGGACAGGAGAAG
DG7	AGWGNAGWANCAWAGG	CS836364 RP	TTGCTTATGTTTTGGTTTTGTC
miR319 Fw	CACCacaaacacacgctcggagc	SALK_054446 LP	CCCTCTTGTGAACAGTTCTGG
miR319 Re	CATGGCGATGCCTTAAATAAAGATAAAC	SALK_054446 RP	TCCAACTTTTCTTCTCGGAC
NAC3 I miR-s	gaTGTTAATGAATCGATCGGCATtctctctttgtattcc	SALK_074723 LP	CTGCTATCATCAGGCAAAACC
NAC3 II miR-a	gaATGCCGATCGATTCAATTAACAtcaagagaatcaatga	SALK_074723 RP	GTTGAAGTAGCCATCAGCGAG
NAC3 III miR*-a	gaATACCGATCGATTGATTAACtTcacaggtcgtgatatg	NAC048 Q- FW	GGTAGCTCAGACCAAACTCACT
NAC3 IV miR*-a	gaAGTTAATCAATCGATCGGTATtctacatatattcct	NAC048 Q-RE	GACTCTTAATCTTCTCGGGTGTATC
ProXVP Fw	caccGAATTcTaactgtcaatgatccgtccagg	ACT2Fw	GCCATCCAAGCTGTTCTCTCC
ProXVP Re	CCATGGACAGGAGTTCCAATTCTCTGCAGC	ACT2Re	TTCTCGATGGAAGAGCTGGT
U6 pro Fw (HindIII)	caccaagcttCATTCGGAGTTTTTGTATCTTGTTCAT	NAC048 FW	CACCcgagaaATGGAGAATCCGGTG
NAC004 sgRNA Re	CTCTAAACCCCCATCTCTCGATTTGATCATC	NAC048 RE	GCTATTATGTTCTTGAGATAGAAGAACATTG
NAC004 sgRNA Fw	AGTGATTGATGATCAAAATCGAGAGATGGGGGTTTT	MYB46 Q-Fw	TCTTCGTCCTGACCTCAAGC
Tracr RNA Re (XmaI)	CCCGGGGCCATTTGTCTGCAGAATTGGCGCAC	MYB46 Q-Re	GTGCTGCAATCTGAGACCAC
NAC005 sgRNA Re	TAAACCCACATCAAGAAAGCCAGAAAAC	MYB83 Q-Fw	AACGTGGATCCTTCTCTCTC
NAC005 sgRNA Fw	GTGATTGTTTCTGGCTTTCTTGATGTGGGTTTT	MYB83 Q-RE	AGCCGAGTAGCTATTTGAGACC
Tracr RNA Re (EcoRI)	GAATTGCGCATTTGTCTGCAGAATTGGCGCAC	SND1 Q-Fw	CAAGCTTGAGCCTTGGGATA
CLE44 pro-FW	CACCGGTATCACTGGATATT	SND1 Q-Re	TGGTCCCGGTGGATACCT
CLE44 pro-RE	TTGCCTTGCAATTATAAAGAAG	XVP Q FW	TCACCTATGTTCTTCCATCTCAAG
VND6 pro-FW	CACCAGCTCGCTCATATAAC	XVP Q-RE	ACCAAGATAGACATCATAGCATACA
VND6 pro-RE	CGAGACTTTCATTTGATCTT	Flag_LB4	CGTGTGCCAGGTGCCACGGAATAGT
		Flag_RB4	TCACGGGTTGGGGTTTCTACAGGAC
		Flag_346F11_LP	TTCTCGCGGTATCAATACTG
		Flag_346F11_RP	GGGTTTTGGAAGAGTACTGGC

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