

RESEARCH PAPER

Nicotiana benthamiana VASCULAR-RELATED NAC-DOMAIN7-2 (*NbVND7-2*) has a role in xylem formation during interfamily grafting

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Abstract

VASCULAR-RELATED NAC-DOMAIN7 (VND7) is a transcription factor gene that plays a critical role in xylem differentiation. The ectopic expression of *VND7* induces the formation of secondary cell walls with spiral patterns in multiple plant cell types. In the present study, we identified four homologs of *VND7* in *Nicotiana benthamiana* and assigned them the names *NbVND7-1* to *NbVND7-4*. In particular, *NbVND7-1* and *NbVND7-2* were highly expressed during *N. benthamiana* and *Arabidopsis thaliana* (*Nb/At*) interfamily grafting. Analysis of the promoter β -glucuronidase (GUS) reporter lines of *NbVND7* genes elucidated the expression of *NbVND7-1* and *NbVND7-2* in xylem tissues of intact and grafted plants, and those of *NbVND7-3* and *NbVND7-4* in internal phloem tissues. Gene network analysis revealed the downstream genes of each *NbVND7* homolog and highlighted the association of *NbVND7-1* and *NbVND7-2* with xylem formation. A β -estradiol-inducible system for *NbVND7-2* demonstrated that *NbVND7-2* promotes ectopic xylem vessel differentiation in *N. benthamiana* seedlings and in the stem tissues at graft junctions. Induction of *NbVND7-2* at graft junctions enhanced ectopic xylem formation in the callus tissues proliferated at the graft boundary, accelerated the initiation of water transport from stock to scion, and enhanced scion stem growth after grafting. This study reveals a role for *NbVND7* genes in xylem formation that can enhance *Nicotiana* interfamily grafting.

Keywords: *Arabidopsis thaliana*, β -estradiol, interfamily grafting, *Nicotiana benthamiana*, VND7, xylem formation.

Introduction

Plant grafting is a common horticultural practice used to propagate plants or produce new plant varieties with desirable traits such as disease resistance, increased fruit production, and improved root systems (Liu, 2006; Wang, 2011; Melnyk, 2017).

Grafting involves connecting the vascular tissues of two or more plant parts, usually the stems or roots, to form a functional unit. Grafting success largely depends on the formation of a vascular connection between the grafted parts that allows water and nutrients to flow between them (Kümpers and Bishopp, 2015; Thomas and Frank, 2019). In recent studies, plant grafting was successfully performed among interfamilial species (Notaguchi *et al.*, 2020; Reeves *et al.*, 2022). Some *Solanaceae* plants exhibit powerful abilities during interfamilial grafting. *Nicotiana benthamiana* grafted onto *Arabidopsis thaliana* (*Nb/At*) is a model for studying the process of plant interfamilial grafting (Notaguchi *et al.*, 2020; Kurotani *et al.*, 2022, 2023). The *de novo* tracheary elements, also known as xylem vessel elements, in the graft junction are responsible for water transport and play critical roles in the interfamilial grafting process (Huang *et al.*, 2023). However, the gene regulatory pathway that promotes xylem formation has not been fully explored in *N. benthamiana*.

Xylem formation is a highly coordinated process that is controlled by genetic factors. In *Arabidopsis*, previous research has revealed a network of transcription factors that regulates the expression of genes involved in xylem differentiation. These transcription factors include members of the NAC (NAM, No Apical Meristem; ATAF, *Arabidopsis* Transcription Activation Factor; CUC, Cup-Shaped Cotyledon), MYB (myeloblastosis), and WRKY transcription factors (Nieminen *et al.*, 2004; Kubo *et al.*, 2005; Nakano *et al.*, 2010; Wang *et al.*, 2010). Within the NAC gene family, Vascular-related NAC Domain (VND) genes play crucial roles in plant development and stress responses (Sun *et al.*, 2018; Ramachandran *et al.*, 2021). VND7 is a master transcription factor specifically involved in the regulation of xylem cell differentiation in *Arabidopsis* (Yamaguchi *et al.*, 2008, 2011; Endo *et al.*, 2015). Owing to the redundancy of VND genes, only the dominant repression of VND7 was found to suppress xylem vessel formation. However, overexpression of VND7 induces ectopic xylem vessel formation in other plant tissues (Kubo *et al.*, 2005). Previous research has shown that manipulation of VND7 expression can lead to changes in plant growth and development. Overexpression of VND7 in *Arabidopsis* has been dramatically shown to result in the formation of ectopic xylem vessels, as well as increased resistance to pathogens and improved tolerance to drought stress (Reusche *et al.*, 2012; Augstein and Carlsbecker, 2022; Cornelis and Hazak, 2022). Overexpression of *PtVNS07*, a homolog of VND7 in poplar, enhances secondary growth and increases wood density (Demura and Fukuda, 2007; Ohtani *et al.*, 2011). In addition, the VND7-inducible system (VND7-VP16-GR) has been widely used in *Arabidopsis* and *in vitro* in tobacco BY-2 cells for investigating the mechanism of xylem formation (Yamaguchi *et al.*, 2010; Goué *et al.*, 2013; Noguchi *et al.*, 2018). Although many previous studies have been performed on VND7, there are no reports explaining the functions of VND7 homologous genes in *N. benthamiana*.

In this study, we have identified the homologs of VND7 in *N. benthamiana* and its downstream genes using network analysis. Relevant β -glucuronidase (GUS) reporter lines were generated to detect the expression patterns of the *N. benthamiana* VND7 (*NbVND7*) genes during interfamilial grafting. We established a β -estradiol induction system for *NbVND7-2* expression using the *NbEF1 α* ubiquitous promoter. The function of *NbVND7-2* in xylem formation was investigated using the induction line, and the importance for *Nicotiana* interfamilial grafting was tested.

Materials and methods

Plant materials and growth conditions

Nicotiana benthamiana (Lab 360) and *Arabidopsis thaliana* (ecotype Col-0) were used in this study. The seeds of *N. benthamiana* and *Arabidopsis* were immersed and shaken in a 5% (w/v) solution of sodium hypochlorite (NaClO) for 5 min, followed by six rinses with sterile water. The washed seeds were kept in sterile water and in darkness at 4 °C for 3 d. For *N. benthamiana*, the treated seeds were sown on half-strength Murashige and Skoog (1/2 MS) medium containing 0.5% (w/v) sucrose and 1% (w/v) agar. The pH of the medium was adjusted to 5.8 using 1 M KOH. After cultivation for 1 week on this medium in growth chambers, the seedlings were relocated to the culture soil containing a 1:1 mixture of Hana-Chan Culture Soil (Hanagokoro, Nagoya, Japan) and vermiculite (Nittai, Osaka, Japan) in a growth room. Seedlings of *N. benthamiana* were grown at 27 °C with 70% relative humidity and 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ continuous illumination. For *Arabidopsis*, the treated seeds were directly surface sown on the culture soil and covered with a transparent plastic film until germination. Seedlings of *Arabidopsis* were grown at 23 °C with 30% relative humidity and 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ continuous illumination. Chrysanthemum (cv. Jinba) was propagated by cutting. A length of 6 cm of the stem was trimmed from the mother plant and was transplanted into culture soil where ~2 cm of the stem was imbedded in the soil. The cut stems were grown at 22 °C with 30% relative humidity under continuous light conditions with 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ illumination. The chrysanthemum cuttings were cultivated for 3 weeks before grafting.

Construction of plasmid vectors

Genomic DNA was extracted from *N. benthamiana* plants using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The promoter sequences of *NbVND7* and *NbEF1 α* were amplified by PCR using TaKaRa Ex Taq DNA Polymerase (TaKaRa Bio, Tokyo, Japan). DNA segments were purified using the MonoFas DNA Purification Kit I (ANIMOS, Saitama, Japan). Total RNA was isolated from *N. benthamiana* plants using the RNeasy Plant Mini Kit (Qiagen). cDNA was synthesized using SuperScript III First-Strand Synthesis SuperMix (Thermo Fisher Scientific, Waltham, MA, USA). The fragment of the *NbVND7-2* coding sequence (CDS) was amplified by PCR using a cDNA template.

DNA fragments were inserted into the plasmid vectors using the NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs, Ipswich, MA, USA). Primers used in this study are listed in [Supplementary Table S1](#). For the GUS fusion construction, the putative promoter sequence amplified from the *N. benthamiana* genome was fused upstream of the GUS reporter gene in the pENTR vector (pENTR/D-TOPO Cloning Kit, Thermo Fisher Scientific) and transferred into the binary vector pGWB1 by LR recombination reaction. For the XVE fusion construction, the *NbEF1 α* promoter, 3 $\times$ VENUS-NLS, and the *NbVND7-2* CDS were fused into the XVE plasmid backbone as described previously

(Zuo *et al.*, 2000), and transferred into the binary vector *pAGM4723* using a modular cloning system (Weber *et al.*, 2011).

Generation of transgenic plants

The relevant plasmid vectors (*pGWB1* and *pLv2*) were transferred to *N. benthamiana* by *Agrobacterium* (LBA4404)-mediated transformation to generate the transformants. Transgenic plants of both GUS fusion lines and XVE fusion lines were screened for antibiotic resistance. For the GUS fusion lines, four T₁ lines of *pNbVND7-1::GUS*, four T₁ lines of *pNbVND7-2::GUS*, 10 T₁ lines of *pNbVND7-3::GUS*, and four T₁ lines of *pNbVND7-4::GUS* were harvested, and representative GUS expression patterns were identified for each construct. *pNbVND7-1::GUS* #2, *pNbVND7-2::GUS* #3, *pNbVND7-1::GUS* #1, and *pNbVND7-4::GUS* #3 were used as representative lines. Homozygous XVE-induced lines were harvested and confirmed in the T₂ generation. *pNbEF1 α -XVE-NbVND7-2* #33-6 and *pNbEF1 α -XVE-3 $\times$ VENUS-NLS* #7-1 were used as the representative lines in this study.

Interfamily grafting

Wedge grafting was performed on the stems of 4-week-old *N. benthamiana* and 5-week-old *Arabidopsis* plants as described previously (Notaguchi *et al.*, 2020). The *N. benthamiana* stems were cut and trimmed into a V-shape. The inflorescent stems of *Arabidopsis* were cut and split from the middle of the stem at the graft site. The V-shaped stem was inserted into a middle-split stem and fixed using a grafting clip (Cat: 0108007104001 for No. 26; Nasunics, Tochigi, Japan) to fix a graft union. The scions were covered with water-sprayed plastic bags and then grown in an incubator at 27 °C under continuous light (30 $\mu\text{mol m}^{-2} \text{s}^{-1}$) for 10 d. Afterwards, the plastic bags were uncovered, and the grafts were transferred to a 23 °C growth room with 30% relative humidity, and were grown under 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ continuous illumination.

Stem grafting was performed on 4-week-old *N. benthamiana* and chrysanthemum plants using grafting cassettes (GRA&GREEN, Nagoya, Japan). The grafts were covered with water-sprayed plastic bags and grown at 27 °C in a growth room under continuous light (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$) for 6 d. Afterwards, the plastic bags were removed from the grafts, and the grafts were transferred to a 22 °C growth room with 30% relative humidity, and were grown under 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ continuous illumination.

Chemical staining and microscopy

The GUS staining solution consisted of 10 mM phosphate-buffered saline (PBS) at pH 7, 0.1% Triton X-100, 10 mM EDTA, 0.5 mg ml⁻¹ X-Gluc, 0.5 mM potassium ferricyanide, and 0.5 mM potassium ferrocyanide. Plant tissues were placed in a 90% acetone solution for 5 min, rinsed in 1 mM PBS, transferred to GUS stain solution, and incubated at 37 °C for 6–12 h. Plant tissues were soaked in 70% ethanol to stop GUS staining, prepared as resin-embedded sections, or microscopically observed. The GUS-stained seedlings were observed using a zoom stereomicroscope (SZX10; Olympus, Tokyo, Japan) equipped with a digital camera (DP22; Olympus). Resin-embedded tissue sections were prepared using Technovit 7100 kits (Kulzer, Wehrheim, Germany) and cut using a rotary microtome (RX-860; Yamato Kohki, Saitama, Japan). The tissue sections were soaked in safranin-O stain solution, which was 2.5% (w/v) dissolved in 99% ethanol, for 5 min and observed under a microscope (BX53, Olympus) equipped with a digital camera (DP73, Olympus) for high-magnification images.

Fresh tissue sections were cut manually using a blade. For tracheary element observation, the tissue sections were soaked in advance with a mixed solution composed of 20 $\mu\text{g ml}^{-1}$ BF-170 (FUJIFILM Wako Chemicals, Osaka, Japan) solution. The excitation wavelength of the confocal laser scanning microscope (FV3000, Olympus) was set at 488 nm

for BF-170, with an emission barrier filter range of 500–550 nm. For cellular VENUS observation, a confocal laser scanning microscope (FV3000, Olympus) was used at an excitation wavelength of 513 nm, with an emission barrier filter range of 525–550 nm.

Chemical treatment of intact and grafted tissues

The β -estradiol (FUJIFILM Wako Chemicals) was dissolved in DMSO to prepare a 100 mM β -estradiol stock solution. A 50 μl aliquot of the β -estradiol stock solution was added into 1 liter of 1/2 MS medium (described above) to prepare the 5 μM β -estradiol medium (0.005% DMSO). Seedlings of β -estradiol-inducible lines were grown on the 1/2 MS medium for the first 7 d and transferred to the 5 μM β -estradiol-containing 1/2 MS medium and then grown for the next 3 d. A 5 μl aliquot of β -estradiol stock solution was added into 100 ml of distilled water to dilute to a 5 μM solution (0.005% DMSO). A 40 μl drop of the 5 μM β -estradiol solution was added to the graft union twice at 0 (soon after grafting) and 2 days after grafting (DAG), three times at 0, 2, and 4 DAG, or six times at 0, 2, 4, 6, 8, and 10 DAG. In the experiment for the measurement of the scion growth, a paste of lanolin (FUJIFILM Wako Chemicals) containing β -estradiol was used. Lanolin was heated to 60 °C, and 200 μl was mixed well with 0.1 μl of 100 mM β -estradiol stock solution or DMSO to obtain a 50 μM β -estradiol (0.05% DMSO) or 0.05% DMSO (v/v) lanolin mixture. A 5 μl aliquot of lanolin mixture was applied to the scion side of the grafted union at 0 DAG.

Measurement of toluidine blue transportation

Toluidine blue dye transport experiments were conducted using the *Nb/At* interfamily grafts. Toluidine blue dye was detected in scion stems at 1, 3, 5, and 7 DAG. The bottom of *Arabidopsis* inflorescence stems was cut and soaked in 0.5% (w/v) toluidine blue solution for 12 h. Cross-sections of scion stems were directly cut by hand with a blade and observed under a microscope (S9D, Leica, Osaka, Japan) equipped with a digital camera (DP22, Olympus).

Gene expression analysis

RNA-seq data were collected in this study under the accession number DRA009936. RNA-seq data were obtained from the DNA Data Bank of Japan (DDBJ; <http://www.ddbj.nig.ac.jp/>). Quantitative reverse transcription-PCR (qRT-PCR) was performed using the KAPA SYBR FAST qPCR Master Mix (2X) Kit (KAPA Biosystems, Wilmington, MA, USA) on a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific) with a standard protocol. Primers used for PCR amplification are listed in Supplementary Table S1.

Network analysis

Whole-genome Bayesian network analysis was conducted as in Huang *et al.* (2023). The normalized datasets of RNA-seq experiments in 24 *Nb/At* interfamily grafting, nine *Nb/Nb* self-grafting, and three intact *N. benthamiana* stems (Notaguchi *et al.*, 2020) were used to perform a Bayesian network estimation with the program SiGN-BN (<http://sign.hgc.jp/signbn/index.html>), implemented on the supercomputer system at the Human Genome Center of the University of Tokyo (<https://supcom.hgc.jp/english>) (Tamada *et al.*, 2010, 2011). After computing the whole-genome network with the SiGN-BN NNSR program, the subnetwork containing 2038 downstream genes of *NbVND7* homologs was extracted at the six levels. The estimated gene network was visualized using the gene network analysis software, Cytoscape (<https://cytoscape.org/>). Gene Ontology (GO) enrichment analysis was performed using DAVID (<https://david.ncicrf.gov>) with *Arabidopsis* gene IDs. Supplementary Table S2 shows the expression profiles of 2038 *NbVND7*

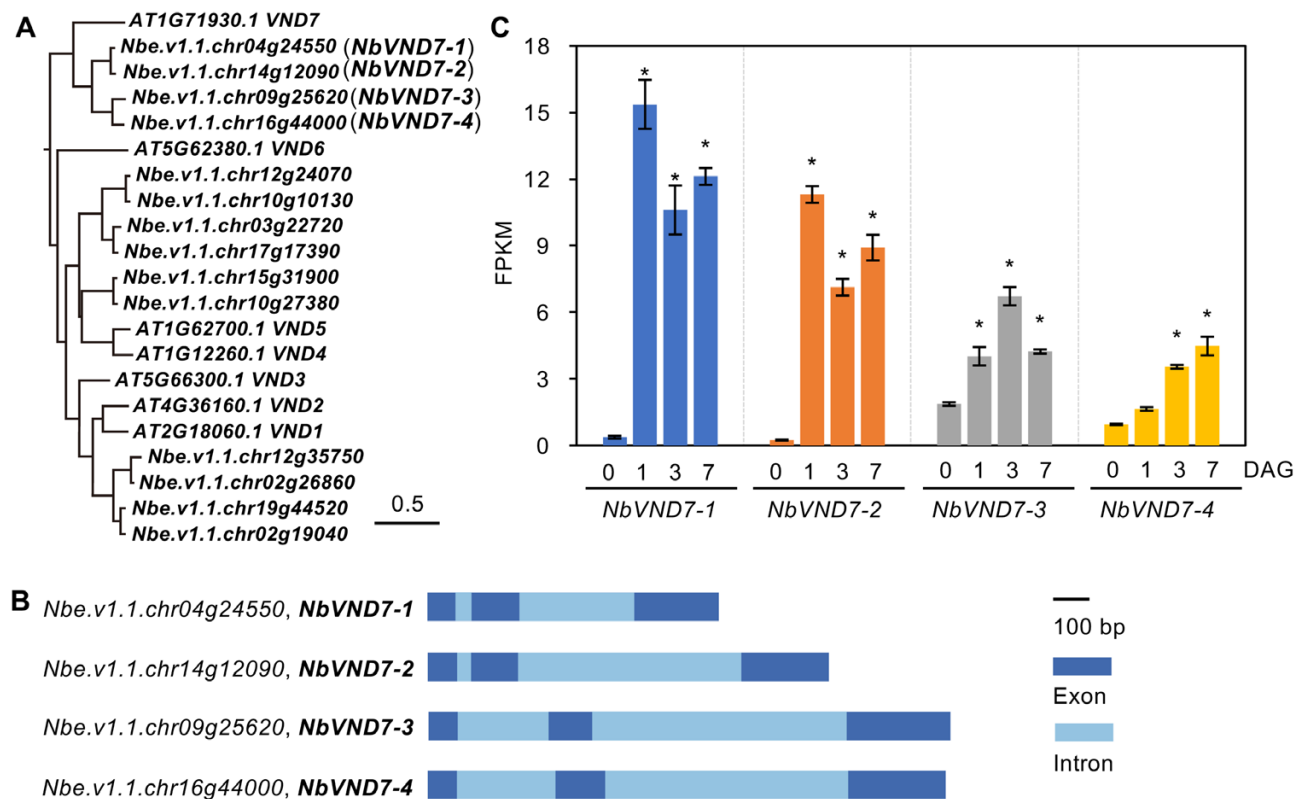


Fig. 1. Analysis of VND7 homologs in *N. benthamiana*. (A) Phylogenetic tree for VND7 homologs from Arabidopsis and *N. benthamiana* (*Nb*) using amino acid sequences in the Neighbor-Joining clustering method with ClustalW software. *NbVND7-1* to *NbVND7-4* represent four paralogous genes of Arabidopsis VND7 in *Nb*. (B) Schematic representation of the *NbVND7* coding sequence (CDS) structures. Sequence information from NbenBase (<https://nbenhamiana.jp>). The exons and introns were predicted using the Sol Genomics Network (<https://solgenomics.net/>). Scale bar=100 bp. (C) Expression of *NbVND7* genes during graft union formation. The transcription levels of genes in *Nb/At* interfamily grafting were estimated by RNA-seq at 0, 1, 3, and 7 days after grafting (DAG). 0 DAG indicates an intact plant without grafting (Notaguchi et al., 2020). FPKM, fragments per kilobase per million mapped fragments, used as a normalized unit to quantify transcript expression levels in RNA-seq data. Asterisks indicate significant differences determined by Student's *t*-test ($P < 0.05$). Error bars indicate the means $\pm$ SE ($n=3$).

downstream genes in the *Nb/At* interfamily and *Nb/Nb* homo grafting. Only data of intact plants corresponding to 0 DAG are presented, and the effect on gene expression by both grafting and the plants getting older could be included in the expression data.

Statistical analysis

Statistical analyses were conducted using the online platform available at https://astatsa.com/OneWay_Anova_with_TukeyHSD/. Student's *t*-test was used to determine the significance of differences. Tukey's Honest Significant Difference (HSD) test was applied for multiple comparisons. Data were assessed for normality and equal variance prior to analysis. A *P*-value < 0.05 was considered statistically significant, and a *P*-value < 0.01 was considered highly significant.

Results

Identification of VND7 homologs in *N. benthamiana*

To identify the VND homolog genes in *N. benthamiana*, we performed a phylogenetic analysis. In the phylogenetic tree, Arabidopsis VND1–VND6 correspond to 10 *Nb*

homologs and Arabidopsis VND7 corresponds to four *Nb* homologs. Thus, the phylogeny of VND genes in *N. benthamiana* is quite different from that in Arabidopsis, and *N. benthamiana* evolved a number of homologous genes closest to Arabidopsis VND7 (Fig. 1). The four Arabidopsis VND7 homologs, *Nbe.v1.1.chr04g24550*, *Nbe.v1.1.chr14g12090*, *Nbe.v1.1.chr09g25620*, and *Nbe.v1.1.chr16g44000* were denoted as *NbVND7-1*, *NbVND7-2*, *NbVND7-3*, and *NbVND7-4*, respectively (Fig. 1A). The amino acid sequences of Arabidopsis VND7 and *NbVND7*s were aligned to explore their similarities and biological functions. In the amino acid alignment, the closest homologs, *NbVND7-1* and *NbVND7-2*, showed a high identity of 94.4%, while the closest homologs, *NbVND7-3* and *NbVND7-4*, showed identity in 73.4%. The first 160 amino acid residues contain subdomains I–V of the NAC domain (Ko et al., 2007; Yamaguchi et al., 2008). Moreover, only the LP box (Ko et al., 2007) was conserved in *NbVND7* genes, and the WQ box was changed (Supplementary Fig. S1A). The prediction of protein structures of *NbVND7*s indicates some differences among these

homologs, but the NAC domains were highly conserved (Supplementary Fig. S1B, C). Based on the *N. benthamiana* genome sequence, we drew schematic structures for NbVND7-1 to NbVND7-4 (Fig. 1B). All NbVND7 genes contain three exons and two introns, NbVND7-1 and NbVND7-2 have shorter intron sequences than NbVND7-3 and NbVND7-4, and 'NbVND7-1 and NbVND7-2' and 'NbVND7-3 and NbVND7-4' are closer to each other. Next, we investigated the evolutionary aspects of NbVND7 homologs using the genome sequences of six *Nicotiana* species (Sierro *et al.*, 2014, 2024; Xu *et al.*, 2017; Kurotani *et al.*, 2023; Ranawaka *et al.*, 2023). All four NbVND7 homolog sequences were found in genomes of the two ecotypes of *N. benthamiana*: a commonly used lab strain and an associated wild accession, QLD (Sierro *et al.*, 2014, 2024; Kurotani *et al.*, 2025). NbVND7-1 and NbVND7-2 deviated significantly from the genes derived from a hybrid ancestor. NbVND7-3 is close to the VND7 homolog gene of *Nicotiana attenuata* which is close to *Nicotiana noctiflora*, an ancestor of *N. benthamiana*. NbVND7-4 is close to the VND7 homolog of *Nicotiana tabacum* and its ancestor, *Nicotiana sylvestris*. The other *N. tabacum* VND7 homolog genes were found to be close to the VND7 homolog genes of *Nicotiana tomentosiformis*, another parent species of *N. tabacum*, and those of its close relative, *Nicotiana otophora*, but they were not close to NbVND7 genes. Based on these facts, we speculated that NbVND7-1 and NbVND7-2 probably originated from NbVND7-4 through duplication and underwent further duplication in a very recent event with NbVND7-1 and NbVND7-2 (Supplementary Fig. S2). We continued to investigate the transcription patterns of NbVND7 genes during Nb/At interfamily grafting based on a previous database (Notaguchi *et al.*, 2020). Although all of the NbVND7 genes were up-regulated, NbVND7-1 and NbVND7-2 were up-regulated with higher levels than NbVND7-3 and NbVND7-4 (Fig. 1C). These results suggest that NbVND7 plays a role in the formation of graft unions.

NbVND7 genes are expressed in graft junctions

To investigate the expression patterns of NbVND7-1–NbVND7-4 in plant tissues, we generated promoter–GUS transgenic lines for each gene, named *pNbVND7-1::GUS* to *pNbVND7-4::GUS* (Supplementary Fig. S3). In 7-day-old seedlings, GUS expression was weak in the leaf veins of all of the lines (Supplementary Fig. S4). In 4-week-old plants, *pNbVND7-1::GUS* and *pNbVND7-2::GUS* directed GUS expression in the xylem tissues of intact stems (Fig. 2A, C), whereas slight GUS staining also appeared in the internal phloem of the *pNbVND7-1::GUS* line (Fig. 2A). By contrast, *pNbVND7-3::GUS* and *pNbVND7-4::GUS* did not direct GUS expression in the xylem of intact stems, but were strongly expressed in the internal phloem (Fig. 2E, G). Next, we established Nb/At interfamily grafts using these GUS fusion lines to verify that NbVND7 genes were induced during grafting.

Each of the *pNbVND7-1::GUS* to *pNbVND7-4::GUS* lines was grafted onto the Arabidopsis stock, and GUS expression at the graft junction was examined 3, 7, and 14 DAG. GUS staining was detected in the callus area of the scions at the graft junctions at 7 and 14 DAG in all four lines (Fig. 2B, D, F, H; Supplementary Fig. S5). Cells stained with GUS were clustered, which may imply that vascular tissue was formed in continuous cells, and cell differentiation in these continuous cells occurred simultaneously.

Network analysis revealed the downstream genes of NbVND7 genes

To investigate the regulation of four NbVND7 genes during grafting, Bayesian network analysis was performed using the same gene annotations to be consistent with the analysis executed in our previous study (Huang *et al.*, 2023). This network contained 2038 genes downstream of NbVND7 genes at six levels. Among them, 1846, 294, nine, and three downstream genes were regulated by NbVND7-1, NbVND7-2, NbVND7-3, and NbVND7-4, respectively (Fig. 3A; Supplementary Table S2). NbVND7-1 harbored most of NbVND7-2 and NbVND7-3 downstream genes. The NbVND7-4 downstream genes are not overlapped with the downstream genes of other NbVND7 genes. Taken together with the phylogenetic analysis, NbVND7-3 and NbVND7-4 may have functional modes different from those of NbVND7-1 and NbVND7-2. To investigate the potential functions of NbVND7-1 and NbVND7-2, we performed a GO term analysis. Downstream genes controlled by either NbVND7-1 or NbVND7-2 were subjected to GO analysis. In the bar chart showing the most significant 15 GO terms in each category, several xylem formation pathways are shown and marked in the biological process and cellular component sections. We hypothesized that NbVND7-1 and NbVND7-2 were involved in xylem formation (Fig. 3B). In particular, 'the programmed cell death (PCD) pathway' in biological processes and 'the cell wall-related pathway' in cellular components indicate the relationship to xylem formation. PCD and cell wall genes play crucial roles in the formation of tracheary elements, xylem vessels, and tracheids by eliminating the cellular content and promoting lignification to create hollow conduits for transportation (Demura and Fukuda, 2007; Yamaguchi *et al.*, 2011). Next, heat maps were created to show the expression profiles of the downstream genes during Nb/At interfamily grafting (Supplementary Fig. S6). The up-regulated genes were concentrated xylem genes, such as the homologous genes of *XYLEM CYSTEINE PEPTIDASE 1/2* (*XCP1/2*), which are involved in the PCD process of xylem vessel cells; the homologous genes of *MYB DOMAIN PROTEIN 83* (*MYB83*), which are involved in secondary cell wall biosynthesis; and the homologous genes of *METACASPASE 9* (*AtMC9*), which are involved in the degradation of vessel cell contents after vacuolar rupture (Supplementary Fig. S6; Supplementary Table S2).

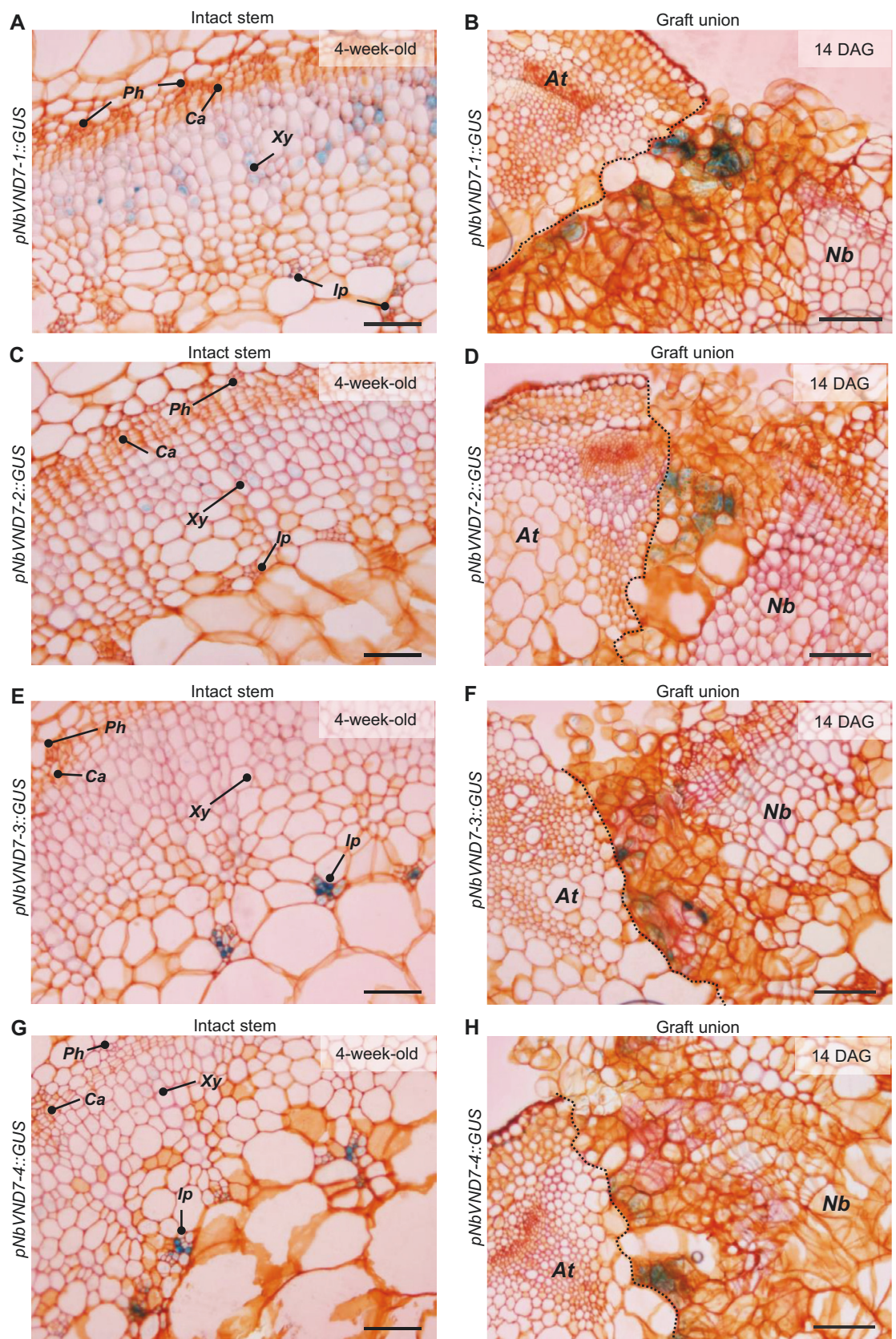


Fig. 2. Expression patterns of *NbVND7* genes. (A) The *NbVND7-1* promoter directs GUS expression in 4-week-old intact plant stems. A putative promoter from *Nbe.v1.1.chr04g24550* was selected for constructing *pNbVND7-1::GUS* for the generation of transgenic plants. The cross-sections

were stained by Safranin-O. (B) The *NbVND7-1* promoter directs GUS expression during *Nb/At* interfamily graft union formation at 14 DAG. Transgenic plants (as mentioned in A) were used for interfamily grafting. The dashed lines indicate the contact surface of *N. benthamiana* and *Arabidopsis* in cross-section. (C–H) The promoters of *NbVND7-2*, *NbVND7-3*, and *NbVND7-4* direct GUS expression in stem and graft unions. Putative promoters of *Nbe.v1.1.chr14g12090*, *Nbe.v1.1.chr09g25620*, and *Nbe.v1.1.chr16g44000* were used to construct *pNbVND7-2::GUS*, *pNbVND7-3::GUS*, and *pNbVND7-4::GUS*, individually. The same experiment was performed as in (A) and (B). Ph, phloem; Ca, cambium; Xy, xylem; Ip, internal phloem; At, *Arabidopsis*; Nb, *N. benthamiana*. Scale bars, 50 μ m (A, C, E, and G); or 100 μ m (B, D, F, and H).

NbVND7-2 induced xylem vessel differentiation

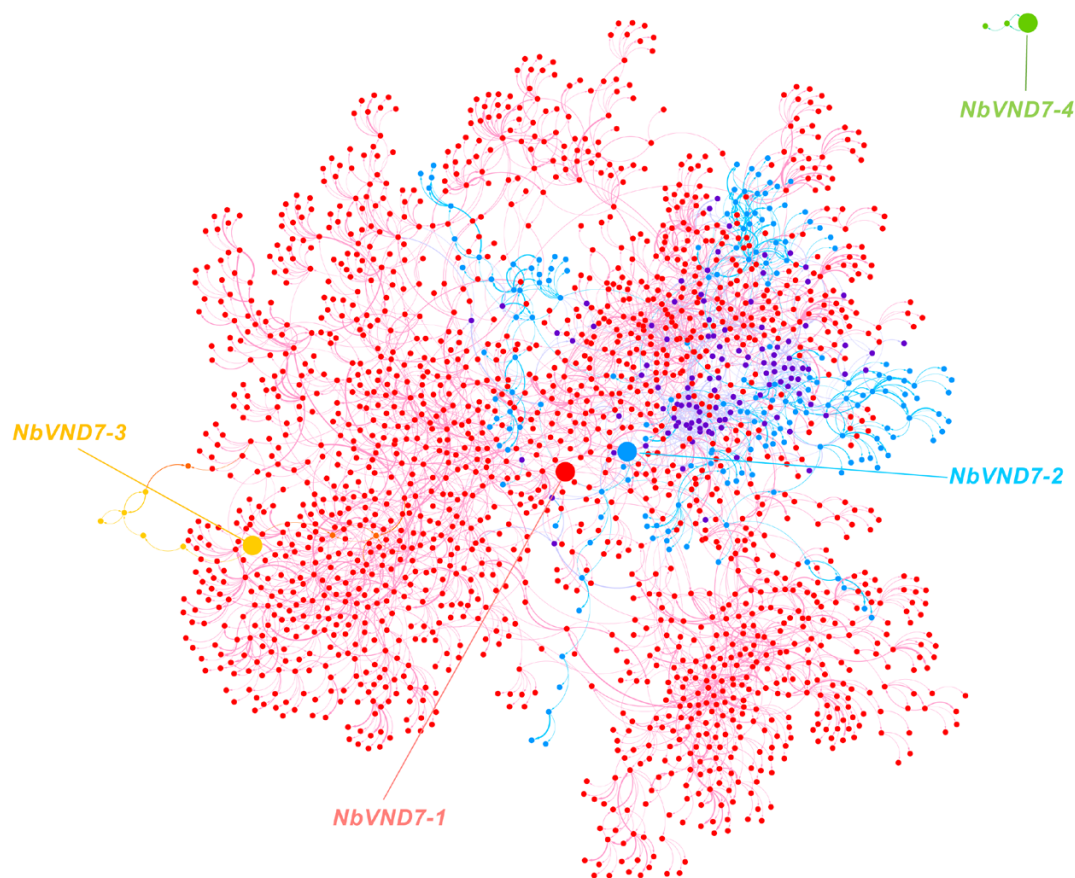
In *Arabidopsis VND7*, a single knockout or knockdown cannot generate detectable phenotypes (Kubo *et al.*, 2005). We also attempted to knock down *NbVND7* genes but did not detect a phenotype in xylem formation. The *NbVND7-2* promoter directed GUS expression highly specifically in the xylem, therefore we generated overexpression transgenic plants to investigate the function of *NbVND7-2*. The XVE-induced gene expression system, using an assembly of the bacterial repressor LexA (X), the acidic transactivating domain of VP16 (V), and the regulatory region of the human estrogen receptor (E) (Zuo *et al.*, 2000), was used for the ectopic overexpression of *NbVND7-2* in *N. benthamiana* (Fig. 4A). In the XVE plasmid vector, a putative promoter of *NbEF1 α* (*Nbe.v1.1.chr17g33070*) was for used directing *NbVND7-2* or *3 $\times$ VENUS-NLS* ectopic expression. *NbEF1 α* was also used as a reference gene because of its stable expression in both plant and grafted tissues (Supplementary Fig. S7). The experiments were conducted on an experimental line harboring *pNbEF1 α -XVE-NbVND7-2* and a control line harboring *pNbEF1 α -XVE-3 $\times$ VENUS-NLS*. In the β -estradiol-treated *pNbEF1 α -XVE-3 $\times$ VENUS-NLS* seedlings, the fluorescence of VENUS was detected in the cell nuclei of leaves, hypocotyls, and roots (Fig. 4B). Then, in the same manner, β -estradiol was used to treat *pNbEF1 α -XVE-NbVND7-2* transgenic plants, and xylem formation was observed using BF-170 dye that can visualize lignin surrounding xylem cells (Nurani *et al.*, 2020). The ectopic xylem vessels with spiral patterns in secondary cell walls were differentiated from the cells of the leaves, hypocotyls, and roots (Fig. 4C). We assessed the dose dependency in these XVE lines. In the *pNbEF1 α -XVE-3 $\times$ VENUS-NLS* plants, 5 nM or higher concentrations of β -estradiol induced a nuclear-localized VENUS fluorescent signal in the root and the hypocotyl. In contrast, 10 nM concentrations of β -estradiol induced ectopic xylem vessel formation in the root of *pNbEF1 α -XVE-NbVND7-2* plants and only 1000 nM concentrations of β -estradiol induced ectopic xylem vessels in the hypocotyl (Supplementary Fig. S8). Among spiral, reticulate, and pitted secondary wall patterns of xylem vessel cells, spiral patterns were prevalent for the ectopic xylem cells differentiated by *NbVND7-2* induction. Consistent with the dose-dependent manner of xylem vessel differentiation in the *pNbEF1 α -XVE-NbVND7-2* seedlings, β -estradiol at concentrations >50 nM inhibited seedling growth (Supplementary Fig. S9). Next, we tested whether the β -estradiol induction system also worked in the graft junction regions in the *Nb/At* grafts. After treatment

twice (at 0 and 2 DAG) with a 40 μ l drop of 5 μ M β -estradiol to the *pNbEF1 α -XVE-3 $\times$ VENUS-NLS* scion near the graft junction, VENUS fluorescence was detected in the nuclei of the scion tissues at the graft boundary at 3 DAG (Fig. 4D). By contrast, the same two β -estradiol treatments of the *pNbEF1 α -XVE-NbVND7-2* scion induced ectopic xylem vessels that were differentiated from callus tissues, and were transdifferentiated from the parenchyma cells in the scion tissues of the graft boundary (Fig. 4E), whereas DMSO treatment did not promote ectopic xylem formation in the same line (see also below). In the *pNbEF1 α -XVE-NbVND7-2/At* grafts, ectopic tracheary elements exhibiting spiral secondary wall patterns were newly differentiated at the graft boundary at 1 d after *NbVND7-2* induction or later (Fig. 4F, G). Taken together, it is suggested that the XVE β -estradiol induction system can activate exogenous gene expression in the intact and grafted *N. benthamiana* plants, and ectopic *NbVND7-2* expression induces xylem vessel differentiation in various types of plant cells.

Overexpression of *NbVND7-2* in the graft union enhanced scion growth

To investigate whether xylem differentiation induced by *NbVND7-2* improves water transport from the stock to the scion in the *Nb/At* grafts, a *pNbEF1 α -XVE-NbVND7-2* *N. benthamiana* transgenic plant was grafted onto *Arabidopsis*. A 40 μ l drop of 5 μ M β -estradiol or the solvent DMSO was used to treat the scion stem near the graft junction twice at 0 and 2 DAG, three times at 0, 2, and 4 DAG, or six times at 0, 2, 4, 6, 8, and 10 DAG. In the case of β -estradiol which was used in six treatments, the scion died (Supplementary Fig. S10A). Transcriptional induction by 0, 0.5, 5, and 50 μ M β -estradiol treatment was dose dependent in *pNbEF1 α -XVE-NbVND7-2* (Supplementary Fig. S10B). Then, we chose an induction condition: adding a 40 μ l drop of 5 μ M β -estradiol twice at 0 and 2 DAG. To assess the water transportation in the *Nb/At* grafts, toluidine blue was used as an indicator of water uptake and the transport from the stock to the scion. Toluidine blue solution was absorbed from the cut stem of the *Arabidopsis* stock and was detected in the *N. benthamiana* scion stem tissues of the graft union area and upper region (Fig. 5A). Toluidine blue occurrence in the *N. benthamiana* scion was investigated at 1, 3, 5, and 7 DAG. In the grafts treated with DMSO, toluidine blue detection started from 5 DAG. However, in the β -estradiol-treated grafts, toluidine blue detection started from 3 DAG and the occurrence of toluidine blue-detectable grafts

A



B

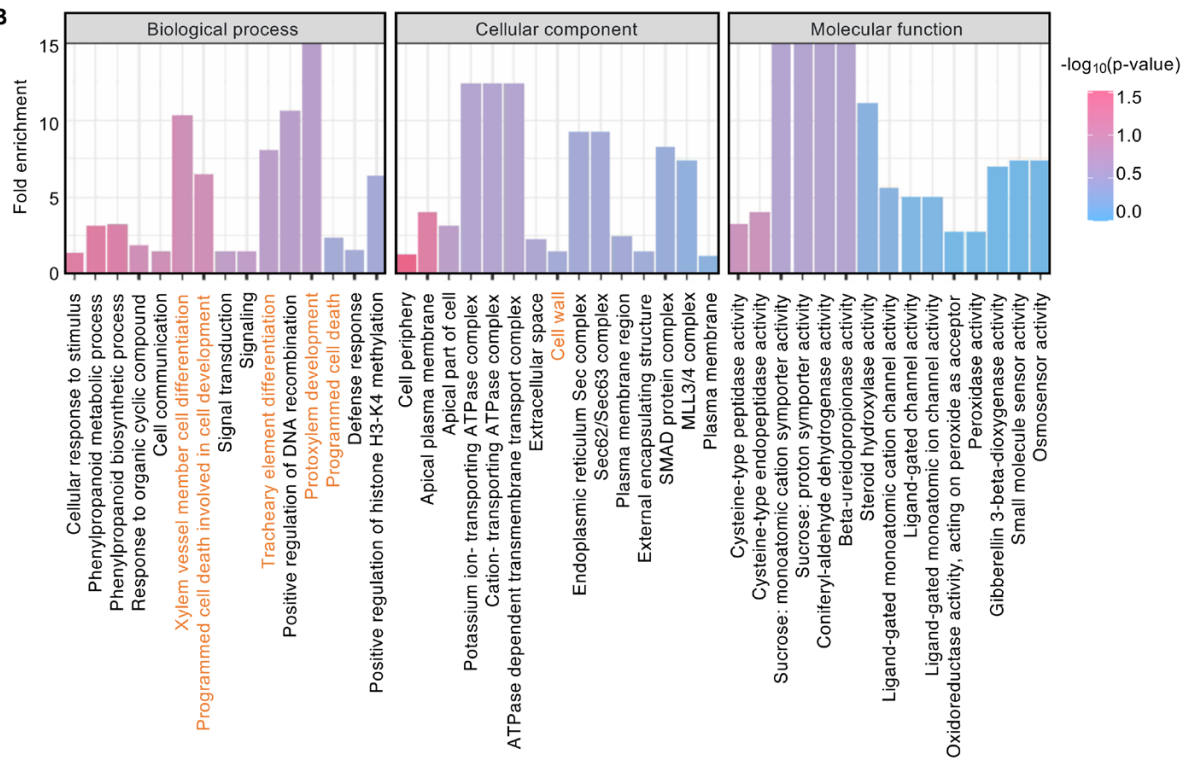


Fig. 3. Analysis of downstream genes of *NbVND7-1*, *NbVND7-2*, *NbVND7-3*, and *NbVND7-4* during *Nb/At* interfamily grafting. (A) A network diagram of the 2038 genes located downstream of the four *NbVND7* genes at six levels is shown. A whole-genome Bayesian network was created from the

gene expression profiles of *N. benthamiana* in Huang *et al.* (2023). Red dots and arrowed lines present the gene network downstream of *NbVND7-1*; blue dots and arrowed lines the gene network downstream of *NbVND7-2*; yellow dots and arrowed lines the gene network downstream of *NbVND7-3*; green dots and arrowed lines the gene network downstream of *NbVND7-4*; purple dots and arrowed lines the gene network downstream of *NbVND7-1* and *NbVND7-2*; and orange dots and arrowed lines the genes that were regulated in *NbVND7-1* and *NbVND7-3* networks. (B) Gene Ontology (GO) enrichment analysis for *NbVND7-1* and *NbVND7-2* downstream genes. The most significant 15 GO terms in each category are shown. Xylem formation-related pathways are marked in orange.

was increased at 5 DAG when compared with DMSO-treated grafts (Fig. 5A). These results imply that water transportation from the stock to the scion was enhanced by ectopic xylem formation through *NbVND7-2* induction at the graft region. We next tested whether *NbVND7-2* induction at the graft region can improve *N. benthamiana* scion growth following interfamily grafting. To maintain the chemical concentration for a long time, lanolin was used as solvent and DMSO or 50 μ M β -estradiol pre-mixed lanolin was pasted once onto the scion stem near the graft union soon after grafting. We first confirmed the effect of lanolin treatment by observation of ectopic xylem vessel cell formation at the graft junction only in estrogen-treated grafts, but not in DMSO-treated grafts (Fig. 5B). Compared with DMSO treatment, β -estradiol treatment significantly increased ectopic xylem formation at the graft junction of the *pNbEF1 α -XVE-NbVND7-2/At* grafts at 1, 3, and 5 DAG (Fig. 5C). We detected *XVE*-induced exogenous *NbVND7-2* expression after β -estradiol treatment at the graft union region (Fig. 5D). To investigate whether the scion growth is influenced by ectopic xylem formation at the graft boundary, we measured scion growth after grafting in DMSO or β -estradiol-treated grafts. The newly grown part of the scion after grafting was longer following β -estradiol treatment at 35 DAG or later compared with the DMSO treatment (Fig. 5E). In the case where a *pNbEF1 α -XVE-3 $\times$ VENUS-NLS* transgenic plant was used as a scion, β -estradiol treatment of the transgenic line did not promote scion growth (Supplementary Fig. S11). These results suggest that the scion growth after grafting is dependent on water transport efficiency, which is influenced by xylem vessel formation, and that the use of *NbVND7-2* to induce xylem formation can improve *N. benthamiana* interfamily grafting. We then examined the effect of *NbVND7-2* induction in another interfamily graft combination, *pNbEF1 α -XVE-NbVND7-2 N. benthamiana* scion and *Chrysanthemum morifolium* stock (*Nb/Cm*). In the *Nb/Cm* grafts, the *Nb* scions grew dramatically taller with β -estradiol treatment compared with the case of DMSO mock treatment (Supplementary Fig. S12).

Discussion

Nicotiana benthamiana is a species of the tobacco genus of the *Solanaceae* family and is genetically similar to other members of the *Solanaceae* family, such as eggplants and tomatoes. Genome sequence analysis revealed the complexity of the *N. benthamiana* genome after branching from the ancestral species, and

comprehensive genome information promotes its wide use as a model plant for dicotyledonous angiosperms (Bally *et al.*, 2018; Kurotani *et al.*, 2023). Recent previous studies revealed that *N. benthamiana* is one of the plants that could establish interfamily grafting. *Glycosyl hydrolase 9B3* (*GH9B3*) encoding β -1, 4-glucanase in *N. benthamiana* was important to achieve cell–cell adhesion at the graft boundary after grafting (Notaguchi *et al.*, 2020; Kurotani and Notaguchi, 2021). Homologs of *GH9B3* in petunia and parasitic plants could also play roles in cell–cell adhesion (Kurotani *et al.*, 2020, 2022), however *GH9B3* paralogs in pepper lack this function (Luo *et al.*, 2022). Functional differences exist among species, even among conserved homologous genes. Phylogenetic analyses identified two genes as potential orthologs of *VND6* and two genes as potential orthologs of *VND7* in tomatoes. Only one of these four genes was expressed in the xylem and was determined to be the most likely functional ortholog of *VND6*, termed *Solanum lycopersicum VND6* (*SlVND6*) (Kajala *et al.*, 2021). In the present study, we identified four *N. benthamiana* genes as potential orthologs of *VND7* using phylogenetic analysis (Fig. 1A). Two of these four genes were expressed in the xylem (Fig. 2), and one gene (*NbVND7-2*) was confirmed to be involved in ectopic xylem formation (Fig. 4C). Two genes, *NbVND7-1* and *NbVND7-2*, were possibly derived from genes that have been duplicated since the establishment of *N. benthamiana*, whereas the other two genes were likely to have retained sequences inherited from their respective ancestral species (Supplementary Fig. S2). Recent genomic studies have revealed the evolutionary relationships between several *Nicotiana* species, and they have explored the conservation and innovation of genes during evolution (Kurotani *et al.*, 2023; Ranakawa *et al.*, 2023; Wang *et al.*, 2023, 2024). For example, only *Dicer-like 2* (*DCL2*) was lost owing to chromosome fragment loss in *N. tabacum*, whereas *Argonaute 2* (*AGO2*), *RNA-dependent RNA polymerase 6* (*RDR6*), and *DCL3* were lost as single genes in *N. benthamiana* (Wang *et al.*, 2024). *Nicotiana benthamiana* has two additional copies of the *VND7* gene, in addition to the two *VND7* genes from the ancestral species that form allotetraploids. Similarly, *N. tabacum*, an allotetraploid but a relatively recent hybrid, showed no gene amplification. Furthermore, the sequence variation found among the three inbred varieties of *N. tabacum* was small, whereas complexity was added in *N. benthamiana*, even among ecotypes (Supplementary Fig. S2). The rate of genome evolution is high in *Nicotiana*, and this means that it is possible that diversification in duplication, mutation, and expression regulation is more likely to occur (Ranakawa *et al.*, 2023;

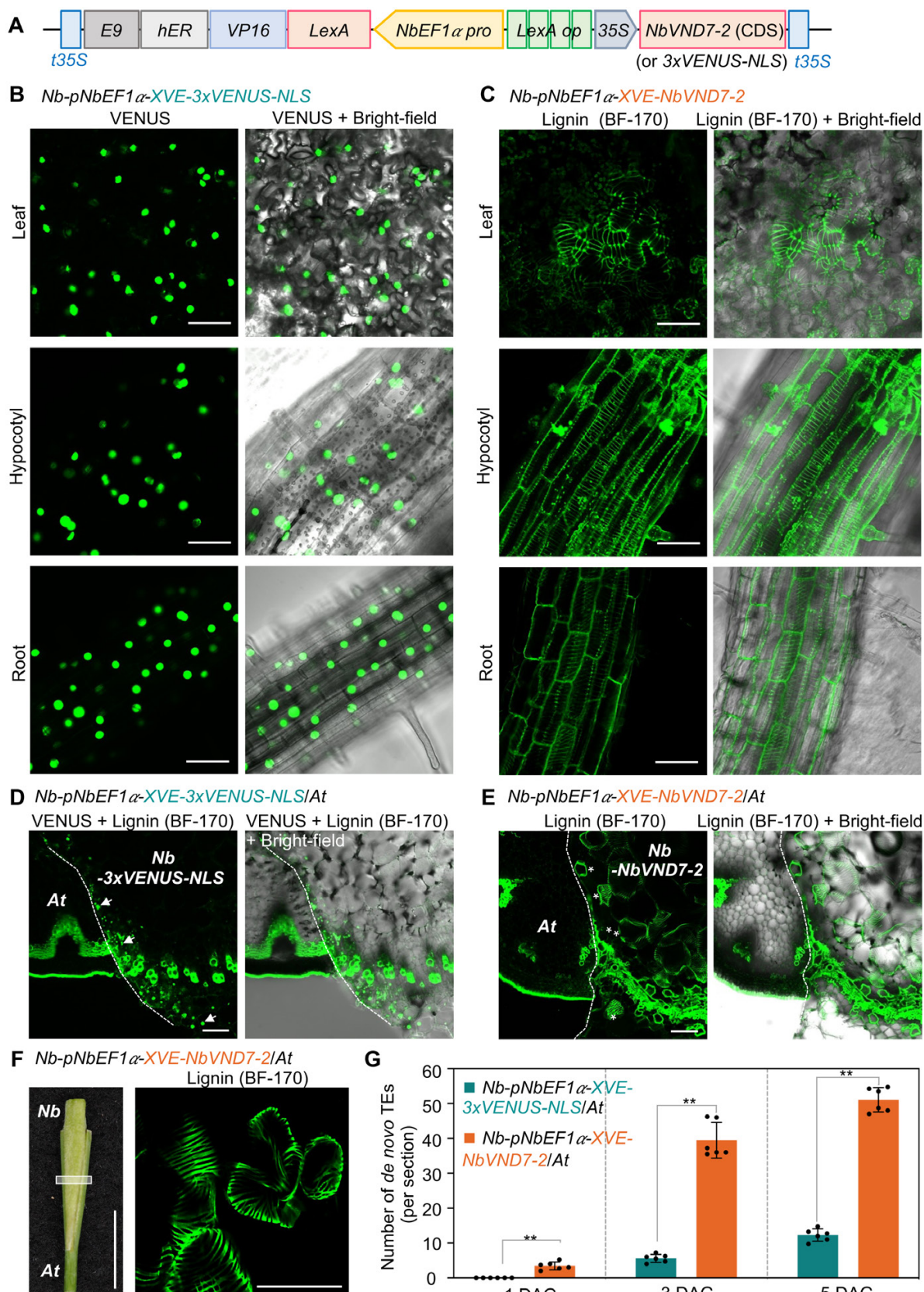


Fig. 4. NbVND7-2 promotes ectopic xylem formation in XVE plants. (A) Schematic diagram of the XVE vectors. Experimental lines harbored *pNbEF1α-XVE-NbVND7-2*, and control lines harbored *pNbEF1α-XVE-3xVENUS-NLS*. NbVND7-2 (CDS), coding sequence of NbVND7-2 (*Nbe.v1.1.chr14g12090*).

3×VENUS-NLS, three tandem modified variant of the yellow fluorescent protein- (VENUS) fused nuclear localization sequence (NLS); *t35S*, 35S terminator derived from cauliflower mosaic virus (CaMV); *E9*, terminator of the pea Rubisco small subunit (*rbcS*) *E9* gene, which functions to direct the polyadenylation of mRNA; *hER*, the human estrogen receptor; *VP16*, transcription activation domain from herpes virus protein; *LexA*, DNA-binding domain (DBD) of the LexA repressor; *NbEF1α pro*, the putative promoter of *Nbe.v1.1.chr17g33070*; *LexA op*, a synthetic operator containing four ColE1 operator sequences, which is activated by binding with the LexA DBD (Ebina et al., 1983; Emelyanov and Parinov, 2008; Gillor et al., 2008); 35S, CaMV 35S minimal promoter. (B and C) β-Estradiol treatment in *Nb-pNbEF1α-XVE-3×VENUS-NLS* and *Nb-pNbEF1α-XVE-NbVND7-2* plants. Nuclear signals were detected in 10-day-old *Nb-pNbEF1α-XVE-3×VENUS-NLS* seedlings. Ectopic xylem vessel cells [tracheary elements (TEs)] were observed in 10-day-old *Nb-pNbEF1α-XVE-NbVND7-2* seedlings. BF-170 was used for staining the xylem secondary cell walls. Scale bar: 50 μm. (D and E) Observation of graft unions with β-estradiol treatment at 3 DAG. The XVE transgenic plants were used in establishing *Nb/At* interfamily grafts. The dashed lines indicate the contact surface of *Nb* and *At*. Arrows indicate the nuclei. Asterisks indicate the ectopic TEs. Scale bars: 100 μm. *Nb*, *N. benthamiana*; *At*, *Arabidopsis*. (F) Ectopic TEs differentiated from the cells at the *Nb/At* graft union. TEs exhibiting spiral secondary wall patterns were found in a BF-170-stained cross-section around the middle area of the graft union (white frame) at 3 DAG. Scale bars: 1 cm in left panel and 100 μm in right panel. (G) The numbers of *de novo* ectopic TEs differentiated at the graft union were counted at 1, 3, and 5 DAG in β-estradiol-treated *Nb-pNbEF1α-XVE-3×VENUS-NLS/At* and *Nb-pNbEF1α-XVE-NbVND7-2/At*. Six grafts for each line and 4–6 cross-sections per graft were examined. The cross-sections were prepared from the middle area of the graft union as shown in (F). Asterisks indicate significant differences determined by Student's *t*-test (***P*<0.01). Error bars indicate the mean ±SE.

Wang et al., 2024). Although bioinformatics methods can estimate the functions of homologous genes in different species, it is still necessary to verify this hypothesis through specific experiments.

The plant vascular system is composed of two main types of tissues: xylem and phloem. Within the phloem, there are two distinct types of tissues: external and internal. The external phloem is located on the outer side of the vascular bundle, whereas the internal phloem is located within the bundle itself (West et al., 1999; Van Bel and Hafke, 2011). The internal phloem can be clearly observed in the stems of *Solanaceae* plants such as tobacco, tomato, and eggplant (Esau, 1938; Thompson and Heimsch, 1964; Xu et al., 2001). In terms of function, both external and internal phloem are involved in the transport of photosynthetic products. However, relevant studies that distinguish between these roles are lacking. Related research on *N. tabacum* revealed that *Sieve Element Occlusion* (SEO) genes encode structural phloem proteins involved in wound sealing. SEO is expressed in both the external and internal phloem (Ernst et al., 2012). In our cross-sectional observations, the putative promoters of *NbVND7-3* and *NbVND7-4* directed GUS, which was clearly expressed in the internal phloem, rather than in the external phloem (Fig. 2). Currently, there are no reports of *VND* genes expressed in the phloem. Thus, we have hypothesized the following: (i) the internal phloem area contains xylem-like structures that are regulated by *NbVND7* and (ii) the formation of an internal phloem is uniquely controlled by *NbVND7* in *N. benthamiana*. These uncertain factors in plant structure need to be confirmed in future studies.

Two main inducible expression systems have been developed for *Arabidopsis VND7* that use different compounds (dexamethasone or estrogen) to induce gene overexpression. Based on expression systems, researchers have verified that the *Arabidopsis VND7* protein can promote the ectopic differentiation of xylem-like cells (Yamaguchi et al., 2010; Zhong et al., 2010; Endo et al., 2015). We redesigned XVE plasmid vectors to investigate whether *NbVND7-2* also can induce ectopic differentiation of xylem vessels (Figs 4, 5). *Arabidopsis VND7* and

NbVND7-2 had 50.6% identity in the alignment of the full-length amino acid sequence, and the high identity part was concentrated in amino acid residues 1–160 (Supplementary Fig. S1). Among the first 160 amino acid residues, *NbVND7-1* and *NbVND7-2* had almost identical sequences. Though we did not directly examine the role of *NbVND7-1* in xylem vessel differentiation in this study, we surmised a similar function for *NbVND7-1* and *NbVND7-2* based on their amino acid sequences. Because of the absence of sequences in regard to the IV and V subdomains of NAC, *NbVND7-3* may play different roles, such as in the internal phloem. We considered that the first 160 amino acid residues, rather than the LP box, were more critical for inducing xylem cell differentiation. Together with the gene network analysis (Fig. 3) and the promoter activity analysis (Fig. 2), we found that *NbVND7-1* and *NbVND7-2* were more strongly associated with xylem vessel differentiation than *NbVND7-3* and *NbVND7-4*; in particular, *NbVND7-2* was specifically involved in xylem formation. The *Arabidopsis VND7* transcription factor regulates downstream genes to form xylem cells (Yamaguchi et al., 2011), and potential downstream genes of *NbVND7* genes were up-regulated in both *Nb/At* interfamily and *Nb/Nb* homo grafts (Supplementary Fig. S6), suggesting that *NbVND7* genes are involved in these grafts. Since the expression level of *NbVND7* genes in *Nb/At* interfamily grafts was higher than that in *Nb/Nb* homo grafts (Notaguchi et al., 2020), it is possible that *NbVND7* genes exert their function more in the interfamily grafts, which is a more stressful situation than homo grafts. In this study, we found a positive effect of *NbVND7-2* induction in interfamily grafting, but the effect in homo grafting is still unknown.

Chemical compounds have been used to improve the success rate and efficiency of grafting processes. These compounds can be applied directly to the grafting site and used as pre-treatments for the scion or rootstock to stimulate callus formation, increase vascular tissue formation, and improve the survival of grafted plants (Maurya et al., 2019; Xu et al., 2022; Zhu et al., 2024). In the actual production of plant grafts, certain

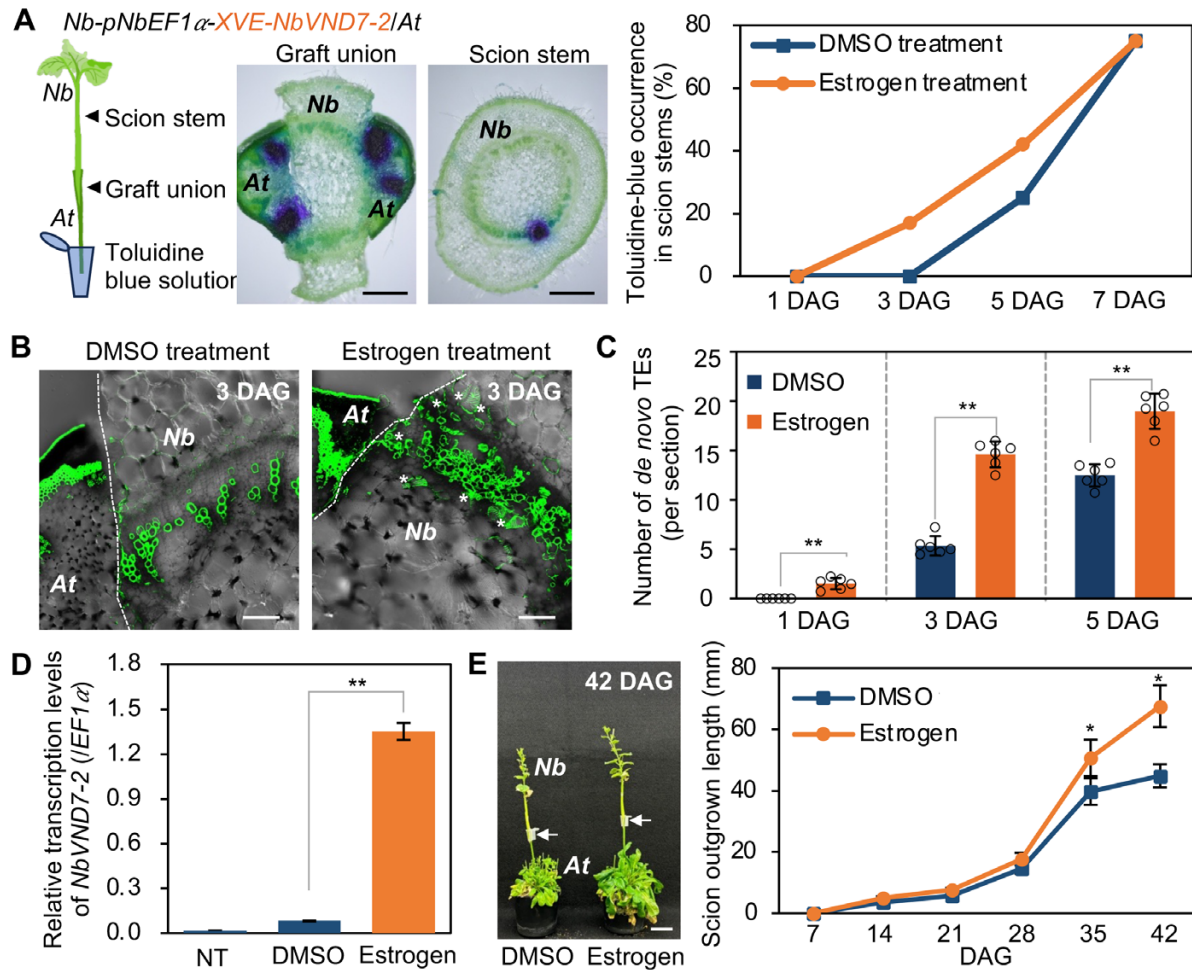


Fig. 5. Ectopic overexpression of *NbVND7-2* enhances scion growth in *Nb/At* interfamily grafts. (A) Measurement of the vascular tissue reconnection in the interfamily grafts of *pNbEF1 α -XVE-NbVND7-2* transgenic *N. benthamiana* (*Nb*) and wild-type *Arabidopsis* (*At*). The schematic diagram presents the graft treated in toluidine blue solution (0.5% w/w). The left photo is a cross-section of the scion stem, and the right photo is a cross-section of the graft junction. Dark blue indicates the point of transportation of toluidine blue. The frequency of toluidine blue detection in the scion was measured at 1, 3, 5, and 7 DAG. Twelve grafts were examined for each time point studied, except for 1 DAG where three grafts were examined. Estrogen treatment was β -estradiol dissolved in DMSO solvent and mixed into lanolin, then painted on the graft union. DMSO treatment was a control. (B) Formation of ectopic tracheary elements (TEs) in β -estradiol-treated *Nb-pNbEF1 α -XVE-NbVND7-2/At* at 3 DAG. Representative cross-sections were stained by BF-170. The dashed lines indicate the contact surface of *Nb* and *At*. Asterisks indicate ectopic xylem vessel cells (TEs). Scale bars: 100 μ m. (C) The number of *de novo* ectopic TEs at the graft union in DMSO- or β -estradiol-treated *Nb-pNbEF1 α -XVE-NbVND7-2/At* at 1, 3, and 5 DAG. Six grafts for each treatment and 4–6 cross-sections per graft were examined. The cross-sections were prepared from the middle area of the graft union. Asterisks indicate significant differences determined by Student's *t*-test (** $P < 0.01$). (D) Transcription levels of exogenous *NbVND7-2* examined by qRT-PCR analysis using samples of the untreated *Nb* stem (NT) and the DMSO- and estrogen-treated *Nb/At* graft unions at 3 DAG. Asterisks indicate significant differences determined by Student's *t*-test (** $P < 0.01$). Error bars indicate the mean $\pm$ SE ($n=3$). (E) Scion growth of the *Nb-pNbEF1 α -XVE-NbVND7-2* line grafted on *At* at 42 DAG. Scale bars: 3 cm. The length of the newly grown part of the *Nb* scion was measured at 7, 14, 21, 28, 35, and 42 DAG and plotted. Arrows indicate the graft unions. Error bars indicate the means $\pm$ SE ($n=33$ –36 for each time point). Asterisks present significant differences determined by Tukey's HSD test (* $P < 0.05$).

compounds, such as auxins, cytokinins, and gibberellins, have been widely studied and applied (Yin et al., 2012; Ding et al., 2013; Cui et al., 2021). Previous research revealed β -estradiol influencing plant growth at different concentrations (Shore et al., 1992; Adeel et al., 2017). However, we did not find an influence of β -estradiol on seedling growth or post-grafting growth of the scion in the *pNbEF1 α -XVE-3 $\times$ VENUS-NLS*

(Supplementary Figs S9, S10). This implied that estrogen itself does not affect plant grafting but it influences grafting by activating an exogenous gene (*NbVND7-2*) in the XVE plasmid vectors. Our previous study revealed that *de novo* tracheary elements in a graft union can enhance graft establishment and scion growth (Huang et al., 2023), and the current study once again demonstrated that xylem cell differentiation

can promote scion growth during interfamily grafting (Fig. 5; Supplementary Fig. S12). In our study, when large amounts of β -estradiol were directly added to the grafted union, the scion from the *XVE-NbVND7-2* line died rapidly within 14 DAG (Supplementary Fig. S10A). This was the same as found in Arabidopsis research: excessive ectopic differentiation of xylem vessels causes plant cells to lose their normal functions, resulting in plant death (Yamaguchi *et al.*, 2010). There is a balance range in plant cell differentiation, which determines whether to promote or inhibit plant growth and development. Additional experiments are still needed to confirm the quota of differentiated xylem cells that are ideal for plant grafting.

Supplementary data

The following supplementary data are available at [JXB online](#).

Table S1. Transcription levels of *NbVND7* downstream genes.

Table S2. Sequences of the primers used in the experiments.

Fig. S1. Structures of VND7 homologs in *N. benthamiana*.

Fig. S2. Evolutionary analysis for *NbVND7* genes.

Fig. S3. Schematic representation of the GUS fusion constructs.

Fig. S4. GUS expression analysis of *pNbVND7::GUS* in 7-day-old seedlings.

Fig. S5. Expression of *NbVND7* genes at graft junctions.

Fig. S6. Expression profiles of downstream genes of *NbVND7* genes during *Nb/At* interfamily and *Nb/Nb* homo grafting.

Fig. S7. *NbEF1 α* expression during *Nb/At* and *Nb/Nb* grafting.

Fig. S8. Dose dependency of β -estradiol induction in *pNbEF1 α -XVE-3 $\times$ VENUS-NLS* and *pNbEF1 α -XVE-NbVND7-2* *N. benthamiana* transgenic seedlings.

Fig. S9. Growth of *pNbEF1 α -XVE-3 $\times$ VENUS-NLS* and *pNbEF1 α -XVE-NbVND7-2* *N. benthamiana* transgenic seedlings on estrogen-containing medium.

Fig. S10. Effective dosage and concentration for estrogen treatment.

Fig. S11. Measurement of scion growth of the *N. benthamiana* *pNbEF1 α -XVE-3 $\times$ VENUS-NLS* line grafted on Arabidopsis.

Fig. S12. Measurement of the scion growth of the *N. benthamiana* *pNbEF1 α -XVE-NbVND7-2* line grafted on *Chrysanthemum morifolium*.

Author contributions

CH, KK, and MN: conceived the study and designed the experiments; CH and KK: analyzed the transcriptome data; KK: conducted the network analyses; CH, KT, EM, and AI: generated the transgenic plants; CH, KT, EM, and AI: performed the grafting experiments and microscopy analysis; CH, KT, KK, and MN: wrote the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Data availability

All of the relevant data and figures in this study can be found in the article and its supplementary data.

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