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RESEARCH PAPER

Modulation of NAC transcription factor NST1 activity by XYLEM NAC DOMAIN1 regulates secondary cell wall formation in Arabidopsis

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Abstract

In Arabidopsis, secondary cell walls (SCW) are formed in fiber cells and vessel cells in vascular tissue for providing plants with mechanical strength and channels for the long distance transportation of water and nutrients. *NAC SECONDARY WALL THICKENING PROMOTING FACTOR1* (*NST1*) acts as a key gene for the initiation of SCW formation through a hierarchical transcription network. In this study, we report that NST activity is modulated by the NAC domain transcription factor XYLEM NAC DOMAIN1 (XND1) during plant growth. Using yeast two-hybrid screening and *in vivo* protein interaction analysis, XND1 was identified as an NST-interacting protein that modulates NST1 activity. XND1 and NST1 were co-localized in the nucleus and the interaction of XND1 with NST1 resulted in inhibition of NST1 transactivation activity. In the process of inflorescence growth, *XND1* was expressed with a similar pattern to *NST1*. Up-regulation of *XND1* in fiber cells repressed SCW formation. The study demonstrates that NST1 activity is modulated by XND1 in the regulation of secondary cell walls formation.

Keywords: Arabidopsis, fiber cells, NAC SECONDARY WALL THICKENING PROMOTING FACTOR 1, protein interaction, secondary cell wall, XYLEM NAC DOMAIN 1.

Introduction

In higher plants, vessel cells and fiber cells in xylem tissue form thickened secondary cell walls (SCWs) to provide for long distance transport of water and nutrients and provide mechanical strength for support (Kumar *et al.*, 2016). During plant growth, formation of the thickened SCW is controlled by multi-tier transcriptional networks, which direct biosynthesis of SCW components including cellulose, hemicellulose, and lignin (Taylor-Teeples *et al.*, 2015). The upper tier players in the transcriptional networks include NAC transcription factors that initiate SCW formation in specific cell types. For example. *VASCULAR-RELATED NAC-DOMAIN6* (*VND6*) and *VND7* are expressed specifically in vessel cells and play a pivotal role in control of vessel SCW formation (Yamaguchi *et al.*, 2008; Ohashi-Ito *et al.*, 2010). *SECONDARY*

Abbreviations: 4CL1, 4-coumarate:CoA-ligase 1; CESA4, cellulose synthase A4; GUS, β-glucuronidase; IRX8, IRREGULAR XYLEM8; NST1, NAC SECONDARY WALL THICKENING PROMOTING FACTOR1; SCW, secondary cell wall; SND1, SECONDARY WALL-ASSOCIATED NAC DOMAIN1; VND6, VASCULAR-RELATED NAC-DOMAIN6; VND7, VASCULAR-RELATED NAC-DOMAIN7; WT, wild type; XND1, XYLEM NAC DOMAIN1.

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WALL-ASSOCIATED NAC DOMAIN1 (*SND1*) and *NAC SECONDARY WALL THICKENING PROMOTING FACTOR1* (*NST1*) are expressed specifically in fiber cells and function as key regulators to initiate fiber cell SCW thickening (Mitsuda *et al*., 2007; Zhong *et al*., 2007). Furthermore, the transcriptional networks for initiation of SCW formation in specific cell types are regulated by environmental or developmental signals through various mechanisms. Several positive or negative regulators have been identified as interacting with these upper tier players and regulate SCW formation in Arabidopsis. For example, the transcription factor E2FC negatively regulated *VND6* and *VND7* expression in the root (Taylor-Teeples *et al*., 2015). VNI2 interacts with and inhibits VND6 and VND7 transcriptional activity (Yamaguchi *et al*., 2010), and *MYB26* and *WRKY12* are reported to act upstream of *NST1* and *NST2* to inhibit their expression (Wang and Dixon, 2012). The basic helix–loop–helix transcription factor MYC2 was found to directly bind to the *NST1* promoter for activation of the *NST1*-directed transcription network under blue light (Zhang *et al*., 2018). However, it is unknown whether NST1 activity is regulated at the protein level.

XYLEM NAC DOMAIN1 (*XND1*) was reported to be preferentially expressed in xylem tissue (Zhao *et al*., 2005). Overexpression of *XND1* under the control of the 35S promoter led to a reduction of xylem SCW formation and caused extreme dwarfism in Arabidopsis (Zhao *et al*., 2008). XND1 is a NAC domain transcription factor. Recently, XND1 protein was shown to interact with a RETINOBLASTOMA-RELATED protein and play a role in inhibition of cell differentiation (Zhao *et al*., 2017). During our screening of the NST1-interacting proteins in Arabidopsis inflorescence stem, XND1 was identified as interacting with NST1. We examined how XND1 acts in associated with NST1 in SCW formation. The results revealed that XND1 directly interacts with NST1 and inhibits the NST1 transactivation activity, which leads to repression of SCW formation in Arabidopsis xylem cells.

Materials and methods

Plant materials and growth conditions

Arabidopsis (Columbia-0) was used as wild type (WT) and grown in a phytotron with a light–dark cycle of 16 h–8 h at 22 °C. *xnd1-6* (Accession number SALK_022552) and *xnd1-3* (Accession number SALK_046891C) were ordered from Arabidopsis Biological Resource Center (ABRC). *XND1* overexpression transgenic plants were generated by transforming WT with *p1300-SND1pro-XND1-3HA* (*SX1* and *SX2*), *p1300-XND1p-XND1-3HA*, and *p1300-35S-XND1-3HA* (*35S-XND1-13*). *SND1* promoter (approx. −1 to −2874 from ATG) and *XND1* promoter (approx. −1 to −2091 from ATG) were PCR-amplified from Arabidopsis genome DNA. *XND1* coding sequences were PCR-amplified from xylem tissue cDNA. All primers used in this study is listed in Supplementary Table S1 at *JXB* online.

Yeast two-hybrid screening

Using Arabidopsis inflorescence stem, an expression cDNA library was constructed in a *pDEST22* vector as described (Zhang *et al*., 2018) and used as screening prey; the N-terminus of NST1 (amino acids 1–192) was cloned into a *pDEST32* vector and transferred to yeast strain *MAV203* (Invitrogen) as screening bait. Then the bait strain was transferred with 10 μg of the library cDNA and sprayed onto 80 Petri dishes (15 cm) which contained SD−LEU−TRP−HIS + 100 mM 3-amino-1,2,4-triazole (3-AT), and grown at 30 °C for 4–10 d. Positive clones were selected twice on SD−LEU−TRP−HIS + 100 mM 3-AT plates. Positive clones were PCR amplified using pDEST22-F/R primers and sequenced. For a protein self-activation test in the yeast two-hybrid system, the coding sequence from the bait gene was cloned into the *pDEST32* vector and co-transferred with *pDEST22* empty vector into yeast *MAV203* and tested on the SD−LEU−TRP−HIS plate supplied with 20, 50 and 100 mM 3-AT. For observation of protein interaction, the coding sequence from the prey gene was cloned into the *pDEST22* vector and co-transferred with corresponding bait into *MAV203*, and tested on the SD−LEU−TRP−HIS plate with 100 mM 3-AT.

Bimolecular fluorescence complementation and protein subcellular localization analysis

For bimolecular fluorescence complementation, the *XND1* and *NST1* coding sequences were cloned into a *p1300-35S-YN* and a *p1300-35S-YC* vector, respectively (designated *Yn-XND1/NST1*, *NST1-Yc*) as described (Gui *et al*., 2016). For analysis of protein subcellular localization, the *XND1* and *NST1* coding sequences were cloned into a *pHB-X-CFP* and a *pHB-X-YFP* vector (Luo *et al*., 2014), respectively, and the constructs yielded were transformed to *Nicotiana benthamiana* leaf according to the description of Gui *et al*. (2016). After incubation for 48 h, abaxial epidermal cells of the leaf were observed under a confocal microscope (Olympus FV1000).

Co-immunoprecipitation

The *XND1* coding sequence was used to generate a *p1300-35s-XND1-3HA* vector. Then, a *4CL1pro-NST1-3FLAG* fragment (Zhang *et al*., 2018) was used for insertion into the *p1300-35s-XND1-3HA* vector to generate the *p1300-4CL1pro-NST1-3FLAG-35s-XND1-3HA* construct. This construct was transferred into Arabidopsis. Arabidopsis plants transformed with *p1300-4CL1pro-NST1-3FLAG* or *p1300-SND1pro-XND1-3HA* were used as a control. Co-immunoprecipitation was carried out as previously described (Gui *et al*., 2016). Briefly, total proteins were extracted from the inflorescence stems of 5-week-old plants. Extraction buffer contained 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.1% Triton X-100, 0.2% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1% protease inhibitor cocktail (Roche). After extraction at 4 °C for 30 min and then centrifugation, supernatant was pre-cleared with protein A/G agarose beads according to the manufacturer's instruction (Abmart, 50 μl ml^{-1}). Then extracted protein was incubated with anti-HA/FLAG-tag mouse mAb-conjugated agarose beads (Abmart, cat. no. M20013S, M20018S, 20 μl per 500 μl protein solution) for about 4 h at 4 °C, washed with wash buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 0.1% Triton X-100, 0.2% Nonidet P-40, 1 mM PMSF) three times. Agarose beads were collected by centrifugation; after removal of the supernatant, 20 μl SDS loading buffer was added to the pellet, and gently mixed; the mixture was boiled for 10 min and centrifuged; and the supernatant was detected by western blot using anti-HA-tag rabbit monoclonal antibody (Cell Signaling Technology, cat. no. 3724S, 1:1000 dilution) or anti-NST1 rabbit polyclonal antibody (Shanghai Youke Biotechnology Co. Ltd).

Western blot and NST1/XND1 immunolocalization

Western blot was performed as described (Yu *et al*., 2013). Total protein from inflorescence stem was reacted with either anti-HA or anti-ACTIN2 (Abmart, 1:1000 dilution) monoclonal antibodies, followed by reaction with goat-anti-mouse antibodies (Thermo Fisher, 1:5000 dilution). 5-Bromo-4-chloro-3′-indolyphosphate *p*-toluidine (BCIP)–nitro-blue tetrazolium chloride (NBT) (Life Technologies) staining was used to visualize the blots.

Protein immunolocalization was performed according to the previous study (Zhang *et al*., 2018). The basal part of the inflorescence stem from

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5-week-old plants transformed with *p1300-XND1p-XND1-3HA* were used for analysis. The stem was fixed in acetone, embedded in paraffin, and cross-sections made. For detection of NST1/XND1 protein, the sections were blocked by BSA and then incubated with anti-NST1 rabbit polyclonal antibody or anti-HA mouse monoclonal antibody in blocking solution (1:200 dilution), and then incubated with alkaline phosphatase-conjugated secondary antibodies (1:5000 dilution, goat anti-rabbit/mouse antibody, Thermo Fisher Scientific, cat. no. 31340). The section reacted with only secondary antibody (goat anti-mouse antibody) was used as the control. Sections were stained with BCIP–NBT at room temperature and observed under a light microscope (Olympus BX51).

Free-hand cross-section

The inflorescence stem at 0.5 cm above the rosette leaves was used for a free-hand section. The section was stained with phloroglucinol–HCl and observed under a light microscope (Olympus, BX53).

Measurement of stem tensile strength and secondary cell walls components

The first internode (close to the rosette leaf) of the inflorescence stem was used for tensile strength measurement as described (Zhang *et al.*, 2018). Relative tensile strength was normalized against WT. For analysis of SCW components, the lower part of the inflorescence stem (about 10 cm) from 5-week-old Arabidopsis was collected for cell wall component determination as described (Zhang *et al.*, 2018).

RNA extraction and quantitative RT-PCR analysis

Total RNA from various tissues of Arabidopsis plants was isolated using the E.Z.N.A. Plant RNA Kit (Promega, R6827-02). The first-strand cDNA was synthesized using TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, AT311-03) for quantitative real-time PCR (qRT-PCR) analysis of transcript abundance. qRT-PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad) and an iQ5 Real-Time PCR Detection System (Bio-Rad). Gene expression was normalized using *ACT2* as an internal control.

XND1 promoter activity analysis

The *XND1* promoter (approx. −1 to −2091 from ATG) sequence was PCR-amplified from Arabidopsis genome and cloned into a *p1301-GUS* vector. After the promoter–β-glucuronidase (GUS) construct was transferred into WT, the transgenic plants were analysed with GUS staining as described (Zhang *et al.*, 2018).

Transactivation assay of XND1 and NST1 interaction

XND1's effect on NST1 transactivation activity was examined using a dual-luciferase (LUC) reporter assay system (Promega) through a transient protoplast transfection. The coding sequence of *XND1* and *NST1* or their truncation was cloned into a pA7 vector under the control of the 35S promoter. Expressed XND1 and NST1 were used individually or together as an effector. The *IRREGULAR XYLEM8* (*IRX8*) promoter (approx. −1 to −1305 from ATG) sequence was cloned into a *pGreenII0800-LUC* vector in front of the LUC gene and used as a reporter. *Renilla* luciferase gene in a *pGreenII0800-LUC* vector was used as an internal control. Protoplasts from Arabidopsis mesophyll cells were isolated and transformed as previously described (Zhang *et al.*, 2018).

Electrophoretic mobility shift assay

An electrophoretic mobility shift assay (EMSA) was used for analysis of XND1's effect on the NST1 DNA binding activity. The coding sequence of *XND1* or *NST1* was cloned into a *pGEX4T1* vector. After transfer into *E.coli* strain *BL21*, recombinant XND1 and NST1 proteins were expressed under induction with 1 mM isopropyl β-D-1-thiogalactopyranoside, and purified using Pierce Glutathione Superflow Agarose (Thermo Fisher Scientific, 25236). XND1 and NST1 were incubated with a Cy5-labled *IRX8* promoter fragment (approx. −184 to −584 from ATG) individually or together in a total volume of 20 µl 1×binding buffer (5×binding buffer: 141.6 µl 5×EMSA buffer (100 mM Tris–HCl, pH 7.9, 25% glycerol, 0.2 mg ml^{-1} BSA), 3.2 µl $MgCl_2$ (2.5 M), 0.8 µl DTT (1 M), 1 µl sperm DNA (1 µg $µl^{-1}$), 13.4 µl H_2O). Unlabeled *IRX8* promoter fragment with a 100-fold concentration was used for competitive protein–DNA binding. After incubated for 15 min at room temperature, the reaction mixture was separated by 6% native PAGE at 4°C for 2 h according as described (Hellman and Fried, 2007). The PAGE image was scanned under a Fujifilm FLA 9000 plus DAGE.

Accession numbers

Sequence data from this article can be found at https://www.arabidopsis.org with the following accession numbers: *XND1* (AT5G64530), *NST1* (AT2G46770), *SND1* (AT1G32770), *VND6* (At5g62380), *VND7* (At1g71930), *IRX8* (AT5G54690), *CESA4* (AT5G44030), and *4CL1* (AT1G51680).

Results

XND1 was identified as a NST1 interacting protein

NST1 is a key transcription factor for initiation of the transcriptional networks of SCW formation. In order to understand how NST1 performs its role in directing SCW formation, we carried out yeast two-hybrid screening to identify interacting proteins. A cDNA expression library was constructed using Arabidopsis inflorescence stem. NST1 belongs to the NAC domain transcription factor family, which contains a NAC DNA binding domain and a WQ-box domain for transactivation (Olsen *et al.*, 2005; Fig. 1A). Because NST1 showed self-activation activity in yeast (see Supplementary Fig. S1), a truncated NST1 (with WQ-box deleted) was used as the bait to hybridize with the cDNA library in yeast. By screening 1×10^6 independent transformants, a collection of 162 candidate genes encoding the interacting proteins was isolated and sequenced (Supplementary Table S2). Among them, XND1, which plays a role in repressing xylem cell wall thickening (Zhao *et al.*, 2008), was identified for possible interaction with NST1.

XND1 is also a NAC domain transcription factor (Zhao *et al.*, 2017), and we truncated XND1 into different fragments to examine their structural interaction with NST1 using a yeast two-hybrid system (Fig. 1A). XND1N1 (amino acids 1–155, containing the NAC domain) showed a faint interaction, while XND1N2 (amino acids 1–174, containing the NAC domain and an extra structure, but with WQ-box deleted) showed a strong interaction with NST1N2 (Fig. 1B, C), suggesting that the fragment from amino acids 156 to 174 is a key structure for the interaction. This fragment in XND1 was then further deleted, yielding $XND1^{\Delta156-174}$. No interaction of $XND1^{\Delta156-174}$ with NST1 was detected (Fig. 1D). On the other hand, NST1 was truncated to examine the structure required for interaction with XND1. NST1 showed an interaction with XND1 at the N-terminus (amino acids 1–249, with WQ-box deleted) while the fragment of amino acids 1–192 (the NAC domain) did not show an interaction with the N-terminus of XND1 (Fig. 1C). In addition, deletion of the fragment of amino acids 180–249 of NST1 eliminated the interaction between XND1 and

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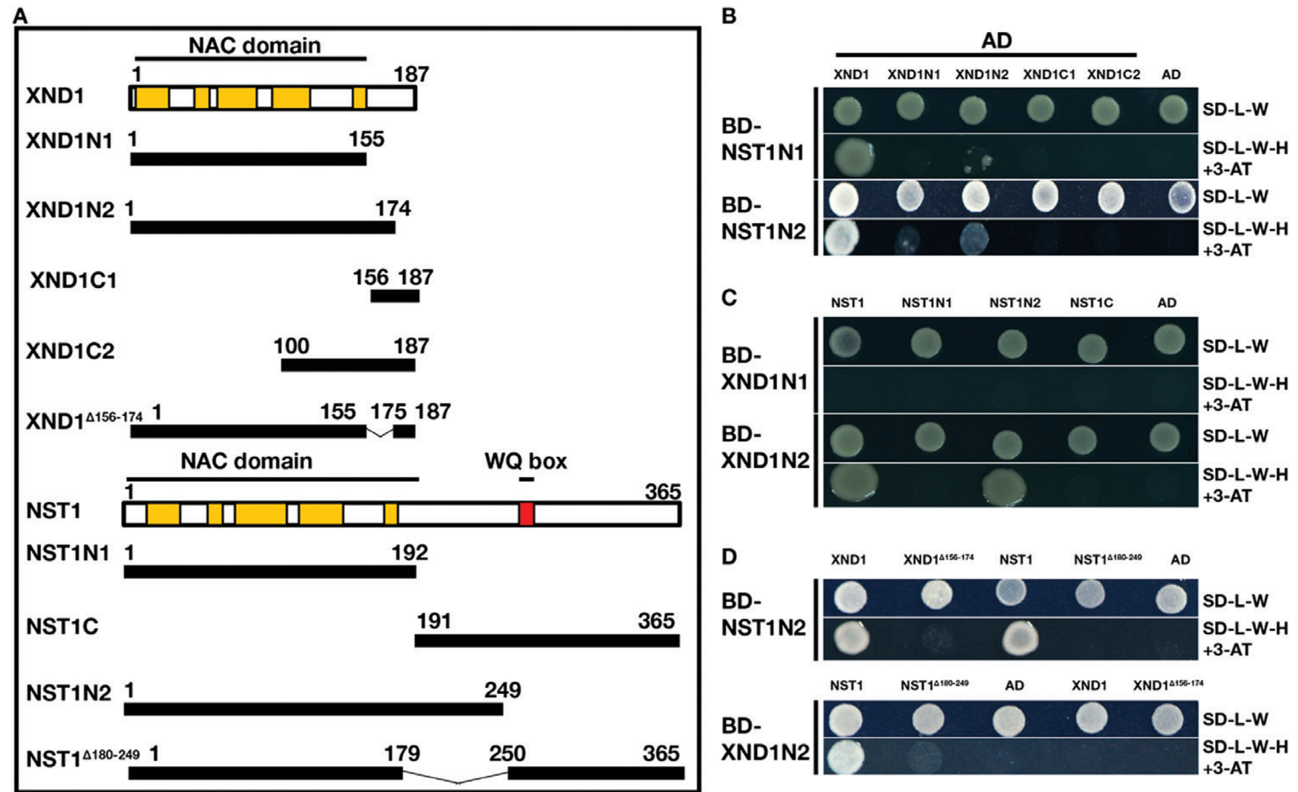

Fig. 1. Analysis of XND1 interaction with NST1. (A) Schematic representation of the structure of XND1 and NST1 and their truncated fragments. (B) Interaction of the XND1 truncated fragments with NST1. (C) Interaction of the NST1 truncated fragments with XND1. (D) The fragments responsible for interaction between XND1 and NST1.

NST1 as well as the NST1 self-interaction (Fig. 1D), indicating that the fragment of amino acids 180–249 of NST1 is essential for its interaction with XND1. Together, the results suggest that the structure between the NAC domain and WQ-box is required for the interaction of NST1 with XND1.

XND1 and NST1 were co-localized in nucleus and interacted together

To examine whether XND1 acts along with NST1 in cells, XND1 and NST1 were fused with cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP), respectively, and expressed in tobacco leaf cells. The NST1–YFP signal was co-localized with 4′,6-diamidino-2-phenylindole in the nucleus and XND1–CFP was detected in the nucleus, along with a weak signal in the cytosol. Co-expression of NST1–YFP and XND1–CFP indicated both were co-localized in the nucleus (Fig. 2A). To test whether this co-localization involves their interaction, a bimolecular fluorescence complementation (BiFC) assay was performed through co-expression of Yn–XND1 with NST1–Yc, mutant Yn–XND1$^{\Delta156-174}$ with NST1–Yc, or Yn–NST1 with NST1–Yc in tobacco leaf cells. Direct interaction between XND1 and NST1 was observed. Additionally, NST1 was detected interacting with itself (Fig. 2B). Furthermore, we generated transgenic Arabidopsis that expressed XND1 labeled with 3HA and NST1 labeled with 3FLAG simultaneously. Proteins were extracted from the inflorescence stems of the transgenic plants and used for co-immunoprecipitation assay. XND1–3HA was co-immunoprecipitated along with NST1–3FLAG by FLAG antibodies. Conversely, NST1–3FLAG was co-immunoprecipitated along with XND1–3HA by 3HA antibodies (Fig. 2C). The results indicated that XND1 interacts with NST1 in Arabidopsis inflorescence stem cells.

XND1 repressed NST1 transactivation activity

XND1 encodes a transcription factor and interacts with NST1. We analysed XND1 and NST1 transcriptional activity through a transcriptional activation assay. *IRREGULAR XYLEM8* (*IRX8*) is a direct target gene in NST1 regulation (Mitsuda *et al.*, 2007; Zhong *et al.*, 2007). Therefore we employed the *IRX8* promoter to analyse NST1 and XND1 transactivation activity using a dual luciferase system. NST1 but not XND1 activated the *IRX8* promoter when NST1 or XND1 was expressed, respectively. When XND1 and NST1 were co-expressed simultaneously, the activation of the *IRX8* promoter was blocked (Fig. 3A), suggesting that XND1 inhibited the NST1 transactivation activity. Moreover, deletion of amino acids 180–249 in NST1 diminished its activation of the *IRX8* promoter, suggesting NST1 dimerization is required for the transcriptional activation. The amino acid fragment 156–174 in XND1 is necessary for its interaction with NST1, and deletion of these amino acids relieved its inhibition of NST1, demonstrating that the XND1–NST1 interaction inhibits NST1's function (Fig. 3B). We investigated the DNA-binding activity of NST1 and XND1 using EMSA. NST1 directly

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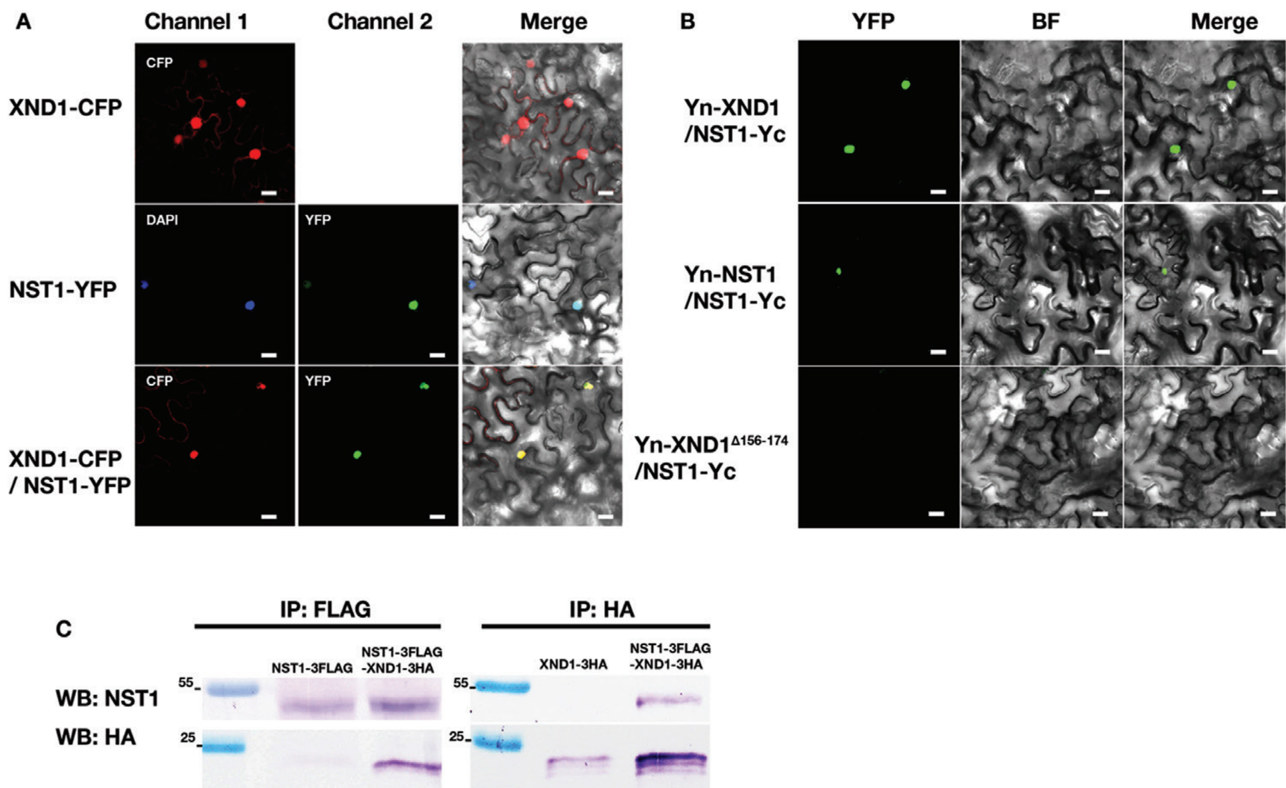

Fig. 2. Verification of NST1 interaction with XND1. (A) Co-localization of XND1 with NST1 in the nucleus. The constructs XND1–CFP and NST1–YFP were transformed into tobacco leaves separately or together. 4′,6-Diamidino-2-phenylindole (DAPI) indicates nuclear location. Scale bar: 20 μm. (B) BiFC analysis of the interaction between XND1 and NST1. Co-transformation of Yn–XND1 and NST1–Yc or Yn–NST1 and NST1–Yc into tobacco leaves. Interaction of NST1 with XND1 generated YFP luminescence, while NST1 and the deleted XND1 (removal of the fragment from amino acids 156 to 174) did not generate luminescence. Scale bar: 20 μm. (C) Co-immunoprecipitation analysis of interaction between XND1 and NST1 in Arabidopsis. Arabidopsis transgenic plants expressing *4CL1P-NST1-3FLAG* (*NST1-3FLAG*), *SND1P-XND1-3HA* (*XND1-3HA*), or *4CL1P-NST1-3FLAG-35S-XND1-3HA* (*NST1-3FLAG-XND1-3HA*) were used for analysis. Total proteins extracted from the inflorescence stem of transgenic plants were immunoprecipitated with anti-FLAG or anti-HA antibodies. The precipitated proteins were tested for NST1 and XND1. Arabidopsis transgenic plants expressing *XND1* or *NST1* individually were used as a control.

bound to the *IRX8* promoter but XND1 did not (Fig. 3C). When XND1 was incubated together with NST1, the NST1 DNA-binding affinity was not compromised (Fig. 3C), suggesting that XND1 did not affect NST1 binding to the *IRX8* promoter DNA. We concluded as a result that XND1 inhibition of NST1 transactivation activity should be through their direct interaction and not through competition for the target DNA binding site.

XND1 *and* NST1 *showed a similar expression profile in vascular tissue*

We analysed the expression profile of *XND1* in various tissues. *XND1* was preferentially expressed in the inflorescense stem, silique, and root. During inflorescence stem growth when SCW formation is highly active, expression of *XND1* displayed a similar profile to that of NST1 (Fig. 4A). In addition, the *XND1* promoter (approx. −1 to −2091 amino acids from ATG) fused with a β-glucuronidase (GUS) reporter gene was transferred to Arabidopsis. The transgenic plants showed GUS activity in the vascular system of leaf and inflorescence stem (Fig. 4B, C), mainly in the cells undergoing SCW thickening (Fig. 4C). Moreover, to determine the cell type localization of XND1 and NST1, we generated plants transformed with *XND1p-XND1-3HA* and detected the protein localization in the serial sections of the inflorescence stem. The result was that both NST1 and XND1 were detected in the fiber cells of the inflorescence stem (Fig. 4D). Together the results indicate that both *XND1* and *NST1* were co-expressed in fiber cells of the inflorescence stem.

XND1 *repressed secondary cell walls formation in Arabidopsis*

To examine the effect of *XND1* on SCW thickening, we first overexpressed *XND1* under control of the 35S promoter. The transgenic plants showed slower growth, shorter and weaker inflorescence stems (see Supplementary Fig. S2A, B), and dwarfism lacking shoot apical growth (Supplementary Fig. S2A enlargements), similar phenotypic alternations to those previously reported (Zhao *et al.*, 2008). In the transgenic plants, SCW formation was obstructed in fiber and vessel cells (Supplementary Fig. S2C–E). Irregular xylem cells (Supplementary Fig. S2D) developed.

To dissect how XND1 interacts with NST1 to regulate SCW formation, we then overexpressed *XND1* under

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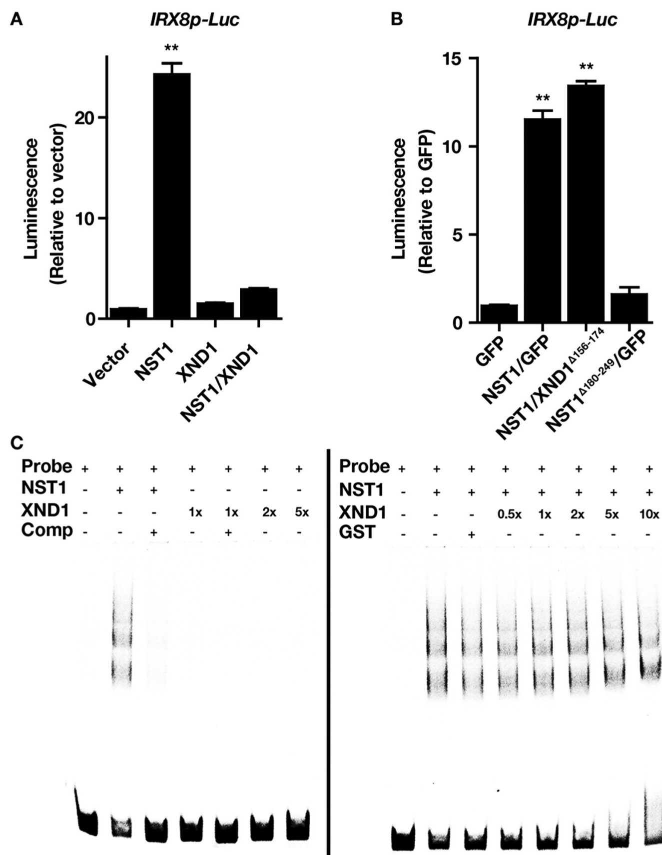

Fig. 3. XND1 antagonizes NST1 function. (A) NST1 activated *IRX8* promoter but XND1 inhibited the NST1 activation of *IRX8* promoter. *pGreenII0800-IRX8pro-LUC* was co-transferred with *pA7-35S-NST1* or *pA7-35S-XND1* or both to the protoplast of Arabidopsis mesophyll cells. Luminescence in the protoplast co-transformed with *pGreenII0800-IRX8pro-LUC* and *pA7-35S* empty vector (vector) was set to 1 as control. Three biological replicates were performed. Results are presented as means ±SD. (B) XND1 inhibited NST1 function through direct interaction. Deletion of the fragment of XND1 that was responsible for NST1–XND1 interaction diminished the XND1 inhibition of NST1 activation. Deletion of the fragment responsible for NST1 homodimerization abolished the NST1 activation of *IRX8* promoter. Luminescence in the protoplast co-transformed with *pGreenII0800-IRX8pro-LUC* and *pA7-35S-GFP* vector (GFP) was set to 1 as control. Three biological replicates were performed. Results are presented as means ±SD. (C) NST1 binding to the *IRX8* promoter fragment (approx. −584 to −184 from ATG) with or without the presence of XND1. The *IRX8* promoter fragment labeled with Cy5 (probe) was incubated with NST1 or XND1 (left) or with both (right). An unlabeled probe was used as a competitor (comp). The protein–probe complex was separated by native PAGE. Glutathione *S*-transferase (GST) protein was used as a negative control.

control of the fiber-specifc *SND1* promoter as *NST1* is expressed in fiber cells (Zhong *et al.*, 2006). The transgenic plants, overexpressing *p1300-SND1P-XND1-3HA*, showed recumbent stems, though the stem length was not notably affected (Fig. 5A, B). The SCW formation in interfascicular fiber cells and vessel cells was mitigated (Fig. 5C). The stem mechanical strength was substantially reduced (Fig. 5D). The representative SCW biosynthesis genes *CELLULOSE SYNTHASE A4* (*CESA4*), *IRX8*, and *4-COUMARATE:COA-LIGASE1* (*4CL1*), which are involved in cellulose, hemicelluloses, and lignin biosynthesis, respectively, were down-regulated (Fig. 5E).

The transgenic plants contained less cellulose and lignin (see Supplementary Fig. S3A, B) and less xylose of hemicelluloses as well (Supplementary Fig. S3C). We also examined SCW thickening in *xnd1* mutants. Two *xnd1* mutants, designated *xnd1-3* and *xnd1-6*, following a previous study (Tang *et al.*, 2018), were used to examine the genotype and *XND1* gene expression. Both mutants were inserted near the ATG start code; they failed to express *XND1* mRNA, but did not show obvious growth alterations (Fig. 6A–D). SCW formation in the mutant was increased in fiber cells and slightly in vessel cells (Fig. 6E, F). Expression of key SCW biosynthesis genes (*CesA4*, *IRX8*, and *4CL1*) was increased in the mutant (Fig. 6G). The results indicated that *XND1* is involved in regulating fiber SCW formation during inflorescence stem growth.

Discussion

NST1 directly interacts with XND1

NST1 encodes a NAC domain transcription factor and plays a key role in initiating SCW biosynthesis in fiber cells. Expression of *NST1* is regulated by various signals through other transcription factor genes. For example, *MYC2* promotes *NST1* expression under blue light (Zhang *et al.*, 2018). Overexpression of LBD30 increased *NST1* gene expression and enhanced SCW thickening in the fiber cell (Liu *et al.*, 2019). *NST1* expression in anther endothecium is induced by *MYB26* (Yang *et al.*, 2007). FUL and IND act upstream of *NST1* to negatively or positively regulate SCW thickening, respectively, in the siliques (Mitsuda and Ohme-Takagi, 2008). After *NST1* transcription is activated, however, little is known of whether NST1 activity is modulated at protein level.

In this study, by screening the expressed protein library from Arabidopsis inflorescence stem, XND1 was identified as a NST1 interacting protein. Co-localization of NST1 with XND1 was observed in the nucleus and such co-localization was demonstrated through direct interaction. The interaction between NST1 and XND1 requires the structure of the $NST1^{180–249}$ fragment and the $XND1^{156–174}$ fragment (Fig. 1), which is located between the NAC domain and WQ-box. The $NST1^{180–249}$ fragment is also responsible for NST1 self-interaction (Fig. 3). Likely, interaction between NST1 and XND1 interferes with NST1 self-interaction in formation of a dimeric structure that is necessary for NST1 functionality (Yamaguchi *et al.*, 2008; Zhao *et al.*, 2014).

Regulation of NST1 activity in secondary cell walls formation

NST1 initiates expression of the SCW formation genes in fiber and anther endothecium cells. Such initiation is likely through an NST1 homodimer transactivating the target genes (Olsen *et al.*, 2005). *NST1* overexpression caused ectopic SCW deposition in the epidermal cells (Mitsuda *et al.*, 2005) and increased SCW thickening in the fiber cells (Zhang *et al.*, 2018). On the other hand, *XND1* is expressed specifically in xylem (Zhao *et al.*, 2005). Overexpression of *XND1* under the control of the 35S promoter suppressed SCW formation

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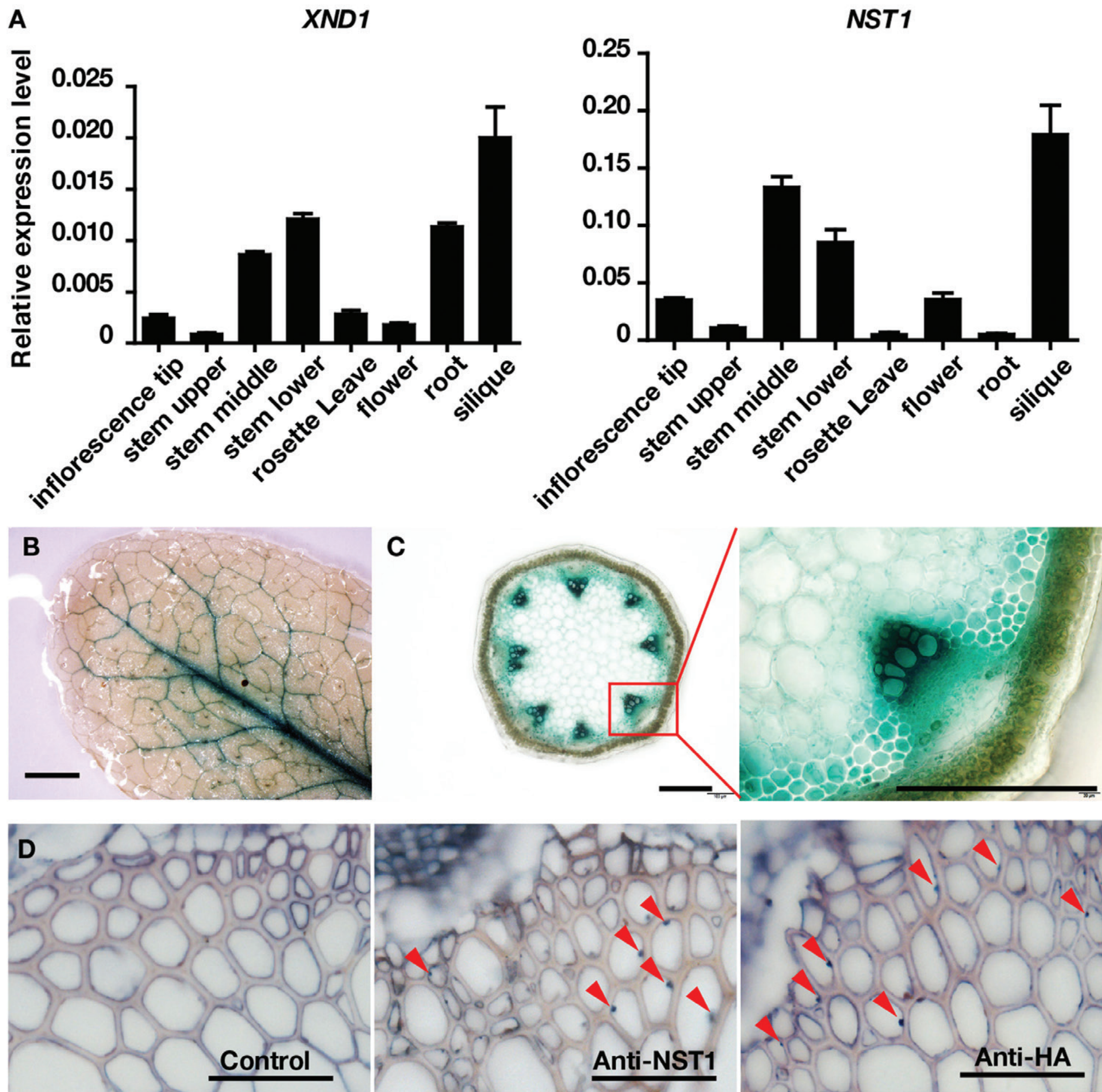

Fig. 4. Preferred expression of XND1 and NST1 in vascular tissue. (A) qRT-PCR analysis of XND1 and NST1 expression in various tissues of the 5-week-old Arabidopsis. Samples were collected from 80 plants. Results are means ±SD of three measurements. (B, C) Activity of the *XND1* promoter in Arabidopsis vascular tissue. GUS activity was stained in the vascular tissue of leaves (B) and inflorescence stem (C), and the enlargement shows the *XND1* promoter activity in fiber cells and vessel cells. Five-week-old transgenic Arabidopsis was used for analysis. Scale bars: 2 mm (B) and 200 μm (C). (D) Localization of XND1 and NST1 in the inflorescence stem. The inflorescence stem from the transgenic plants expressing *XND1p-XND1-3HA* at 5 weeks of age was sampled. Serial sections of the stem were blotted with anti-NST1 rabbit polyclonal antibody or anti-HA mouse monoclonal antibody to detect the location of NST1 and XND1 protein. Secondary antibody blotting only was used as control. Red arrowheads show the location of the target protein, likely in the nucleus. Scale bar: 40 μm.

in xylem cells and retarded plant growth (see Supplementary Fig. S2; Zhao *et al.*, 2008). Both *NST1* and *XND1* are NAC-domain transcription factors, playing opposite roles in regulating SCW formation. In our study, NST1 directly bound to the *IRX8* promoter and activated its transcriptional activity but XND1 did not directly bind to the promoter to affect the transcriptional activity. However, the presence of both NST1 and XND1 together substantially inhibited the promoter transcriptional activity indicating that the NST1–XND1 complex, as opposed to competition for DNA binding, inhibited the promoter activity.

In addition to XND1's interaction with NST1, we also detected weak interaction between XND1 and SND1, and XND1 and VND7 (see Supplementary Fig. S4). Overexpression of XND1 inhibits SCW thickening in the xylem vessel cells (Zhao *et al.*, 2008). Whether the inhibition is through XND1 directly inhibiting VND7's function remains to be investigated. As a key transcription switch for SCW formation, *NST1* has

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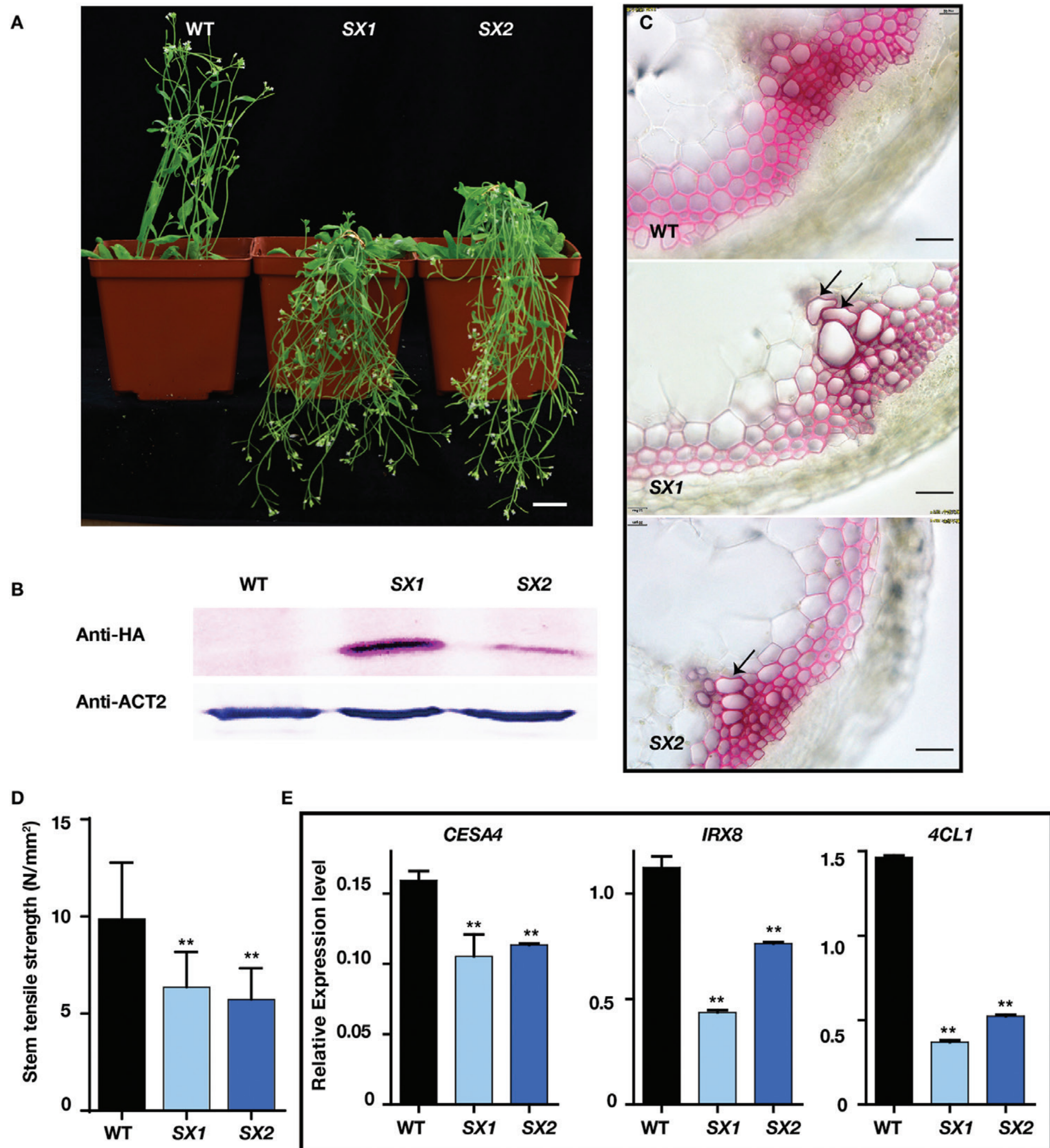

Fig. 5. Expression of *XND1* under control of the *SND1* promoter repressed SCW formation in inflorescence stem. (A) Five-week-old Arabidopsis transgenic plants overexpressing *XND1* under the control of *SND1* promoter. (B) Detection of XND1 protein in the transgenic plants. (C) Cross-sections of the inflorescence stem were stained with phloroglucinol–HCl. Arrows show the irregular vessel cells in the *XND1* transgenic plants. Scale bar: 40 μm. (D) Tensile strength of the inflorescence stem. Each measurement included 20 plants, and three replicates were performed. Student's *t*-test (**P<0.01) was used for statistical analyses. Results are presented as means ±SD. (E) qRT-PCR analysis of the *CESA4*, *IRX8*, and *4CL1* expression in the inflorescence stem. Samples were collected from 80 plants. Results are means ±SD of three measurements.

been much studied for its expression at the transcriptional level, but little is known of how NST1's activity is modulated in regulation of SCW formation at the protein level. The present study provides evidence to indicate that modulation of the NST1 activity may act as a mechanism to tune SCW biosynthesis.

NST1 activity may serve as a check-point in regulation of secondary cell walls formation during plant growth

Previous study showed that NST1 could also bind to the *XND1* promoter directly and activate *XND1* expression (Zhong *et al.*, 2010). In this study, XND1 was found to

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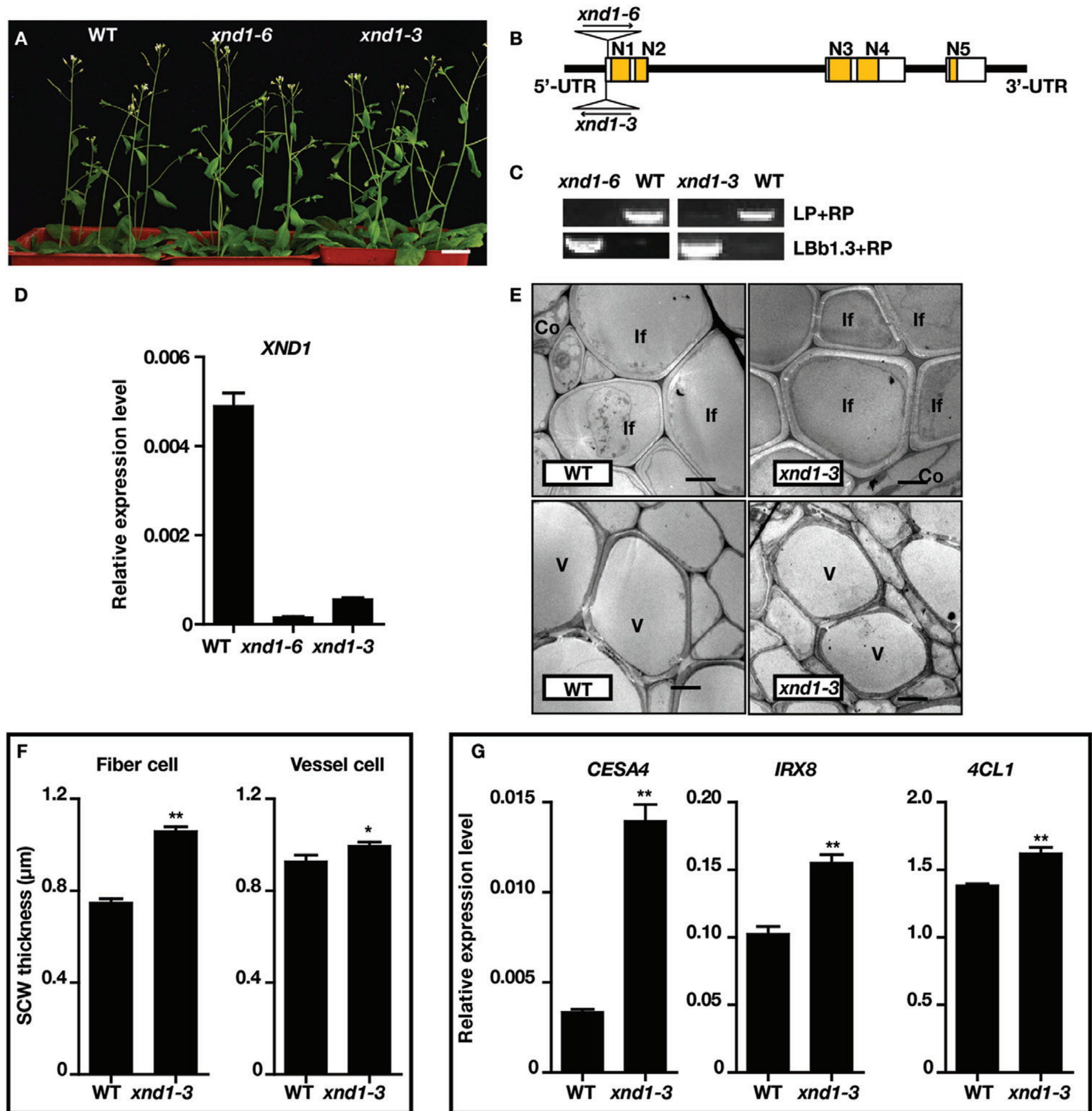

Fig. 6. Increase of SCW formation in *xnd1* mutants. (A) Four-week-old *xnd1* mutants. Scale bar: 2 cm. (B) Schematic representation of the T-DNA insertion in *xnd1-3* and *xnd1-6* mutants. (C) PCR analysis of the mutant genotype. (D) qRT-PCR analysis of XND1 expression in the mutant. Samples were collected from 20 plants. Results are means ±SD of three measurements. (E) Transmission electron micrographs of the cross-section of the inflorescence stem. Interfascicular fiber cells (upper) and vessels (lower). Scale bar: 5 μm. Co, cortex; If, interfascicular fiber; V, vessel. (F) Measurements of SCW thickness in (E). In each genotype, more than 20 cells in each plant were analysed. Three replicates were carried out. Student's *t*-test (**P*<0.1, ***P*<0.01) was used for statistical analyses. Results are presented as means ±SD. (G) Expression of *CESA4*, *IRX8*, and *4CL1* in the inflorescence stem. Samples were collected from 80 plants. Results are means ±SD of three measurements.

interact with NST1 and inhibit its transactivation activity. During plant growth, we found that expression of *NST1* was high in the middle part of the inflorescence stem where SCW formation was at an active stage, while in the bottom part where SCW formation had decelerated, *XND1* expression was raised but *NST1* expression was decreased (Fig. 4A). This suggests that the NST1–XND1 complex may form a negative feedback to turn down the transcription program of SCW formation at the end of inflorescence stem growth. In addition to regulation of *NST1* expression, NST1 activity is also modulated for tuning SCW formation during plant growth and development.

In summary, NST1 is a key transcription factor in the initiation of SCW formation (Mitsuda *et al.*, 2005; Zhong *et al.*, 2007). *XND1* was previously reported to repress SCW formation in xylem cells (Zhao *et al.*, 2008). This study identified

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XND1 as interacting with NST1 and demonstrated that the interaction of XND1 with NST1 results in inhibition of the NST1 transactivation activity. Thus the NST1–XND1 complex may act as a regulating device to tune SCW formation during growth of Arabidopsis inflorescence stem.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. XND1 and NST1 show self-activation activity in yeast two-hybrid system.

Fig. S2. Overexpression of *XND1* inhibited SCW formation in Arabidopsis inflorescence stem.

Fig. S3. Overexpression of *XND1* under control of the *SND1* promoter inhibited secondary cell wall biosynthesis.

Fig. S4. XND1 interaction with SND1 and VND7 in yeast.

Table S1. List of primers used in this research.

Table S2. Candidate genes encoding the NST1-interacting proteins identified by yeast two-hybrid assays.

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Author contributions

QZ and LL designed the research; QZ, FL, YZ and JH performed the experiments; QZ and LL analysed the data and wrote the paper. All authors read and approved the article.

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