

Chapter 4

XND1, a member of the NAC domain family, functions as a negative regulator of xylem differentiation in Arabidopsis

Abstract

The NAC domain superfamily of transcription factors is plant-specific and contains more than 100 members in the Arabidopsis genome (Ooka et al., 2003). NAC genes play roles in cell elongation, lateral organ development, and organ separation. From a comparative analysis of Arabidopsis xylem and phloem-cambium transcript profiles, we noted the expression of six xylem-biased NAC domain genes (Zhao et al., 2005). Overexpression (CaMV 35S-driven) of *XND1*, the most highly expressed xylem-biased NAC gene, resulted in extreme dwarfism in >30% of transformants recovered in five independent experiments. Dwarfism was associated with high levels of *XND1* transcript, dark green color and the absence of tracheary elements (TEs) from the entire plant body for up to two weeks post-emergence. By four weeks post-emergence discontinuous xylem had formed in the roots of the dwarf 35S-*XND1* plants but the complete absence of TEs from aerial organs persisted in the most severely affected individuals. Plant death ensued at about five weeks of age for all of the extreme dwarfs. Vascular bundles lacking TEs also excluded the vital stain Evans blue, indicating that the programmed death of TEs was blocked by *XND1* overexpression. Using GUS reporter lines as genetic backgrounds to further investigate the cell type-specific effects of *XND1* overexpression, we found that overexpression of *XND1* inhibited TE-specific GUS expression but not phloem-specific GUS expression. Two T-DNA insertion lines (*xnd1-1* and *xnd1-2*) and one transposon insertion line (*xnd1-3*) were isolated and analyzed. Under normal conditions and continuous light, *xnd1* plants did not differ significantly from wild type. However, under ABA and cold treatment, preliminary results indicated that *xnd1* plants were ABA and cold hypersensitive. Yeast-two hybrid screening of a xylem cDNA library with a truncated XND1 as bait led to isolation of a novel RING finger protein, At3g62970. These findings suggest that XND1 functions as a negative regulator of xylem differentiation and that this regulation may be integrated with the ubiquitin/26S proteasome pathway.

Introduction

The NAC proteins are plant-specific transcription factors (Souer et al., 1996; Reichmann et al., 2000) that possess a consensus sequence known as the NAC domain in the N-terminal region and a highly divergent C-terminus. The NAC designation is derived from the NO APICAL MERISTEM (NAM) gene from petunia and the Arabidopsis genes ATAF1/ATAF2 and CUP-SHAPED COTYLEDON2 (CUC2) (Aida et al., 1997). The NAC domain comprises five subdomains referred to as A to E (Kikuchi et al., 2000; Duval et al., 2002) or N1 to N5 (Xie et al., 1999; Hegedus et al., 2003).

Recent studies illustrate the wide range of inducible responses and developmental programs that involve NAC proteins. *nam* mutants in petunia fail to develop shoot apical meristems and cotyledons are often laterally fused. In the most extreme cases, cotyledons are fused to become a single disc. In situ hybridizations showed that *nam* is expressed at the boundaries of a variety of primordia and meristems, suggesting that NAM plays a role in specification of the position, rather than the identity, of primordia and meristems (Souer et al., 1996). Similarly, in Arabidopsis, *cuc1 cuc2* double mutants (mutated *CUP-SHAPED COTYLEDON*) completely lack an embryonic SAM and two cotyledons are fused along both edges to form one cup-shaped structure. *cuc3* mutants also have fused cotyledons and floral organs (Aida et al., 1997; Takada et al., 2001; Vroemen et al., 2003). More severe organ fusion was found in the *cupuliformis* (*cup*) mutant of Antirrhinum (Weir et al., 2004). The *NAP* (*NAC-LIKE, ACTIVATED BY AP3/PI*) was identified as an immediate target of the floral homeotic genes *APETAL3* and *PISTILLATA* by differential display (Sablowski et al., 1998). *NAP* is expressed mainly beneath the inflorescence meristem as the meristem develops sepals and at the bases of stamen filaments, and was hypothesized to function in the transition between growth by cell division and cell expansion in stamens and petals. NAC1 is induced by auxin and functions downstream of TIR as a component of auxin signal transduction required for lateral root development (Xie et al., 2000). NAC1 ubiquitination can be catalyzed by SINAT5, an Arabidopsis RING finger E3 (ubiquitin ligase), indicating that 26S proteasome-mediated turnover of NAC1 is a mechanism for downregulating auxin signals important to lateral roots (Xie et al., 2002). Other NACs have been found to be differentially regulated in response to biotic

and abiotic stresses, such as wounding, insect feeding, *sclerotinia sclerotiorum* infection, cold shock and dehydration (Hegedus et al., 2003), and during senescence (John et al., 1997; Guo et al., 2004).

Here we report on the function of *XND1*, a NAC domain gene expressed in xylem and linked with stress and ABA response and leaf senescence. Preliminary results indicated that transcript nulls for *XND1* are ABA and cold hypersensitive, exhibiting reduced growth under ABA and cold treatment. Overexpression of *XND1* results in severe stunting, premature death, and repression of TE differentiation. By yeast-two hybrid screening, a novel RING finger protein, At3g62970, was found to interact with XND1. Together these findings indicate that XND1 functions to negatively regulate xylem cell differentiation, that senescence- and stress-induced repression of differentiation of xylem cells is necessary for normal plant growth, and that XND1 ubiquitination may be catalyzed by At3g62970, a putative RING finger protein.

Materials and Methods

XND1p-GUS

The *XND1p-GUS* construct is described in Zhao et al. (2005).

MYR1p-XND1-GFP* and *35S-XND1-GFP

The *XND1* coding region (without the stop codon) was amplified from a xylem cDNA library (Zhao et al., 2000) by PCR using an upstream *BamH I* (underlined) linker primers 5'-GGATCCATGAATCTACCACCGGGATT-3' and a downstream *EcoR V* linker primer 5'-GATATCCGGTAAGCTTACTTCGTCAA-3'. The resulting PCR products were cloned into pGEM. Plasmid DNA pGEM-*XND1* (without the stop codon) was digested with *BamH I/EcoR V*, the released fragment was ligated with mpBI121-*MYR1p-GFP* or mpBI121-*35S-GFP* (see the construction of *MYR1p-GFP* and *35S-GFP* in Chapter 3 and Appendix I, respectively) digested with *BamH I/Sma I*, thus generating mpBI121-*MYR1p-XND1-GFP* and mpBI121-*35S-XND1-GFP*.

35S-XND1

The *XND1* coding region (with the stop codon) was amplified from a xylem cDNA library (Zhao et al., 2000) by PCR using an upstream *BamH I* linker primer 5'-GGATCCATGAATCTACCACCGGGATT-3' and a downstream *EcoR V* linker primer 5'-GATATCTCTACGGTAAGCTTACTTCTC-3'. The resulting PCR products were cloned into pGEM. Plasmid DNA pGEM-*XND1* (with the stop codon) was digested with *BamH I/EcoR V*, the released fragment was ligated with pFGC5941 digested with *BamH I/Sma I*. The resulting plasmid DNA was digested with *Xho I/BamH I*, blunted, and re-ligated, which removed the CHSA intron and Omega between the *Xho I* and *BamH I* sites, thus generating pFGC5941-*35S-XND1*.

pBGKT7-*XND1* and -*XND1* fragments

Plasmid DNA pGEM-*XND1* (with the stop codon, sense orientation) (see the construction of *35S-XND1*) was digested with *BamH I/Sal I* (the *Sal I* site is located on the vector), the released fragment was ligated with pBGKT7, the DNA-BD vector in BD Matchmaker Two-Hybrid System (Clontech, Palo Aito, CA) digested using the same sets of restriction

enzymes. To make the in-frame fusion of XND1 to GAL4 DNA-binding domain, the resulting plasmid DNA was digested with *BamHI*, blunted, and re-ligated, then digested with *NcoI*, blunted, and re-ligated, thus generating pGBKT7-*XND1*. *XND1*₁₋₁₁₉ encoding the first 119 amino acids for NAM domain, *XND1*₁₋₁₅₅ encoding the first 155 amino acids for NAC domain, and *XND1*₁₋₁₇₄ encoding the first 174 amino acids, were amplified from plasmid DNA pGBKT7-*XND1* by PCR using T7 primer and downstream *SalI* linker primers 5'-GTCGACATCCGGGAGGGAATATTCTT-3', 5'-GTCGACTTTGCTCATACTCTGCATAT-3', and 5'-GTCGACACACTTCATCCAAACATGAGA-3', respectively. The PCR products were cloned into pGEM. The resulting plasmid DNA pGEM-*XND1*₁₋₁₁₉, -*XND1*₁₋₁₅₅, and -*XND1*₁₋₁₇₄ were digested with *SalI/EcoRI*, the released fragments were ligated with pGBKT7-*XND1* digested with the same sets of restriction enzymes, thus replacing the full-length *XND1* with these fragments encoding the first 119, 155, and 174 amino acids, respectively. *XND1*₁₅₄₋₁₈₇ encoding the last 34 amino acids of XND1, was amplified from plasmid DNA pGBKT7-*XND1* by PCR using an upstream *NcoI* linker primer 5'-CCATGGAGCAA AATTGCAGTGAGGA-3' and a downstream *EcoRV* linker primer 5'-GATATCTTACGGTAAGCTTACTTCTC-3'. The PCR products were cloned into pGEM. The resulting plasmid DNA pGEM-*XND1*₁₅₄₋₁₈₇ was digested with *NcoI/EcoRV*, the released fragments were ligated with pGBKT7 digested by *NcoI/SmaI*, thus producing pGBKT7-*XND1*₁₅₄₋₁₈₇.

Phloroglucinol staining

Phloroglucinol staining was performed according to Galavazi (1965), i.e., tissues were vacuum-infiltrated and soaked in 80% ethanol till chlorophyll is extracted, then dehydrated thoroughly in 100% ethanol (3 changes). 1% phloroglucinol in methyl benzoate was prepared by heating 0.1g phloroglucinol in 10 ml methyl benzoate at 65-70°C for 24 hr, then filtered after it became cool. 5 parts of 1% phloroglucinol was acidified by 1 part of concentrated HCl. Tissues were stained using the upper layer of the acidified phloroglucinol solution for 10-30 min, then rinsed in 2 changes of pure methyl benzoate for 1 hr.

Evans Blue Staining

Evans blue staining was performed according to Gaff and Okong'O-Ogola (1971), i.e., roots of Arabidopsis seedlings were cut in water and then submerged in a 0.5% (W/V) Evans blue solution for 15 min at room temperature. Roots were then washed with distilled water and seedlings were directly photographed under a dissection microscope.

Preparation of Arabidopsis DNA for PCR purposes

Genomic DNA from Arabidopsis was isolated according to the simplified CTAB-extraction procedure in supplements material for Lukowitz et al. (2000).

Semi-quantitative RT-PCR

For the analysis of *XND1* expression in *xnd1-1* and *xnd1-2* mutants, and *XND1* overexpression driven by CaMV 35S promoter, total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen). RT-PCR was carried out according to the RETROscript Kit instruction manual (Ambion, Austin, Texas). PCR amplification was performed in 25- μ l standard reaction with the following primers: for *XND1*, 5'-GGATCCATGAATCTACCACCGGGATT-3' and 5'-GATATCTTACGGTAAGCTTACTTCTC-3'; for *ACT7*, 5'-GGCCGATGGTGAGGATATTC-3 and 5'-CTGACTCATCGTACTCACTC-3'. For PCR, after denaturation (5 min, 94°C), samples were subjected to 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, for the number of cycles indicated on figures, following by 10 min at 72°C. Actin cDNA was amplified for a loading control. PCR products were separated on 1% agarose gels and visualized by ethidium bromide staining.

Identification of *XND1* knockout mutants

xnd1-1 (Accession number SALK_022552), *xnd1-2* (Accession number GABI_162D11), and *xnd1-3* (Accession number GT_5_12667) mutants were generated at The Salk Institute Genomic Analysis Laboratory (<http://signal.salk.edu/cgi-bin/tdnaexpress>), Max Planck Institute for Plant Breeding Research (<http://www.mpiz-koeln.mpg.de/>), and The John Innes Centre (<http://www.jic.ac.uk/corporate/index.htm>), respectively. Insertions of the T-DNA in *xnd1-1* and *xnd1-2* or transposon in *xnd1-3* were confirmed by sequencing

the PCR products amplified from genomic DNA using a pROK2 T-DNA left border primer 5'-GCGTGGACCGCTTGCTGCAACT-3 and an *XND1* antisense primer, 5'-GATATCTTACGGTAAGCTTACTTCTC-3' corresponding to the stop codon for *xnd1-1* or a pAC161 T-DNA left border primer 5'-CCCATTTGGACGTGAATGTAG-3 and an *XND1* sense primer 5'-GGATCCATGAATCTACCACCGGGATT-3' corresponding to the translation start site for *xnd1-2* or a transposon specific primer (Ds3-1) 5'-ACCCGACCGGATCGTATCGGT-3' and the *XND1* sense primer mentioned above for *xnd1-3*, respectively. Homozygous *xnd1* were identified by PCR analysis using primers amplifying the *XND1* putative promoter (see the production of *XND1p-GUS*) or the coding region (see the production of *35S-XND1*), respectively (these primer combinations gave no product) and using the pROK2 T-DNA left border primer and the *XND1* antisense primer for *xnd1-1* or the pAC161 T-DNA left border primer and the *XND1* sense primer for *xnd1-2*, or the transposon specific primer and the *XND1* sense primer for *xnd1-3*, respectively (these primer combinations gave PCR products).

To detect whether a hybrid transcript exists in *xnd1-1* or *xnd1-2*, RT-PCR was performed using the pROK2 T-DNA left border primer and the *XND1* antisense primer or another pAC161 T-DNA left border primer (5'-TTCCCGGACATGAAGCCATT-3') and the *XND1* sense primer, respectively. The hybrid transcripts were confirmed by sequencing these resulting PCR products.

Yeast two-hybrid screening

Yeast two-hybrid screening was performed according to the user manual for BD Matchmaker™ Library Construction and Screening Kits (BD Biosciences, Palo Alto, CA).

Results

***XND1* is a xylem-specific NAC domain gene**

By performing a genome-wide comparative analysis of xylem and phloem-cambium transcripts using the 24K GeneChip, we identified 33 xylem-biased transcription factors in addition to 18 phloem-cambium-biased transcription factors (Zhao et al., 2005; Chapter 3). Among the xylem-biased transcription factors, six NAC domain family members (At1g02250, At1g32770, At2g46770, At4g28500, At4g28530, and At5g64530) were noted. Of these, a T-DNA insertion mutation of the At2g46770 gene (Allele Symbol: *emb2301*) was reported by the SeedGenes Project (<http://www.seedgenes.org>) as embryo defective, exhibiting a globular-stage arrest phenotype. At5g64530 was identified recently as part of the leaf senescence transcriptome (Guo et al., 2004) and showed the highest expression levels among all xylem-biased NAC proteins. At5g64530 was designated *XND1* for Xylem NAC Domain 1 (Zhao et al., 2005) and was selected for further characterization.

The xylem-localized expression of *XND1*, predicted from transcript profiling of isolated secondary xylem was consistent with expression of the reporter β -glucuronidase driven by the putative promoter of *XND1* (*XND1p*), a 1.1-kb DNA fragment upstream from the *XND1* translation start. More than 100 independent transgenic (herbicide-resistant) plants, grown under normal conditions (see “Materials and Methods”), were analyzed and the majority showed no detectable GUS activity in the seedling or presenescence stages. For the small number of GUS-positive transgenic seedlings, GUS activity was observed in metaxylem between the protoxylem poles in the root and in cotyledons. GUS activity was detected in the largest percentage of T1 plants when 8 weeks old plants were tested. In this case GUS activity localized to the xylem parenchyma and cambium of vascular bundles of senescing leaves, and to the xylem of the youngest lateral roots (Zhao et al., 2005). Given that *XND1* was identified as part of the leaf senescence transcriptome (Guo et al., 2004), and that GUS activity driven by the putative promoter was easily detected in senescing leaves, and that our xylem transcript profile was derived from senescing plants, *XND1* expression may be strongly linked with senescence. Previous investigations indicated that senescence is regulated by the combined action of several hormones:

cytokinin retards senescence while ABA, ethylene, methyl jasmonate, brassinosteroids and salicylic acid promote senescence (for review, see Lim et al., 2003). We treated *XND1p-GUS* seedlings with ABA and noted that both the percentage of GUS-positive seedlings and the level of GUS activity increased (Fig. 4.1).

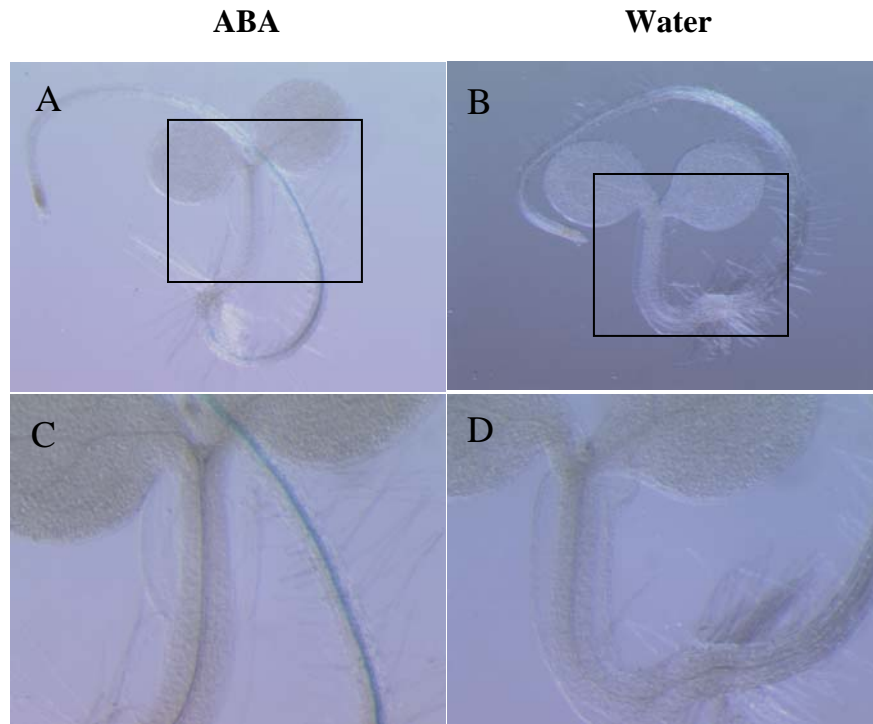


Figure 4.1. Histochemical staining for GUS activity in transgenic *Arabidopsis* plants harboring the *XND1p-GUS* construct with (A and C) or without (B and D) ABA treatment. Sections inside boxes in A and B are shown magnified in C and D, respectively.

The structure of the *XND1* gene

Based on its complete sequence (GenBank accession number NM_125849), the *XND1* cDNA is 930 bp in length and encodes a protein of 187 amino acids. A phylogenetic analysis for NAC domains from rice NACs, *Arabidopsis* NACs, and the other known NAC family proteins supported the classification of NAC proteins into two groups. All the previously characterized NAC family proteins were members of Group I while *XND1* was

placed in Group II (Ooka et al., 2003). Even though XND1 is very different from all the founding members of this family, i.e., XND1 shares only 32.6, 29.9, 33.2, and 31.6% identity in the amino acid sequence with NAM, CUC2, ATAF1, and ATAF2, respectively, the *XND1* gene, like the majority of the NAC genes, is composed of three exons. The lengths of *XND1* exons are 275, 278, and 377 bp separated by two introns of 629 and 147 bp. Most of the first, the second, and the beginning of the third exons encode the conserved NAC domain, which spans amino acids 3 to 155, whereas the majority of the small third exon encodes the highly divergent C-terminal domain. The NAC domain consists of the five conserved subdomains, A-E by Kikuchi et al. (2000) or motifs N1-N5 by Xie et al. (1999) that characterize the NAC family. A PROSITE (<http://us.expasy.org/prosite/>) search detected a serine-rich region between amino acids 117 and 139, i.e., between motifs N4 and N5 (or subdomains D and E) (Fig. 4.2).

A

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1 MSEL LQ - - - - - LPPGFRFHPTDEELVMHYLCRKCASQSI AVPI IA ATAF1
1 MKSELN - - - - - LPAGFRFHPTDEELVKFYLCRKCASEQISAPV IA ATAF2
1 MDIPYYHYDHGGDSQY LPPGFRFHPTDEELI THYLLR KVL DGC FSSRA IA CUC2
1 ME - NYQHFD CSDSN - - LPPGFRFHPTDEELI TYYLLKKV L DSNFTGRA IA NAM
1 MN - - - - - LPPGFRFFPTDEELVVHFLHRKASLLPCHPDV IP XND1

41 EIDL YKYD PWE L PGL AL YGEKE WYFFS PRDR KY P NGSRPN R SAGS GYWKA ATAF1
41 EIDL YKFN PWE L PEMS LY GEKE WYFFS PRDR KY P NGSRPN R AAGT GYWKA ATAF2
51 EVDL NKCEP WQL PGR AKMGEKE WYFFS LRDR KY P TGLR TN R ATEAGYWKA CUC2
48 EVDL NKCEP WEL PEKAKMGEKE WYFFS LRDR KY P TGLR TN R ATEAGYWKA NAM
37 DLDLYHYDPWDL PGLKALGEGRQWYFYSRKTQ - - - - - ERVTSNGYWGS XND1

91 TGADKPIGLPKP - - - VGIKKALV FYAGKAPKGEK TNWIMHE YRLA DVDRS ATAF1
91 TGADKPIGKPKT - - - LGIKKALV FYAGKAPKGIK TNWIMHE YRLANVDRS ATAF2
101 TGKDREIIFS SKTCA L VGMKKT LV FYKGRAPKGEKSNWVMHE YRLE GKFSY CUC2
98 TGKDREI IYS SKTSA L VGMKKT LV FYRGRAPKGEKSNWVMHE YRLD GKFA Y NAM
79 MGDDEPIYTSSTHKKVGIKKYLT FYL G - - - - - DSQTNWIMQEYSLPDSSSS XND1

138 VR - KKKN SLR - - - - - LDDWVLCRI YNKK - - - - - ATAF1
138 ASV NKKN SLR - - - - - LDDWVLCRI YNKK - - - - - ATAF2
151 HFI SRSSK - - - - - DEWVTSRV FQKTT LASTGAV SEGGGGGGA T CUC2
148 HYI SRSSK - - - - - DEWVTSRV FQKSC - STVGT T SNGG - - - - - NAM
125 SSRS SKRSSRASSSSSHKPDYSKWVICRV YEQNC - - - - - SEEDDD DGT E XND1

160 - - - - GATERRGPPPPV VYGDEIMEEKPKVTEMVMPPPPQQ TSEFAYFD T ATAF1
161 - - - - GTMEKYFPA - - - - - DEKPRTTTMAEQSSSP - - - - - FDT ATAF2
189 VSVSSGTGPSKKT KV PSTISRNYQE QPSSPSSVSLPPLLDPT T T LGYTDS CUC2
179 - - - - - KKR LNSSSFNNMYQE - VSSPSSVSLPPLLES - - - - - NAM
168 LS - - - - - XND1

205 SDSV - PKLHTT DSSCSE - - - QVVS - - - - - PEFTSEVQSEPKWKDWSAV ATAF1
189 SDSTYPTLQEDDSSSSSGHGHWVS - - - - - PD - VLEVQSEPKWGELEDA ATAF2
239 S - CSYDSRSTNTT V TASAITEHVS CFSTV - - - - - PTTTALGLDVNS CUC2
209 - - - PYNNTATSA - - - AASKKEHVS CFSTISTPSF - - DPSSVFDISSNSNT NAM
170 - - - - - CLDEV - - - - - XND1

244 SNDNNTLD FGF - - - - - NYIDATVDNAFGGGGSSN ATAF1
231 LEAFDTSM - FGS - - - - - S - MEL LQPDAF - - - - - VPQ ATAF2
280 FSRLPPPLGFD - - FDPFPR FVSRNVSTQSNFRSFQENFNQFPYFGSSSAS CUC2
251 LHS LPAP - SFSAILDPSST FSRNSVF - - PSLRSLQENL - HLP L FSGG - - - NAM
175 - - - - - FLSLDDDLDEVSLP XND1

274 QMFPLQDMFMYM - - - - QKPY ATAF1
255 FLYQSDYFTSFQDPPPEQKPF LNWSFAPQG ATAF2
328 TMTSAVNLPSFQGGGGVSGMNYWLPATAEENESKVGVLHAGLDCIWN Y CUC2
294 - - TSAMH - - - - - GGFSSPLANWVPVETQKVD - - - - - HSELDCMWSY NAM
187 XND1

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B

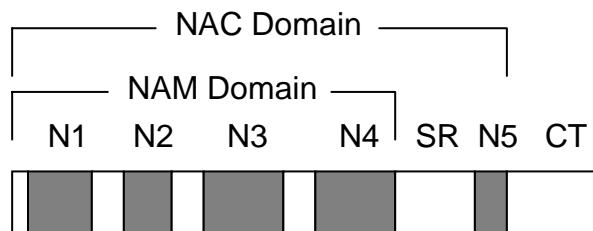


Figure 4.2. The structure of XND1.

A, Multiple sequence alignment of XND1 and other characterized NAC domain-containing proteins. Accession numbers for ATAF1, ATAF2, CUC2, and NAM are NM_100054, NM_147856, AB002560, and X92205, respectively. Residues that are identical to XND1 are highlighted in blue. The locations of the 5 conserved amino acid motifs (N1 to N5) are shown.

B, A schematic diagram of the structure of XND1. The 5 conserved amino acid motifs, N1 to N5, based on the alignment with other known NAC domain-containing proteins are shown by gray boxes. The serine-rich region between motifs N4 and N5 is marked as SR, and the C-terminus as CT. The NAM domain in InterPro (<http://www.ebi.ac.uk/interpro/>) consists of amino acid motifs N1 to N4. The NAC domain consists of amino acid motifs N1 to N5. The multiple sequence alignment was generated with Lasergene.

Nuclear localization of XND1-GFP fusion protein

Previous analysis of NAC protein sequences predicted a nuclear localization signal sequence (NLS) in subdomains N3 and N4 (Kikuchū et al., 2000). Nuclear localization of NAC1 was confirmed using a GFP-NAC1 fusion protein (Xie et al., 2000). No nuclear localization elements were found in XND1 using PredictNLS (<http://cubic.bioc.columbia.edu/predictNLS/>). To test whether XND1 is localized to the nucleus, transgenic plants carrying a *XND1-GFP* fusion gene expressed under the control of *MYR1* promoter or 35S promoter were generated. For comparison, GFP alone as transcriptional fusion with 35S promoter or *MYR1* promoter was used as a control. Roots of several 4-week-old independent transgenic lines were examined using confocal microscopy. The preliminary results indicated that the XND1-GFP fluorescence was localized in the nucleus, whereas in the control seedling, GFP (driven by *MYR1* promoter) fluorescence was observed throughout the cytosol of phloem cells (Fig. 4.3).

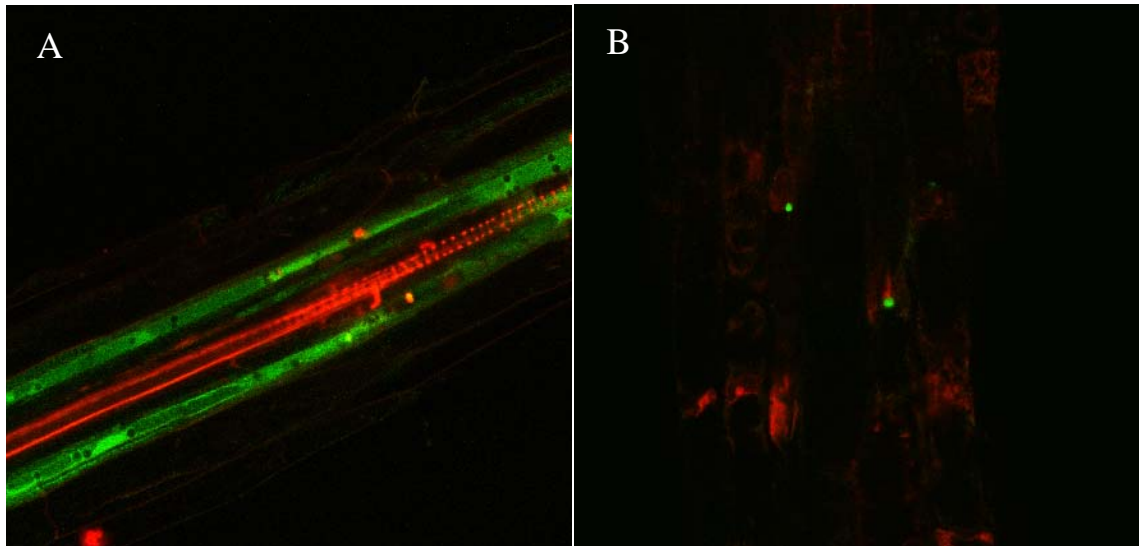
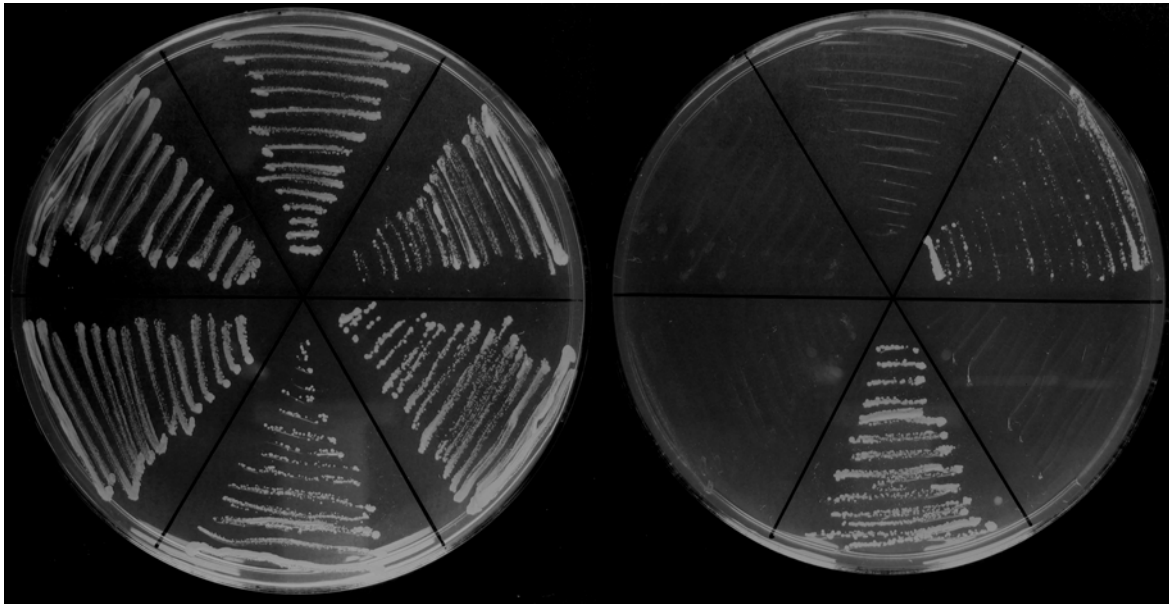


Figure 4.3. Confocal micrographs of fluorescence of GFP (A) or XND1-GFP (B) driven by *MYR1* promoter. Xylem and cell walls were stained by propidium iodide.

The C-terminus of XND1 has transactivation activity

Previous studies demonstrated that NAC family members, such as NAM, CUC2, and GRAB1/2, function by binding DNA and activating gene transcription, i.e., as transcription factors (Aida et al. 1999; Xie et al., 1999). The transcription activation domain was located within the C-terminal region (Xie et al., 2000; Duval et al., 2002; Robertson, 2004; Taoka et al., 2004). Since the C-terminus is highly variable among NAC domain proteins and XND1 is highly divergent compared with all characterized NACs, we therefore made a series of constructs by fusing XND1 full-length and truncated versions to the GAL4 DNA-binding domain and tested their ability to activate gene expression in yeast. The yeast strain used was AH109 that contained four reporters (*ADE2*, *HIS3*, *MEL1*, and *lacZ*) under the control of three distinct GAL4 upstream activating sequences (UASs) and TATA boxes. The results indicated that yeast transformed with XND1₁₋₁₈₇-BD, i.e., full-length XND1 fused in frame to the GAL4 DNA-binding domain, could grow on selective media. Three C-terminal-truncated XND1-BD fusions, XND1₁₋₁₁₉-BD, XND1₁₋₁₅₅-BD and XND1₁₋₁₇₄-BD, were unable to activate gene expression in yeast and could not support yeast growth in the absence of histidine. The XND1 C-terminus consisting of only 34 residues, XND1₁₅₄₋₁₈₇ (encoding amino acids 154-187), was sufficient for transactivation and could promote yeast growth in the absence of histidine. Thus as is true

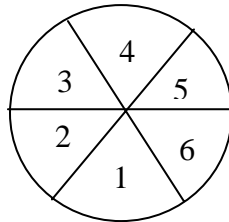
for all characterized NAC proteins, a relatively small C-terminal domain of XND1 contains residues critical for transcriptional activation, whereas the conserved NAC domain was by itself unable to activate gene expression in yeast.



-Trp

-Trp -His

C



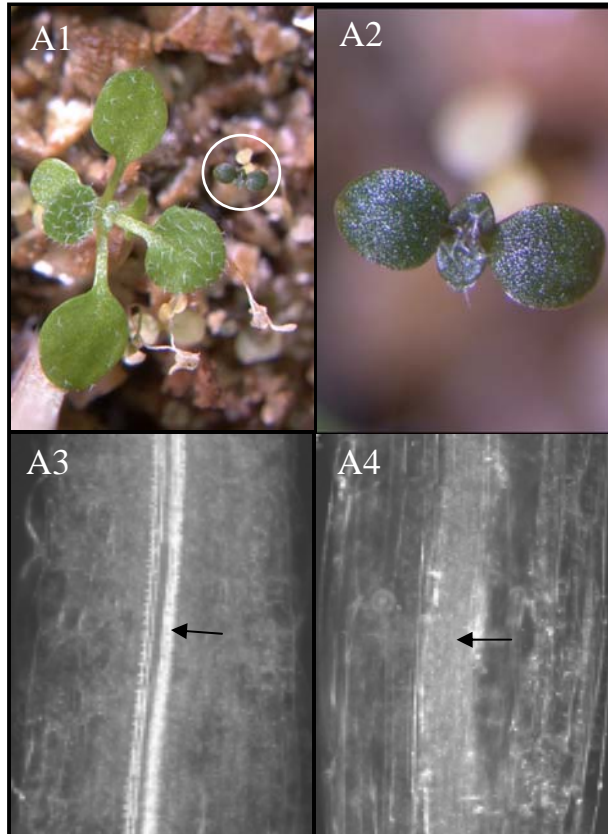
1. XND1₁₋₁₈₇/BD
2. XND1₁₋₁₇₄/BD
3. XND1₁₋₁₅₅/BD
4. XND1₁₋₁₁₉/BD
5. XND1₁₅₄₋₁₈₇/BD
6. BD

Figure 4.4. Identification of the transactivation domain of XND1. XND1 full-length (amino acids 1-187) and truncated versions encoding the N-terminal 174 amino acids (longer than NAC domain), 155 amino acids (NAC domain), 119 amino acids (NAM domain), and the C-terminal 34 amino acids (154-187) were fused in frame to the GAL4 DNA-binding domain in pGBKT7. Yeast cells (AH109) were transformed with the indicated plasmids, and streaked on plates lacking Trp (A. -Trp) or Trp and His (B. -Trp -His). C. The scheme shows the array of yeast transformants containing the indicated plasmids.

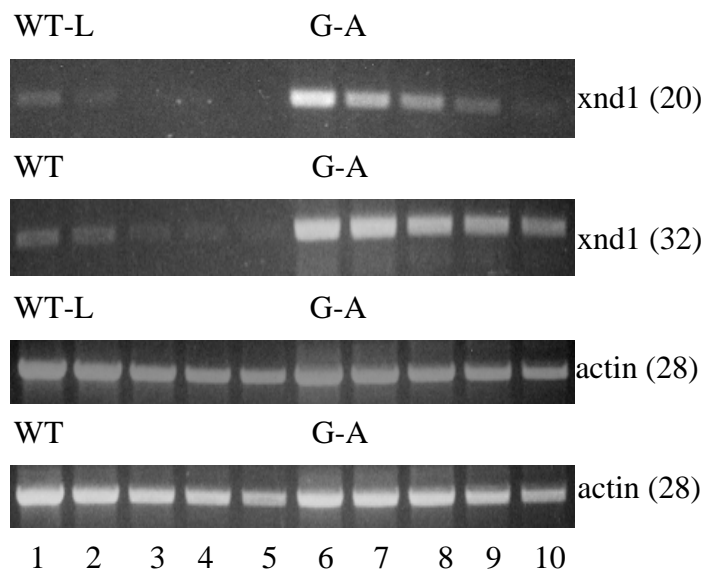
***XND1* overexpression results in dwarfing and suppression of TE differentiation**

To investigate the function of *XND1*, we examined the effects of overexpression of *XND1* in *Arabidopsis*. Overexpression of *XND1* driven by the CaMV 35S promoter resulted in extreme stunting in > 30% of transformants recovered in five independent experiments producing more than 500 T1 transgenic plants. Dwarf seedlings were dark green, and after four to five weeks of growth—beyond which the vast majority of mutants did not survive—most had not expanded beyond 2 mm, measured across both cotyledons. Out of 44 severely stunted 35S-*XND1* plants only three survived to produce a small number of seeds. None of the T2 progeny from these survivors produce seeds. In wild type plants, mature TEs are detectable by 2.75 days post-germination (Busse and Evert, 1999). However, more than 80% of the dwarf 35S-*XND1* plants contained no detectable TEs in any part of the plant body up to two weeks post-emergence. The rest possessed only few TEs, mainly restricted to the base of the cotyledons and discontinuous with root TEs. To determine whether the observed phenotypic effects were caused by *XND1* overexpression, *XND1* mRNA levels in dwarf, TE-minus plants were compared with that of developmentally similar wild type-like transformants and wild type plants using reverse transcriptase-mediated (RT) PCR. Semi-quantitative RT-PCR analysis confirmed the dwarf, TE-minus phenotype was correlated with the highest levels of *XND1* expression, i.e., ~10-fold higher than *XND1* levels in wild type-like transformants and ~2000-fold higher than *XND1* levels in wild type plants. Three homozygous *XND1* insertion lines (discussed below) did not exhibit any of the aforementioned abnormalities in vascular tissue development or dwarfing, further supporting the conclusion that the dwarf, TE-minus phenotype was due to high-level expression of *XND1* but not to cosuppression.

A



B1



B2

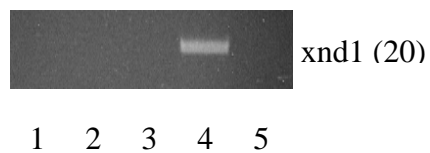


Figure 4.5. A, Overexpression of the xylem-specific NAC domain superfamily gene *XND1* results in dwarfing and suppression of TE differentiation. A1, Two types of herbicide-resistant *35S-XND1* plants are shown: those exhibiting apparently wild type-like growth (larger plant) and those exhibiting extreme dwarfism (plant inside white circle). A2, Enlarged image of the dwarf plant shown in A1. Plants shown in A1 and A2 are two weeks old. A3, Five-day-old (same developmental stage as dwarf *35S-XND1* plants) wild type seedling showing normal development of TEs in the hypocotyl (arrow). A4, Typical ten-day-old dwarf *35S-XND1* plant containing no detectable TEs in the vascular cylinder of the hypocotyl (arrow). A3 and A4 are dark field images of whole-mount hypocotyls. B, Ethidium bromide-stained gels showing products of a semi-quantitative RT-PCR for *35S-XND1* dwarf (growth arrested, G-A), *35S-XND1* wild type-like (WT-L), and wild type (WT) plants. The primers (*xnd1* or *actin*) used for PCR are indicated to the right of each gel panel. B1, cDNA from G-A (lanes 6-10) and WT-L or WT (lanes 1-5) plants was diluted 1-, 2-, 4-, 8- and 16-fold except cDNA from G-A plants, diluted 128-, 256-, 512, 1024, and 2048-fold, for the G-A versus WT comparison. B2, Ethidium bromide-stained gel showing PCR products from RT-minus controls from wild type (lane 1), *35S-XND1* wild type-like (lane 2), and dwarf plants (lane 3), RT-plus positive control from dwarf plants (lane 4), and water-only negative control (lane 5). Numbers of PCR cycles used are indicated in parentheses to the right of each panel. A wide range of cDNA dilutions and PCR cycle combinations was evaluated before these representative experiments were selected for presentation.

***XND1* overexpression specifically suppresses xylem differentiation without preventing phloem differentiation**

To determine whether disruption of TE differentiation caused by overexpression of *XND1* was an effect of general suppression of growth and differentiation or specifically disrupted xylem differentiation, we introduced *35S-XND1* into *XCP2p-GUS*, *XSP1p-GUS*, and *MYR1p-GUS* backgrounds. *XCP1* is a xylem-specific papain-like cysteine peptidase and localizes to TEs (Funk et al., 2002). *XSP1* expression is xylem-biased (Zhao et al., 2005) and *XSP1p-GUS* expression is restricted to TEs (C. Zhao and E. Beers, unpublished data). Expression of *MYR1* (Thelander et al., 2002) is restricted to phloem as is GUS activity

driven by the *MYR1* promoter (*MYR1p*) (Zhao et al., 2005). Among 76 dwarf *35S-XND1* plants stained for GUS activity, approximately 80% did not express the xylem-markers *XCP2p-GUS* or *XSP1p-GUS*, the remaining 20% expressed a very low level of GUS compared with the backgrounds used for transformation. In contrast, expression of the phloem marker *MYR1p-GUS* was detectable in all dwarf *35S-XND1* plants (Fig. 4.6).

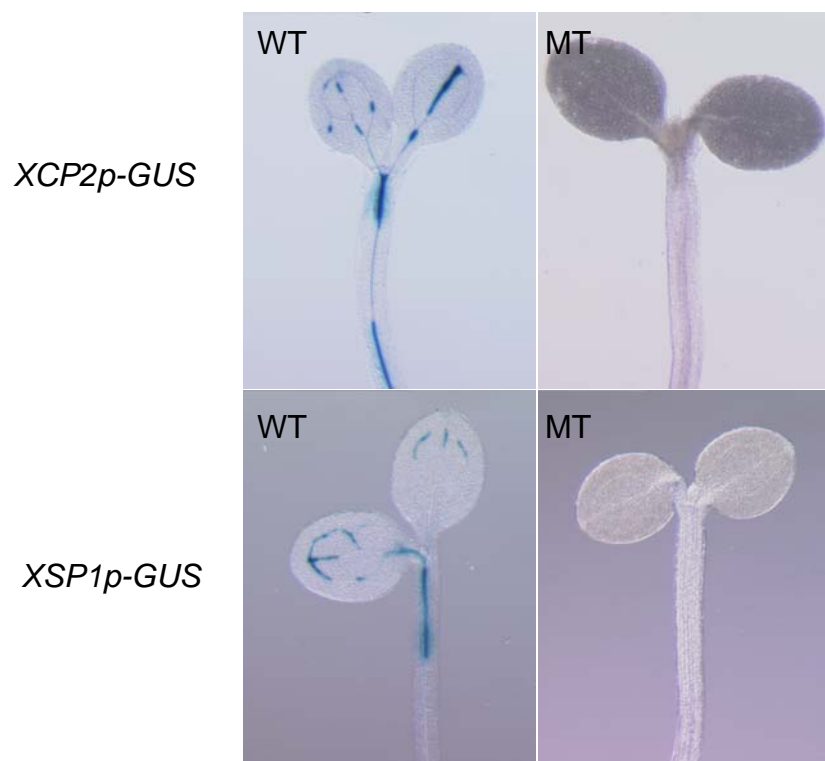
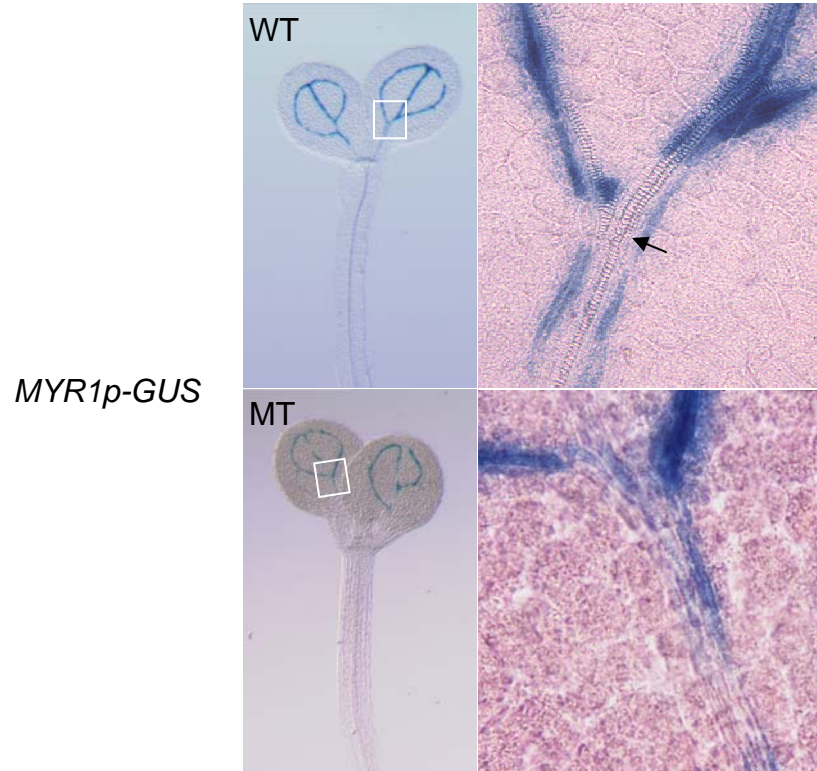


Figure 4.6. Constitutive expression of *XND1* prevents TE differentiation and associated *XCP2* and *XSP1* gene expression (based on *GUS* activity) but does not prevent phloem differentiation (based on *MYR1p-GUS* expression). Transgenic backgrounds are indicated for each panel. Sections inside boxes are shown magnified in adjacent panels. TEs are easily detected in *MYR1p-GUS* (WT) cotyledons (arrow) but are absent from *MYR1-GUS*; *35S-XND1* (mutant, MT) cotyledons. Seedling transgenic for *GUS* only (i.e., without *35S-XND1*) were 5 days old. Double transgenic seedlings were 14 days old but developmentally equivalent to seedlings transgenic for *GUS* only.

***XND1* overexpression blocks cell death**

The TE differentiation program includes elongation and/or expansion, secondary wall thickening, and programmed cell death. To test whether *XND1* overexpression suppressed cell death in addition to secondary cell wall patterning and expression of TE markers, dwarf *35S-XND1* plants were stained with the vital stain Evans blue which is excluded from living cells. As expected, Evans blue entered the dead vessels of the xylem of wild type seedlings and was transported throughout the entire vascular system (Fig. 4.7, left panel). In contrast, when two *35S-XND1* plants, one with TEs in the root only (Fig. 4.7, center panel) and one completely lacking TEs (Fig. 4.7, right panel) were placed in Evans blue, the movement of Evans blue was observed only in vascular tissue with detectable TEs, suggesting that *XND1* overexpression blocked cell death in the vascular system.

The results indicated that *XND1* overexpression suppressed xylem TE differentiation but not phloem differentiation. Whether the dwarfing component of the *35S-XND1* phenotype is a direct consequence of the reduction in TE differentiation or derives from some other *XND1*-mediated pathways is not yet known.

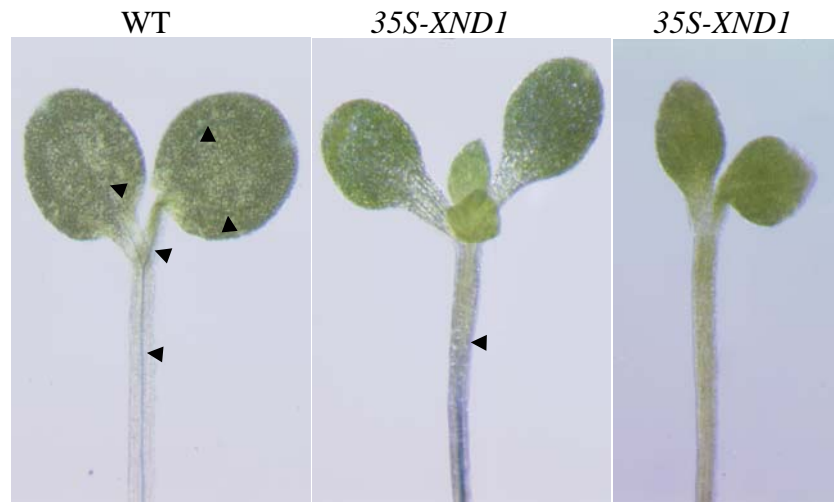


Figure 4.7. Vascular cell death is blocked in dwarf *35S-XNDI* plants. Evans blue is continuous throughout the xylem of a wild type plant (left panel, arrowheads). A *35S-XNDI* seedling (center panel) exhibiting xylem (Evans blue) discontinuity beginning at the hypocotyl-root junction (arrowhead) is compared with another more extreme *35S-XNDI* seedling completely lacking detectable TEs, and consequently, the ability to transport Evans blue (right panel). The wild type seedling was 5 days old. The *35S-XNDI* seedlings were four weeks old.

A mutation in *XNDI* reduces growth under ABA and cold treatment

To obtain a loss-of-function mutation in the *XNDI* gene, we identified two T-DNA insertion lines and one transposon insertion line: the first T-DNA insertion line, SALK_022552, from the Arabidopsis Biological Resource Center (ABRC, <http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.htm>), the second T-DNA insertion line, GABI_162D11, from Max Planck Institute for Plant Breeding Research (<http://www.mpiz-koeln.mpg.de/>), and the transposon insertion line, GT_5_12667, from the Nottingham Arabidopsis Stock Centre (NASC, <http://arabidopsis.info/home.html>). Sequence analysis revealed that the T-DNA insertion site in SALK_022552 (based on the T-DNA left border/plant genomic DNA junction) is located in the putative promoter region of *XNDI*, 31 bp upstream from the putative transcriptional start site. Three bp of microhomology, CCT, was found at the transition

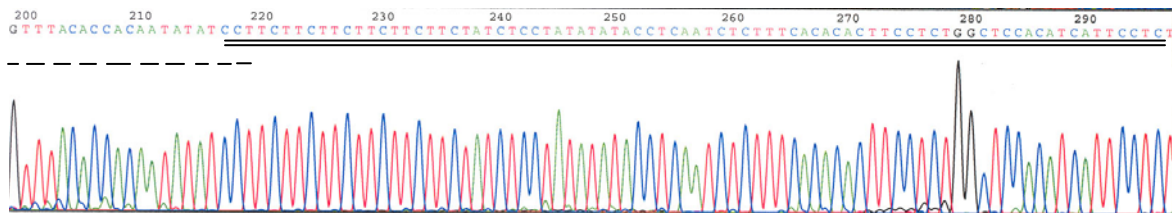
point (Fig. 4.8A1). We named this *xnd1* insertion allele *xnd1-1*. A very low level of *XND1* mRNA (approximately eight-fold lower than that in wild type) was detected in homozygous *xnd1-1* plants by semi-quantitative RT-PCR (Fig. 4.8D1). Further RT-PCR assays using T-DNA left border primer and an *XND1* antisense primer corresponding to the stop codon and sequence analysis indicated that a hybrid transcript existed. This transcript comprised the T-DNA left border, Arabidopsis genomic DNA between the T-DNA left border/Arabidopsis genomic DNA junction and the putative transcriptional start site, and most of *XND1* native transcript (i.e., from the putative transcription start site to the stop codon, the two introns were spliced out). Similar T-DNA/genomic DNA hybrid transcripts have been reported (Wang et al., 2003). Since this hybrid transcript contained the native complete coding sequence, i.e., from the first ATG to the stop codon, it is possible that this hybrid transcript could be translated, i.e., that the mutant is leaky.

T-DNA insert in GABI_162D11 is located in the third exon, 44 bp upstream from the stop codon. Four bp of filler sequence (Windels et al., 2003), CACT, was found between the end point of the T-DNA left border and the starting point of the plant DNA (Fig. 4.8A2). We named this *xnd1* insertion allele *xnd1-2*. No transcript containing the complete coding sequence was detected in homozygous *xnd1-2* plants by semi-quantitative RT-PCR (Fig. 4.8D2). Since the T-DNA/plant DNA junction was located in *XND1* C-terminus and the genome contained the complete promoter for *XND1*, it was possible that a hybrid transcript containing most of *XND1* and part of T-DNA left border could be formed. To test this hypothesis, we performed further RT-PCR using a T-DNA left border primer, 20 bp from the junction between T-DNA left border and plant genomic DNA, and an *XND1* sense primer corresponding to the translation start site. Sequence analysis of the resulting PCR products indicated that a hybrid transcript containing most of *XND1* (i.e., from the translation start site to the junction between T-DNA left border and plant genomic DNA, the two introns were spliced out) and part of T-DNA left border existed. It is not yet known whether this hybrid transcript could be translated *in planta* or not, and whether this hybrid protein, if translated, could function normally or not.

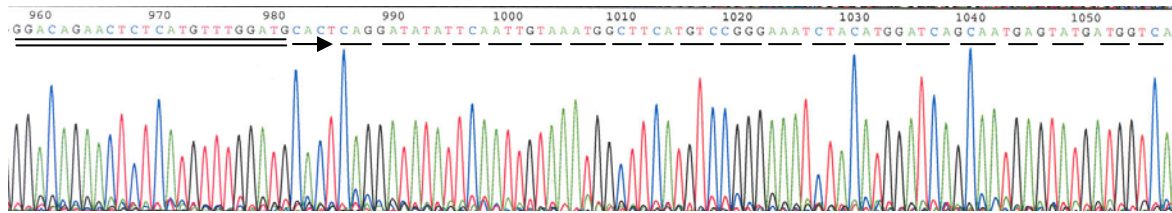
The transposon insertion line, GT_5_12667, generated by the mobilization of a gene-trap Ds transposable element, contains a *GUS* gene with a three-frame splice acceptor (Sundaresan et al., 1995). The insertion site near the three-frame splice acceptor (3'Ds) in GT_5_12667 is located in the second intron, 5 bp downstream from the 5' splice site of intron 2. Two bp of microhomology, TA, was observed at the insertion site (Fig. 4.8A3). We named this *xnd1* insertion allele *xnd1-3*.

Outwardly, the *XND1* knockout mutants did not differ from wild type plants under normal conditions. Consistent with the findings that *XND1* was identified as part of the leaf senescence transcriptome (Guo et al., 2004) and as a xylem-biased gene in secondary xylem (Zhao et al., 2005), that GUS activity was easily detected in xylem of senescing leaves, and that ABA, a known promoter of senescence, could activate GUS expression driven by the *XND1* promoter in seedlings, we analyzed *xnd1* mutants grown under 4°C or treated by ABA. Preliminary data showed that *xnd1* mutants are ABA and cold hypersensitive, exhibiting reduced growth (data not shown).

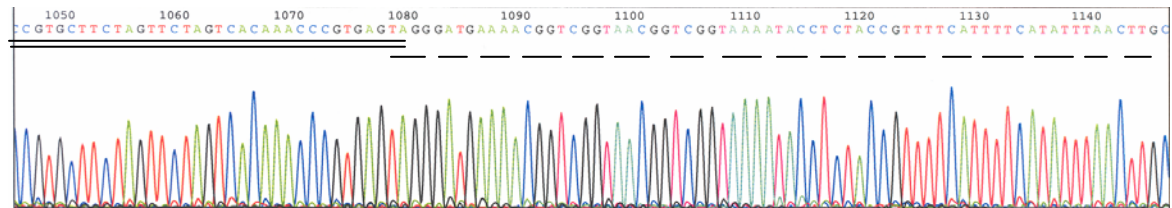
A1



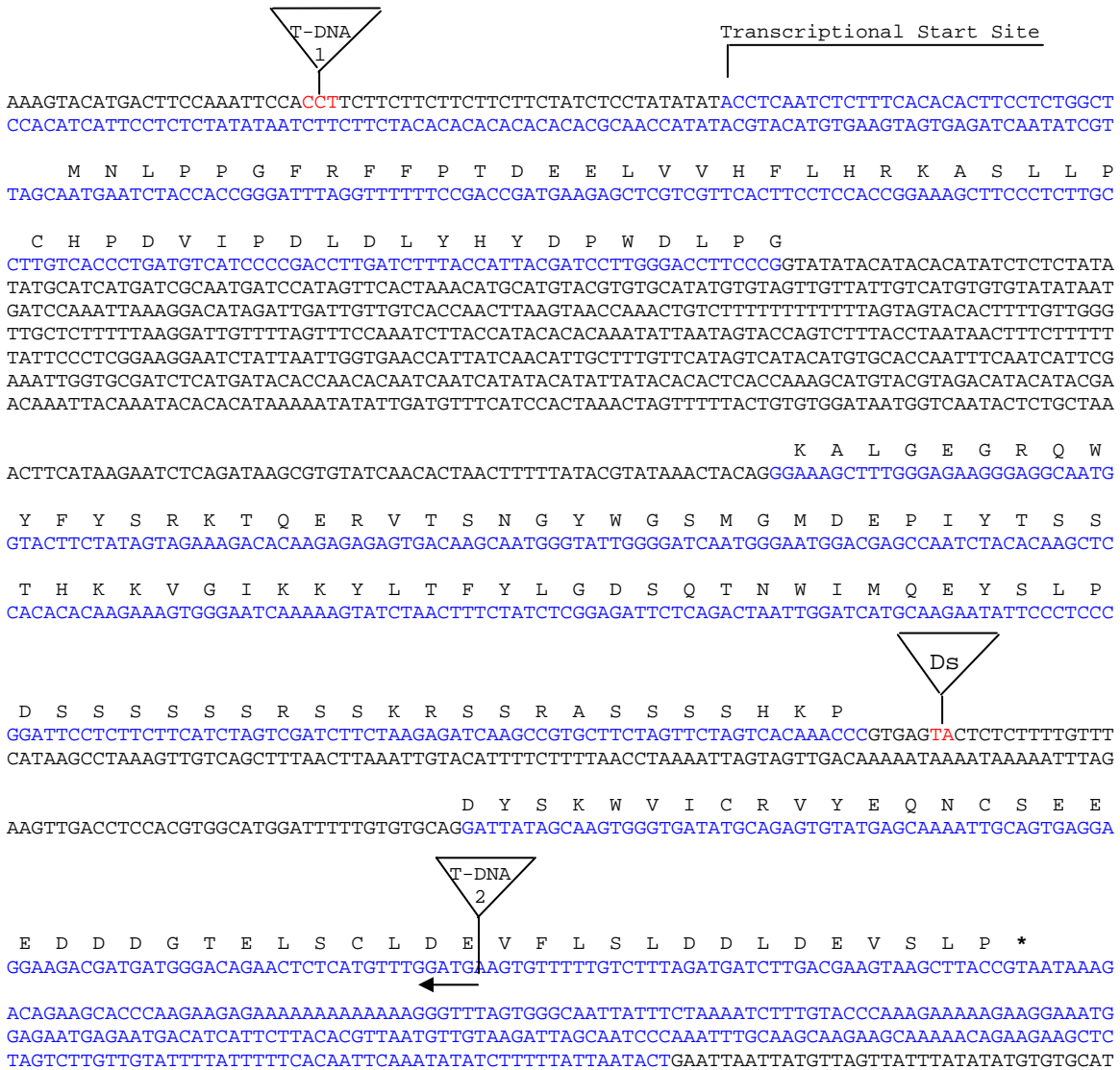
A2



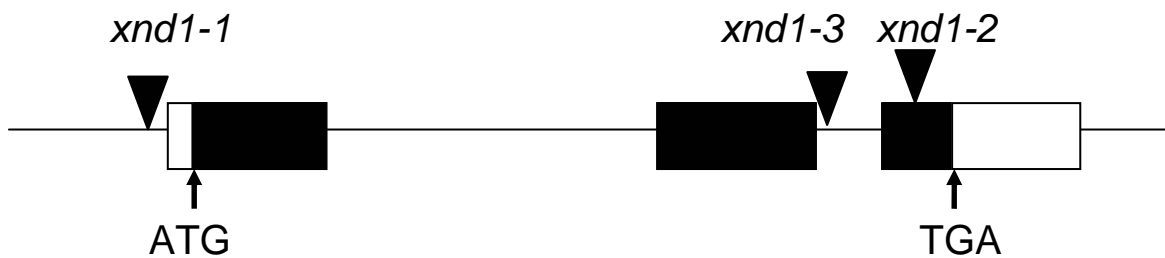
A3



B



C



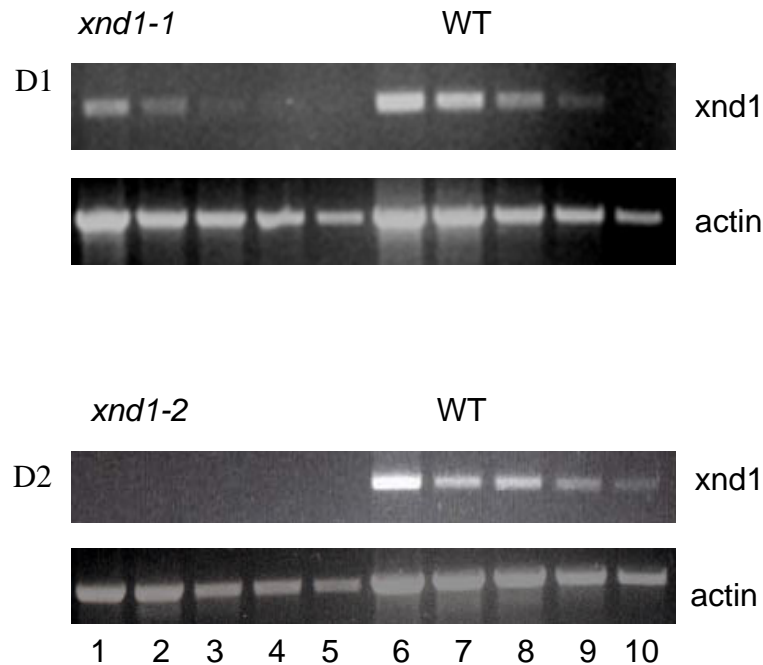
D

Figure 4.8. Characteristics of the *xnd1* mutations.

A, Sequence analysis of junctions between T-DNA left border (dashes in A1 and A2) or Transposon (dashes in A3) and Arabidopsis genomic DNA (double lines) in the *xnd1* insertion allele *xnd1-1*(A1), *xnd1-2* (A2), and *xnd1-3* (A3). Microhomology, the CCT, positions 217-219 indicated by overlapping dashes and double lines in A1; filler DNA, the CACT, positions 982-985 indicated by arrow in A2; and microhomology, the TA, positions 1079-1080 indicated by overlapping dashes and double lines in A3 were shown. B and C, The positions of T-DNA or transposon insertion sites (based on the sequences of junctions between T-DNA left border, or transposon and Arabidopsis genomic DNA) in the *xnd1* insertion allele *xnd1-1*(T-DNA1), *xnd1-2* (T-DNA2), and *xnd1-3* (Ds). DNA based on TAIR (www.arabidopsis.org) and deduced protein sequences of *XND1* were generated using JavaScript DNA translator 1.1 (http://www.bioinformatics.vg/bioinformatics_tools/JVT.shtml). Exons are highlighted in blue. Three bp of microhomology (CCT) at the junction between T-DNA left border and Arabidopsis genomic DNA in *xnd1-1*, and 2 bp microhomology (TA) at the insertion site in *xnd1-3* are highlighted in red. The starting point of Arabidopsis genomic DNA for T-DNA left border/plant DNA junction in *xnd1-2* is indicated by arrow. T-DNA insertion

and the putative transcriptional start sites are marked (B). Protein-coding exons are represented by black boxes, and untranslated regions by white boxes; introns by lines; T-DNA or transposon insertions by black triangles. ATG, translation initiation codon; TGA, translation termination codon (C).

D, Ethidium bromide-stained gels showing products of a semi-quantitative RT-PCR for homozygous *xnd1-1*, *xnd1-2*, and wild-type (WT). The primers (*xnd1* or *actin*) used for PCR are shown to the right of each gel panel. cDNA (lanes 1-5 or 6-10) was diluted 1-, 2-, 4-, 8-, and 16-fold. The numbers of PCR cycles used are 28 for *actin*, *XND1* in *xnd1-1* versus WT, and 32 for *XND1* in *xnd1-2* versus WT.

Identification of XIP7, an XND1-interacting protein that contains a RING-H2 domain

To further understand the function of XND1, we performed a yeast two-hybrid screen using a truncated XND1₁₋₁₇₄ (the first 174 amino acids) as bait. Six colonies were isolated from the 5×10^5 colonies screened. Nine different AD/library plasmids were isolated because some colonies contained more than one AD/library plasmid. By re-transforming bait and prey into the yeast strain AH109, four AD/library plasmids (Yeastx-2, -4, -6, and -7) were found to be able to interact with bait in yeast. DNA sequence analysis showed that all of these four AD/library plasmids (Yeastx-2, -3, -6 and -7) encoded the same protein (At3g62970), a novel RING-finger protein, fused in frame to the GAL4 activation domain, and specified by unique transcripts. Yeastx-6 encoded the full-length At3g62970 reported in TAIR databases (<http://www.arabidopsis.org>). Yeastx-7 encoded the full-length At3g62970 reported in PlantsUBQ databases (<http://plantsubq.sdsc.edu>), which contained 11 additional amino acids at the N-terminus compared with the At3g62970 reported in TAIR database. Yeastx-2 encoded the same length of transcript as Yeastx-7, but their DNA sequences reflected the loss of one nucleotide at different positions in the recombination region, i.e., they were different recombinant molecules. It is not clear how these losses occurred, during recombination or as the result of errors in BD SMART III Oligo. Yeastx-4 contained two other nucleotides at the 5' end compared with that in Yeastx-7 (Fig. 4.9A). It is possible that the At3g62970 reported in PlantsUBQ databases is

a full-length protein. We have named this protein XIP7 (for XND1-interacting protein in Yeastx-7).

The structure of the *XIP7* gene

The longest insert in AD/library plasmid for *XIP7* was 1088 bp, encoding a 287 amino acid protein. Computer analysis using InterPro (<http://www.ebi.ac.uk/interpro>) and Pfam (<http://www.sanger.ac.uk/Software/Pfam/>) showed that the predicted protein contained a cysteine-rich RING motif defined by the consensus sequence C-X₂-C-X₉₋₃₉-C-X₁₋₃-H-X₂₋₃-C/H-X₂-C-X₄₋₄₈-C-X₂-C (where X can be any amino acid, and numbers indicate the number of residues) (Borden, 2000), and a CHY zinc finger motif (Pfam accession number: PF05495). CHY zinc finger motif is often associated with C3HC4 type zinc finger motif. Two putative C2H2 type zinc fingers containing the consensus sequence C-X₁₋₅-C-X₁₂-H-X₃₋₆-H/C (where X can be any amino acid, numbers indicate the number of residues, Pfam accession number: PF00096), were found between the CHY and RING motifs, and at the C-terminus (Fig. 9), respectively. Neither a nuclear localization signal nor a coiled-coil region was found in XIP7 by PredictNLS (<http://cubic.bioc.columbia.edu/predictNLS>) and COILS (http://www.ch.embnet.org/software/COILS_form.html), respectively. A BLAST search showed that the Arabidopsis genome contains other 5 homologs (At1g74760, At3g18290, At5g18650, At5g22920, and At5g25560) that contained both CHY and RING motifs.

finger motifs are underlined. Genomic DNA (based on TAIR, www.arabidopsis.org) upstream from the 5' end of cDNA is highlighted in red. B, The schematic diagram showing the structure of XIP7. The CHY motif is shown by a green box, the RING motif by a gray box, C2H2 motifs by blue boxes.

Discussion

***XND1* is expressed in xylem and in association with ABA response and leaf senescence**

Genome-wide expression profiling indicated *XND1* is xylem-specific (Zhao et al., 2005). To test this predicted xylem-specific expression, we fused the putative *XND1* promoter with the GUS reporter gene. Under normal conditions, the majority of transgenic plants showed no detectable GUS activity in the seedling or presenescence stages. In the small number of GUS-positive seedlings, GUS activity was detected in metaxylem between the protoxylem poles. In two-month-old plants, GUS activity was easily detected in the xylem of the youngest lateral roots and the xylem parenchyma of senescing leaves, consistent with a recent report, in which *XND1* was identified as part of the leaf senescence transcriptome (Guo et al., 2004). Based on published reports that plant hormones, such as abscisic acid, ethylene, methyl jasmonate, brassinosteroids, and salicylic acid, are involved in the regulation of senescence (for review, see Lim et al., 2003), we treated *XND1p-GUS* seedlings with ABA, a known promoter of senescence in Arabidopsis (Pourtau et al., 2004) and found preliminarily that the percentage of GUS-positive seedlings and the level of GUS activity increased, suggesting that *XND1* could be activated by ABA. It is not yet known whether *XND1* expression could be regulated by other hormones.

***XND1* overexpression suppresses xylem differentiation.**

Even though initial studies on NAC genes focused on shoot apical meristems (Souer et al., 1996), recent investigations implicate NAC domain genes as regulators of a variety of organ and tissue development programs. For example, *NAC1* play important roles in lateral root formation (Xie et al., 2000) and *NAP* may function in the transition between growth by cell division and cell expansion in stamens and petals (Sablowski et al., 1998). By comparing Arabidopsis xylem and phloem-cambium transcript profiles, we noted six xylem-biased NAC domain family members (Zhao et al., 2005) and *XND1* showed the highest expression level among these xylem-biased NAC domain genes. Overexpression of *XND1* driven by the CaMV 35S promoter resulted in the suppression of TE differentiation and severe stunting in more than 30% of transformants. Using different

GUS reporter lines as genetic backgrounds, we showed that overexpression of *XND1* suppressed only TE-specific GUS expression but not phloem-specific GUS expression, suggesting that the observed phenotype was due to specific effects on xylem and not a general inhibition of plant growth and development.

The suppression of XND1 on xylem differentiation may be regulated by the ubiquitin/26S proteasome pathway

Ubiquitin-dependent protein degradation has an essential role in development of all organisms (for review, see Frugis and Chua, 2002; Sullivan et al., 2003; Pickart and Cohen, 2004). Ubiquitin attachment to target proteins involves three enzymes referred as the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2), and the ubiquitin ligase (E3). Several classes of proteins, such as HECT, RING, U-box domain families, can function as E3s (for review, see Smalle and Vierstra, 2004). Database searches followed by manual curation identified 469 predicated RING domain-containing proteins in the Arabidopsis genome, accounting for approximately 2% of the total genome. These predicted RING domain-containing proteins have been divided into 8 groups. *In vitro* ubiquitination assays have shown that the majority of RING domain-containing proteins representative of the different groups possess E3 ubiquitin ligase activity (Stone et al., 2005). Among these 469 predicted RING domain-containing proteins, A few with known biological function, such as COP1 (Holm et al., 2002), CIP8 (Hardtke et al., 2002), and SINAT5 (Xie et al., 2002), have been demonstrated to have E2-dependent ubiquitin ligase activity.

By performing a yeast-two hybrid screening of a xylem cDNA library with a truncated XND1 as bait, we identified a novel RING finger protein, XIP7 (At3g62970), which contains a cysteine-rich RING motif defined by the consensus sequence for the canonical RING type (RING-H2 group) proteins (Stone et al., 2005), a CHY zinc finger motif, and two putative C2H2 type zinc fingers. Previous investigations indicated that the RING domain could interact with an NAC protein. For example, Xie et al. (2002) used a truncated NAC1 as bait to carry out yeast two-hybrid assays and identified SINAT5, an *Arabidopsis* homologue of the RING-finger *Drosophila* protein SINA. Greve et al. (2003)

used RHA2alpha, a small RING-H2 protein, as bait in yeast two-hybrid screens and identified ANAC (for abscisic acid-responsive NAC). These results suggest that XND1 may interact with XIP7 *in vivo*. Considering that the majority of the tested RING domain-containing proteins possess E3 ubiquitin ligase activity (Stone et al., 2005), it is possible that XIP7 may be involved in the ubiquitin/26S proteasome pathway.

We have partially evaluated three homozygous *XND1* insertion lines. Under normal conditions, homozygous *XND1* insertion lines, *xnd1*, did not exhibit any obvious phenotype distinct from that of wt controls. Our findings and those of others (Guo et al., 2004) indicated that *XND1* expression is restricted to xylem, responsive to ABA, and upregulated during senescence. Although the precise function of XND1 is not yet known, results from gain-of-function and yeast two-hybrid experiments suggest a model where XND1 functions to limit xylem differentiation during senescence as a means of reducing the sink strength of senescing tissues. Such a reduction in xylem differentiation would favor export of sucrose to developing seeds over sucrose consumption as a precursor for secondary cell wall biosynthesis in xylem. The apparent tissue-specific and senescence-linked transcriptional level regulation of *XND1* may be augmented by posttranslational regulation mediated by the ubiquitin/26S proteasome pathway via XIP7 activity.

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Appendix I

Construction of the modified pBI121, mpBI121

mpBI121 was constructed by inserting the *Bar* cassette from pCB302 (a gift from David J. Oliver, Iowa State University; Xiang et al., 1999) and a portion of pFGC5941 (<http://www.arabidopsis.org/servlets/TairObject?type=vector&id=500300075>) containing a polycloning site into the *EcoR I*, *Hind III/BamH I* sites of pBI121 (Jefferson et al., 1987), respectively, using the following strategy.

Plasmid DNA pCB302 was digested with *Hind III*, blunted using Klenow (Promega, Madison, WI), and then digested with *BamH I*, the released fragment containing *Pnos-Bar-Tnos* (the *Bar* cassette) was ligated with pGEM-XSP1p (antisense orientation in pGEM) (see the construction of pGEM-XSP1p in this appendix) digested with *Spe I* (the *Spe I* site is located on the vector), blunted, and then digested with *BamH I*. The purpose of using pGEM-XSP1p was to introduce a *BamH I* site into pGEM for efficient cloning. The resulting plasmid DNA pGEM-*Bar* was digested with *EcoR I*, the released fragment containing the *Bar* cassette was ligated with pBI121 digested with *EcoR I*, and dephosphorated by Alkaline Phosphatase, Calf Intestinal (CIAP) (Promega). Plasmid DNA pBI121-*Bar* was named mpBI121.1. Plasmid DNA pFGC5941 was digested with *Hind III/BamH I*, releasing two fragments. The smaller one containing *Tocs* was ligated with mpBI121.1 digested with *Hind III/BamH I*, thus replacing CaMV 35S promoter with *Tocs*. Plasmid DNA mpBI121-*Tocs* was named mpBI121.2 (Figure A1). Plasmid DNA mpBI121.2 was digested with *Hind III/Spe I*, blunted, and re-ligated to remove *Tocs*. The resulting plasmid DNA was named mpBI121.3.

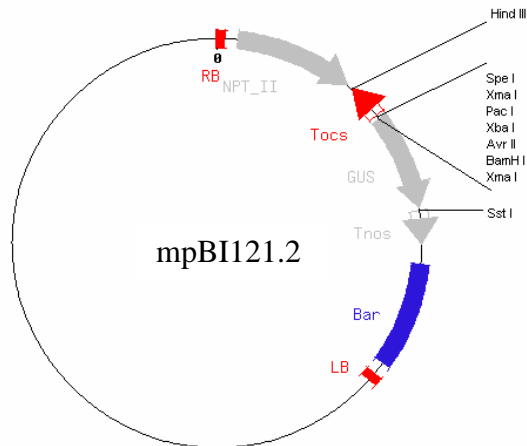


Figure A1. Simplified mpBI121.2 map made with NetPlasmid 1.1 (<http://www.justbio.com>) showing its T-DNA region. The *Bar* cassette (blue box) from pCB302 was inserted into the *EcoR I* site of pBI121. The *Tocs* (red arrow) from pFGC5941 replaced the CaMV 35S promoter. All restriction sites listed on the map are unique except *Xma I* (*Sma I*) that cuts the vector 2 times.

Construction of pGEM-*XSP1p*

The putative promoter of *XSP1* (Zhao et al., 2000), 1.827-kb region (from -1818 to +9-bp relative to the *XSP1* translation start), was amplified from genomic DNA by PCR using an upstream *Hind III* (underlined) linker primer 5'-AAGCTTGGACCGTAAGAGATCTATAG-3' and a downstream *BamH I* linker primer 5'-GGATCCTTATCCTTGTGGTATTGTG-3'. The resulting PCR products were cloned into pGEM, thus yielding pGEM-*XSP1p*.

Construction of mpBI121-35S-*GFP* and mpBI121.2-*GFP*

sm*GFP* was amplified from plasmid DNA pSLJ75515-*RTM1pro-smGFP/RTM1* (a gift from Dr. Jim Carrington, Oregon State University) (Chisholm et al., 2001) by PCR using an upstream *BamH I* (*italic*)/*Sma I* (underlined) linker primer 5'-GGATCCCCGGGATGAGTAAAGGAGAAGAACTTT-3' and a downstream *Sst I*

(*italic*)/*Xho I* (underlined) linker primer 5' -

GAGCTCACCTCGAGTTGTATAGTTCATCCATGCCA-3' that created restriction sites for fusion constructs at both smGFP N- and C-termini. The resulting PCR products were cloned into pGEM to yield pGEM-*smGFP*. A *BamH I*/*Sst I* fragment containing *smGFP* was excised from pGEM-*smGFP* and cloned into mpBI121.1 and mpBI121.2 using the same sets of restriction enzymes, thus replacing *GUS* with *GFP* and producing mpBI121-35S-*GFP* and mpBI121.2-*GFP*, respectively (Fig. A2).

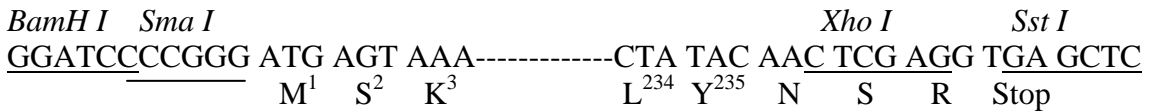


Figure A2. DNA sequences around the translation start and stop codons of *smGFP* in mpBI121-35S-*GFP* and mpBI121.2-*GFP*. Amino acids are numbered according to smGFP (GenBank accession number U70495).

Construction of the modified pFGC5941, mpFGC5941

The modified pFGC5941 was generated by cleaving pFGC5941 with *EcoR I*/*BamH I*, blunted with Klenow, and re-ligated to remove 35S and the CHSA intron. The resulting plasmid DNA was cleaved with *Hind III*/*Spe I*, blunted with Klenow, and re-ligated to remove OCS 3' region, thus producing the modified pFGC5951.

Transformation of *Agrobacterium* GV3101 and *Arabidopsis*

Transformation of *Agrobacterium* GV3101 and *Arabidopsis* was performed according to the protocol I modified from that used in Dr. Winkel-Shirley lab at Department of Biology of VT (the modified protocol omits the liquid N₂ freezing step and decreases 2XTY from 400 ml to 100 ml). i.e., 200 μl of competent cells were placed on ice till just thawed. 1 μg of plasmid DNA was added to the competent cells. Cells were gently mixed, placed on ice for 30 min, then heat-shocked for 2 min in a water bath at 37°C, and immediately re-placed on ice for 2 min. 0.8 ml of 2XTY was added to the tubes containing cells transformed with plasmid DNA. The cells were incubated for 2 hr at 28°C with vigorous shaking (200rpm). Pelleted *Agrobacterium* cells were resuspended in

100 μ l of 2XTY and spread on a LB/antibiotic plate. Plate was incubated at 28°C for 2 days. Single colony was picked and grew in 6 ml of 2XTY with appropriate antibiotics at 28°C with vigorous shaking for 1 day (or till saturated), 100 ml of fresh 2XTY with antibiotics were added and the culture was incubated overnight. The overnight culture was pelleted and resuspended in infiltration medium (5% sucrose, 0.03% Silwet L-77), resulting in an OD₆₀₀ reading of 0.8. Inflorescence of plants was dipped into infiltration medium for 10 minutes and dipped plants were placed under a cover overnight to maintain high humidity. Plants grew normally till seeds become mature. Transformants were selected using antibiotic or herbicide selectable marker.

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