Analysis on interaction mechanisms of transcription factors regulating xylem vessel cell differentiation

（道管分化を制御する転写因子群の作用機構の解析）

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by

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Summary

Woody biomass is the most abundant biomass on land area, and mainly consists of xylem which plays roles in conducting water and mineral, and providing mechanical support. Xylem vessel elements and fiber cells which are the components of xylem, form thick secondary cell wall between normal cell wall (primary cell wall) and plasma membrane. Moreover, the secondary cell wall consists of cellulose, hemicellulose, and lignin which are utilized as materials of huge valuable biomolecules, such as bioethanol, biopolymer, medicines. Therefore, it is quite important to unveil regulatory mechanisms by which secondary cell wall is formed in xylem vessel elements and fiber cells for contributing to solve various global environmental problems.

As previous research, various factors which regulate differentiation of xylem vessel formation were identified. VND7, an *Arabidopsis thaliana* NAC domain transcription factor, was isolated as a master regulator of xylem vessel element differentiation. In addition, another NAC domain transcription factor, VNI2 was isolated as an interacting factor with VND7. As a result of interaction, VNI2 negatively regulates xylem vessel formation by binding and inhibiting VND7 transcriptional activity. However, the molecular mechanism how VNI2 inhibits VND7 is still unclear.

In this thesis, to understand regulatory mechanism of secondary cell wall formation, I focused on obtaining the insights regarding interaction between VND7 and VNI2. At first, I confirmed a repression motif-like (EAR repression motif-like) sequences of VNI2 is not responsible for the inhibition by using protoplast transient expression assay. In addition, I prepared and analyzed C-terminus deletion series of the

VNI2. I found that 10 amino acids of VNI2 effectively inhibits transcriptional activity of VND7, resulting in severe defects in xylem vessel formation. These results clearly demonstrated that the 10 amino acid sequence is important for VNI2 function.

It has been reported that VND7 upregulates a number of target genes. In this dissertation, I also found that VND7 negatively regulates *VNI2* expression, indicating the mutual regulation between VND7 and VNI2. Furthermore, the obtained results suggested that *VNI2* expression is regulated by plural transcription factors during xylem vessel differentiation.

Taken together of the obtained results with the previous published reports, I proposed the existence of complicated regulation in secondary cell wall formation and provide new strategies to effectively increase production and utilization of plant biomass leading to protection of environment and sustainable society.

Chapter 1

General introduction
xylem vessel cell differentiation

Plant biomass is renewable, eco-friendly, clean energy source produced by plants which convert sunlight into plant material through photosynthesis. Utilization of the plant biomass is essential for solving world society problems such as environmental pollution, climate change, energy crisis (Figure 1.1; Tursi et al. 2019).

Plant cell walls are the main source that provide plant biomass (Maleki et al. 2020; Carpita et al. 2020). The cell walls are classified into primary cell walls and secondary cell walls (Figure 1.2). The primary cell walls, which all plant cells surround outside of the plasma membrane and provide protection for biotic and abiotic stresses and mechanical support. The secondary cell walls synthesized in specialized cells such as xylem vessel elements and fiber cells, are formed between the primary cell wall and the plasma membrane (Figure 1.2, 1.3). The thickened secondary cell walls contain huge valuable biomaterials such as cellulose, hemicellulose, and lignin (Kumar et al. 2015; Cosgrove et al. 2012). Therefore, regulation of the secondary cell wall formation is one of important research areas in the plant molecular biology.

The xylem is one of the plant vascular tissues that provide physical support and transport water, nutrients from root to whole of the plant body. The major components of xylem include parenchyma, fibers, and xylem vessel elements. The xylem vessels are characterized by secondary cell wall deposition and programmed cell death (Figure 1.3; Schuetz et al. 2012; Rybel et al. 2016).

One of the great achievements of research on the xylem vessel is establishment of the hormone-mediated in vitro xylem vessel element formation system using Arabidopsis suspension cultured cells (Kubo et al. 2005). In the system, ~50% of the subcultured cells were synchronously differentiated into xylem vessel elements within 7 days in the presence of 1 μM brassinolide and 10 mM boric acid (Kubo et al. 2005). By using

microarray expression analysis, *VASCULAR-RELATED NAC-DOMAIN PROTEIN 1-7* (*VND1-7*) encoding NAC domain transcription factor family were isolated as genes whose expressions were transiently elevated during the in vitro xylem vessel element formation system (Kubo et al. 2005). Ectopic expression of *VND* genes induces trans-differentiation into xylem vessel elements, indicating that VND family is the master regulator of xylem vessel element differentiation (Figure 2.1.1; Kubo et al. 2005; Zhou et al. 2014; Endo 2015). Transcriptome analyses revealed that VND members directly or indirectly regulate a number of genes involved in xylem vessel formation (Kubo et al. 2005; Ohashi-Ito et al. 2010; Zhong et al. 2010; Yamaguchi et al. 2011). Promoter analysis showed that all 7 *VND* genes are specifically expressed in the differentiating xylem vessel elements although expression patten of each gene is not completely same (Kubo et al. 2005; Yamaguchi et al. 2008). Particularly, *VND7* is expressed all types of differentiating xylem vessel elements (Yamaguchi et al. 2008). In addition, *VND7* expression is upregulated by all VNDs (Endo et al. 2015). These data suggested that VND7 is the main regulator of the xylem vessel element differentiation in Arabidopsis Recently, genetic approaches using multiple mutants demonstrated that other VNDs has specific roles in xylem vessel formation in response to environmental stimuli (Tian et al. 2018; Ramachandran et al. 2021).

Because the key role of the VND7 during the xylem vessel differentiation, various tight regulatory mechanisms of the VND7 function at its expression level or protein level were identified (Soyano et al. 2008; Yamaguchi et al. 2008; 2010b; Endo et al. 2015; Kawabe et al. 2018; Ohashi-Ito et al. 2018; Ohtani et al. 2018; Zhong et al. 2021). VND-INTERACTING2 (VNI2), a NAC domain protein, was discovered by yeast two hybrid in which VND7 was used as a bait (Yamaguchi et al. 2010b). VNI2 negatively

regulates xylem vessel formation by inhibiting VND7 function (Figure 2.1.3). Promoter analysis showed that *VNI2* is expressed in the early stage of the xylem vessel differentiation while *VND7* expression is sifted to the later stage (Figure 2.1.2). The biological meaning of the negative regulation was proposed that VNI2 regulates VND7 function in the early stage of the xylem vessel element differentiation (Figure 2.1.4; Yamaguchi et al. 2010b). But the molecular mechanism how VNI2 inhibits VND7 function is still unclear (Figure 2.1.5). Furthermore, ectopic expression of *VNI2* shows defects in xylem vessel formation, resulting in severe dwarf and sterile phenotype (Yamaguchi et al. 2010b), suggesting that robust regulation of the VNI2 is also important during the xylem vessel formation. It is also shown that VNI2 is regulated by proteasome-mediated degradation (Yamaguchi et al. 2010b). However, how *VNI2* expression is regulated during the xylem vessel differentiation is also unclear.

NAC domain transcription factor:

The NAC domain transcription factor family is one of the largest plant specific family. The NAC domain transcription factor family is involved in various biological events such as organ and tissue development, cell differentiation, and biotic or abiotic stress responses. The NAC domain transcription factor family conserved NAC domain at its N-terminus. The NAC domain contributes to nuclear localization, DNA binding, and dimer formation. By contrast, the diverse C-terminal region functions in transcriptional activity (Christianson et al. 2009; Yamaguchi and Demura 2010; Nuruzzaman et al. 2013; Jensen and Skriver 2014; Nakano et al. 2015; Shao et al. 2015).

Transcriptional cascade of the xylem vessel differentiation:

VND7 directly activates genes involved in secondary cell wall biosynthesis [CELLULOSE SYNTHEASE A CATALYTIC SUBUNIT8 (CesA8)/IRREGULAR XYLME1 (IRX1)], programmed cell death [*XYLEM CYSTEINE PEPTIDASE 1* (*XCP1*) and *XCP2*], and transcription factors [*LBD30*/*ASL19*/*JLO*, *MYB46*, and *MYB83*] (Yamaguchi et al. 2011; Zhong et al. 2010). LBD30 and VND7 form a positive feedback loop (Soyano et al. 2009; Ohhashi-Ito et al. 2020). MYB83 and MYB46 regulate secondary cell wall biosynthesis genes (Ko et al. 2014; Nakano et al. 2015). Figure 1.4

The aim of this thesis:

To understand molecular mechanism of regulation of the xylem vessel element differentiation, I investigated as following: 1. Molecular mechanism how VNI2 inhibits VND7 function. 2. Whether *VNI2* expression is regulated during the xylem vessel elements differentiation. As a results, during the investigation of the inhibition mechanism of VNI2, I found that 10 amino acids region of the VNI2 showed effective inhibition on the VND7 function (**Chapter 2**). Transgenic plants ectopically expressing full length or the truncated *VNI2* confirmed that identified amino acid sequence effectively inhibited xylem vessel formation and plant growth (**Chapter 2**). The amino acids of the region are highly conserved in VNI2 orthologs (**Chapter 3**). Based on the motif analysis, I focused on the relationship between VNI2 and protein phosphatase (**Chapter 3**). During the *VNI2* promoter analysis, I found that VND7 negatively regulates *VNI2* expression (**Chapter 4**). Interestingly, MYB83, a downstream transcription factor of VND7, positively regulates *VNI2* expression (**Chapter 4**). These results indicated *VNI2* expression is not only negatively but also positively regulated during xylem vessel differentiation. Moreover, VND7 and VNI2 showed mutually regulate each other. Taken

together with the obtaining results provided new insights of complicated molecular regulation of the xylem vessel differentiation, and important information for new strategies to produce ideal plant biomass.

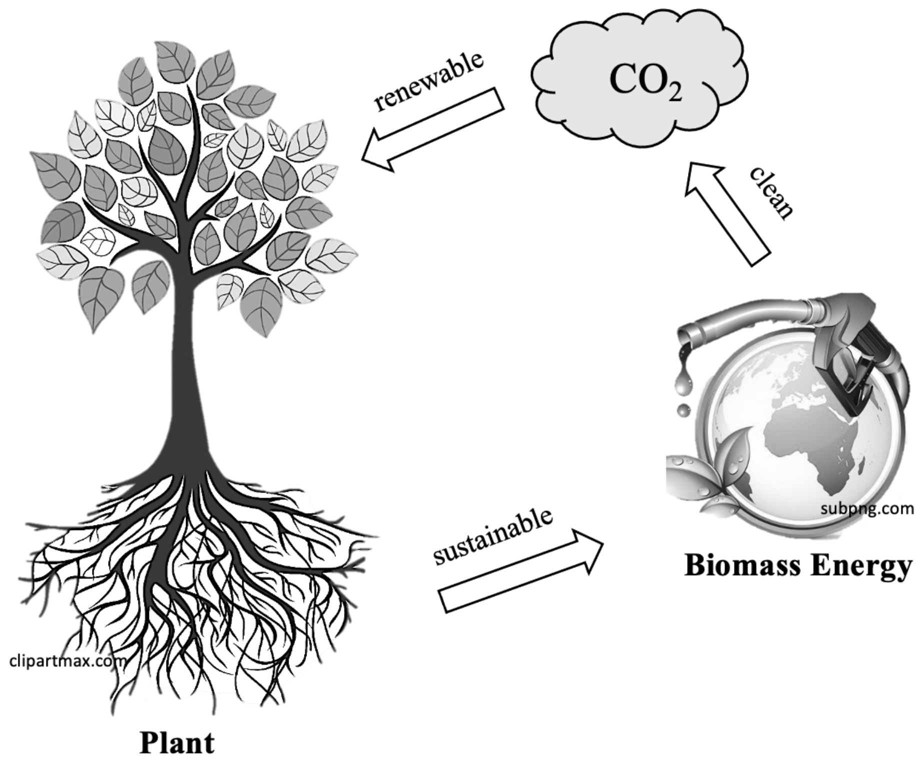


Figure 1.1 Schematic image of plant biomass which is clean, renewable, sustainable energy sources.

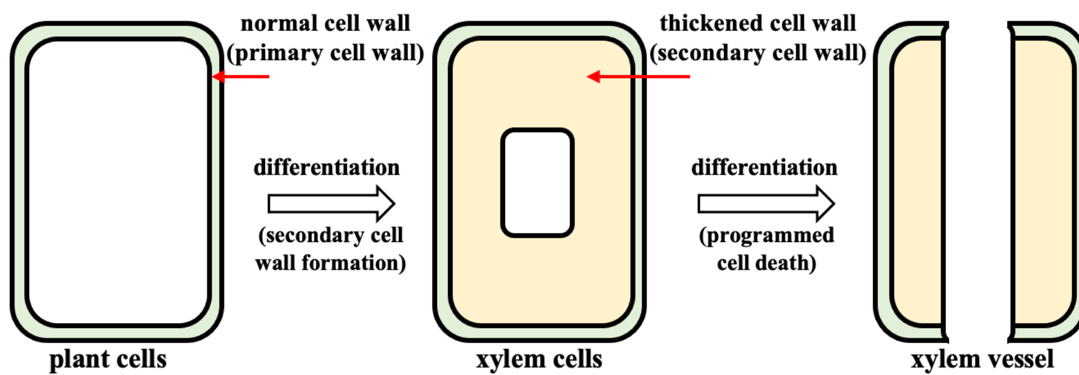


Figure 1.2 Xylem vessel cell differentiation.

Compared to normal plant cells, the xylem cells are differentiated cells that cell wall is thickened (secondary cell wall). The thickened cell wall contains huge valuable biomolecules such as lignin and hemicellulose. The ylem vessel is formatted by next step cell differentiation, programmed cell death.

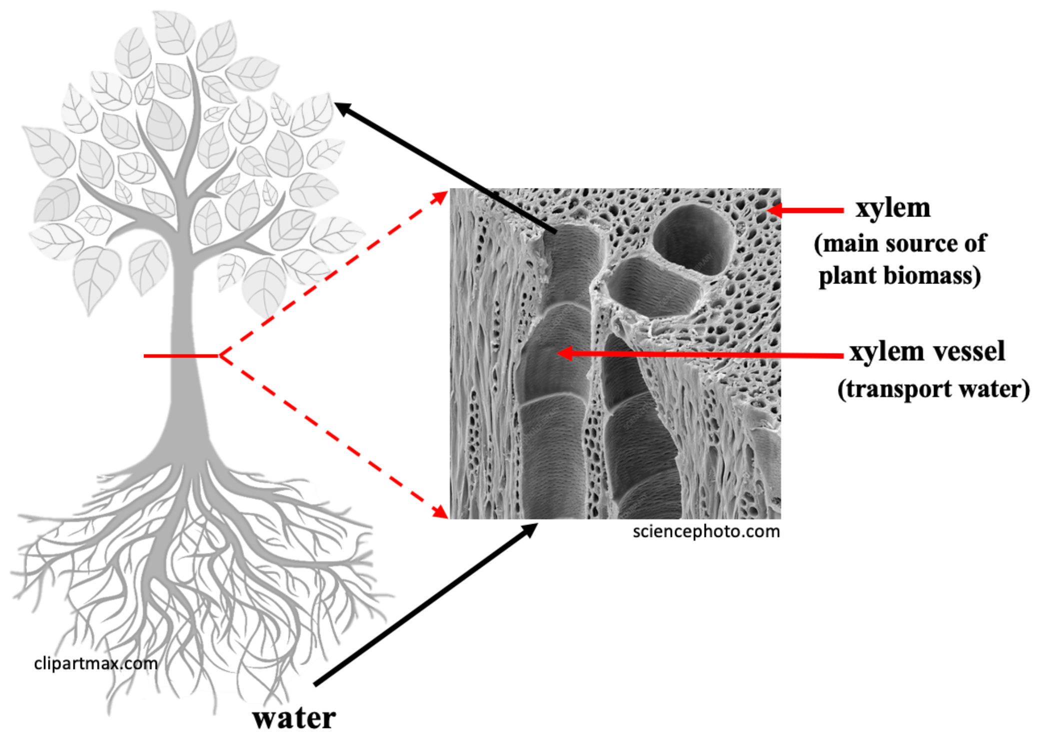


Figure 1.3 The xylem is the main sources that provide plant biomass.
The main part used as biomass of plant is the xylem. The xylem cells are differentiated cells that cell wall is thickened (secondary cell wall). The xylem vessel is an important structure as a components of xylem tissue for water transduction as a part of the plant vascular system.

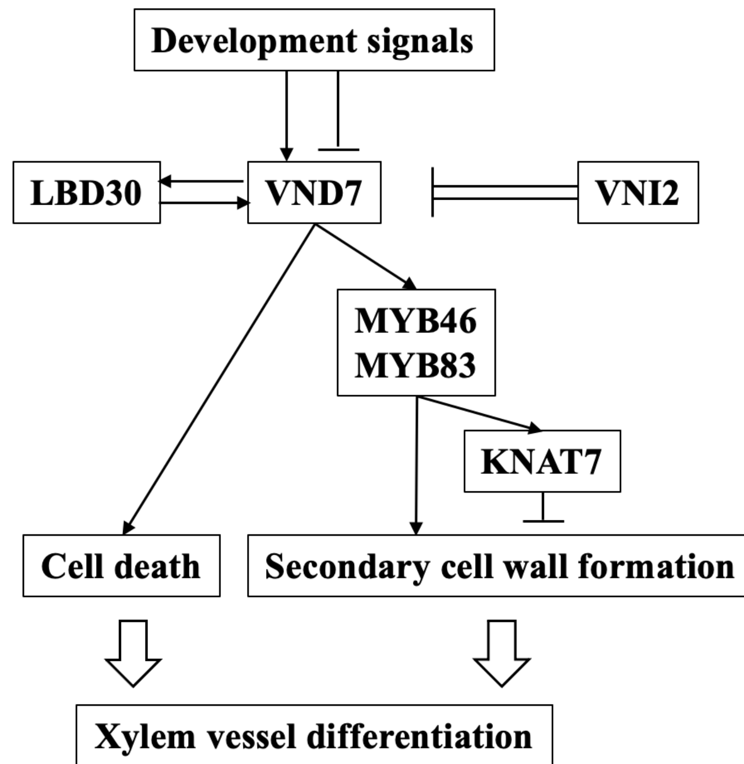


Figure 1.4 The regulatory network of the xylem vessel differentiation.

After receiving developmental signals, transcription factor cascade activates downstream secondary cell wall biosynthesis genes. VND7 directly activates programmed cell death for xylem vessel differentiation.

Chapter 2

201-210 region of VNI2 contributes to effective inhibition of VND7 activity during xylem vessel formation

2.1 Introduction

Xylem vessels transport water and minerals from root to whole of the plant body. Study of xylem vessel elements, component of xylem vessel used to be a useful model to understand determination of plant cell fate because in vitro trans-differentiation systems have been established from *Zennia elegans* mesophyll cells (Fukuda and Komamine, 1980) and Arabidopsis suspension cultured cells (Kubo et al. 2005).

Transcriptomic analysis found that VASCULAR-RELATED NAC-DOMAIN1 (VND1) to VND7, encoding NAC domain transcription factors were transiently elevated during the in vitro xylem vessel element formation system in Arabidopsis suspension cultured cells (Kubo et al. 2005). Overexpression of *VNDs* genes induced trans-differentiation into xylem vessel elements, indicating that VND members are the master switches of xylem vessel element differentiation (Kubo et al. 2005; Yamaguchi et al. 2010a; Zhou et al. 2014; Endo et al. 2015). Within the VNDs family, VND7 was shown to be a master regulator to regulate differentiation of all types of cells into xylem vessels in shoots and roots (Yamaguchi et al. 2008). VND7 directly regulates the expression of a broad range of genes for xylem vessel formation (Yamaguchi et al. 2011). Because of the key role of the VND7 during xylem vessel differentiation, some regulation mechanisms, by expression or protein level, of the VND7 were also gradually reported. At protein level, Proteasome-mediated degradation regulates stability of VNI2 protein (Yamaguchi et al. 2008). In addition, S-NITROSOGLUTATHIONE REDUCTASE (GSNOR) S-nitrosylated VNI2 (Kawabe et al. 2018). VND-INTERACTING2 (VNI2), a NAC domain transcription factor, was isolated as an interacting protein with VND7 by yeast two hybrid assay (Yamaguchi et al. 2010b). Further research showed that VNI2

binds to and inhibits VNDs family. Promoter analyses demonstrated that *VNI2* expression was detected in the early stage of the xylem vessel differentiation while *VND7* expression was observed at the late stage. Extension of *VNI2* expression during xylem vessel element differentiation induced severe defects in xylem vessel formation and plant growth. Therefore, the role of VNI2 seems be to regulate transcriptional activities of VNDs at the early stage of the cell differentiation (Yamaguchi et al. 2010b). Mutation of the *VNI2* gene do not show obvious defects in xylem vessel differentiation, suggesting that there is other factors to take acting as VNI2. However, molecular mechanism how VNI2 inhibits VND7 is still unclear. Because VNI2 also interacts other many transcription factors which regulating various plant development (unpublished data). Therefore, the molecular mechanism how VNI2 inhibits VND7 will give us valuable important information on VNI2.

Previous research showed that VNI2 contains a PEST motif, known as a target of protein degradation, at its C-terminal region (Yamaguchi et al. 2010b). The PEST motif contributes to the VNI2 inhibition ability through affecting protein stability of VNI2. Therefore, the VNI2 protein lacking a part of the PEST motif was more stable than full-length VNI2 resulting in stronger inhibition of VND7 activity (Yamaguchi et al. 2010b). This also suggested that the responsible region for the inhibition should be different from the PEST motif. Interestingly, in the PEST motif of VNI2, there is a sequence similar to an ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR (ERF)-associated amphiphilic repression (EAR) motif which is a transcriptional repression motif conserved in a number of plant transcriptional repressors (Ohta et al. 2001). Transcription factors fused the EAR motif dominantly become transcriptional repressors (Hiratsu et al. 2003). Therefore, it was possible that the EAR repression

motif-like sequences of the VNI2 may be associated with the inhibition mechanism.

In Chapter 2, to understand mechanism by which VNI2 inhibits VND7, I tried to confirm whether the EAR repression motif-like sequences of the VNI2 is responsible for the inhibition. Next, I looked for responsible region of VNI2 function, and found that 10 amino acid sequences of the $VNI2^{201\text{-}210}$ are acting as effective inhibition on VND7. Transgenic plant phenotypes also showed the effectiveness of the $VNI2^{201\text{-}210}$ on inhibition of xylem vessel formation.

As a summary of Chapter 2, VNI2 inhibits VND7 transcriptional activation activity by various inhibition levels. This may be the ideal regulation of a master regulator which regulating plant important development.

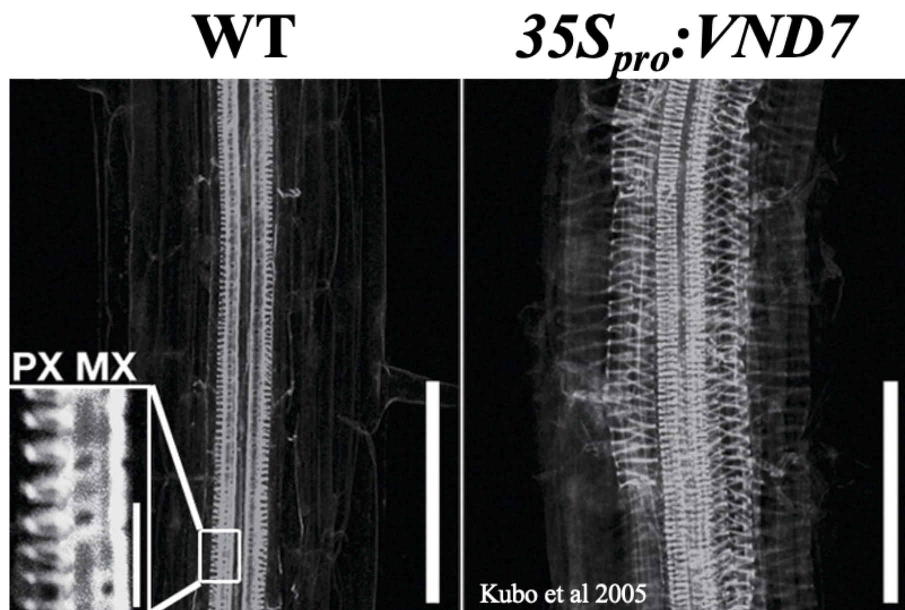


Figure 2.1.1 VND7 is a key regulator of the xylem vessel differentiation.

Based on the Kubo et al. (2005) Genes Dev. 19: 1855–1860. Overexpression of *VND7* induced ectopic secondary cell wall formation.
(PX) Protoxylem vessel; (MX) metaxylem vessel. Bars: 100 μm

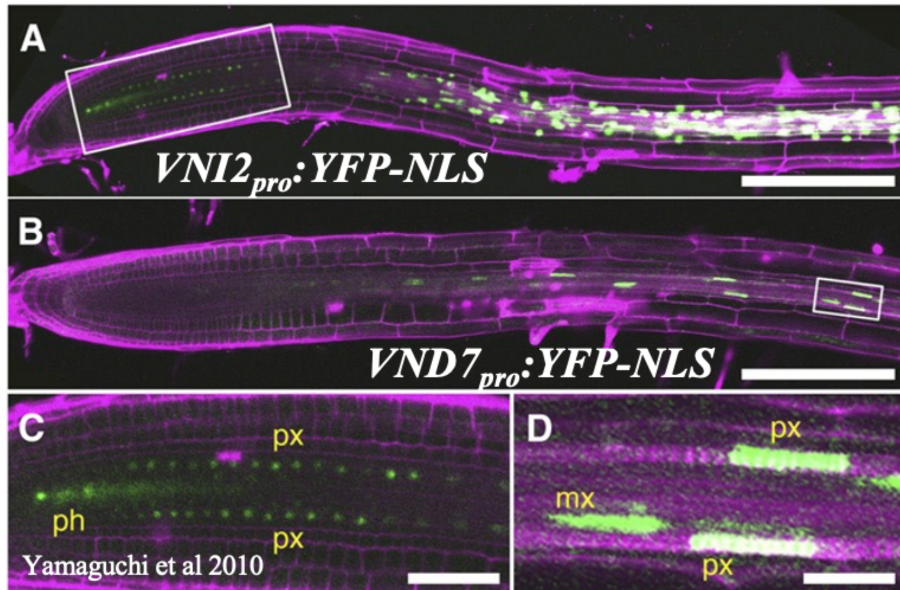


Figure 2.1.2 Expression patterns of the *VNI2*.

Based on the Yamaguchi et al. (2010) Plant Cell 22: 1249–1263. *VNI2* expression was detected in the vessel precursors before elongation, whereas only *VND7* was expressed at later stages in the elongating protoxylem vessel precursors.

C and D show magnified views of the region indicated by the white frames in A and B, respectively. mx, metaxylem; ph, phloem; px, protoxylem.

Bars = 200 mm in A and B, 50 mm in C, and 20 mm in D.

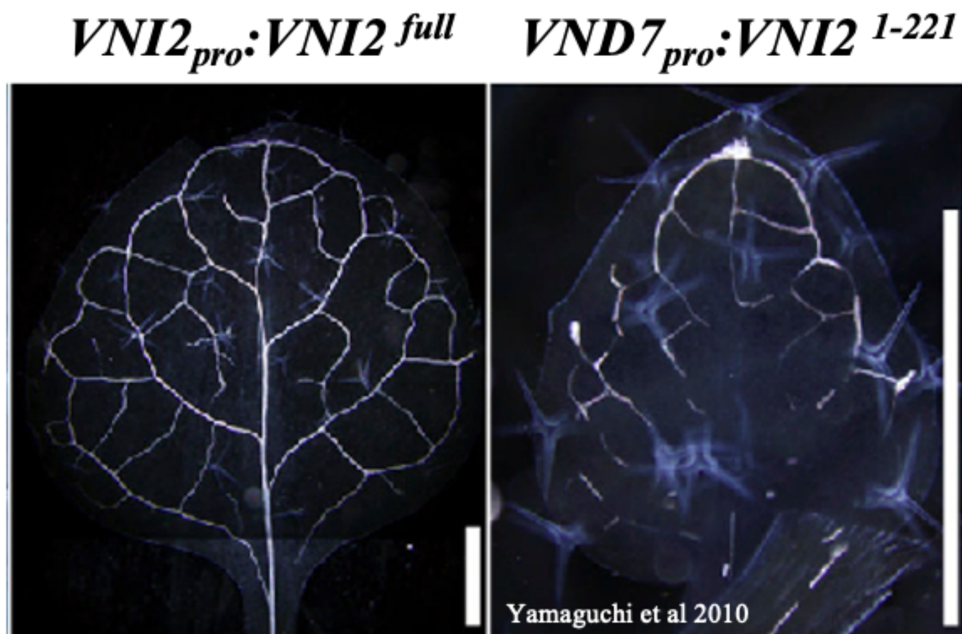


Figure 2.1.3 VNI2 negatively regulates xylem vessel formation by inhibiting VND7.

Based on the Yamaguchi et al. (2010) Plant Cell 22: 1249–1263. VNI2 binds and inhibits VND7 transcriptional activation activity. Because *VNI2* expression occurs at the early stage of the xylem vessel formation, VNI2 protein is regulated by its protein stability, when use *VND7* promoter to extend stable VNI2 expression to the late stage, the vessel defects were observed.

Bars = 1 mm.

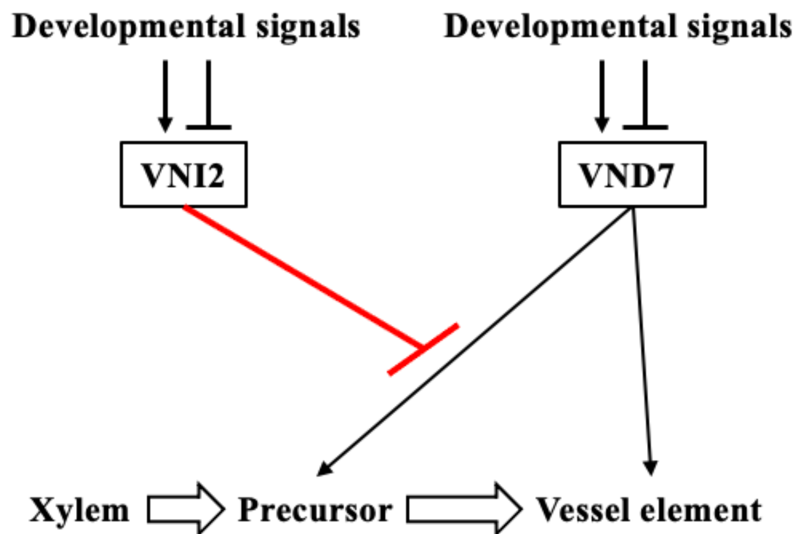


Figure 2.1.4 The role of VNI2 during the xylem vessel differentiation.

VNI2 regulates VND7 transcriptional activation activity at the early stage of the xylem vessel differentiation. The cell fate is irreversible once it is decided to differentiate into xylem vessel element. Thus, the regulation of the VND7 activity is important for proper development of xylem vessels, and plant growth.

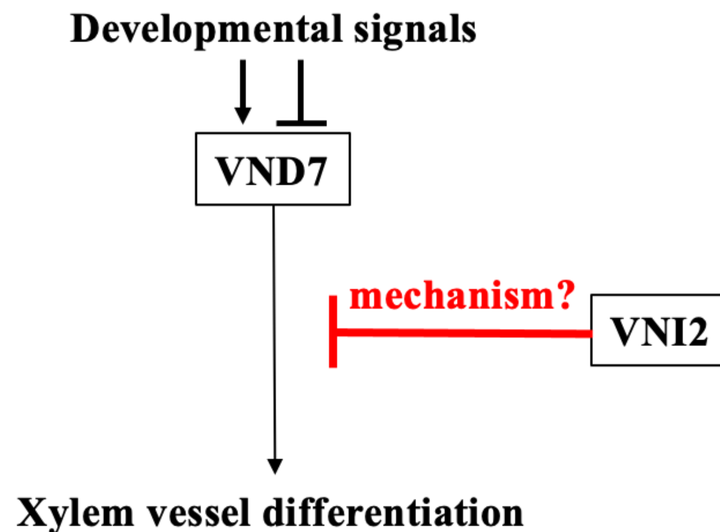


Figure 2.1.5 The aim of Chapter 2.

The aim of the Chapter 2 research is to investigate the molecular mechanism how VNI2 inhibits VND7 transcriptional activation activity. The result will be helpful to us to understand regulation mechanism of the xylem vessel differentiation. Because VNI2 also interacts with other many proteins (unpublished data). So, the result also gives us important information to understand the mechanisms of plant development regulated by VNI2.

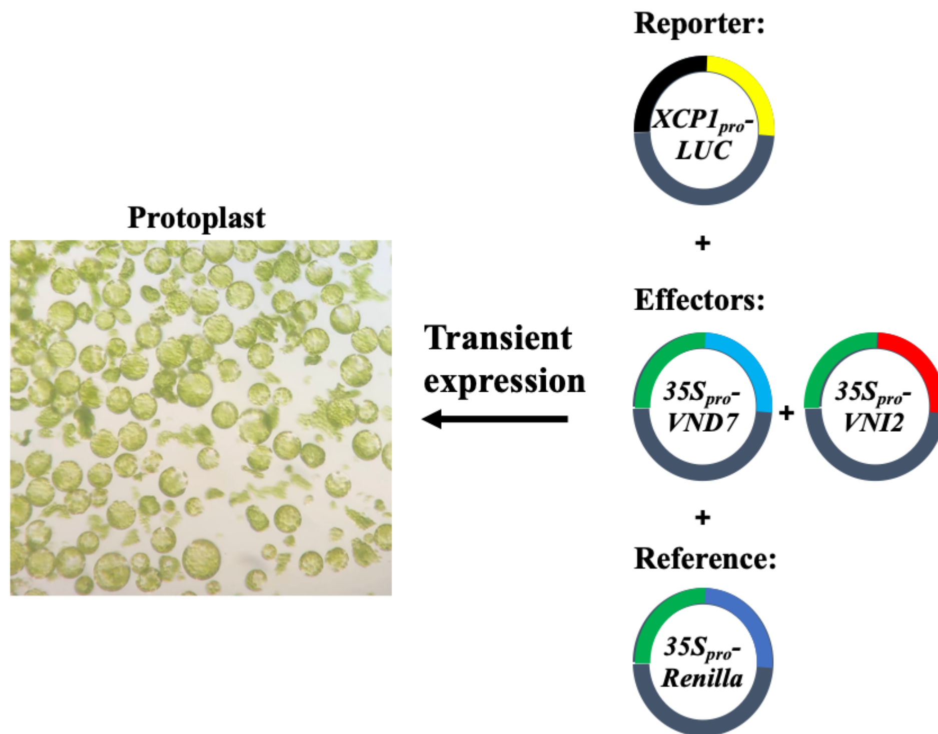


Figure 2.1.6 Schematic diagrams of protoplast transient gene expression analysis.

XCP1 promoter, a direct target of the VND7, is used as a reporter plasmid. The transcriptional activity level is evaluated by Firefly LUC activity. *Renilla* LUC activity is used for normalization.

2.2 Materials and methods

Materials for plasmid preparation

LB liquid (1 L)

Reagents	Amount
Yeast extract	5 g
Hipolypeptone	10 g
NaCl	5 g

Autoclaved at 121°C for 15min.

LB media (1 L)

Reagents	Amount
Yeast extract	5 g
Hipolypeptone	10 g
NaCl	5 g
Agar	15 g

Autoclaved at 121°C for 15min and then transfer to plates.

LB media (1 L) antibiotics

Reagents	Amount
Ampicillin (100 mg/ml)	1 ml
Kanamycin (50 mg/ml)	1 ml
Spectinomycin (50 mg/ml)	1 ml

Materials for transient gene expression

200 mM MES (pH 5.7) 1 L

MES 42.65 g
Adjust with KOH (pH 5.7) and then water up to 1 L.
Sterilized with a filter membrane.

1 M $CaCl_2$ 300 ml

$CaCl_2$:2H_2O 44.10 g
Water up to 300 ml.
Autoclaved at 121°C for 15 min.

Digestion Buffer 500ml

Reagents	Amount
Mannitol	36.43 g
KCl	0.75 g
1 M $CaCl_2$	5 ml
200 mM MES	50 ml
water up to	500 ml

Sterilized with a filter membrane.

W5 Buffer 1L

Reagents	Amount
NaCl	8.77 g
KCl	0.37 g
1 M $CaCl_2$	125 ml
200 mM MES	50 ml
water up to	1 L

Sterilized with a filter membrane.

MMg Solution 500ml

Reagents	Amount
Mannitol	36.44 g
$MgCl_2$ $6H_2O$	1.55 g
200 mM MES	10 ml
water up to	500 ml

Sterilized with a filter membrane.

PEG Solution 40ml

Reagents	Amount
PEG 4000 (Sigma)	16 g
Mannitol	1.44 g
1 M $CaCl_2$	4 ml
water up to	40 ml

Enzyme solution for protoplast preparation

Reagents	Amount
Cellulose “Onozuka” R10 (Yakulto)	0.2 g
Macerozyme R10 (Yakulto)	0.05 g
β-mercaptoethonol	14 ul

Digestion buffer up to 20 ml.

Materials for plant growth

GM medium 1 L

Reagents	Amount
MS plant salt mixture (Wako)	1 packet
Sucrose	5 g
5% MES-KOH	10 ml
500 X Vitamin mix	2 ml
Gellan gum	6 g

Autoclaved at 121°C for 15 min and then transfer to plates.

500 X Vitamin mix

Reagents	Amount
Myo-Inositol	50 g
Thiamine hydrochloride	10 g
Pyridoxine hydrochloride	0.5 g
Nicotinic Acid	0.5 g

Stored at -30°C

PPM Solution 100 ml

Reagents	Amount
PPM	3 ml
1g/L $MgCl_2$	5 ml
Distilled water up to	100 ml

Plasmid construction

To obtain the entry clones, the amplified DNA fragments by polymerase chain reaction (PCR) were subcloned into the pDONR207 or pENTR/D-TOPO vectors (Thermo Fisher Scientific). The entry clones were integrated into GATEWAY destination vectors by using LR Clonase (Thermo Fisher Scientific). The GATEWAY destination vectors were used as following; pA35G, containig the cauliflower mosaic virus *35S* (*CaMV35S*) promoter was used for effector constructs; pAGL, which contains a firefly *LUCIFERASE* gene was used for reporter; pBG and pBGH*, which contains the *NOS* terminator and the 3'-UTR region of *HSP18.2* (Nagaya et al. 2010), respectively, were used for generation of transgenic plants. The nucleotide sequences information of a multi-cloning site (MCS), a control fragment, and primers are shown in Supplementary Table.

Dual luciferase transient expression assay

As a reference, *Renilla reniformis LUCIFERASE* driven by *CaMV35S* promoter was used. Protoplasts were prepared from 3 to 4-week-old *Arabidopsis* leaves. The effector, reporter, and reference constructs were introduced into the protoplasts using the PEG transformation method (Figure 2.1.6; Sakamoto et al. 2016). Detection of the Luciferase activities were used by Dual-Luciferase Reporter Assay System (Promega) and a Mithras LB941 Multimode Microplate Reader (Berthold).

Generation of transformants

The constructed plasmids pBG-$VNI2_{pro}$:$VNI2^{Full}$, pBG-$VND7_{pro}$:$VNI2^{Full}$, pBGH*-$VND7_{pro}$:$VNI2^{1\text{-}210}$, pBGH*-$VND7_{pro}$:$VNI2^{1\text{-}200}$ were transformed into *Agrobacterium tumefaciens* strain GV3101::pMP90. The Agrobacterium containing the plasmids were

transformed into Arabidopsis plant (Col-0) using a floral-dip method. Transgenic Arabidopsis seedlings were selected with a germination medium containing 10 μg/L Bialaphos for 10 days, and then transferred to antibiotic-free medium for an additional 4 days at the incubator under a continuous light condition. The total 14-day-old transgenic seedlings were transferred in Jiffy-7 pots (Jiffy) under a continuous light condition to observe the plant growth. For observation of xylem vessel formation, the 14-day-old seedlings were fixed with 90% (v/v) acetone and then washed three times with 0.1 M phosphate buffer (pH 7.0). The first leaves, hypocotyls, and roots were observed under a microscope (DMR 025525.732; Leica) with a digital camera (BFC 480; Leica).

2.3 Results

2.3.1 The EAR-like motif does not contribute to VNI2 function

VNI2 encoding an Arabidopsis NAC domain transcription factor is isolated as interacting protein with VND7 which is the key regulator of xylem vessel element differentiation (Yamaguchi et al. 2010b). VNI2 inhibits the transcriptional activity of VND7 by forming protein complex. By previous research, a PEST motif at the C-terminal region of VNI2 seems to be a target of protein degradation (Yamaguchi et al. 2010b). Interestingly, an ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR (ERF)-associated amphiphilic repression (EAR) motif (L/FDLNL/F(X)P)-like sequences are also overlapped within the PEST motif. The EAR motif is an active repressor motif and existed in a number of plant transcriptional repressors (Figure 2.3.1; Ohta et al. 2001). Therefore, it is possible that the EAR motif-like sequences of VNI2 were responsible for inhibition of VND7. To verify the possibility that the EAR motif-like sequence is the reason for the inhibition, I prepared constructs in which amino acid residues in the EAR motif-like of VNI2 were substituted into alanine (Figure 2.3.2). A firefly *LUCIFERASE* (LUC) gene driven by *XYLEM CYSTEINE PEPTIDASE 1* (*XCP1*) promoter was prepared as reporter construct. XCP1 is a direct target of VND7, and involved in programmed cell death during xylem vessel element differentiation. *VND7*, *VNI2* or substituted *VNI2* driven by the cauliflower mosaic virus *35S* promoter (*CaMV35S*) were used as the effectors. By using the prepared constructs, protoplast transient expression assay was performed (Figure 2.3.2). After introduction of the constructs into protoplasts of *Arabidopsis* mesophyll cells, LUC activity was measured. As previously report, VND7 upregulated LUC activity driven by the *XCP1* promoter compared to the MCS construct.

When protoplast is transformed by both VND7 and VNI2 constructs, the LUC activity is reduced because of the inhibition. Next, when I used EAR repression motif-like mutated VNI2 against to the VND7, the inhibition level has no significant difference with the normal VNI2. Therefore, I concluded that the EAR motif-like sequence is not required for the inhibition.

2.3.2 201-210 region of VNI2 contributes to effective inhibition of VND7 activity

As above result, the EAR-like sequence of VNI2 is not associated with the inhibition. I thought a hypothesis that the functional region may N-terminal region from the PEST motif of VNI2. Therefore, I prepared C-terminally truncated VNI2 such as $VNI2^{1\text{-}210}$ and $VNI2^{1\text{-}200}$ (Figure 2.3.3). As a results of the transient expression assay, $VNI2^{1\text{-}210}$ showed stronger inhibition than full length VNI2 on VND7 transcriptional activity. This may be because of $VNI2^{1\text{-}210}$ is completely deleted PEST motif and protein is more stable than full length VNI2. However, $VNI2^{1\text{-}200}$ showed weaker inhibition than full length VNI2. These results indicated that the 201-210 amino acid region of the VNI2 confer the effective inhibition of the VND7 activity.

2.3.3 The region 201-210 of VNI2 strongly affects *in planta*

By above results, to investigate whether the 10 amino acid sequence affects plant development, I generated transgenic plant. Because as previous report, *VNI2* expression precedes *VND7* expression (Yamaguchi et al. 2010b). Therefore, I used *VND7* promoter to extend to the later stage of the differentiation as well as *VNI2* own promoter, and generated $VNI2_{pro}{:}VNI2^{Full}$, $VND7_{pro}{:}VNI2^{Full}$, $VND7_{pro}{:}VNI2^{1\text{-}210}$, and

$VND7_{pro}$:VNI2$^{1\text{-}200}$ (Figure 2.3.4). Within the T1 generation, more than 80% of *$VND7_{pro}$:VNI2$^{1\text{-}210}$* plants exhibited dwarf phenotype without bolting and fertility. The dwarf seedlings also showed severe defects in vessel formation in leaves and roots (Figure 2.3.5). The defects in xylem vessel formation was observed even in the hypocotyl of the strongest *$VND7_{pro}$:VNI2$^{1\text{-}210}$* line (Figure 2.3.5). By contrast, the tendency of defects in plant growth and xylem vessel formation in *$VND7_{pro}$:VNI2$^{1\text{-}200}$* plants were milder than those of *$VND7_{pro}$:VNI2$^{1\text{-}210}$* line (Figure 2.3.4; 2.3.5). The dwarf seedlings exhibited milder defects in vessel formation than *$VND7_{pro}$:VNI2$^{1\text{-}210}$* plants. The obtaining results in the Chapter 2 strongly suggested that the 10 amino acid sequence of VNI2 is critical for its function .

A

1 160. PEST 252

NAC domain

B

213 228

PEST RTTDLNLLPSSPSSDH

C

Repressors	EAR motif ($^{L}/_{F}$DLN$^{L}/_{F}$[x]P)
VNI2	TTTDLNLLPSSP
ERF3	IDLDLNLAPPME
AtERF4	LDLDLNLPPPSE
OsERF3	FDLDLNRPPPVE
ZAT10	RGFDLNIPPIPE
ZAT1	SLIDLNL-PAPS
WZF1	RAFDLNI-PAVP

Figure 2.3.1 The EAR repression motif-like sequences of the VNI2.

(A) Schematic diagram of VNI2. VNI2 contains a PEST motif, target of protein degradation, at its C-terminal region. (B) Alignment of the PEST motif of VNI2. Within the PEST motif, an EAR repression motif-like sequence (black) is also overlapped . (C) Alignments of the EAR motifs of the transcriptional repressors.

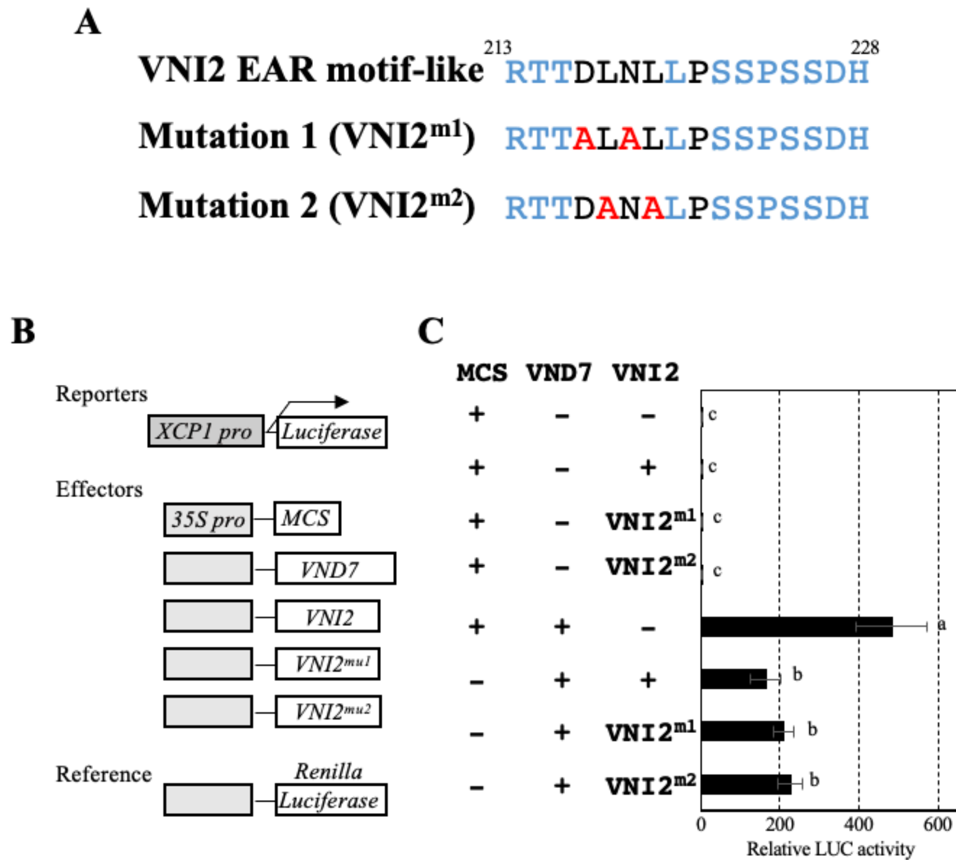


Figure 2.3.2 Mutation in the EAR motif-like sequences of the VNI2.

(A) The mutation in the EAR motif-like sequences of the VNI2. The residues 216 (aspartic acid) and 218 (asparagine) were replaced by alanine residues in VNI2 mutation 1 while the leucine residues at position 217 and 219 were replaced by alanine in VNI2 mutation 2. (B) Schematic diagrams of constructs for the transient assay. (C) The result of the transient assay. Error bars indicate standard deviations (n = 4). Different alphabets indicate significant differences at $P < 0.05$, as determined by one-way ANOVA with Tukey's HSD test.

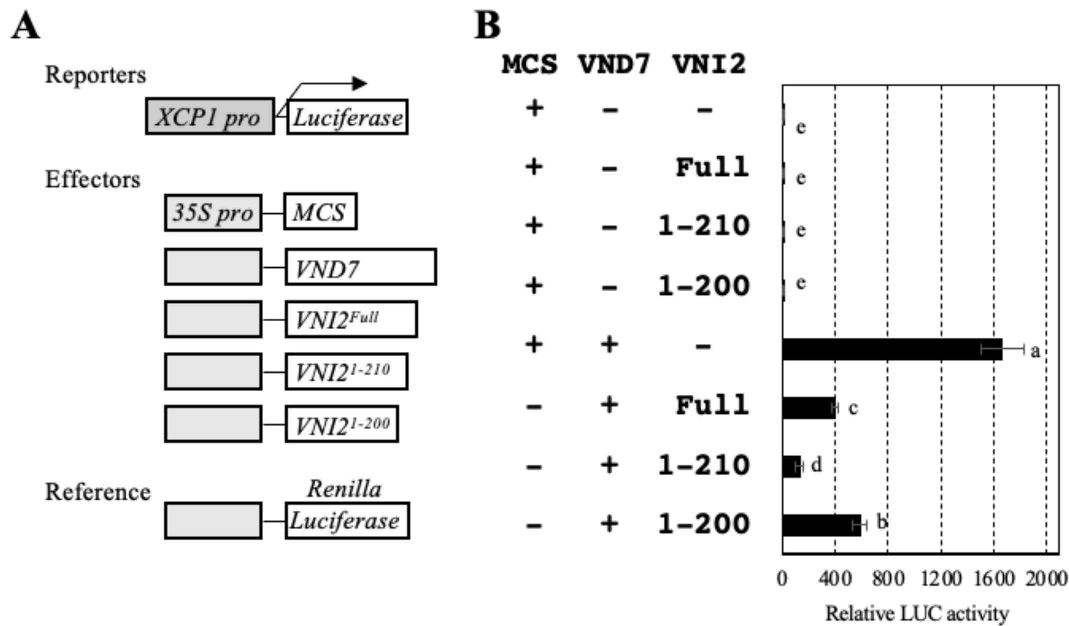


Figure 2.3.3 Comparison of the inhibitory effects between full length and C-terminally truncated VNI2 proteins.

(A) Schematic diagrams of full length and truncated VNI2 using for the transient assay. (B) The result of the transient assay. rror bars indicate standard deviations (n = 4). Different alphabets indicate significant differences at $P < 0.05$, as determined by one-way ANOVA with Tukey's HSD test.

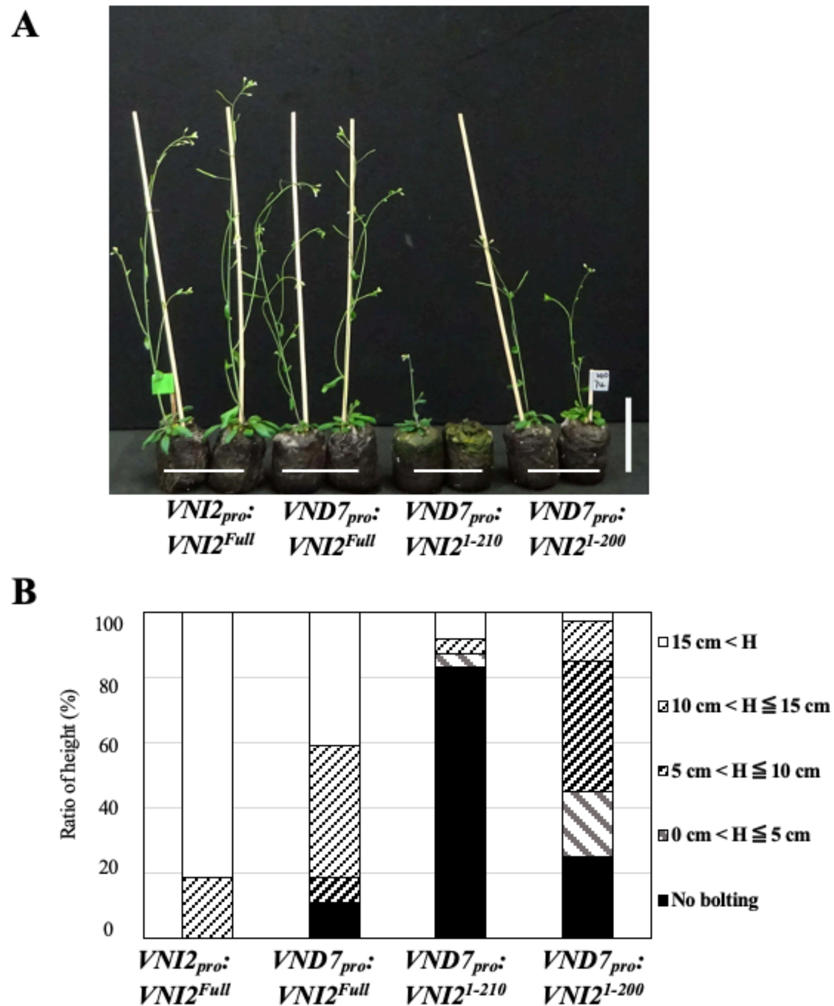


Figure 2.3.4 The dwarf phenotype of transgenic plants expressing C-terminally truncated VNI2.

(A) Thirty-five-day-old T1 generation of transgenic plants. Bar = 5 cm. (B) Ratio of heights of the 35-day-old transgenic plants.

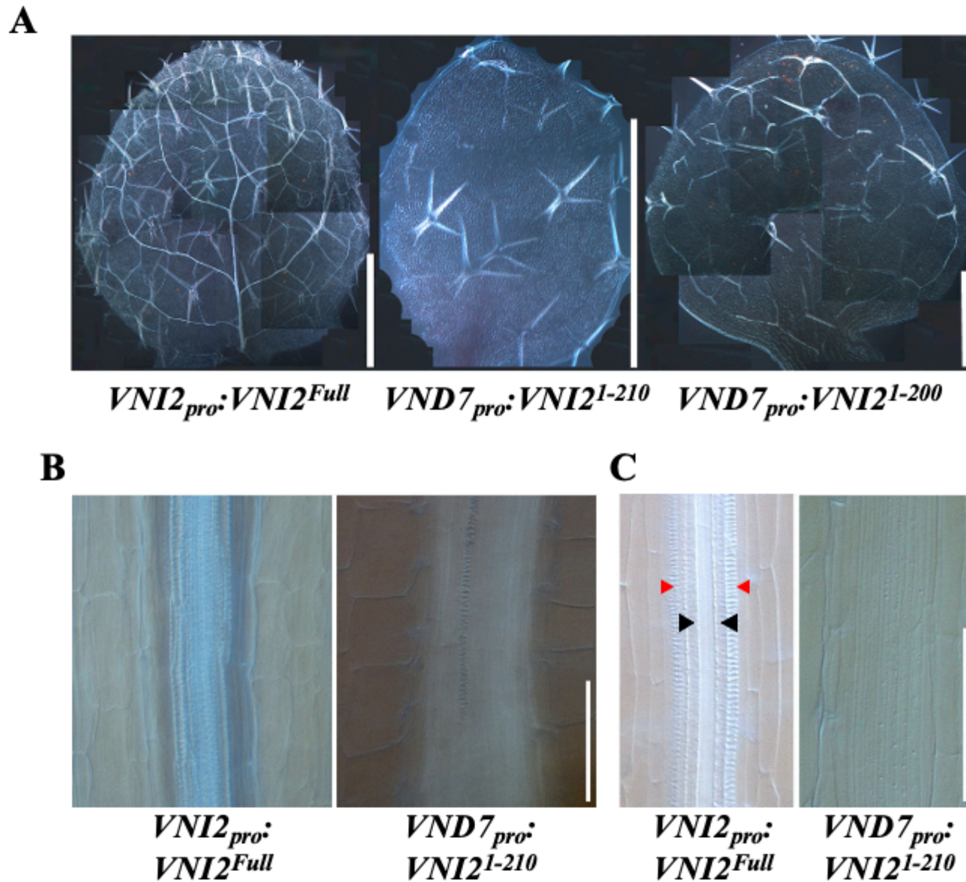


Figure 2.3.5 Defects in xylem vessel formation in transgenic plants expressing C-terminally truncated VNI2.

(A) Dark-field images of the first true leaves of 14-day-old plants. Bars = 1 mm. (B) Xylem vessel formation in hypocotyls of 14-day-old plants. Bar = 50 µm. (C) Xylem vessel formation in roots of 14-day-old plants. In control plants, protoxylem vessel elements (red arrowheads) and metaxylem vessel elements (black arrowheads) were differentiated. Bar = 50 µm.

2.4 Discussion

VND7 is a key regulator of the xylem vessel formation (Kubo et al. 2005; Yamaguchi et al. 2008; Yamaguchi et al. 2010a). VND7 seems to activate by forming homodimers or heterodimers with the other NAC domain transcription factors (Yamaguchi et al. 2008; 2015). In addition, another NAC domain transcription factor VNI2 inhibits VND7 by protein complex formation (Yamaguchi et al. 2010b).

A number of transcriptional repressors contain repression motifs, such as EAR motif and R/KLFGV motif (Ohta et al. 2001; Ikeda and Ohme-Takagi, 2009). Mutations in these motifs decline the repression activities (Ohta et al. 2001). Although VNI2 also contained an EAR motif-like sequence (Figure 2.3.1), the transient expression analysis using effectors containing the substituted VNI2 clearly indicated that the EAR-like motif of VNI2 is not responsible for inhibition of VND7.

As well as VNI2, XYLEM NAC DOMAIN1 (XND1), is a NAC domain transcription factor that negatively regulates differentiation of xylem vessel element (Zhao et al. 2007). Recently, it has been reported that XND1 interacts with and inhibits VND7 as in the case of VNI2 (Zhang et al. 2020; Zhong et al. 2021). Zhong et al. (2021) found that XND1 contains nuclear export signal (NES), and retains VND proteins to the cytoplasm (Zhong et al. 2021). By contrast, VNI2 is shown to be localized into the nucleus (Yamaguchi et al. 2010b), and does not contain predicted NES sequences. Therefore, VNI2 does not regulate subcellular localization of VND7 resulting in repression of VND7.

It was shown that VNI2 protein is degraded by proteasome (Yamaguchi et al. 2010b). The PEST motif at the C-terminal of VNI2 is targeted by the protein degradation.

Previous research showed that C-terminally truncated VNI2$^{1-221}$ which lacks part of the PEST motif is more stable than full length VNI2, resulting in strongly inhibiting VND7 (Yamaguchi et al. 2010b). In addition, overexpression of VNI2$^{1-221}$ shows severe discontinuous xylem vessel formation resulting in growth defect (Yamaguchi et al. 2010b). In my case, VNI2$^{1-210}$ lacking the whole PEST motif also showed strong inhibition of transcriptional activation activity of VND7 compared to full length VNI2. Moreover, extension of VNI2$^{1-210}$ expression exhibited severe defects in xylem vessel formation and plant growth as in the case of VNI2$^{1-221}$. Therefore, it is likely that VND2$^{1-210}$ protein is stable resulting in exhibiting strong inhibition. The VNI2$^{1-200}$ had weaker inhibition activity than full length VNI2. Interestingly, the *VND7pro:VNI2$^{1-200}$* plants also showed defects in xylem vessel formation and plant development although the tendencies were milder than *VND7pro:VNI2$^{1-210}$* plants. Therefore, VNI2$^{1-200}$ may be stable than full length VNI2. Full length VNI2 should be unstable but seems to effectively inhibit VND7 activity compared to VNI2$^{1-200}$. Therefore, full length VNI2 inhibits VND7 stronger than VNI2$^{1-200}$ in transient expression assay. On the other hand, VNI2$^{1-200}$ weakly inhibited VND7 for a long period resulting in visible defects in xylem vessel formation in the transgenic plants.

In Chapter 2, through the characterization of VNI2, I found the 10 amino acid sequence that effectively inhibits VND7 (Figure 2.4.1). However, it is still unclear why the 10 amino acid sequence works. Thus, it is necessary to analyze the amino acid sequence in more detail.

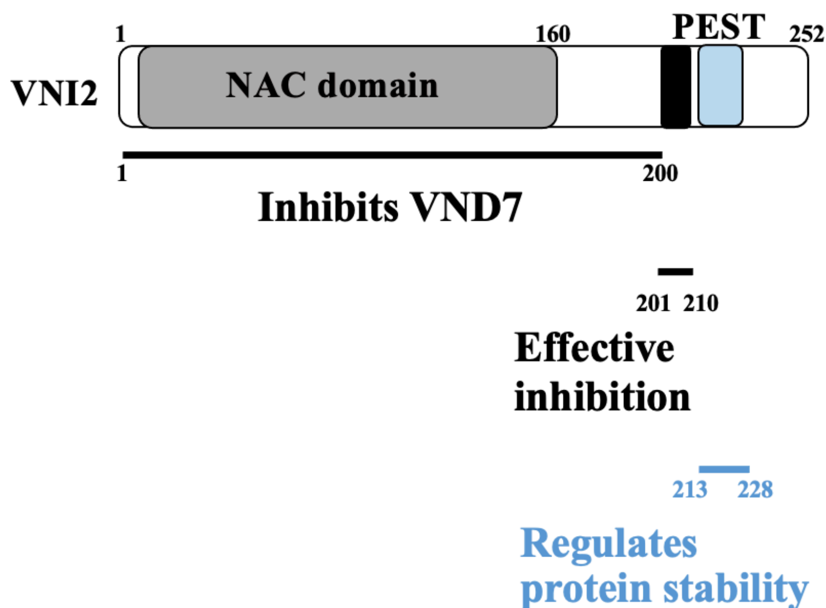


Figure 2.4.1 The summary of the Chapter 2.
As previous research, the PEST motif of the VNI2 regulate its protein stability. The regulation of the protein stability could be used as regulation of the inhibition level on the VND7. In this Chapter, I found the region 201-210 of VNI2 regulates effective inhibition on transcriptional activation activity of VND7.

Chapter 3

The function of the VNI2 201-210 region

3.1 Introduction

VNI2 negatively regulates xylem vessel differentiation by inhibiting the master regulator VND7. As I showed in Chapter 2, although the EAR repression motif-like sequences of the VNI2 seemed to be the reason of the inhibition, the sequences were not responsible for the inhibition. The transient gene expression analysis and transgenic plant showed truncated VNI2$^{1\text{-}210}$ strongly inhibit transcriptional activation activity VND7. Previous research reported that VNI2$^{1\text{-}221}$, which is lack of half of the PEST motif protein, is more stable and inhibits VND7 stronger than full length VNI2 (Yamaguchi et al. 2010b). VNI2$^{1\text{-}210}$ is complete deletion of the PEST motif. Therefore, the protein stability may be the reason of the strong inhibition. In contrast to VNI2$^{1\text{-}210}$, VNI2$^{1\text{-}200}$ showed weaker inhibition than full length VNI2. Therefore, the region 201-210 of VNI2 indicated effectiveness of the inhibition. But how the region confers the effective inhibition is still unclear (Figure 3.1.1).

In Chapter 3, to investigate how the region 201-210 of the VNI2 effectively inhibits VND7 transcriptional activation activity, I tried to analyze the 10 amino acids. Search of database showed that the 201-210 amino acid sequence is highly conserved in the VNI2 ortholog. The transient gene expression analysis using the region mutated VNI2 also confirmed that importance of the 201-210 region for effective inhibition. According to the online protein functional motif prediction search, the 201-210 region contains several possible functional motifs such as protein kinase and, phosphatase recognition motifs, that may act for the strong inhibition. Protein phosphatase regulates many important plant developments (Uhrig et al. 2013). The dephosphorylation by phosphatase cause the protein active or inactive.

To investigate whether the region does function as the prediction, I performed yeast two hybrid using VNI2 and protein phosphatases. Unfortunately, I could not obtain positive results by several phosphatase family member. So, there is also possibility the other member of the phosphatase families may interact with and dephosphorylate VNI2.

As a summary of Chapter 3, the amino acid sequence of 201-210 of VNI2 may take some function of the effectively inhibition on the VND7. There is also other possibilities that the region may affect VNI2 protein stability or binding affinities to VND7 protein.

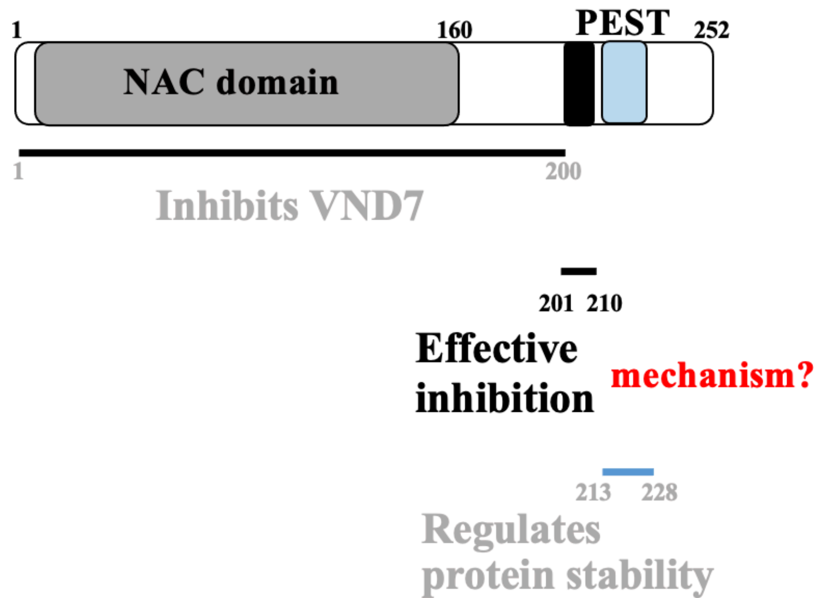


Figure 3.1.1 Schematic diagram of the aim of the research in Chapter 3.
Previous research reported that PEST motif of VNI2 regulates its protein stability. The protein stability indirectly regulates the inhibition level. As shown in Chapter 2, the region 201-210 of the VNI2 showed effective inhibition on the VND7 transcriptional activation activity. But how the region shows effectiveness was unclear. In Chapter 3, I tried to investigate the mechanism.

3.2 Materials and methods

Ortholog analysis

Phytozome (https://phytozome-next.jgi.doe.gov) online database was used for searching and obtaining the VNI2 orthologs.

Plasmid construction

The preparation of the plasmid for the transient gene expression in this Chapter is as same as the Materials and methods of Chapter 2. The primer sequence information are described in the Supplementary Table. *VNI2 201-210 mutant* fragment was integrated into the pA35G. The coding sequence of *VNI2* and TOPPs in the entry clones were integrated into the GATEWAY destination vectors, pBD-GAL4-GWRFC and pAD-GAL4-GWRFC, respectively, for yeast two-hybrid assay.

Dual luciferase transient expression assay

The transient gene expression analysis was performed as described in the Materials and methods in Chapter 2. I used more than three biological replicates, and repeated the experiments more than three times.

Online protein functional domain prediction

For prediction of function of the 201-210 region, the online Eukaryotic Linear Motif (ELM) resource (http://elm.eu.org) website was used.

Yeast two hybrid

pBD-GAL4- VNI2 and pAD-GAL4-TOPP plasmids were transformed into *S. cerevisiae* strain AH109 with S. c. EasyComp^{TM} Transformation kit (Invitrogen) according to the manufacture's protocol. Transformants were incubated at 30 °C on SD medium lacking with tryptophan and leucine for 3 days. The transformed cells were streaked onto SD medium lacking tryptophan, leucine and histidine and incubated at 30 °C for 3 days. MCS were used as negative controls. 0.2 mM 3AT was added to eliminate false positive effect.

3.3 Results

3.3.1 The 201-210 amino acid sequence is highly conserved

As Chapter 2 showed, the 201-210 region of VNI2 effectively inhibits VND7 transcriptional activation activity. To investigate whether the region is also conserved in the VNI2 ortholog or not, I searched the orthologs from plant species of which the genome sequence has been released using Phytozome. Within the genome of *Capsella rubella*, *Brassica rapa*, *Populus trichocarpa*, *Citrus clementina*, and *Eucalyptus grandis*, putative VNI2 orthologs in which the 10 amino acid sequence is highly conserved (Figure 3.3.1), suggesting that the amino acid sequence of 201-210 has important role in other species. By contrast, there are not VNI2 orthologs in *Oryza sativa* and *Amborella trichopoda*.

3.3.2 The conserved amino acid sequences are important

To investigate whether the conserved amino acids are important for control of VND7, the transient expression assay was carried out. Two amino acid residues were substituted into alanine of the truncated VNI2$^{1\text{-}210}$ (VNI2$^{1\text{-}210I204AF206A}$) was used as an effector. As a result, VNI2$^{1\text{-}210I204AF206A}$ inhibited the transcriptional activation activity of VND7 weaker than VNI2$^{1\text{-}210}$ (Figure 3.3.2), suggesting that the conserved sequences play critical roles in the inhibition.

3.3.3 The 201-210 region contains possible function motifs

By above results, to investigate whether region 201-210 takes exact function which is necessary for the effective inhibition, I predicted possible functional motifs by the online

Eukaryotic Linear Motif (ELM) resource (http://elm.eu.org) website. As a result, the region contained several possible functional motifs (Figure 3.3.3). A protein phosphatase 1 catalytic subunit (PP1c) interacting RVXF motif (RVXF motif: [RK]{0,1}[VI][^P][FW]; VNI2 200 KTKPIFFD 207) were contained on the 201-210 region (Figure 3.3.3). At region just close to the 201-210, VNI2 contained GSK3 phosphorylation recognition site (GSK3 site: ([ST])...[ST]; VNI2 190 EIITTDQT 197; VNI2 194 TDQTDDKT 201). In addition, inverted version of SUMOylation motif ([SDE].{0,5}[DE].(K).{0,1}[AIFLMPSTV]; VNI2 195 DQTDDKT 201), and a phosphothreonine motif (..(T)..[ILV]; VNI2 199 DKTKPIF 205) are also predicted. It is possible that some of the predicted motifs regulate the VNI2 function.

3.3.4 Searching of interactors with the 201-210 region of VNI2

Type one protein phosphatase 1 (PP1) family regulates many important plant developments through catalytic subunit (PP1c) interacting RVXF motif (Uhrig et al. 2013). The dephosphorylation by the phosphatases activates or inactivates the target proteins (Figure 3.3.4). To investigate whether the VNI2 RVXF like-motif interacts with the phosphatase, I performed yeast two hybrid assay. Unfortunately, three of the nine Arabidopsis PP1 phosphatase family member (TOPP1, TOPP4 and TOPP7; Figure 3.3.6; Figure 3.3.7) showed negative results. The result suggested, it is necessary to investigate whether other members of the phosphatase family interact with VNI2.

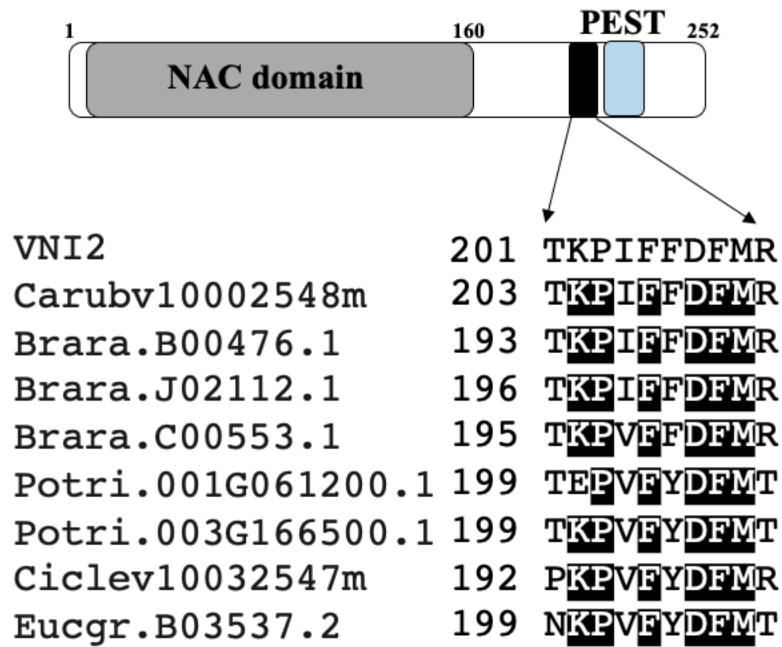


Figure 3.3.1 The region 201-210 of VNI2 is highly conserved.

Alignments of the 201-210 amino acid sequences of the VNI2 and its orthologs.

Carubv: *Capsella rubella*, Brara: *Brassica rapa*, Potri: *Populus trichocarpa*, Ciclev: *Citrus clementina*, Eucgr: *Eucalyptus grandis*.

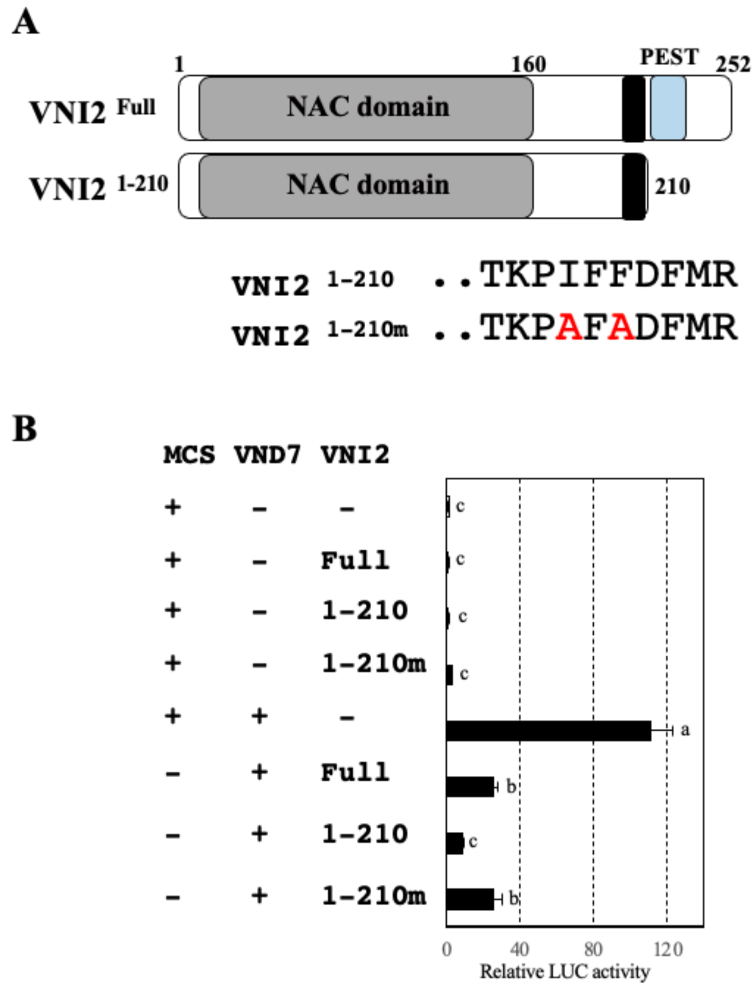
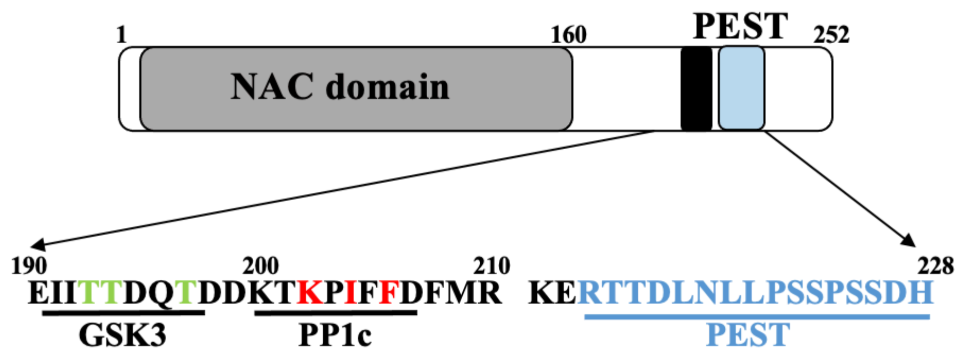


Figure 3.3.2 Mutation in the 201-210 region of VNI2 reduces inhibition activity.
(A) Schematic diagrams of full length and mutated VNI2, the amino acid residues 204 (isoleucine) and 206 (phenylalanine) were mutated by alanine residues in VNI2 1-210m. (B) The result of the transient assay. Error bars indicate standard deviations (n = 4). Different alphabets indicate significant differences at $P < 0.05$, as determined by one-way ANOVA with Tukey's HSD test.



200-207: Protein phosphatase 1 catalytic subunit (PP1c) interacting motif

199-205: Phosphothreonine motif (FHA domain).

195-201: SUMOylation motif.

190-197/194-201: GSK3 phosphorylation recognition site.

Figure 3.3.3 Schematic diagram of the putative motifs around the region 201-210 of VNI2.

By using the Eukaryotic Linear Motif (ELM) resource (http://elm.eu.org), several possible motifs were predicted, such as protein phosphatase 1 catalytic subunit (PP1c) interacting RVXF motif (RVXF motif: [RK]{0,1}[VI][^P][FW]), GSK3 phosphorylation recognition site (GSK3 site:...([ST])...[ST]; VNI2 190 EIITTDQT 197), inverted version of SUMOylation motif ([SDE].{0,5}[DE].(K).{0,1}[AIFLMPSTV]; VNI2 195 DQTDDKT 201), and Phosphothreonine motif (..(T)..[ILV]; VNI2 199 DKTKPIF 205)

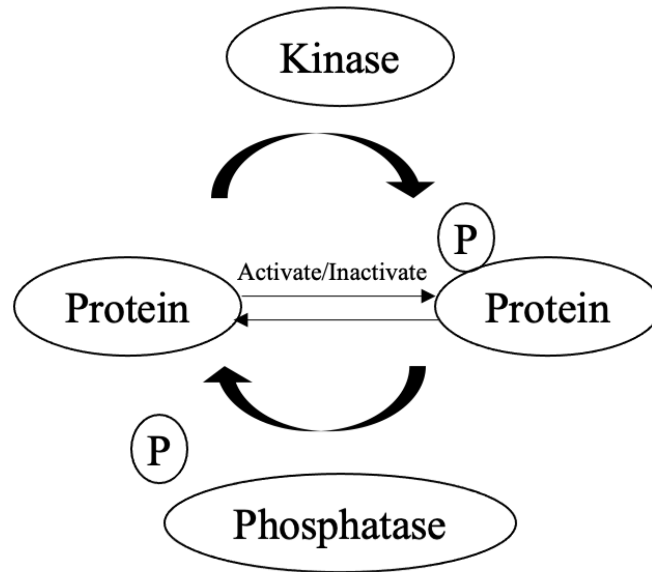


Figure 3.3.4 Protein phosphorylation and dephosphorylation.
Phosphorylation by kinase activates or inactivates target protein. The phosphorylated protein is dephosphorylated by phosphatase.

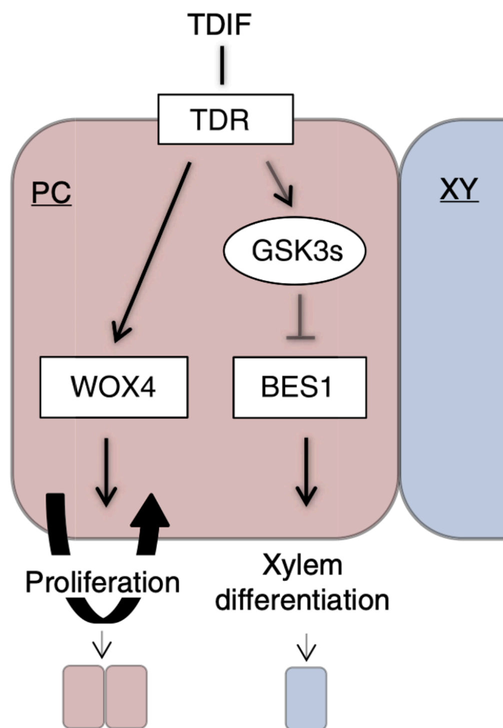


Figure 3.3.5 GSK3 kinase regulates secondary cell wall formation.
Based on the Kondo et al. (2013) Nature Communications 5:3504, glycogen synthase kinase 3 proteins (GSK3s) are downstream components of the tracheary element differentiation inhibitory factor (TDIF) signaling pathway that negatively regulates xylem differentiation from procambial cells.

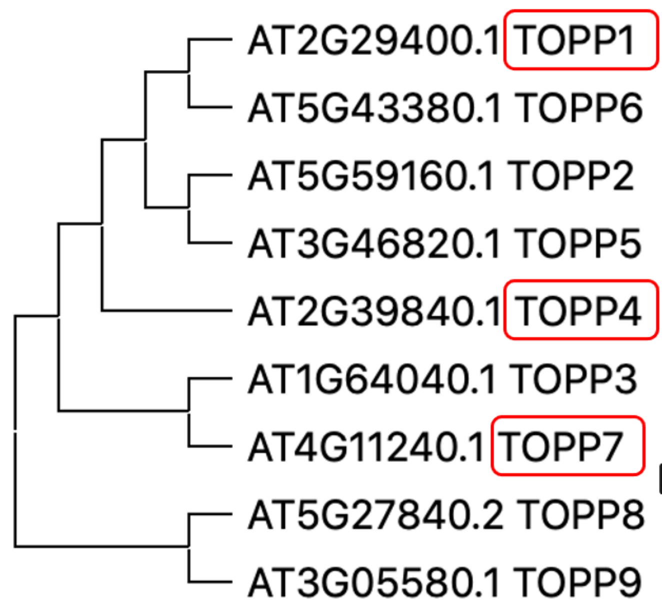


Figure 3.3.6 Arabidopsis type one protein phosphatases.

Arabidopsis type one protein phosphatases contains nine members:

TOPP1 (AT2G29400), TOPP2 (AT5G59160), TOPP3 (AT1G64040), TOPP4 (AT2G39840), TOPP5 (AT3G46820), TOPP6 (AT5G43380), TOPP7 (AT4G11240), TOPP8 (AT5G27840), TOPP9 (AT3G05580). TOPP1, TOPP4 and TOPP7 (marked with red) were selected for yeast two hybrid assay,.

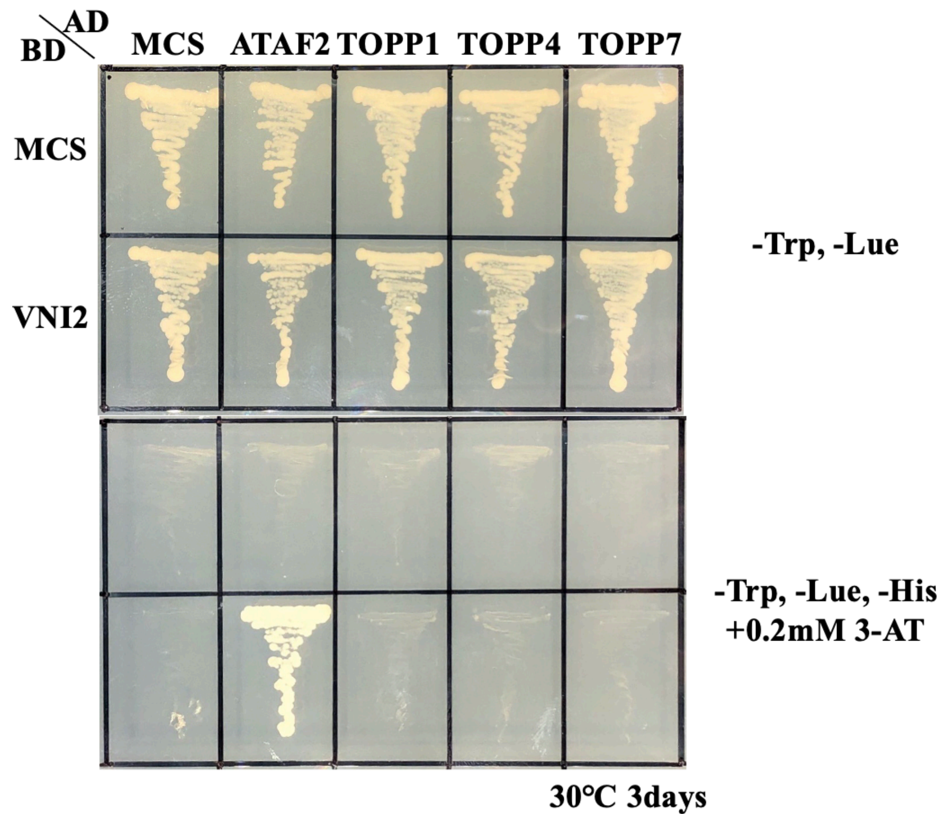


Figure 3.3.7 VNI2-phosphatase interaction.

Yeast cells expressing VNI2 and TOPPs fused with GAL4-BD and GAL4-AD, respectively, were grown on control (-Trp, -Leu) or restrictive (-Trp, -Leu, -His +0.2mM 3-AT) media. As results, none of TOPP1, TOPP4 and TOPP7 showed positive interaction with VNI2. ATAF2, a regulator of leaf senescence, shows interaction with VNI2 by previous report. So, ATAF2 was used as positive control.

3.4 Discussion

In the Chapter 2, I found that the region 201-210 of the VNI2 shows effective inhibition on the VND7 transcriptional activation activity. In this Chapter, I showed the amino acids sequences of the 201-210 are highly conserved in the VNI2 orthologs. The transient assay using VNI2 mutated at the I204 and F206 suggested the importance of the conserved sequence for the effective inhibition. Interestingly, the region also contained some possible functional motifs. Especially, some important motifs, such as the protein phosphatase 1 catalytic subunit (PP1c) interacting RVXF motif (RVXF motif: [RK]{0,1}[VI][^P][FW])-like sequences are contained in the region (Figure 3.3.3; VNI2 200 KTKPIFFD 207). The protein phosphatase regulates various plant development by target protein dephosphorylation (Bian et al. 2020; Durian et al. 2016). Protein phosphatase 1 positively regulates stomatal opening in *Vicia faba* and Arabidopsis (Takemiya et al. 2006; 2012). In addition, an Arabidopsis type1 protein phosphatase, TOPP4 regulates protein stability of PHYTOCHROME INTERACTING FACTOR5 (PIF5) during photomorphogenesis (Yue et al. 2016). As well as VND7, VNI2 is regulated by proteolysis (Yamaguchi et al. 2008; 2010b). Therefore, it is possible the phosphorylation and dephosphorylation of VNI2 my affect inhibition of VND7 through protein stability of VNI2. Moreover, some other motifs, such as GSK3 phosphorylation recognition site, SUMOylation motif, and Phosphothreonine motif are also predicted around the 10 amino acid sequence (Figure 3.3.3). It have been reported that GSK3 kinase negatively regulates xylem cell differentiation (Kondo et al. 2014). Therefore, further research are necessary whether the predicted motifs work for regulation of VNI2. As the yeast two hybrid result, the three of nine Arabidopsis phosphatase 1 family

member did not show the interaction with VNI2 (Figure 3.3.7). It is also necessary to investigate the interaction of the other members, or interactions uding different approaches. In addition, according to a phosphorylation database (PhosPhAt), there are putative phosphorylation sites (T193, T194, and T197). It is interesting whether these sites are phosphorylated and involved in VNI2 function.

The region 201-210 may also related to VNI2 protein stability or binding affinity to VND7. As the previous results, protein stability of the VNI2 correlates to its inhibitory function. Therefore, it is important to verify the relationship between the conserved amino acid sequence and protein stability.

In Chapter 3, I confirmed the region 201-210 of VNI2 is important for effective inhibition on VND7 transcriptional activation activity (Figure 3.4.1). I also showed several possible inhibition mechanisms of the VNI2. Therefore, further research to confirm which kind of inhibition mechanism that VNI2 having is necessary.

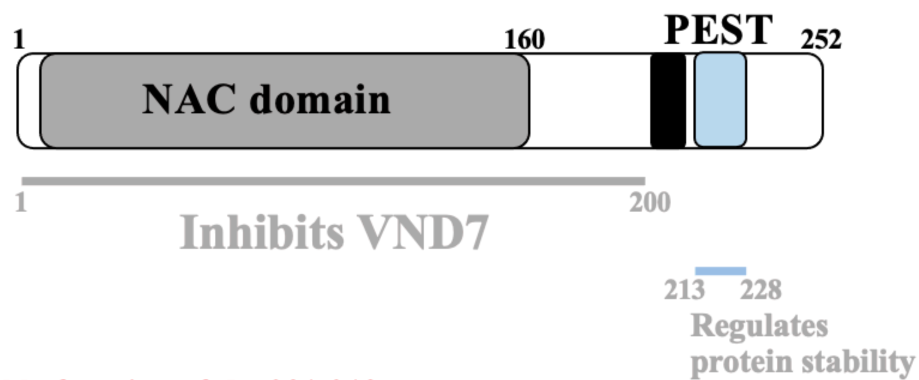


Figure 3.4.1 A schematic putative hypothesis of regulation of VNI2 by phosphorylation and dephosphorylation.
As a summary of this Chapter, the function of the 201-210 region of VNI2 which for effective inhibition is investigated. The region may indirectly regulate the inhibition effectiveness by such as VND7-VNI2 protein binding or VNI2 protein stability. Alternatively, the region may directly regulate the inhibition effectiveness by taking the predicted function such as PP1 phosphatase interaction or GSK3 kinase interaction.

Chapter 4

VNI2 expression is regulated during the xylem vessel differentiation

4.1 Introduction

As previous Chapters mentioned, VND7 is a master switch of xylem vessel element differentiation. Overexpression of *VND7* induces ectopic xylem vessel elements (Yamaguchi et al. 2008). At protein structure, the N-terminal of VND7 protein is the conserved NAC domain which do function for binding of downstream genes promoter. By contrast, the entire C-terminal of the VND7 shows transcriptional activation activity. Indeed, the C-terminal deletion of the VND7 reduced its transcriptional activation activity level (Yamaguchi et al. 2008). VND7 directly regulates a number of genes involved in secondary cell wall biosynthesis [CELLULOSE SYNTHEASE A CATALYTIC SUBUNIT8 (CesA8)/IRREGULAR XYLME1 (IRX1)], programmed cell death [*XYLEM CYSTEINE PEPTIDASE 1* (*XCP1*) and *XCP2*], and transcription factors [*LBD30*/ *ASL19*/ *JLO*, *MYB46*, and *MYB83*] (Yamaguchi et al. 2011; Zhong et al. 2010). In addition, there is a transcriptional network in xylem vessel element differentiation. For examples, LBD30 and VND7 form a positive feedback loop (Soyano et al. 2009; Ohhashi-Ito et al. 2020). MYB46 and MYB83 regulates some other MYB transcription factors and KNOTTED ARABIDOPSIS THALIANA7 (KNAT7) (Zhong et al. 2008; McCarthy et al. 2009; Liu et al. 2015).

As I demonstrated, VNI2 binds to and inhibits VND7 resulting in negatively regulating xylem vessel formation (Yamaguchi et al. 2010b). Expression of *VNI2* is observed earlier stage of differentiating xylem vessels than that of *VND7* (Yamaguchi et al. 2010b). Extension of the *VNI2* expression to the later stage of the differentiating stage exhibits defects in xylem vessel formation. Therefore, it is likely that *VNI2* expression is regulated precisely during xylem vessel formation. It has been reported that *VNI2*

expression is upregulated by NAC domain transcription factors ATAF2 (Nagahage et al. 2020) or ORE1 (Rauf et al. 2013). However, it is still uncertain how *VNI2* expression is regulated during the xylem vessel formation (Figure 4.1.2).

In Chapter 4, to understand how *VNI2* expression is regulated during the xylem vessel element differentiation, I investigated whether several transcription factors in volved in the differentiation regulates *VNI2* expression. Transient gene expression showed that VND7 downregulates *VNI2* expression whereas MYB83 significantly upregulated *VNI2* expression. These data suggested that *VNI2* expression is not only negatively but also positively regulated during the xylem vessel formation. Moreover, VND7-VNI2 mutually regulate each other.

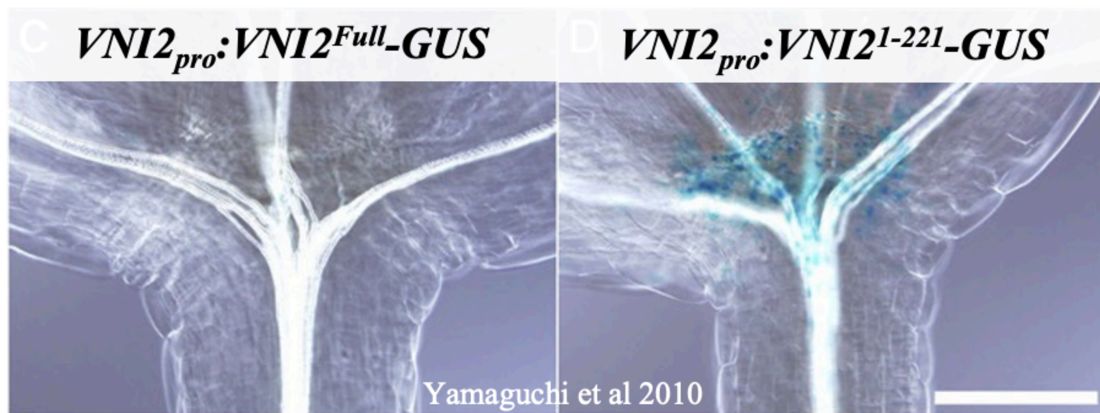


Figure 4.1.1 VNI2 protein is regulated by protein degradation.

Referred from the Yamaguchi et al. (2010) Plant Cell 22: 1249–1263. GUS staining of the shoot apical regions in transgenic plants carrying *$VNI2_{pro}$:$VNI2^{full}$-GUS* and *$VNI2_{pro}$:$VNI2^{1–221}$-GUS*.

Bar = 200 mm.

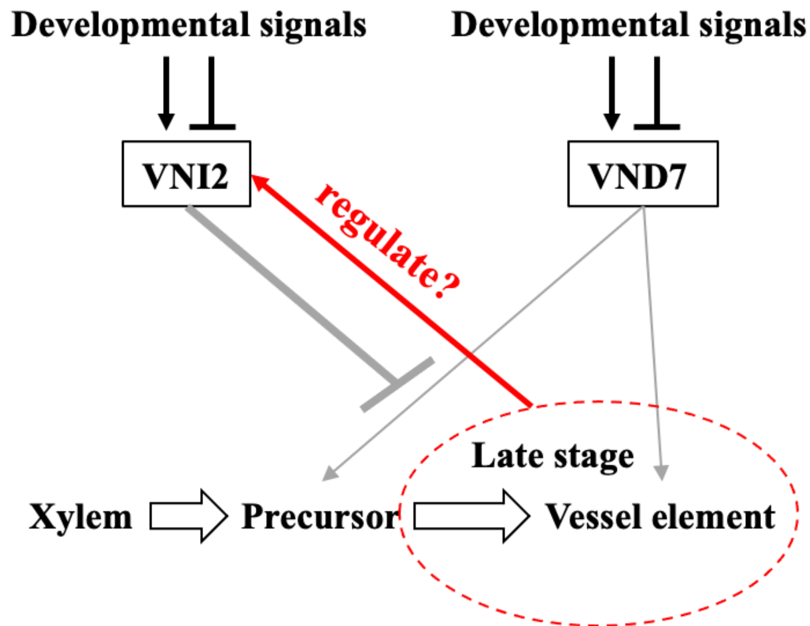


Figure 4.1.2 Schematic diagram of the aim of this chapter.

During xylem vessel element differentiation, *VNI2* expression is initiated earlier than *VND7* expression. Then, VNI2 seems to protect from progression of the later stage of xylem vessel element differentiation by inhibiting VND7. In this chapter, I focused on whether *VNI2* expression is regulated during the xylem vessel formation.

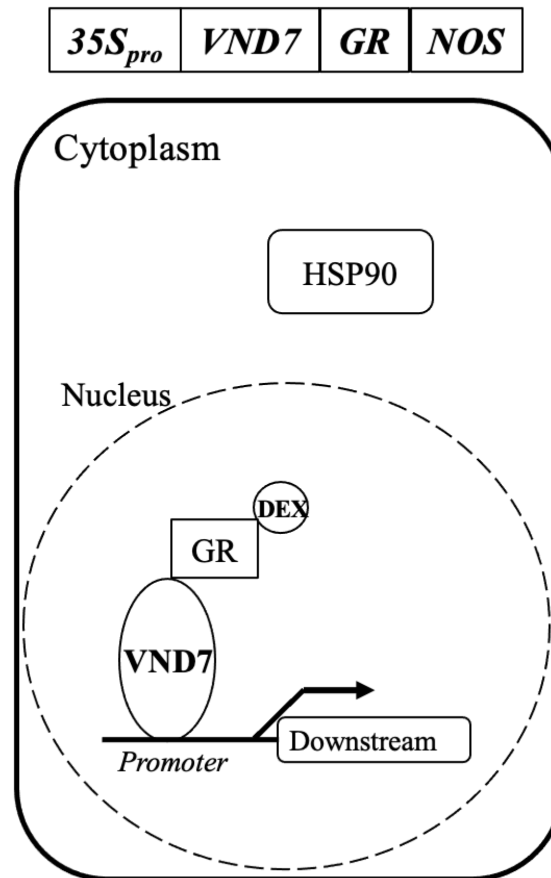


Figure 4.1.3 Glucocorticoid-mediated transcriptional induction system.
Schematic diagrams of the glucocorticoid-mediated transcriptional induction system. In this system, VND7 transcriptional activation activity is activated by a glucocorticoid derivative, DEX, treatment. After DEX treatment, VND7 relocates to the nucleus and activates.
35Spro: CaMV35S promoter, *GR*: hormone-binding domain of rat glucocorticoid receptor, *NOS*: terminator of Nopaline synthase.

4.2 Materials and Methods

Plasmid Construction

The preparation of the plasmid for the transient gene expression in this Chapter is as same as the Materials and methods of Chapter 2. The nucleotide sequences of primers used in this study are provided in Supplementary Table. The coding sequence of VND7 were amplified with PCR using primers containing the recognition sequence for BamHI (S1 Table). After digestion with BamHI, the amplified fragment was subcloned into the BamHI sites of the VP16-GR vector (Gifted from Dr. Nam-Hi Chua). The resultant construct was designated as pVND7-GR containing VND7 fused to rat glucocorticoid receptor (GR) domain under controls of *CaMV35S* promoter.

Dual luciferase transient expression assay

The transient gene expression analysis was performed as the Materials and methods of Chapter 2. I use three or four biological replicates, and I repeated the experiments more than three times.

Generation of Transformants

Generation method of transgenic plant is as same as Materials and methods of Chapter 2. Transgenic Arabidopsis seedlings were selected with a germination medium in the presence of 50 mg L^{-1} kanamycin.

Expression Analysis

Ten-day-old T3 homozygous single insertion seedlings of VND7-GR, VP16-GR grown

on the germination medium without antibiotics were soaked with sterilized water containing with or without 10 μM DEX, for 12 h. Total RNA was extracted by using the RNeasy Plant Mini Kit (Qiagen), and treated with DNase I (Qiagen) to remove genomic DNA contamination. cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen). Quantitative RT-PCR was performed using KAPA SYBR FAST qPCR Kits (Nippon Genetics) and the 7300 Real-Time PCR system (Applied Biosystems). Relative expression values are obtained by delta delta Ct method. To determine statistical significance, one-way ANOVA followed by Tukey's HSD test was performed. Each expression analysis was repeated two times. The primers used for the expression analyses are listed in supplementary table.

4.3 Results

4.3.1 *VNI2* expression is negatively regulated by VND7

To investigate whether *VNI2* expression is regulated during the xylem vessel formation, a transient expression assay was performed using the Arabidopsis protoplasts obtained from mesophyll cells. At first, I investigated whether VND7 regulates *VNI2* expression, because VND7 is the master regulator of the transcriptional cascade of the xylem vessel differentiation. Reporter constructs harbored a firefly *LUCIFERASE* (*LUC*) gene driven by *VNI2* promoter as well as promoters of *MYB46* and *XCP1* which are direct targets of VND7 (Yamaguchi et al. 2011). The effector construct contained coding region of *VND7* driven by the cauliflower mosaic virus *35S* promoter (*CaMV35S*). These constructs as well as reference construct containing *Renilla LUC* gene driven by *CaMV35S* promoter were transfected into the protoplasts. VND7 elevated expression of the reporter gene under controls of the *MYB46* and *XCP1* promoters (Figure 4.3.1). By contrast, when the *VNI2* promoter was used as the reporter, VND7 rather downregulated the *LUC* expression (Figure 4.3.1). These results indicated that VND7 as master regulator of the xylem vessel differentiation negatively regulates *VNI2* expression.

4.3.2 VND7 regulates *VNI2* expression through the C-terminal domain

As shown in Yamaguchi et al. (2008), VND7 lacking C-terminal region loses its transcriptional activation activity. Thus, I performed the transient expression assay using a C-terminally truncated VND7 (VND7$^{1\text{-}161}$) in which only NAC domain was included as an effector (Figure 4.3.2), to investigate relationship between the C-terminal region of VND7 and downregulation of *VNI2* expression. VND7$^{1\text{-}161}$ just slightly upregulated the

LUC expression under controls of the *MYB46* and *XCP1* promoters, as previously reported (Yamaguchi et al. 2008). Moreover, VND7$^{1\text{-}161}$ did not effectively downregulated the firefly *LUC* expression under controls of the *VNI2* promoter (Figure 4.3.2), suggesting that VND7 regulates *VNI2* through the C-terminal region.

4.3.3 400 bp of the *VNI2* promoter region is responsible for the negative regulation

To investigate which region of the *VNI2* promoter is responsible for the negative regulation by VND7, 5' deletion series of the *VNI2* promoter was used as a reporter of the transient expression assays (Figure 4.3.3). VND7 negatively regulated the reporter expression when VNI2 *proD* (from -791 to +9 bp) and the longer promoter regions were used as reporter constructs (Figure 4.3.3). By contrast, when *proE* (from -391 to +9 bp) were used as the reporter, the significantly negative regulation of the reporter gene expression was not observed (Figure 4.3.3). These results indicated that the 400 bp region (-791 to -391 bp) contains the responsible sequence for negative regulation by VND7.

4.3.4 *VNI2* expression is both positively and negative regulated during xylem vessel formation

As previous reports, VND7 activates many downstream transcription factors associated with the xylem vessel formation (Yamaguchi and Demura 2010; Nuruzzaman et al. 2013; Nakano et al. 2015; Shao et al. 2015; Figure 1.4). To investigate whether the negative regulation of the *VNI2* by VND7 is directly or by its downstream transcription factors, transcription factors involved in xylem vessel formation were used as effectors of the transient expression assay. Within the transcriptional cascade, LBD30/ASL19 forms a

positive feedback loop with VND7 while MYB83 is a downstream transcription factor of the VND7. KNAT7, which is a negative regulator of secondary cell wall formation in xylem vessel elements, is regulated by MYB83 (Liu et al. 2014; McCarthy et al. 2009; Wang et al. 2019). In this condition, I showed that VND7 downregulated the reporter gene expression under controls of the *VNI2* promoter (Figure 4.3.4). However, any other transcription factors did not downregulate the LUC activity (Figure 4.3.4). Rather, the LUC activity was upregulated when MYB83 was used as the effector (Figure 4.3.4). These results suggested that the *VNI2* expression is not only negatively but also positively regulated during the xylem vessel formation.

4.3.5 Downregulation of *VNI2* was not observed in planta

To investigate whether the downregulation of *VNI2* expression by VND7 occur in planta, I generated a transgenic Arabidopsis plants using a chemical-inducible system. In the transgenic plants, VND7 fused to the hormone-binding domain of glucocorticoid receptor (GR) was expressed under the control of the *CaMV35S* promoter (VND7-GR). The GR fused proteins are localized in the cytosol without glucocorticoid condition (Figure 4.3.5). In the presence of glucocorticoid derivative, dexamethasone (DEX), the structure of GR changes resulting in localized to the nucleus. In several T3 homozygous single insertion lines, transdifferentiation into xylem vessel elements was observed dependent on the DEX treatment, indicating that VND7-GR is activated dependent on DEX treatment manner. Ten-day-old seedlings of the VND7-GR plants were soaked in water in the absence or presence of DEX for 12 hours, and then total RNA was extracted. Quantitative RT-PCR analysis showed that expression levels of *MYB46* and *XCP1*, direct targets of VND7, were significantly elevated after DEX treatment. Inconsistent with the

transient expression analyses, *VNI2* expression was slightly but significantly upregulated by DEX treatment in the VND7-GR plants (Figure 4.3.6). Previous transcriptome analyses also demonstrated that overexpression of *VND7* did not downregulate *VNI2* expression, suggesting that overexpression of *VND7* may have different effects on *VNI2* expression in whole plants and protoplasts.

4.4 Discussion

In this chapter, I found that VND7 negatively regulates *VNI2* expression. It is different from previous reports that VND7 have been known as a transcriptional activator (Yamaguchi et al. 2008). Additionally, it has been reported that VND7 has a transcriptional activation domain at its C-terminal region (Yamaguchi et al. 2008). Interestingly, I found that the C-terminal region of the VND7 also showed important for the regulation of *VNI2* expression. Therefore, there are two possibilities how VND7 able to negatively regulates *VNI2* expression. One of the possibilities is that VND7 upregulates unknown downstream transcriptional repressors which negatively regulate *VNI2* expression. Alternatively, VND7 directly negatively regulates *VNI2* expression since VND7 has a bifunctional activity. I also confirmed the 400 bp region of the *VNI2* promoter is responsible for the negative regulation. Therefore, further studies on the 400 pb promoter region to identify the DNA sequences will give us the exact mechanism how VND7 negatively regulates *VNI2* expression.

As another interesting point of this Chapter, MYB83 elevates *VNI2* expression (Figure 4.3.4). Differentiation of xylem vessel elements accompanies secondary cell wall biosynthesis and then programmed cell death (Nakano et al. 2015; Escamez et al. 2016). VND7 directly or indirectly regulates expression of genes involved in secondary cell wall formation and programmed cell death (Zhong et al. 2010; Yamaguchi et al. 2010b; Yamaguchi et al. 2011) while MYB83 and MYB46 directly or indirectly regulate only genes regulating secondary cell wall biosynthesis (McCarthy et al. 2009; Ko et al. 2014; Nakano et al. 2015). It is likely that precise regulation of *VNI2* expression is required for

proper controls of secondary cell wall biosynthesis and programmed cell death during the xylem vessel element differentiation.

About on the regulation of the *VNI2* expression, it is also a new finding that a transcription factor negatively regulates *VNI2*. NAC domain transcription factors ORE1 and ATAF2 encoding NAC domain transcription factors promote leaf-senescence. It has been shown that ORE1 and ATAF2 upregulate *VNI2* expression during progression of leaf-senescence (Rauf et al. 2013; Nagahage et al. 2020). Information about the transcriptional factors regulating *VNI2* has been limited. Therefore, with this Chapter results, it will be helpful to understand how *VNI2* expression is regulated.

The most meaningful point which I found in this chapter is the mutual regulation between VND7 and VNI2. *VNI2* expression is sifted to earlier than *VND7* expression (Yamaguchi et al. 2010b). At the earlier stage, VNI2 inhibits VND7 and the other VND families to repress the irreversible progression. By contrast, at the later stage, VND7 may downregulates *VNI2* expression to promote the irreversible cell fate. As shown in chapter 2, ectopic expression of *VNI2* driven by VND7 promoter to extend to the later stages of the differentiation showed defects in xylem vessel formation and plant growth (Figure 2.3.5). It has been also reported that VNI2 is regulated by the post-translational step (Yamaguchi et al. 2010b). These data strongly suggest that controls of VNI2 expression and/or function are important for proper xylem vessel formation and plant growth (Yamaguchi et al. 2010b).

Unlike the transient gene expression result, the VND7-GR line did not showed downregulation of the *VNI2* expression. Through transient expression assays using protoplasts, a number of transcription factors were identified as putative positive regulators of *VND7* expression. Overexpression of the transcription factors upregulated

secondary cell wall related genes while *VND7* expression was not markedly changed. These results implied that *VND7* expression is tightly regulated in a tissue/cell type-specific manner. Recently, transcriptome analysis of single cells from roots in which *VND7* expression was induced by chemical treatment was reported (Turco et al. 2019). Compared to *XCP1*, the expression level of *VNI2* was not strongly correlated with that of *VND7*. Further analysis using a uniform cell population may be necessary to investigate whether VND7 regulates *VNI2* expression during xylem vessel differentiation.

In future, to unveil the regulatory mechanism of VNI2 expression, it is necessary to identify the cis-sequence contributing to the downregulation and upregulation of *VNI2* by VND7 and MYB83, respectively.

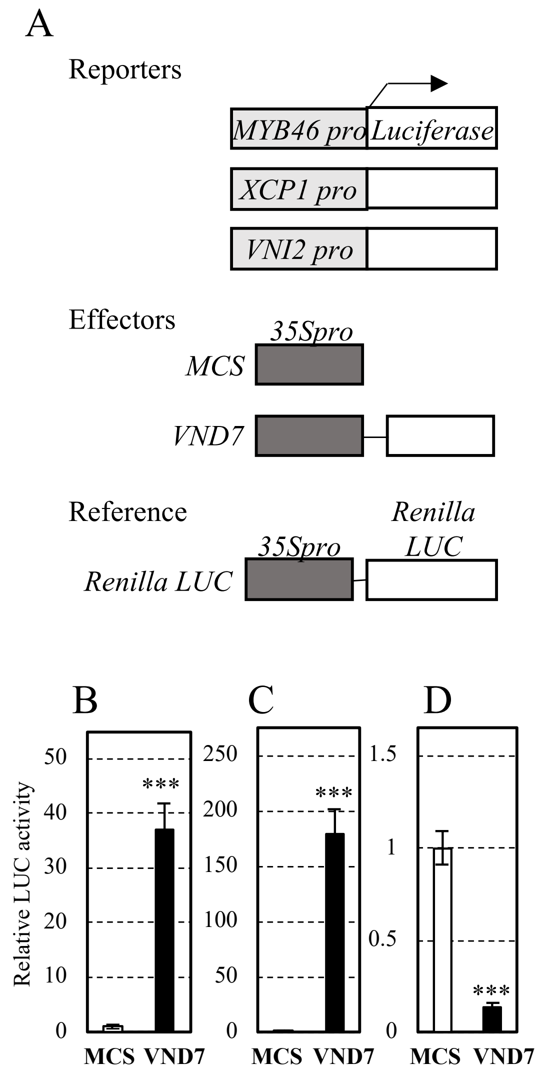


Figure 4.3.1 VND7 downregulates *VNI2* expression in protoplasts.

(A) Schematic diagrams of constructs using for the transient gene expression assay. (B-D) Results of the transient gene expression assays using promoters of *MYB46* (B), *XCP1* (C), and *VNI2* (D). Error bars indicate standard deviations (n = 4). Asterisks indicate the statistical differences compared to the control. (Student's t-test, ***$P < 0.001$)

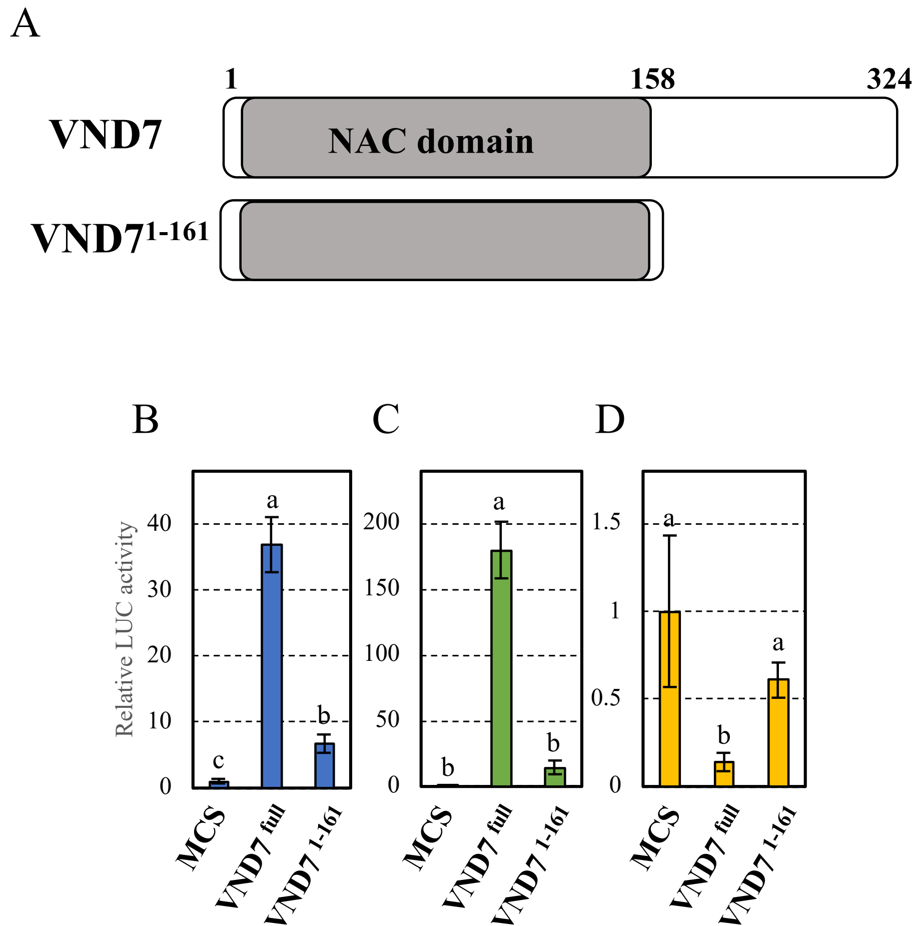


Figure 4.3.2 VND7 regulates *VNI2* expression through its C-terminal domain.

(A) Schematic diagrams of full length and the C-terminally truncated VND7. (B-D) Results of the transient gene expression assays using promoters of *MYB46* (B), *XCP1* (C), and *VNI2* (D). Error bars indicate standard deviations (n = 4). Different alphabets indicate statistical differences at $P < 0.05$, calculated by one-way ANOVA with Tukey's HSD test.

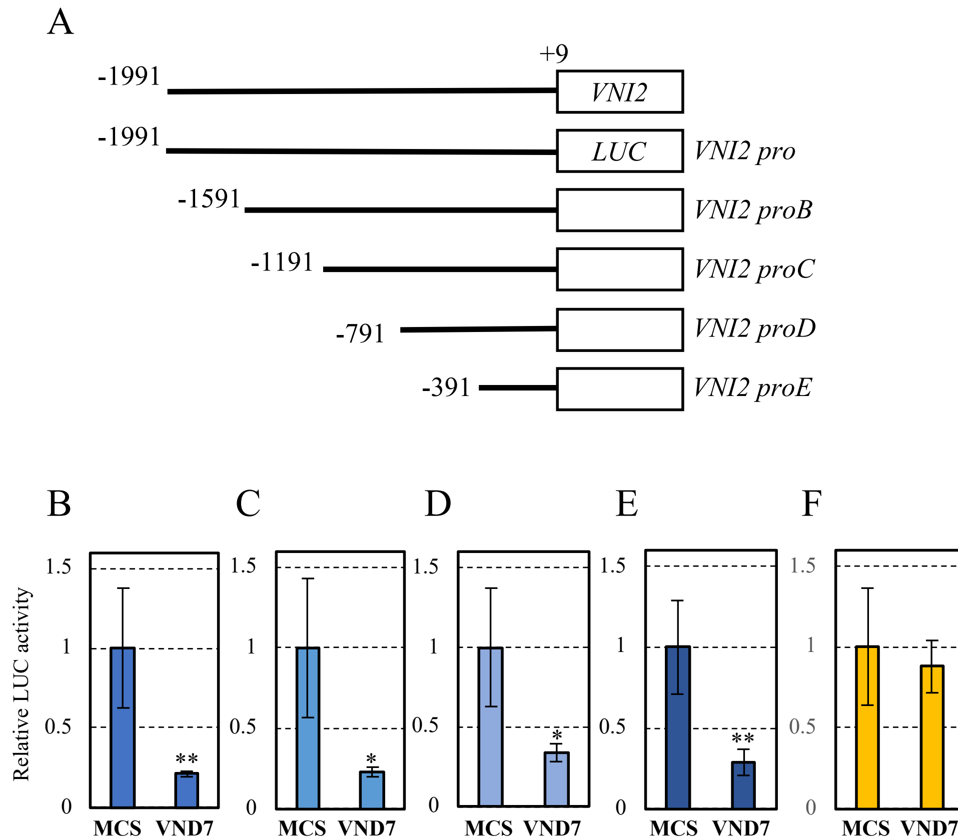


Figure 4.3.3 A transient reporter assay using the fragmented *VNI2* promoter.
(A) Schematic diagrams of *VND2* promoter used for the transient expression assays. (B-F) Results of the transient expression assays using *VNI2pro* (B), *VNI2proB* (C), *VNI2proC* (D), *VNI2proD* (E), and *VNI2proE* (F). Error bars indicate standard deviations (n = 4). Asterisks indicate the statistical differences compared to the control vector (Student's t-test, **: $P < 0.01$, *: $P < 0.05$).

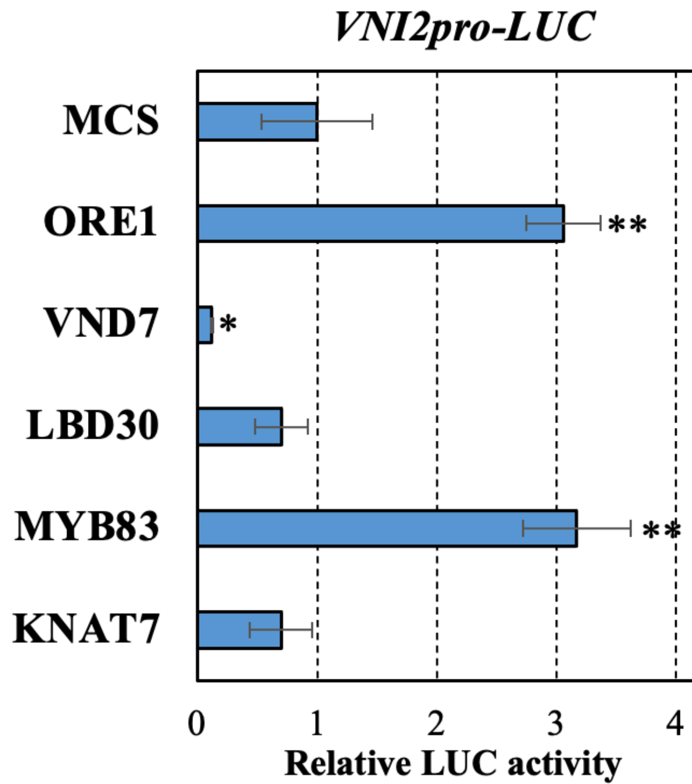


Figure 4.3.4 A transient expression assay with various transcription factors regulating xylem vessel formation.

Indicated transcription factors were used as effectors. Error bars indicate standard deviations (n = 4). Asterisks indicate statistical differences compared to the control (one-way ANOVA with Dunnett's test, **: $P < 0.01$, *: $P < 0.05$).

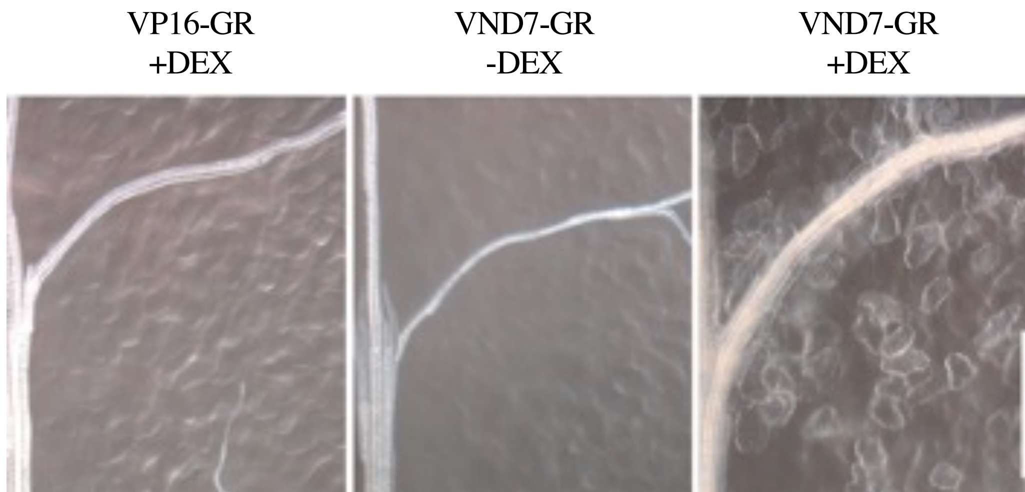


Figure 4.3.5 Trans-differentiation into xylem vessel elements induced by activation of VND7-GR.

For investigation of the downstream gene expression of VND7, glucocorticoid-mediated transcriptional induction system is used. Leaves of transgenic plants expressing a control VP16-GR or VND7-GR were soaked into water in the absence (-) or in the presence (+) of 10 μM DEX for 38 hours.
Bar = 100 μm.

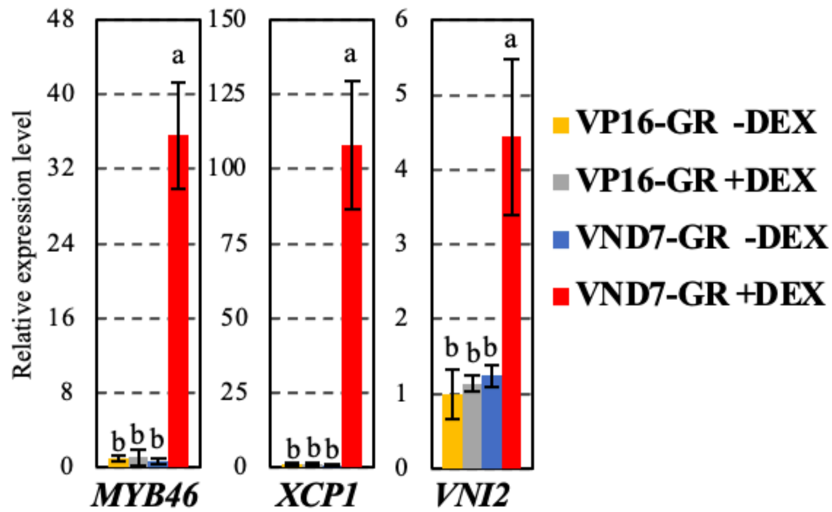


Figure 4.3.6 Expression analysis using transgenic plants expressing VND7-GR chimera gene.

Quantitative RT-PCR analysis was performed using the whole seedling of 10-day-old plants treated with or without DEX for 12 hours. mRNA levels for each gene were normalized to *UBQ10* mRNA. Error bars indicate standard deviations (n = 3). Different alphabets indicate statistical differences at $P < 0.05$, calculated by one-way ANOVA with Tukey's HSD test.

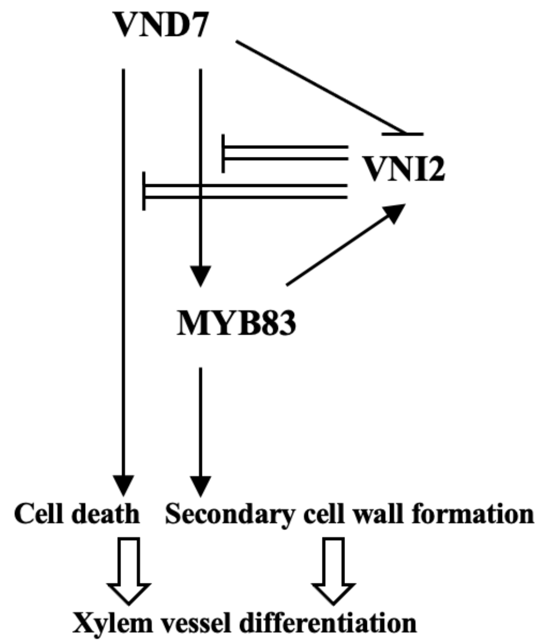


Figure 4.4.1 The summary of Chapter 4.

VNI2 expression is regulated positively and negatively during the xylem vessel element differentiation. Moreover, several new findings have also been discovered such as VND7 could be a negative regulator, an exact region in the *VNI2* promoter is exist for its regulation. The most important insight is existence of the mutual regulation between VND7 and VNI2.

Chapter 5

General Discussion

Plant growth and development are tightly regulated by outside environmental signals and inside regulatory network. The plant hormones are fundamental to this process (Gray et al. 2004). The in vitro xylem vessel element differentiation system was also established by hormonal-mediated induction (Kubo et al. 2005). A number of genes are identified which regulate xylem vessel element differentiation (Yamaguchi et al. 2011; Zhong et al. 2010). However, our knowledge about the complicated regulatory network is still limited during xylem vessel element differentiation.

The NAC domain transcription factor VND-INTERACTING2 (VNI2) was targeted for research since it negative regulates xylem vessel formation by inhibiting the master regulator VND7 (Yamaguchi et al. 2010b). Ectopic expression of *VND7* or *VNI2* showed severe defects in normal xylem vessel formation (Kubo et al. 2005; Yamaguchi et al. 2010b; Endo et al. 2015) suggesting that the precise VNI2-VND7 regulation is important for xylem vessel formation.

In this thesis, I tried to make clear the molecular mechanism of the VNI2-VND7 interaction. By previous report (Yamaguchi et al. 2010b), VNI2 protein stability is controlled by protein degradation. Indeed, protein stability of VNI2 affects its inhibitory function. It is likely that the unstable feature of VNI2 protein is important to regulate proper progression of the xylem vessel element differentiation. Moreover, as **Chapter 2** results, VNI2 also contained a region which for effective inhibition on the VND7 activity. Therefore, it is possible that VNI2 able to inhibit VND7 activity independent on the protein stability through the identified sequence. In the **Chapter 3,** I tried to understand the possible molecular mechanism how VNI2 effectively inhibits VND7 transcriptional activation activity. The predicted functional motif such as GSK3 kinase interaction motif might be possible to link to the VNI2 inhibition mechanism. Because the GSK3 kinase

negatively regulates xylem cell differentiation (Kondo et al. 2014). Therefore, the further research, such as identification of the phosphorylation sites, or interaction of PP1 and VNI2 in vitro and in vivo will give us the new insight of the regulation of the xylem vessel differentiation. The **Chapter 4** showed another interesting insight that VND7 and VNI2 mutually regulate their functions. The results also indicated the existence of the elaborate regulation of the *VNI2* expression during xylem vessel element differentiation. In addition, the *VNI2* expression is regulated both positively and negatively in the differentiation process, indicating that regulation of the *VNI2* expression seems to be complicated during the xylem vessel differentiation.

As described above, I obtained a number of novel findings which contribute to unveiling the molecular mechanism of xylem vessel element differentiation. Our laboratory found that VNI2 interacts with a number of transcription factors, which are known to be various biological processes. Therefore, the obtaining results also provide for understanding the processes. However, I also showed various questions which is necessary to solve for understanding the regulatory mechanisms. For examples, how does the 201-210 region of VNI2 protein works the effective inhibition of VND7? It is likely that isolation of interacting protein with the 10 amino acid sequence and identification of phosphorylation sites of VNI2 should be useful approaches to solve the question. It is also necessary to identify the *cis*-elements involved in regulation of *VNI2* expression of VND7 and MYB83. I have already succeeded in narrowing down to 400 bp region. Thus, EMSA analysis should be expected to be effective. Further analyses should be necessary.

Supplementary table

Primer	Primer Sequence（5’-3’）	Purpose
VND7-F	CACCATGGATAATATAATGCAATCGTCAAT	For amplifying CDS sequence of VND7 gene
VND7-R	TTACGAGTCAGGGAAGCATCCAAG	For amplifying CDS sequence of VND7 gene
VNI2 full-F	CACCATGAAGTCGGAGCTAAATTTACCA	For amplifying CDS sequence of VNI2 full gene
VNI2 full-R	TTACCCCTGTGGAGCAAAACTCCAATTC	For amplifying CDS sequence of VNI2 full gene
VNI2 D216AN218A-F	AAAGAAAGAACAACAGCTTTGGCCCTTTTGCCGAGC	For amplifying CDS sequence of VNI2 D216AN218A
VNI2 D216AN218A-R	CGGCAAAAGGGCCAAAGCTGTTGTTCTTTCTTT	For amplifying CDS sequence of VNI2 D216AN218A
VNI2 L217AL219A-F	GAAAGAACAACAGATGCGAACGCTTTGCCGAGCTCTCCT	For amplifying CDS sequence of VNI2 L217AL219A
VNI2 L217AL219A-R	AGGAGAGCTCGGCAAAGCGTTCGCATCTGTTGTTCTTTC	For amplifying CDS sequence of VNI2 L217AL219A
VNI2 1-210-R	TCATCTCATGAAATCAAAGAAGATTGGTTTTGT	For amplifying CDS sequence of VNI2 1-210
VNI2 1-200-R	TCATTTATCATCTGTTTGGTCTGTTGTAATTAT	For amplifying CDS sequence of VNI2 1-200
VNI2pro-F	CACCGAAATCGATCATTTTTTTATTTTAATTTAG	For amplifiying promoter sequence of VNI2 gene
VND7pro-VNI2-F	ATAGGATCATCGTGGATGGATAATGTCAAA	For amplifying fusion fragments of VND7 promoter: VNI2 coding region
VND7pro-VNI2-R	TTTGACATTATCCATCCACGATGATCCTAT	For amplifying fusion fragments of VND7 promoter: VNI2 coding region
VNI2 1-210 (204206A)-F	TAAAACAAAACCAGCCTTCGCCGATTTCATGAGA	For amplifying CDS sequence of VNI2 I204AF206A
VNI2 1-210 (204206A)-R	TCTCATGAAATCGGCGAAGGCTGGTTTTGTTTTA	For amplifying CDS sequence of VNI2 I204AF206A
XCP1 promoter F	CACCGTAAGTTAACCACATTTGCTTTTGCT	For amplifying promoter sequence of XCP1 gene
XCP1 promoter R	AAAAGCCATAGCCAAATTTGTTCACTG	For amplifying promoter sequence of XCP1 gene
VND71-161F	CACCATGGATAATATAATGCAATCGTCAAT	For amplifying CDS sequence of VND71-161 gene
VND71-161R	TTATGGAATTGGCTTCCTAAATGCTCGACA	For amplifying CDS sequence of VND71-161 gene
LBD30F	CACCATGAGCAGTAGCGGAAACCCTAGCAGC	For amplifying CDS sequence of LBD30 gene
LBD30R	TCATTCTCGTTTTATCACTGACGAGGCA	For amplifying CDS sequence of LBD30 gene
MYB83F	CACCATGATGATGAGGAAACCGGACATTA	For amplifying CDS sequence of MYB83 gene
MYB83R	TCAATCGACTTGGAAATCAAGGAAGGGA	For amplifying CDS sequence of MYB83 gene
KNAT7F	CACCATGCAAGAAGCGGCACTAGGTATGAT	For amplifying CDS sequence of KNAT7 gene
KNAT7R	TTAGTGTTTGCGCTTGGACTTCAAGGAAGT	For amplifying CDS sequence of KNAT7 gene
ORE1F	CACCATGGATTACGAGGCATCAAGAATCGTCGAA	For amplifying CDS sequence of ORE1 gene
ORE1R	TCAGAAATTCCAAACGCAATCCAATTCTTC	For amplifying CDS sequence of ORE1 gene
VNI2 promoter F	CACCGAAATCGATCATTTTTTTATTTTAATTTAG	For amplifying promoter sequence of VNI2 gene
VNI2 promoter R	ATTATCCATGGTGGTTCCAAACAAAGAGAG	For amplifying promoter sequence of VNI2 gene
VNI2 promoterB F	CACCGGATATTTCAGATGGGATATCAAATT	For amplifying promoter sequence of VNI2proB gene
VNI2 promoterB R	ATTATCCATGGTGGTTCCAAACAAAGAGAG	For amplifying promoter sequence of VNI2proB gene
VNI2 promoterC F	CACCAATGGAAAGCACTTAGAGGGATAATG	For amplifying promoter sequence of VNI2proC gene
VNI2 promoterC R	ATTATCCATGGTGGTTCCAAACAAAGAGAG	For amplifying promoter sequence of VNI2proC gene
VNI2 promoterD F	CACCTATATGTAAATATAGACGAAGAGTGC	For amplifying promoter sequence of VNI2proD gene
VNI2 promoterD R	ATTATCCATGGTGGTTCCAAACAAAGAGAG	For amplifying promoter sequence of VNI2proD gene
VNI2 promoterE F	CACCATGGACCCAACTTCTCACTTATACTT	For amplifying promoter sequence of VNI2proE gene
VNI2 promoterE R	ATTATCCATGGTGGTTCCAAACAAAGAGAG	For amplifying promoter sequence of VNI2peoE gene
MYB46 promoter F	CACCACCTCACACTACTGTATTCTTACTTTTAAT	For amplifying promoter sequence of MYB46 gene
MYB46 promoter R	CTTCCTCATATTTTTGGTTGAGTTAATTGT	For amplifying promoter sequence of MYB46 gene
Multiple cloning site (MCS) F	CACCTAGTGGATCCCCCGGGCTGCAGGAATTCGATATCAAGC TTATCGATACCGTCGACCTCGTGATG	For amplifying CDS sequence of MCS
Multiple cloning site (MCS) R	CATCACGAGGTCGACGGTATCGATAAGCTTGATATCGAATTC CTGCAGCCCGGGGGATCCACTAGGTG	For amplifying CDS sequence of MCS

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