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Original Paper

Arabidopsis NAC domain proteins VND-INTERACTING1 and ANAC103 interact with multiple NAC domain proteins

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Abstract The *Arabidopsis thaliana* NAM, ATAF1/2 and CUC2 (NAC) domain transcription factor VND-INTERACTING1 (VNI1) was previously isolated as an interacting factor of VASCULAR-RELATED NAC-DOMAIN PROTEIN7 (VND7), a key regulator of xylem vessel differentiation, in a yeast two-hybrid screening. Here, we characterized VNI1 and its closest homolog, ANAC103, at the molecular level. Both VNI1 and ANAC103 interacted in vitro not only with VND proteins but also with other NAC domain proteins, such as NAC1 and CUC2. A transient expression assay showed that both VNI1 and ANAC103 are transcriptional activators. *ANAC103* promoter activity was detected in vascular tissues, as well as in the trichomes, guard cells, and margins of young leaves. These data suggest that VNI1 and ANAC103 promote the differentiation of various types of cells by modulating the transcriptional activities of a wide range of NAC domain transcription factors.

Key words: *Arabidopsis thaliana*, NAC domain protein, protein complex, transcription factor, xylem vessel differentiation.

The NAM, ATAF1/2 and CUC2 (NAC) domain protein family is a plant-specific transcription family. Genomic sequence analysis revealed that approximately 100, 140, and 160 genes encoding NAC domain proteins exist in Arabidopsis, *Oryza sativa* (rice), and *Populus trichocarpa* (poplar), respectively (Fang et al. 2008; Hu et al. 2010; Ooka et al. 2005), suggesting that the functions of NAC proteins have diversified during the evolution of each species. In support of this notion, NAC domain proteins were found to be involved in many key biological processes, such as apical meristem maintenance (Aida et al. 1997; Souer et al. 1996), root formation (Willemsen et al. 2008; Xie et al. 2000), biotic and abiotic responses (Nakashima et al. 2007; Puranik et al. 2012), and iron homeostasis (Ogo et al. 2008). Research conducted in the past decade has shown that the members of a certain class of NAC transcription factors, including VASCULAR-RELATED NAC-DOMAIN PROTEIN (VND), act as master regulators of xylem cell differentiation (reviewed in Demura and Ye 2010; Ohashi-Ito and Fukuda 2010; Yamaguchi and Demura 2010; Zhang et al. 2011).

VND genes were isolated from an in vitro transdifferentiation system in which Arabidopsis suspension-cultured cells were induced to develop into xylem vessel elements (Kubo et al. 2005). Overexpression of *VND7* induces transdifferentiation of various types of cells into xylem vessel elements, while functional suppression of VND7 inhibits xylem vessel formation (Kubo et al. 2005; Yamaguchi et al. 2008; 2010a). Transcriptome analysis demonstrated that VND7 directly regulates a broad range of genes involved in xylem vessel differentiation (Yamaguchi et al. 2011; Zhong et al. 2010). VND-INTERACTING1 (VNI1) and VNI2, which encode NAC domain proteins, were shown to interact with VND7 by yeast two-hybrid analysis (Yamaguchi et al. 2010b). Detailed functional analyses revealed that VNI2 is a transcriptional repressor that

Abbreviations: AD, activation domain; ANAC, *Arabidopsis thaliana* NAC; BD, binding domain; CaMV35S, Cauliflower mosaic virus 35S promoter; CDS, coding sequence; GUS, β-glucuronidase; MCS, multi cloning site; NAC, NO APICAL MERISTEM, ATAF1/2 and CUC SHAPED COTYLEDONE; PCR, poly chain reaction; VND, VASCULAR-RELATED NAC DOMAIN PROTEIN; VNI, VND-INTERACTING PROTEIN; XCP1, XYLEM CYCTEINE PEPTIDASE1.

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negatively regulates xylem vessel element differentiation by inhibiting the transcriptional activation activities of members of the VND family during the early stages of cell differentiation (Yamaguchi et al. 2010b). Furthermore, VNI2 is involved in senescence and environmental stress responses (Yang et al. 2011) and interacts with a geminiviral replication initiator protein (Suyal et al. 2014). However, the molecular function of VNI1 is unknown.

Here, we performed a basic molecular characterization of VNI1 and its closest homolog, ANAC103. Like VNI1, ANAC103 formed a dimer complex with VND7. In contrast to VNI2, VNI1 and ANAC103 were found to be transcriptional activators. An in vitro pull-down assay showed that VNI1 and ANAC103 bound not only to VND7, but also to other NAC domain proteins. These data suggest that VNI1 and ANAC103 modulate the transcriptional activities of various types of NAC domain proteins during biological processes.

Materials and methods

Vector construction

To generate the Gateway destination vector for the transient expression assay, p35S-GAL4-BD, containing the GAL4 DNA-binding domain under the control of the *cauliflower mosaic virus 35S promoter* (*CaMV35S*) (Ohta et al. 2000), was digested with SmaI and ligated into an EcoRV-digested Gateway Reading Frame Cassette B (Life Technology, http://www.lifetechnologies.com). The resultant vector was named pA35BDG. The coding sequences (CDSs) of NAC domain transcription factors and the promoter region of ANAC103 were amplified by polymerase chain reaction (PCR) with gene-specific primer sets (Table S1). The amplified fragments were subcloned into the pENTR/D-TOPO vector (Life Technology) to generate the entry clones. The entry clones were integrated into Gateway destination vectors for yeast two-hybrid analysis (pBD-GAL4-GWRFC and pAD-GAL4-GWRFC; Yamaguchi et al. 2008), transient expression assay (pA35BDG), in vitro pull-down assay (pDEST14 and pDEST17; Life Technology), and promoter analysis (pBGGUS; Kubo et al. 2005). The GATEWAY destination vectors containing the multi-cloning site (MCS) fragment (Yamaguchi et al. 2008) were used as controls.

Yeast two-hybrid assay

Two vector constructs harboring CDSs fused to GAL4-BD and the GAL4 activation domain (GAL4-AD) were transformed into budding yeast (*Saccharomyces cerevisiae*) strain AH109 (Clontech, http://www.clontech.com) using the S.c. EasyComp Transformation Kit (Life Technology). The transformants were streaked on minimal medium lacking tryptophan and leucine or tryptophan, leucine and histidine, and incubated at 30°C. pBD-wt and pAD-wt express fragment C of bacteriophage lambda cI repressor protein fused to GAL4-BD and GAL4-AD, respectively (Agilent Technologies, http://www.home.agilent.com). cI protein naturally forms homodimers. Therefore, transformed cells harboring pBD-wt and pAD-wt were used as the positive controls.

Dual luciferase transient expression assay

The effector, reporter, and reference plasmids were delivered to Arabidopsis rosette leaves just before bolting by using the Idera GIE-III particle bombardment system (Tanaka, http://www.kktanaka.co.jp/iderapage.htm). The condition of bombardment was 80 kPa of vacuum pressure, 320 kPa of helium pressure, 6.5 cm of target distance, 2 μm diameter of tungsten particles, and 0.4 μg of each plasmid per shot. After overnight incubation on the wet filter paper under dark condition, the leaves were powdered using a multi-beads shocker (BioMedical Science Co., Ltd., Tokyo, Japan). Luciferase activities were assayed with the Dual-Luciferase Reporter Assay System (Promega, http://www.promega.com) using a Mithras LB940 Multimode Microplate Reader (Berthold, http://berthold.com).

In vitro pull-down assay

The CDSs of *VNI1* and *ANAC103* integrated into the pDEST17 vector were transformed into *E. coli* strain BL21-AI. His-tagged recombinant proteins were purified with Ni-agarose resin (Qiagen, http://www.qiagen.com). The CDSs of *VND* and other NAC domain transcription factors integrated into the pDEST14 vector were expressed using the TNT-Coupled Reticulocyte Lysate System (Promega) and [^{35}S]Met. His-tagged VNI1 and ANAC103 proteins immobilized with Ni-agarose resin were incubated with the [^{35}S]Met-labeled recombinant proteins. The proteins retained on the resin were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Expression analysis

The *ANAC103* promoter region (from −1824 to +9 nt relative to the start codon) integrated into the pBGGUS vector was transformed into *Agrobacterium tumefaciens* strain GV3101/pMP90, which was used to generate transgenic Arabidopsis Col-0. Twelve-day-old T1 generation plants were used for analysis. GUS staining was performed as described in Kubo et al. (2005).

Results and discussion

ANAC103 interacts with VND7 in yeast cells

A previous phylogenetic analysis of Arabidopsis NAC domain proteins revealed that ANAC103 is the closest homolog of VNI1 (Yamaguchi et al. 2010b). The amino acid sequences of the full-length protein and NAC domain of ANAC103 are 44% and 78% identical, respectively, to those of VNI1. To investigate whether ANAC103, like VNI1, interacts with VND7, we performed a yeast two-hybrid assay. The NAC domain consists of five well-conserved subdomains, termed subdomain I to V. When the N-terminal region of VND7 (amino acid residues 1 to 147; VND7$^{1-147}$),

containing NAC subdomains I to IV, was used as bait for yeast two-hybrid screening, various forms of truncated VNI1 lacking the N-terminal region were isolated (Yamaguchi et al. 2010b). The shortest clone isolated was VNI1$^{138–489}$, which included only NAC subdomain V and the unconserved transcriptional regulatory domain. Thus, we used a C-terminal region of ANAC103, ANAC103$^{138–373}$, which corresponds to VNI1$^{138–489}$, as prey. When VND7$^{1–146}$ fused to the GAL4 binding domain (GAL4-BD-VND7$^{1–147}$) was co-transformed with ANAC013$^{138–373}$ fused to the GAL4 activation domain (GAL4-AD-ANAC103$^{138–373}$), the yeast cells grew on selective medium lacking histidine, as did GAL4-AD-VNI1$^{138–489}$ (Figure 1). By contrast, transformants co-expressing GAL4-BD-VND7$^{1–146}$ and any other NAC domain protein (NAC1$^{153–324}$, CUC2$^{149–375}$, VND3$^{144–292}$, or VND7$^{142–324}$) fused to GAL4-AD failed to grow on the selective medium (Figure 1). These results indicate that VNI1 and ANAC103 bind to VND7 in an unusual manner; they do not need their entire own NAC domain region to interact with VND7, as shown for VNI1 in Yamaguchi et al. (2010b). It has been reported that NAC domain proteins form homodimers or heterodimers with other NAC domain proteins through the entire NAC domain regions (Earnst et al. 2004; Hegedus et al. 2003; Xie et al. 2000). Likewise, VND7 forms homodimers and heterodimers with other VND proteins via whole regions of the NAC domain (Yamaguchi et al. 2008). Thus, VNI1 and ANAC103 could influence VND-VND dimerization by interacting with their NAC domains and affect their transcriptional activities.

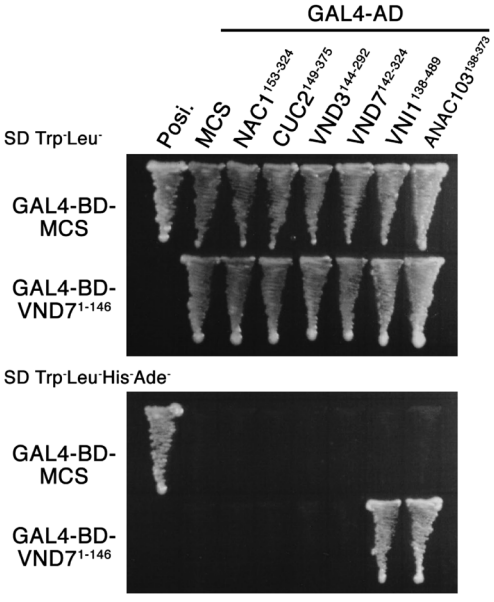

Figure 1. VNI1 and ANAC103 interact with VND7 in yeast cells. Vectors harboring the empty multi-cloning site (MCS) or truncated NAC domain proteins fused to GAL4-BD or GAL4-AD were introduced into yeast strain AH109 and the yeast was grown on media lacking tryptophan and leucine (top) or tryptophan, leucine, histidine and adenine (bottom) at 30°C for 4 days. Posi indicates the positive controls for the interaction.

VNI1 and ANAC103 interact with multiple NAC domain transcription factors

To investigate the interactions between VNI1 and ANAC103 and VND and other NAC domain proteins, we performed in vitro pull-down assays. Polyhistidine-tagged full-length VNI1 and ANAC103 proteins (His-VNI1 and His-ANAC103) were immobilized on Ni-conjugated agarose resin with [^{35}S]Met-labeled NAC domain proteins synthesized in rabbit reticulocyte lysate (Figure 2). Consistent with the results of our yeast two-hybrid assay, VNI1 efficiently bound to VND7. Surprisingly, in addition to VND7, VNI1 also bound to other NAC domain proteins, including VND1, VND2, VND3, NAC1, and CUC2. By contrast, binding of VNI1 to itself or to ANAC103 was weak (Figure 2). His-ANAC103 bound to the same NAC proteins as did His-VNI1, but with lower affinity. These data suggest that VNI1 and ANAC103 interact with a wide range of NAC domain proteins.

VNI1 and ANAC103 act as transcriptional activators

VNI2, which interacts with VND7, was shown to function as a transcriptional repressor (Yamaguchi et al. 2010b). To investigate whether VNI1 and ANAC103 function as transcriptional activators or repressors, we carried out dual luciferase transient transfection assays. We prepared a reporter construct containing the firefly

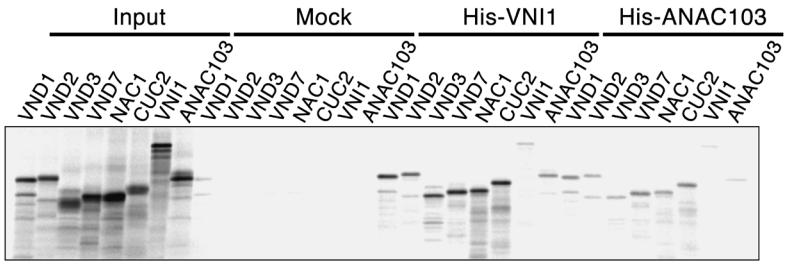

Figure 2. VNI1 and ANAC103 interact with NAC domain proteins in vitro. [^{35}S]methionine-labeled recombinant proteins (shown as "input") were incubated with Ni-agarose without conjugation (mock), or with Ni-agarose conjugated with polyhistidine-tagged VNI1 (His-VNI1) or ANAC103 (His-ANAC103). Proteins bound to the beads were separated by SDS-PAGE and subjected to autoradiography.

(*Photinus pyralis*) luciferase gene linked to the GAL4-upstream activator sequence (GAL4-UAS) and effector constructs containing chimeric genes of *VND7*, *VNI2*, *VNI1*, or *ANAC103* coding sequences (CDSs) under the control of the *cauliflower mosaic virus 35S* (*CaMV35S*) promoter and fused to GAL4-BD (Figure 3A). These constructs were introduced into Arabidopsis leaves by particle bombardment. As previously reported, expression of GAL4-BD-VND7 but not GAL4-BD-VNI2 up-regulated luciferase activity (Figure 3B, Yamaguchi et al. 2008, 2010b). When GAL4-BD-VNI1 or GAL4-BD-ANAC103 was used in the assay, the luciferase activity was significantly increased compared with the control (Figure 3B). Thus, VNI1 and ANAC103 have transcriptional activation activities.

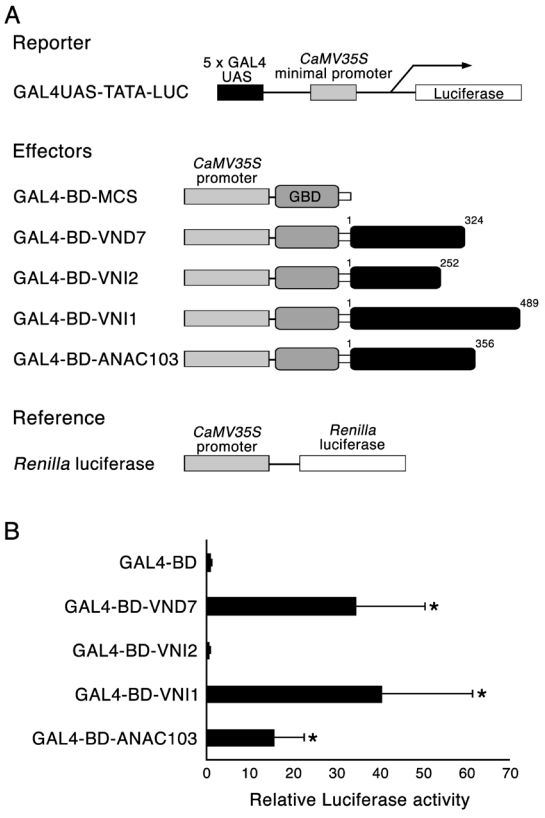

Figure 3. VNI1 and ANAC103 have transcriptional activation activities. (A) Schematic diagrams of the reporter, effector, and reference constructs used in dual luciferase transient transfection assays. The reporter construct contained the firefly luciferase reporter gene under the control of five repeats of the upstream activation sequence (UAS) of GAL4 fused to a minimal *CaMV35S* promoter. The effector constructs contained GAL4-BD bound to an empty MCS (GAL4-BD-MCS) or to CDSs corresponding to full-length *VND7*, *VNI2*, *VNI1* and *ANAC103* driven by the *CaMV35S* promoter. The reference construct harbored *Renilla* luciferase driven by the *CaMV35S* promoter. (B) Results of our dual luciferase transient transfection assay. The effector, reporter, and reference constructs were co-introduced into Arabidopsis leaves by particle bombardment. Firefly luciferase activities were normalized by *Renilla* luciferase activities. Results are presented as means±SD ($n=3$). Asterisks indicate statistically significant differences (Student's t-test, $p<0.05$) compared to the vector control, GAL4-BD-MCS.

VNI2 inhibits the transcriptional activation activity of VND7 by forming a complex with this protein (Yamaguchi et al. 2010b). As described above, VNI1 and ANAC103 interact with various NAC domain proteins (Figure 2). It is possible that VNI1 and ANAC103 promote the activities of these proteins.

Spatial expression pattern of ANAC103

We previously reported that *VNI1* is expressed in the root apical regions (Yamaguchi et al. 2010b). To monitor the spatial expression pattern of *ANAC103*, we generated transgenic plants expressing the β-glucuronidase (GUS) reporter gene under the control of the *ANAC103* promoter (*ANAC103pro:GUS*). GUS signals were observed in the vascular tissue of leaves and roots (Figure 4A and 4B). *VND7* and/or its target genes, *XYLEM CYCTEINE PEPTIDASE1* (*XCP1*), are expressed at differentiating all types of xylem vessels (Yamaguchi et al. 2008, 2011). These data indicate that expression pattern of *ANAC103* is at least partially overlapped with that of *VND7*. In addition, strong *ANAC103pro:GUS* expression was detected in the trichomes, leaf margins, and guard cells of leaves (Figure 4C–E). These patterns suggest that ANAC103 is involved in several types of cell differentiation processes. Recently, it was reported that *ANAC103* expression is induced by endoplasmic reticulum stress in Arabidopsis (Sun et al. 2013). Therefore, ANAC103 may regulate cell differentiation and/or the cellular stress response through binding with other NAC domain proteins.

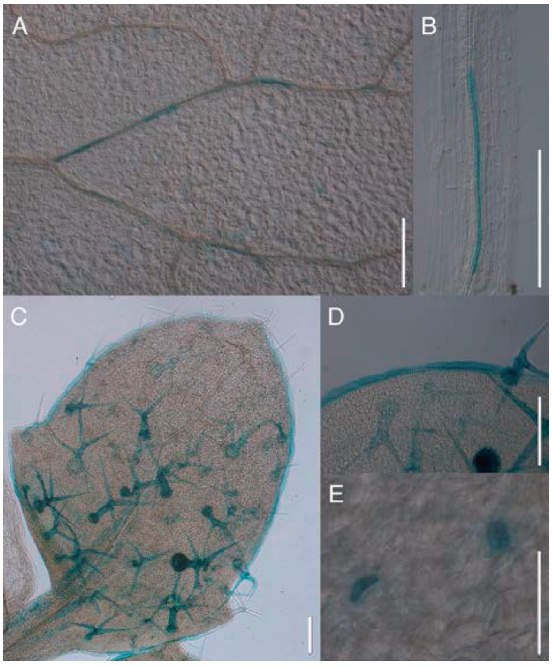

Figure 4. Expression pattern of *ANAC103pro:GUS* in 12-day-old seedlings. GUS signal in the vascular tissue of the leaf (A) and root (B), and in the trichome (C), margin (D), and guard (E) cells of the leaf. Bar=200 μm (A–D) and 50 μm (E).

Taken together, our results provide insight into the molecular functions of VNI1 and ANAC103. Both of these proteins function as transcriptional activators and as modulators of a wide range of NAC domain transcription factors. The expression patterns of *VNI1* and *ANAC103* suggest that VNI1 is involved in the regulation of root meristem function, and that ANAC103 participates in cell differentiation. Further studies should aim to identify the proteins that interact with VNI1 and ANAC103 *in planta*, as this would provide further insight into the transcriptional regulation system in plant cells.

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Table S1. Oligonucleotides used in this study

Name	**Sequence**	**Purpose**
VNDIP1-for1	CACCATGGGGAAAACTCAACTCGCTCCTGGATTT	Amplification of *VNI1*
VNDIP1-rev5	TTACATTTTTGGTCTATGTCTCATGGAAGC	Amplification of *VNI1*
VNDIP1-for2	CACCATGCTGACGCAGATGAATGTTCCTCAG	Amplification of *VNI1*(138-489)
VNDIP1L-for1	CACCATGGGGAAAACTAACTTGGCACCTGGTTTT	Amplification of *ANAC103*
VNDIP1L-rev2	TTAATCGTCCTTAGTCTGACCGTTGCTTCT	Amplification of *ANAC103*
VNDIP1L-for2	CACCATGCTGGCTCAAAAGAATGTTCCTCAG	Amplification of *ANAC103*(138-373)
AtNAC89-for1	CACCATGGATAATATAATGCAATCGTCAAT	Amplification of *VND7*
VND7-rev9	TTACTGAACCGGGGCAAGCTCGGA	Amplification of *VND7*(1-146) with stop codon
NAC1-for1	CACCATGGAGACGGAAGAAGAGATGAAGGAAAGT	Amplification of *NAC1*
NAC1-rev3	TCAGCAATTCCAAACAGTGCTTGGAATACCGAT	Amplification of *NAC1*
NAC1-for2	CACCATGAATCATTCTCTGAGCTCTCCAAAG	Amplification of *NAC1*(153-324)
CUC2-for1	CACCATGGACATTCCGTATTACCACTACGACCAT	Amplification of *CUC2*
CUC2-rev3	TCAGTAGTTCCAAATACAGTCAAGTCCAGCATG	Amplification of *CUC2*
CUC2-for2	CACCATGTCTTACCATTTCATCTCAAGAAGC	Amplification of *CUC2*(149-375)
AtNAC95-for1	CACCATGATGAAGGTTGATCAAGATTATTC	Amplification of *VND3*
VND3-rev1	TTAGTCTTCTCCACTCATCAAAAA	Amplification of *VND3*
VND3-for2	CACCATGCACCAGAACTCTCCTCCACAGGAA	Amplification of *VND3*(144-292)
VND7-rev1	TTACGAGTCAGGGAAGCATCCAAG	Amplification of *VND7*(142-324)
VND7-for3	CACCATGCTTGCCCCGGTTCAGGAGGAAGG	Amplification of *VND7*(142-324)
VNDIP2-for1	CACCATGGATAATGTCAAACTTGTTAAGAATGGT	Amplification of *VNI2*
VNDIP2-rev2	TCATCTGAAACTATTGCAACTACTGGTCTC	Amplification of *VNI2*
AtNAC91-for1	CACCATGGAGCCAATGGAATCTTGTAGCGT	Amplification of *VND1*
VND1- rev1	TCAATTATCAAATACGCAAATCCC	Amplification of *VND1*
AtNAC93-for1	CACCATGGAATCGGTGGATCAATCATGTAG	Amplification of *VND2*
VND2- rev1	TCAAACATGTAAATCCCTATATAA	Amplification of *VND2*
VNDIP1L-Pro-for1	CACCACTCTTCACACATATACTCAATTTTCATAA	Amplification of *ANAC103* promoter
VNDIP1L-Pro-rev1	TTTCCCCATTGGAAGACAAGGGGAAAACTT	Amplification of *ANAC103* promoter