NAC transcription factor family members are differentially expressed in rice seedlings in response to *Rice Tungro Spherical Virus*

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Abstract

The NAC genes encode plant-specific transcriptional factors known to play diverse roles in various plant developmental processes. Rice cultivar Taichung Native 1 (TN1) is susceptible to *rice tungro spherical virus* (RTSV). Backcross line TW16 was developed by TN1 and RTSV-resistant cultivar UtriMerah. Propagation of RTSV is supported in TN1 but not in TW16, although both TN1 and TW16 remain asymptomatic. Here, we compared the gene expression profiles of TN1 and TW16 infected by RTSV to identify the OsNAC gene expression patterns associated with the accumulation and suppression of RTSV. Among 112 OsNAC genes examined by a microarray, approximately 31% and 55% of them were found differentially expressed by RTSV in TN1 and TW16, respectively. Four NAC genes showed continuously expression in TW16 at all days post-inoculation (dpi). Six NAC genes showed higher expression in the TW16 than the TN1 at different dpi with RTSV. Most of the genes in subgroups ONAC2 (50%), ONAC3 (56%), and ONAC7 (80%) were more highly activated in TW16 than in TN1 after RTSV infection. These results suggested that OsNAC genes might be related to the responses induced by the RTSV infection. Whereas, a number of genes in the NAC subgroups NAC1 and were ONAC1 down-regulated for RTSV infection. These OsNAC genes might be associated to the health stage maintenance of the host plants, which may help to clarify the function of these key genes in network pathways.

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Introduction

The NAC gene family was named after three transcription factors: NAM (no apical meristem, Petunia), ATAF1–2 (Arabidopsis thaliana activating factor), and CUC2 (cup-shaped cotyledon, Arabidopsis), which share the same DNA-binding domain (Aida et al., 1997). Rice tungro disease (RTD) is a serious constraint in the production of rice in South and Southeast Asia. Rice plants affected by RTD show symptoms such as stunting and yellow to orange discoloration of leaves (Hibino, 1983). RTD is caused by two viruses, rice tungro spherical virus (RTSV) and rice tungro bacilliform virus (RTBV). These two viruses are transmitted mainly by green leafhoppers (GLH, Nephotettixvirescens). RTBV can be transmitted by GLH only in the presence of RTSV (Hibino 1983). RTBV is primarily responsible for causing the disease symptoms, whereas RTSV plays as a helper virus for insect transmission of RTBV, and also enhances the disease symptoms affected by RTBV (Hibino 1983). RTSV has a single-stranded polyadenylated plus-sense RNA genome of around 12 kb encapsidated in polyhedral particles (Hull 1996). RTSV alone generally does not cause evident symptoms in rice, except mild stunting (Hibino 1983). Utri Merah is an Indonesian rice cultivar resistant to RTSV and RTBV (Encabo et al., 2009). Lee et al. (2010) reported that RTSV resistance in Utri Merah is controlled by a single recessive locus (tsv1) mapped around 22.1 Mb of chromosome 7. The tsv1 locus contains a gene encoding translation initiation factor 4 gamma (eIF4G), one of the key components of translation initiation in rice (Lee et al., 2010). It is suggested that the mutations in the eIF4G gene in UtriMerah are associated with resistance to RTSV.

In this study, we investigated the expression of NAC family genes in the seedlings of two near-isogenic rice plants susceptible (TN1) and resistant (TW16) to RTSV using a customized whole genome oligoarray system to profile the transcriptomes of OsNAC genes during RTSV infection. The result indicated that several NAC genes or subgroups of this gene family are associated with a host defense system which is activated in RTSV-susceptible TN1 by the propagation of the virus, but not in resistant TW16. We selected some NAC genes and confirmed our array data by RT-PCR analysis. Taken together, these results offer a solid basis for future functional genomic research of OsNAC genes.

Materials and methods

Plant materials and virus accumulation

TW16 is a backcross line (BC5F7–8) developed from donor cultivar UtriMerah (International Rice Germplasm Collection accession number 16682) and recurrent parent Taichung Native 1 (TN1) (Lee et al., 2010). TN1 is susceptible to RTSV, whereas TW16 is resistant to RTSV (Encabo et al., 2009). The RTSV accumulation in plants was estimated by ELISA as reported by Shibata et al. (2007). All the tests were performed triple times (biological replications).

Preparation of RNA

Preparation of RNA was described previously Nuruzzaman et al. (2010).

Microarray analysis

Cyanine-3 (Cy3) and cyanine-5 (Cy5)-labeled target complementary RNA (cRNA) samples were prepared from 850 ng total RNA using the low-input RNA labeling kit (Agilent Technologies, USA) in accordance with the manufacturer’s instructions. Transcriptome profiles specific to infected plants were examined by the direct comparison of transcription activities between RTSV-infected and mock-inoculated plants on the customized oligoarray. Hybridization solution was prepared containing 825 ng of each of the Cy3- and Cy5-labeled cRNA preparations using an in situ Hybridization Kit Plus (Agilent Technologies, USA). The fragmented cRNAs were added to the hybridization buffer, applied to the microarray, and hybridized for 17 h at 60°C. The microarray experiments were performed in triplicate with independent samples. The Cy3 and Cy5 signal intensities were normalized using rank-consistency filtering and the LOWESS method, processed by Feature Extraction version 9.5 (Agilent Technologies, USA). Expression patterns of all samples were transformed into log2-based numbers and normalized using EXPANDER version 4.1 (Shamir et al., 2005) according to the quantile method for standardization of array slides.
Differentially expressed gene (DEG) was defined as a gene whose log₂ (mean expression intensity in infected plant/in control plant) was ≥0.585 or ≤-0.585, and the difference in the gene expression change between infected and control plants was significant by a paired t-test (p = 0.05, permutations, all possible combinations; FDR collection, adjusted Bonferroni method). Data processing was performed with MeV version 4.4 (Saeed et al., 2006). Gene expression data used in this study (GSE16142) is available at NCBI Gene Expression Omnibus GEO (www.ncbi.nlm.nih.gov/geo/info/linking.htm).

**RT-PCR analysis**

DEGs were analyzed using RT-PCR and methods of these PCR were described by Nuruzzaman et al. (2010).

**Results**

**RTD disease symptoms caused by RTSV and RTBV infections**

In this study, we focused on RTSV virus infection mentioned in methods. RTD is caused by two viruses RTSV and RTBV.

Rice plants affected by RTSV and RTBV at different dpi showed disease symptoms such as stunting and yellow orange discoloration of leaves (Figure 1).

**Expression of NAC genes in susceptible and resistant plants during RTSV infection**

To gain insight into the comprehensive roles of the OsNAC gene family members in response to RTSV, their expression patterns were investigated in infected rice seedlings by microarray analysis. Only the genes whose expression change was at least 1.5 folds (increased or decreased) were considered to have responded to RTSV (Figure 2).

Out of 151 OsNAC genes identified in the rice genome (Nuruzzaman et al., 2010), 112 were examined by our customized microarray, and 52 genes were found to be differentially expressed. About 46% (52) of the 112 differentially expressed OsNAC genes were detected commonly in both TN1 and TW16 (Figure 2).
Fig. 2. Differential expression of OsNAC genes under RTSV infection (log₂ ratio); color bar at top shows level of expression. Red indicates expressed genes and green indicates unexpressed ones.
The OsNAC genes had been previously classified into 16 subgroups (Nuruzzaman et al., 2010). With the exception of Os04g52810 and Os11g31380, DEGs belonging to subgroups NAC1 (60%) and ONAC1 (50%) were down-regulated in both TN1 and TW16 after RTSV infection. Many DEGs in subgroups ONAC2 (50%), ONAC3 (56%), and ONAC7 (80%) were more highly regulated in TW16 than in TN1 after RTSV infection (Figure 2). Five genes Os05g34310, Os07g37920, Os11g03300/SNAC10, Os11g08210/OsNAC5, and Os12g03040 belonging to the SNAC subgroup were up-regulated after RTSV infection in TW16, whereas the SNAC genes appeared to be predominantly suppressed in TN1 (Figure 2).

Selection of the most promising putative RTSV infection responsive candidate genes and subgroups
To identify putative candidate genes that are responsible for virus infection responses in the seedlings, this study focused on genes that exhibited high levels of expression in infected plants compared to control plants. Four genes Os01g15640 (TIP), Os08g23880 (ONAC2), Os09g12380 (ONAC3), and Os01g64310 (ONAC7) were found to be continuously up-regulated in TW16 by RTSV during the observation (Figure 2). In the rice seedlings, six genes Os01g64310 (TIP), Os04g52810 (NAC1), Os11g08210/OsNAC5 (SNAC), Os08g23880 (ONAC2), Os09g12380 (ONAC3), and Os01g64310 (ONAC7) exhibited higher expression levels (≥ 2 folds) in TW16 after RTSV infection (Figure 2). In addition, in the seedlings during RTSV infection, we noted that most of the genes (80%) assigned to the ONAC7 subgroup were highly expressed when compared with control (Figure 2).

Expression analysis by RT-PCR
In figure 4, gene primers are shown and rice actin gene (LOC_Os11g06390) was used as an internal
control, whose expression remained nearly constant compare to microarray data under all experimental conditions.

**Discussion**

Viruses are a major agricultural constraint, thus, understanding the responses of crops such as rice, to virus infection is important for agricultural production. To establish infection in plants, viruses need host factors for their replication and for cell-to-cell and long-distance movement.

Our goals in this study were to (i) know the expression patterns of members of the OsNAC gene family in plants susceptible and resistant to RTSV, and (iii) select the best putative candidate genes for further functional analysis, which in turn may aid to elucidate gene functions and gene networks. Some OsNAC gene family members showed a strong response to RTSV infection in both resistant and susceptible plants, indicating their association with responses to RTSV infection.

![Expression of the OsNAC gene family in response to RTSV](image)

**Expression of the OsNAC gene family in response to RTSV**

Virus infection affects plant growth development and morphogenesis processes, and the disturbance of gene expression by virus infection may lead to the development of disease symptoms such as dwarfism and yellow orange on leaves (Hibino 1983). In this gene family, 52 (46%) non-redundant genes were up-regulated during RTSV infection (Figure 2). We observed that the number of genes up-regulated was highest at 6 dpi in TW16 during RTSV infection, followed by 15 and 9 dpi (Figure 3).

The number of OsNAC genes with up-regulated expression was higher in TW16 than TN1 (Figures 2,3), which points that defense systems were activated in RTSV infection. One of the host defense systems against virus infection is the gene silencing system. The expression of genes involved in the gene silencing system is often activated by viral infection (Diaz-Pendo & Ding 2008). Changes in gene expression in large numbers of OsNAC genes coincide with symptoms induced by RTSV infection of rice tested in this study, leading us to hint that members of this family contribute heavily to the plant response to virus infection.
Among the OsNAC genes responding during RTSV infection, for example Os01g15640, Os08g23880, Os11g08210/OsNAC5, and Os11g03300/SNAC10 were continuously activated preferentially in RTSV-resistant TW16. Moreover, six genes such as Os10g42130 (TIP) and Os04g52810 (NAC1) exhibited higher expression levels (≥ 2 folds) in TW16 after RTSV infection (Figure 2). Therefore it is possible that these genes are involved in the regulation of rice seedling growth and development.

Role of different subgroups of OsNAC gene family
OsNAC genes play crucial roles in various developmental processes, including signaling, stress responses and plant defenses. About 35% of SNAC genes were up-regulated in resistant TW16 during RTSV infection (Figure 2). From our microarray analysis, we observed that OsNAC genes (Os11g08210/OsNAC5 and Os11g03300/SNAC10) that were up-regulated during virus infection include those previously reported to be induced by abiotic stress such as cold temperature, drought, submergence, and different hormonal treatments (Nuruzzaman et al., 2010, 2013). Around 33% genes of the NAM/CUC3 subgroup were induced during RTSV infection. Many genes of subgroups ONAC3 (55%), ONAC5 (67%), and ONAC7 (80%) were up-regulated during RTSV infection (Figure 2). In our study, six genes Os10g15640 (TIP), Os04g52810 (NAC1), Os03g01870 (NAC2), Os11g08210/OsNAC5 (SNAC), Os08g23880 (ONAC2), Os09g12380 (ONAC3), and Os01g64310 (ONAC7) appeared to highly activated in TW16 than in TN1 after RTSV infection (Figure 2). Together, these results reported here suggest that up-regulation of the subgroups ONAC3, ONAC5, and ONAC7 genes or specific candidate genes may be involved in regulating the seedling development and in the response to RTSV infection. While, many genes in the NAC subgroup NAC1 were down-regulated for RTSV virus infection. These OsNAC genes might be related to the health stage maintenance of the host plants. Therefore through monitoring the changes of NAC genes’ transcriptional data, it might be possible to discriminate the functional roles of host NAC genes after virus infection.

There is high homology with known genes and tight clustering of members in each subgroup reported by Nuruzzaman et al. (2010). It is assumed that members of this subgroup may be involved in morphogenesis related to virus infection, plant growth and development processes. Although phylogenetic analysis provides important support for candidate gene selection, it alone cannot precisely indicate gene function but this study can help to understand the function and relationships of NAC transcription factors.

Responses of OsNAC genes during different treatments
We found that RTSV-responsive OsNAC genes were among those we previously reported to be activated by at least one of the treatments with naphthalene acetic acid NAA, GA₃, SA, ABA or JA, and abiotic treatments (cold temperature, drought, and submergence) in rice seedlings (Nuruzzaman et al., 2010). In this study, Os11g31380 (ONAC1), Os10g09820 (ONAC5), Os05g37080 (ONAC7), and Os11g08210/OsNAC5 (SNAC) genes were induced after RTSV infection (Figure 2) and these genes were reported to be also activated by NAA, KT, ABA, and SA treatments (Nuruzzaman et al., 2010). Therefore, these key genes (Os11g31380 and Os10g09820) might be involved in the defense system during RTSV infection and abiotic stress conditions. Before, we revealed that Os03g21030 gene was induced in the in leaf under severe and mild drought conditions and ABA treatments (Nuruzzaman et al., 2010), and we observed the same genes (Os03g21030 and Os11g08210/OsNAC5) responding to infection by RTSV in this study. By these results, we suppose that there are OsNAC genes which function in ABA signaling pathways that are involved in the defensive response against virus infection.

The defense mechanism of OsNAC genes in plants
Gene expression patterns have been published for genes encoding proteins containing protein kinase, leucine-rich, NB-ARC, and EF-hand domains, which might function in signal transduction for defense systems (Tameling & Baulcombe 2007; Li et al., 2009).
Os11g31380, Os10g09820, and Os05g37080 genes belonging to ONAC1, ONAC5, and ONAC7 subgroups, respectively, were reported to be involved in tissue specificities, and the responses to ABA, GA3, SA, auxin, and light (Nuruzzaman et al., 2010). Some reports suggested that CaNAC1, BnNACs, and OsNAC6 members of subgroup SNAC, share common functions in the plant induction response to virus/pathogen infection and abiotic stresses (Nuruzzaman et al., 2010, 2013).

Conclusions
In conclusion, the application of a comprehensive 44K oligoarray platform with different near-isogenic rice plants enabled us to determine gene expression profiles during infection of rice seedlings under RTSV infection. Interestingly, many genes in the NAC subgroups NAC1 and ONAC1 were down-regulated at all days tested during RTSV infection, while the genes in subgroups ONAC2, ONAC3, and ONAC7 were more highly expressed in TW16 compare to TN1 during RTSV infection. Some subgroups showed a high level of expression in virus infection, suggesting that they might have undergone functional divergence. Present work to function a number of selected genes through overexpression and mutant analyses is underway in our laboratory towards the optimization of molecular breeding schemes for the OsNAC gene family in rice.

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