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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* genesor subgroups of this gene familyare 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 in 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 $\log_2$–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 $\log_2$ (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 (□ = 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).

Fig. 1. Tungro disease phenotype in infected rice plants by RTSV and RTBV.

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).

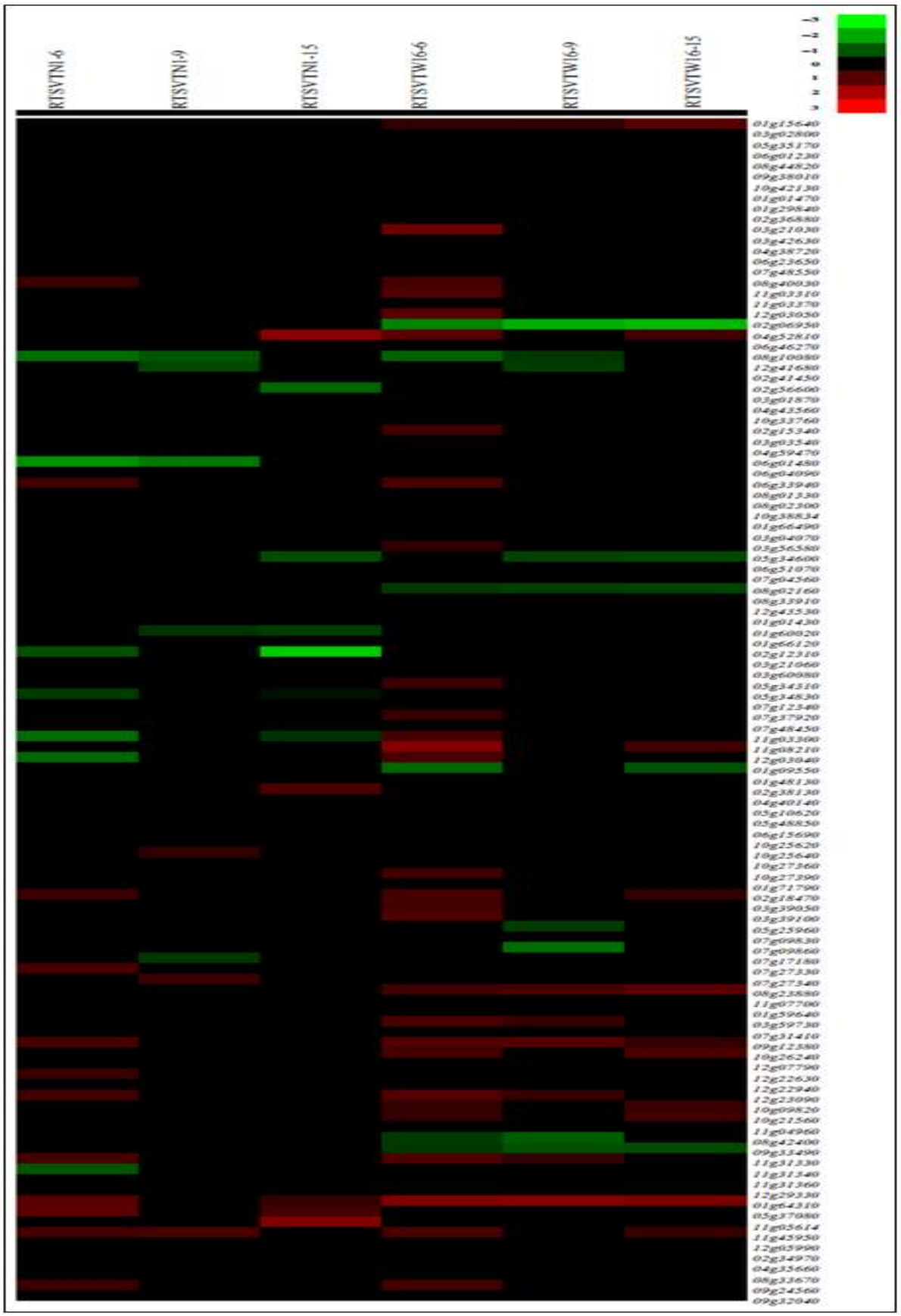

Fig. 2. Differential expression of *OsNAC* genes under RTSV infection (log_2 ratio); color bar at top shows level of expression**.** Red indicates expressed genes and green indicates unexpressed ones.

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).

The greatest number of up-regulated genes (29) was found in resistant rice TW16 at 6 dpi. The lowest number of up-regulated genes (3) was observed in susceptible TN1at 9 dpi (Figure 3).

The greatest number of down-regulated genes (9) was found in TW16 at 9 dpi, whereas the lowest number (5) was found in TN1 and TW16 at 9 and 15 dpi, respectively (Figure 3). Overall, the *OsNAC* genes appeared to be more highly activated in TW16 than inTN1 after RTSV infection (Figure 3).

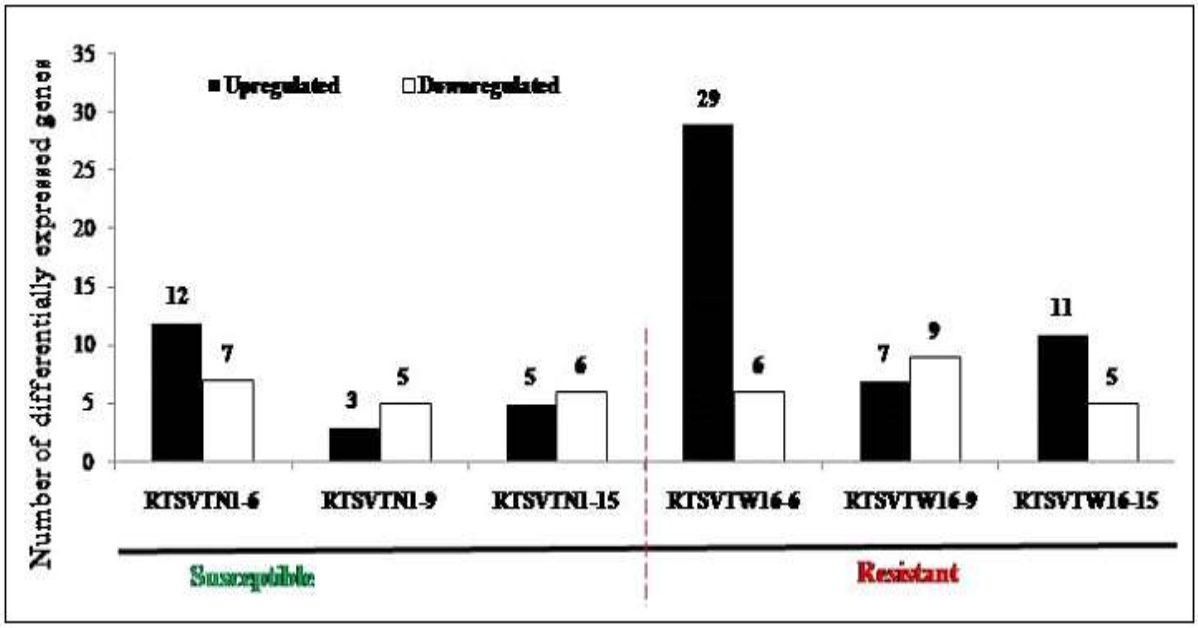

Fig. 3. Number of differentially expressed genes (DEGs) in susceptible and resistant near-isogenic rice plants (TN1 and TW16). Infected with RTSV at different dpi. *Y*-axis represents the number of DEGs and treatments RTSV are indicated on the *X*-axis.

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 *Os10g42130* (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.

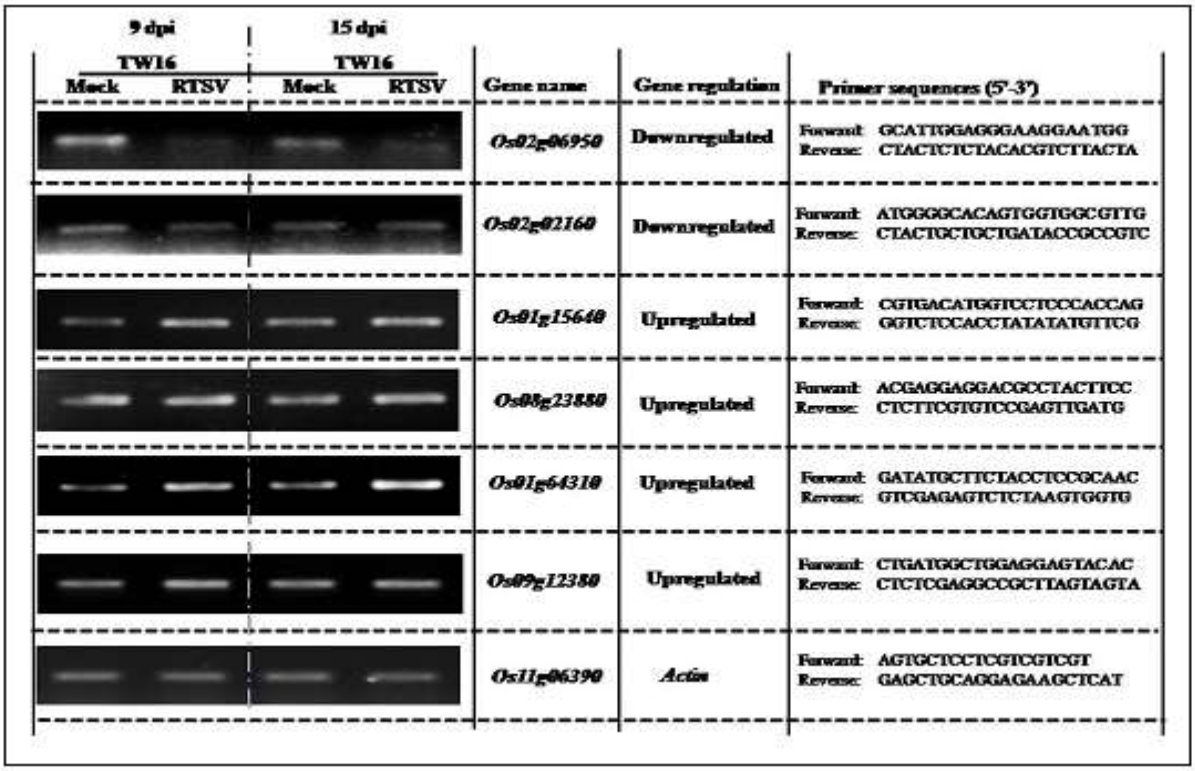

Fig. 4. Evaluation for the expression levels of selected DEGs by RT-PCR under *rice tungro spherical virus* infection.

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* (NAC22), *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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