OsNAC6, a member of the NAC gene family, is induced by various stresses in rice

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Members of the NAC gene family encode plant-specific transcription factors and are widely distributed in plant species. The OsNAC6 gene is one of many NAC genes in rice and has high similarity to genes in the ATAF subfamily. Here we show that OsNAC6 is induced by cold, salt, drought and abscisic acid (ABA). We found that OsNAC6 is also induced by wounding. The response of OsNAC6 to wounding is very rapid and strong. OsNAC6 was also induced by jasmonic acid (JA), a plant hormone that activates defense responses against herbivores and pathogens. Our results imply that OsNAC6, besides having a role in plant adaptation to abiotic stresses, also integrates signals derived from both abiotic and biotic stresses.

Key words: jasmonic acid (JA), NAC gene family, rice, transcription factor, wounding

Although higher plants have evolved mechanisms to survive under a variety of abiotic and biotic stresses, crop production is often damaged by various stresses. In order to improve the stress tolerance of crops, the mechanisms by which they adapt to stresses need to be elucidated. Genes in the NAC family are plant-specific and are known to possess diverse roles as transcription factors in plant development and in the recognition of environmental stimuli (Olsen et al., 2005). These genes contain a highly conserved domain in the N-terminal region. This region was defined as the NAC protein domain by Aida et al. (1997). Some NAC family genes, such as the NAM of petunia (Souer et al., 1996), and CUC1 and CUC2 (Aida et al., 1997) of Arabidopsis, are also involved in shoot development. Another NAC gene, NAP of Arabidopsis has been isolated as an immediate target of the organ-identity genes AP3 and PI (Sabolowski et al., 1998). NAC1 of Arabidopsis is involved in auxin signaling in lateral root formation (Xie et al., 2000). Genes in the ATAF subfamily of the NAC family, which include ATAF1 and ATAF2 of Arabidopsis and StNAC of potato, are induced by wounding (Collinge et al., 2001).

The ATAF subfamily includes stress-responsive genes. OsNAC6 of rice is a member of the ATAF subfamily. Rice has 75 NAC genes (Ooka et al., 2003). Cold, salt and drought stresses induce an accumulation of ABA that plays a crucial role in adaptation to abiotic stress (Finkelstein et al., 2002; Leung and Giraudat, 1998; Zeevaart and Creelman, 1988). Many ABA-inducible genes contain a conserved, ABA-responsive, cis-acting element named ABA-responsive element (ABRE, PyACGTGGC) in their promotor regions (Guiltnan et al., 1990). ABREs are recognized by transcription factors containing a basic leucine zipper structure (Choi et al., 2000; Finkelstein and Lynch, 2000; Hobo et al., 1999; Uno et al., 2000).

In a previous microarray analysis, we found that OsNAC6 was highly induced by cold treatment. The fact that OsNAC6 has six ABREs within 500 nucleotides upstream of the transcription start site suggests that OsNAC6 is involved in stress-responsive ABA-dependent signaling. In this study, we show that OsNAC6 is also induced by various treatments and hormones. Transcription factors, such as OsNAC6, that are induced by various stresses are rare in rice. Here, we suggest that OsNAC6 participates in the recognition of various abiotic and biotic stimuli.
Six-day-old rice (Oryza sativa cv. Nipponbare) seedlings were grown hydroponically at 28°C. The control plants were kept in the light at 28°C. Plants that were treated with hormones and salt stress were grown hydroponically in a solution containing 250 mM NaCl and 100 mM ABA (Sigma Chemical, USA) in the light. Cold treatment consisted of transferring plants grown at 28°C to a 4°C lighted refrigerator. Plants were subjected to dehydration by placing them in empty petri dishes under the light. Plants were subjected to wounding by squeezing them with needle-point flower holders. About fifty rice seedlings were sprayed with 2.5 ml of a solution of 1mg/ml jasmonic acid (JA) (Sigma Chemical, USA) in 1mM sodium phosphate buffer. For a control, leaves were sprayed with an equal volume of 1mM sodium phosphate buffer. After rice seedlings were subjected to a stress treatment, they were frozen immediately in liquid nitrogen and were stored at –80°C.

Rice total RNA was extracted with a RNeasy Plant Mini kit (QIAGEN). Electrophoresis of RNA and Northern hybridization were performed as described by Suisho et al. (1997). Each blot was hybridized only with the specific probe and equal loading of RNA was confirmed by an rRNA blot that was included in the same experimental set. Each probe for OsNAC6 (GenBank Acc. no. AB028185) was made by PCR using primers (3’ UTR probe forward, 5’-GGCAGCGACCCCCCTCTCC-3’; 3’ UTR probe reverse, 5’-GGGCTAGCTTTCTGCC-3’; intron probe forward, 5’-ATTGGAGAGCTTGGCTCATC-3’; intron probe reverse, 5’-GCTAAACCATCCACAA-3’). A probe for OsNAC4 (GenBank Acc. no. AB028183) was made by PCR using primers (3’ UTR probe forward, 5’-GGTACTTTG-CAGTCCATC-3’; 3’ UTR probe reverse, 5’-TTGGCAGAT-TACCAAATGC-3’). The experiments were repeated two more times and the same results were obtained.

Transcripts levels of OsNAC6, and rice Bowman Birk Inhibitor 3-1 gene (rbbi) (GenBank Acc. no. AB098712) were measured by quantitative real-time RT-PCR using specific primers for AB028185 (forward, 5’-CATGGCCGGTGAACTTGGAC-3’; reverse, 5’-TCCTGCATCTGTCAGGTCAG-3’), and for AB098712 (forward, 5’-TTGCTCGATCATTCA-GAG-3’; reverse, 5’-GGGACAGGGGACAAATTA-3’). The time course accumulation of rbbi was monitored as positive controls for the effectiveness of treatments of jasmonic acid. Quantitative real-time RT-PCR (qRT-PCR) was performed using a Light Cycler (Roche Diagnostics, Germany) and the QuantiTect SYBR Green RT-PCR kit (Qiagen, Valencia, CA, USA) according to the manufacturers’ protocols. For each sample, qRT-PCR was observed using specific primers to amplify 17S RNA (forward, 5’-TCTCTACCGATGATTAG-GTCC-3’; reverse, 5’-CTTGTACGACTTCTCCCTCC-3’) to normalize the amounts of RNA purification.

The microarray analysis revealed that cold treatment resulted in at least a three-fold increase of mRNAs for seven transcription factors: CCAAT-binding transcription factor subunit A (GenBank Acc. no. AK108142), H-protein promoter binding factor (GenBank Acc. no. AK066984), lip19 for leucine zipper protein (GenBank Acc. no. AK065180), MADS box protein (GenBank Acc. no. AU162282), OsNAC4, OsNAC6, and WRKY12 (GenBank Acc. no. AK121190). OsNAC6 and OsNAC4 are evolutionarily close among NAC genes of rice (Fig. 1A). To clarify whether OsNAC6 and OsNAC4 are actually induced by cold treatment in our experimental system, we performed a Northern blot analysis on total RNA isolated from six-day-old rice seedlings with or without cold (4°C) treatment, using a probe for the 3’ UTR region of each gene (Fig. 1B). The Northern blot analysis showed that OsNAC6 was strongly induced by cold, and failed to detect transcripts of OsNAC4 (Fig. 1B).

Many cold-responsive genes are also induced by salt, drought and ABA (Narusaka et al., 2003). OsNAC6 is also thought to be induced by these treatments. To confirm this, we extracted RNA from rice seedlings exposed to salt, drought, or ABA. A Northern blot analysis indicated that OsNAC6 was induced by these treatments (Fig. 1C). ABA is known to play an important role in the regulation of stress-responsive genes. Under cold, salt, or drought stress conditions, plants accumulate increased amounts of ABA (Xiong et al. 2002).

As shown by a Northern hybridization (Fig. 2), OsNAC6 was induced by wounding stress. This was confirmed by qRT-PCR (data not shown). We observed a rapid and transient induction of OsNAC6 mRNA that peaked at 0.5 h after wounding. This result agrees with the findings that the expressions of StNAC of potato, and ATAF1 and ATAF2 of Arabidopsis, all of which have high homologies with OsNAC6, were upregulated by wounding (Collinge et al., 2001).

The Northern analysis revealed two distinct bands of about 2.4 kb and about 1.5 kb. The position of the lower band was consistent with the length of the mature mRNA of OsNAC6. The upper band did not appear when the plants were exposed to other stresses such as cold, salt, drought and ABA. We assumed that the upper band is the result of incomplete splicing of the second intron. We could not determine whether the upper band contains the first intron because the first intron is too short to distinguish the size difference on the Northern image. If the upper band contains the first intron, this incomplete splicing generates a new termination codon inside the first intron, and if the upper band does not contain the first intron, such incomplete splicing generates a new termination codon inside the second intron. These results suggest that the longer transcripts are not translated into functional proteins.
OsNAC6 induced by various stresses

Fig. 1. Time course of OsNAC6 transcripts accumulation upon abiotic stresses. (A) A dendrogram of NAC genes based on the amino-acid sequences of their NAC domains. The tree was made by the neighbor-joining method. The dendrogram shows that NAC genes fall into several subfamilies, i.e., the ATAF, NAM, and OsNAC3 subfamilies (Kikuchi et al., 2000). (B) Northern hybridization analysis of the effect of cold treatment on OsNAC6 and OsNAC4 transcripts. Six-day-old rice seedlings grown in constant light at 28°C were transferred to 4°C in the light. The control plants were kept in the light at 28°C and collected after 48 hours. Rice seedlings were collected at the indicated times. 0 means just before the treatment. 5 µg of total RNA was loaded onto each lane. The probe was specific for the 3'-UTR. Equal loadings of total RNA were checked by ethidium bromide (EtBr) staining. (C) Six-day-old rice seedlings grown in constant light at 28°C were treated by each stress. Rice seedlings were collected at the indicated times. 0 means just before the treatment. For salt treatment, rice was grown with 250 mM NaCl. For drought treatment, rice was grown without water. For ABA treatment, rice was grown with 100 µM ABA. For the control sample, rice seedlings without treatment were collected during the same time course. 5 µg of total RNA was loaded onto each lane. Hybridization was performed using the probe for 3'-UTR of OsNAC6. Equal loadings of total RNA were checked by ethidium bromide (EtBr) staining.

Fig. 2. Time course of OsNAC6 transcripts accumulation following wounding. Time course accumulation of OsNAC6 transcripts was analysed by Northern hybridization. The probes were specific for the 3'-UTR and second intron of OsNAC6. Rice seedlings were collected at the indicated times. 0 means just before the treatment. Equal loadings of total RNA extracted from these samples were checked by ethidium bromide (EtBr) staining. M, RNA marker.
Many wound-responsive genes are induced through jasmonic acid-dependent signal transduction cascades. JA was found to enhance OsNAC6 transcript levels in rice seedlings rapidly within a few hours and transiently (Fig. 3A). A gene that is known to be induced by JA, rice Bowman-Birk inhibitor gene (rbbi) (Qu et al., 2003), was used as a positive control for JA treatment (Fig. 3B).

![Fig. 3. Time course of transcripts level of OsNAC6 following JA treatment.](image)

**A**

![Relative mRNA level](image)

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![Fig. 3. Time course of transcripts level of OsNAC6 following JA treatment.](image)

(B) Time course of transcripts level of rbbi following JA treatment.

It is important to clarify the difference between the regulatory systems for signaling pathways for resistance to herbivores or diseases and the regulatory systems for signaling pathways for resistance to other stresses such as cold, salt, drought and ABA. ABA is generally thought to serve as a second messenger in response to cold, salt and drought stresses. ABA is reported to have a positive role with jasmonate-induced defenses against herbivores (Anderson et al., 2004).

In Arabidopsis, Fujita et al. (2004) suggested that the dehydration-responsive expression of RD26 which encodes an NAC transcription factor was regulated mainly by ABA. On the contrary, NaCl signaling for RD26 expression was found to occur through an ABA-independent pathway (Fujita et al., 2004). They also showed that RD26 was induced by 50 µM methyl jasmonate. In tomato (Lycopersicon esculentum), ABA is necessary for the wound-induced expression of proteinase inhibitor genes (Carrera and Prat, 1998), but it does not appear to be a primary signal in wound signaling (Birkenmeier and Ryan, 1998).

In this study, we showed that OsNAC6 is strongly induced by cold, salt, drought and ABA. These results agree well with the results of Rabbani et al. (2003). We showed here that OsNAC6 is also induced by wounding and JA.

We are unaware of any other transcription factors in rice that are induced by both abiotic and biotic factors. The OsNAC6 responses to wounding and JA may be activated through signal transduction cascades that do not involve ABA. It is possible that the expression of OsNAC6 is controlled synergistically by ABA and JA. A preliminary microarray analysis revealed that another member of ATAF subfamily of rice (OsNAC5) is also induced by salt treatment. Our results suggest that genes belonging to ATAF subfamily are generally related to stress response.

**REFERENCES**


Fujita, M., Fujita, Y., Maruyama, K., Seki, M., Hiratsu, K., Ohme-Takagi, M., Tran, L. S. P., Yamaguchi-Shinozaki, K.


