Effects of High Nitrogen Supply on the Susceptibility to Coolness at the Young Microspore Stage in Rice (Oryza sativa L.): Gene Expression Analysis in Mature Anthers

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Abstract: Changes in gene expression patterns by high nitrogen (High-N) and High-N plus cooling at the young microspore stage (High-N-cooling) in rice mature anthers were analyzed by semiquantitative RT-PCR with gene specific primers. Gene expression of alpha-expansin 18 (EXPA18) was repressed under High-N-cooling. Beta-expansin 1 (EXPB1), putative aldehyde dehydrogenase (ALDH) and Fructokinase II (FKII) were upregulated under High-N. EXPB1 and FKII were highly expressed under High-N-cooling. Comprehensive examination of gene expression patterns of 26 alpha-expansins (EXPs) and 16 beta-expansins (EXPBs) showed that all expansins (EXPs) except EXPA12 were expressed in the anthers. Gene expression of EXPs did not change under High-N except EXPA1, EXPB1 and EXPB5 which were upregulated. In total, 18 EXPs and 6 EXPBs were repressed under High-N-cooling, and among these, EXPA18, EXPA19 and EXPA20 had high similarities in the amino acid sequences, suggesting that these three genes may constitute a distinct functional gene subfamily related to the decrease in the pollen germination ability.

Key words: Anther, Cool temperature susceptibility, Expansin, Gene expression, High nitrogen, PCR, Pollen germination.

In northern Japan, cool temperatures in summer often cause a serious reduction in the yield of rice mainly because of unsuccessful fertilization due to damaged pollen grains. A sufficient nitrogen supply is necessary for optimal plant growth and yield in rice (Shiga, 1984; Goto et al., 2006; Fukushima, 2007). In cool summers, however, more sterile spikelets are observed in rice plants grown with a high nitrogen (High-N) supply (Sasaki and Wada, 1975; Amano and Moriwaki, 1984; Satake et al., 1987). This enhanced reduction in yield by cool temperatures under High-N supply is a major problem in rice production in northern Japan, but the physiological mechanisms that underlie this enhanced sterility have not been elucidated.

Exposure to a cool temperature at the young microspore stage, the most sensitive stage to cooling during the reproductive period (Hayase et al., 1969), lowers the number of microspores and pollen grains (Satake, 1991), and a High-N supply enhances these decreases (Tatsuta, 1999; Hayashi et al., 2000). The number of pollen grains per anther is highly correlated with fertility (Nishiyama, 1982) and the decrease in the number of engorged pollen grains caused by cooling is the major factor for the floral impotency. This decrease is enhanced by High-N supply (Tatsuta, 1999; Hayashi et al., 2000). At the flowering time, the number of pollen grains on stigma is an important factor for fertility, and about 40 grains are necessary for fertilization (Satake and Shibata, 1992), suggesting that if more than 40 engorged pollen grains survived after cooling would shed on stigma, the spikelets could be fertile. In cooled rice plants, however, the fertility is low even when almost the same numbers of pollen grains are shed on stigma (Satake, 1989). Furthermore, plants exposed to cool temperatures that were grown under High-N show lower fertility than the plants under normal nitrogen even though they have almost the same number of engorged pollen grains on stigma (Hayashi et al., 2000). The pollen germination ratio is also decreased by cooling at the young microspore stage and this decrease is enhanced under High-N conditions (Hayashi et al., 2006). These results suggest that the increase in sterile spikelets due to the cool temperature and High-N is caused by the enhanced decreases in the number of engorged pollen grains and the germination ratio.

In the previous report, the proteome analysis of the rice mature anthers was used to examine the changes in proteins under High-N and/or High-N plus cooling at the young microspore stage (High-N-cooling), and changes in 11 proteins involved in cell elongation,
stress responses and sugar metabolism were observed (Hayashi et al., 2006). It is noteworthy that 3 out of these 11 proteins are identified as expansin (EXP). EXPs are involved in cell elongation functioning in cell-wall loosening (Cosgrove, 2000). The hypothetical action of expansins is transient release of a short segment of matrix glycans attached to cellulose microfibrils, and in consequence, cellulose and matrix polymers slide relative to one another (Cosgrove, 1998). In maize, pollen allergen protein Zea-m1, consists of at least 4 beta-expansins (EXPBs), has the function of loosening silk cell wall and is assumed to play a role in pollen tube elongation (Cosgrove et al., 1997; Li et al., 2003; Wei et al., 2004) and EXP-like protein is present on the wall of the pollen tube tip (Suen et al., 2003).

In this study, besides the candidate genes found in proteome analysis, we analyzed the gene expression patterns of almost all rice alpha-expansins (EXPs) and EXPBs in the mature anthers by semiquantitative RT-PCR with gene specific primers, and the changes under High-N and High-N-cooling. In total, 18 EXPs and 6 EXPBs were downregulated under High-N-cooling. EXPAI and EXPB1 were upregulated under High-N and High-N-cooling. The molecular and phylogenetic characteristics of these EXPs and their involvements in enhanced sterility of rice plants are discussed.

### Materials and Methods

#### 1. Plant Materials

An early-mature rice variety (*Oryza sativa* L. *japonica* cv. Hayayuki) was used. Twenty seeds were sown in a circular pattern on vermiculite in plastic sieves in 18 cm-diameter and 20 cm-height pots (Satake et al., 1969; Satake and Koike, 1983). Plants were grown in an artificially lit chamber (Plant Growth Chamber, Conviron, Canada) under an illumination of 300 µmol photons m⁻² s⁻¹ just above the rice plants under a 12 hr day length and a day/night temperature regime of 24/19°C until the young microspore stage. Plants were grown with tap water for ten days, and then with a culture solution including a standard level of nitrogen (10 ppm of N) (Satake and Koike, 1983). In order to equalize the growth of the main stems, tillers were removed as they appeared. Genes, whose expressions

| Gene Name | Primer Sequence 5’ to 3’ | Product Size bp | Accession No. | RT-PCR temp. ºC | cycle |
|-----------|---------------------------|-----------------|---------------|-----------------|-------|
| CDPK11    | CACCTGCTGCGGCAGCTTTTAC   | 215             | X81393        | 52              | 32    |
|           | GTAAAGCTGCGGCAGGTGC       |                 |               |                 |       |
| HSP82     | GTTTATAGGAGCCATGGCT       | 334             | Z11920        | 55              | 30    |
|           | TCTCACCTGCTTCTCTGAGCCCT   |                 |               |                 |       |
| putative ALDH | GGAGCGAAATGGTTACTTGTG   | 352             | AF148877      | 52              | 30    |
|           | ATCCGACATCTGTACTCGTCCCC   |                 |               |                 |       |
| FK II     | CGCCAGACGAAGAAAGAACGCAG   | 360             | AF249947      | 55              | 30    |
|           | CTTTTGAGTGCAAGATGGCTC     |                 |               |                 |       |
| EXP5      | CATCTGCCTGGTAGTTGCAGT     | 425             | AF247162      | 55              | 35    |
|           | GTTCATAAGGCAGCACACAGGG    |                 |               |                 |       |
| EXP6      | CTTAGGAGGCGAGCATTCTAG     | 378             | AF247163      | 55              | 35    |
|           | GGAGTGGTAGAGAAAGAAGAGGTT  |                 |               |                 |       |
| EXP7      | TGAGGAAGAGGAGGGGTT        | 464             | AF247164      | 55              | 35    |
|           | TGAGGAAGAGGAGGGGTT        |                 |               |                 |       |
| EXP10     | GCCAAACATACACTCGAGCAAGC   | 256             | AF247165      | 55              | 35    |
|           | CATCAAGGCTCTGAGTGCC       |                 |               |                 |       |
| EXPB15    | TTCTCCATCCGCCTACGCTC      | 268             | AF391108      | 55              | 35    |
|           | CCTCGACATCGGCTGTTATA      |                 |               |                 |       |
| EXPB17    | TACACCTCCGGCCTCAACTCC     | 331             | AF391110      | 55              | 35    |
|           | AACGCGTCTTCTCTTTCGCG      |                 |               |                 |       |
| EXPB18    | CGACGGTGGAACTAATAGTGCAGC  | 214             | Os05g0246300  | 55              | 35    |
|           | TGTTAAATCTAGCTGGCCGCTCC   |                 |               |                 |       |

Primer sets for EXPs not shown in this table were adopted from Lee and Kende, 2001 and Lee and Kende, 2002. RT-PCR conditions; 35 cycles of 55°C for EXPs but 30 cycles of 55°C for EXPB1. 27 cycles of 55°C for Act1.
are changed by High-N and by High-N-cooling, are assumed to be involved in enhanced sterility of rice plant. On the basis of this, three treatments were set as follows: 1) standard nitrogen (Standard-N; 10 ppm), 2) High-N (80 ppm) and 3) High-N-cooling, following the method of Hayashi et al. (2006). From the spikelet differentiation stage, plants were grown under Standard-N or High-N. To obtain uniform anthers, the third to the fifth spikelets from the top on the first and the second primary branches of the main stem were examined by the methods of Satake and Hayase (1970) and Satake et al. (1987). The stages of the anthers were checked using a microscope (BX50; Olympus, Tokyo, Japan). At the young microspore stage, plants for High-N-cooling treatment were placed in a 12°C chamber for 3 days, then, transferred back to the 24/19°C chamber. Nutrient solution supply was discontinued after the start of flowering. Spikelets were detached from the plants about 2 hr before flowering and the mature anthers from which filaments were carefully removed were immediately frozen in liquid nitrogen and kept at −80°C until analysis (Hayashi et al., 2006).

2. Semiquantitative RT-PCR

Total RNA were extracted from 100 μg fresh weight of frozen samples of the anthers with RNeasy Plant Mini Kit (QIAGEN, GmbH, Germany) according to the manufacturer’s protocol. The first strands of the cDNA mixture were generated from 1.0 μg of total RNA and 10 pmol of Oligo d(T)20 primer. Reverse transcription was done for 30 min at 42°C using ReverTra Ace-α- (Toyobo, Osaka, Japan). The resulting cDNA solution was then diluted 10 fold with TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). The PCR reaction mixture (20 μL) contained 1.0 μL of diluted reverse transcribed first strands of cDNA in 15 mM Tris-HCl (pH 8.0), 50 mM KCl, 0.2 μM each of two primers, 200 μM dNTPs mixture (Applied Biosystems, Foster City, CA, USA) and 0.04 unit of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA).

Gene expression levels of calcium dependent protein kinase 11 (CDPK11), heat shock protein 82 (HSP82), putative aldehyde dehydrogenase (ALDH), fructokinase II (FKII) and EXPs were analyzed since the abundances of these proteins in rice mature anthers are changed by High-N and High-N-cooling (Hayashi et al., 2006). Genes were searched through BLAST program on DDBJ of these proteins in rice mature anthers are changed by High-N and by High-N-cooling, are examined by the methods of Satake and Hayase (1970) and Satake et al. (1987). The stages of the anthers were checked using a microscope (BX50; Olympus, Tokyo, Japan). At the young microspore stage, plants for High-N-cooling treatment were placed in a 12°C chamber for 3 days, then, transferred back to the 24/19°C chamber. Nutrient solution supply was discontinued after the start of flowering. Spikelets were detached from the plants about 2 hr before flowering and the mature anthers from which filaments were carefully removed were immediately frozen in liquid nitrogen and kept at −80°C until analysis (Hayashi et al., 2006).

amplification cycles and temperature conditions. The PCR conditions for all EXPs were fixed to 35 cycles of 55°C but 30 cycles of 55°C for EXPB1.

To confirm the uniformity of cDNA synthesis, we amplified cDNAs for actin by 27 cycles of 55°C. PCR products were separated by electrophoresis in 1.5% agarose gel and visualized with the ethidium bromide. The template cDNA are from the anthers about 2 hr before glumes opened. Standard-N; 10 ppmN, High-N; 80 ppmN, High-N-cooling; High-N plus cooling (12°C for 3 d at the young microspore stage).

3. Phylogenetic analysis

The sequences of EXPs examined by RT-PCR were adopted from the database of Expansin central (http://www.bio.psu.edu/expansins/) and RAP-DB (Rice Annotation Project, 2008; The Rice Annotation Project, 2007). Multiple alignment of deduced amino acid sequences of EXPs from rice and Arabidopsis were done by the ClustalW ver. 1.83 program on DDBJ (Sugawara et al., 2008; Thompson et al., 1994) and the results were displayed using the TreeView program ver. 1.6.6 (Page, 1996).

Results

1. Gene expression analysis

In previous proteome analysis of rice mature anthers, the changes in the following proteins were observed by High-N and High-N-cooling conditions; EXPA18, EXPB1, EXPB13, HSP82, CDPK11, putative ALDH, and FKII (Hayashi et al., 2006).
The RT-PCR images of Fig. 2. Semiquantitative RT-PCR analysis of rice EXPAs. The RT-PCR images of EXPAs 18 and Act1 were identical to those in Fig. 1. Standard-N; 10 ppmN, High-N; 80 ppmN, High-N-cooling; High-N plus cooling (12ºC for 3 days at the young microspore stage).

The expression patterns of these candidate genes found in mature anthers by proteome analysis, were analyzed by semiquantitative RT-PCR with gene specific primers. All semiquantitative RT-PCR experiments were carried out at least three times for each gene, and one series of data is shown in Figs. 1, 2 and 3. Gene expression of EXPA18 was repressed under High-N-cooling. EXPB1, putative ALDH and FKII were upregulated under High-N, and EXPB1 and FKII were intensified under High-N-cooling. On the other hand, gene expression of EXPB13, CDPK11 and HSP92 were not changed under High-N or High-N-cooling (Fig. 1).

The rice genome contains 33 EXPAs and 18 EXPBs (http://www.bio.psu.edu/expansins/). From these EXPs, gene expression patterns of 26 EXPAs and 16 EXPBs in the anthers were observed (Figs. 2, 3). The RT-PCR images of EXPA18, EXPB1, EXPB13 and Act1 were identical to those in Fig. 1.

The genes expressions of all EXPAs except EXPA12 in the anthers were observed (Fig. 2). Gene expressions of most of EXPAs were not changed by High-N, but EXPA1 was upregulated. Gene expressions of EXPA1, EXPA2, EXPA4, EXPA5, EXPA6, EXPA7 and EXPA10 were not changed by High-N-cooling. It was noteworthy that 18 EXPAs (EXPa3, EXPA8, EXPA9, EXPA11, EXPA13, EXPA14, EXPA15, EXPA16, EXPA17, EXPA18, EXPA19, EXPA20, EXPA21, EXPA22, EXPA23, EXPA24, EXPA25, EXPA26) out of 26 expressed EXPAs were repressed under High-N-cooling (Fig. 2).

All EXPAs examined were expressed in the anthers (Fig. 3). Similar to the expression patterns of EXPAs, the gene expression of EXPBs was unchanged under High-N except for the upregulation of EXPB1 and EXPB5. Six EXPBs (EXPB5, EXPB7, EXPB11, EXPB12, EXPB14 and EXPB17) were repressed under High-N-cooling (Fig. 3).

2. Phylogenetic analysis

Not only EXPA18, EXPB1 and EXPB13, but also 25 EXPAs out of 26 EXPAs and 16 EXPBs were expressed in the anthers. Among these, 18 EXPAs and 6 EXPBs were repressed under High-N-cooling. To elucidate the evolutionary relationship among these EXPs, we performed phylogenetic analysis based on the deduced amino acid sequences of rice EXPAs and EXPBs, and the phylogenetic tree were generated, which was divided into 3 groups for EXPAs and 4 groups for EXPBs (Fig. 4).

The group A1 contained 15 EXPAs including EXPA18, and 13 EXPs in this group were downregulated under High-N-cooling. In the group A1, EXPA18, EXPA19 and EXPA20 were clustered in the same clade and these three EXPAs were all downregulated under High-N-cooling. Since the expression of a large number of EXPAs in group A1 were downregulated by High-N-cooling, phylogenetic analysis was executed between rice EXPAs in group A1 and fully assigned 26 Arabidopsis EXPAs. As a consequence, only Arabidopsis EXPA11 (AtEXPAN11) was phylogenetically linked to group A1 among 26 AtEXPAs (data not shown). According to the phylogenetic analysis of the group A1 between rice and Arabidopsis, AtEXPAN11 was more closely related to EXPA1 and EXPA12 than EXPA18 (Fig. 4).
Discussion

The decrease in the number of pollen grains in the anther and in the pollen germination ratio by cooling at the young microspore stages is enhanced by High-N supply (Tatsuta, 1999; Hayashi et al., 2000; Hayashi et al., 2006). To clarify the physiological aspects involved in this enhancing effect of High-N supply, we examined the mature anthers about 2 hr before glumes opening since it was difficult to obtain enough pollen grains.

In previous proteome analysis, 11 proteins in the anthers are altered under High-N and/or High-N-cooling conditions (Hayashi et al., 2006). In this study, the gene expression patterns of 7 of the 11 candidate genes, namely EXPA18, EXPB1, EXPB13, CDPK11, putative ALDH, HSP82 and FKII were analyzed.

EXPA18, EXPB1 and putative ALDH proteins are increased under High-N. EXPA18 is decreased, but FKII is strongly increased by High-N-cooling (Hayashi et al., 2006). In this study, gene expression of EXPB1 and putative ALDH were upregulated under High-N, while EXPA18 was downregulated and EXPB1 and FKII were upregulated under High-N-cooling (Fig. 1). The changes in EXPA18, EXPB1, putative ALDH and FKII proteins in the anthers were assumed to be regulated at the transcriptional level.

EXPB13, CDPK11 and HSP82 proteins are increased under High-N. CDPK11 and HSP82 are decreased but EXPB13 is increased by High-N-cooling (Hayashi et al., 2006). The expression patterns of these genes were not changed by High-N or High-N-cooling (Figs. 2, 3). This suggests the post-transcriptional modulation of these genes, such as the regulation of translation, protein maturation and protein turnover.

At the flowering time, pollen grains swell rapidly for anther dehiscence (Matsui et al., 1999). Pollen grains shed on stigma start to germinate and elongate pollen tubes. EXPs might be involved in these events because cell wall loosening is thought to be essential for pollen grain swelling, germination and pollen tube elongation. At the time of the pollen tube penetration, glucanase and xylanase in pollen coat are thought to break the stigma wall, and EXP-like protein is present on the wall of the pollen tube tip in maize (Suen et al., 2003).

Zea-m1 protein, which is abundant in maize pollen grains and consists of at least 4 EXPBs, is assumed to play a role in pollen tube elongation by loosening silk cell walls (Cosgrove, 2000; Wei et al., 2004). From the abundance of EXPB1 protein in mature anthers (Hayashi et al., 2006) and the close similarity between the amino acid sequences of EXPB1, EXPB13 and Zea-m1, rice EXPB1 and EXPB13 may have functions similar to Zea-m1 and may be related to the decrease in fertility. The protein and gene expression of EXPB1 was, however, upregulated by High-N and was not downregulated by High-N-cooling. Imin et al. (2004) observed partially degraded EXPB1 proteins in the trinucleate stage anthers after cooling at the young microspore stage, and suggested the protein abnormality. These results suggest that, EXPB1 is highly expressed under High-N-cooling, but the partially degraded EXPB1 protein caused by cooling might have some inhibitory effects on loosening of stigma cell walls and subsequent pollen tube elongation.

Different members of the large EXP gene family are expressed in different tissues. EXP1A1, EXP4, EXP5, EXP10, EXP13 and EXP16 are expressed both in rice leaves and roots (Cosgrove, 2000; Lee and Kende, 2001; Shin et al., 2005). EXP5, EXP10, EXP18 and EXP26 are expressed in rice anthers (Kerim et al., 2003; Shin et al., 2005; Dai et al., 2007) and EXP26 is considered as anther specific (Shin et al., 2005). EXP2, EXP3, EXP4, EXP6, EXP11 and EXP12 are expressed in upper ground tissues and EXP2, EXP3, EXP4 and EXP6 are also expressed in roots (Lee and Kende, 2002). These results were obtained under normal conditions and the effects of stress on the gene expression have not been reported. Therefore, we analyzed the expression patterns of members of the EXP gene family in the anthers under High-N and High-N-cooling.

Comprehensive gene expression analysis showed that all EXPs examined (EXP1A to EXP26) except...
EXPA12 were expressed in the anthers and 18 EXPs were repressed by High-N-cooling (Fig. 2). As mentioned above, EXPA5, EXPA10, EXPA18 and EXPA26 are expressed in rice anthers (Shin et al., 2005). Among these 4 EXPs, EXPA18 and EXPA26 were downregulated by High-N-cooling. Gene expression of EXPBs in anthers had not been well studied. In the present study, 16 EXPs were expressed and 6 EXPs were repressed by High-N-cooling (Fig. 3).

Totally, 18 EXPs and 6 EXPBs were repressed by High-N-cooling, but EXPB1 was upregulated by High-N-cooling. The downregulation of large numbers of EXPs by High-N-cooling suggested that these EXPs may be involved in the decreases in pollen germination and fertilization. Under High-N-cooling, HSP82 and CDPK11 were downregulated (Hayashi et al., 2006). Arabidopsis AtHSP81, which belongs to the same HSP90-family as rice HSP82, is expressed in pollen grains (Yabe et al., 1994). CDPK is transcribed in mature and germinating pollen and is required for germination (Taylor, 1997) and is shown to participate in cold stress signaling (Abbasi et al., 2004). These properties of HSP and CDPK, suggest the involvement of HSP82 and CDPK11 in the downregulation of EXPs in rice anther under cooling stress.

To elucidate the evolutionary relationship among these EXPs, a phylogenetic analysis was undergone. The generated phylogenetic tree was divided EXPs into 3 groups and EXPBs into 4 groups. Since the majority of EXPs in group A1 including EXPA18 were repressed by High-N-cooling, phylogenetic analysis was executed between rice EXPs in group A1 and Arabidopsis EXPs, and only AtEXPA11 was phylogenetically linked to group A1. AtEXPA11 is similar to rice EXPA18 (61% identity), however, AtEXPA11 had much higher similarities to EXPA1 (79% identity) whose gene expression was increased by High-N-cooling and EXPA12 (73% identity) whose gene expression was not detected in the anthers, indicating that AtEXPA11 is functionally not orthologous to EXPA18 (Fig. 4).

The rice EXP genes examined in this study are located on all chromosomes except chromosomes 9 and 11. Chromosome 3 harbors the largest number of EXPs, 10 EXPAs including EXPA18, EXPA19, EXPA20 and 7 EXPBs (Expansin Central; http://www.bio.psu.edu/expansins/). EXPA18, EXPA19 and EXPA20 are arranged in tandem on the long arm of chromosome 3 and showed high similarity with each other. In addition to these sequence similarities and gene arrangements, EXPA18 protein and these three genes were repressed by High-N-cooling. Therefore, we assumed that EXPA18, EXPA19 and EXPA20 form a distinct subfamily, which may be similarly regulated by environmental conditions in rice and play a crucial role in responding to the damages in pollen germination under High-N.

In monocotyledous plants including rice, A1-type EXPs may have diversified from a common ancestor of monocotyledons and dicotyledons, to adapt to environmental stresses such as cool temperatures during anther development and pollen germination under different nitrogen conditions.

Further study is needed on the regulated expression mechanisms of respective EXPs, especially EXPA18 subfamily and EXPB1, in pollen grains and germinated pollen grains, and on the participations of these EXPs in the depression of pollen germination under High-N-cooling.

In this study, the gene expression patterns of rice anther genes regarding the effects of High-N on cool temperature damages were analyzed. In total, 25 EXPAs and 16 EXPBs were expressed in the anthers. EXPA1 and EXPB1 were upregulated under High-N and 18 EXPAs including EXPA18 and 6 EXPBs were downregulated under High-N-cooling. Since many EXPs were repressed under High-N-cooling, these EXPs are considered to be involved in the enhanced decreases in pollen germination ratio under High-N-cooling.

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