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To cite this article: Takami Hayashi, Tomoya Yamaguchi, Katsuhiro Nakayama & Setsuo Koike (2009) 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, Plant Production Science, 12:3, 271-277, DOI: [10.1626/tpps.12.271](https://doi.org/10.1626/tpps.12.271)



To link to this article: <https://doi.org/10.1626/tpps.12.271>

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 Published online: 03 Dec 2015.

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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* (*EXPAs*) 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 *EXPAs* 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,

Received 2 October 2008. Accepted 4 December 2008. Corresponding author: T. Hayashi (takami@affrc.go.jp, fax +81-19-643-3467).

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Abbreviations : ALDH, aldehyde dehydrogenase; CDPK, calcium dependent protein kinase; EXP, expansin; EXPA, alpha-expansin; EXPB, beta-expansin; FK, fructokinase; High-N, high nitrogen; High-N-cooling, high nitrogen plus cooling; HSP, heat shock protein; RT-PCR, reverse transcriptase polymerase chain reaction; Standard-N, standard nitrogen.

Table 1. Gene-specific primer sets used for the RT-PCR amplifications.

Gene Name	Primer Sequence 5' to 3'	Product Size	Accession No.		RT-PCR	
		bp	cDNA	Genomic DNA	temp. °C	cycle
<i>CDPK11</i>	CACCTGCTGGCAGCTTTTAC GTAAAAAGCTGCCGAGGTGC	215	X81393		52	32
<i>HSP82</i>	GTTTATGGAGGCACTGGCTG TCTCAGTGGTCTTCTCAGTCC	334		Z11920	55	30
putative <i>ALDH</i>	GGAGCGAAATGGTCTACTTGTGC ATCCCATCTTGTACTCGTCCC	352	AF148877		52	30
<i>FK II</i>	CGCCAACGACGAGAAGAACG CTTGGTGGTGCAGATGGCTC	360	AF429947		55	30
<i>EXPA5</i>	CATCGTCGTGGTAGTTGCAGT GTTTATAAGCAGCACACAGG	425	AF247162		55	35
<i>EXPA6</i>	CTTCGAGGGCAGGCAGTTCTAG GGAGTGAGTAGCAAACAAGC	378	AF247163		55	35
<i>EXPA7</i>	TGCAGGAAGAAGGGAGGGGTT TGCAGGAAGAAGGGAGGGGTT	464	AF247164		55	35
<i>EXPA10</i>	GGCAAAACATACACTGGCAAGC CATCAAGCCTCTGTAGTGC	256	AF247165		55	35
<i>EXPB15</i>	TTCTCCATCCGCCTCACGTC CCTCTGCATCTCGCCGTTATTA	268		AF391108	55	35
<i>EXPB17</i>	TACACCTCGCGCCTCAACTTC AACGCTCTCTTCTCCTTCGG	331		AF391110	55	35
<i>EXPB18</i>	CGACGGTGAATACTAATGATCGC TGGTAAATCATCTGCGCCTCC	214	Os05g0246300		55	35

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*.

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* (*EXPA*s) 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 *EXPA*s and 6 *EXPBs* were downregulated under High-N-

cooling. *EXPA1* 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 on 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 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ 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

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 pmoles 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 (Sugawara et al., 2008) from amino acid sequence. Gene specific primer sets were shown in Table 1.

For *EXPs*, 26 *EXPA*s and 16 *EXPB*s were examined. Primer sequences of *EXPs* which are not shown in Table 1 were adopted from Lee and Kende (2001) and Lee and Kende (2002). The primer sets of *actin (Act1; AB047313)* were from Yamaguchi et al. (2002). The temperature cycling parameters were as follows: 95°C for 10 min; 30–35 cycles of 94°C for 1 min, 52–55°C for 1 min, 72°C for 12 min. Table 1 shows the number of

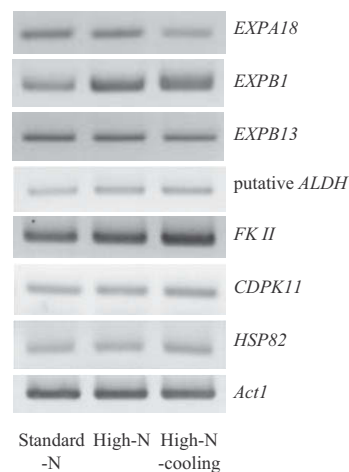


Fig. 1. Semiquantitative RT-PCR analysis of genes selected based on proteome analysis of rice mature anther.

EXPA; *alpha-expansin*, *EXPB*; *beta-expansin*, *ALDH*; *aldehyde dehydrogenase*, *FK*; *fructokinase*, *CDPK*; *calcium dependent protein kinase*, *HSP*; *heat shock protein*.

The first strand of the cDNA mixtures were generated from the total RNA of mature anther. The PCR products were electrophoresed in agarose gel and visualizes 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).

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 gels, stained with ethidium bromide, and visualized with the BioDoc-It System (UVP, Upland, CA, USA).

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).

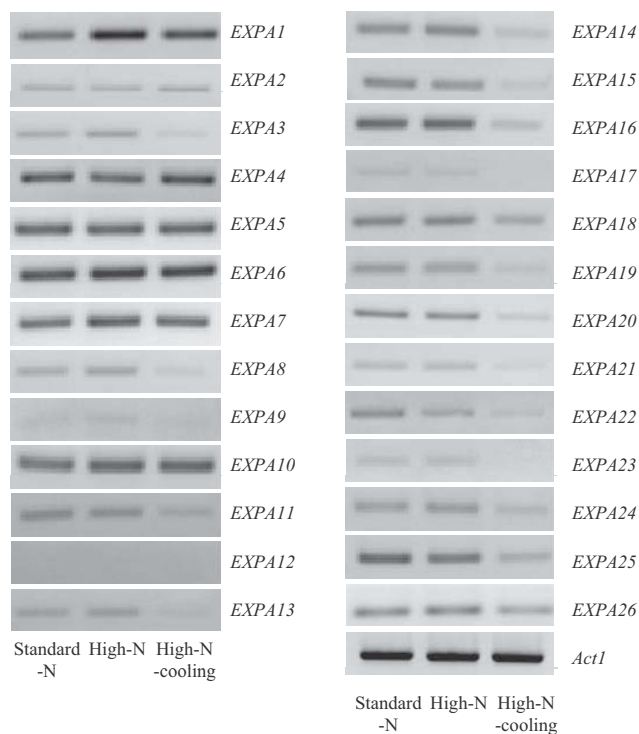


Fig. 2. Semiquantitative RT-PCR analysis of rice *EXPA*.

The RT-PCR images of *EXPA18* and *Act1* were identical to those in Fig. 1. 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).

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 *HSP82* were not changed under High-N or High-N-cooling (Fig. 1).

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

The gene expressions of all *EXPA*s except *EXPA12* in the anthers were observed (Fig. 2). Gene expressions of most of *EXPA*s 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 *EXPA*s (*EXPA3*, *EXPA8*, *EXPA9*, *EXPA11*, *EXPA13*, *EXPA14*, *EXPA15*, *EXPA16*, *EXPA17*, *EXPA18*, *EXPA19*, *EXPA20*, *EXPA21*, *EXPA22*, *EXPA23*, *EXPA24*, *EXPA25*, *EXPA26*) out of 26 expressed *EXPA*s

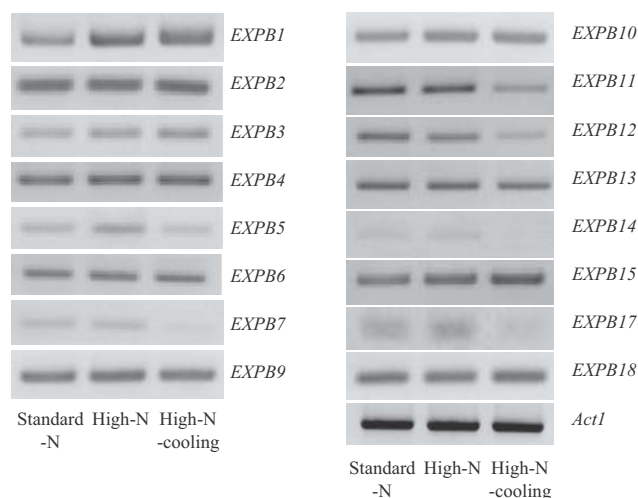


Fig. 3. Semiquantitative RT-PCR analysis of rice *EXPB*.

The RT-PCR images of *EXPB1*, *EXPB13* and *Act1* were identical to those in Fig. 1. Standard-N; 10 ppmN, High-N; 80 ppmN, High-N-cooling; High-N plus cooling (12°C for 3 days at the young microspore stage).

were repressed under High-N-cooling (Fig. 2).

All *EXPB*s examined were expressed in the anthers (Fig. 3). Similar to the expression patterns of *EXPA*s, the gene expression of *EXPB*s was unchanged under High-N except for the upregulation of *EXPB1* and *EXPB5*. Six *EXPB*s (*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 *EXPA*s out of 26 *EXPA*s and 16 *EXPB*s were expressed in the anthers. Among these, 18 *EXPA*s and 6 *EXPB*s 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 *EXPA*s and *EXPB*s, and the phylogenetic tree were generated, which was divided into 3 groups for *EXPA*s and 4 groups for *EXPB*s (Fig. 4).

The group A1 contained 15 *EXPA*s 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 *EXPA*s were all downregulated under High-N-cooling. Since the expression of a large number of *EXPA*s in group A1 were downregulated by High-N-cooling, phylogenetic analysis was executed between rice *EXPA*s in group A1 and fully assigned 26 *Arabidopsis* *EXPA*s. As a consequence, only *Arabidopsis* *EXPA11* (AtEXPA11) was phylogenetically linked to group A1 among 26 AtEXPA11 (data not shown). According to the phylogenetic analysis of the group A1 between rice and *Arabidopsis*, AtEXPA11 was more closely related to *EXPA1* and *EXPA12* than *EXPA18* (Fig. 4).

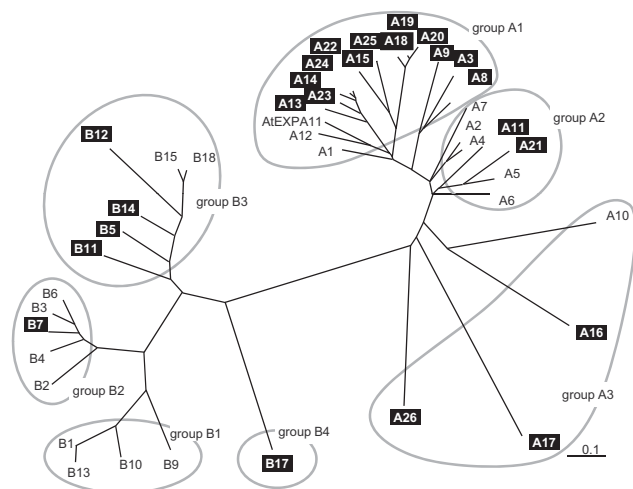


Fig. 4. Phylogenetic tree of rice EXPs.

A; EXPA, B; EXPB. AtEXPA11; *Arabidopsis* EXPA11. White letters in the black background indicated EXPs downregulated under High-N-cooling. EXP family was divided into 3 groups of EXPAs and 4 groups of EXPBs. The sequences of EXPs were adopted from the Expansin Central (<http://www.bio.psu.edu/expansins/>), and RAP-DB (Rice Annotation Project, 2008; The Rice Annotation Project, 2007). A multiple sequence alignment was performed with ClustalW ver. 1.83 program on DDBJ (Sugawara et al., 2008; Thompson et al., 1994) and the results were displayed using the TreeView program (Page, 1996). Scale bar; 0.1 amino acid substitution per site.

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. *EXPA1*, *EXPA4*, *EXPA5*, *EXPA10*, *EXPA13* and *EXPA16* are expressed both in rice leaves and roots (Cosgrove, 2000; Lee and Kende, 2001; Shin et al., 2005). *EXPA5*, *EXPA10*, *EXPA18* and *EXPA26* are expressed in rice anthers (Kerim et al., 2003; Shin et al., 2005; Dai et al., 2007) and *EXPA26* is considered as anther specific (Shin et al., 2005). *EXPB2*, *EXPB3*, *EXPB4*, *EXPB6*, *EXPB11* and *EXPB12* are expressed in upper ground tissues and *EXPB2*, *EXPB3*, *EXPB4* and *EXPB6* 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 *EXPAs* examined (*EXPA1* to *EXPA26*) except

EXPA12 were expressed in the anthers and 18 *EXPAs* 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 *EXPBs* were expressed and 6 *EXPs* were repressed by High-N-cooling (Fig. 3).

Totally, 18 *EXPAs* 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 *EXPAs* into 3 groups and *EXPBs* into 4 groups. Since the majority of *EXPAs* in group A1 including *EXPA18* were repressed by High-N-cooling, phylogenetic analysis was executed between rice *EXPAs* in group A1 and *Arabidopsis EXPAs*, 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

monocotyledonous plants including rice, A1-type *EXPAs* 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.

Acknowledgements

We are grateful to Mr. K. Hayasaka, Mr. E. Kumagai, Mr. Y. Otsubo, Mr. K. Tamura, Mr. E. Yoshida and Mr. A. Tanifuji in our research center for technical assistance. We are also grateful to Dr. L. Lopez, in Iwate University, for his comments on the paper.

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* In Japanese with English abstract.

** In Japanese with English summary.

*** In Japanese.