Susceptibility to Coolness at the Young Microspore Stage under High Nitrogen Supply in Rice (*Oryza Sativa* L.).

Proteome Analysis of Mature Anthers

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Abstract: In vitro pollen germination experiment using agar plates showed that the growth under high nitrogen conditions enhanced the damage to pollen germination ability caused by the cooling at the young microspore stage. To clarify the physiological factors related to this damage to pollen germination, we performed the comparative proteome analysis of mature anthers and identified proteins that were changed by high nitrogen conditions or high nitrogen plus cooling conditions. Proteins were extracted from mature anther samples and separated by two-dimensional polyacrylamide gel electrophoresis. By comparing anther protein maps of the samples collected from the plants grown under standard nitrogen conditions, high nitrogen conditions and high nitrogen plus cooling conditions, we found 11 protein spots, which varied with the treatment. These protein spots were identified based on the rice proteome database and/or peptide mass fingerprinting (PMF) analysis after digestion with trypsin. Digested samples were analyzed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry to produce PMF data. Database searches using these PMF data revealed the identities of 9 proteins. Seven of these proteins were polypeptides involved in cell elongation, stress responses and sugar metabolism. The relation between the fluctuations of these proteins and the decrease in pollen germination are discussed.

Key words: Cool temperature, High nitrogen, Pollen germination, Proteome analysis, Sterility, Two-dimensional polyacrylamide gel electrophoresis.

In northern Japan, cool temperature in summer often causes a serious decrease in the yield of rice plants mainly because of unsuccessful fertilization due to damaged pollen grains. A sufficient nitrogen supply is necessary for optimal plant growth and yield in rice. In cool summers, however, a higher number of sterile spikelets are observed in rice plants that are grown under high nitrogen conditions. This decrease in yield is a major problem in rice production, but the physiological mechanism of the increased sterility is unclear.

The number of pollen grains per anther is highly correlated with fertility (Nishiyama, 1982). Cool temperatures at the young microspore stage, the most sensitive to cool temperatures during the reproductive period (Hayase et al., 1969), lead to decrease in the number of microspores and pollen grains (Satake, 1991), and high nitrogen conditions enhance this decrease (Tatsuta, 1999; Hayashi et al., 2000). At anthesis, the number of pollen grains on the stigma is an important factor of the fertility, and about 40 grains is enough for fertilization (Satake and Shibata, 1992). In cooled plants, the fertility of plants grown under high nitrogen conditions was lower than that of plants grown under standard nitrogen conditions even though they had almost the same number of engorged pollen grains on the stigma (Hayashi et al., 2000). This suggests that high nitrogen supply affected the activities of mature pollens which have a normal appearance. In this study, in vitro pollen germination experiment was carried out using agar plate to exclude the effects of stigma conditions. Then, to investigate the physiological changes caused by cooling under high nitrogen conditions, we performed the proteome analysis on mature anthers and searched for proteins that may participate in lowering of pollen germination ability.

Materials and Methods

1. Plant Materials

Hayayuki, a cold tolerant, early ripening japonica rice variety from Hokkaido, was used. Twenty seeds per pot were sown in a circular pattern (Satake et al., 1969). Plants were grown under 12-hour photoperiod.
at 300 \(\mu\text{mol photons m}^{-2}\text{s}^{-1}\) and day/night temperature regime of 24/19°C. Tillers were removed when they elongated, remaining the main stem. Plants were grown in a nutrient culture solution (Satake and Koike, 1983) that included a standard level of nitrogen (10 ppm). At the spikelet differentiation stage, half of the pots were transferred to high (80 ppm) nitrogen conditions. To obtain uniform samples, we used the third to the fifth spikelets from the top on the first and the second primary branches of the main culms.

When spikelets mentioned above were at the young microspore stage, plants for cooling were transferred to a cool chamber (12/12°C). The duration of cooling treatment was three days for pollen germination experiment. For proteome analysis, cooling treatment was given for five days to enhance the changes in protein. After cooling, pots were transferred back to the 24/19°C chamber. Nutrient solution supply was discontinued after the end of flowering.

2. **In vivo pollen germination**

Stigmas were removed from closed flowers about 4 hours after anthesis to observe the pollen germination and pollen tube elongation. They were fixed and immersed in 50% ethanol. For visualization of callose in pollen tubes, stigmas were stained with 0.05% aniline blue (Carland et al., 1999). We observed 10 or more stigmas from each treatment with a fluorescence microscope (BX60 with filter set BP 330 385, DM 400, BA 420; Olympus, Tokyo, Japan).

3. **In vitro pollen germination**

Pollen grains were germinated on the medium containing 1% agar, 20% sucrose and 20% K\(_2\)B\(_4\)O\(_7\) (Kariya, 1989). Pollen grains in anthers were directly shed on the medium in petri dishes (35 mm \(\phi\)) as soon as the flower began to open, then incubated for 20 min at 20°C. We used 8 or more flowers from each treatment. After incubation, pollen grains were stained with iodine/potassium iodide solution. The numbers of total fully stained pollen grains and germinated pollen grains were counted under a microscope (BX50; Olympus, Tokyo, Japan).

4. **2D-electrophoresis and protein identification**

Spikelets were detached from the plants about two hours before flowering, then mature anthers from which filaments were carefully removed were collected and kept at ~80°C. One hundred to 150 anthers were homogenized in lysis buffer containing 8 M urea, 2% NP-40, 4% Ampholine (pH 3.5-10 and pH 5-8; equal volumes), 5% mercaptoethanol, and 5% PVP-40 and centrifuged twice at 13000g for 5 min. The supernatant was used for the two-dimensional polyacrylamide gel electrophoresis (2-DE). Protein extraction and 2-DE were carried out based on the protocol of Rakwal and Komatsu (2000). The proteins on preparative gels were stained with coomassie brilliant blue (CBB) solution. The protein spots whose density changed by the nitrogen conditions or by high nitrogen plus

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**Fig. 1.** Pollen germination on the stigma. Cooling (12°C for 3 days) at the young microspore stage suppressed pollen germination and pollen tube elongation.

**Fig. 2.** Effects of high nitrogen conditions and cooling on the germination of pollen grains. Plants were cooled (12°C) at the young microspore stage. The number of spikelets used; 21 for standard nitrogen conditions, 21 for standard nitrogen plus cooling, 11 for high nitrogen conditions and 8 for high nitrogen plus cooling conditions. Bar; standard error.
cooling conditions were examined. At least three gels from the different experiment sets were compared. Proteins were identified using the Rice Proteome Database (http://gene64.dna.affrc.go.jp/RPD/) or by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) (Voyager DE-PRO, Applied Biosystems, USA) and PMF analysis (Mascot Search, Matrix Science, http://www.matrixscience.com/home.html).

Results

1. Pollen germination

In the plants grown under high nitrogen conditions without cooling, pollen tubes elongated well. Whereas in cooled plants, a few germinated pollen grains were observed and pollen tubes were shorter (Fig. 1). The reduction of pollen germination ratio by cooling was also observed in the experiment using agar plates. After a 3-day cooling, the pollen germination ratio was 51% in plants grown under standard nitrogen conditions and 25% in the plants grown under high nitrogen conditions, decreasing to a half of that under standard nitrogen conditions (Fig. 2).

2. Proteome analysis

We performed the proteome analysis to investigate the physiological aspects of mature anthers and to identify proteins that may participate in pollen germination. Over 1000 protein spots were detected on 2D-electrophoresis (Fig. 3). We found 11 spots whose densities changed under the high nitrogen and high nitrogen plus cooling treatments. Nine protein spots (spots 732, 761, 783, 442, 839, 912, 722, 634 and 802) were identified by database search but two protein spots (spots 505 and 545) did not have matched protein data. Seven proteins were polypeptides that are involved in cell elongation (spots 732, 761 and 783), stress responses (spots 442, 839 and 912) and sugar metabolism (spot 722) (Table 1).

Three protein spots were identified as expansins that are involved in cell elongation. All of them were upregulated under high nitrogen conditions. Spot 783 identified as β-expansin 13 (EXPB13) was upregulated by cooling. Spot 732 that identified as α-expansin 18 (EXPA18) was downregulated by cooling. Spot 761 identified as EXPB1 was not changed by cooling (Table 1 and Fig. 6).

With respect to stress response, 3 spots were identified. They were also upregulated under high nitrogen conditions and two of them, spot 442 and spot 912, were downregulated by cooling. Spot 442 was identified as calcium-dependent protein kinase isoform 11 (CASKII1) and spot 912 as heat shock protein 82 (HSP82). Spot 839 that was not changed by cooling was identified as putative aldehyde dehydrogenase (ALDH) (Table 1 and Figs. 4, 5, 6).

Spot 722 was identified as putative fructokinase II that is involved in sugar metabolism. It was not
changed under high nitrogen conditions and increased by cooling (Table1 and Fig. 4).

Discussion

The number of pollen grains on the stigma is correlated with the rate of fertility. However, even in the plants that have the same number of pollen grains on the stigma, the fertility is lower in cooled plants (Satake, 1989) and high nitrogen supply enhances this decrease in fertility (Hayashi et al., 2000). These results suggest that damages to pollen grains caused by cool temperature at the young microspore stage are enhanced under high nitrogen conditions.

Pollen grains were germinated on agar plates containing culture medium to exclude the effects of stigma. The germination percentage of pollen from

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**Table 1. Changes in anther proteins caused by high nitrogen and cooling treatments.**

| Spot No. | Standard N | High N | High N Cooled | Accession | Matched Protein | Organism |
|----------|------------|--------|---------------|-----------|-----------------|----------|
| Cell elongation | | | | | | |
| 732* | ± | ++ | + | AF394553 | α-expansin 18 | Oryza sativa |
| 761 | - | ++ | + | Q9LD01 | β-expansin 1 | Oryza sativa |
| 783* | ± | + | ++ | AF391106 | β-expansin 13 | Oryza sativa |
| Stress responses | | | | | | |
| 442 | + | ++ | ± | P53684 | Calcium-dependent protein kinase, isoform 11 | Oryza sativa |
| 839* | + | ++ | ++ | AF148877 | Putative aldehyde dehydrogenase | Oryza sativa |
| 912 | + | ++ | ± | Z15018 | Heat shock protein 82 | Oryza sativa |
| Sugar metabolism | | | | | | |
| 722 | ± | ± | ++ | Q944F5 | Putative fructokinase II | Oryza sativa |
| Others | | | | | | |
| 505 | + | ++ | + | not identified | | |
| 545 | - | - | + | not identified | | |
| 634* | ± | - | + | AC1454 | Protein gp18 from bacteriophage A118 homolog | Listeria innocua |
| 802* | +++ | + | ++ | P31417 | Fatty acid binding protein 2 | Manduca sexta |

1) Names based on the rice proteome database. Asterisks indicate identifications based on the rice proteome database.
2) Standard nitrogen conditions (Standard N); 10 ppm, High nitrogen conditions (High N); 80 ppm.
3) “-” indicates that the protein spot is absent, ±: weak, +: present, ++: large, +++: larger.
3-day-cooled plants was lower when the plants were grown under high nitrogen conditions than under standard nitrogen conditions. This also indicated that high nitrogen conditions enhanced the damages caused by cooling, in agreement with the previous observations in the experiments on stigma (Hayashi et al., 2000). We assumed that high nitrogen supply induces changes in the physiological conditions of pollen grains and additional cooling causes the damages in pollen germination.

To clarify the physiological aspects, proteome analysis is useful to visualize the protein pattern using 2-DE and to identify proteins expressed in tissues by N-terminal amino acid sequencing and PMF analysis of the protein spots.

We performed comparative proteome analysis of mature anthers for the plants grown under standard nitrogen conditions, high nitrogen conditions and high nitrogen plus cooling conditions. High nitrogen conditions themselves did not affect the pollen germination, but additional cooling treatments decreased the pollen germination ability. We searched for protein spots changed by high nitrogen conditions and then changed by cooling. Over 1000 protein spots were detected on the 2-DE gels, but the number of protein spots changed by the treatments was extremely small, 0.1% of total protein spots. The protein spots that are involved in nitrogen metabolism were not changed by the treatments, but in the protein spots that are involved in cell elongation, stress responses, and sugar metabolism were changed.

Expansins play a role in plant growth, cell enlargement, cell wall loosening and cell wall reconstruction (Cosgrove, 1998) and the high expansin content was found in the most rapidly growing regions of tissues and organs of rice (Cho and Kende, 1997; Lee and Kende, 2001). The pollen grains swell rapidly at the anthesis and this swelling is a part of the driving force of anther dehiscence (Matsui et al., 1999). Expansins may also take part in this pollen grain swelling. Thirty-three kinds of α-expansins and 18 kinds of β-expansins have been reported in rice (D.J. Cosgrove. http://www.bio.psu.edu/expansins/index.htm). Expression of these rice expansin genes were investigated in vegetative tissues (Lee and Kende, 2001, 2002), but there are only a few observations in reproductive tissues (Kerim et al., 2003; Imin et al., 2004). Zea m1 that is abundant in pollen grain in maize has been identified as expansin and considered to help pollen tube development by loosening the stigma and style cells (Cosgrove et al., 1997; Wu et al., 2001). The amino acid sequence of Zea m1 is similar to rice EXPB1 and EXPB13. EXPB1 protein increases in rice anther at the heading (Kerim et al., 2003). In our observation, EXPB1 (spot 761) was not detected under standard nitrogen conditions. However, large spots were found both in the anthers under high nitrogen conditions and high nitrogen plus cooling conditions. No differences were observed in intensity of spot 761 between cooled and not cooled plants, in agreement with the results of Imin et al. (2004).

To our knowledge, this is the first report on the changes in expression of EXPA18 and EXPB13 protein in rice anther and neither protein was detected in vegetative tissues (Lee and Kende, 2001, 2002). EXPA18 was decreased by cooling, whereas EXPB13 was increased. These results suggest that EXPA18 might be related to pollen germination.

CDPK, HSP and ALDH are related to stress responses. OsCDPK11 protein has been reported to accumulate in spikelets (Frattini et al., 1999). In this study, the changes in CDPK11 protein in anther were observed for the first time. CDPKs are involved in signal transductions in response to stimulus. Rice has 29 genes encoding CDPKs that are classified into four groups (Asano et al, 2005) and CDPK11 is classified into group I, whose gene expression is upregulated by a low temperature, salt stress and gibberellins, as in OsCDPK7 and OsCDPK13 (Saijo et al., 2000; Yang et al., 2003; Abbasi et al., 2004). It has been reported that OsCDPK7 (Saijo et al., 2000) and OsCDPK13 (Yang et al., 2003) are involved in cold tolerance in rice seedlings but we could not find references on the involvement of CDPK11. The increase in CDPK11
under high nitrogen conditions may suggest that a high nitrogen condition itself is a kind of stress stimulus and that CDPK11 acts as a stress response factor. The decrease in CDPK11 after cooling may suggest that CDPK11 also act as a stress response factor not only in a very short term but also in a long term in plant tissue. HSPs were found as proteins induced by high temperature stress. Recently, HSPs are revealed as chaperons that play a role in protein folding (Vierling, 1991). Small molecular heat shock proteins participate in stress resistance (Sabehat et al., 1998). The role of HSP82 that was decreased by cooling in our result is unknown. Plants have many ALDH genes and some of them are induced by stresses such as osmotic stress, low temperature and anaerobic conditions (Nakazono et al., 2000; Kirch et al., 2004). The increase in these proteins, which are related to stress responses, suggests that the high nitrogen condition itself might induce some stress responses in rice anther. CDPK11 and HSP82 were downregulated in cooled anther, indicating that the decreases of these proteins may be related to the cooling damages.

Fructokinase is an important enzyme that is involved in energy production and also in storing sugars (Pego and Smeekens, 2000). Sink organs receive sucrose transported from source organs, and then sucrose is used for their development or is converted into starch (Schaffer and Petreikov, 1997). The amount of fructokinase protein is highest several days before anthesis and decreases at the anthesis (Kerim et al., 2003), suggesting the role of this enzyme on the production of starch grains in pollen. Our observation that the small amount of fructokinase protein in not cooled anthers was consistent with the results of Kerim et al. (2003). On the other hand, higher amount of fructokinase protein was observed in cooled anthers, while these anthers contained a smaller number of engorged pollen grains. The increase in fructokinase protein may indicate the changes of sugar or starch metabolism in mature anther caused by cooling treatments and may be related to damages of pollen grains in the cooled anthers.

Conclusions

In this study, we confirmed that high nitrogen conditions enhance the cooling damage on pollen germination. Therefore we analyzed the proteins participating in this damage using the proteome method to clarify the physiological factors. We found 11 spots changed by high nitrogen conditions and high nitrogen plus cooling conditions on 2-DE gels. Seven of these proteins were identified as proteins involved in cell elongation, stress responses and sugar metabolism. Taken together, these results indicate that the cell elongation and sugar metabolism may be involved in lower pollen germination ability. The increase in proteins that are related to stress responses suggest that high nitrogen conditions themselves might be some stress conditions and that these stress responses caused by high nitrogen conditions may enhance the damages caused by cool temperature. Considering the longer duration of cooling treatment in the experiment of proteome analysis, these proteins may also contain those that irrelevant to the pollen germination. Further observation such as gene expression analysis and genetic analysis of sense or antisense transformants of expansins, and analysis of enzymatic activities of fructokinase in anthers are needed to clarify the relationship between these proteins and pollen germination.

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