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Analysis of Factors Related to Enhanced Sterility Due to Coolness at the Young Microspore Stage under High Nitrogen Supply in Rice (Oryza sativa L.).

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Analysis of Factors Related to Enhanced Sterility Due to Coolness at the Young Microspore Stage under High Nitrogen Supply in Rice（*Oryza sativa* L.）.

Takami HAYASHI*1)

Abstract : Effects of high nitrogen（High-N）supply combined with cooling on increased sterility in rice plant were examined. High-N enhanced the suppression of pollen germination caused by cooling and the results of comparative proteome analysis and gene expression analysis revealed that a number of expansins（*EXP*s）were downregulated by High-N plus cooling（High-N-cooling）. EXPs act on cell wall loosening and are involved in pollen tube penetration. As many *EXP*s were repressed under High-N-cooling, it appears that these EXPs may be involved in the enhanced damages on pollen grains, and, consequently, the enhanced sterility. It is concluded these findings about the effects of High-N on cool-temperature damages in rice plants will contribute to forming a physiological basis for future research on the prevention of such damage under various nutrient conditions and to the development of damage reduction technologies.

Key Words : Anther, Expansin, Gene expression, High nitrogen, Pollen germination, Proteome, Rice, Cool temperature, Sterility, Young microspore stage

多窒素施肥がイネ小胞子初期の冷温による不稔を助長する要因の解析：林　高見*1)

抄　録：本研究では、冷温によるイネの不稔を窒素施肥が助長する要因を明らかにするため、冷害の主な要因の一つである花粉の発育障害に注目した。多窒素条件では小胞子初期の冷温によって小胞子数や充実花粉数の減少が助長されるだけでなく，柱頭上の受粉数と発芽花粉数が著しく減少することを見出した。そこで花粉発芽に注目し、成熟葯を用いて障害の生理的側面を検討した。比較プロテオーム解析および遺伝子発現解析から、多くのエクスパンシン（*EXP*）遺伝子の発現が多窒素・冷温によって抑制されることが示された。EXPは細胞壁を緩める働きをもち、花粉管伸長にも関与する。多窒素・冷温によって多くの*EXP*遺伝子の発現が抑制されたことから、これらの*EXP*は花粉発芽の抑制に関与し、結果として多窒素栽培における冷温障害の助長に関係することが示唆された。以上のように冷温障害における多窒素の影響について重要な知見が得られた。これらの知見は、冷温障害発生と栄養条件との関係の解明や、被害軽減技術の開発に向けた手がかりになると考える。

キーワード：葯、エクスパンシン、遺伝子発現、多窒素、花粉発芽、プロテオーム、イネ、冷温、不稔、小胞子初期

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Ⅰ Introduction

Rice is one of humankind's major sources of carbohydrates. In 2007, world cereal production was 2.2×10^{9}t, of which rice accounted for 30%, 6×10^{8}t（overseas statistical information, http://www.toukei.maff.go.jp/world/index.html）. In the same year, rice production in Japan was 8.7×10^{6}t, of which 3.7×10^{6}t was produced in the Tohoku region（Statistics of Agriculture, Forestry and Fisheries; http://www.maff.go.jp/j/tokei/index.html）. This large amount of rice production in the Tohoku region is associated with the long day length in the summer season and the large diurnal range of temperature（Uchijima, T. 1983）, and the highly improved cultivation technique, breeding and farming technology（Nishiyama, 1985）.

One drawback of the Tohoku region is the high risk of cool summer temperatures, mainly caused by cool air from high pressure systems originating in the Sea of Okhotsk（Bokura, 2001）. Rice farming in the Tohoku region was advanced by the 16th century（Uchiyamada and Watanabe, 1996）. In Japan, a famine year was recorded 64 times from 1600 to 1867, meaning that cool temperature reduces crop production once every 4.1 years during this period（Nishiyama, 1985）. In Iwate Prefecture, it was 59 times from 1611 to 1817（Seki, 1986）.

In 1993, Japan was hit by a historically cool summer, which reduced rice production nationwide. Yield losses in northern Japan were particularly catastrophic. The Japanese Government thus urgently imported 2.6×10^{6}t of rice（Statistics Bureau, http://www.stat.go.jp/data/getujidb/index.htm）, which was equivalent to a quarter of Japan's average annual rice production. Research by the Tohoku National Agricultural Experiment Station and the prefectural governments to record and analyze this event showed that the extraordinary yield reduction was a result of sterility, caused by continuous cool temperatures from the end of July to the middle of August（Mayumi, 1994）, a period that extended across the booting stage, when the rice plant is most sensitive to cool temperature. These studies also showed that the larger nitrogen supply in 1993 reduced the yield under cool weather（Kodama, 1994; Miyamori, 1994; Takeda, 1994; Tamagawa, 1994）.

Nitrogen is one of the most important elements for living organisms. About 90% of the dry weight of plants consists of carbon and oxygen, and only about 3% consists of nitrogen（Tadano, 2002）; however, nitrogen is involved in all physiological processes of plant development, proliferation and differentiation, and is a major limiting factor in plant growth（Yoneyama, 2002）. Historical research has shown that Japan's capacity for rice production was 1 t ha-1 to 1.5 t ha^{-1} until the mid 18th century. It was in the 1960s that rice production began to increase dramatically due to the application of chemical nitrogen fertilizer（Nishio, 2005）. Nitrogen improves rice growth and yield（Shiga, 1984; Goto *et al.*, 2006;

Fukushima, 2007). Paradoxically, if sufficient amount of nitrogen is used based on normal weather patterns, it severely decreases yield in unexpected cool summer because of sterile spikelets (Sasaki and Wada, 1975; Amano and Moriwaki, 1984; Satake *et al.*, 1987; Watanabe and Takeichi, 1991). The amount of nitrogen supply tends to decreasing in 1990's because the taste of rice deteriorates when nitrogen levels of plants are high (Ishima *et al.*, 1974, Yamashita and Fujimoto, 1974). The fact that, however, cool-temperature damage still occurred in 1993 indicates that much remains to be learned about the effects of nitrogen on cool-temperature damage.

Cool-temperature damages in rice plants are categorized into two types: damage due to delayed growth and damage due to floral impotency (Nishiyama, 1985). Like the case in 1993, floral impotency causes irreparable damage, and much research has been done on floral impotency caused by cool weather.

Cool weather at the booting stage causes pollen abortion. The young microspore stage, tetrad stage and early microspore stage just after the microspores are released from the tetrad, in particular, are the stages when rice is most sensitive to cool temperature (Hayase *et al.*, 1969; Satake and Hayase, 1970). Cool temperature during these critical stages severely represses pollen development, and the decrease in the number of engorged pollen grains is thought to be a major factor in sterility (Nishiyama, 1982; Satake *et al.*, 1988; Satake, 1991).

Satake and Shibata (1992) proposed four factors involved in sterility caused by cool-temperature damage: 1, the number of differentiated microspores; 2, the percentage of developed pollen grains; 3, the percentage of shed pollen grains; 4, the fertilization efficiency of shed pollen grains. Among these, they noted that the number of pollen grains is the major factor. When the nitrogen supply intensifies cool-temperature damage, the repression of pollen development is considered to be an important factor in sterility.

In this study, the combined effects of high nitrogen and cooling on the factors that affect fertility were examined, such as the numbers of microspores, engorged pollen grains, pollen grains shed on the stigma and germinated pollen grains on the stigma (Chapter Ⅱ). To clarify the physiological aspects of the damage to anthers caused by High-N-cooling, protein expression patterns were observed by comparative proteome analysis (Chapter Ⅲ) and gene expression analysis (Chapter Ⅵ).

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Ⅱ Effects of high nitrogen supply and cooling at the young microspore stage on factors related to sterility

A sufficient supply of nitrogen is necessary for optimal plant growth and yield in rice（Shiga, 1984; Kobayashi and Horie, 1994; Goto *et al.*, 2006; Fukushima, 2007）. In cooler-than-average summers, however, higher numbers of sterile spikelets are found in rice plants grown under sufficient nitrogen conditions, resulting in a decrease in yield. This yield decrease is a major problem in rice production, and the physiological mechanisms of the increased sterility are still uncertain.

The young microspore stage is the time during the reproductive period when rice is most sensitive to cool temperatures（Hayase *et al.*, 1969; Satake and Hayase, 1970）. Cool temperature at this stage decreases the numbers of microspores and engorged pollen grains（Nishiyama, 1982; Satake *et al.*, 1988, Satake, 1991）, and the numbers of engorged pollen grains are highly correlated with fertility（Nishiyama, 1982; Satake *et al.*, 1988）.

Satake *et al.*（1987）clearly showed that High-N from the spikelet differentiation stage to the young microspore stage greatly increased sensitivity to coolness at the young microspore stage. Tatsuta（1999）reported that High-N decreased anther length and numbers of engorged pollen grains, but did not examine the effects of cooling. Lee（1990）showed that cool temperature decreases the number of engorged pollen grains as the nitrogen level increases.

From these results, researchers have concluded that the sterility caused by cool temperature at the critical stage is mainly due to a decrease in the numbers of engorged pollen grains. With High-N, the decrease in pollen grain numbers is also thought to be a major factor in increasing sterility.

Shading causes a decrease in fertility（Shimazaki, *et al.*, 1964; Wada *et al.*, 1972）; however, the effects of shading vary among rice cultivars（Wada *et al.*, 1972）. Since cool summer weather is often accompanied by some degree of low insolation, the effects of shading on the cool-temperature damage should be clarified. Therefore, in this study, the combined effects of High-N, cooling and shading on the factors that affect fertility, such as anther length, and also the numbers of microspores, engorged pollen grains, pollen grains shed on the stigma and germinated pollen grains on the stigma were investigated.

Materials and Methods

Experiment 1 Effects of High-N and cooling on flower components

An early-maturing rice variety（*Oryza sativa* L. *japonica* cv. Hayayuki）from Hokkaido was used in this experiment. Twenty seeds were sown in a circular pattern on vermiculite in plastic sieves in pots 18 cm in diameter and 20 cm in height, following the methods of Satake *et al.*（1969）and Satake and Koike（1983）（Fig. 1）. Plants were grown using tap water until the third leaf stage, after which a culture solution（Satake and Koike, 1983）including a standard level of nitrogen（Standard-N: 10 ppm of N）was used. The culture solution was changed once every 2 to 3 days. Plants were grown in an artificially lit chamber（Plant Growth Chamber, Conviron, Winnipeg, Manitoba, Canada）under an illumination of 300 μmol photons m^{-2} s^{-1} just above the plants for a 14-hour day length and with a day/night temperature regime of 24/19℃ until the young microspore stage. Tillers were removed as they appeared. Stages were checked using a microscope. To obtain uniform samples, the third to the fifth spikelets from the top on the first and the second primary branches of the main stem were examined following the methods of Satake and Hayase（1970）and Satake *et al.*（1987）（Fig. 2）. From the spikelet differentiation stage to the date when the specified spikelets were at the young microspore stage, half the pots were grown under High-N（80 ppm of N）conditions. Thereafter, all plants were grown under Standard-N conditions.

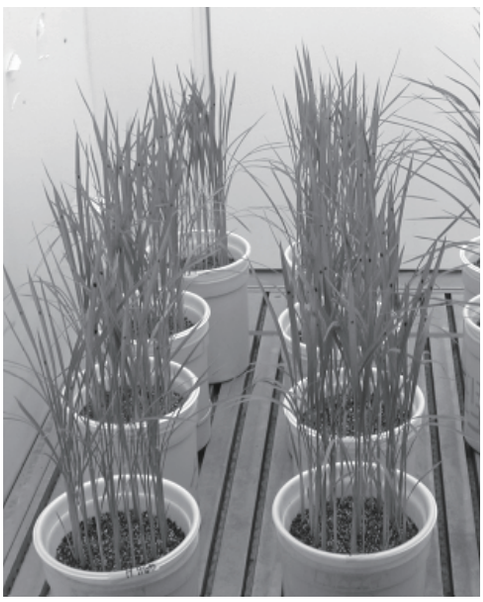


Fig. 1 Twenty plants in a circular pattern grown with water culture.

Water culture was used to control nitrogen level. Plants were grown under standard-N (10 ppm) until the spikelet differentiation stage when half the plants were grown under High-N until the young microspore stage, after which all plants were grown under standard-N. Nitrogen supply was stopped at the end of flowering.

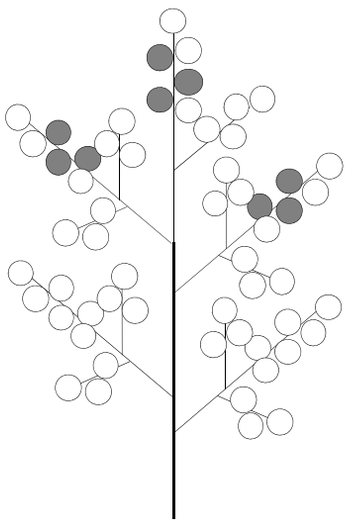


Fig. 2 Spikelets at the uniform stage on a panicle.

To obtain uniform samples, the third to the fifth spikelets from the top on the first and the second primary branches of the main stem (gray circles) were examined following the methods of Satake and Hayase (1970) and Satake et al. (1987).

At the young microspore stage, plants that were to undergo cool-temperature treatment were transferred to a cool chamber (12/12℃) for 4 days, and then transferred back to the 24/19℃ chamber. The nutrient solution supply was discontinued after the end of flowering.

To determine the number of microspores per anther, spikelets from 5 to 7 panicles were fixed with 50% (v/v) ethanol at the young microspore stage, or, for cooled plants, at 1 day after the end of the cool treatment. One day after heading, spikelets were fixed with 50% (v/v) ethanol to determine the numbers of pollen grains.

Anthers were excised from all of the fixed spikelets, and the number of microspores per anther was determined from an average of 8 to 10 anthers randomly selected from the excised anthers. The number of pollen grains per anther was determined from an average of 20 to 25 anthers. Each anther was dissected and stained with iodine/potassium iodide solution (Satake *et al.*, 1988), and microspores and fully stained engorged pollen grains were counted. To determine average anther length, 30 anthers were measured per treatment. Fertility was determined by spikelets sampled from 20 to 30 panicles.

Experiment 2 Combined effects of High-N, shading and cooling on flower components

For shading treatments, Hayayuki plants were grown under the same conditions as in experiment 1 except for light conditions, which in this experiment were 419 instead of 300 μmol photons m^{-2} s^{-1}. Two days after the start of the High-N treatment, half the pots from both Standard-N and High-N plants were shaded with white mesh cloth until the start of cooling. The light condition under shading was 107 μmol photons m^{-2} s^{-1}, which was 74% lower than the unshaded condition. At the young microspore stage, plants were cooled for 3 days with a temperature regime of 12/12℃, and then transferred back to the

24/19℃ chamber. The nutrient solution supply was discontinued after the end of flowering. Flower components were observed as in experiment 1.

Stigmas were removed from the closed spikelets about 4 hours after flowering to determine the numbers of engorged pollen grains and germinated pollen grains on the stigma. Nine to 19 stigmas from 8 to 10 panicles were placed on slide glasses. After adding 50%（v/v）ethanol, stigmas were stained with acetocarmine solution（Nacalai Tesque, Kyoto, Japan）, and the numbers of fully stained pollen grains and germinated pollen grains with elongated pollen tubes were counted under a microscope（BX40; Olympus, Tokyo, Japan）.

Experiment 3 *In vivo* and *in vitro* pollen germination

For microscopic observation of *in vivo* and *in vitro* pollen germination, Hayayuki were grown under the same conditions as in experiment 1. At the young microspore stage, plants for cooling were transferred to a chamber where they were cooled（12/12℃）for 3 days, and then transferred back to the 24/19℃ chamber. The nutrient solution supply was discontinued after the end of flowering.

Stigmas for pollen tube observation were fixed and immersed in 50%（v/v）ethanol, and callose walls of pollen tubes were stained with 0.05%（w/v）aniline blue（Carland *et al.*, 1999）. Ten or more stigmas from each treatment were observed with a fluorescence microscope（BX60 with filter set BP 330 385, DM 400, BA 420; Olympus, Tokyo, Japan）.

Pollen germination on an agar plate was observed so that the effects of stigma conditions could be eliminated. As soon as the flowers began to open, 8 or more spikelets were removed and gently shaken above a medium containing 1%（w/v）agar, 20%（w/v）sucrose and 20 ppm $K_2B_4O_7$（Kariya, 1989）in petri dishes（35 mm ϕ）. After incubating for 20 min at 20℃ and staining with iodine/potassium iodide solution（Fig. 3）, total numbers of fully stained pollen grains and germinated pollen grains were counted under a microscope（BX50; Olympus, Tokyo, Japan）.

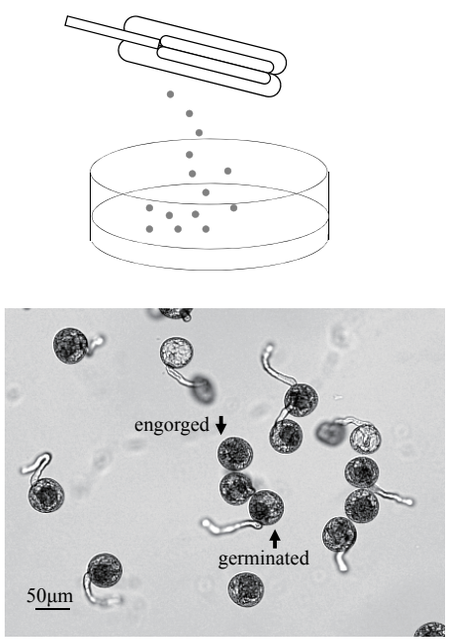


Fig. 3 *In vitro* pollen germination on agar plates.

Anthers were removed from the spikelets when the spikelets began to open. Pollen grains were sprinkled on the agar plate containing 1% agar, 20% sucrose and 20 ppm $K_2B_4O_7$（Kariya, 1989） and incubated at 20℃ for 20 min. The pollen germination was observed under a microscope. Engorged pollen grains and germinated pollen grains（arrows）were counted.

Results

Experiment 1 Effects of High-N and cooling on flower components

Without cooling, fertilities were close to 100%, regardless of the nitrogen conditions. After cooling, fertility declined and was much lower under High-N（11%）than under Standard-N（41%）. High-N decreased microspore numbers by 23%. Cooling further decreased these numbers, resulting in a total decrease of 34%, but cooling alone caused only a 9% decrease under Standard-N. High-N decreased engorged pollen grain numbers, but this decrease did not result in low fertility. After cooling, engorged pollen grain numbers decreased by 66% under Standard-N and by 83% under High-N（Fig. 4）.

Experiment 2 Combined effects of High-N, shading and cooling on flower components

Shading decreased numbers of microspores under both Standard-N and High-N conditions. High-N decreased microspore numbers as in experiment 1, which were lowest under High-N plus shading (Fig. 5).

Without cooling, High-N decreased engorged pollen grain numbers more than did shading. The ratio of numbers of engorged pollen grains to numbers of microspores was smallest under High-N plus shading. Cooling decreased engorged pollen grain numbers by 29% under Standard-N, and the numbers were further decreased under High-N for a total decrease of 40%. Shading decreased these numbers by only 13% under Standard-N, while shading plus cooling decreased these numbers by 39%. High-N enhanced the decrease caused by shading plus cooling, resulting

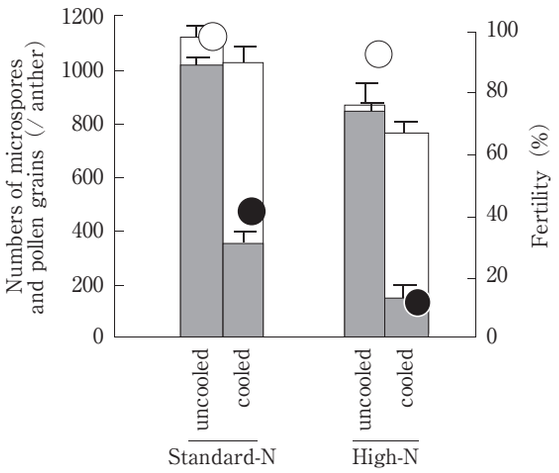


Fig. 4 Effects of nitrogen level and cooling treatments on fertility, and numbers of microspores and pollen grains per anther.

Percentage of fertility is indicated by open circles for uncooled plants and solid circles for cooled plants. Open bars show microspore numbers and gray bars show pollen grain numbers. Standard-N：standard nitrogen conditions (10 ppm), High-N：high nitrogen conditions (80 ppm), narrow bars：standard errors.

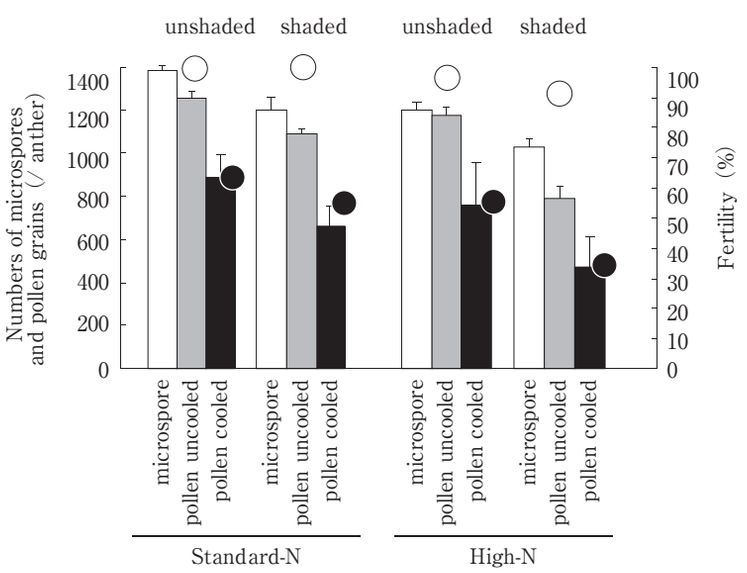


Fig. 5 Effects of nitrogen level, cooling and shading treatments on fertility, numbers of microspores and pollen grains per anther

Shading decreased numbers of microspores and pollen grains. Shading enhanced the decrease in pollen grain numbers under High-N.

Percentage of fertility is indicated by open circles for uncooled plants and solid circles for cooled plants. Bars showed the numbers of microspores and pollen grains： open bars indicate microspores, gray and black bars indicate pollen grains for uncooled and cooled plants, respectively. Standard-N：10 ppm, High-N：80 ppm. Narrow bars：standard errors.

Numbers of microspores in cooled plants were not counted.

Table 1 Effects of nitrogen level, light conditions and cooling treatment on anther length.

Light conditions	Nitrogen conditions	Anther length (mm)			
		Experiment 1		Experiment 2	
		not cooled	cooled (12℃/4days)	not cooled	cooled (12℃/3days)
unshading	Standard-N	2.09 ± 0.02	1.97 ± 0.05	2.28 ± 0.02	2.09 ± 0.02
	High-N	1.98 ± 0.02	1.67 ± 0.06	2.27 ± 0.02	1.97 ± 0.02
shading	Standard-N			2.15 ± 0.02	1.98 ± 0.03
	High-N			2.00 ± 0.02	1.88 ± 0.03

Values are shown as means ± standard error. Standard-N；10 ppmof N. High-N；80 ppm of N.

Table 2 Effects of nitrogen level, light conditions and cooling treatment on the number of pollen grains on stigma in experiment 2.

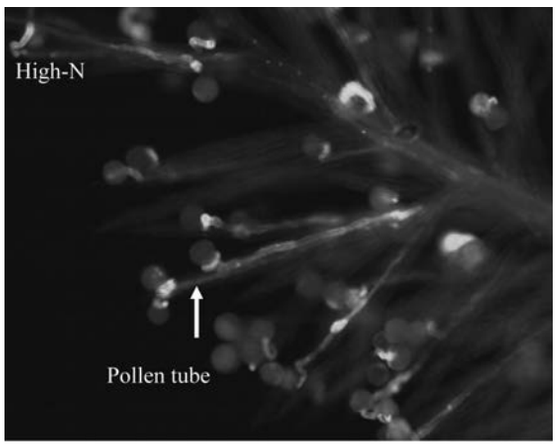
Light conditions	Nitrogen conditions	Temperature treatment	Number of pollen grains (/stigma)		Germinated /Shed (%)
			Shed	Germinated	
unshading	Standard-N	not cooled	272 ± 33	30 ± 3	12 ± 2
		cooled	245 ± 38	20 ± 4	10 ± 1
	High-N	not cooled	132 ± 21	14 ± 3	11 ± 2
		cooled	97 ± 20	3 ± 1	4 ± 1
shading	Standard-N	not cooled	191 ± 21	13 ± 2	7 ± 1
		cooled	133 ± 25	9 ± 3	5 ± 1
	High-N	not cooled	219 ± 38	22 ± 4	12 ± 2
		cooled	115 ± 20	5 ± 1	5 ± 1

Values are shown as means ± standard error. The cooling treatment was started at the young microspore stage and treated at 12℃ for 3days. Standard-N；10 ppm of N. High-N；80 ppm of N.

in a 62% decrease. Fertility was nearly 100% under Standard-N and over 90% under High-N, even in shaded uncooled plants. Cooling decreased fertility by 36% under Standard-N and by 42% under High-N, whereas cooling plus shading decreased fertility by 45% under Standard-N and by 64% under High-N (Fig. 5).

High-N and cooling decreased anther length, which is a parameter of pollen grain number. In experiment 1, High-N decreased anther length by 5% and cooling further decreased it by 16% (Table 1) ; the decreases in anther length were smaller than the decreases in engorged pollen grain numbers. Without shading, High-N did not change anther length, whereas High-N-cooling resulted in a 13% decrease. Shading decreased anther length by 6% under Standard-N and by 12% under High-N (Table 1).

Table 2 shows the numbers of engorged pollen grains and germinated pollen grains on the stigma. High-N under unshaded conditions decreased the numbers of both pollen grains and germinated pollen grains shed on the stigma. Cooling enhanced these decreases. Pollen grain numbers on the stigma were over 90, even under High-N combined with cooling and shading, but very few germinated pollen grains were observed. Without cooling, the ratio of germinated pollen grain numbers to pollen grain numbers shed on the stigma was around 10%, while after cooling, this ratio was around 5%, except for under Standard-N. Plants of High-N plus shading conditions showed a smaller decrease in the numbers of pollen grains shed on the stigma and germinated pollen grains than did plants subjected to High-N without shading.



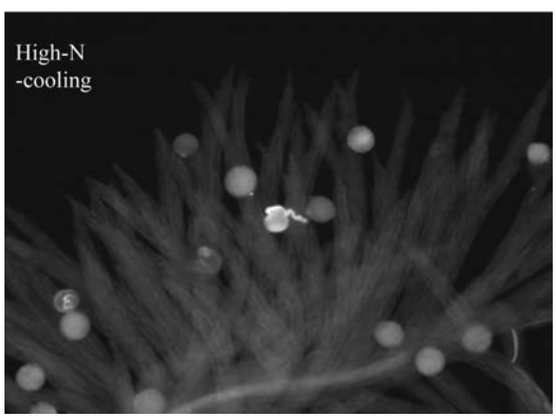


Fig. 6 Pollen germination on the stigma under High-N.

High-N: 80 ppm; High-N-cooling: High-N and 12℃ cooling for 3 days at the young microspore stage. Pollen tubes were stained by aniline blue (green fluorescence). Cooling at the young microspore stage suppressed pollen germination and pollen tube elongation.

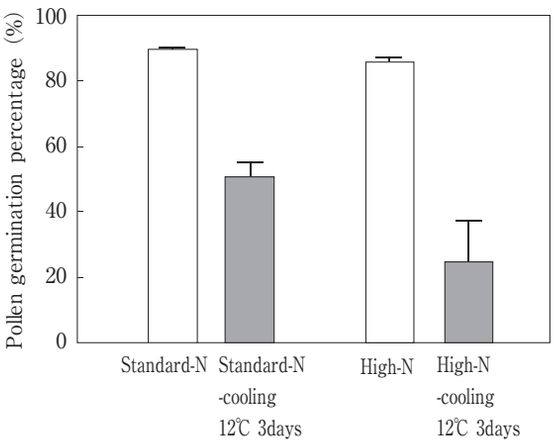


Fig. 7 Effects of High-N and cooling on pollen grain germination

Standard-N：10 ppm；High-N: 80 ppm；High-N-cooling：High-N and 12℃ cooling for 3 days at the young microspore stage. Numbers of spikelets used：21 for Standard-N, 21 for Standard-N plus cooling, 11 for High-N, 8 for High-N-cooling. Bar：standard error.

$$\text{pollen germination percentage} = \frac{\text{germinated pollen grains}}{(\text{engorged pollen grains} + \text{germinated pollen grains})} \times 100$$

Experiment 3　pollen germination

Under High-N condition, many of pollen grains germinated and elongated pollen tubes well in plants grown without cooling. After 3 days of cooling, few pollen grains germinated and pollen tubes were stunted (Fig. 6).

A reduction in the pollen germination ratio by cooling was also observed in the experiment with agar plates. After cooling, the pollen germination ratio was 51% in plants grown under Standard-N and 25% in plants grown under High-N, decreasing to half of that under Standard-N (Fig. 7).

Discussion about the combined effects of High-N, cooling and shading on the factors that affect fertility

High-N has been reported to reduce engorged pollen grain numbers and anther lengths (Tatsuta, 1999; Gunawardena *et al.*, 2003) and to increase numbers of sterile spikelets (Yamada and Kono, 1976; Gunawardena *et al.*, 2003). Pollen grain numbers are limited by the number of differentiated microspores, and environmental conditions affect microspore formation. For example, microspore numbers increase as water temperature increases (Satake, 1989), and they decrease with High-N application (Lee, 1990). In the variety Sangpung, a 3% reduction in microspore numbers was observed under High-N (Lee, 1990). In this study, the decrease in microspore numbers as a result of High-N ranged from 23% (experiment 1) to 13-14% (experiment 2). Along with the reduction in microspore numbers, anther length, numbers of engorged pollen grains and germinated pollen grains on the stigma (Tables 1,

2）were also decreased. However, these decreases in microspore numbers did not appear to be large enough to affect percentage fertility, which was close to 100% without cooling（Figs. 4, 5）, in agreement with the results of Lee（1990）.

In contrast, High-N-cooling had a greater effect on pollen grain development than that of High-N alone. The decrease in engorged pollen grain numbers was accompanied by a large decrease in fertility（Figs. 4, 5）.

In experiments using the variety Sangpung, Lee（1990）showed that a 3-day treatment at 12℃ reduced fertility by 13% under Standard-N and 19% under High-N, while the same temperature regime in the present experiment led to 36% and 42% decreases, respectively（Fig. 5）. Cooling for 4 days had a much greater effect on fertility, reducing it by 58% under Standard-N and by 88% under High-N（Fig. 4）.

Cool summer weather is often accompanied by low intensity of sunlight. Kiyosawa and Aimi（1959）noted no effect of shading on the reduction of fertility owing to cooling, while Wada *et al.*（1972）showed that the effects of shading on cool injury differed among genotypes. In the case of "Hayayuki", shading alone did not decrease percentage fertility, though it decreased the numbers of microspores and of engorged pollen grains per anther. Shading plus cooling caused larger decreases in engorged pollen grain numbers, resulting in lower fertility（Fig. 5）. These results suggest that study of the microspore development under shading in varied cultivars is important to clarify the combined effects of shading and cooling.

It is interesting to consider the similarity between the male sterility caused by cooling and the cytoplasmic male sterility（CMS）used for plant breeding. Based on the results of a reciprocal crossing experiment, the cool tolerance of Hayayuki has been thought to exist in the nucleus（Sawada 1978）. In Norin-PL8, a highly cool-tolerant breeding line developed by the cross Silewah/Hokkai241, the cytoplasm is not associated with cold tolerance（Saito, 2006）. Hokkai241 was developed by the cross Norin-22/Hayayuki. From these observations, it appears likely that the sterility caused by cooling in Hayayuki is generated by a different mechanism from that of CMS.

Nishiyama（1983）reported that 143 engorged pollen grains per anther were necessary to obtain 10% fertility, 392/anther for 50% fertility and 640/anther for 90% fertility. Using the rice cultivars Hayayuki and Norin 20, Satake（1991）estimated that 360 engorged pollen grains per anther were necessary for 50% fertility and 650/anther for 90% fertility. In this study, in the absence of cooling, spikelets showed over 90% fertility and had over 800 engorged pollen grains per anther, corresponding to the results of Satake（1991）and Nishiyama（1983）. With cooling, the relationship between percentage fertility and engorged pollen grain numbers observed in experiment 1 was similar to the results of Satake（1991）and Nishiyama（1983）. However, in experiment 2, percentage fertility was lower than that estimated by engorged pollen grain numbers, indicating that these numbers alone are not sufficient to estimate percentage fertility.

Moriwaki（1959）found that sterility and pollen grain numbers on the stigma were correlated. Satake and Shibata（1992）reported that more than 40 grains were shed on the stigma when fertility was higher than 90%. In this study, pollen grain numbers shed on the stigma were near or over 100 in all cases. These numbers were much larger than those reported by Satake and Shibata（1992）.

It is interesting to note that, even in the plants with similar pollen grain numbers shed on the stigma, fertility was lower in cooled plants, and was much lower in plants under High-N-cooling. Satake（1989）also showed that when pollen grain numbers shed on the stigma were almost the same, fertility of cooled plants was lower than that of uncooled plants. These results suggest that pollen grains in cooled plants,

which seemed to be normal, might have lower activity.

In summary, High-N starting from the spikelet differentiation stage and continuing to the young microspore stage decreased microspore and engorged pollen grain numbers, and shading caused further decreases. Cooling under both Standard-N and High-N slightly decreased microspore numbers but notably decreased engorged pollen grain numbers in the anthers of High-N plants. Further, High-N-cooling conspicuously suppressed pollen germination and pollen tube elongation. These results suggest that the reduction in fertility under High-N is the result of a reduction in engorged pollen grain numbers and pollen viability.

Ⅲ Proteome analysis of rice mature anthers

At anthesis, the number of pollen grains on the stigma is an important factor in fertility, and about 40 grains are enough for fertilization (Satake and Shibata, 1992). In cooled plants, however, the fertility of plants grown under High-N was lower than that of plants grown under Standard-N, even though they had almost the same numbers of pollen grains on the stigma (Table 2). This result suggested that High-N affected the activity of mature pollen of normal appearance. In this chapter, to investigate the physiological changes caused by cooling under High-N, comparative proteome analysis was performed on mature anthers and searched for proteins that might reduce the ability of pollen to germinate.

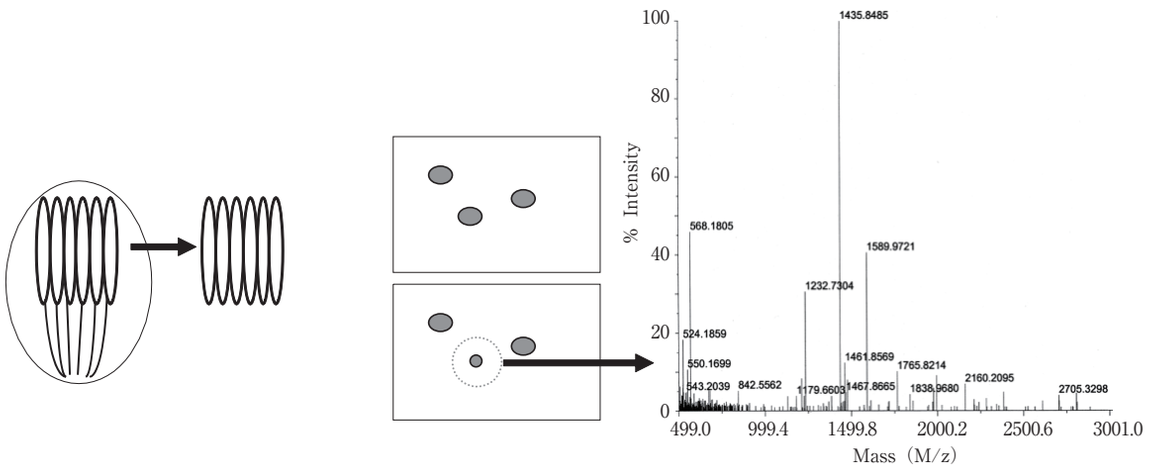
Materials and Methods

Plant materials

The plant materials and the growing conditions were the same as in experiment 1 in Chapter II. On the assumption that physiological changes brought about by High-N and by High-N-cooling at the young microspore stage are involved in enhanced sterility of rice plants, three treatments were set as follows: 1) Standard-N (10 ppm of N), 2) High-N (80 ppm of N) and 3) High-N-cooling. To obtain uniform samples, the third to the fifth spikelets from the top on the first and the second primary branches of the main culms were used. When the spikelets mentioned above were at the young microspore stage, plants for cooling were transferred to a cool chamber (12/12℃) for the High-N-cooling treatment. The cooling treatment lasted for 3 days in the pollen germination experiment. For proteome analysis, cooling lasted for 5 days to enhance the changes in proteins. After cooling, pots were transferred back to the 24/19℃ chamber. The nutrient solution supply was discontinued when flowering ended.

Proteome analysis

Spikelets were detached from plants about 2 hours before flowering, and then mature anthers from which filaments had been carefully removed were collected and kept at −80℃ (Fig. 8-1). One hundred to 150 anthers were homogenized in lysis buffer containing 8 M urea, 2% (v/v) NP-40, 4% (v/v) ampholine (pH 3.5-10 and pH 5-8; equal volumes), 5% (v/v) mercaptoethanol and 5% (v/v) PVP-40, and then centrifuged twice at 13,000 g for 5 min. The supernatant was used for 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 was changed by High-N or High-N-cooling were examined (Fig. 8-2). At least 3 gels from the different experimental sets were compared. Proteins were identified using the Rice Proteome Database (http://gene64.dna.affrc.go.jp/RPD/) or by peptide mass fingerprinting (PMF) analysis (Mascot Search, Matrix Science, http://www.matrix-



8－1. Obtaining uniform anthers

About 2 hours before flowering, mature anthers from which filaments were carefully removed were collected and kept at －80℃.

8－2. Differential analysis of the electrophoresis images

Protein spots changed by treatments were cut out. After the digestion by trypsin, partially degraded proteins were analyzed by MALDI-TOF MS.

Fig. 8 Uniform anthers and peptide mass fingerprinting analysis

8-1. Anthers were removed from the spikelets about 2 hours before flowering. 8-2. Protein spots that were changed by High-N or High-N-cooling were picked up and partially degraded, then the protein mass spectrum analyzed by MALDI-TOF MS (Voyager DE-PRO, Applied Biosystems). Proteins were identified from the mass spectrum data by PMF analysis.

science.com/home.html）from mass spectra from matrix-assisted laser desorption/ionization-time of flight mass spectrometry（MALDI-TOF MS）(Voyager DE-PRO, Applied Biosystems, Carlsbad, CA, USA).

Results

Over 1000 protein spots were detected on 2-DE gel images（Fig. 9）, and 11 spots whose densities changed under High-N and High-N-cooling treatments were found. Nine of the 11 protein spots（spots 732, 761, 783, 442, 839, 912, 722, 634 and 802）were identified by a database search, but 2 protein spots（spots 505 and 545）did not have matched protein data. Among the 9 proteins identified, 7 were categorized into 3 groups: those involved in 1）cell elongation（spots 732, 761 and 783）, 2）stress responses（spots 442, 839 and 912）and 3）sugar metabolism（spot 722）(Table 3).

The 3 protein spots involved in cell elongation were identified as expansins（EXPs）. All of these were upregulated under High-N, whereas under High-N-cooling, spot 783, identified as β-expansin 13（EXPB13）, was upregulated, spot 732, identified as α-expansin 18（EXPA18）, was downregulated, and spot 761, identified as EXPB1, was not changed (Table 3, Fig. 12).

The 3 spots involved with stress response were also upregulated under High-N, and 2 of them, spot 442 and spot 912, were downregulated by High-N-cooling. Spot 442 was identified as calcium-dependent protein kinase isoform 11（CDPK11）, and spot 912 was identified as heat shock protein 82（HSP82）. Spot 839, which was not changed by High-N-cooling, was identified as putative aldehyde dehydrogenase（ALDH）(Table 3, Figs. 9, 10, 11). Spot 722, identified as fructokinase II（FKII）, was not changed under High-N and was increased by High-N-cooling (Table 3, Fig. 9).

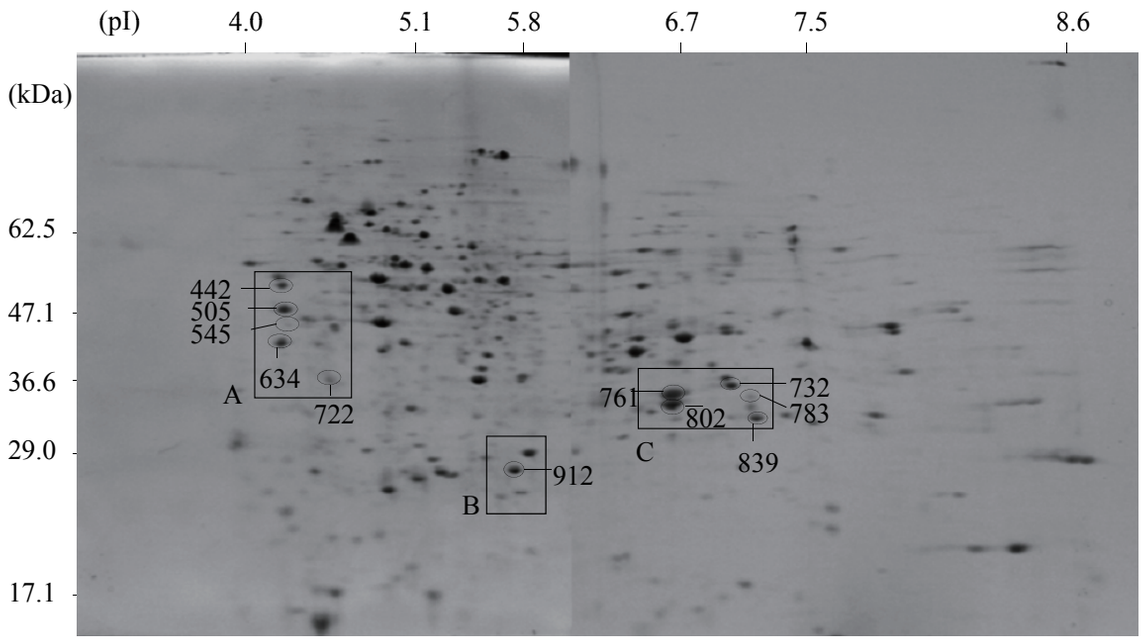


Fig. 9 2-DE image of rice mature anther proteins.

Over 1,000 protein spots were separated. Protein spots changed by treatments were circled and numbered based on the rice proteome database (http://gene64.dna.affrc.go.jp/RPD/). A to C indicated areas described in Figure 10 to 12.

Table 3 Changes in anther proteins caused by high nitrogen and cooling treatments.

Spot No.	Standard -N	High-N	High-N -cooling	Accession No.	Matched Protein	Organism
Cell elongation						
732*	+	++	+	AF394453	α-expansin 18 (EXPA18)	*Oryza sativa*
761	–	++	++	Q9LD01	β-expansin 1 (EXPB1)	*Oryza sativa*
783*	+	+	++	AF391106	β-expansin 13 (EXPB13)	*Oryza sativa*
Stress responses						
442	+	++	+	P53684	calcium-dependent protein kinase, isoform 11 (CDPK11)	*Oryza sativa*
839*	+	++	++	AF148877	putative aldehyde dehydrogenase (putative ALDH)	*Oryza sativa*
912	+	++	+	Z15018	heat shock protein 82 (HSP82)	*Oryza sativa*
Sugar metabolism						
722	+	+	++	Q944F5	fructokinase II (FKII)	*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*

Protein spots were numbered based on the rice proteome database (http://gene64.dna.affrc.go.jp/RPD/main.html). Proteins were identified by PFM analysis, but protein spots with asterisks were identified with the rice anther gel image of the rice proteome database. Standard-N；10 ppm of N, High-N；80 ppm of N, High-N-cooling；High-N and treated at 12℃ for 5 days. "-" indicated that the protein spot was absent, +；weak, +；present, ++；large, +++；larger.

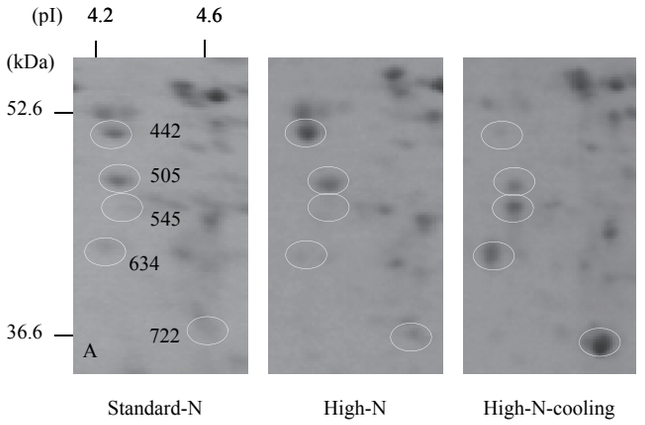


Fig. 10 Comparison of some of the proteins in the area A in Fig. 9 that were differentially displayed by treatments（circled）.

Protein spots were numbered based on the rice proteome database（http://gene64.dna.affrc.go.jp/RPD/）.

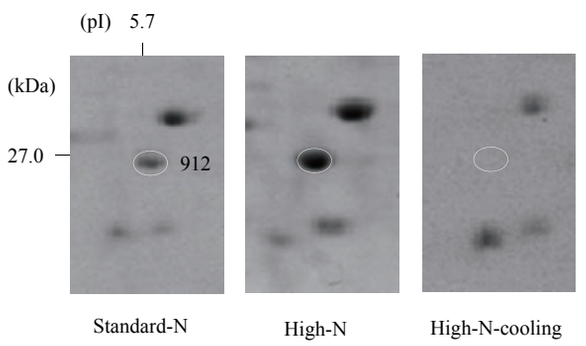


Fig. 11 Comparison of some of the proteins in the area B in Fig. 9 that were differentially displayed by treatments（circled）.

Protein spots were numbered based on the rice proteome database（http://gene64.dna.affrc.go.jp/RPD/）.

Discussion of the combined effects of High-N and cooling on the protein expression patterns in mature anthers

Numbers of engorged pollen grains per anther are highly correlated with fertility（Nishiyama, 1982）, and the decrease in numbers of engorged pollen grains caused by cooling is the major factor in floral impotency. High-N supply decreases anther length and engorged pollen grain numbers（Lee, 1990; Tatsuta, 1999）, and High-N strongly enhanced the decrease in engorged pollen grain numbers caused by cooling（Figs. 4, 5）. Interestingly, even in plants with almost the same pollen grain numbers on the stigma, fertility was lower in cooled plants and became much lower with High-N-cooling（Table 2）. High-N-cooling strongly reduced the germination ratio of pollen grains（Fig. 7）. These results suggest that High-N enhances pollen grain damage caused by cool temperature at the young microspore stage and that High-N induces changes in the physiological conditions of pollen grains and additional cooling damages to pollen germination.

It is necessary to identify the proteins which are changed by treatments to clarify the physiology of what occurs under High-N-cooling. Proteome analysis of mature anthers was used to clarify the changes in the protein expression patterns with 2-DE and to identify separated proteins by N-terminal amino acid

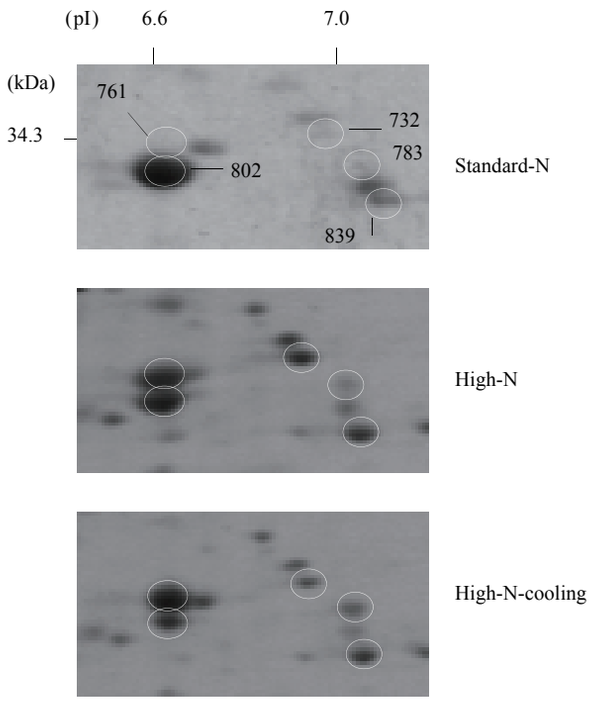


Fig. 12 Comparison of some of the proteins in the area C in Fig.9 that were differentially displayed by treatments (circled).

Protein spots were numbered based on the rice proteome database (http://gene64.dna.affrc.go.jp/RPD/).

sequencing and PMF analysis. Comprehensive 2-DE analysis of mature anthers was performed for plants grown under Standard-N, High-N and High-N-cooling. Over 1000 protein spots were detected on the 2-DE gels, but the treatments changed an extremely small number of protein spots (0.1% of total protein spots). Changes in protein spots involved in nitrogen metabolism were not observed, but changes in protein spots involved in cell elongation (EXPs), stress responses (CDPK11, putative ALDH, HSP82) and sugar metabolism (FKII) were detected.

EXPB1 (spot 761) was not detected under Standard-N, but large EXPB1 spots were found in anthers under both High-N and High-N-cooling. There were no differences in intensity of EXPB1 spots between cooled and uncooled plants. This result is in good agreement with that of Imin *et al.* (2004).

These are first evidences on changes in expression of EXPA18 and EXPB13 protein in rice anthers by stress conditions. It was reported neither of proteins are expressed in vegetative tissues (Lee and Kende, 2001; Lee and Kende, 2002). High-N-cooling decreased EXPA18 but increased EXPB13. These results support the idea that EXPA18 might be related to pollen germination.

CDPK, HSP and ALDH are categorized to proteins that are associated with stress responses. OsCDPK11 protein has been reported to accumulate in spikelets (Frattini *et al.*, 1999). In the present study, changes in CDPK11 protein in anthers were observed for the first time. CDPKs are involved in signal transductions in response to stimuli. Rice has 29 genes encoding CDPKs that are classified into 4 groups (Asano, 2005), and OsCDPK11 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). Both OsCDPK7 and OsCDPK13 are involved in cold tolerance in rice seedlings (Saijo *et al.*, 2000; Yang *et al.*, 2003). The increase in CDPK11 under High-N suggests that

High-N itself may be a kind of stress stimulus and that CDPK11 acts as a stress response factor. The decrease in CDPK11 after cooling indicates that CDPK11 may also act as a stress response factor not only in the very short term but also in the long term in plant tissues. HSPs were found as proteins induced by high temperature stress. Recently, HSPs were revealed as chaperones that play a role in protein folding（Vierling, 1991）. Small molecular HSPs participate in stress resistance（Sabehat *et al.*, 1998）. The role of HSP82, which was decreased by cooling in this study, remains to be clarified. Plants have many ALDH genes, some of which 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 High-N itself might induce some stress responses in rice anthers. CDPK11 and HSP82 were downregulated in cooled anthers, indicating that the decreases of these proteins might be related to cooling damage.

FK is an important enzyme involved in energy production and also in sugar storage（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 FK protein is highest several days before anthesis and decreases at anthesis（Kerim *et al.*, 2003）, suggesting a role for this enzyme in the production of starch grains in pollen. Our observation that a small amount of FKII exists in uncooled anthers is consistent with the results of Kerim *et al.*（2003）. On the other hand, higher amounts of FKII protein were observed in cooled anthers which contained a smaller number of engorged pollen grains. The increase in FKII may indicate that cooling treatments changed the sugar or starch metabolism in mature anthers, which may be related to pollen grain damage in cooled anthers.

In summary, the proteins participating in cooling damage were analyzed using the proteome method. Eleven spots on 2-DE gels were found to be changed by High-N and High-N-cooling, 7 of which were identified as proteins involved in cell elongation, stress responses or sugar metabolism. Taken together, these results indicate that cell elongation and sugar metabolism may be involved in the lower pollen germination ability after cooling at the young microspore stage. The increases in proteins related to stress responses suggest that High-N itself might be a stress condition, and the stress responses caused by High-N may enhance cool-temperature damage.

Ⅳ Gene expression analysis in rice mature anthers

Experiments reported in Chapter II showed that High-N-cooling at the young microspore stage strongly reduced the pollen germination ratio and that the number of engorged pollen grains was notably low. Because High-N alone did not decrease the pollen germination ratio, it is possible that the changes in metabolism under High-N, which do not affect pollen germination under normal temperature, enhance cooling damage and reduce pollen viability.

In Chapter III, changes in proteins under High-N and/or High-N-cooling, and changes in 11 proteins involved in cell elongation, stress responses and sugar metabolism were examined by proteome analysis of rice mature anthers. It is noteworthy that 3 of these 11 proteins were identified as EXPs, which are involved in cell elongation by loosening cell walls（Cosgrove, 2000）. The hypothetical action of EXPs is a transient release of a short segment of matrix glycans attached to cellulose microfibrils, and, in consequence, the sliding of cellulose and matrix polymers occurs（Cosgrove, 1998）. In maize, pollen allergen protein Zea m 1, which consists of at least 4 beta-expansins（EXPBs）, functions by loosening the silk cell wall and is assumed to play a role in pollen tube elongation（Cosgrove *et al.*, 1997; Wu *et al.*, 2001; Li

et al., 2003; Wei *et al.*, 2004). In addition, an EXP-like protein is present on the wall of the pollen tube tip (Suen *et al.*, 2003). It is therefore speculated that EXPs may play similar roles also in rice plants.

In this chapter, besides candidate genes found in proteome analysis, the gene expression patterns of almost all rice alpha-expansin genes (*EXPA*s) and *EXPB*s in the mature anthers were analyzed by semiquantitative reverse transcription polymerase chain reaction (RT-PCR) with gene-specific primers, focusing on the changes under High-N and High-N-cooling. In total, 18 *EXPA*s and 6 *EXPB*s were down-regulated under High-N-cooling, and *EXPA1* and *EXPB1* were upregulated under High-N and High-N-cooling. Molecular phylogenetic characteristics of these EXPs and their involvement in enhanced sterility of rice plants are discussed.

Materials and Methods

Plant Materials

Plant materials and the growing conditions were the same as in experiment 1 in Chapter II. Genes whose expressions were changed by High-N and by High-N-cooling were assumed to be involved in enhanced sterility of the rice plant. On this basis, 3 treatments were set up: 1) Standard-N (10 ppm of N), 2) High-N (80 ppm of N) and 3) High-N-cooling. Plants were cooled at the young microspore stage for 3 days at 12 ℃. Spikelets were detached from the plants about 2 hours before flowering, and the mature anthers from which filaments had been carefully removed were immediately frozen in liquid nitrogen and kept at −80℃ until analysis.

Semiquantitative RT-PCR

Total RNA was extracted from 100 μg fresh weight of frozen samples of the anthers with an RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan) 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 Oligo d (T) 20 primer. Reverse transcription was done for 30 min at 42℃ 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 uL) 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 units of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA).

Genes for RT-PCR were selected based on proteome analysis of rice mature anthers (Table 3). Gene expressions of calcium dependent protein kinase 11 (*CDPK11*), heat shock protein 82 (*HSP82*), putative ALDH, fructokinase II (*FKII*) and *EXP*s were analyzed. Genes encoding these proteins were searched through BLAST program on DDBJ (Sugawara *et al.*, 2008) with deduced amino acid sequences. Gene-specific primer sets are shown in Table 4. For *EXP*s, 26 *EXPA*s and 16 *EXPB*s were examined. Primer sequences of *EXP*s that are not shown in Table 4 were taken 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: 95℃ for 10 min; 30-35 cycles of 94℃ for 1 min, 52-55℃ for 1 min, 72℃ for 12 min. The number of amplification cycles and temperature conditions are shown in Table 4. The PCR conditions for all *EXP*s were fixed to 35 cycles of 55℃ except for 30 cycles of 55℃ for *EXPB1*. To confirm the uniformity of cDNA synthesis, cDNAs for actin were amplified at 27 cycles of 55℃. PCR products were separated by electrophoresis in 1.5% agarose gel, stained with ethidium bromide and visualized with the BioDoc-It System (UVP, Upland, CA, USA).

Table 4 Gene-specific primer sets used for the RT-PCR amplifications.

Gene Name	Primer Sequence 5' to 3'	Product Size bp	Accession No. cDNA	Accession No. Genomic DNA	RT-PCR temp. ℃	RT-PCR cycle
CDPK11	CACCTGCTGGCAGCTTTTAC GTAAAAGCTGCCGCAGGTGC	215	X81393		52	32
HSP82	GTTTATGGAGGCACTGGCTG TCTCAGTGGTCTTCTCAGTCC	334	Z11920		55	30
putative *ALDH*	GGAGCGAAATGGTCTACTTGTGC ATCCCCATCTTGTACTCGTCCC	352	AF148877		52	30
FK II	CGCCAACGACGAGAAGAACG CTTGGTGGTGCAGATGGCTC	360	AF429947		55	30
EXPA5	CATCGTCGTGGTAGTTGCAGT GTTCATAAGCAGCACACAGG	425	AF247162		55	35
EXPA6	CTTCGAGGGCAGGCAGTTCTAG GGAGTGAGTAGCAAACAAGC	378	AF247163		55	35
EXPA7	TGCAGGAAGAAGGGAGGGGTT TGCAGGAAGAAGGGAGGGGTT	464	AF247164		55	35
EXPA10	GGCAAAACATACACTGGCAAGC CATCAAGCCTCTGTAGTGC	256	AF247165		55	35
EXPB15	TTCTCCATCCGCCTCACGTC CCTCTGCATCTCGCCGTTATTA	68		AF391108	55	35
EXPB17	TACACCTCGCGCCTCAACTTC AACGCTCTCTTCTCCTTCGG	331		AF391110	55	35
EXPB18	CGACGGTGAACTACTAATGATCGC TGGTAAATCATCTGCGCCTCC	214	Os05g0246300		55	35

Primer sets for *EXP*s which were not shown in this table were adopted from Lee and Kende（2001）and Lee and Kende（2002）. RT-PCR conditions；35 cycles of 55℃ for *EXP*s but 30 cycles of 55℃ for *EXPB1*. 27 cycles of 55℃ for *Act1*.

Phylogenetic analysis

The sequences of *EXP*s examined by RT-PCR were taken 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

Gene expression analysis of *EXP*s

In the previous proteome analysis of rice mature anthers from plants grown under High-N and High-N-cooling, changes in the following proteins were observed: EXPA18, EXPB1 and EXPB13, HSP82, CDPK11, putative ALDH and FKII（Table 3）. For these candidate genes found by proteome analysis, gene expression patterns in mature anthers were analyzed by semiquantitative RT-PCR with gene specific primers. All semiquantitative RT-PCR experiments were carried out at least 3 times for each gene, and one series of data is shown in Figs. 13, 14 and 15. 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* was not changed under High-N or under High-N-cooling（Fig. 13）.

The rice genome contains 33 *EXPA*s and 18 *EXPB*s（http://www.bio.psu.edu/expansins/）. From these *EXP*s, gene expression of 26 *EXPA*s and 16 *EXPB*s in the anthers were examined（Figs. 14, 15）.

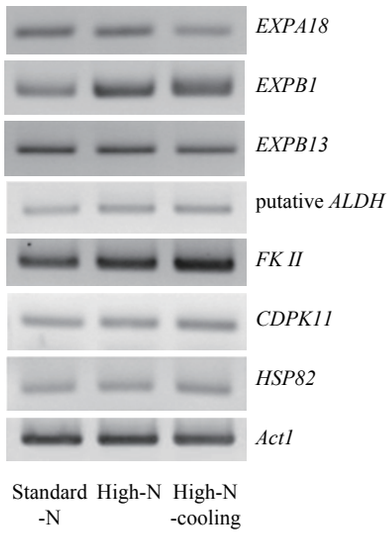


Fig. 13 Semiquantitative RT-PCR analysis of genes selected based on proteome analysis of rice mature anther.

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 visualized with the ethidium bromide. The template cDNA are the anthers about two hours before glumes opening. Standard-N; 10 ppm, High-N; 80 ppm, High-N-cooling; High-N and 12℃ for 3 days at the young microspore stage.

The RT-PCR images of *EXPA18*, *EXPB1*, *EXPB13* and *Act1* were identical to those in Fig. 13.

For *EXPA*s, gene expressions of all *EXPA*s except *EXPA12* in the anthers were observed (Fig. 14). High-N did not change gene expressions of most of *EXPA*s, but *EXPA1* was upregulated. High-N-cooling did not change gene expressions of *EXPA1*, *EXPA2*, *EXPA4*, *EXPA5*, *EXPA6*, *EXPA7* or *EXPA10*. It is noteworthy that 18 of 26 expressed *EXPA*s (*EXPA3, EXPA8, EXPA9, EXPA11, EXPA13, EXPA14, EXPA15, EXPA16, EXPA17, EXPA18, EXPA19, EXPA20, EXPA21, EXPA22, EXPA23, EXPA24, EXPA25, EXPA26*) were repressed under High-N-cooling (Fig. 14).

All *EXPB*s examined were expressed in the anthers (Fig. 15). Similar to the expression patterns of *EXPA*s, High-N did not change gene expressions of *EXPB*s except *EXPB1* and *EXPB5*, which were upregulated under High-N. High-N-cooling repressed 6 *EXPB*s (*EXPB5, EXPB7, EXPB11, EXPB12, EXPB14* and *EXPB17*) (Fig. 15).

Phylogenetic analysis of EXPs

Including *EXPA18*, *EXPB1* and *EXPB13*, a total of 25 *EXPA*s out of 26 *EXPA*s and 16 *EXPB*s were also expressed in the anthers. Among these genes, 18 *EXPA*s and 6 *EXPB*s were repressed under High-N-cooling. To infer the evolutionary relationship among these *EXP*s, phylogenetic analysis were undergone based on the deduced amino acid sequences of rice EXPAs and EXPBs. The phylogenetic tree showed that these EXPs were divided into 3 groups for EXPAs and 4 groups for EXPBs (Fig. 16). Group A1 contains 15 EXPAs including EXPA18, and 13 *EXP*s in this group were downregulated under High-N-cooling. In group A1, EXPA18, EXPA19 and EXPA20 were clustered in the same clade, and these 3 *EXPA*s were all downregulated under High-N-cooling. As expressions of a large number of *EXPA*s in group A1 were downregulated by High-N-cooling, phylogenetic analysis was executed between rice EXPAs in group A1 and 26 fully assigned Arabidopsis EXPAs. Only Arabidopsis EXPA11 (AtEXPA11)

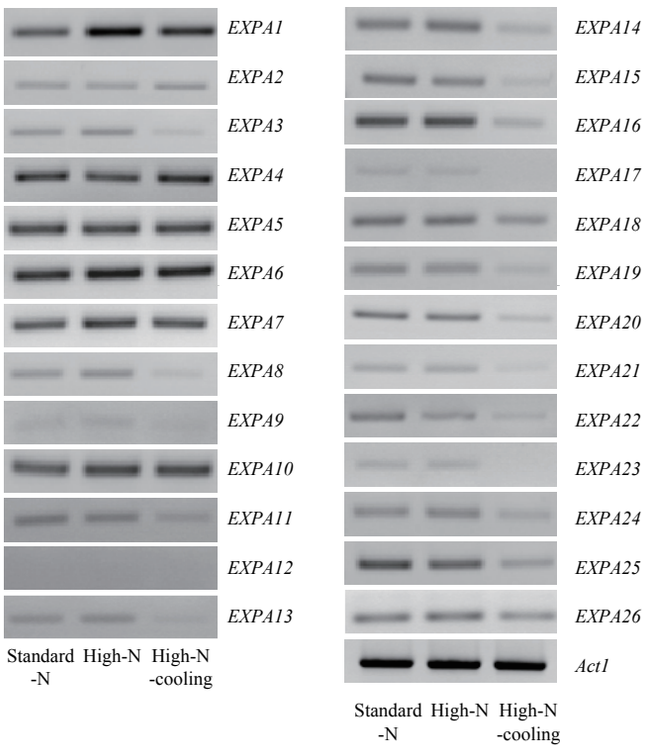


Fig. 14　Semiquantitative RT-PCR analysis of rice *EXPA*.

The RT-PCR image of *EXPA18* and *Act1* was identical to that in Fig. 13, respectively. Standard-N; 10 ppm, High-N; 80 ppm, High-N-cooling; High-N and 12℃ for 3 days at the young microspore stage.

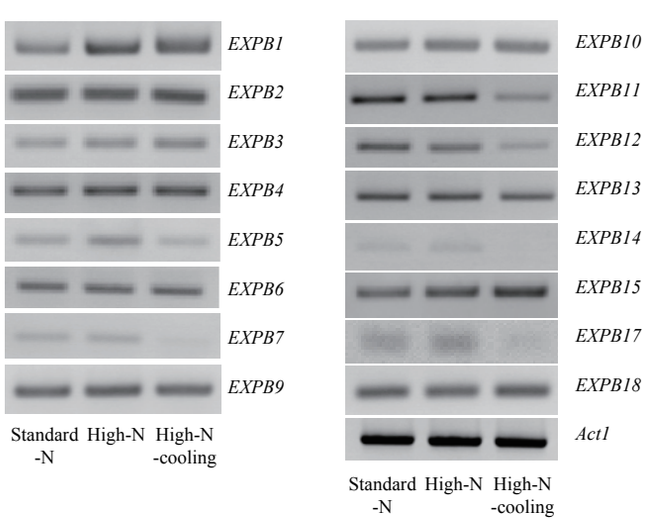


Fig. 15　Semiquantitative RT-PCR analysis of rice *EXPB*.

The RT-PCR image of *EXPB1*, *EXPB13* and *Act* was identical to that in Fig. 13, respectively. Standard-N; 10 ppm, High-N; 80 ppm, High-N-cooling; High-N and 12℃ for 3 days at the young microspore stage.

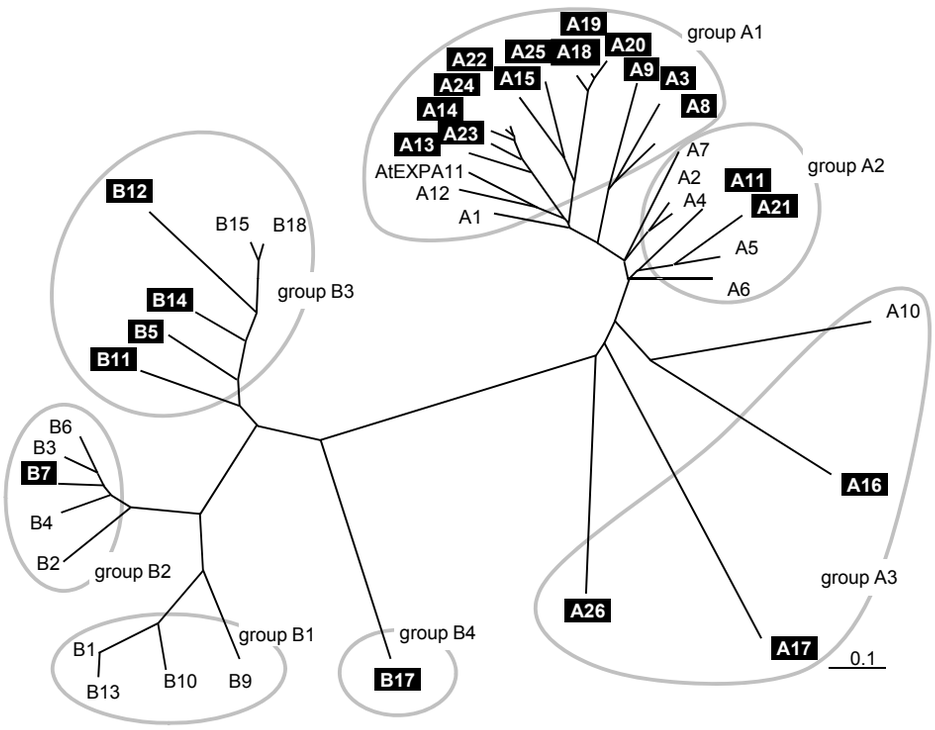


Fig. 16 Phylogenetic tree of rice EXPs.

A; EXPA, B; EXPB. AtEXPA11; Arabidopsis EXPA11. White letters in the black background indicated *EXP*s down-regulated 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; Itoh et al., 2007).
A multiple sequence alignment was performed with ClustalW ver. 1.83 program (Sugawara et al., 2008) and the results were displayed using the TreeView program (Page, 1996). Scale bar; 0.1 amino acids substitutions per site.

was phylogenetically linked to group A1 among 26 AtEXPAs (data not shown). According to the phylogenetic analysis of group A1 between rice and Arabidopsis, AtEXPA11 was more closely related to EXPA1 and EXPA12 than to EXPA18 (Fig. 16).

Discussion of the combined effects of High-N and cooling on the gene expression in mature anthers

High-N enhances the repression of pollen development and pollen viability by cooling at the young microspore stage (Chapter II). To clarify the physiological aspects involved in this enhancing effect of High-N, a comparative proteome analysis of the mature anthers was undertaken. Eleven proteins in the anthers altered under High-N and/or High-N-cooling were observed (Chapter III).

Because of the similarity between the expression patterns of protein and genes, changes in EXPA18, EXPB1, putative ALDH and FKII proteins in the anthers are assumed to be regulated at the transcriptional level. However, the gene expression patterns of *EXPB13*, *CDPK11* and *HSP82* were not similar to the pattern of proteins, suggesting the possible post-transcriptional modulation of these genes, such as the regulation of translation, protein maturation and protein turnover.

At flowering, pollen grains swell rapidly for anther dehiscence (Matsui *et al.*, 1999), and pollen grains shed on the stigma start to germinate and elongate pollen tubes. It is assumed that 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 pollen tube penetration, glucanase and xylanase in the pollen coat are thought to break down the stigma wall, and an EXP-like protein is reported to be present on the wall of the pollen tube tip in maize（Suen *et al.*, 2003）.

As described in the introduction, Zea m 1 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 *et al.*, 1997; Wu *et al.*, 2001; Li *et al.*, 2003; Wei *et al.*, 2004）. Based on the abundance of EXPB1 protein in mature anthers（Table 3, Fig. 12）and the close similarity between the amino acid sequences of EXPB1, EXPB13 and Zea m 1, it is likely that rice EXPB1 and EXPB13 have similar functions to those of Zea m 1 and may be related to decreased fertility. Protein and gene expression of EXPB1 was, however, upregulated by High-N and was not downregulated by High-N-cooling in the present study. Imin *et al.*（2004）observed partially degraded EXPB1 proteins in trinucleate stage anthers after cooling at the young microspore stage and suggested 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 in rice have been observed to be expressed in different tissues. *EXPA1*, *EXPA4*, *EXPA5*, *EXPA10*, *EXPA13* and *EXPA16* are expressed both in leaves and roots（Cosgrove, 2000; Lee and Kende, 2001; Shin *et al.*, 2005）, whereas *EXPA5*, *EXPA10*, E*XPA18* and *EXPA26* are expressed in 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* and *EXPB6* are expressed in both above-ground tissues and roots, while *EXPB11* and *EXPB12* are expressed only in above-ground tissues（Lee and Kende, 2002）. These results, however, were obtained under normal conditions, and the changes in gene expression under stress conditions are not well known. Therefore, expression patterns of members of the *EXP* gene family in the anthers under High-N and High-N-cooling were examined.

Comprehensive gene expression analysis showed that all *EXPA*s examined（*EXPA1* to *EXPA26*）except for *EXPA12* were expressed in anthers, and High-N-cooling repressed 18 *EXPA*s（Fig. 14）. As mentioned above, *EXPA5*, *EXPA10*, *EXPA18* and *EXPA26* are expressed in rice anthers（Shin *et al.*, 2005）. Among these 4 *EXP*s, High-N-cooling downregulated *EXPA18* and *EXPA26*. Gene expression of *EXPB*s in anthers has not been well studied. In this study, 16 *EXPB*s were expressed, and High-N-cooling repressed 6 of them（Fig. 15）.

High-N-cooling repressed a total of 18 *EXPA*s and 6 *EXPB*s but upregulated EXPB1. The downregulation of large numbers of *EXP*s by High-N-cooling indicates that these *EXP*s may be involved in decreases in pollen germination and fertilization. Under High-N-cooling, HSP82 and CDPK11 were downregulated（Table 3, Figs. 10, 12）. 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 both mature and germinating pollen. It is required for pollen germination（Taylor, 1997）and has been shown to participate in cold stress signaling（Abbasi *et al.*, 2004）. These properties of HSP and CDPK suggest that HSP82 and CDPK11 may be involved in the downregulation of EXPs in rice anthers under cooling stress.

To infer the evolutionary relationship among these EXPs, a phylogenetic analysis was undergone. The generated phylogenetic tree was divided into 3 groups for EXPAs and 4 groups for EXPBs. As High-N-cooling repressed most *EXPA*s in group A1, including *EXPA18*, a phylogenetic analysis was executed between rice EXPAs in group A1 and Arabidopsis EXPAs, and only AtEXPA11 was phylogenetically linked to group A1. AtEXPA11 was similar to rice EXPA18（61% identity）; however, AtEXPA11 had much stronger 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. 16）.

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 *EXP*s, 10 *EXPA*s including *EXPA18*, *EXPA19*, *EXPA20* and 7 *EXPB*s（Expansin Central; http://www.bio.psu.edu/expansins/）. Especially, *EXPA18*, *EXPA19* and *EXPA20* are arranged in tandem on the long arm of chromosome 3 and showed high similarity to each other. In addition to these sequence similarities and gene arrangements, High-N-cooling repressed gene expression of all 3 of these *EXP*s and downregulated EXPA18 protein. Therefore, it is hypothesized that EXPA18, EXPA19 and EXPA20 form a distinct subfamily that may be similarly regulated by environmental conditions in rice and play a crucial role in responding to the repression of pollen germination under High-N-cooling. It is also possible that monocotyledonous plants, including rice, have diversified group A1-type EXPs from a common ancestor of monocotyledons and dicotyledons, to adapt to environmental stresses such as cool temperature during anther development and pollen germination under different nitrogen conditions.

In summary, when gene expression patterns of rice anther genes in relation to the effects of High-N on cool-temperature damage were analyzed, a total of 25 *EXPA*s and 16 *EXPB*s were expressed in the anthers. *EXPA1* and *EXPB1* were upregulated under High-N, and 18 *EXPA*s, including *EXPA18*, and 6 *EXPB*s were downregulated under High-N-cooling. As High-N-cooling repressed numbers of *EXP*s, it appears that these EXPs may be involved in the enhanced decreases in the pollen germination ratio under High-N-cooling.

V Discussion

Conquering the cool-temperature damages caused by frequent cool weather in the summer season has been a big challenge for rice cultivation in northern Japan. In 1993, Japan experienced the coolest weather it had had in a century, which caused a catastrophic decrease in the rice yield, especially on the Pacific Ocean side of northern Japan, because of spikelet sterility. In the special research following this extraordinary event, it was concluded that the yield reduction was a result of the sterility caused by the continuous cooling during the booting stage, which is the stage most sensitive to coolness（Mayumi, 1994）. Study results also confirmed that the decrease in the yield caused by cool weather correlated with the increase in nitrogen level（Kodama, 1994; Miyamori, 1994; Takeda, 1994; Tamagawa, 1994）.

Satake *et al.*（1987）clearly demonstrated that High-N supply from the spikelet differentiation stage to the young microspore stage increased the sensitivity to coolness at the critical stage. The anther length and the number of pollen grains decrease under High-N conditions（Lee, 1990; Tatsuta, 1999）. As the nitrogen level increases, the number of engorged pollen grains in cooled plants decreases（Lee, 1990）. A variety of studies showed that cool temperature at the young microspore stage is the period most harmful to rice reproduction and causes a decrease in the number of engorged pollen gains, resulting in increased sterility（Hayase *et al.*, 1969; Satake and Hayase, 1970; Nishiyama, 1982; Satake *et al.*, 1988; Satake, 1991; Satake and Shibata, 1992）. From these reports, it is speculated that the decrease in the suppression of pollen development is a major factor in increased sterility also in the case of High-N.

Often cool summer weather is accompanied by a low amount of solar radiation; however, the effects of shading vary among rice cultivars（Shimazaki, *et al.*, 1964; Wada *et al.*, 1972）. In the present study, the influence that both High-N and shading exerted on the cool-temperature damage was examined focusing on pollen development.

1 Effects of high nitrogen supply and cooling at the young microspore stage on factors related to sterility

The effects of High-N-cooling at the young microspore stage on the factors involved in sterility, such as the numbers of microspores, engorged pollen grains and pollen grains on the stigma as well as the pollen germination ratio were analyzed with Hayayuki, an early-maturing japonica rice variety that has been used for research on cool-temperature damage. The number of microspores is the limiting factor for the number of pollen grains and cool tolerance increases as the microspore increases（Satake, 1989）. It was reported that High-N decreases the number of microspores（Lee, 1990）. In the present study, the number of microspores was reduced by High-N and/or shading, and it was strongly reduced by High-N plus shading even under normal temperatures. These results suggest that the pollen production ability is lowered by High-N and shading by decreasing the number of microspores.

The number of engorged pollen grains was markedly decreased by cooling at the young microspore stage. These decreases were enhanced by High-N and shading. The decreases in the number of engorged pollen grains were more notable than the decreases in the number of microspores. As the number of engorged pollen grains was decreased, the fertility declined.

Cooling decreased both the number of engorged pollen grains shed on the stigma and the number of germinated pollen grains on the stigma. High-N caused further decreases. The number of engorged pollen grains on one stigma was close to 100 even under High-N-cooling plus shading, and this was enough pollen for setting a seed. Even in plants with similar numbers of pollen grains shed on the stigma, the fertility in cooled plants was lower, and it was much lower under High-N and shading. These results suggest that pollen grains in cooled plants, which seemed to be normal, might have lower activity.

After gaining these results, the viability of pollen grains in plants grown under High-N and High-N-cooling conditions was solely focused on. Pollen germination and pollen tube elongation were notably suppressed by High-N-cooling. To exclude the effect of the stigma and to quantify the pollen viability, the pollen germination was tested on an agar plate, and conspicuous suppression of pollen germination by High-N-cooling was observed.

Taken together, the enhanced suppression of pollen development and pollen viability are likely to be involved in the intensified reduction in fertility under High-N-cooling. The suppression of pollen germination by High-N-cooling was notable and may have a direct effect on sterility.

2 Proteome analysis and gene expression analysis

Recently, the map-based sequencing of the rice genome was completed, and about 37,000 genes were detected（Rice Genome Sequencing Project, 2005）. From the gene expression analysis, we can identify genes that may contribute to physiological phenomena. Because the transcript levels of genes are, however, sometimes inconsistent with the levels of translated proteins（Hirano, 2001）, it is desirable to examine of both gene expression and protein expression in the tissue. Proteome analysis is a powerful tool and has been remarkably developed in the last 10 years. In proteome analysis, a large number of proteins are analyzed by separation and purification of proteins and identification of protein by protein or genome database（Hirano, *et al.*, 2004）.

To clarify the changes in physiological aspects of the damages on pollen grains caused by High-N-cooling, protein expression patterns in mature anthers were observed by comparative proteome analysis. In mature anthers, only 11 spots whose densities were changed by High-N and High-N-cooling treatments were observed, and 7 out of 11 protein spots were identified as known rice proteins. Three proteins were expansins（EXPs）that are involved in cell elongation. Alpha-EXP18（EXPA18）and beta-EXP1（EXPB1）

were increased by High-N. EXPA18 was decreased and EXPB13 was increased by High-N-cooling. Three other proteins involved in stress responses, namely, calcium-dependent protein kinase 11 (CDPK11), putative aldehyde dehydrogenase (ALDH) and heat shock protein 82 (HSP82), were increased by High-N. Although CDPK11 and HSP82 were decreased by High-N-cooling, Fructokinase II (FKII), which is involved in sugar metabolism, was increased by the same treatment. These results indicate that these proteins might be associated with a repression in the pollen activity.

As noted above, the level of protein expression is not always consistent with the level of gene expression, so the gene expression patterns of those candidate genes detected from the proteome analysis were analyzed. From the similarities between the patterns of gene expression and protein expression, the changes in EXPA18, EXPB1, putative ALDH and FKII proteins in the anthers are assumed to be regulated at the transcriptional level. Gene expression of EXPB13, CDPK11 and HSP82 were not changed by High-N or High-N-cooling. This may suggest the post-transcriptional modulation of these genes, such as the regulation of translation, protein maturation and protein turnover. Taken together, changes in cell elongation and sugar metabolism might be involved in damages to pollen grains. Since these 3 proteins known to be involved in stress responses were increased by High-N, it is also speculated that High-N itself might act as a kind of stressor.

It is interesting to note that 3 out of 7 changed proteins were EXPs. As EXPs have cell wall loosening activity, it seems likely that EXPs are involved in pollen grain swelling, germination and pollen tube elongation. Since the cell wall loosening is thought to be essential for these processes, expression patterns of members of the *EXP* gene family, 26 *EXPA*s and 16 *EXPB*s, in the anthers under High-N and High-N-cooling were examined. All 26 *EXPA*s and 16 *EXPB*s examined were expressed in the anthers, except *EXPA12*, and 18 *EXPA*s and 6 *EXPB*s were found to be repressed under High-N-cooling.

The downregulation of large numbers of *EXP*s by High-N-cooling suggest that these *EXP*s might be involved in the decreases in pollen germination and fertilization. In addition, HSP82 and CDPK11 were downregulated by High-N-cooling. Arabidopsis *AtHSP81*, which belongs to the same HSP90 family as rice HSP82, is expressed in pollen grains (Yabe *et al*., 1994). CDPK is reported to be transcribed in both mature and germinating pollens and to be required for germination (Taylor, 1997). It is also reported to participate in cold stress signaling (Abbasi *et al*., 2004). These properties of HSP and CDPK suggest that HSP82 and CDPK11 may be involved in the downregulation of EXPs in rice anthers under cooling stress. Among these downregulated *EXP*s, *EXPA18*, *EXPA19* and *EXP20* had high similarities in the deduced amino acid sequences, suggesting that these three genes may constitute a distinct functional gene subfamily related to the decrease in the pollen germination ability caused by High-N-cooling.

In this study, the effects of High-N and High-N-cooling on increased sterility in rice plants were examined, and it was revealed that a number of *EXP*s were downregulated by High-N-cooling. It appeared that these EXPs might be involved in the enhanced decreases in pollen germination ratio, and consequently, cool-temperature damage under High-N-cooling. These results were obtained using mature anther samples, including various sporophytic anther tissues such as anther walls and vascular bundles, and gametophytic pollen grains. These individual tissues have a diverse range of transcriptional activity. Studies on the expression patterns and localization of transcripts and products of these genes in the germinated and/or sterile pollen grains are needed to clarify the role of EXPs, CDPK11 and HSP82 in rice pollen grains. Further studies on EXPs may contribute to improving the fertility potential of rice plants.

As the world population is increasing rapidly, food production must increase, which includes the production of rice, a major food crop. To improve the productivity of rice, it is of great importance to improve the cultivating technology in various environments. Even in tropical and subtropical areas, the

double-cropping of rice is occasionally difficult because of the cool temperature during the dry season (Farrell, 2006; Nishiyama, 1985). The early cropping of rice can also be subjected to cool weather in spring (Nishiyama, 1985). Nitrogen application systems must be renovated to guarantee both high yield and less damaging effects due to unpredictable cool weather. The findings obtained in the present study regarding the effects of High-N on cool-temperature damages in rice plants will form an important basis for future research to improve rice tolerance against cool weather in various soil conditions, as well as providing useful information for other areas of plant reproduction science.

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