Sterility Caused by Cooling Treatment at the Flowering Stage in Rice Plants

II. The abnormal digestion of starch in pollen grain and metabolic changes in anthers following cooling treatment

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As reported in the previous paper\textsuperscript{a}, sterility caused by cooling at the flowering stage in rice plants increased as cooling was prolonged and as the flower approached anthesis. Sterility was associated with a decrease in the number of pollen grains that germinated on the stigma. This suggests that matured pollen grains which are ready for anthesis may lose the ability to germinate during cooling treatment.

This study was undertaken to investigate the digestion of starch in pollen grains and metabolic changes in anthers following cooling treatment.

Materials and Methods

Culture and cooling treatment

Twenty seeds of rice variety Hayayuki were directly sown in a circular pattern in a 4-liter plastic pot. Plants were grown in a naturally lit room at 24/19°C day/night. Only the panicles of main stems with the same heading date were used. Flowering started 3 days after heading. The cooling treatment was at 12°C in a naturally lit room. It was started at 08:30 a.m. 2 days after heading (1 day before the start of flowering) and ended at 08:30 a.m. after a different number of days depending on the experiment. After cooling treatment, plants were moved to the room controlled at 24/19°C and allowed to flower. Spikelets used in the experiments were the most matured ones which were 1 day before flowering at the start of treatment. The order of flowering reported in the previous paper\textsuperscript{a} was applied to decide which spikelets were examined.

Pollen observation

Spikelets from 2 days before anthesis to the beginning of anthesis were fixed with formalin-acetic acid-50\% ethanol (5:5:90, v/v/v) (FAA) solution and pollen grains from them were observed after staining with iodine-potassium iodide (I\sub{2}-KI) solution. Pollen grains were classified into 5 types according to the digestion pattern of starch, as shown in Fig. 1. The percentage of each type was counted by observing 60 anthers from 10 spikelets.

Carbohydrate

Sixty anthers were homogenized in 80\% ethanol. Reducing and total sugars were determined in the extract by the Somogyi-Nelson method\textsuperscript{[12]} and anthrone method\textsuperscript{[14]}, respectively. Starch in the residue was solubilized by heating in a boiling water bath for 15 min, and 3.6 units of salivary amylase (one unit of the enzyme can liberate 0.1 mg of glucose from soluble starch per hour at 37°C) was added into the suspension. Reducing sugars in the clear suspension were determined by the Somogyi-Nelson method.

Enzyme assays

Crude extract was prepared by homogenizing 250 anthers in 1 ml distilled water and by centrifuging at 3000 rpm for 10 min. Each assay was incubated at 37°C for 30 min. as follows: Amylase assay: 160μl 2% soluble starch solution, 40μl extract and 100μl 0.1 M acetate buffer (pH5.0). Glucose was determined by the Somogyi-Nelson method. Acid phosphatase assay: 20μl 2mM disodium p-nitrophenol phosphate, 50μl extract and 200μl 0.2M phosphate buffer (pH6.0). The yellow color of p-nitrophenol was measured.
at 420 nm. Amylophosphorylase assay: 160 μl, 5% soluble starch solution, 150 μl 0.1 M glucose-1-phosphate, 50 μl extract and 280 μl phosphate buffer (pH 6.3). Inorganic phosphate was measured by amidol method 49.

Respiration

Respiratory activity was measured with micromanometer at 30°C using the same method of Nishiyama 50. Thirty anthers were used for each measurement.

Adenosine triphosphate (ATP) level

ATP was assayed using firefly luciferin-luciferase 51. HEPES buffer (10 ml) containing 36 anthers was boiled for 20 min. and cooled to 0°C in an ice bath, and 0.5 ml of the exarax was mixed with 0.5 ml enzyme solution and the fluorescence was measured for 60 sec.

Results

1. Digestion of starch in pollen grains

Fig. 1 shows the normal and abnormal digestion patterns of starch in pollen grains. Starch in normally engorged pollen grains decreased just before anther dehiscence at the end opposite to the germ pore and pollen grains become sugar type ones. Starch in cooled pollen grains began to decrease around the whole of the cell wall, including the germ pore site.

Fig. 2 shows that pollen grains were filled with starch 1 day before anthesis and that the starch in these grains was digested over a few hours just before anther dehiscence.

Fig. 3 shows that during the cooling treatment the percentage of pollen grains filled with starch decreased, while the percentage of pollen grains with abnormally digested starch increased during cooling treatment. The percentage of abnormal digestion of starch was not so large (ca. 32%) at the end of 5 day treatment. After the cooling treatment, however, the proportion of cells with abnormal digestion of starch increased rapidly. This effect was more pronounced after the longer treatment with cooling. The proportion of normal pollen grains which included engorged (0), partly digested (+1) and fairy digested (+2) pollen grains corresponded to the spikelet fertility.

As shown in Fig. 4, the correlation between the percentage of fertility and that of normal pollen grains at anther dehiscence was highly

![Fig. 2. Time course of starch engorgement in normal pollen grains. -3h, -27h and -51h mean 3, 27 and 51 hours before anther dehiscence, respectively.](image-url)
positive, with $r = 0.836^{***}$ (n = 19).

2. Changes in the metabolic activities of anthers following cooling treatment

Fig. 5 shows decrease in the fertility of spikelets with the time for which they had been cooled at 12°C. As shown in Fig. 6, starch content decreased with treatment for 4 days or more. The increase of starch for the first 2 days suggests that engorgement of starch proceeded to some extent even after the start of treatment. In contrast to the decrease in starch, total sugars increased during the treatment. Total carbohydrate (starch and sugars) decreased after the 2 day treatment.

Enzyme activities related to carbohydrate metabolism are shown in Fig. 7. Amylophosphorylase is related to starch degradation and synthesis depending on inorganic phosphate levels, and acid phosphatase is concerning with inorganic phosphate and carbohydrate metabolism. No significant changes in the three enzyme activities were found following cooling treatment.

Fig. 8 shows a change in the respiratory activity increased to a maximum after 4 day treatment and then decreased after 6 and 8 day treatments. Even after 8 day treatment, however, the respiratory activity was still higher than the initial level.

Fig. 9 shows changes in ATP level in anthers during cooling treatment. ATP level reached to a maximum after 4 day treatment as 1.8 times higher than the initial level and at the end of 8 day treatment ATP level remained higher than the initial one.

Discussion

In general, pollen grains in which starch was digested just before anther dehiscence at the end opposite to the germ pore have a high

![Diagram](image)

Fig. 3. State of starch engorgement and starch digestion in pollen grains at the end of cooling treatment and at anther dehiscence.

C, 3T and 5T mean control (not treated), at 12°C for 3 days and at 12°C for 5 days, respectively.

- +2: fairly digested.
- +1: partly digested.
- 0: engorged.
- -1: abnormal digestion proceeded partly.
- -2: abnormal digestion proceeded fairly.
- -0: percentage of fertility.
potential for germination. In normally engorged pollen grains, starch was rapidly digested at the end opposite to the germ pore 3-4 hours before anther dehiscence (Figs. 1, 2, 3). During the cooling treatment, starch in the pollen grains was only gradually digested. After the treatment, digestion of starch proceeded rapidly not only at the end opposite to the germ pore but also over the whole of the inside of the pollen wall (Figs. 1, 3).

The results of microscopy suggest that during and after the cooling treatment, enzyme activities involved in starch catabolism and sugar metabolism were retained and starch was consumed as a substrate for respiration. As shown in Fig. 6, the starch content of cooled pollen grains gradually decreased, and soluble sugar content concomitantly increased. Amylase, amyl phosphorylase and acid phosphatase activities were not altered during this time (Fig. 7).

The respiratory activity of the cooled
Fig. 8. Changes in the respiratory activity of anthers following cooling treatment.

Fig. 9. Changes in ATP level in anthers during cooling treatment.

Anthers increased up to 4 days after the start of treatment and then returned to the level of uncooled anthers at the end of 8 day treatment (Fig. 8).

These data supported the previously mentioned suggestion that functions involving respiration are retained during cooling treatment and the digestion of starch and increase in soluble sugar content could result in a temporary increase in respiration.

Nishiyama reported that cooling treatment at the young microspore stage reduced acid phosphatase activity of anthers but not that of amylophosphorylase. The discrepancy concerning acid phosphatase activity may be attributed to the different sensitivity of this enzyme to cool temperature depending on the development stage of these cells in the anthers.

ATP level in anthers cooled for 4 days increased by 1.8 times over the initial level. Although it decreased with prolonged treatment, at the end of 8 day treatment ATP level remained still higher than the initial one (Fig. 9). The reason for the distinct increase can be explained by an idea that even under cool condition oxidative phosphorylation still continued to some extent and the depression of synthetic pathways reduced a consumption of ATP.

Seo et al. reported that ATP levels in anthers decreased by one third after the end of a cooling treatment at 15°C for 5 days. Their data do not agree with ours', although their changes in ATP level during treatment at 15°C were not shown. The reason why the responses of ATP level to cooling differed in the two sets of experiments is uncertain. It should be noted, however, that the anthers used in their experiment were 3 days younger than the ones we used. A number of papers have dealt with the relation between ATP levels and chilling stress. The decrease of ATP level caused by chilling stress is not a general finding. KABAKI et al. detected no decrease of ATP level in the radicles of rice seedlings treated at chilling temperatures above 6°C for 24 and 120 hours. Hardening at 15°C caused an increase in ATP after 2 days in the leaves and roots of cotton seedlings and 12°C for 4 days in the leaves of Phaseolus vulgaris.

The percentage of fertility decreased with prolonged cooling treatment, and this decrease had positive correlation to the increase of the percentage of abnormally digested pollen grains (Figs. 3, 4). This result strongly suggests that abnormal digestion of starch in pollen grains is a cause of the reduced ability of pollen to germinate, resulting in spikelet sterility.

The reason why the abnormal digestion of starch takes place in cooled pollen grains remains uncertain, but during cooling treatment the cellular compartmentation of pollen grains might be damaged and the integrity of enzymatic reactions which were strictly required at the time of pollen germination might be perturbed.
Summary

Rice plants at the flowering stage were cooled at 12°C for different length of day from 2 to 8 days under natural light in the phytotron. The abnormal digestion of starch in pollen grains was observed microscopically and metabolic activities of anthers were examined.

In normal pollen grains, after the end of starch engorgement, starch was rapidly digested at the end opposite to the germ pore 3-4 hours before anther dehiscence, and the more than 70% of pollen grains became sugar type ones at anther dehiscence (Figs. 1, 2, 3). In cooled pollen grains starch was digested around the inside of the pollen cell wall, being different from the locality that was observed in normally digested pollen grains (Fig. 1). This abnormal digestion of starch partly took place during the treatment and proceeded rapidly after the treatment (Fig. 3).

During the treatment, the starch content of anthers decreased, and soluble sugar content concomitantly increased (Fig. 6). This result agreed with the microscopical examinations. Enzyme activities involved in carbohydrate metabolism showed no changes (Fig. 7), and respiratory activity (Fig. 8) and ATP (Fig. 9) of anthers increased temporarily following the treatment. These results suggested that the system involving respiration and carbohydrate metabolism preserved their activities even during the cooling treatment. Thus, starch was partly consumed as a substrate for respiration during the treatment and rapidly digested after the treatment. The data, however, could not explain the reason why the digestion of starch occurred abnormally.

The percentage of fertility decreased with prolonged treatment, and this decrease was positively correlated to the increase of percentage of abnormally digested pollen grains (Figs. 3, 4).

The experiment strongly suggests that the abnormal digestion of starch caused by cooling treatment is a cause of the reduced ability of pollen for germination.

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References

[和文摘要]

イネの開花期冷温処理による不稔

第2報 花粉の糖化異常と薬の生理活性

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開花期のイネをファイトロン自然光室で12℃、2～8日間冷温処理し、花粉内でんぶん糖化異常の顕微鏡観察と薬の生理活性の測定を行った。

正常な花粉の発育過程では、でんぶんが花粉の中心から端に充満したのち、発芽孔の反対側ででんぶんが一部消し（糖化）する（第1図）。このでんぶん糖化は開顕直前の3～4時間の間に急速におこり、正常な条件では開顕開始時において70％以上が糖化型花粉であった（第2、3図）。冷温処理された花粉のでんぶん糖化は花粉壁内側の周辺全体からおこり、正常な糖化とは明らかに異なる（第1図）。この糖化異常は処理中に一部おこるが、処理終了後開顕開始までの間に急速に進んだ（第3図）。

薬によりでんぶん含量は処理日数の増加に伴って減少し、可溶性糖含量は一時的に増加した（第6図）。これは処理中の花粉でんぶん糖化の顕微鏡観察の結果とよく符号した。ATP含量は冷温処理によって低下しなかった（第9図）。これらの事実は、冷温処理によっても呼吸と炭水化物代謝に関する機能は保持されていることを示し、処理中に呼吸基質としてでんぶんが一部消費されること、また処理終了後の急速な糖化現象を支持している。しかし、糖化が正常型と異なって何故異常となるかは、この結果からは説明できなかった。

処理日数の増加による稔実歩合の低下と糖化異常花粉歩合との正の相関関係が認められ（第3、4図）。冷温処理による糖化異常が花粉の発芽能力の低下の原因と考えられた。