Proteins and Carbohydrates in Developing Rice Panicles with Different Numbers of Spikelets
— Cultivar difference and the effect of nitrogen topdressing —

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Abstract: Proteins and carbohydrates in developing rice panicles were analyzed to see whether these parameters control spikelet number in rice. Two rice cultivars and 2 levels of nitrogen topdressing were used to obtain panicles with different numbers of spikelets. A japonica rice cultivar, Nipponbare, with topdressing (H) had 1.8 times more spikelets per panicle than that without topdressing (L). Moreover, the number of spikelets per panicle in an indica rice cultivar, Takanari, without topdressing was 2.7 times larger than that in Nipponbare-L. Panicles with more spikelets (LP) in Nipponbare-H and Takanari-L showed slower growth than those with fewer spikelets (SP) in Nipponbare-L in an early stage. LP, however, increased markedly in size thereafter, eventually exceeding SP, in length and fresh weight. Soluble protein content was higher in LP than SP in an early stage, but this difference was hardly detected in a late stage. No clear difference was observed in sugars or starch between LP and SP. Analysis of soluble and insoluble proteins by SDS-polyacrylamide gel electrophoresis showed that bands corresponding to insoluble proteins with a molecular weight about 42 kDa were present at higher intensities in LP than in SP. These results suggest that the spikelet number in rice is controlled by the soluble protein content in an early stage and insoluble proteins with a molecular weight of 42 kDa during panicle development, but not by the carbohydrates in developing panicles.

Key words: Carbohydrates, Cultivar difference, Nitrogen, *Oryza sativa* L., Panicle, Proteins, Rice, Spikelet number.

Rice grain yield is mostly determined by the number of spikelets per unit area and by the percentage of ripened grains (Matsushima, 1980). Since rice grains are formed using carbohydrates produced by photosynthesis, it is highly important to improve the percentage of ripened grains. In many cases, however, spikelet number per unit area limits grain yield (Yoshida, 1981), indicating that it is also necessary to increase the number of spikelets for high yield.

There is little difference in spikelet number per unit area between old and new Japanese cultivars (Takeda et al., 1984a; Maruyama et al., 1988). New Korean cultivars and high-yielding indica cultivars have a higher number of spikelets per panicle and per unit area than Japanese cultivars (Takeda et al., 1984b; Maruyama et al., 1988). Despite the difference in spikelet number per panicle between the high-yielding indica and Japanese cultivars investigated morphologically elsewhere (Yamagishi et al., 1992; Fukushima, 1999; Kobayasi et al., 2002), the physiological basis of the difference remains unclear.

Nitrogen fertilization especially nitrogen topdressing at neck-node initiation effectively increases the number of spikelets per unit area (Matsushima, 1980; Senanayake et al., 1996). A close relationship was found between the number of spikelets and the amount of nitrogen absorbed by rice plants until spikelet differentiation (Wada, 1969). The efficiency of nitrogen for spikelet formation, however, varies with the region of Japan where rice plants grow (Murayama, 1969; Hasegawa et al., 1994). This difference in the response of spikelet formation to nitrogen was ascribed to the differences in nitrogen concentration during panicle formation (Hasegawa et al., 1994; Kobayasi and Horie, 1994), suggesting that carbohydrates and other factors also are related to spikelet formation. Nevertheless, nonstructural carbohydrates in stems hardly affect spikelet differentiation (Kobayasi et al., 2001).

Since the amounts of nitrogen and nonstructural carbohydrates were measured in shoots or stems in previous investigations (Murayama, 1969; Hasegawa...
et al., 1994; Kobayasi and Horie, 1994), these parameters do not necessarily reflect the metabolism of developing panicles. More precise investigation is needed to analyze causal relationships between panicle constituents and spikelet number. We measured proteins and carbohydrates in developing rice panicles with different numbers of spikelets, using different cultivars and different levels of nitrogen topdressing.

Materials and Methods

1. Plants and growth conditions
An experiment was conducted in a greenhouse at National Institute of Crop Science in 2001, using a japonica rice cultivar (Oryza sativa L., cv. Nipponbare) and an indica cultivar (Oryza sativa L., cv. Takanari). The seeds of each cultivar were sown in a nursery box filled with soil on April 21. Three seedlings at the 4th leaf stage were transplanted into a 9-liter plastic pot filled with paddy soil on May 18. The soil in the pot was basally fertilized with 0.3 g each of nitrogen, phosphate and potash. The half pots of Nipponbare were top-dressed with 0.3 g nitrogen per pot on July 2. This was about 7 days before panicle initiation. Other pots of Nipponbare and pots of Takanari were without topdressing. The water level in pots was maintained at 3 cm above the soil surface throughout the experiment. Air temperature in the greenhouse was controlled within the range from 15 to 30°C.

2. Measurement of panicle characteristics
Panicle tissues were sampled in the morning at 3- to 4-day intervals from panicle initiation to heading. The beginning of panicle initiation was roughly estimated by plant age in leaf number, and actual panicle initiation was determined by the precise observation of shoot apex under a microscope. The panicle tissue of main stems from 12 plants was dissected carefully from shoot apex with a fine needle under a microscope, and then the length and fresh weight of tissue were quickly measured. After measurement, the sample was stored at −85°C, until further analysis.

Fully developed panicles were sampled from main stems of 18 plants in each treatment after heading. The numbers of visible rachis branches and spikelets per panicle were counted, and used as the numbers of surviving rachis branches and spikelets. The number of degenerated rachis branches and spikelets was also estimated according to Matsushima (1980). The number of differentiated rachis branches was calculated as the number of degenerated rachis branches plus the number of surviving ones. The number of differentiated spikelets was calculated in the same way as rachis branches.

3. Measurement of proteins
The amount of proteins in developing panicles was measured as described elsewhere (Maruyama et al., 1990). Panicle tissues were homogenized in a prechilled extraction buffer that contained 50 mM Tris-HCl (pH 7.5), 1 mM Na₂EDTA, 8 mM MgCl₂ and 2% (v/v) 2-mercaptoethanol, and the homogenate was centrifuged at 18,000 g for 20 min at 4°C. The supernatant was removed to another tube, and an aliquot (50 µl) was subjected to analysis by SDS-PAGE of the soluble protein fraction. The rest of the soluble protein fraction was mixed with trichloroacetic acid, and left to stand for 30 min. After centrifugation at 18,000 g for 20 min at 4°C, the pellet was reextracted with 0.1 M NaOH that contained 2% (w/v) SDS at 40°C for 60 min. The protein content of the fraction was determined by the method of Lowry et al. (1951), with bovine serum albumin as a standard.

Insoluble proteins in the precipitate were washed 3 times with the extraction buffer without 2-mercaptoethanol to remove soluble proteins. Insoluble proteins were extracted from the washed pellet after suspension in 50 mM Tris-HCl (pH 6.8) that contained 8 M urea and 3.2% (w/v) SDS. The suspension was kept at 40°C for 60 min, and then centrifuged at 18,000 g for 20 min at 25°C. Fifty microliters of the supernatant were taken for the analysis by SDS-PAGE, and the rest was subjected to measurement of insoluble proteins.

4. Measurement of carbohydrates
Sugars and starch in developing panicles were measured as described elsewhere (Wang et al., 1999). Panicle tissues were placed in 20 mM HEPES-NaOH buffer (pH 7.4) and heated at 90°C for 5 min. The heated sample was homogenized on ice with fine quartz sand, and then centrifuged at 18,000 g for 10 min at 4°C. The supernatant was used for the analysis of soluble sugars. Glucose, fructose, and sucrose in the supernatant were determined by coupled enzymatic assay (Bergmeyer and Bernt, 1974).

The precipitate was washed 3 times with 80% methanol to remove soluble sugars. The washed pellet was suspended in distilled water, and the suspension was heated at 90°C for 2 hr. The gelatinized starch was digested with amyloglucosidase at 55°C for 30 min, and resultant glucose was determined according to the method described above (Bergmeyer and Bernt, 1974). Starch content was expressed as the amount of glucose on fresh weight of panicles.

5. Analysis of protein patterns
Proteins were separated by SDS-PAGE according to the method of Laemmli (1970), on a 12.5% acrylamide slab gel. Fifty microliters of the soluble protein fraction was mixed with the same volume of sample buffer that contained 125 mM Tris-HCl (pH 6.8), 4% (w/v) SDS, 20% (w/v) glycerol, and 4% (v/v) 2-mercaptoethanol, and heated at 90°C for 5 min. For insoluble proteins, the fraction was mixed with the same volume of
sample buffer that contained 125 mM Tris-HCl (pH 6.8), 4% (w/v) SDS and 4% (v/v) 2-mercaptoethanol, and incubated at 45°C for 30 min. Samples of both soluble and insoluble proteins were loaded onto gels, with bromophenol blue as a tracking dye, and the electrophoresis was conducted with a constant current of 20 mA at room temperature. After electrophoresis, polypeptides on the gels were detected by staining with a Silver Stain Kit (Wako Chemicals, Osaka, Japan).

**Results**

1. **Number of spikelets**

Table 1 shows the numbers of primary and secondary rachis branches and the number of spikelets per panicle. The numbers of differentiated rachis branches and spikelets decreased in the order of Takanari without topdressing (Takanari-L), Nipponbare with topdressing (Nipponbare-H) and Nipponbare without topdressing (Nipponbare-L). Although there were several degenerated rachis branches and spikelets, the numbers of surviving rachis branches and spikelets decreased in the same order as differentiated ones. As a result, the number of surviving spikelets in Nipponbare-H was about 1.8 times and that in Takanari-L was about 2.7 times larger than that in Nipponbare-L.

2. **Growth of panicles**

Fig. 1 shows the changes in length and fresh weight of developing panicles. Panicle developing stages coincided with days before heading (DBH) in different cultivars and in different levels of nitrogen topdressing. Panicles differentiated primary rachis branches 25 DBH, secondary rachis branches 22 DBH,

| Plot       | No. of primary rachis branches | No. of secondary rachis branches | No. of spikelets |
|------------|--------------------------------|----------------------------------|------------------|
|            | Differentiated | Surviving | Differentiated | Surviving | Differentiated | Surviving |
| Nipponbare-L | 8.9 ± 0.2 b  | 7.8 ± 0.2 c  | 14.5 ± 0.4 c  | 9.3 ± 0.3 c  | 70.6 ± 1.6 c  | 67.2 ± 1.5 c  |
| Nipponbare-H | 9.3 ± 0.2 b  | 9.0 ± 0.2 b  | 32.3 ± 1.8 b  | 21.2 ± 0.9 b  | 122.9 ± 3.1 b | 117.6 ± 3.2 b |
| Takanari-L  | 12.6 ± 0.2 a  | 12.6 ± 0.2 a  | 57.6 ± 1.1 a  | 33.2 ± 1.5 a  | 186.5 ± 6.1 a | 179.5 ± 5.7 a |

L, Without nitrogen topdressing; H, topdressing with 0.3 g nitrogen per pot. Values are mean ± SE of 18 panicles. Figures followed by a different letter are significantly different at 5% LSD.

Fig. 1. Changes in length (A) and fresh weight (B) of developing rice panicles in different cultivars and in different levels of nitrogen topdressing.

The vertical line indicates standard error of 12 panicles.

Fig. 2. Changes in soluble (A) and insoluble (B) proteins of developing rice panicles in different cultivars and in different levels of nitrogen topdressing.

The vertical line indicates standard error of 3 panicles.
Panicles of Nipponbare-L, with few spikelets (SP), elongated rather more rapidly than those of Nipponbare-H and Takanari-L, with many spikelets (LP), from 20 to 10 DBH (Fig. 1A).

LP of Nipponbare-H and Takanari-L, however, grew markedly thereafter, and the length of these panicles exceeded the SP of Nipponbare-L from 10 DBH to heading. A similar trend was observed in the fresh weight of panicles among the 3 plots (Fig. 1B).

3. Proteins in developing panicles

Fig. 2 shows the changes in soluble and insoluble proteins of developing panicles. Both soluble and insoluble protein content decreased as panicles developed. The soluble protein content in LP of Nipponbare-H and Takanari-L was higher than that in SP of Nipponbare-L from 20 to 10 DBH (Fig. 2A). The difference between SP and LP was, however, hardly detected after 10 DBH. In contrast, no clear difference was observed in insoluble protein content with DBH among the 3 plots (Fig. 2B).

Fig. 3 shows the relationships between fresh weight and soluble or insoluble proteins in developing rice panicles at spikelet differentiation.

Lanes 1-3 and 4-6 correspond to the soluble and the insoluble protein fractions. Lanes 1 and 4, Nipponbare without topdressing 19 DBH; lanes 2 and 5, Nipponbare with 0.5 g nitrogen topdressing 19 DBH; lanes 3 and 6, Takanari without topdressing 18 DBH. Samples equivalent to 5 µg of soluble proteins were loaded in lanes 1-3, and samples equivalent to 7 µg of insoluble proteins in lanes 4-6. Arrowheads A and B indicate the insoluble proteins with a molecular weight of about 42 kDa.

4. Carbohydrates in developing panicles

Fig. 4 shows the changes in sugars and starch of developing rice panicles in different cultivars and in different levels of nitrogen topdressing.

The vertical line indicates standard error of 3 panicles.

and spikelets from 19 to 15 DBH, which was similar to Matsushima (1957). Panicles of Nipponbare-L, with few spikelets (SP), elongated rather more rapidly than those of Nipponbare-H and Takanari-L, with many spikelets (LP), from 20 to 10 DBH (Fig. 1A). LP of Nipponbare-H and Takanari-L, however, grew markedly thereafter, and the length of these panicles exceeded the SP of Nipponbare-L from 10 DBH to heading. A similar trend was observed in the fresh weight of panicles among the 3 plots (Fig. 1B).

3. Proteins in developing panicles

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Fig. 3 shows the relationships between fresh weight and soluble or insoluble protein content in developing panicles. When the panicle fresh weight was less than 100 mg, the soluble protein content in LP was higher than that in SP (Fig. 3A), but there was little difference in insoluble protein content with panicle fresh weight among the 3 plots (Fig. 3B).

4. Carbohydrates in developing panicles

Fig. 4 shows the changes in sugars and starch in
developing panicles. The sugar content decreased as the panicle developed. Starch content was lower than sugar content, but slightly increased during panicle development. No clear difference was seen in sugar and starch content of developing panicles among Nipponbare-L, Nipponbare-H, and Takanari-L.

5. Protein patterns in developing panicles

The distribution of protein components in soluble and insoluble protein fractions was compared by SDS-PAGE with silver staining for panicles obtained at spikelet differentiation from 19 to 18 DBH (Fig. 5). The difference in protein pattern was scarcely detected in soluble proteins between SP of Nipponbare-L and LP of Nipponbare-H and Takanari-L. The distribution of protein components in LP was also basically similar to that of insoluble proteins in SP, except for higher intensities of insoluble proteins with a molecular size of about 42 kDa (42 kDa-insoluble proteins, hereafter; A and B in Fig. 5).

Discussion

Panicle differentiation and development were morphologically investigated for japonica (Matsushima, 1957), and indica rice (Senanayake et al., 1991). In contrast, physiological investigation of panicle development has been limited in rice plants, because young panicle tissues are too small to be analyzed. In the present study, we measured proteins, sugars and starch in developing panicles by sensitive methods, although the sensitivity was not sufficient for panicle tissues from neck-node initiation to rachis branch differentiation stages. Direct measurement of proteins and carbohydrates in developing panicles during spikelet differentiation provides information for discussing causal factors controlling spikelet number.

Takeda et al. (1984b) reported that new Korean cultivars have more spikelets per unit area than Japanese cultivars. This difference is ascribed to the number of spikelets per panicle (Takeda et al., 1984b), particularly those on secondary and tertiary rachis branches (Maruyama et al., 1988). These findings are supported by the results obtained with the high-yielding indica cultivars (Maruyama et al., 1988). These cultivars have more spikelets per unit amount of nitrogen absorbed until heading than Japanese cultivars (Yamamoto et al., 1991), suggesting that the cultivar difference in spikelet number is not ascribable to nitrogen content in shoots. We found, however, that the soluble protein content and the amount of 42 kDa-insoluble proteins in developing panicles in Takanari, with many spikelets, are higher than those in Nipponbare, with few spikelets. It thus appears that the distribution of nitrogen to developing panicles in high-yielding indica cultivars is larger than that in Japanese cultivars.

Wada (1969) reported that the number of differentiated spikelets is related to the amount of nitrogen absorbed by rice plants until spikelet differentiation. Kobayasi and Horie (1994) pointed out that change in nitrogen content from panicle initiation to spikelet differentiation also affects the number of differentiated spikelets. Nevertheless, Kobayasi et al. (2001) stated that the content of nonstructural carbohydrates in stems scarcely affected the number of differentiated spikelets. Although nitrogen topdressing increased the soluble protein content and 42 kDa-insoluble proteins, it hardly changed the contents of sugars and starch in developing panicles. This suggests that nitrogen topdressing increases the number of spikelets per panicle through maintaining a high soluble protein content and increasing the 42 kDa-insoluble proteins during panicle development.

Our study suggested that the number of spikelets per panicle is related to soluble protein content in developing panicles, irrespective of cultivar and nitrogen topdressing. Takanari-L and Nipponbare-H showed rather slower growth of LP during spikelet differentiation than that of SP in Nipponbare-L. The slower growth of LP may keep the level of soluble proteins higher than that of SP. Numbers of spikelets per panicle are, however, not completely controlled by soluble protein content in developing panicles. In fact, Takanari-L had more spikelets than Nipponbare-H, although the soluble protein content in developing panicles in Takanari-L was comparable to that in Nipponbare-H. Our study also indicated that the number of spikelets per panicle is related to the amount of 42 kDa-insoluble proteins in developing panicles, irrespective of cultivar and nitrogen topdressing. Although the characteristics of the 42 kDa-insoluble proteins remain unclear, they may have important roles in determining the number of spikelets per panicle.

In conclusion, our results suggest that the number of spikelets in rice is controlled by the soluble protein content and 42 kDa-insoluble proteins, but not by the carbohydrates in developing panicles. Further study is needed to clarify the mechanism of maintaining soluble proteins in developing panicles and characteristics of insoluble proteins closely related to the number of spikelets.

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