Size and Activity of Shoot Apical Meristems as Determinants of Floret Number in Rice Panicles

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Abstract: The sink capacity (floret number per unit land area) is currently a serious constraint to grain yield production in japonica rice. The size and activity of the early reproductive shoot apex (incipient panicle) are potential determinants of the number of florets generated on the panicle. This hypothesis was tested using eight field-grown japonica rice cultivars (IR65598-112-2, IR65564-44-51, Nipponbare, Akenohoshi, Dobashi 1, Koshihikari, Kochihikiki and Nakateshinsenbon). Using morphometric microscopy, we found that the initial size of the reproductive shoot apex was highly correlated with the number of primary branches, but not with the number of florets per primary branch. The cell division activity of the early reproductive apex examined by in situ hybridization analyses using the histone H4 gene probe as a marker for DNA-replicating cells varied with the cultivar. Akenohoshi had twice as many DNA-replicating cells as Nipponbare and the cell division activity was highly correlated with the number of florets per primary branch, but not with the primary branch number. We concluded that the primary branch number was determined by the initial size of the reproductive apex, and that the floret number per primary branch was determined by the cell division activity in the following apex growth. This result provides the first evidence of a relationship between cell division activity and floret formation in the rice panicle.

Keywords: Floret, Histone H4, Panicle, Primary branch, Rice, Shoot apical meristem, Yield.

Floret differentiation is fundamental to rice yield. Recent rice breeding efforts in Japan have dramatically improved the source capacity, and, consequently, the sink capacity has become a serious constraint to rice grain yield production (Takeda et al., 1984; Yamamoto et al., 1991; Saitoh et al., 1993). In theory, the rice sink capacity can be increased by manipulating both the panicle number and the individual panicle size (floret number per panicle). However, the latter strategy might be more promising because existing super high-yielding indica rice cultivars such as Tongil (Korea) and Qui-chao 2 (China) have large panicles (Maruyama et al., 1988; Yamamoto et al., 1991). A similar breeding effort is in progress at the International Rice Research Institute (IRRI), Philippines, to develop the so-called "new plant type" (NPT) characterized by markedly large panicles and very few tillers (Peng et al., 1999; Khush, 2000). Despite its agronomic importance, information on how floret number is determined during panicle development is still limited.

It takes approximately one month for a rice panicle to progress from initiation to flowering. During this period, branches are formed on early reproductive shoot apices, followed by the formation of florets on the branches and the development of floral organs (palea, lemma, anthers and ovary) in the florets (Matsushima, 1957). The branches and florets are generated in a narrow stage (i.e., almost exclusively in the first 10 days) of early reproductive development. Morphological studies revealed that cultivars with large panicles generally have large shoot apices at this stage (Yamagishi et al., 1992; Fukushima, 1999a, 1999b; Mu et al., 2001; Kobayasi et al., 2002). This result suggests that the initial size of a reproductive apex may determine the number of branches and florets by acting as the "space" for the differentiation of these structures. However, this "apex size" scheme cannot explain the panicle gigantism in some cultivars. For example, despite its large panicles, the high-yielding japonica rice cultivar, Akenohoshi, has a normal-sized shoot apex at the onset of reproductive growth (Yamagishi et al., 1996). This fact leads us to examine the activity of cell division on shoot apex that would be another factor to determine the number of branches and florets.

Traditionally, the activity of cell division in shoot apices have been studied by labeling experiments in which S-phase (i.e., DNA-replicating) nuclei were fed with [3H]thymidine and then detected autoradiographically. However, this RI method is very limited to use. In the last decade, a number of genes whose expressions are regulated in cell cycle-dependent manner have been cloned, and in situ detection of these genes have been proven to be a...
powerful tool for identification of cells that are in a specific phase of cell cycle, among which are histone genes. Histones are nuclear proteins that participate in chromatin formation. The synthesis of the histone proteins is highly correlated to DNA replication. Recent advance in plant histone researches (see Meshi et al, 2000 for a review) have facilitated to use its gene expression as a useful marker for visualizing S-phase cells via in situ hybridization analysis. Such methods have been successfully applied for identification of S-phase cells in tomato (Brandstädter et al., 1994), soybean (Kouchi et al., 1995), and snapdragon (Fobert et al., 1996) shoot apices.

In this study, the growth of transitional shoot apices was quantitatively characterized in eight japonica rice cultivars, including Akenohoshi. We evaluated the cell division activity of the apical meristems using the histone H4 expression gene as a molecular marker.

**Materials and Methods**

### 1. Plant materials and growth conditions

Eight japonica cultivars (IR65598-112-2, IR65564-44-51, Nipponbare, Akenohoshi, Dobashi 1, Koshihikari, Kochihibiki and Nakateshinsenbon) were grown in a paddy field at the University Farm of the University of Tokyo, Nishitokyo, in 1996, 1999 and 2000. IR65598-112-2 and IR65564-44-51 were NPT lines from IRRI (Khush, 2000), the others originated in Japan. Other than IR65564-44-51, all lines were grown in 1996. Four selected cultivars, IR65564-44-51, Nipponbare, Akenohoshi and Dobashi 1, were grown in 1999 and 2000. The growth duration and the date of panicle initiation are almost the same in these four cultivars. The 28-day-old seedlings (200 plants of each cultivar in 1996 and 600 plants of each cultivar in 1999 and 2000) were transplanted with 30 cm row spacing and 15 cm hill spacing (one plant per hill) on 4 June (1996), 20 May (1999) and 20 May (2000). A chemical compound fertilizer (50:33:55 kg NPK ha⁻¹) was basally applied with no topdressing. At maturity, eight plants from each line were sampled and the panicles of the main tiller examined for panicle characters.

### 2. Measurement of panicle characters

The rice panicle is composed of primary and secondary branches, both of which have florets. The main axis carries primary branches on its successive nodes, while the primary branches carry secondary branches on their proximal portions (the distal portions generate florets instead of branches-see below). Florets are generated on these branches (i.e., on the secondary branches and distal portions of the primary branches) as a single-floret spikelet. These structures exhibit an array of genetic and environmental variations. For example, using the panicles of 65 japonica rice cultivars from around the world, Yamagishi et al. (2003) reported that the number of primary branches and total florets in the main-tiller panicle ranged from 8 to 20 and from 99 to 375, respectively. Besides the existing branches and florets, some were aborted during panicle development. These aborted branches and florets, which appeared as rudimentary small protrusions on the mature panicles, enabled the estimation of lost florets due to abortion (Matsushima, 1957). We counted the primary branches and florets, both existing and aborted. The total number of branches or florets that have once been differentiated (whether they survived or were aborted) was calculated as follows: differentiated branches (florets) = existing branches (florets) + aborted branches (florets)

### 3. Morphometric microscopy of shoot apices

To examine the dynamics of apex growth during early panicle development, we sampled transitional shoot apices in 1996, 1999 and 2000. After removing the daughter tillers, the apices of the main tillers were excised, fixed in FAA (formalin: acetic acid: 50% ethanol=5 : 5 : 90) overnight, dehydrated in a tert-butyl alcohol series and embedded in paraffin wax (Sass 1958). Longitudinal sections 10 μm thick were cut and stained with safranin, iron-tannic acid and orange G (Sharman, 1943). The developmental stage of each apex was identified and only apices of three selected stages (flag leaf initiation, the first bract initiation and the second bract initiation) were photographed for measurements (see below) under an optical microscope. The flag leaf initiation stage and the first bract initiation stage were identified with the number of already differentiated leaves and the size of the primodium, because the number of leaves produced on the main stem are constant in each cultivar when the cultivation management are the same. The second bract initiation stage was identified with the serial sections in each apice. The height and width of the apical meristems were measured, and cells on the photographed median longitudinal sections were counted. A shoot apical meristem was defined as the dome just above the youngest leaf primordium (up to the stage of flag leaf formation) or the first bract of the panicle (after the stage of bract formation). Assuming it half ellipsoid (Smith and Rogan, 1979), the volume of the shoot apical meristem was calculated using the following formula:

\[ V = \frac{2}{3} \pi r^2 h \]

where V, r and h are the volume, basal radius and height of the apical meristem, respectively.

### 4. In situ hybridization analysis of the histone H4 gene on shoot apices

The cell division activity of shoot apical meristems was evaluated for apices collected in 2000 by in situ
hybridization using the rice histone H4 antisense probe. Histones are nuclear proteins that participate in chromatin formation. Because their synthesis is highly correlated with DNA replication, the histone expression gene can be used as a marker for visualizing S-phase cells via in situ hybridization.

The hybridization experiment was conducted following the method described by Chono et al. (2001). Tissues were fixed with FAA, embedded in paraffin and sectioned as described above. The sections were mounted on silane-coated slides, dewaxed, pretreated with Proteinase K and probe-hybridized. The hybridized sections were then washed, RNase-treated and signal detected immunohistochemically using BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (nitroblue tetrazolium) as coloring substances. The slides were dehydrated, mounted with Eukitt. Under an optical microscope, the developmental stage of each apex was identified and three apices of the second bract initiation in each cultivar were photographed for measurements. The histone H4-labeled and non-labeled cells in the apical meristems were counted on the photographs.

Results

1. Panicle characters

All examined panicle characters varied with the cultivar and, occasionally, with the year (Table 1). The number of florets per panicle was highest in IR65598-112-2, followed by IR65564-44-5165-1 and Akenohoshi. Florets per panicle were smallest in Kochihibiki and Nakateshinsenbon. The number of primary branches ranged from nine (Nakateshinsenbon) to 21 (IR65598-112-2) in 1996 and floret number per primary branch ranged from 11.7 (Kochihibiki) to 27.8 (IR65598-112-2). Differences in the floret number per primary branch were mainly due to the difference in the number of secondary branches on the individual primary branches.

2. Size of the early reproductive shoot apex

Figure 1 shows apices at the initiation stage of the flag leaf (Fig. 1a), the first bract (Fig. 1b) and second bract (Fig. 1c) in Nipponbare. The volume of the apices increased with time, and the rate of increase by phase proceeding differed among the cultivars (Table 2). The rate was highest in Akenohoshi (326%, averaged over two years) and lowest in Koshihikari (202%) and Nakateshinsenbon (202%) (Table 2). Cell size was almost constant in all cultivars and stages (data not shown).

3. Cell division activity of the early reproductive shoot apex

The marked difference in the rate of increase of the apex volume among cultivars led us to examine cell division activity in the apex by in situ hybridization analyses using the rice histone H4 gene probe. Fig. 2 shows the apices at the second bract initiation stage, where S-phase cells were labeled by the histone H4 expression gene. Cells in the S-phase that expressed the histone H4 gene were predominantly found in the shoot apex and leaf primordia. A marked difference in the ratio of S-phase cells in the apex was observed among cultivars. In Akenohoshi, 40% of the cells revealed on the section were labeled with histone H4.
Fig. 1. The apices at the initiation of the flag leaf (a), first bract (b), and second bract (c) in Nipponbare. FL and B1 are the flag leaf and the first bract, respectively. The scale bar in each figure show 50µm.

Table 2. The volume of apices on the flag leaf, the first bract, and the second bract initiation stage. The values in parentheses are the relative values of the volume comparing with that on the flag leaf initiation stage. Values are given as means ±S.D.

| Cultivar     | Year | Flag leaf initiation stage | First bract initiation stage | Second bract initiation stage |
|--------------|------|-----------------------------|-----------------------------|-------------------------------|
|              |      | Volume of apices (x10^3μm^3) | Sample number | Volume of apices (x10^3μm^3) | Sample number | Volume of apices (x10^3μm^3) | Sample number |
| IR65598-112-2| 1996 | 3.585±0.309(100)             | 10                         | 5.520±0.906(154)              | 3             | 8.668±0.843(242)              | 6             |
|              | 1999 | 2.761±0.289(100)             | 13                         | 3.948±0.639(143)              | 3             | 6.772±1.412(245)              | 4             |
|              | 2000 | y/a                         | y/a                        | y/a                          | y/a                  | y/a                          | y/a                  |
| Nipponbare   | 1996 | 1.845±0.201(100)             | 4                          | 2.357±0.335(129)              | 3             | y/a                          | y/a                  |
|              | 1999 | 1.616±0.325(100)             | 12                         | 1.985±0.304(123)              | 5             | 3.901±0.666(241)              | 3             |
|              | 2000 | y/a                         | y/a                        | y/a                          | y/a                  | 4.443±0.673                  | 3             |
| Akenoboshi   | 1996 | 2.571±0.157(100)             | 4                          | 3.670±0.214(143)              | 3             | 8.478±0.795(330)              | 3             |
|              | 1999 | 1.696±0.331(100)             | 15                         | 2.301±0.515(136)              | 5             | 5.441±0.382(321)              | 3             |
|              | 2000 | y/a                         | y/a                        | y/a                          | y/a                  | 6.612±1.676                  | 3             |
| Dobashi 1    | 1996 | 2.826±0.327(100)             | 7                          | y/a                          | y/a                  | 6.707±1.333(237)              | 5             |
|              | 1999 | 1.928±0.265(100)             | 8                          | 2.612±0.250(136)              | 3             | 6.480±0.552(336)              | 3             |
|              | 2000 | y/a                         | y/a                        | y/a                          | y/a                  | 7.506±1.741                  | 3             |
| Koshihikari  | 1996 | 1.951±0.205(100)             | 3                          | 2.393±0.452(123)              | 3             | 3.935±0.642(202)              | 3             |
| Kochihibiki  | 1996 | 1.736±0.249(100)             | 5                          | 2.399±0.328(138)              | 3             | y/a                          | y/a                  |
| Nakateshinsenbon | 1996 | 1.641±0.230(100)             | 6                          | 2.202±0.122(134)              | 3             | 3.314±0.529(202)              | 5             |

a/ : not determined.
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whereas less than 20% were labeled in Nipponbare (Fig. 3). This indicated that the cell division activity of the apical meristems at the second bract initiation stage was much higher in Akenohoshi.

4. Correlations of apex size with cell division activity and panicle characters of the apex

The number of primary branches showed a high positive correlation with the size of the early reproductive apex (Fig. 4). The regression coefficient was highest in the apex at the flag leaf initiation stage ($r = 0.843$, $P = 0.011$) and lowest in the apex at the second branch initiation stage ($r = 0.759$, $P = 0.0265$). In contrast, there was no high significant correlation between floret number per primary branch and apex size at any stage of panicle development.

Similarly, a high positive correlation ($r = 0.989$, $P = 0.0105$) was found between the cell division activity (shown as the frequency of S-phase cells) and floret number per primary branch (Fig. 5b). However, there was no correlation between cell division activity and the number of primary branches (Fig. 5a).

Discussion

The variation in total floret number of a panicle is attributed to the difference in two morphological components: the number of primary branches and the number of florets per primary branch. Our results suggest that morphogenetic control of these two components might differ.

1. The initial size of the reproductive shoot apex determines the number of primary branches, but not the number of florets per primary branch

In many plant species, the size of a shoot apex at the onset of reproductive development affects the number of branches and florets by giving the "space" for their differentiation. For example, in sunflower plants across cultivars and environments, the number of florets in a capitulum is closely correlated with the area of the earliest capitulum (Palmer and Steer, 1985). Such an interesting finding led us to compare panicle characters with the early reproductive shoot apex size. Our result showed that the number of primary branches appeared to be correlated with the size of the early reproductive apex, which is consistent with previous reports (e.g., Matsuba, 1991). Furthermore, a detailed analysis revealed that this correlation was higher at the apex where the flag leaf had just initiated (the stage at which no traces of branch formation were
recognizable) than at any of the succeeding stages in which branch formation took place (Fig. 4). This indicates that the number of primary branches may have been physiologically predetermined (cells might have been destined to become a branch) before any morphological sign of branch formation occurred.

Unlike primary branch formation, floret formation on individual primary branches was poorly correlated with the size of the early reproductive apex. This result becomes clearer when indica-japonica cultivar, Milyang 23 developed in Korea, is included. The volume of apices of Milyang 23 in 1999 grown in the same condition was $1.17 \times 10^5 \mu m^3$ on flag leaf initiation stage and $1.95 \times 10^5 \mu m^3$ on the second bract initiation stage, though the mean number of primary branches was 11.4 and that of florets per primary branch was 19.6. This result is reasonable because the primary branches and the florets are formed at a slightly different stage of panicle development. All primary
branches are formed on the shoot apex, whereas the florets are formed on individual primary branches.

2. The number of florets per primary branch is determined by the cell division activity of the reproductive shoot apex

The finding that the initial size of the reproductive shoot apex determines the number of primary branches, but not the number of florets per primary branch, raises a new question—what factor(s) is involved in the determination of floret number within each primary branch? To answer this question, we examined the cell division activity of the early reproductive apex and found that it was poorly correlated with the number of primary branches, but highly correlated with the number of florets per primary branch (Fig. 5). This fact suggests that (1) floret and primary branch formation are differentially controlled and (2) the floret formation on individual primary branches largely depends on the cell division activity of their mother cells. This is the first evidence of a relationship between cell division activity and floret formation in quite early stage, before differentiation of primary branch, of rice panicle initiation, though Fukushima (1999b) mentioned that the number of florets per primary branch was defined by the size of primary branch primordia.

Generally, cell division activity is controlled by the duration of the cell cycle. During plant development, a dramatic change in the duration of the cell cycle occurs at the transition from the vegetative to the reproductive phase. The cell cycle in the shoot apex shortens and, therefore, the growth rate of the apex increases at the reproductive phase. A wheat shoot apex, for example, has a cell cycle of about 40 h in the vegetative stage, which is shortened to about 20 h after the onset of panicle initiation (Lyndon, 1976). Although little is known on how the plant cell cycle is regulated in a phase-dependent manner, studies on rice panicle development may provide such information. Akenohoshi has an introgressed indica genome segment near the telomeric end of the short arm of chromosome 1, where a quantitative trait locus (QTL) affecting floret number has been repeatedly mapped with diverse mapping populations (Nagata et al., 2001). It would be tempting to hypothesize that this indica genome segment might confer numerous florets by acting as an accelerator of the cell cycle for the reproductive apex. Development of a near-isogenic line for this genomic region is necessary to test this hypothesis.

3. Toward the improvement of sink function: the manipulation of panicle architecture

Besides total floret number, architectural features (such as patterns of branching, arrangement of florets, etc.) of the panicle also affect productivity. This is because both the abortion and grain filling of individual florets depends on the order of the branch on which the floret is positioned (Matsushima, 1957). In general, florets attached to the secondary branches abort more frequently (Kobayasi et al., 1997). They are also less filled when they survive (Maruyama et al., 1988; Yamamoto et al., 1991) compared with those attached directly to the axis of the primary branches. Ishii (1992) reported that in Nipponbare grown in a standard culture condition, only 80% of the differentiated florets on the secondary branches survived, of which 54% were filled, but all florets on the primary branches survived, and 93% were filled. Although increasing both primary branches and florets per primary branch could increase floret number per panicle, the former strategy is more advantageous and practical in improving rice productivity. The present results demonstrated that the number of primary branches was determined by the size of the shoot apex at the time of reproductive transition, which, in turn, was determined by the preceding vegetative apex.
growth. This implies that an understanding of how a shoot apex grows during the vegetative stage is crucial in manipulating the number of primary branches.

Although many factors affect vegetative grass apex growth, density-dependent interference between plants and/or tillers seem to be the most dominant. Low planting density results in an increase of the vegetative apex size in rice (Yamazaki, 1963). In quack grass (Agropyron repens (L.)), outgrowth of the rhizomes (underground tillers) was accompanied by a decrease in the shoot apex size of the main tiller. This decrease was reversed by the removal of the rhizome (Smith and Rogan, 1980). These reports suggest that tiller bud outgrowth and maintenance of the shoot apex were competing for photoassimilates. That is, in other words, the reduction of tiller outgrowth result in the giant shoot apex, which would bring the high branching ability in a panicle. Despite their low-tillering habits, such a phenomenon could explain the numerous primary branches of the two NPT lines (IR65598-112-2 and IR65564-44-5165-1) and Dobashi 1. Recently, QTLs that control tillering have been identified from diverse rice lines (Yan et al., 1998).

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