Pollen-mediated transgene flow is a major concern for the production of genetically modified (GM) rice. Cleistogamy is a useful tool for preventing this form of gene flow. We previously identified the cleistogamous rice mutant superwoman1-cleistogamy (spw1-cls) and determined its molecular genetic mechanism. In the present study, we cultivated spw1-cls over five years to examine effects of cleistogamy on agronomic traits. Simultaneously, we cultivated cleistogamous backcross lines created by continuous backcrossing with “Yumeaoba” (a japonica cultivar) as the recurrent parent and by application of a DNA marker. In these experimental cultivations, spw1-cls and its backcross lines showed almost equal or slightly lower, but acceptable, agronomic traits compared with each control line. We also conducted natural crossing tests in paddy fields to assess the gene containment capability of spw1-cls. In a series of field experiments, there was no natural crossing between spw1-cls (pollen donor) and pollen recipient lines, but the wild-type donor and recipient lines were crossed. Thus, the cleistogamy of the spw1-cls mutation is able to inhibit natural crossing effectively, without significantly loss of commercial benefits, such as yield. We conclude that spw1-cls cleistogamy is a practical tool for gene containment in GM rice cultivation.

**Key Words:** rice, cleistogamy, gene containment, pollen dispersal, agronomic traits, dCAPS marker, natural crossing.

**Introduction**

Commercial cultivation of genetically modified (GM) crops started in 1996 and the area of GM crop cultivation has increased each year since then. In 2010, the total area under GM crop cultivation was $1 \times 10^8$ ha across 29 countries (James 2010). Although GM crops confer significant benefits including resistance to herbicides and biotic/abiotic stresses, they also raise concerns about potential environmental impacts. Furthermore, new types of GM crops, including GM cereals, that produce pharmaceutical agents or industrial raw materials are in development and will be released in the near future. Consideration of public concerns is especially necessary to realize co-existence of such GM and non-GM crops (Konagaya et al. 2008). Gene flow is one such major concern. Gene flow is the transfer of genes from one plant to another, and gene flow from GM crops is termed “transgene flow” (Daniell 2002, Gressel 2010, Hüskens et al. 2010). There are three mechanisms by which genes can flow from GM crops: pollen dispersal (mediated by wind, insects, or birds), seed contamination and vegetative propagule-mediated gene flow (Hüskens et al. 2010). To inhibit pollen-mediated gene flow, it is necessary to physically suppress pollen movement, or construct temporal/spatial barriers between pollen donors and recipients. Cleistogamy (pollination without flower-opening) is an effective mechanism that prevents transgene flow through pollen dispersal from GM crops (Daniell 2002, Hüskens et al. 2010). Although it is difficult to introduce cleistogamy to allogamous crops such as maize, this is not the case for autogamous crops like rice.

Cleistogamy has been well studied in barley, and many cleistogamous barley cultivars have been raised in Japan. Genetic analyses of barley cleistogamy have revealed its mode of inheritance, showing that the responsible genetic locus occurs on chromosome 2HL (Kurauchi et al. 1994, Turuspekov et al. 2004). In barley, the sizes of lodicules (petal equivalents in grasses that force the lemma and palea apart at anthesis) and auxin responses are related to cleistogamy and are under the control of the genes cleistogamy1 (Cly1) and Cly2 (Honda et al. 2005). The Cly1 gene was identified by map-based cloning. It encodes a putative transcription factor with two AP2 domains and a putative miR172 target site (Nair et al. 2010). The cly1 phenotype is associated with nucleotide substitution within the miR172 target site, indicating that de-repression by miR172 causes upregulation of the Cly1 transcript, leading to cleistogamy (Nair et al. 2010).

Cleistogamous varieties have also been developed in...
Agronomic traits and gene containment capability of cleistogamous rice lines

Fig. 1. Grains and brown rice from two cleistogamous mutants. left: spw1-cls, right: d7. Scale bar = 1 cm. Grains of d7 are small in all dimensions; those of spw1-cls are normal.

oilseed rape (Renard and Tanguy 1997). Although these varieties do not always have complete cleistogamy and the mechanism of cleistogamy remains to be clarified, they are effective in suppressing the rate of outcrossing; hence, they may well be beneficial when used in combination with other mechanisms employed in a containment strategy (Leflon et al. 2010).

A few varieties of wheat and soybean are cleistogamous (Chhabra and Sethi 1991, Takahashi et al. 2001, Ueno and Itoh 1997), but they have not been used as practical tools for gene containment because the mechanism and stability of the cleistogamy has not been investigated.

The first reported cleistogamous mutant in rice was d7, which is also known as ‘Heiidaikoku’ or ‘cleistogamous dwarf’ (Nagao and Takahashi 1954). Paleae and lemmas of the d7 mutant are fused at their bases so that they are unable to open during blooming. Although d7 has a lower flower-opening rate (~30%) than the wild type (~90%), it also has agronomically unfavorable (possibly pleiotropic) effects, namely compact short panicles, small grains and reduced fertility (Nagao and Takahashi 1954, Fig. 1). Another cleistogamous mutant is “lodicleless spikelet” (ld), which has unknown agronomic traits and phenotypic developmental mechanisms (Maeng et al. 2006). More recently, Yoshida et al. (2007) identified the practical cleistogamous mutant superwoman1-cleistogamy (spw1-cls) in a Taichung 65 (T65) population mutagenized with N-methyl-N-nitrosourea (MNU). SUPERWOMAN1 (SPW1) is one of the class B MADS-box genes. It specifies identities of lodicules and stamens (Nagasawa et al. 2003). In spw1-cls, SPW1 has a single base change leading to an amino acid substitution in the MADS-box domain. This mutation reduces the interaction ability between SPW1 and its dimerization partners (MADS2 and MADS4); the mutation affects lodicule identity but not that of the stamen. Lodicules of spw1-cls are transformed into organs resembling the marginal region of the palea (mrp). These organs have no swelling ability, which causes the cleistogamy (Yoshida et al. 2007). Unlike d7, spw1-cls has agronomic characters very similar to those of the wild type. Consequently, the allele may have potential in the development of rice cultivars suitable for gene containment (Yoshida et al. 2007).

In this study, we cultivated spw1-cls over five years and examined the influences of cleistogamy on agronomic traits. We also selected backcross lines with the spw1-cls mutation using a dCAPS marker and cultivated these lines over two years to further evaluate effects of the spw1-cls mutation on agronomic traits. Finally, we performed natural crossing tests on spw1-cls in rice paddy fields to examine its capability in gene containment.

Materials and Methods

Plant materials

The cleistogamous rice mutant spw1-cls was selected from an M2 population of the rice japonica cultivar T65 mutagenized with MNU (Yoshida et al. 2007). We established cleistogamous backcross lines by continuous backcrossing with the japonica rice cultivar Yumeaoba as the recurrent parent. All experimental cultivations were performed under standard conditions in experimental paddy fields at the Hokuriku Research Center, NARO Agricultural Research Center (Niigata, Japan).

Agronomic trait analysis

The heading date for each line was calculated as the average date of first panicle heading in each plant. For individual plants, we counted the days from seeding date to heading date. Longest culm length was measured from the ground to the highest panicle neck. Panicle numbers were counted on each plant. Longest panicle length for each plant was measured from the neck of the longest panicle to the top. We counted the number of spikelets on the longest panicle of individual plants. Percentage of ripened grain was calculated from the number of unhulled brown rice grains trapped in a 1.8-mm screen to the number of spikelets on the longest panicle. Brown rice dry grain weight was calculated as the average of 20 brown rice grain weights corrected by the moisture content of brown rice determined with a Riceter M grain moisture tester (Kett Electric Laboratory, Tokyo, Japan).

Selection using a dCAPS marker

We designed a dCAPS marker that distinguished the single base change mutation in spw1-cls. The sequences of forward and reverse primers were cls-dCAPS f: 5′-ACCGG ATCGGAAACCGACC-3′ and cls-dCAPS r: 5′-GGAAG GGTGCGAAGAGCTCGTGTAATCTGTTGCCTGGTGAAG GAAACACATG-3′, respectively. To prepare the genomic DNA samples, we ground frozen leaves with glass beads in a commercial mixer (CapMix; 3M ESPE, Seefield, Germany) in 1.5-ml microcentrifuge tubes. We used the
alkali treatment and boiling method described by Klimyuk et al. (1993). We used TaKaRa Taq Hot Start Version (Takara Bio, Shiga, Japan) and TaKaRa PCR Thermal Cycler Dice Standard (Takara Bio), programmed for a first denaturation step of 3 min at 94°C, followed by 35 cycles at 94°C for 30 s, 60°C for 30 s, 72°C for 30 s for amplification and a final extension step of 72°C for 7 min. The 14 μl PCR reaction mixtures contained 0.75 units Taq polymerase, 0.2 mM of each dNTP and 0.2 mM of each primer, to which was added 1 μl of leaf extract as template DNA. We added 10 units of the restriction enzyme NcoI (New England Biolabs Japan Inc., Tokyo, Japan) with the prescribed amount of buffer to the PCR products and digested the amplified DNA at 37°C for 2 h. The digested products were separated by 2.4% agarose gel electrophoresis, and gels were stained with ethidium bromide and photographed.

Natural crossing test

Natural crossing tests were performed in 2008 and 2010 in experimental paddy fields at the Hokuriku Research Center, NARO Agricultural Research Center. We cultivated spw1-cls and T65 (non-glutinous cultivars) as the pollen parent lines; we also raised the Raichou-mochi (2008 and 2010) and Kagura-mochi (2008) cultivars as seed parent lines. These are japonica and glutinous cultivars with heading dates similar to that of T65. Each experiment had a donor block containing 49 plants of the pollen parent line in the center. The donor block was surrounded by four recipient plots containing 30 (2008) or 44 (2010) plants of the seed parent line (Fig. 2). Each plant was transplanted into an array with 15 cm hill distances and 15 cm row widths. North winds prevailed in the paddy fields during the heading and flowering stages of pollen and seed parent lines. After seed parent lines had ripened, we harvested all brown rice from each recipient plot and searched for xenia grains derived from crossings with the pollen parent divided by the total number of brown rice grains examined.

We prepared DNA samples for genotyping from xenia grains by using a modified version of the rapid DNA preparation method of Monna et al. (2002) as follows: each grain was placed separately in a 2.0-ml microcentrifuge tube and homogenized with 0.4 ml of TPS buffer (100 mM Tris-Cl, 10 mM EDTA and 1 M KCl, pH = 8.0) using a Mixer Mill MM 300 (QIAGEN KK, Tokyo, Japan) for 2 min at 30 rpm. Each sample was centrifuged for 10 min at room temperature and the supernatant was transferred to a new 1.5-ml microcentrifuge tube. A 0.4 ml volume of chloroform/isoamyl alcohol (24 : 1 v/v) was added and contents were thoroughly mixed. The sample was centrifuged for 10 min at room temperature and the aqueous layer was transferred to a new 1.5-ml microcentrifuge tube. A 0.4 ml volume of isopropanol was added and the contents were mixed well. The sample was centrifuged for 10 min at room temperature. The pellet was rinsed with 70% ethanol and dried. DNA was dissolved in 0.1 ml of 1/10 TE buffer (1 mM Tris-Cl and 0.1 mM EDTA, pH = 8.0). The PCR reaction was performed using the TaKaRa Taq Hot Start Version (Takara Bio) and TaKaRa PCR Thermal Cycler Dice Standard (Takara Bio) programmed as follows: a first denaturation step at 92°C for 2 min, 34 cycles at 94°C for 10 s, 58°C for 10 s, 72°C for 30 s and a final extension step at 72°C for 3 min. The 10 μl PCR reaction mixture contained 0.25 units of Taq polymerase and 0.2 mM of each dNTP; 5 μl of template DNA solution were added. We used 5 sets of single nucleotide polymorphism (SNP) markers (Hayashi et al. 2004) that detect the SNPs of the rice genome and can distinguish the genotype of T65 and others (Tabuchi et al. unpublished data). PCR products were separated by 2.4% agarose gel electrophoresis. We calculated the crossing rate for each plot from the number of xenia grains derived from crossings with the pollen parent divided by the total number of brown rice grains examined.

Results

The lodicule plays an important role in the flowering of rice. Two scale-shaped lodicules are located between the lemma and stamens at the base of the rice spikelet. Lodicules start to swell when rice flowering begins. Mechanical pressure generated by the rapidly swelling lodicules pushes the neighboring lemma downward. At the same time, the hook holding the lemma and palea is unfastened and the spikelet opens. Sixty to 150 min later, lodicules shrink as they lose water and the spikelets close (Hoshikawa 1989).

In spw1-cls spikelets, lodicules are transformed into elongated organs similar to mrp and they have no swelling ability (Yoshida et al. 2007, Fig. 3A). Consequently, spw1-cls spikelets did not open, and pollination was completed without the stamens extending out of the spikelets (Fig. 3B). Other than the opening of spikelets, all pollination processes including elongation of anther filaments, dehiscence of anthers and dispersal of pollen grains to the stigma occurred.
as usual and subsequent ripening proceeded normally (Fig. 3C). Withered anthers remained inside the spikelets while the ripening process proceeded, but they did not influence ovary development. After ripening was complete, withered anthers remained in the hull (Fig. 3D), but were almost completely removed by the hulling process. Polishing of the brown rice completely removed dried anthers (Fig. 3E). In appearance, polished rice from spw1-cls was indistinguishable from T65, the chasmogamous wild-type line of spw1-cls.

Influence of cleistogamy on agronomic traits

To examine influences of cleistogamy on agronomic traits, we cultivated spw1-cls in an experimental paddy field over five years (Table 1). T65 was cultivated for comparison purposes. Almost all spw1-cls plants grew normally, although a few exhibited dwarfism, poor growth in the early developmental stage, or sterility. We excluded these abnormal individuals from our analysis of the effect of cleistogamy.

The heading dates of spw1-cls matched those of T65 or were slightly later. The number of days from seeding to heading in spw1-cls exceeded those of T65 by 0.5 to 2.5 days. The longest spw1-cls culm length was less than (in 2006 and 2008), longer than (in 2010), or almost equal to (in 2007 and 2009) those of T65. Panicle numbers of spw1-cls were fewer than (in 2006), more than (in 2007 and 2009), or almost equal to (in 2008 and 2010) those of T65. Longest panicle lengths and numbers of spikelets on the longest panicle in spw1-cls tended to exceed those of T65. The ripened grain ratio and the brown rice dry grain weight of T65 exceeded those of spw1-cls throughout the five years.

Overall, agronomic traits of spw1-cls closely matched those of T65, but some differences were observed in yield-related traits (panicle number, number of spikelets on longest panicle, percentage of ripened grain and brown rice dry grain weight). Although the percent ripened grain and the dry grain weight of brown rice in spw1-cls were smaller than those in T65, the differences were not large.

Development of dCAPS marker and backcross lines

During crossbreeding, DNA markers were the most efficient way of introducing the spw1-cls mutation into other cultivated lines. Accordingly, we designed a dCAPS marker that recognized the spw1-cls point mutation by using the spw1-cls genome sequence we identified (see Materials and Methods). This marker discriminated genotypes of the mutant homozygote, the heterozygote, and the wild type homozygote (Fig. 4A), allowing efficient selection at early developmental stages. Indeed, we readily created cleistogamous backcross lines of the “Yumeaoba” cultivar by continuous backcrossing with the dCAPS marker. These lines were tentatively designated as “Yumeaoba-cls.” The lodicules of Yumeaoba-cls were elongate and transformed into mrp-like organs identical to those of spw1-cls (Fig. 4B). Consequently, the spikelets of Yumeaoba-cls did not open.

Agronomic traits of backcross lines with the spw1-cls mutation

Yumeaoba-cls was cultivated in the experimental paddy field over two years to evaluate effects of the spw1-cls mutation on agronomic traits (Table 2). The Yumeaoba cultivar, which is the recurrent parent of Yumeaoba-cls, was cultivated concurrently for comparison purposes. The generations of Yumeaoba-cls examined in 2009 and 2010 were BC3:F3 and BC2:F2, respectively.

The heading date and the days from seeding to heading were almost identical for Yumeaoba-cls and Yumeaoba.
indicate lodicules in each spikelet. Scale bar
mozygous lines. Heterozygous lines have all three fragments, and
parison of spikelets between Yum eaoba-cls (left) and Yumeaoba
wild-type homozygous lines have only the 157 bp fragment. (B) Com-
mutation. (A) Example of procedur e for detecting genetic lines with
Yumeaoba. Panicle number and longest panicle length of

Fig. 4. Selection of cleistogamous backcross lines with the spw1-cls
mutation. (A) Example of procedure for detecting genetic lines with the
spw1-cls mutation by using a dCAPS marker. There are 109 bp and
48 bp fragments, but a 157 bp fragment is absent in the mutant ho-
mozygous lines. Heterozygous lines have all three fragments, and
wild-type homozygous lines have only the 157 bp fragment. (B) Com-
parison of spikelets between Yumeaoba-cls (left) and Yumeaoba
(right). Lemmas and paleae have been removed. Yellow arrowheads
indicate lodicules in each spikelet. Scale bar = 2.0 mm.

Table 1. Agronomic traits of spw1-cls

| Line name | n= | Heading date | Days from seeding to heading (days) | Longest culm length (cm) | Panicle number (number/plant) | Longest panicle length (cm) | Number of spikelets on longest panicle (number/plane) | Percentage of ripened grain (%) | Brown rice dry grain weight (mg) |
|-----------|----|--------------|------------------------------------|-------------------------|-------------------------------|-----------------------------|---------------------------------------------------|-------------------------------|---------------------------------|
| 2006 year |     |              |                                    |                         |                               |                             |                                                   |                               |                                 |
| spw1-cls  | 135| 8/13         | 119.1 ± 2.22                      | 87.1 ± 4.98             | 8.7 ± 2.68                    | 22.6 ± 1.22                 | 116.3 ± 17.16                      | 77.1 ± 13.90                     | 19.0 ± 0.70                      |
| T65*      | 45 | 8/10         | 116.7 ± 2.09                      | 88.2 ± 3.73             | 9.1 ± 1.30                    | 22.4 ± 0.82                 | 100.4 ± 14.08                      | 86.6 ± 9.49                      | 21.4 ± 1.41                      |
| 2007 year |     |              |                                    |                         |                               |                             |                                                   |                               |                                 |
| spw1-cls  | 25 | 8/11         | 118.8 ± 1.52                      | 90.3 ± 4.14             | 11.7 ± 3.11                   | 23.1 ± 1.04                 | 134.6 ± 14.02                      | 83.4 ± 8.30                      | 19.9 ± 0.57                      |
| T65       | 60 | 8/9          | 116.8 ± 1.75                      | 90.3 ± 3.59             | 9.8 ± 1.58                    | 22.1 ± 0.90                 | 97.2 ± 13.88                       | 88.7 ± 7.26                      | 21.8 ± 1.16                      |
| 2008 year |     |              |                                    |                         |                               |                             |                                                   |                               |                                 |
| spw1-cls  | 243| 8/15         | 117.6 ± 2.33                      | 95.6 ± 5.60             | 10.2 ± 2.39                   | 23.1 ± 1.19                 | 118.6 ± 15.65                      | 77.6 ± 4.74                      | 20.3 ± 0.66                      |
| T65       | 38 | 8/14         | 116.7 ± 2.57                      | 98.4 ± 3.87             | 10.4 ± 2.18                   | 22.4 ± 0.95                 | 106.3 ± 16.50                      | 86.2 ± 5.50                      | 23.4 ± 1.12                      |
| 2009 year |     |              |                                    |                         |                               |                             |                                                   |                               |                                 |
| spw1-cls  | 102| 8/15         | 119.0 ± 1.76                      | 99.1 ± 4.31             | 10.5 ± 1.94                   | 23.0 ± 1.00                 | 119.6 ± 12.89                      | 80.2 ± 4.65                      | 20.6 ± 0.52                      |
| T65       | 40 | 8/13         | 116.5 ± 1.96                      | 99.3 ± 2.88             | 9.8 ± 2.16                    | 21.5 ± 1.20                 | 106.8 ± 17.25                      | 91.2 ± 6.12                      | 22.6 ± 1.06                      |
| 2010 year |     |              |                                    |                         |                               |                             |                                                   |                               |                                 |
| spw1-cls  | 41 | 8/10         | 115.0 ± 1.72                      | 89.8 ± 2.49             | 8.7 ± 1.65                    | 22.9 ± 1.07                 | 105.1 ± 12.71                      | 76.7 ± 4.90                      | 19.7 ± 0.59                      |
| T65       | 40 | 8/10         | 114.4 ± 2.19                      | 86.4 ± 2.83             | 8.8 ± 1.79                    | 21.8 ± 1.01                 | 91.8 ± 14.96                      | 92.5 ± 2.94                      | 20.5 ± 0.81                      |

Values are means ± standard deviations.

* T65, which is the parental cultivar of spw1-cls, was used as a control.

Yumeaoba-cls were, respectively, fewer than but not sub-
stantially different from those of Yumeaoba. The number of
spikelets on the longest panicle and the percentage of rip-
ened grains of Yumeaoba-cls were both larger than those of
Yumeaoba in 2009; however, the rank order was reversed in
2010. The brown rice dry grain weight of Yumeaoba-cls was
higher than that of Yumeaoba in both years.

Thus, although there were small differences among lines,
agronomic traits of Yumeaoba-cls were not much different
from those of Yumeaoba. In particular, differences of yield-
related traits between Yumeaoba-cls and Yumeaoba were
smaller than those between spw1-cls and T65.

Natural crossing test of spw1-cls

To determine the gene containment capability of spw1-
cls, we conducted natural crossing tests in the experimental
paddy field during 2008 and 2010 (see Material and Meth-
ods). The heading dates (in this test, the dates when approx-
imately half of the panicles were heading) of the pollen par-
ents in the north, east, south and west plots, respectively,

In 2008, 19, 14, 18 and 9 xenia grains produced by cross-
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spw1-cls, we conducted natural crossing tests in the experimental
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imately half of the panicles were heading) of the pollen par-
ents in the north, east, south and west plots, respectively,
seed parent lines differed by 2 to 6 days in 2008
approximately half of the panicles were heading) of the pollen par-
ods). The heading dates (in this test, the dates when approx-
imately half of the panicles were heading) of the pollen par-
ents should have overlapped sufficiently for our
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approximately half of the panicles were heading) of the pollen par-
ents should have overlapped sufficiently for our
crossing tests.
Agronomic traits and gene containment capability of cleistogamous rice lines

Table 2. Agronomic traits of Yumeaoba-cls lines

| Line name          | n= | Heading date | Days from seeding to heading (days) | Longest culm length (cm) | Panicle number (number/plant) | Longest panicle length (cm) | Number of spikelets on longest panicle (number/panicle) | Percentage of ripened grain (%) | Brown rice dry grain weight (mg) |
|--------------------|----|--------------|-----------------------------------|--------------------------|-------------------------------|-----------------------------|-------------------------------------------------------|-------------------------------|---------------------------------|
| 2009 year          | 40 | 8/6          | 109.1 ± 1.88                      | 81.6 ± 3.37              | 7.7 ± 1.82                    | 20.3 ± 1.10                 | 148.7 ± 26.06                                        | 84.4 ± 7.04                   | 25.0 ± 0.72                     |
| Yumeaoba-cls (BC\(_2\)F\(_3\)) | 36 | 8/6          | 109.4 ± 1.50                      | 76.9 ± 2.57              | 8.6 ± 1.50                    | 20.2 ± 0.90                 | 139.3 ± 23.22                                        | 81.3 ± 15.42                  | 23.7 ± 1.10                     |

| 2010 year          | 40 | 8/2          | 106.2 ± 1.45                      | 67.7 ± 1.98              | 6.5 ± 0.72                    | 22.2 ± 0.98                 | 159.7 ± 19.39                                        | 81.6 ± 6.73                   | 22.9 ± 0.95                     |
| Yumeaoba-cls (BC\(_2\)F\(_3\)) | 36 | 8/1          | 105.7 ± 1.32                      | 70.4 ± 3.26              | 7.2 ± 0.92                    | 22.2 ± 1.28                 | 170.2 ± 25.67                                        | 88.7 ± 3.72                   | 21.9 ± 0.64                     |

Values are means ± standard deviations.

|   | Crossing rate with donor (%) |   |   |   |   |   |   |   |   |   |
|---|-------------------------------|---|---|---|---|---|---|---|---|---|
|   | T65                           |   |   |   |   |   |   |   |   |   |
|   | Raichou-mochi                 |   |   |   |   |   |   |   |   |   |
|   | North                         | 14,727 | 19 | 0 | 19 | 0.000 |
|   | East                          | 13,018 | 14 | 0 | 14 | 0.000 |
|   | South                         | 18,186 | 18 | 0 | 18 | 0.000 |
|   | West                          | 14,906 | 9  | 0 | 9  | 0.000 |
|   | T65                           |   |   |   |   |   |   |   |   |   |
|   | Raichou-mochi                 |   |   |   |   |   |   |   |   |   |
|   | North                         | 8,593  | 13 | 3 | 10 | 0.035 |
|   | East                          | 9,764  | 6  | 3 | 3  | 0.031 |
|   | South                         | 13,468 | 29 | 27| 2  | 0.200 |
|   | West                          | 9,629  | 7  | 5 | 2  | 0.052 |
|   | spw1-cls                      |   |   |   |   |   |   |   |   |   |
|   | Kagura-mochi                  |   |   |   |   |   |   |   |   |   |
|   | North                         | 14,150 | 8  | 0 | 8  | 0.000 |
|   | East                          | 15,470 | 13 | 0 | 13 | 0.000 |
|   | South                         | 10,212 | 8  | 0 | 8  | 0.000 |
|   | West                          | 13,449 | 11 | 0 | 11 | 0.000 |
|   | T65                           |   |   |   |   |   |   |   |   |   |
|   | Kagura-mochi                  |   |   |   |   |   |   |   |   |   |
|   | North                         | 18,378 | 6  | 2 | 4  | 0.011 |
|   | East                          | 17,110 | 10 | 7 | 3  | 0.041 |
|   | South                         | 15,822 | 20 | 17| 3  | 0.107 |
|   | West                          | 15,168 | 12 | 6 | 6  | 0.040 |
|   | spw1-cls                      |   |   |   |   |   |   |   |   |   |
|   | (8/5)                         |   |   |   |   |   |   |   |   |   |
|   | Raichou-mochi                 |   |   |   |   |   |   |   |   |   |
|   | North                         | 20,302 | 1  | 0 | 1  | 0.000 |
|   | East                          | 22,727 | 0  | 0 | 0  | 0.000 |
|   | South                         | 18,634 | 0  | 0 | 0  | 0.000 |
|   | West                          | 20,672 | 0  | 0 | 0  | 0.000 |
|   | T65                           |   |   |   |   |   |   |   |   |   |
|   | Raichou-mochi                 |   |   |   |   |   |   |   |   |   |
|   | North                         | 20,345 | 0  | 0 | 0  | 0.000 |
|   | East                          | 21,635 | 2  | 1 | 1  | 0.005 |
|   | South                         | 20,644 | 4  | 4 | 0  | 0.019 |
|   | West                          | 20,246 | 3  | 2 | 1  | 0.010 |

Values are means ± standard deviations.

Donor (Heading date) Recipient (Heading date) Experimental plot n= Number of xenia Genotype Crossing rate with donor (%)

| 2008 year |  |  |  |  |  |  |  |  |  | |
|-----------|---|---|---|---|---|---|---|---|---|---|
| spw1-cls  | 8/11 | Raichou-mochi | (8/7) | North | 14,727 | 19 | 0 | 19 | 0.000 |
| T65       | 8/10 | Raichou-mochi | (8/7) | East | 13,018 | 14 | 0 | 14 | 0.000 |
| spw1-cls  | 8/7  | Kagura-mochi | (8/5) | North | 14,150 | 8  | 0 | 8  | 0.000 |
| T65       | 8/11 | Kagura-mochi | (8/5) | East | 15,470 | 13 | 0 | 13 | 0.000 |
| spw1-cls  | 8/5  | Raichou-mochi | (8/2) | North | 20,302 | 1  | 0 | 1  | 0.000 |
| T65       | 8/9  | Raichou-mochi | (8/1) | North | 20,345 | 0  | 0 | 0  | 0.000 |

Values are means ± standard deviations.

Donor (Heading date) Recipient (Heading date) Experimental plot n= Number of xenia Genotype Crossing rate with donor (%)

| 2010 year |  |  |  |  |  |  |  |  |  | |
|-----------|---|---|---|---|---|---|---|---|---|---|
| spw1-cls  | 8/5  | Raichou-mochi | (8/2) | North | 20,302 | 1  | 0 | 1  | 0.000 |
| T65       | 8/9  | Raichou-mochi | (8/1) | North | 20,345 | 0  | 0 | 0  | 0.000 |

a Date when about 50% of panicles were heading.

b Crossing rate is the proportion of xenia brown rice with the T65 genotype in the total brown rice count.

experimental plots with spw1-cls as the pollen parent line was 0.000%. In the another experiment in 2008 with T65 as the pollen parent line, we discovered 13, 6, 29 and 7 xenia grains in the north, east, south and west plots, respectively. Genotype testing using DNA markers indicated that 3 of 13, 3 of 6, 27 of 29 and 5 of 7 xenia grains in the north, east, south and west plots, respectively, were derived from crossing with the pollen parent T65. The crossing rates in these experimental plots were 0.031–0.200%. In the experiment with spw1-cls as the pollen parent line and Kagura-mochi as the seed parent line, we discovered 8, 13, 8 and 11 xenia grains in the north, east, south and west plots, respectively.
but none of them was derived from spwl-cls (DNA marker genotyping test). Therefore, the crossing rate with spwl-cls as the pollen parent line was 0.000%. In the experiment with T65 as the pollen parent line, we discovered 6, 10, 20 and 12 xenia grains in the north, east, south and west plots, respectively. Among these, 2 of 6, 7 of 10, 17 of 20 and 6 of 12 in the north, east, south and west plots, respectively, were derived from crosses with T65. The crossing rates in these plots were 0.011–0.107%.

The natural crossing rate was low in 2010. We discovered only 1 xenia grain in the north plot with spwl-cls as the pollen parent line, but this grain was not the product of a cross with spwl-cls. In the experiment with T65 as the pollen parent line, we discovered 2, 4 and 3 xenia grains in the east, south and west plots, respectively. Of these 1 of 2, 4 of 4 and 2 of 3 in the east, south and west plots, respectively, were derived from crosses with T65. The crossing rates in these plots were 0.005–0.019%.

In summary, throughout the two years, crossing always occurred in experiments with T65 as the pollen parent and rates were comparable in the north, east and west plots; the crossing rate was reproducibly higher in the south plot, which was to the lee of the prevailing winds (data not shown). In contrast, we did not find grains derived from crosses with spwl-cls in any of the plots in experiments with spwl-cls as the pollen parent line.

Discussion

Development of cleistogamous rice lines through introduction of the spwl-cls mutation into common chasmogamous cultivars would be unsatisfactory if the cleistogamous lines were phenotypically different from the original cultivars, particularly if agronomic traits were inferior. It is essential that there is little difference between the cleistogamous and the original chasmogamous cultivars, except for the non-flowering character.

Persistence of anthers inside the caryopsis is one of the inevitable features of cleistogamous rice; this characteristic is usually absent in chasmogamous cultivars. Anther persistence might reduce brown rice quality by deforming or staining the endosperm. Anthers remained in the hull throughout the ripening stages of spwl-cls. However, withering anthers of spwl-cls do not affect ripening processes (Yoshida et al. 2007, Fig. 3C). We also demonstrated that brown and polished rice of spwl-cls are not damaged by persistent anthers. Furthermore, it was possible to remove all remaining anthers by hulling and polishing (Fig. 3E). Thus, spwl-cls maintains brown rice quality and the rice can be handled in the same manner as common cultivars.

Using relatively coarse evaluation procedures, we demonstrated that cleistogamy introduced by the spwl-cls mutation had little effect on many agronomic traits (Yoshida et al. 2007). In this study, over a five-year cultivation period, there were some differences in yield-related traits between spwl-cls and T65; however the estimated yield of spwl-cls (calculated from yield-related traits) was similar that of T65 and was acceptable (Table 1). In this respect, the spwl-cls mutation has more promise than the d7 mutation, which is accompanied by a dwarfing phenotype and significantly unfavorable agronomic traits, especially low yield (Nagao and Takahashi 1954, Fig. 1). In addition, the spwl-cls lines and individuals are likely to have various second-site mutations that occurred concurrently with the spwl-cls mutation in the original M1 generation mutant, because they varied in some characteristics (i.e., dwarfism and sterility). It is possible that these mutations lowered the yield of spwl-cls.

Because we identified the position of nucleotide change responsible for spwl-cls, we were able to design DNA markers that detected the spwl-cls mutation. As a model case for using such DNA markers, we created the cleistogamous backcross lines Yumeaoba-cls using a dCAPS marker (Fig. 4). In Japan, research on GM rice for livestock forage is in progress. Because “Yumeaoba” is one potential cultivar suitable for whole crop silage (Miura et al. 2006), we selected this variety as the recurrent parent of the cleistogamous backcross line. Yumeaoba-cls had stable cleistogamy in the paddy fields through two years of experiments. Thus, we were able to successfully introduce the spwl-cls mutation to other cultivars by using a reliable DNA marker. The spwl-cls mutation was functional in other genetic backgrounds. Although the number of backcrosses was limited, the yield-related traits of Yumeaoba-cls of the BC2F3 generation in 2009 and the BC2F3 generation in 2010 were closer to those of the recurrent parent Yumeaoba (Table 2), compared with the yield difference between spwl-cls and T65. The creation of Yumeaoba-cls lines that would be more isogenic with Yumeaoba is likely possible, if we were to increase the number of backcrosses for removing the second-site mutations. On the other hand, the introduced spwl-cls mutation is not expected to have secondary effects on agronomic traits expressed in the original cultivars.

Our natural crossing tests in the paddy fields clearly showed that spwl-cls is able to suppress outcrossing (Table 3). In all the experiments with T65 as the pollen parent line, we found significant numbers of xenia grains derived from crossings with the pollen parent. The number of xenia grains in the south experimental plot located to the lee of prevailing winds was higher than in the other (i.e., north, east and west) plots. However, we found no xenia grains in experiments with spwl-cls as the pollen parent line under conditions prevailing when T65 was the pollen parent. Although xenia grains with non-T65 genotype were discovered, we judged that they were derived from crossings other than crossing with spwl-cls, because the experimental paddy field where the natural crossing tests were conducted was surrounded by other paddy fields where various cultivars and lines other than T65 were cultivated. Crossing rates of rice decrease as distances between plants increase (Sato and Yokoya 2008, Tanno et al. 2011). In our experiment, pollen parent lines and seed parent lines were very close to one another. In lines positioned closest together, panicles of
individual parents were able to make physical contact. In spite of this close contact, *spw1-cls* never crossed with the seed parent lines. Thus, there was almost total suppression of crossing capability in *spw1-cls*, at least under the experimental conditions we used. We consider that the difference in whole crossing rate between 2008 and 2010 was related to the length of overlapped heading periods of donor and recipient lines. The temperature in summer 2010 was remarkably high and caused more rapid growth and earlier heading of “Raichou-mochi” than T65 and *spw1-cls*. These conditions decreased the overlap period of heading, and thus suppressed the crossing rate in 2010. The number of non-T65 genotype xenia grains employing *spw1-cls* as the pollen donor was greater than that employing T65. We hypothesized that this could have been caused by the absence of pollen dispersal from the donor block. Because of the lack of competition in pollination between the pollen donor and other varieties cultivated in neighboring fields, the natural crossing other than with *spw1-cls* would have increased.

Although corn, soybean and cotton are the most widely cultivated commercial GM crops worldwide, GM rice cultivars will soon be developed and cultivated widely. GM rice with the *spw1-cls* mutation will suppress crossing with non-GM rice cultivars and inhibit gene flow by pollen dispersal. In addition, the mutant also inhibits the non-GM to GM gene flow, which would otherwise eliminate useful characteristics of GM rice. It is important maintain the quality of GM rice, particularly those GM rice traits that are of medical use. The *spw1-cls* mutation would also be useful in maintaining “purity of lines” of non-GM rice cultivars. For example, keeping glutinous and non-glutinous lines separate is an issue of concern (Kamagata et al. 1988, 1990). Currently, suppression of such natural crossings is done by separating the heading dates of the cultivars and maintaining adequate distances between cultivated paddy fields of glutinous and non-glutinous cultivars. This entails much effort and is demanding on space. However, the use of glutinous rice cultivars with the *spw1-cls* mutation would make it possible to cultivate glutinous and non-glutinous cultivars in adjacent paddy fields without consideration of heading dates.

Although the natural crossing rate of rice is usually affected by the shapes and sizes of stamens and pistils (Kato and Namai 1987a, 1987b), cleistogamy in *spw1-cls* is unaffected by these factors because the lemma and palea enclose the inner floral organs and do not open. Thus, the *spw1-cls* mutation is suitable for cultivars with all shapes and sizes of floral organs.

The molecular lesion of *spw1-cls* causes a missense mutation in the *SPW1* gene encoding a B-class MADS-box protein. The *SPW1* protein forms a heterodimer with MADS2 or MADS4 proteins thus exerting B-class activity to specify lodicule and stamen identities. The *spw1-cls* mutation causes reduction in protein-protein interaction between *SPW1* and MADS2/4, with consequently reduced B-class activity that results in the transformation of the lodicule to an mrp-like organ (Yoshida et al. 2007). Yoshida et al. reported that protein-protein interaction between SPW1<sup>cls</sup> and MADS2/4, which causes cleistogamy, is restored by reducing temperature in a yeast two-hybrid system. Therefore, it would be important to determine whether the cleistogamy of *spw1-cls* is maintained through the range of climates under which rice is cultivated, particularly where summers are cool. Despite this qualification, we have demonstrated significant advantages and stability of the *spw1-cls* mutation and the cleistogamous phenotype. The *spw1-cls* mutation is not expected to affect commercial traits, and we were able to reliably introduce the mutation to any cultivar by using DNA markers. We also showed that *spw1-cls* cleistogamy effectively inhibits natural crossing in paddy fields. We have thus developed the most practical mechanism for suppressing transgene flow and/or maintaining purity of genetic lines.

**Acknowledgments**

We thank K. Tsukada, K. Yukawa, N. Ichimura, K. Asano, M. Sekizawa and M. Iizuka for their excellent assistance in various experiments, K. Hayashi and M. Kimizu for helpful discussion and encouragement and T. Kotake, M. Ichihashi, T. Genba, S. Yuminamochi and K. Koide for their help in the rice cultivation. This work was supported by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan (Research Project for Genomics for Agricultural Innovation).

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