閉花受粉性イネ突然変異体の解析とその利用
Analysis and Utilization of a Cleistogamous Mutant of Rice

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Introduction

Until more information about the environmental and agronomic impact of genetically modified (GM) crops is available, gene containment is necessary to prevent the spread of transgenes into the environment (DANIELL, 2002). Cultivated rice in Japan is essentially autogamous, with an outcrossing frequency of less than 1%, but its flowers still open at anthesis and release pollen grains into the environment. Therefore, the possibility of outcrossing with wild relatives or non-GM rice has raised public concern regarding the environmental impact of GM rice. Several approaches have been proposed to minimize or eliminate gene flow through pollen, but no practical method has yet been developed in rice.

One possible approach, cleistogamy (self-fertilization without flower opening), would minimize the risk of transgene spread. In rice, cleistogamous mutants and transgenic plants have been reported; however, there have been no commercially practical cleistogamous rice lines identified or developed so far. In collaboration with the University of Tokyo and Kyushu University, we identified a novel cleistogamous mutant with a missense mutation in the floral homeotic gene SPW1, and we found that the mutation does not affect other agronomic traits (YOSHIDA et al., 2007). In this chapter, we describe the molecular and physiological characterization of this mutant and discuss strategies for its utilization.

Mechanistic view of rice flower opening: the important role of the lodicule

To develop and utilize cleistogamous rice, we first need to understand the mechanism of flower opening in rice, and then to inhibit the flower-opening machinery without affecting other physiological processes. The flower of rice, also known as the floret, comprises one lemma, one palea, two lodicules, six stamens, and one pistil (Fig. 1).

Each stamen further consists of a filament and an anther, in which more than 1000 pollen grains are produced. The spreading of the tips of the lemma and palea is called flower opening or anthesis. The driving force behind flower opening is generated by the lodicule, the least conspicuous floral organ in rice. The lodicule is a small and roundish scale-like organ located inside the flower at the base of the lemma. The lodicules start to swell immediately before flower opening and push the lemma outward to make the flower open (HOSHIKAWA, 1989). Based on comparative observations of its location in the flower, surface cell structure, and expression patterns of floral genes, the lodicule is regarded as a homolog of the dicot petal (KYOZUKA and SHIMAMOTO, 2002). Its appearance is quite different from that of the...
beautiful petals in roses or cherry blossoms; however, its role in flower opening is the same. The lodicule possesses a number of vascular bundles that allow water uptake, which causes the lodicule to swell. At the same time, the filament begins to elongate and lift the anther toward the top of the flower. Immediately before the onset of flower opening, the anthers dehisce and disperse the pollen grains onto the stigmas within the same floret. After dehiscence, the filaments continue to elongate and move the anthers outside the glumes (i.e., the lemma and palea). Finally, the lodicules stop swelling and begin to shrink, closing the glumes. Once the lodicules shrink, they lose the ability to swell, thus the flower never opens again. At the same time, filament elongation stops, and the nearly empty anthers are left outside the closed glumes. Therefore, it is possible to evaluate the frequency of flower opening by counting numbers of florets with anthers left outside the glumes.

As a method to produce fertilized seeds while at the same time preventing pollen dispersal into the environment, cleistogamy would be an efficient strategy, at least in theory. It is unclear whether creating a practical type of cleistogamous rice is really possible, but several reports provide useful information relevant to this question. For example, it has long been known that rice flowers do not open on rainy days, but the anthers dehisce inside the closed flowers and disperse the pollen grains onto the stigma, self-fertilizing the ovule (HO-SHIKAWA, 1989). Therefore, rice appears to have the potential to become cleistogamous. There have also been several studies on cleistogamous rice mutants such as d7; however, these mutants have not been of practical use because they also have undesirable agronomic characters such as dwarfism (NAGAO and TAKAHASHI, 1963). On the other hand, cleistogamous varieties and landraces are already available in other gramineous species such as barley and wheat. Cleistogamous barley varieties have smaller lodicules than those of non-cleistogamous varieties; therefore, it is suggested that their lodicules are less able to push the lemma outward and cause flower opening (TURUSPEKOV et al., 2004). These observations suggest that it would be possible to make practical cleistogamous rice if the morphology or identity of the lodicule could be modified.

One of the possible strategies to create cleistogamous rice would be to transform the identity of the lodicule into that of another floral organ. B-class MADS-box genes are involved in the specification of lodicules and stamens in grass species (AMBROSE et al., 2000; NAGASAWA et al., 2003). In Arabidopsis, B-class MADS-box proteins comprise two major subfamilies, APETALA3 (AP3) and PISTILLATA (PI). Members of these two subfamilies combine to form a heterodimer (AP3-PI) that exerts B-class activity and specifies petal and stamen identity (JACK et al., 1992; GOTO and MEYEROWITZ, 1994; RIECHMANN et al., 1996). Rice has one AP3 ortholog, SUPERWOMAN1/MADS16 (SPW1), and two PI orthologs, MADS2 and MADS4 (KANG et al., 1998; NAGASAWA et al., 2003; PRASAD and VIJAYRAGHAVAN, 2003; YADAV et al., 2007; YOSHIDA et al., 2007; YAO et al., 2008). Therefore, it would be theoretically possible to create cleistogamy by manipulating expression of these B-class genes.

Identification of spw1-cls, a practical cleistogamous mutant in rice

We performed screening for cleistogamous mutants at the experimental farm of the University of Tokyo, using a mutated population of ssp. japonica cv. Taichung 65 generated by the research group at Kyushu University. From an M2 population induced by MNU (N-methyl-N-nitrosourea) treatment, we selected several lines that segregated for plants in which anthers were not observed outside the glumes at the flowering stage. Further examination revealed that most of these lines had underdeveloped stamens and were male-sterile. However among these lines, we identified one line in which the cleistogamy segregated as a single-gene recessive trait, and we tentatively named the mutant cleistogamy (cls) (YOSHIDA et al., 2007). As described above, the stamens of wild-type flowers elongated out of the glumes upon flower opening and remained outside of the glumes for several days. In contrast, cls mutant flowers did not open and no stamens were observed outside the glumes (Fig. 2). Consequently, the pollen grains of this mutant were theoretically not dispersed.
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Fig. 2. Panicle phenotype of the cleistogamous rice mutant *spw1-cls*.
Panicles of the wild-type (left) and the *spw1-cls* mutant (right). Stamens were not observed outside the glumes of the mutant.

into the environment. However, it will be necessary to confirm whether pollen grains that do not land where they can fertilize an ovule survive long enough to fall from the flower and enter the environment. When this mutation was introduced into "Kasalath" (an indica variety), the cleistogamous trait was expressed stably despite the differences in genetic background between Kasalath and Taichung 65.

The *cls* mutant would not be of practical use if the causal gene caused pleiotropic effects on other agronomic traits, as in the case of *d7*. We also wondered if the stamens remaining inside the glumes might cause a malformed grain shape. Therefore, we cultivated this mutant at the experimental farms at Hokuriku Research Center (Jo-etsu, Niigata, Japan) and at the University of Tokyo (Tanashi, Tokyo, Japan), and examined the agronomic traits and grain shape of the *cls* mutant. The heading date, plant height, culm number, panicle length, flower number, seed fertility, grain weight, and grain shape of *cls* did not differ significantly from those of the wild-type (YOSHIDA et al., 2007). Thus, *cls* appears to affect specifically the mechanism of flower opening and is therefore a promising allele for practical gene containment in rice.

When we examined the florets of *cls* in detail, the stamens and pistil of *cls* were normal, but its lodicules were elongated compared to those of the wild-type (Fig. 3). The number of vascular bundles in the lodicules of *cls* was also reduced compared to wild-type lodicules. A series of morphological observations suggested that the *cls* lodicules partially acquire the identity of the glume; therefore, the cleistogamy of *cls* appears to result from a partial homeotic conversion of lodicules into glumes. This defect seems to inactivate lodicule swelling and thus prevent flower opening.

**Molecular characterization of the *spw1-cls* mutation**

The *cls* mutation was mapped to the long arm of chromosome 6 using molecular markers. This region contains the *SPW1* gene, which (as described above) is a B-class MADS-box gene involved in the specification of lodicule and stamen identity. Two recessive alleles of *SPW1*, *spw1-l* and *spw1-2*, both cause the homeotic transformation of lodicules into glume-like organs and of stamens into carpels. Therefore, the flowers of *spw1-l* and *spw1-2* do not open and are sterile (NAGASAWA et al., 2003). The flowers of *cls* somewhat resemble *spw1-l* and *spw1-2* flowers, in that their lodicules become elongated like glumes. Examination of the nucleotide sequence of the *SPW1* gene in *cls* revealed a single base change leading to an amino acid change (isoleucine-45 to threonine; I45T) in the MADS-box domain. A complementation test further revealed that the cleistogamous phenotype of *cls* could be rescued to that of
the wild-type by introducing a genomic DNA fragment of the wild-type SWP1 (gSWP1), whereas introduction of the same genomic region containing the I45T mutation (gSWP1\textsuperscript{145T}) failed to complement the cleistogamous phenotype. Therefore, whereas swp1-1 and swp1-2 are thought to be severe null alleles, cls is a novel weak allele of SWP1 and was thus renamed as superwoman1-cleistogamy (spw1-cls; YOSHIDA et al., 2007). In SWP1 RNAi lines, a wide range of phenotypes from Spw\textsuperscript{−} (the phenotype of swp1-1 and swp1-2) to Cls\textsuperscript{−} (the phenotype of swp1-cls) was observed. In addition, introduction of gSWP1\textsuperscript{145T} into swp1-1 phenocopied the Cls\textsuperscript{−} phenotype. These results suggest that lodicule and stamen development have differential sensitivity to the level of B-class protein activity. They further suggest the possibility of producing cleistogamous plants by engineering one or more B-class floral organ identity genes and thus transforming lodicule identity.

As in Arabidopsis, the proper functioning of SWP1 in rice would require heterodimerization with two PI orthologs (MADS2 and MADS4). The 45th amino acid of SWP1 is located in the β-strand of the MADS domain and is thought to be involved in the dimerization of MADS proteins. The MADS-box proteins possess a conserved cluster of small hydrophobic amino acids (I, L, V, A, or F) at or around amino acid 45, whereas swp1-cls has a hydrophilic amino acid (T) in this position. In a yeast two-hybrid system, the wild-type SWP1 strongly interacted with both MADS2 and MADS4, suggesting formation of two types of heterodimers: SWP1\textsuperscript{WT}-MADS2 and SWP1\textsuperscript{WT}-MADS4. However, SWP1\textsuperscript{145T} showed much weaker interaction with MADS2 than did wild-type SWP1. SWP1\textsuperscript{145T} interacted even less with MADS4; almost no interaction was detected, suggesting the critical role of a hydrophobic amino acid at this position for the dimerization of MADS-box proteins. Therefore, the SWP1\textsuperscript{145T} protein is defective in dimerization with the counterpart B-class proteins (YOSHIDA et al., 2007; YAO et al., 2008).

\textit{In situ} hybridization analysis revealed that MADS2 was expressed in both lodicule and stamen primordia, whereas MADS4 was expressed in stamen primordia but not in lodicule primordia (YOSHIDA et al., 2007), suggesting that the SWP1-MADS2 heterodimer is the one that is important for lodicule formation. In contrast, both the SWP1-MADS2 and SWP1-MADS4 heterodimers are involved in stamen development. Thus, it is presumed that the low level of the SWP1\textsuperscript{145T}-MADS2 heterodimer present in swp1-cls is insufficient to allow the establishment of complete lodicule identity, but sufficient for the specification of stamen identity, because SWP1\textsuperscript{145T} failed to interact with MADS4, which is normally expressed in stamen primordia.

Taken together, the lodicule-specific phenotype of swp1-cls is likely caused by a combination of differential requirements of B-class protein activity in lodicule and stamen, differential expression of MADS2 and MADS4 in lodicule and stamen, and differential interaction affinity of SWP1\textsuperscript{145T} for MADS2 and MADS4.

\textbf{Toward the effective utilization of cleistogamy in rice}

The occurrence of outcrossing in rice, even at a low frequency, has caused concern about transgene contamination from GM rice in both feral rice (crop-to-wild) and cultivated rice (crop-to-crop). Therefore, gene containment technologies are necessary in rice to suppress gene flow and enable the coexistence of GM rice with non-GM rice or wild relatives in both experimental and commercial cultivation. As described above, swp1-cls is a promising genetic resource for developing a practical gene containment technology in rice. With that goal in mind, we have been introducing the swp1-cls mutation into several Japanese cultivars using DNA markers to make near-isogenic lines (OHMORI and YOSHIDA, unpublished data). These lines can be used not only for gene containment in GM rice, but also for other situations in which outcrossing is unfavorable. For example, it would be useful for suppressing outcrossing between purple/red rice cultivars or cultivars with modified levels of specific seed contents and normal cultivars. It would be also useful for maintenance of purity during foundation seed production.

However, because swp1-cls is a weak mutation based on a reduced level of protein–protein interaction, it is likely to be a temperature-sensitive mutant. Indeed,
yeast two-hybrid experiments revealed that low temperature caused an increase in the binding activity between SPW1<sup>-14ST</sup> and MADS2 (YOSHIDA et al., 2007). These results suggest that when the temperature at the booting stage is cool, increased B-class heterodimer formation in the spw1-cls mutant may lead to partial recovery of lodicule morphology, resulting in flowers that open at the usual flowering time. Therefore, we have been examining the stability of the cleistogamy in spw1-cls for several years in several regions of Japan with a range of environmental conditions (from Hokkaido to Okinawa). In collaboration with many laboratories in Japan, we found that spw1-cls flowers open at a certain rate in the northern area of Japan (i.e., in Hokkaido and part of the Tohoku region; Ohmori et al., unpublished data). Further investigation should allow us to define detailed conditions to regulate flower opening/non-opening of spw1-cls. By applying such knowledge, guidelines for use of this mutation in different environments and in various genetic backgrounds can be established.

Because of the potential instability of spw1-cls, screening for more stable cleistogamous mutants is required. In addition, molecular biological techniques that artificially induce cleistogamy should be pursued and could be useful in cereal crops other than rice. Recently, several novel findings on molecular genetic mechanisms regulating lodicule development have been published, such as identification of the barley cleistogamy gene Cly1/HvAP2 (NAIR et al., 2010) and the rice MFO1 gene, which regulates floral morphology, including lodicule development (OHMORI et al., 2009). Such basic studies would facilitate our understanding of lodicule development and enhance the development of cleistogamous cereal crops.

Cleistogamy is a novel character that has not yet been used in commercial rice but would provide a number of benefits. It is anticipated that further studies will lead to various agronomic applications of this novel and intriguing character.

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10. NAIR, S.K., WANG, N., TURUSPEKOV, Y., POUKHEIRAN-


閉花受粉性イネ突然変異体の解析とその利用

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日本で栽培されているイネの大部分は自殖性が高く、花（小花）が開くときにおしぼれ小花の外まで伸び出て花粉を飛散させるため、低頻度ではあるものの、自然交雛が起きる。花粉飛散による遺伝子組換えイネと一般的イネとの自然交雛を抑制するための技術の一つとして、閉花受粉性（閉花せずに出粉・穂稔する性質）の利用が有効と考えられる。本稿では、筆者らが発見した閉花受粉性イネ突然変異体の特徴、原因遺伝子の作用機構、さらに今後の利用に向けた取り組みなどについて紹介する。

イネの小花は、雌穂と内穂、2つの鱗被、6本の雄蕊、1本の雌蕊、によって構成される。このうち、開花を引き起こす原動力となるのが「鱗被」である。鱗被は外穂の基部にある丸い小さな器官であるが、開花の直前から急激に膨らみ始め、外穂を外側に押し出すため、開花が起きる。開花時に花の外に抽出した薬は、穂が閉じた後にも外部に取り残される。一度閉じた穂は二度と開かないため、穂には含まれた薬の有無によって、開花の有無を判断することができる。鱗被の形態だけを変化させることができれば、イネにおいても実用的な閉花受粉性を付与することが可能であると考えられたため、鱗被の形態形成に着目し、「台中65号」の突然変異体群を用いて開花受粉性突然変異体のスクリーニングを行った。まず、開花期に穂の外に薬が出ていないものを数系統選抜したところ、多くのものは不稔であったものの、花を開かずに正常に出穂をした1系統を見出し、「閉花受粉性」を意味する「cleistogamy」（略称：cls）と名付けた。出穂日、草丈、穂数、穂長、1穂粒数、穂実率、粒重、粒の形状や外観などの農業形質について、clsと原種の農業特性には、顕著な差がなく、clsは実用的な閉花受粉性イネを開発するための有望な遺伝資源と考えられた。clsの花では、雄蕊や雌蕊には変化がないが、鱗被が平らで細長い穂狀の器官に変化し、内部の維管束数も減少していた。こうした変化により鱗被が膨潤できず、開花しなくなったものと考えられる。

閉花受粉性の原因は、SUPERWOMAN1 (SPW1)遺伝子のアミノ酸置換変異であったため、この閉花受粉性イネを superwoman1-cleistogamy(spw1-cls)と呼ぶことにした。SPW1は鱗被と雄蕊の形作りに関わる遺伝子群の発現を制御するMADSボックス型転写因子をコードしており、spw1-1、spw1-2などの機能欠失型アリルでは、鱗被が穂状器官に変化するだけでなく、雄蕊も雌蕊へと変化し、完全不稔となる。同じ遺伝子の変異が原因であるにもかかわらず、なぜspw1-clsでは雌蕊が正常に形成され、正常に稔実するのだろうか？転写因子であるSPW1タンパク質は、DNA結合ドメインであるMADSドメインなどを介してヘテロ二量体を形成し、標的DNAに結合する。酵母ツーハイブリッドアッセイの結果などから、cls型SPW1タンパク質は、MADSドメイン内の保存性の高い45位アミノ酸が鍵の位置から親水性に変異しているため、二量体形成能が低下し、転写因子としての機能が低下するものと考えられた。また、SPW1のパートナータンパク質をコードする2つの遺伝子のうち、OsMADS2は鱗被と雄蕊の両方の形成に関与するのに対し、OsMADS4は主に維管束形成に関与するため、これらパートナー遺伝子の発現パターンおよび、cls型SPW1タンパク質との相互作用能の違いなどによって、鱗被にだけ形態変化を生じるものと考えられる。
えられた。
spwl-cls 変異体は実用的な開花受粉性遺伝資源として期待されるが、アミノ酸変換による弱い変異が原因であるため、穂の形成時期に冷涼となる地域では温度感受性を示し、野生型と同様に開花する可能性が考えられた。実際、日本国内の環境の異なる地域で栽培を行い、開花受粉性の安定性を検討したところ、北海道や東北などでは spwl-cls は一定の割合で開花することが明らかとなり、spwl-cls を安定的に利用するためには、開花を引き起こす詳細な条件などの知識を積み重ねていく必要があると考えられた。また、新規開花受粉性突然変異体の探索や、人為的に閉花受粉性イネを作出する技術を開発していくことも重要である。

一方では、spwl-cls 変異体をさまざまな実用品種と交配し、開花受粉性準同質遺伝子系統の育成を進めている。こうした系統は、遺伝子組換えイネの母本としてだけでなく、紫黒米や赤米と言った有色素米品種、特定の成分含量を変化させた機能性品種、あるいは原種や原種品種で種子の純度維持が強く求められる品種など、花粉飛散による交雑に対して注意が必要とされる場面への応用も考えられる。イネにおいて、開花受粉性はこれまでになかった新しい形質である。今後、さらなる研究の進展により、多様な場面において閉花受粉性イネが利用されていくことを期待したい。