Introduction

In conventional breeding of autogamous crops such as rice (*Oryza sativa*), wheat (*Triticum aestivum*), and soybean (*Glycine max*), breeders generally use pedigree breeding (Breseghello and Coelho 2013). They cross two materials, then perform genetic fixation by selfing, phenotype-based selection, and evaluation of regional adaptability. Finally, the selected line is registered as a cultivar. This procedure requires approximately one decade and the typical number of usable materials is two. To breed the best possible cultivar, breeders must cross the top cultivars. After several decades, repeating this breeding cycle decreases the genetic diversity of the breeding population and therefore the breeding effect. Indeed, the yield improvements of autogamous crops have stagnated after the 1990s (Tanaka and Tabei 2014).

In contrast, the increase in the yield of maize, an allogamous crop, due to breeding has still not leveled off (USDA National Agricultural Statistics Service, https://www.nass.usda.gov/index.php). Maize cultivars are usually F1 hybrids derived from crossing between two parental lines. The increase in the yield of maize is driven mainly by the improvement of parental lines by recurrent selection (RS)-based breeding, rather than by finding new crossing combinations for new F1 hybrid cultivars. In RS, maize breeders repeat the cycles of outcrossing and selection after selfing in breeding populations. Multiple successive rounds of selection increase the frequency of desirable combinations of alleles and improve the genetic gain in the breeding population. Breeders can use many breeding materials (founders), and there is frequent outcrossing among them. Thus, RS-based breeding continuously improves maize yield.

To implement the maize-like breeding methods for an autogamous crop, recurrent selection using genetic male sterility has been proposed and attempted to practice in autogamous crop breeding (Brim and Stuber 1973, Doggett and Eberhart 1968, Fujimaki 1980). However, these breeding systems have not become a major breeding method in autogamous crops because they require much additional labor. As a more efficient system, recurrent selection using...
transgenic male sterility (RSUTMS) was proposed (Tanaka 2010). RSUTMS is based on the development and application of dominant transgenic male sterility (TMS) with positively and negatively selectable marker traits. A dominant TMS gene cassette facilitates efficient outcrossing without manual emasculation, while the positively and negatively selectable marker gene cassettes allow to distinguish between male-sterile and fertile individuals. The introduction of such gene cassettes can create a positively and negatively selectable TMS plant to facilitate RS in autogamous crops.

Some TMS rice lines have been developed (Abe et al. 2018, Akasaka et al. 2018, Lu et al. 2000), but their flowering habits are not suitable for RS-based breeding because of their delayed heading, flowering, and daily flowering time relative to the wild type (Abe et al. 2018). Similar delays were observed in cytoplasmic male sterility (MS) in hybrid rice, which caused failure of opening of 20% of female parent spikelets (Yan and Li 1987), partial or full panicle enclosure (Singh and Shirisha 2003, Tien et al. 2013, Yin et al. 2007), and asynchronous flowering (Lou et al. 2014). These features decrease outcrossing fertility.

The development of a gene cassette to increase outcrossing fertility of plants with dominant TMS would provide a useful tool for the novel breeding system in rice. Plants with dominant TMS cannot produce viable pollen grains and therefore require wild-type pollen for fertility. For this reason, all individuals with dominant TMS must be heterozygous for the TMS gene cassette. Consequently, the newly developed gene cassette to increase outcrossing fertility must be dominant when introduced together with the TMS gene cassette by the same vector into the same locus. If the new cassette is recessive, it will not be functional with dominant TMS.

A dominant gene could be under control of a native promoter, or the promoter could be improved, for example, to increase the expression level and to narrow the tissue specificity. In contrast, the use of a recessive gene must be combined with RNA interference technology or similar techniques; however, it is widely recognized that it is difficult to perfectly suppress the gene activity by these techniques, and the effectiveness of these techniques tends to be unstable in long-term applications (Mitsuhara et al. 2002). The availability of a dominant cassette to increase rice outcrossing fertility will make RSUTMS more efficient and applicable to large-scale rice breeding.

Stigma exsertion can improve outcrossing fertility in rice (Kato and Namai 1987). The exserted stigma can stay viable and accept pollen for 6 days (Xu and Shen 1987, Yan and Li 1987, Yan et al. 2009). This trait increases the opportunity for pollination in female parents of hybrid cultivars (Kato and Namai 1987) and overcomes the barrier of non-synchronous flowering between the parents (Lou et al. 2014).

As a quantitative trait, stigma exsertion is controlled polygenically and has no cytoplasmic effect (Li and Chen 1985, Virmani and Athwal 1974). It has high heritability and can be improved by breeding (Virmani 1994, Yan et al. 2009). Many QTLs for this trait have been reported (Li et al. 2014, Lou et al. 2014, Miyata et al. 2007, Rahman et al. 2016, Uga et al. 2003, Yamamoto et al. 2003). The most common and most powerful QTL for stigma exsertion is frequently detected near the centromere in the short arm of chromosome 3 (Li et al. 2014, Miyata et al. 2007, Yamamoto et al. 2003). The responsible gene of this QTL has been cloned as the Grain Size 3 gene (GS3) (Fan et al. 2006, Takano-Kai et al. 2009, 2011). GS3 is a major gene for seed length (Fan et al. 2006); it also affects stigma length and stigma exsertion in rice (Takano-Kai et al. 2011), but the long and narrow grain and stigma exsertion–type allele of GS3 is recessive.

Reduction of stigma exsertion is a domestication trait in rice (Huang et al. 2012). In wild rice species, stigma exsertion facilitates outcrossing and maintains the diversity of natural populations. This diversity plays a key role in adaptation to environmental fluctuations as a wild species. On the other hand, stigma exsertion is not desirable in cultivars because it disturbs the uniformity and stability achieved by selfing. Generally, during domestication, many functional (dominant) alleles of wild species mutate to loss-of-function (recessive) alleles. Wild relatives would preserve the dominant alleles and would thus seem to be promising resources for detection of such alleles, including those for stigma exsertion.

There is a wide range of variability for stigma exsertion among wild rice species (Marathi and Jena 2015, Uga et al. 2003). In this study, we screened wild rice species and detected two clearly dominant QTLs for stigma exsertion, which have the potential to improve RSUTMS in the future.

Materials and Methods

Database screening of donor accessions
To obtain suitable genetic material for QTL analysis, we searched the Oryzabase database (https://shigen.nig.ac.jp/rice/oryzabase/) using 23 image files of the flowers of wild rice accessions on the “Flower” page under “Image Gallery”. The screening criteria were as follows: 1) Clear stigma exsertion. 2) More closely related to O. sativa to give lower crossing isolation and more frequent genetic recombination. 3) Rounder grain shape, which is desirable without long grain type of the allele of GS3 as a background at the QTL analysis to detect the other QTLs.

Construction of populations for genetic analysis
Because we aimed to detect dominant QTLs, genetically fixed materials were not suitable, so we prepared F2 and BC1F1 populations. The F1 individuals were generated by
crossing the *japonica* cultivar ‘Akidawara’ and *O. rufipogon* accession ‘W0120’ in a simplified biotron breeding system (Tanaka et al. 2016). Because wild rice, especially the perennial types such as *O. rufipogon*, has a high probability of outcrossing in nature (Huang et al. 2012, Khush 1997), ‘W0120’ might have heterozygous genome regions. Therefore, only one F1 individual was arbitrarily selected and selfed to produce the F2 population for QTL analysis. The BC1F1 population, used to validate the detected dominant QTLs, was derived by backcrossing ‘Akidawara’ with the same F1 plant used for the preparation of the F2 population.

**Phenotypic evaluation of stigma exsertion**

Because wild species and their progeny generally have deep seed dormancy, all seeds were soaked in 1% H2O2 for 2 days before sowing. Seedlings were grown in nursery boxes for 4 weeks, and each individual was transplanted into a horticultural pot and grown in summer in a NARO research field in Kannondai, Tsukuba, Ibaraki, Japan (36°13′N, 140°64′E). To synchronize heading as much as possible, short-day treatment was applied in the biotron for 2–3 weeks.

Stigma exsertion was evaluated at 7–10 days after heading in all spikelets of one spike per individual. Spikelets with stigma exsertion and total spikelets were counted, and the ratio of spikelets with stigma exsertion to total spikelets (stigma exsertion ratio, SER, %) was calculated.

**DNA marker detection**

Total DNA was extracted from a young leaf (3–4 weeks old) of each individual with a DNA Sui Sui-S extraction kit v. 1.01 (Rizo Inc., Tsukuba, Japan). Indel markers were used because they are codominant, have high density, and are convenient for direct detection by PCR (Liu et al. 2013, Pâcurar et al. 2012). All markers were chosen from among those listed in Yonemaru et al. (2015) with consideration of the GC content of the amplicon, melting temperature of primers, and marker position in the rice genome. In total, 216 markers were tested for their polymorphisms, and markers polymorphic between the parental accessions were used for genotyping.

PCR was performed in a final volume of 10 μl containing 5 μL GoTaq Green Master Mix (Promega Corporation, Madison, WI, USA), 5 μM each of a PCR-primer pair, 0.1 μM each of a PCR-primer pair and 2 ng of template DNA. Touchdown PCR was performed as follows: initial denaturation for 3.5 min at 94°C; 34 cycles of 30 s at 94°C, 60 s at annealing temperature (described below), and 30 s at 72°C; 10 min at 72°C. The annealing temperature was 62°C in the first cycle; lowered by 0.5°C per cycle during cycles 2 to 14; and retained at 55°C for the last 20 cycles. PCR products were electrophoresed in agarose gel in TAE buffer and stained with ethidium bromide.

**Construction of a linkage map and QTL analysis**

The F2 population included 114 individuals. A linkage map was constructed in Antmap v. 1.2 software (Iwata and Ninomiya 2006). Grouping of markers into linkage groups was performed using the nearest-neighboring locus method with a threshold grouping criterion of 0.4. Genetic distances were calculated using the Kosambi mapping function (Kosambi 1944). QTL analysis was performed in Windows QTL Cartographer 2.5 software (Wang et al. 2006) using composite interval mapping with a manual threshold value of LOD = 2.0. Each detected QTL was named according to QTL nomenclature (McCouch et al. 1997).

**QTL validation**

In the BC1F1 population (188 individuals), marker genotypes were expected to be either homozygous for the ‘Akidawara’ type or heterozygous between ‘Akidawara’ and the donor. The expected segregation ratio was 1:1. A t-test was used to check the significance of differences in SER between genotypes of the flanking markers in the BC1F1 population. ANOVA and Tukey’s honestly significant difference (HSD) test were used for multiple comparisons between genotype combinations. The dominant effect of QTLs, calculated as the ratio of the average phenotypic value of heterozygotes to the average phenotypic value of both parents, was evaluated using the genotypes of markers nearest to the QTLs detected in the F2 population.

**Evaluation of the shape of BC1F2 grains**

BC1F2 seeds were harvested from 116 BC1F1 individuals and used for grain shape evaluation. The length, width, and ratio of length to width of all grains of each line were measured in SmartGrain v. 1.1 software (Tanabata et al. 2012).

**Results**

**Database screening of donor accessions**

Among 23 images of the flowers of wild rice accessions available in Oryzabase, 19 images were suitable for evaluation of the level of stigma exsertion. By eye, we selected 8 accessions with clear stigma exsertion (Table 1). They included two accessions (‘W0120’ and ‘W1945’) of *O. rufipogon*, which is most closely related to *O. sativa*. We chose ‘W0120’ for its rounded grain shape and clear stigma exsertion.

**Table 1. Wild rice accessions with stigma exsertion identified from flower images in the Oryzabase database**

| Acc. No. | Species | Genome | Country of origin | Grain shape            |
|---------|---------|--------|-------------------|------------------------|
| W0001   | *O. ridleyi* | HJJJ   | Thailand          | Solder                 |
| W0120   | *O. rufipogon* | AA     | India             | Somewhat slender       |
| W1185   | *O. glumaepatula* | AA | Suriname           | Somewhat slender       |
| W1194   | *O. grandiglumis* | CCDD   | Brazil            | Sharp-pointed          |
| W1197   | *O. latifolia* | CCDD   | Colombia          | Sharp-pointed          |
| W1413   | *O. longistaminata* | AA | Sierra Leone       | Somewhat slender       |
| W1945   | *O. rufipogon* | AA     | Thailand          | Solder                 |
| W2199   | *O. glumaepatula* | AA | Brazil            | Solder                 |
Stigma exertion ratio of ‘W0120’ and the F1 plant

The SER of ‘W0120’ was 89.3% (average of 11 individuals), and the SER of a F1 plant derived from a cross between ‘W0120’ and ‘Akidawara’ was 64.5% (average of 7 spikes). On the other hand, the SER of ‘Akidawara’ was 18.8% (average of 20 plants).

Linkage mapping and QTL analysis

Of the 216 markers evaluated, 105 were polymorphic; of these, we selected 92 on the basis of their polymorphisms and distribution (Supplemental Table 1). The SER in the F2 population showed a continuous variation from 0% to 90% (average, 36%).

The linkage map included 12 linkage groups and 92 markers with no gaps; the total length was 1555 cM (Fig. 1). Five QTLs for SER were detected in the F2 population on chromosomes 2, 3, 4, 8, and 11 (Fig. 1, Table 2). Except for the QTL on chromosome 4, the ‘W0120’ alleles increased SER. Two major QTLs were detected: qSER3, near the end of the short arm of chromosome 3, and qSER8, on the short arm of chromosome 8. Both had stronger dominance effects than the other QTLs. Contributions to phenotypic variation were 17% by qSER3 and 13% by qSER8. The region of GS3, which is the most commonly detected

Table 2. QTLs for stigma exertion ratio detected in the F2 population from a cross between ‘Akidawara’ and ‘W0120’

| QTLs  | Chromosome Position (cM) | LOD | R2(%)a | Add.b | Dom.c | The Nearest Markers | Marker Position | Nearby previously detected QTL or the nearest marker to the peak |
|-------|------------------------|-----|--------|-------|------|---------------------|----------------|--------------------------------------------------|
| qSER2 | 2                      | 6.0 | 2.0    | 9.8   | 10.1 | C5-indel1361        | 827575         | qPES-2 (Deng et al. 2010) |
| qSER3 | 3                      | 0.8 | 3.2    | 14.5  | 5.9  | C5-indel2400        | 537699         | R1468B (Yamamoto et al. 2003) |
| qSER4 | 4                      | 21.6| 2.0    | 1.5   | 12.1 | C5-indel3632        | 5050627        | |
| qSER8 | 8                      | 12.5| 2.3    | 6.6   | 11.6 | C5-indel8795        | 855285         | qSSE-11 (Yamamoto et al. 2003) |
| qSER11| 11                     | 1.0 | 2.2    | 4.5   | 9.9  | C5-indel8795        | 855285         | qDSE-11 (Rahman et al. 2016) |

a Phenotypic variance explained by QTL.
b Affected by additive effect.
c Degree of dominance.
Dominant QTLs for stigma exsertion ratio in rice

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would seem to be a great reservoir of dominant genes. In this study, we screened wild relatives of rice and selected ‘W0120’ as an ideal accession with clear stigma exsertion. We expected that ‘W0120’ may have a major dominant QTL, but found that the trait was controlled by several QTLs, similar to previous reports (Li et al. 2014, Lou et al. 2014, Miyata et al. 2007, Rahman et al. 2016, Uga et al. 2003, Yamamoto et al. 2003).

An F_1 individual from a cross between ‘W0120’ and ‘Akidawara’ showed clear stigma exsertion, which indicated the existence of at least one clearly dominant QTL. In fact, we detected two such QTLs, but none in the GS3 region in which Cai and Morishima (2002) detected a major QTL for grain shape. There is no polymorphism in the GS3 exon between ‘W0120’ and japonica-type rice cultivars in TASUKE (Kumagai et al. 2013, http://ricegenomes.dna.affrc.go.jp/), but there are many polymorphisms in the promoters and introns. The absence of a QTL in the GS3 region in our study indicates that these polymorphisms did not considerably affect GS3 function. The absence of QTL detection in the GS3 region must have increased the detectability of other QTLs. The screening of the materials with rounder shape as donor is assumed to be one of the reasons for the successful detection of QTLs outside of the GS3 region.

Fig. 2. Validation of the two dominant QTLs for stigma exsertion ratio (SER) in the BC1F_1 population. (A) qSER3 (C5-indel2400), (B) qSER8 (C5-indel6873). ■ ‘Akidawara’ type; □ heterozygotes between ‘Akidawara’ and ‘W0120’. ▼ SER of ‘Akidawara’; ◁ SER of ‘W0120’.

Strategy to detect dominant QTLs using F_2 and BC1F_1 populations

The type of population considerably affects the results of QTL region for stigma exsertion in rice (Li et al. 2014, Miyata et al. 2007, Yamamoto et al. 2003), was not detected in this population.

QTL validation

The frequency of the distribution of SER in the BC1F_1 population varied from 0% to 89% (average, 27.8%). Genotypes of the markers nearest to the QTLs on chromosomes 3 (C5-indel2400) and 8 (C5-indel6873) were clearly related to the SER phenotype (Fig. 2), with P-values of 0.0001 and 0.001, respectively, by t-test. The dominant effect was 1.55 for C5-indel2400 and 1.43 for C5-indel6873. The theoretical segregation ratio in the BC1F_1 population is 1:1, but homozygotes for the ‘Akidawara’-type alleles of both markers appeared more often than heterozygotes, and the distortions of segregation ratio were significant. Individuals with the heterozygous genotypes of both qSER3 and qSER8 showed 38.9% SER (Supplemental Fig. 1).

Fig. 3. Relationship between the genotypes of indel markers nearest to qSER3 and qSER8 and grain length in the BC1F_2 seeds derived from BC 1F_1 in Fig. 2. (A) C5-indel2400, the nearest marker to qSER3; (B) C5-indel6873, the nearest marker to qSER8. ■ ‘Akidawara’ type; □ heterozygotes between ‘Akidawara’ and ‘W0120’. ▼ SER of ‘Akidawara’; ◁ SER of ‘W0120’.

Relationships between the major detected QTLs for SER and grain shape

Evaluation using BC1F_2 seeds showed that C5-indel2400 and C5-indel6873 were related to grain length and the ratio of length to width in the BC1F_1 population (Fig. 3), but not to grain width. We could not detect any clear relationship between SER and grain shape traits.

Discussion

Effectiveness of screening of wild rice species

Because the outcrossings promoted by stigma exsertion reduces the uniformity and sustainability of cultivars of autogamous crops, we assumed that genes related to this trait are easily lost during domestication. Because many genes related to the domestication mutate from dominant to recessive during domestication (Li et al. 2006), wild relatives would seem to be a great reservoir of dominant genes. In this study, we screened wild relatives of rice and selected ‘W0120’ as an ideal accession with clear stigma exsertion. We expected that ‘W0120’ may have a major dominant QTL, but found that the trait was controlled by several QTLs, similar to previous reports (Li et al. 2014, Lou et al. 2014, Miyata et al. 2007, Rahman et al. 2016, Uga et al. 2003, Yamamoto et al. 2003).

An F_1 individual from a cross between ‘W0120’ and ‘Akidawara’ showed clear stigma exsertion, which indicated the existence of at least one clearly dominant QTL. In fact, we detected two such QTLs, but none in the GS3 region in which Cai and Morishima (2002) detected a major QTL for grain shape. There is no polymorphism in the GS3 exon between ‘W0120’ and japonica-type rice cultivars in TASUKE (Kumagai et al. 2013, http://ricegenomes.dna.affrc.go.jp/), but there are many polymorphisms in the promoters and introns. The absence of a QTL in the GS3 region in our study indicates that these polymorphisms did not considerably affect GS3 function. The absence of QTL detection in the GS3 region must have increased the detectability of other QTLs. The screening of the materials with rounder shape as donor is assumed to be one of the reasons for the successful detection of QTLs outside of the GS3 region.

Rice chromosomal segment substitution lines (CSSLs, Ebirani et al. 2005, Kubo et al. 2002, Takai et al. 2014) are excellent materials in which to detect QTLs. However, to detect new QTL alleles with large effects, starting from phenotype-based screening will be effective, rather than using the already-developed CSSLs.

Strategy to detect dominant QTLs using F_2 and BC1F_1 populations

The type of population considerably affects the results of
QTL analysis. In recent years, genetically fixed materials such as recombinant inbred lines (RILs) and CSSLs have often been used for QTL analysis of autogamous crops. The traits of these materials can be evaluated repeatedly with high reliability. However, genetically fixed materials are unsuitable for the detection of dominant QTLs. Although an F$_2$ population is suitable for producing a large number of populations and in which dominant effects can be evaluated, it is not suitable for high-sensitivity QTL analysis because evaluation using multiple individuals cannot be performed. On the other hand, production of a BC1F$_1$ population requires a large number of crossings, but it can be used for more precise analysis of dominant QTLs because of the genome-wide distribution of heterozygous regions, which eliminates the effects of recessive QTLs, and because of the theoretical 1:1 segregation ratio. We chose a strategy that included an initial QTL analysis using an F$_2$ population and then QTL validation using a BC1F$_1$ population. Using the F$_2$ population, we detected two QTLs with clear dominant effects but low LOD values, and validated them using a BC1F$_1$ population. Thus, the above strategy was efficient for our purpose.

**Relationship between the detected QTLs and grain shape**

We detected qSER3 and qSER8, two clearly dominant QTLs for stigma exsertion. qSER8 was mapped near qPES-8, which was detected as a QTL for stigma exsertion using a doubled haploid population derived from japonica and indica varieties (Deng et al. 2010). Although qSER8 and qPES-8 were detected in different materials, they might be the same QTL. qSER3 was detected almost at the end of the short arm of chromosome 3, where the QTL related to stigma exsertion was reported by Yamamoto et al. 2003. These two QTLs may be different or different alleles, because the previously detected QTL should a very small degree of dominance (Yamamoto et al. 2003).

Both QTL regions related to stigma exsertion were also related to grain length in the BC1F$_1$ population. It is unknown whether this is due to pleiotropic effects or to linkage. As GS3 also affects both stigma exsertion and grain length, this gene and the two detected QTLs might affect stigma exsertion through a common mechanism. Near the qSER3 region, OsCDPK1 affects grain shape (Ho et al. 2013), OsFIE1 (Folsom et al. 2014) and OsFIE2 (Nallamilli et al. 2013), located near the qSER8 region, also affect grain shape. However, these genes seem to be distinct from the QTLs detected in this study because they are located far from the peaks of our QTLs. In addition, qSER3 and qSER8 affect only the longitudinal axis direction of the grain, whereas the three previously identified genes influence grain size.

**Potential application of detected QTLs to improving seed productivity of hybrid rice**

Introducing the QTLs for stigma exsertion into the seed parents of hybrid rice cultivars by backcrossing with ‘W0120’ as a donor can improve hybrid seed productivity. However, because the effect is dominant, the seed parent trait will be expressed in F$_1$ cultivars. In introducing the QTLs into the seed parents of hybrid cultivars, breeders should pay attention to influencing the stigma exsertion ratio of hybrid cultivars. Also, if the introduced QTLs are pleiotropic, they will affect the grain shape of F$_1$ cultivars. Although long grain might increase yield, recessive genes such as GS3 may be easier to use for improving seed productivity without changing the grain traits of F$_1$ cultivars.

**Toward application of detected dominant QTLs to a new rice breeding method**

qSER3 and qSER8 may be used to increase seed productivity in RSUTMS through cloning of the respective genes via fine mapping and improving the flowering habit. These genes can be used with native promoters, but to improve stigma exsertion only in male-sterile individuals, an effective pistil-specific promoter may be needed, which can be identified using a gene expression profile database (Akasaka et al. 2018). Using one of the two genes may be sufficient to improve stigma exsertion.

For RS in autogamous crop species, an efficient outcrossing system is required, but the flowering characteristics of rice are not suitable for efficient outcrossing, especially in temperate japonica cultivars. The constructs for RSUTMS contain a dominant MS gene cassette and positively and negatively selectable marker trait gene cassettes. The constructs can potentially be improved by introducing additional gene cassettes. Detection and application of genes that make autogamous crops allogamous such as those detected in this study can improve the flowering habit of MS rice plants for RSUTMS.

In many crop breeding programs, a reduction in the diversity of breeding populations limits breeding effects, even in the era of advanced genome analysis technology. To overcome this limitation, an efficient outcrossing-based breeding system is required. We expect that MS with a flowering habit improved by a QTL detected in this study will be useful for RSUTMS and will help to overcome the limitation of autogamous rice breeding.

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