Introduction

Breeding of autogamous crop species commonly starts with bi-parental crossings, and subsequent genetic fixation by selfing, phenotypic screening, and selection of desirable fixed lines. This method has two advantages: 1) the clarity of the relationship between the cross combinations, breeding objectives, and the strategies of screening and selection, and 2) the ease of obtaining high-quality phenotype data for the fixed lines. However, this method has critical disadvantages. Because the breeders have to repeatedly cross already well-improved materials to breed the best cultivar, the genetic diversity of the breeding population quickly decreases, which in turn leads to less effective breeding. Fujimaki (1980) pointed out the disadvantages of this method as follows: 1) limited use of the full range of available genetic resources, 2) restricted potential for genetic recombination, 3) difficulty in obtaining successive improvements. In fact, the increase in the yields of autogamous crops has slowed drastically since the 1990’s (FAOSTAT, http://www.fao.org/faostat/en/#data, Tanaka and Tabei 2014).

In contrast, the yield of allogamous maize has grown continuously over the last several decades without signs that it is reaching a peak (USDA National Agricultural Statistics Service, https://www.nass.usda.gov/index.php). The breeding of the parental strains of maize F1 cultivars has been driven by recurrent selection-based population improvements, and uses repetitive cycles of selfing and outcrossing...
among genetically diverse populations. This breeding system is powerful because breeders can add selective pressure continuously on the outcrossing populations with many type of genome fragments derived from diverse materials. In addition, genetic recombinations occur very frequently in the population, because most genomic regions are heterozygous. In the maize breeding programs of private companies in mainly US, genomic selection (GS), which uses genome-wide markers, has enabled the continuous yield increases. GS-based breeding of livestock animals has also contributed to the dramatic improvement of their traits, especially in the production life of dairy cattle (García-Ruiz et al. 2016, Meuwissen et al. 2001). To use GS effectively for autogamous crop species, however, it is necessary to develop novel breeding systems that can realize effective outcross-based population breeding.

A previous study has proposed that dominant male-sterility with negatively and positively selectable trait markers is an ideal tool for facilitating outcrossing of autogamous crops (Tanaka 2010). Although there are some reports of dominant male-sterility (Ni et al. 2017, Yang et al. 2017), the frequency of emergence of dominant male-sterility is low, and it has been difficult to develop a tightly-linked marker for this trait. In contrast, transgenic technology can provide a very tightly-linked marker if marker genes are introduced with the dominant male-sterility gene into the genome by the same vector construct. Since there is no counterpart sequence of the introduced sequence on the homologous chromosome, there is very little risk of linkage break-up.

The development of dominant male-sterility is not technologically difficult when we employ a construct containing an anther-specific promoter driving a lethal gene such as barnase, encoding ribonuclease from Bacillus amyloliquefaciens (Acc. No. M14442, EC 3.1.27, Paddon and Hartley 1985). This type of dominant male-sterility has been developed in many plants, such as oilseed rape (Brassica napus L., Mariani et al. 1990), wheat (Triticum aestivum, De Block et al. 1997), oilseed mustard (Brassica juncea, Jagannath et al. 2001), maize (Zea mays, Sun et al. 2008), eggplant (Solanum melongena, Cao et al. 2010), pine (Pinus radiata, Zhang et al. 2012) and eucalyptus (Eucalyptus occidentalis, Zhang et al. 2012), and pelargoni- um (Pelargonium zonale, García-Sogo et al. 2012). There are some reports on development in rice, however, in many cases, the developed recombinants have problems in flowering habits, such as flowering rate and flowering time (Abe et al. 2018, Lu et al. 2000). Because this tendency is also found in non-transgenic male-sterile rice derived by mutation (Tamaru 1994), it is presumed that this tendency is a general issue of male-sterility in rice. Since pollen fertilization ability of rice is lost within 30 min (Song et al. 2001), excellent flowering characteristics is a key for efficient out-crossing fertility in rice. To obtain practical male-sterility by transgenic technology, the timing and organ-specificity of lethal gene expression are important. Therefore, the development of a highly anther-specific promoter is desired.

The rice genome was sequenced completely with extremely high precision prior to the genomes of other crops (International Rice Genome Sequencing Project 2005), and databases for genomic sequences and genes (Sakai et al. 2013), expression profiles of genes (Kawahara et al. 2016, Sato et al. 2011), and detected QTLs (Yonemaru et al. 2010) have been published. Here, comprehensive screening of the expression profile database ‘RiceXPro’ was conducted to identify the best anther-specific promoters. Thirty-eight genes specifically expressed in anthers were identified, and the ability of their near-upstream sequences to induce dominant male-sterility with desirable flowering habit was evaluated.

Materials and Methods

The rice cultivar ‘Nipponbare’ was used as wild type in all experiments in this study.

Comprehensive screening for anther-specific promoters in ‘RiceXPro’

Fig. 1 shows the flow of screening for Anther-Specific Promoters (ASPs) in this study. First, we accessed the data set designated as RXP_000 in the rice expression profile database RiceXPro (Sato et al. 2011, 2013, http://ricexpro.dna.affrc.go.jp) published by the National Institute of Agrobiological Sciences and screened it five times (100–300 genes per screening) according to the intensity of expression at four stages of anther development (phases 1–4 in RiceXPro). Genes with expression profiles in the following five categories were identified: I) very high anther-to-pistil expression ratio; II) extremely high expression in phase 4; III) high expression in phase 2 or 3; IV) moderate expression peaking in phase 3 or 4, and V) high expression peaking in phase 3 or 4. To identify genes with anther-specific expression, we then screened the combined list of the above genes (overlaps removed) for no or extremely low expression in other tissues (leaf blade, leaf sheath, root, stem, pani- cle, lemma/palea, ovary, embryo, and endosperm) based on visual appearance in the ‘Raw Signal Intensity Bar Graph’. Thus, a subset of candidate genes with anther-specific expression were identified.

We analyzed the sequences of these anther-specific genes by using the annotation databases Rice TOGO Browser (Nagamura et al. 2011, http://agri-trait.dna.affrc.go.jp) and RAP-DB (Sakai et al. 2013, http://rapdb.dna.affrc.go.jp) to select those where 1) the distance to the gene upstream was >800 bp, and 2) the near-upstream sequences (containing promoter region) did not have many restriction enzyme sites or GC-rich repeat regions. Finally, we selected 38 near-upstream sequences of the genes that fulfilled the above-mentioned conditions, and labelled the sequences as ASPs in all five categories, respectively (Table 1).

Amplification and modification of ASP sequences

ASP fragments were obtained by PCR amplification
from rice genomic DNA extracted from seedlings by using diatomaceous earth and a spin filter (Tanaka and Ikeda 2002) or a DNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands). Primer sets for PCR amplification were designed based on the ASP candidate sequences with additional XbaI and BamHI sites (Supplemental Table 1). PCR amplifications were performed using a PrimeSTAR (TaKaRa, Shiga, Japan) or KOD FX Neo (Toyobo Life Science, Osaka, Japan) with 0.35 ng/μL final concentration of template DNA, 0.4 mM dNTPs, and 0.3 μM each primer. Touchdown PCR (Don et al. 1991) with PrimeSTAR DNA polymerase was performed as follows: 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 with KOD FX Neo DNA polymerase was performed as follows: 2 min at 94°C, 32 cycles of 10 s at 98°C, and 5 min at 68°C. ASP304 sequence was obtained by nested PCR; the PCR product from the first primer set was used as a template. Sequences of ASP102 and ASP114 were synthesized by a gene synthesis service (GenScript Inc., Piscataway, NJ, USA) (Supplemental Fig. 1). PCR products of ASBs were purified with a QIAquick Gel Extraction Kit (Qiagen), and adenine base was added to the 3′ end by using EX Taq polymerase (TaKaRa). PCR products were subcloned into pGEM-T Easy vector (Promega Corporation, Madison, WI, USA) and their sequences were confirmed by Sanger sequencing. All XbaI, BamHI, AscI, MluI, and EcoRI restriction enzyme sites in the ASP sequences were mutagenized by PCR using the PrimeSTAR Mutagenesis Basal Kit (TaKaRa) or designed primers (Table 2, Supplemental Table 1).

Vector construction and rice transformation

The binary vector used in this study was constructed using a pZH2Bi-KXB vector (Kuroda et al. 2010, Fig. 2). Each ASP sequence was connected with the extracellular ribonuclease gene, barnase to drive anther-specific cell death. To cancel out the influence of leaky expression of the barnase gene in non-anther tissues, we inserted a barstar cassette in the same construct; this cassette harbored the Cauliflower mosaic virus (CaMV) 35S promoter, a barnase-specific inhibitor gene “barstar” (Abe et al. 2018), and a double terminator (DT) consisting of the CaMV 35S terminator and nos terminator (Luo and Chen 2007). Each
Table 1. List of 38 candidates of anther-specific expressed genes from ‘RiceXPro’ (Sato et al. 2013)

| Category | Accession No. | Description | Feature number in RiceXPro | Mean of gene expression values in every phase of anther development | Promoter name |
|----------|---------------|-------------|----------------------------|---------------------------------------------------------------|---------------|
| I)       | Os01g0579000  | AK064700    | Conserved hypothetical protein | 2735 10 78961 165 5 | ASP02         |
|          | Os02g0120500  | AK119580    | Basic helix-loop-helix (bHLH) transcription factor, Tapetum development and degeneration | 14476 1977 34991 25939 6473 | ASP04         |
|          | Os03g0296000  | CI284136    | Similar to DNA binding protein | 13718 26 77268 1312 58 | ASP05         |
|          | Os04g0453700  | AK106823    | Similar to Serine proteinase (Fragment) | 12015 79 84 325190 594 | ASP09         |
|          | Os04g0737100  | AK106787    | Similar to Mandelonitrile lyase-like protein | 44587 856 5639 30067 4 | ASP10         |
|          | Os05g0277200  | AK106886    | Similar to Beta-1,3-galactosyltransferase sqv-2 | 23845 254 90369 939 7827 | ASP11         |
|          | Os12g0472000  | CI225548    | Protein kinase, catalytic domain domain containing protein | 8136 3 4 6307 512 | ASP23         |
| II)      | Os01g0594900  | AK070921    | Conserved hypothetical protein | 31977 3 6 20 173260 | ASP102        |
|          | Os01g0929600  | AK070978    | Similar to Anther specific | 35595 5 11 7 183660 | ASP103        |
|          | Os03g0136400  | AK121484    | Similar to Inorganic phosphate transporter | 26234 5 63 230 22083 | ASP104        |
|          | Os04g0521300  | AK064703    | Similar to OSIGBa0092M08.3 protein | 13449 14 17 8 167380 | ASP105        |
|          | Os05g0181200  | AK105519    | Similar to Phytochrome P450-like protein | 14707 97 7 13 26127 | ASP106        |
|          | Os06g0228800  | AK106814    | Amino acid transporter, transmembrane domain containing protein | 10820 18 12 15 29850 | ASP107        |
|          | Os06g0635300  | CI260272    | Similar to gastric triacylglycerol lipase | 41436 44 51 19 57967 | ASP108        |
|          | Os06g0730000  | CI494903    | Similar to Sarine carboxypeptidase II-like protein | 30731 17 10 21 34884 | ASP109        |
|          | Os07g0359900  | AK120983    | Amino acid transporter, transmembrane domain containing protein | 21295 18 22 49 93525 | ASP110        |
| III)     | Os01g0219500  | AK106863    | Plant lipid transfer protein/Par allergen family protein | 28222 12 66676 2275 183 | ASP201        |
|          | Os04g0267600  | AK071614    | Cyclin-like F-box domain containing protein | 24788 25 35116 1122 127 | ASP202        |
|          | Os05g0289100  | CI516481    | Hypothetical conserved gene | 32458 2416 40032 4889 9933 | ASP203        |
|          | Os05g0574000  | CI260287    | Lipase, class 3 family protein | 10361 563 147060 213750 251 | ASP204        |
|          | Os08g0413000  | CI273950    | Similar to Valosin-containing protein (Fragment) | 22773 10 33 3333 5276 | ASP205        |
|          | Os10g0345900  | AK120983    | Amino acid transporter, transmembrane domain containing protein | 21295 18 22 49 93525 | ASP206        |
| IV)      | Os02g0219000  | AK064689    | Interferon-related developmental regulator domain containing protein | 44600 4 3 4743 12 | ASP301        |
|          | Os03g0135900  | CI514768    | Hypothetical conserved gene | 44274 1465 11320 5188 70 | ASP302        |
|          | Os04g0267600  | AK071614    | Cyclin-like F-box domain containing protein | 15024 3 5 4096 2918 | ASP303        |
|          | Os05g0891000  | CI516481    | Hypothetical conserved gene | 6394 3 3 3308 263 | ASP304        |
|          | Os05g0740000  | CI620878    | Lipase, class 3 family protein | 23262 402 2697 6691 5576 | ASP305        |
|          | Os08g0123600  | CI399987    | Conserved hypothetical protein | 29232 293 303 3333 5276 | ASP306        |
|          | Os10g0424100  | AK109240    | Similar to Anther-specific protein | 17305 29 44 7299 48 | ASP307        |
| V)       | Os01g0112400  | AK106825    | Major intrinsic protein family protein | 14558 10 22 173380 148 | ASP401        |
|          | Os02g0250500  | AK107896    | Conserved hypothetical protein | 35214 3 5 490 18265 | ASP402        |
|          | Os03g0313000  | AK069332    | Similar to Aldose 1-epimerase-like protein | 33725 3 396 3 26546 | ASP403        |
|          | Os03g0826600  | CI43867     | Similar to ATCHX19 (CATION/H+ EXCHANGER 19) | 481 4 7 24773 5 | ASP404        |
|          | Os08g0413000  | CI273950    | Similar to Valosin-containing protein (Fragment) | 42490 7 14 34062 11049 | ASP405        |
|          | Os11g0582500  | AK0983      | Protease inhibitor, lipid transfer protein (LTP), Postmeiotic anther development | 42529 4 7 72911 67658 | ASP406        |

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*I) genes with very high anther-to-pistil expression ratio; II) genes with extremely high expression in ‘phase 4’; III) genes with high expression in ‘phase 2 or 3’; IV) genes with moderate expression peaking in ‘phase 3 or 4’, and V) genes with high expression peaking in ‘phase 3 or 4’ (see Materials and Methods).

*Mean values of “Raw Signal Intensity” for anther in ‘View plot data’ in ‘RiceXPro’.

*In ‘RiceXPro’, the developmental stage of anther is classified into the following four phases according to anther size: phase 1, 0.3–0.6 mm; phase 2, 0.7–1.0 mm; phase 3, 1.2–1.5 mm; and phase 4, 1.6–2.0 mm.

*Same gene as anther-specific expressed gene PT42 registered in the US Patent US5639948A “Stamen-specific promoters from rice” (Michiels et al. 1997).

*Identical to Osc6, which was isolated as an anther-specific expressed gene (Tsuchiya 1992).
sterility when connected upstream of barnase (Abe et al. 2018, Konagaya et al. 2008), was constructed in the same manner as for the ASP constructs.

The binary vectors were introduced into Agrobacterium tumefaciens strain EHA105, and then used for transformation under the culture conditions described previously (Ozawa and Takaiwa 2010). About 20 individuals per construct were produced and cultivated in the simplified Biotron Breeding System (sBBS) (Tanaka et al. 2016) under condition of 27°C during the 10-h-light period (230 μmol photons m⁻² s⁻¹, from 7:00 to 17:00), 25°C during the 14-h-dark period, and 600 ppm CO₂.

Observation of anther shapes and pollen

Spikelets were sampled from the panicles a few days after heading. Three or more individuals per construct were investigated. Anthers were stained overnight at room

Table 2. Information on the promoter regions of the anther-specific expressed genes

| Promoter name | Amplified sequence size (bp) | Region of amplified promoter | Mutagenized restriction enzyme site | Position of mutagenesis | Modifications |
|---------------|------------------------------|-----------------------------|-------------------------------------|-------------------------|--------------|
| ASP02         | 1202                         | chr01:22,239,039..22,400,110 (– strand) | MlaI | 2 | A→T |
| ASP04         | 2004                         | chr02:1,076,181..1,078,184 (– strand) | XbaI | 1502 | A→C |
| ASP05         | 1534                         | chr03:10,365,265..10,366,804 (– strand) | BamHI | 565 | G→A |
| ASP09         | 926                          | chr04:27,221,751..27,222,679 (+ strand) | | | |
| ASP10         | 502                          | chr04:28,869,880..28,870,381 (+ strand) | | | |
| ASP11         | 1070                         | chr05:20,947,056..20,948,125 (+ strand) | | | |
| ASP23         | 1889                         | chr12:13,601,137..13,603,025 (+ strand) | | | |
| ASP102        | 1963                         | chr01:23,312,537..23,314,499 (+ strand) | | | |
| ASP103        | 907                          | chr01:40,798,447..40,799,353 (+ strand) | | | |
| ASP104        | 835                          | chr02:2,013,430..2,014,264 (+ strand) | | | |
| ASP105        | 1325                         | chr01:40,798,447..40,799,353 (+ strand) | | | |
| ASP107        | 1626                         | chr04:33,134,195..33,135,820 (+ strand) | | | |
| ASP108        | 921                          | chr05:4,884,501..4,885,421 (+ strand) | | | |
| ASP110        | 1242                         | chr06:6,695,017..6,696,258 (+ strand) | | | |
| ASP111        | 1613                         | chr06:25,755,592..25,757,204 (+ strand) | | | |
| ASP114        | 951                          | chr06:31,109,826..31,110,776 (+ strand) | | | |
| ASP201        | 1951                         | chr01:6,539,046..6,540,996 (+ strand) | | | |
| ASP202        | 2191                         | chr04:20,111,441..20,113,631 (+ strand) | | | |
| ASP204        | 2276                         | chr06:22,328,886..22,331,163 (+ strand) | | | |
| ASP205        | 2214                         | chr08:24,531,119..24,533,332 (+ strand) | | | |
| ASP206        | 2272                         | chr12:7,328,101..7,330,372 (+ strand) | | | |
| ASP207        | 1349                         | chr04:26,390,979..26,392,327 (+ strand) | | | |
| ASP208        | 1991                         | chr03:27,230,812..27,232,802 (+ strand) | | | |
| ASP301        | 2072                         | chr02:6647952..6645881 (+ strand) | BamHI | 2008 | A→T |
| ASP302        | 2411                         | chr03:25470682..25468272 (+ strand) | XbaI | 1257 | TTCT→CCGC |
| ASP303        | 2415                         | chr04:11064467..11062053 (+ strand) | BamHI | 780 | CC→AA |
| ASP304        | 2158                         | chr05:12570317..12572474 (+ strand) | BamHI | 1036 | T→G |
| ASP305        | 2483                         | chr05:28595736..28598218 (+ strand) | | | |
| ASP307        | 2416                         | chr08:1293225..1290810 (+ strand) | XbaI | 646 | TAG→CGC |
| ASP308        | 2431                         | chr09:18454621..18457051 (+ strand) | | | |
| ASP309        | 2487                         | chr10:15033604..15036090 (+ strand) | XbaI | 781 | TA→AC |
| ASP401        | 1394                         | chr01:648121..646728 (+ strand) | | | |
| ASP402        | 1919                         | chr02:18954598..18956516 (+ strand) | | | |
| ASP403        | 1851                         | chr03:15104287..15102437 (+ strand) | | | |
| ASP404        | 2053                         | chr03:3480840..34802892 (+ strand) | | | |
| ASP406        | 1926                         | chr08:19762731..19760806 (+ strand) | | | |
| ASP407        | 1565                         | chr11:22015277..22016841 (+ strand) | XbaI | 896 | T→A |

* Sequence position on the ‘Nipponbare’ IRGSP-1.0 reference genome.
* Counted from the beginning of the promoter.

ASP sequence in pGEM vector was digested by XbaI and BamHI, and inserted upstream of barnase in the pZH2Bi-KXB vector. As a control, a construct using the BoA9 promoter, which has already been confirmed to induce male-
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The presence or not of active pollen and the degree of pollen staining were examined using a Microphot-FXA EPI-FL3 microscope (Nikon, Tokyo, Japan).

Checking the sterility characteristics of transformants

For each individual transformant, seed settings in a main culm panicle were counted to confirm the sterility. We judged “sterility” as fewer than three set seeds, because cross pollination can happen in a close planting under sBBS conditions. The subset of transformants with confirmed sterility and stable growth were pruned back and re-grown in a closed greenhouse or under sBBS again for confirmation of male-sterility by female-fertility test. The upper parts of spikelets in some panicles of transformants were cut off, and put in a bag together with the flowering panicles of the pollen parent (i.e., wild type). The bags were shaken every 30 min under sBBS, or every 1 h in a closed greenhouse, between 11:30 and 14:30 over the period of flowering. For each transformant, after about one month, the panicles were harvested and the number of seeds was counted; then, for each construct, the percentage of sterility was calculated as (number of investigated plants — number of fertile plants) × 100.

Investigation of flowering habits

Three of the constructs (ASP108, ASP208, and ASP304, Table 1) which produced transformants with male-sterility, female fertility, and normal growth, were used to produce transformants again, and compared to equivalent constructs containing the BoA9 promoter instead of the ASP. About 10 individuals per construct were cultivated under sBBS, and their main culm panicles were investigated from their heading date onwards: opened spikelets were counted every hour from 9:00 to 17:00 until an opened spikelet was not observed for over three days. The following phenotypes were compared between transformants: 1) number of days between heading and flowering; 2) number of days between the onset of flowering and the flowering peak; 3) flowering period; 4) flowering rate from 13:00 to 15:00 (the peak flowering time in wild type); and 5) flowering rate (number of opened spikelets/all spikelets) (Supplemental Fig. 2).

Results

In silico screening of ASPs from RiceXPro database

In our comprehensive series of screens of the rice expression profile database RiceXPro, we identified a total of 106 genes based on (a) very high expression in anthers relative to pistils and (b) no or extremely low expression in other tissues. The number of genes in the five categories of expression during anther development (see Materials and Methods for details) were as follows: category (I), 23 genes; category (II), 14 genes; category (III), 8 genes; category (IV), 44 genes; category (V), 22 genes; 5 overlaps were removed. We then performed a further screen using TOGO Browser and RAP-DB to identify which of these anther-specific genes had the most potentially useful upstream sequences for use in expression cassettes. As a result, we identified a total of 38 ASPs to use in further experiments: 7 from category (I), 10 from category (II), 7 from category (III), 8 from category (IV), and 6 from category (V) (Table 1). The flow chart of screening used in this study is shown in Fig. 1.

Production of transformants and phenotype screening

Each ASP was cloned and some were mutagenized by PCR to remove restriction enzyme sites as necessary (Table 2, Supplemental Fig. 1). The BoA9 promoter, which is known to induce male-sterility when directing expression of barnase gene (Abe et al. 2018, Konagaya et al. 2008), was cloned and used as a control. Each ASP or BoA9 promoter was connected with barnase, to construct binary vectors composed of the following three cassettes aligned in tandem: the hygromycin resistance cassette, anther-specific barnase gene-expressing cassette, and CaMV 35S promoter-driven barstar gene-expressing cassette (Fig. 2). Using the prepared construct, rice was transformed via the Agrobacterium method.

Constructs containing ASP103 or ASP307 failed to regenerate plants from hygromycin-resistant calli after selection. Regenerated plants were obtained from six ASP constructs (ASP05, ASP09, ASP10, ASP107, ASP114, and ASP303), but they did not grow normally and most of them died immediately after transplantation. For the remaining 30 ASP constructs, transformants grew normally until heading. However, for 18 of these constructs, most individuals suddenly died around the time of heading, so ≤10 out of ~20 regenerated individuals could be investigated for sterility (Table 3). Finally, for a total of 12 ASPs, namely ASP04, ASP108, ASP110, ASP111, ASP204, ASP207, ASP208, ASP304, ASP305, ASP308, ASP401, and ASP407, we confirmed that most of the regenerated individuals grew normally.

Phenotypic features of transformants

1) Anther and pollen

The anthers of transformants harboring ASP04, ASP204, ASP206, ASP207, ASP208, or ASP407 were white and degenerated, and pollen grains could hardly be observed inside (Fig. 3). Conversely, the anthers of transformants produced by the other 23 constructs were yellow, and pollen grains were observed inside them, as in wild type.

2) Sterility characteristics

Transformants harboring ASP04, ASP204, ASP206, ASP207, ASP208, or ASP407 were observed to have no pollen grains (i.e., complete sterility) in all individuals (Table 3). In addition, transformants harboring ASP108, ASP109, ASP301, or ASP304 were sterile in most individuals even though pollen grains were observed in their anthers (Table 3); these pollen grains stained with Alexander’s solution, but were inferior to wild type in terms of their amount and fullness (Fig. 3). We judged the
transformants derived from the 19 other ASP constructs to be non-sterile or unclassifiable because numerous set seeds were observed or too few individuals (≤3) survived past heading, respectively. Finally, we identified seven ASPs, namely ASP04, ASP108, ASP204, ASP207, ASP208, ASP304, and ASP407, as promising promoters inducing normal growth and effective sterility phenotypes.

3) Confirmation of male-sterility by female-fertility test

Artificial crossing with wild-type pollen demonstrated that all individuals derived from the above seven ASP constructs showed female-fertility (i.e., cross-fertility; Table 4); with the exception of one individual derived from the ASP108 construct. Because transformants derived from these seven constructs produced almost no seeds by selfing (Table 3), but showed female fertility, we judged them to show male-sterility, presumably induced by the respective ASP and barnase.

Flowering habits of male-sterile transformants

From among the seven most promising ASP constructs, we selected ASP108 and ASP304 (which generate could pollen-producing transformants), and ASP208 (which could generate pollen-less transformants) for further investigation; ASP208 was chosen because the pollen-less transformants derived from four other ASP constructs (ASP04, 204, 207, and 407) showed a very poor flowering rate compared with those derived from ASP208 in the preliminary investigation. A construct containing the BoA9 promoter (Abe et al., 2018, Konagaya et al. 2008) in place of the ASP was used as a control. Wild type and transformants harboring the ASP108, ASP304, ASP208, or BoA9 constructs (about 10 individuals of each) were compared in terms of the following five survey items (1) number of days between heading and flowering, (2) number of days between the onset of flowering and the flowering peak, (3) flowering period, (4) flowering rate from 13:00 to 15:00 (the peak flowering time of wild type), and (5) flowering rate (Supplemental Fig. 2).

For each construct used, the flowering rate, the number of days between heading and flowering, and the flowering period of the individual transformants varied widely (Fig. 4, Supplementary Fig. 2), with the exception that the number

| Phase with maximum expression in anther | Construct | Pollens | No. of investigated plants | No. of fertile plants | Sterility (%) |
|----------------------------------------|-----------|---------|---------------------------|----------------------|--------------|
| Phase 2                                | ASP02     | unidentified | 3                        | 3                    | 0            |
|                                        | ASP04     | unidentified | 14                       | 0                    | 100          |
|                                        | ASP11     | unidentified | 2                        | 1                    | 50           |
|                                        | ASP201    | identified   | 10                       | 3                    | 70           |
|                                        | ASP202    | identified   | 3                        | 2                    | 33           |
|                                        | ASP204    | unidentified | 20                       | 0                    | 100          |
|                                        | ASP206    | unidentified | 4                        | 1                    | 75           |
|                                        | ASP207    | unidentified | 17                       | 0                    | 100          |
|                                        | ASP302    | unidentified | 1                        | 0                    | 100          |
| Phase 3                                | ASP23     | identified   | 5                        | 5                    | 0            |
|                                        | ASP208    | unidentified | 17                       | 0                    | 100          |
|                                        | ASP301    | identified   | 5                        | 1                    | 80           |
|                                        | ASP304    | identified   | 18                       | 1                    | 94           |
|                                        | ASP305    | identified   | 21                       | 19                   | 10           |
|                                        | ASP309    | identified   | 3                        | 1                    | 67           |
|                                        | ASP401    | identified   | 16                       | 7                    | 56           |
|                                        | ASP404    | identified   | 1                        | 0                    | 100          |
|                                        | ASP406    | identified   | 2                        | 0                    | 0            |
| Phase 4                                | ASP102    | identified   | 5                        | 3                    | 40           |
|                                        | ASP104    | identified   | 4                        | 4                    | 0            |
|                                        | ASP105    | identified   | 3                        | 3                    | 0            |
|                                        | ASP108    | identified   | 18                       | 1                    | 94           |
|                                        | ASP109    | identified   | 9                        | 1                    | 89           |
|                                        | ASP110    | identified   | 16                       | 10                   | 38           |
|                                        | ASP111    | identified   | 14                       | 5                    | 64           |
|                                        | ASP205    | identified   | 1                        | 1                    | 0            |
|                                        | ASP308    | identified   | 19                       | 18                   | 5            |
|                                        | ASP402    | identified   | 3                        | 0                    | 100          |
|                                        | ASP403    | identified   | 1                        | 1                    | 0            |
|                                        | BoA9      | unidentified | 19                       | 0                    | 100          |
|                                        | Nipponbare| identified   | 2                        | 2                    | 0            |

Single underlines indicate the representative constructs that typically generate pollen-producing sterile transformants. Double underlines indicate the representative constructs that typically generate pollen-less sterile transformants (Fig. 3).

* Categorization according to “Mean gene expression values” in Table 1.

b Number of individuals with normal growth from about 20 regenerated individuals.

Table 3. Phenotypic features and sterility characteristics of transformants

| Phase with maximum expression in anther | Construct | Pollens | No. of investigated plants | No. of fertile plants | Sterility (%) |
|----------------------------------------|-----------|---------|---------------------------|----------------------|--------------|
| Phase 2                                | ASP02     | unidentified | 3                        | 3                    | 0            |
|                                        | ASP04     | unidentified | 14                       | 0                    | 100          |
|                                        | ASP11     | unidentified | 2                        | 1                    | 50           |
|                                        | ASP201    | identified   | 10                       | 3                    | 70           |
|                                        | ASP202    | identified   | 3                        | 2                    | 33           |
|                                        | ASP204    | unidentified | 20                       | 0                    | 100          |
|                                        | ASP206    | unidentified | 4                        | 1                    | 75           |
|                                        | ASP207    | unidentified | 17                       | 0                    | 100          |
|                                        | ASP302    | unidentified | 1                        | 0                    | 100          |
| Phase 3                                | ASP23     | identified   | 5                        | 5                    | 0            |
|                                        | ASP208    | unidentified | 17                       | 0                    | 100          |
|                                        | ASP301    | identified   | 5                        | 1                    | 80           |
|                                        | ASP304    | identified   | 18                       | 1                    | 94           |
|                                        | ASP305    | identified   | 21                       | 19                   | 10           |
|                                        | ASP309    | identified   | 3                        | 1                    | 67           |
|                                        | ASP401    | identified   | 16                       | 7                    | 56           |
|                                        | ASP404    | identified   | 1                        | 0                    | 100          |
|                                        | ASP406    | identified   | 2                        | 0                    | 0            |
| Phase 4                                | ASP102    | identified   | 5                        | 3                    | 40           |
|                                        | ASP104    | identified   | 4                        | 4                    | 0            |
|                                        | ASP105    | identified   | 3                        | 3                    | 0            |
|                                        | ASP108    | identified   | 18                       | 1                    | 94           |
|                                        | ASP109    | identified   | 9                        | 1                    | 89           |
|                                        | ASP110    | identified   | 16                       | 10                   | 38           |
|                                        | ASP111    | identified   | 14                       | 5                    | 64           |
|                                        | ASP205    | identified   | 1                        | 1                    | 0            |
|                                        | ASP308    | identified   | 19                       | 18                   | 5            |
|                                        | ASP402    | identified   | 3                        | 0                    | 100          |
|                                        | ASP403    | identified   | 1                        | 1                    | 0            |
|                                        | BoA9      | unidentified | 19                       | 0                    | 100          |
|                                        | Nipponbare| identified   | 2                        | 2                    | 0            |
Comprehensive screening of anther-specific promoters for transgenic male-sterile rice

**Discussion**

**Comprehensive and effective screening of promoters in an expression profile database**

In this study, we identified candidate anther-specific promoters *in silico* by efficient screening of an expression profile database. This method has the following three advantages.

The first is that by targeting all the expressed genes in the database we can screen comprehensively for effective promoter candidates. The rice genome was sequenced in 2004 (IRGSP 2005), and since then various database tools such as ‘RiceXPro’, ‘TOGO browser’, ‘RAP-DB’, and ‘Q-TARO’ (Yonemaru et al. 2010) have been published. Here, we identified multiple ASP candidates of which ASP201 was identical to the promoter of *PT42*, which is registered in the US patent “Stamen-specific promoters from rice” (Michiels et al. 1997), and ASP407 was identical to the promoter of *Osc6*, which is listed as a “gene expressed in rice anthers” in Tsuchiya et al. (1992). Our identification of known anther-specific promoters in rice confirms the comprehensiveness of our strategy.

The second advantage is that by working *in silico*, it is possible to efficiently utilize research resources such as time, cost, and labor. Conventionally, to acquire tissue-specific promoters it is necessary to 1) extract RNA from the target tissue, 2) perform cDNA synthesis, 3) analyze the tissue-specificity of expression by Northern blotting etc. using the obtained cDNAs as probes, 4) screen clones of genomic fragments corresponding to cDNA from the genomic library, 5) evaluate the near-upstream sequences as specific...
Here, by using genomic information resources including expression profiles, we could obtain specific candidate sequences by performing only PCR and subcloning, and we could proceed directly to the evaluation of each candidate to obtain suitable tissue-specific promoters.

The third advantage is that the strategy can be flexibly applied to the screening of promoters that are expressed in various tissues, environments, developmental stages, and/or daily time periods; for instance, in ‘RiceXPro’, datasets of expression at various time periods in a day at various developmental stages are available. In addition, stress response expression data in a database such as ‘TENOR’ (Kawahara et al. 2016) could be used to acquire stress-responsive promoters.

The utilization of a promoter that was identified by field transcriptomic analyses, and whose tissue-specificity was confirmed by using ‘RiceXPro’ (Okada et al. 2017), has been reported previously. However, the current study is the first to utilize this expression database for in silico screening to obtain tissue-specific promoters for use in transgenes. The results demonstrate that we could efficiently obtain desirable expression promoters by this strategy. Because of the above-mentioned three advantages of this strategy, this research will become an important milestone in attempts to acquire new tissue- or stage-specific promoters in the genomic era.

**Characterization of transgenic male-sterile rice**

In ‘RiceXPro’, the developmental stages of anther in ‘Nipponbare’ are described by their length, based on the

| Construct | Individual No. | Total spikelets (a) | No. of set seeds (b) | Cross-fertility (b/a) (%) |
|-----------|----------------|--------------------|----------------------|--------------------------|
| ASP04     | 1              | 41                 | 4                    | 9.8                      |
|           | 2              | 107                | 1                    | 0.9                      |
| ASP108    | 1              | 70                 | 12                   | 17.1                     |
|           | 2              | 62                 | 0                    | 0.0                      |
|           | 3              | 110                | 54                   | 49.1                     |
| ASP204    | 1              | 31                 | 15                   | 48.4                     |
|           | 2              | 26                 | 6                    | 23.1                     |
|           | 3              | 25                 | 1                    | 4.0                      |
|           | 4              | 17                 | 7                    | 41.2                     |
| ASP207    | 1              | 41                 | 16                   | 39.0                     |
|           | 2              | 124                | 74                   | 59.7                     |
|           | 3              | 59                 | 3                    | 5.1                      |
|           | 4              | 84                 | 54                   | 64.3                     |
| ASP208    | 1              | 68                 | 20                   | 29.4                     |
|           | 2              | 105                | 17                   | 16.2                     |
|           | 3              | 78                 | 25                   | 32.1                     |
|           | 4              | 184                | 22                   | 12.0                     |
|           | 5              | 75                 | 13                   | 17.2                     |
|           | 6              | 109                | 15                   | 13.8                     |
| ASP304    | 1              | 49                 | 20                   | 40.8                     |
|           | 2              | 14                 | 3                    | 21.4                     |
| ASP407    | 1              | 155                | 82                   | 52.9                     |
|           | 2              | 98                 | 57                   | 58.2                     |
|           | 3              | 33                 | 12                   | 36.4                     |
|           | 4              | 35                 | 9                    | 25.7                     |
|           | 5              | 79                 | 34                   | 43.0                     |
| BoA9      | 1              | 74                 | 27                   | 36.5                     |
|           | 2              | 70                 | 17                   | 24.3                     |
|           | 3              | 69                 | 5                    | 7.2                      |
|           | 4              | 71                 | 37                   | 52.1                     |

Fig. 4. Flowering rates of Nipponbare (wild type) and male-sterile transformants. The survey items are illustrated in Supplemental Fig. 2.
observations of Itoh et al. (2005): i.e., phase 1, formation of tapetum; phase 2, meiosis; phase 3, formation of uninnucleate gametophytes; and phase 4, formation of mature pollen. Here, male-sterile transformants harboring ASPs predicted to be expressed mainly in the period from formation of tapetum to meiosis according to the RiceXPro data (e.g., ASP04, ASP204, and ASP207, Table 1) showed phenotypes of no pollen grains and anthers that were white and degenerated. The male-sterile transformants harboring ASP208, which is predicted to be expressed mainly in the period from meiosis to formation of uninnucleate gametophytes, also showed the phenotype of no pollen grains. In contrast, male-sterile transformants harboring ASPs predicted to be highly expressed from formation of uninnucleate gametophyte to mature pollen (e.g., ASP304 and ASP108, Table 1) showed phenotypes with pollen grains and anther shape and flowering characteristics similar to wild type (Table 3, Fig. 3). Our observation that the male-sterile transformants harboring ASP108, ASP109, ASP301, or ASP304 produced pollen grains with normal starch accumulation (Fig. 3), raises the possibility that their pollen tube could not elongate normally, as in the CW-cytoplasmic male-sterility line (Fujii and Toriyama 2005).

Our finding that flowering characteristics differed largely between individuals within the transformant population of each ASP construct indicates that it is important to select individual transformants for creation of breeding lines. Among transformants harboring ASP304, 5 out of 12 individuals showed incomplete male-sterility indicating that attention may be required when using this promoter. In contrast, transformants harboring ASP108 or ASP304 produced pollen grains, but showed more stable and higher rates of male-sterility than those harboring the known male-sterility promoter PT42 (ASP201, Supplemental Table 2). Our observation that many of the transformants harboring ASP108 showed better flowering characteristics than those containing the BoA9 promoter (Figs. 4, 5) indicates that ASP108 is a particularly promising promoter for the production of male-sterile plants that can efficiently produce outcrossed seeds. In addition, transformants with white and degenerate anthers without normal pollen (e.g., those harboring ASP208), have the advantage that it is easy to discriminate whether they are male-sterile or not at the time of flowering (Fig. 3), so their male-sterility can be reliably identified before pollination.

Many ASPs that are highly expressed during the formation of mature pollen according to ‘RiceXPro’ did not induce male-sterility in the current study. Since transgenes are commonly heterozygous in the T0 generation, when the barnase transgene is activated by the ASP during the formation of mature pollen, half of the pollen might be inactivated by the lethal gene, and half the pollen might remain active. Although these promoters are unsuitable for production of male-sterile plants, they might be effective for inactivating pollen and thus might be useful for the development of SPT (Seed Production Technology; https://www.pioneer.com/home/site/about/news-media/media-kits/seed-production-technology/).
Future applications of ASP promoters in breeding

In this study, a comprehensive screening of anther-specific expressed genes in the rice genome resulted in the discovery of seven promoters that can be used to induce male-sterility. Among these promoters, ASP108 appeared particularly promising for the development of male-sterile rice with excellent flowering habits. However, not all transformants carrying ASP108 exhibited excellent flowering habits; for instance, maximum flowering synchronization rate among the 10 individual transformants was about 15%, therefore selection of individual transformants as a practical breeding tool is important. Furthermore, efforts to increase the outcrossed seed fertility ratio, for example, by introducing the stigma exsertion trait or open hull trait, would be advantageous in the future.

By using the ASPs obtained in this study, such as ASP108, efficient development of male-sterile rice has become possible. Based on this technology, we anticipate that it will be possible to develop male-sterile rice lines that are ideal for recurrent selection by introducing gene cassettes with a male-sterility sequence linked to a selectable marker gene. The current research should pave the way to a new era of crop breeding that can effectively utilize the genome information available for autogamous crops species, using transgenic male-sterility (Tanaka 2010).

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