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Research Paper

Development of transgenic male-sterile rice by using anther-specific promoters identified by comprehensive screening of the gene expression profile database 'RiceXPro'

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Because genomic selection is designed for the population breeding of allogamous species, a successive outcrossing system is required for efficient use of genomic selection in autogamous crops, such as *Oryza sativa* L. (rice). Transgenic and dominant male-sterility is a suitable tool for efficient outcrossing of autogamous crops. Though there have been some reports of dominant male-sterile rice developed using transgenic technology, the flowering habit was substandard. Here, to isolate promoters that, when linked to a lethal gene, induce dominant male-sterility while retaining a good flowering habit, we identified 38 candidate genes with anther-specific expression by using the 'RiceXPro' database. We then evaluated the abilities of the near-upstream regions of these genes to induce male-sterility when linked to the lethal gene *barnase* and introduced into the rice cultivar 'Nipponbare'. Seven of the 38 promoters induced clear dominant male-sterility; promoters expressed in the later stage of anther development induced male-sterility while retaining better flowering habits when compared to ones expressed in the early stage. These seven promoters could potentially be used to facilitate development of an efficient outcross-based breeding system in rice.

Key Words: flowering habits, male-sterility, rice (*Oryza sativa* L.), outcrossing, *barnase*, RiceXPro, anther-specific promoter.

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 F_1 cultivars has been driven by recurrent selection-based population improvements, and uses repetitive cycles of selfing and outcrossing

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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 pelargonium (*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 outcrossing 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, panicle, 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

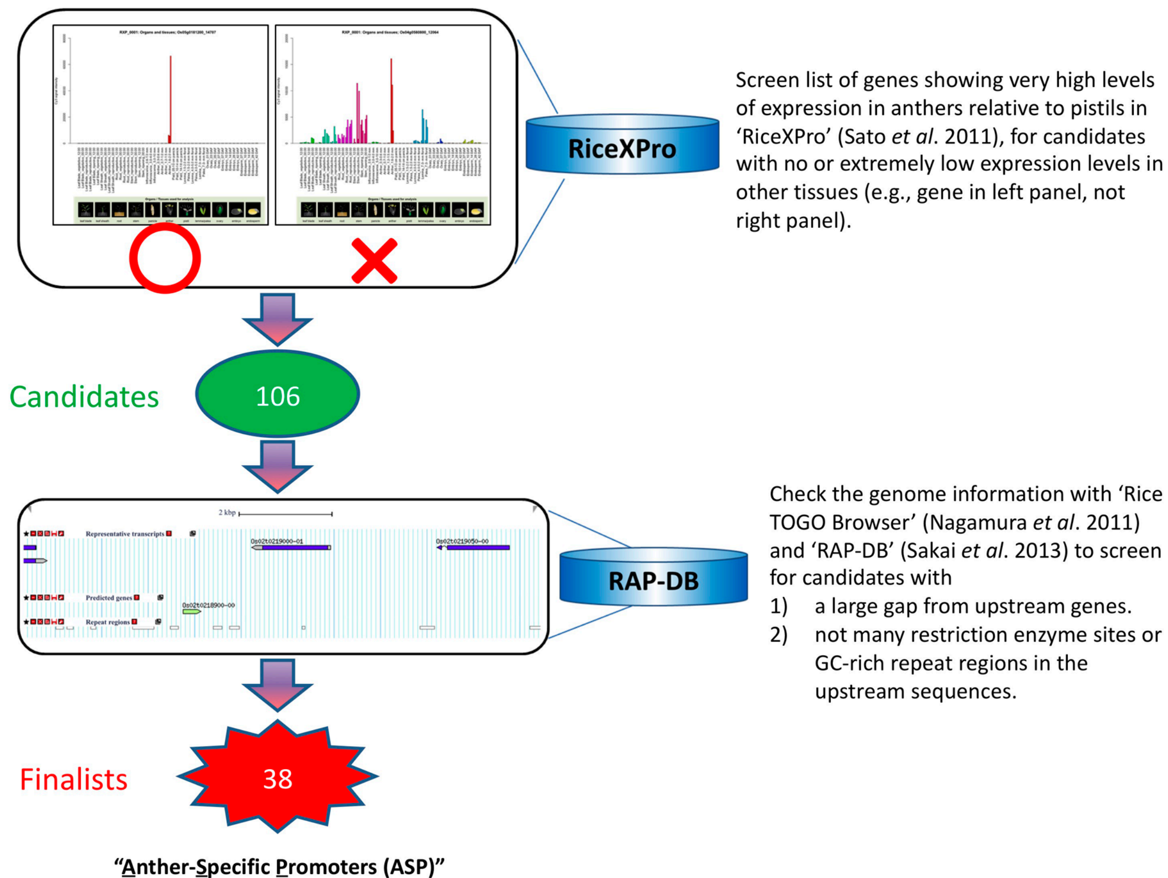

Fig. 1. Overview of screening for anther-specific promoters. Screen list of genes showing very high levels of expression in anthers relative to pistils in 'RiceXPro' (Sato *et al.* 2011) for candidates with no or extremely low expression levels in other tissues (e.g., gene in left panel, not right panel). Check the genome information with 'Rice TOGO Browser' (Nagamura *et al.* 2011) and 'RAP-DB' (Sakai *et al.* 2013) to screen for candidates with 1) a large gap from upstream genes. 2) not many restriction enzyme sites or GC-rich repeat regions in the upstream sequences.

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 *Xba*I and *Bam*HI 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 ASPs 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 *Xba*I, *Bam*HI, *Asc*I, *Mlu*I, and *Eco*RI 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[a]	Accession No.		Description	Feature number in RiceXPro	Mean of gene expression values[b] in every phase of anther development[c]				Promoter name
					Phase 1	Phase 2	Phase 3	Phase 4	
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
	Os04g0543700	AK106823	Similar to Serine proteinase (Fragment)	12015	79	84	325190	794	ASP09
	Os04g0573100	AK106787	Similar to Mandelonitrile lyase-like protein	44587	856	5639	30067	4	ASP10
	Os05g0427200	AK106896	Similar to Beta-1,3-galactosyltransferase sqv-2	23845	254	90369	939	7827	ASP11
	Os12g0427000	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 1	26234	5	63	230	22083	ASP104
	Os04g0415900	C99446	Similar to OSIGBa0092M08.3 protein	13449	14	17	8	167380	ASP105
	Os04g0650200	AK109786	Lipase, GDSL domain containing protein	15427	16	14	30	57019	ASP107
	Os05g0181200	AK105519	Similar to Phytochrome P450-like protein	14707	97	7	13	26127	ASP108
	Os06g0228800	AK106814	Amino acid transporter, transmembrane domain containing protein	10820	18	12	15	29850	ASP109
	Os06g0635300	CI260272	Similar to gastric triacylglycerol lipase	41436	44	51	19	57967	ASP110
	Os06g0730000	CI494903	Similar to Serine carboxypeptidase II-like protein	30731	17	10	21	34884	ASP111
	Os10g0345900	AK120983	Amino acid transporter, transmembrane domain containing protein	21295	18	22	49	93525	ASP114
III)	Os01g0219500	AK106863	Plant lipid transfer protein/Par allergen family protein[d]	28222	12	66676	2275	183	ASP201
	Os04g0398900	AK107729	Similar to H0209H04.6 protein	24788	25	35116	1122	127	ASP202
	Os06g0574900	AK109218	Conserved hypothetical protein	32458	2416	40032	4889	9933	ASP204
	Os08g0496800	AK120942	Similar to RAFTIN1a protein (RAFTIN1a anther protein)	32668	1265	70569	146420	148440	ASP205
	Os12g0233900	AK058390	FAS1 domain domain containing protein	18425	18	53290	589	8	ASP206
	Os04g0528200	AK064693	Similar to OSIGBa0115K01-H0319F09.17 protein	40623	30	50296	275	17	ASP207
	Os03g0683500	CI507674	Conserved hypothetical protein	10361	563	147060	213750	251	ASP208
IV)	Os02g0219000	AK064689	Interferon-related developmental regulator domain containing protein	44600	4	3	4743	12	ASP301
	Os03g0653900	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
	Os05g0289100	CI516481	Hypothetical conserved gene	6394	3	3	3308	263	ASP304
	Os05g0574000	CI260287	Lipase, class 3 family protein	23262	402	2697	6691	5576	ASP305
	Os08g0123600	CI399987	Conserved hypothetical protein	22773	10	33	585	90	ASP307
	Os09g0480900	AK109240	Similar to Anther-specific protein	29232	293	303	3333	5276	ASP308
	Os10g0424100	—	Similar to BTB/POZ domain containing protein	17305	29	44	7299	48	ASP309
V)	Os01g0112400	AK106825	Major intrinsic protein family protein	14558	10	22	173380	148	ASP401
	Os02g0520500	AK107896	Conserved hypothetical protein	35214	3	5	490	18265	ASP402
	Os03g0381000	AK069332	Similar to Aldose 1-epimerase-like protein	33725	3	396	3	26546	ASP403
	Os03g0828600	CI143867	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	ASP406
	Os11g0582500	D10983	Protease inhibitor, lipid transfer protein (LTP), Postmeiotic anther development[e]	42529	4	75	72911	67658	ASP407

[a] 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).
[b] Mean values of "Raw Signal Intensity" for anther in "View plot data" in 'RiceXPro'.
[c] 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.
[d] Same gene as anther-specific expressed gene *PT42* registered in the US Patent US5639948A "Stamen-specific promoters from rice" (Michiels *et al.* 1997).
[e] Identical to *Osc6*, which was isolated as an anther-specific expressed gene (Tsuchiya *et al.* 1992).

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

Promoter name	Amplified sequence size (bp)	Region of amplified promoter[a]	Mutagenesis		
			Mutagenized restriction enzyme site	Position of mutagenesis[b]	Modifications
ASP02	1202	chr01:22,398,039..22,400,110 (– strand)			
ASP04	2004	chr02:1,076,181..1,078,184 (– strand)			
ASP05	1534	chr03:10,365,265..10,366,804 (– strand)			
ASP09	926	chr04:27,221,751..27,222,679 (+ strand)			
ASP10	502	chr04:28,869,880..28,870,381 (+ strand)	*Mlu*I	2	A→T
ASP11	1070	chr05:20,947,056..20,948,125 (– strand)			
ASP23	1889	chr12:13,601,137..13,603,025 (– strand)	*Xba*I	1502	A→C
ASP102	1963	chr01:23,312,537..23,314,499 (– strand)			
ASP103	907	chr01:40,798,447..40,799,353 (– strand)			
ASP104	835	chr03:2,013,430..2,014,264 (– strand)	*Bam*HI	565	G→A
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)			
ASP109	1242	chr06:6,695,017..6,696,258 (– strand)	*Bam*HI	1192	A→T
ASP110	1613	chr06:25,755,592..25,757,204 (– strand)			
ASP111	951	chr06:31,109,826..31,110,776 (+ strand)			
ASP114	1443	chr10:10,356,456..10,357,898 (+ strand)	*Bam*HI	203/1018	TC→AT/C→G
ASP201	1951	chr01:6,539,046..6,540,996 (+ strand)	*Bam*HI	1036	T→G
ASP202	2191	chr04:19,711,441..19,713,631 (– 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)	*Bam*HI	2008	A→T
ASP302	2411	chr03:25470682..25468272 (– strand)	*Xba*I	1257	TTCT→CCGC
ASP303	2415	chr04:11064467..11062053 (– strand)	*Bam*HI	780	CC→AA
ASP304	2158	chr05:12570317..12572474 (+ strand)			
ASP305	2483	chr05:28595736..28598218 (+ strand)			
ASP307	2416	chr08:1293225..1290810 (– strand)			
ASP308	2431	chr09:18454621..18457051 (+ strand)			
ASP309	2487	chr10:15033604..15036090 (+ strand)	*Xba*I	646	TAG→CGC
ASP401	1394	chr01:648121..646728 (– strand)			
ASP402	1919	chr02:18954598..18956516 (+ strand)			
ASP403	1851	chr03:15104287..15102437 (– strand)			
ASP404	2053	chr03:34800840..34802892 (+ strand)		781	TA→AC
ASP406	1926	chr08:19762731..19760806 (– strand)			
ASP407	1565	chr11:22015277..22016841 (+ strand)	*Xba*I	896	T→A

[a] Sequence position on the 'Nipponbare' IRGSP-1.0 reference genome.
[b] Counted from the beginning of the promoter

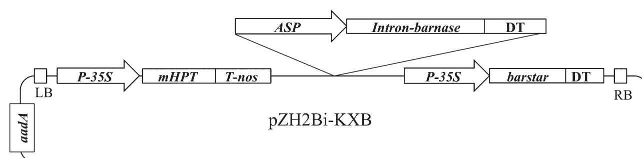

Fig. 2. Binary vector used in this study, pZH2Bi-KXB. *ASP*, anther-specific expressed gene promoter region; *aadA*, spectinomycin resistance protein; *P-35S*, CaMV *35S* promoter; *mHPT*, modified hygromycin phosphotransferase; *T-nos*, nopaline synthase terminator; DT, *35S* and *nos* double terminator; LB, T-DNA left border; RB, T-DNA right border.

ASP sequence in pGEM vector was digested by *Xba*I and *Bam*HI, 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-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 CO_2.

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

temperature according to Alexander (1969). 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/ number of investigated 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, ASP302, 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, ASP302, 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

Table 3. Phenotypic features and sterility characteristics of transformants

Phase with maximum expression in anther[a]	Construct	Pollens	No. of investigated plants[b]	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	2	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
	ASP407	unidentified	19	0	100
	BoA9	unidentified	20	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**).
[a] Categorization according to "Mean gene expression values" in **Table 1**.
[b] Number of individuals with normal growth from about 20 regenerated individuals.

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

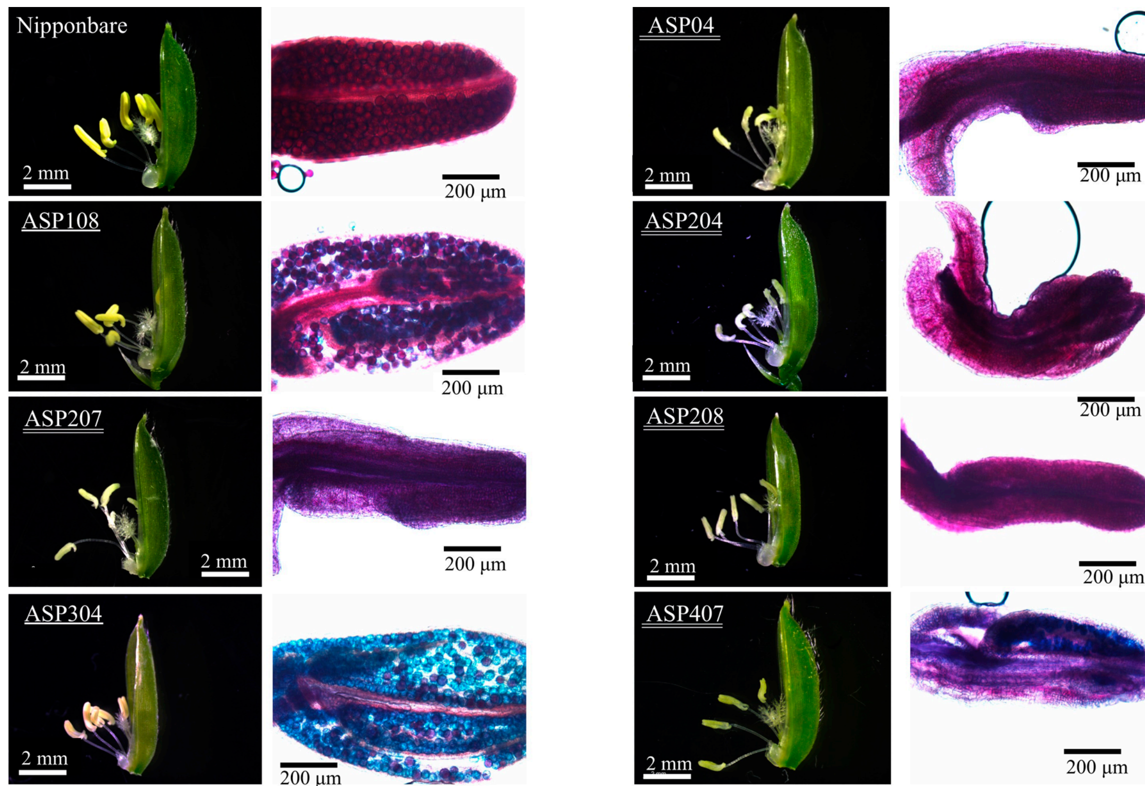

Fig. 3. Spikelets (left) and pollen grains (stained with Alexander's solution; right) of Nipponbare (wild type) and transformants. Left panels: Single underlines indicate the representative constructs that typically generate pollen-producing sterile transformants. Double underlines indicate the representative constructs that typically generate pollen-less sterile transformants. Right panels: blue and red staining indicates non-active and active pollen, respectively.

of days between heading and flowering was consistently two days or less for ASP108 transformants (male-sterility with pollen grains), which was close to the one day or less observed for wild type (**Fig. 5**). For all constructs, most transformants showed a flowering rate from 13:00 to 15:00 of 0%–10%, but some individual ASP304 transformants showed a flowering rate of 50% at this time (**Supplemental Fig. 2C**); a possible explanation for this is that, in this experiment, 5 out of 12 ASP304 transformants displayed incomplete male-sterility (**Supplemental Fig. 2C**, **Supplemental Table 2**). In the transformants harboring ASP208 or BoA9 (i.e., the pollen-less transformants), the flowering rates of many individuals were low compared to the rates observed for the pollen-producing transformants (**Fig. 4**). In BoA9, only two individuals (No. 5 and No. 7) could be examined in their peak of flowering, because the others showed no flowering or a remarkably low number of flowering spikelets per day, and the flowering time was too long (**Supplemental Table 2**).

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

Table 4. Cross-fertile of transformants

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	17.3
	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

promoters, and so on. 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

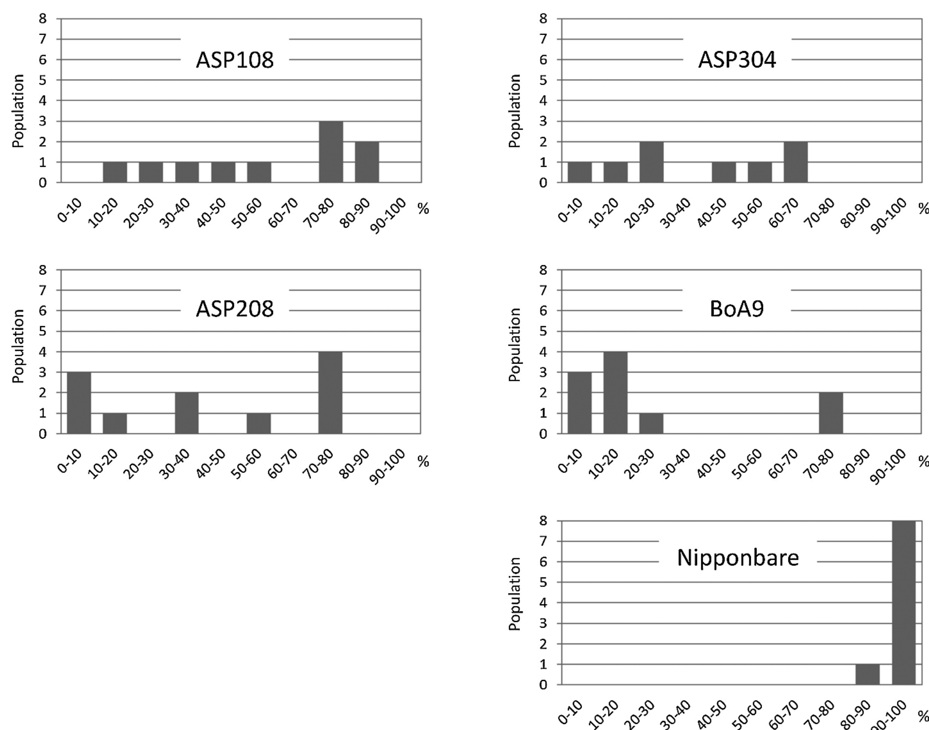

Fig. 4. Flowering rates of Nipponbare (wild type) and male-sterile transformants. The survey items are illustrated in **Supplemental Fig. 2**.

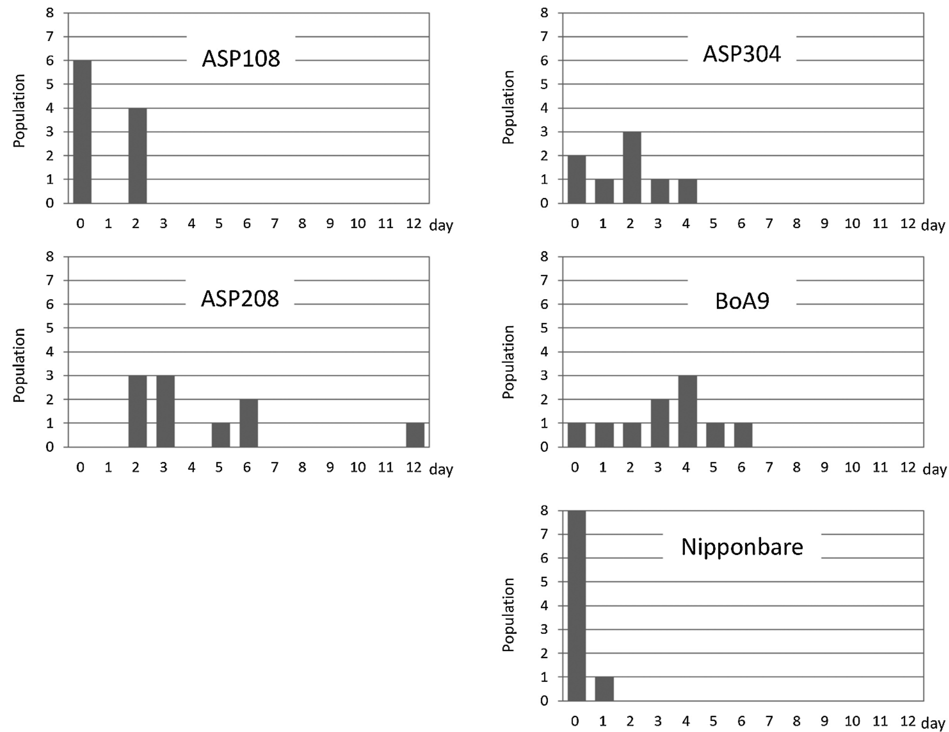

Fig. 5. Number of days between heading and flowering.

observations of Itoh *et al.* (2005): i.e., phase 1, formation of tapetum; phase 2, meiosis; phase 3, formation of uninucleate 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 uninucleate gametophytes, also showed the phenotype of no pollen grains. In contrast, male-sterile transformants harboring ASPs predicted to be highly expressed from formation of uninucleate 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 T_0 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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Literature Cited

Abe, K., M. Oshima, M. Akasaka, K. Konagaya, Y. Nanasato, A. Okuzaki, Y. Taniguchi, J. Tanaka and Y. Tabei (2018) Development and characterization of transgenic dominant male sterile rice toward an outcross-based breeding system. Breed. Sci. 68: 248–257.

Alexander, M.P. (1969) Differential staining of aborted and nonaborted pollen. Stain Technol. 44: 117–122.

Cao, B., Z. Huang, G. Chen and J. Lei (2010) Restoring pollen fertility in transgenic male-sterile eggplant by *Cre/lox*p-mediated site-specific recombination system. Genet. Mol. Biol. 33: 298–307.

De Block, M., D. Debrouwer and T. Moens (1997) The development of nuclear male sterility system in wheat. Expression of the *barnase* gene under the control of tapetum specific promoters. Theor. Appl. Genet. 95: 125–131.

Don, R.H., B.J. Cox, B.J. Wainwright and J.S. Mattick (1991) 'Touchdown' PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res. 19: 4008.

Fujii, S. and K. Toriyama (2005) Molecular mapping of the fertility restorer gene for ms-*CW*-type cytoplasmic male sterility of rice. Theor. Appl. Genet. 111: 696–701.

Fujimaki, H. (1980) Recurrent population improvement in rice breeding facilitated with male sterility. Gamma Field Symposia 19: 91–102.

García-Ruiz, A., J.B. Cole, P.M. Van Raden, G.R. Wiggans, F.J. Ruiz-López and C.P. Van Tassell (2016) Changes in genetic selection differentials and generation intervals in US Holstein dairy cattle as a result of genomic selection. Proc. Natl. Acad. Sci. USA 113: E3995–4004.

García-Sogo, B., B. Pineda, E. Roque, T. Antón, A. Atarés, M. Borja, J.P. Beltrán, V. Moreno and L.A. Cañas (2012) Production of engineered long-life and male sterile Pelargonium plants. BMC Plant Biol. 12: 156–171.

International Rice Genome Sequencing Project (2005) The map-based sequence of the rice genome. Nature 436: 793–800.

Itoh, J., K. Nonomura, K. Ikeda, S. Yamaki, Y. Inukai, H. Yamagishi, H. Kitano and Y. Nagato (2005) Rice plant development: from zygote to spikelet. Plant Cell Physiol. 46: 23–47.

Jagannath, A., P. Bandyopadhyay, N. Arumugam, V. Gupta, P.K. Burma and D. Pental (2001) The use of a spacer DNA fragment insulates the tissue-specific expression of a cytotoxic gene (*barnase*) and allows high-frequency generation of transgenic male sterile lines in *Brassica juncea* L. Mol. Breed. 8: 11–23.

Kawahara, Y., Y. Oono, H. Wakimoto, J. Ogata, H. Kanamori, H. Sasaki, S. Mori, T. Matsumoto and T. Itoh (2016) TENOR: Database for comprehensive mRNA-seq experiments in rice. Plant Cell Physiol. 57: e7.

Konagaya, K., S. Ando, S. Kamachi, M. Tsuda and Y. Tabei (2008) Efficient production of genetically engineered, male-sterile *Arabidopsis thaliana* using anther-specific promoters and genes derived from *Brassica oleracea* and *B. rapa*. Plant Cell Rep. 27: 1741–1754.

Kuroda, M., M. Kimizu and C. Mikami (2010) A simple set of plasmids for the production of transgenic plants. Biosci. Biotechnol. Biochem. 74: 2348–2351.

Lu, G.H., H.T. Sun, J.L. Zhang and M.M. Hong (2000) Induction of male sterility by the integration of chimeric *RTS-barnase* gene into rice (*Oryza sativa* L.) genome. Acta Physiol. Sinica 26: 171–176.

Luo, Z. and Z. Chen (2007) Improperly terminated, unpolyadenylated mRNA of sense transgenes is targeted by RDR6-mediated RNA silencing in *Arabidopsis*. Plant Cell 19: 943–958.

Mariani, C., M. De Beuckeleer, J. Truettner, J. Leemans and R.B. Goldberg (1990) Induction of male sterility in plants by a chimaeric ribonuclease gene. Nature 347: 737–741.

Meuwissen, T.H.E., B.J. Hayes and M.E. Goddard (2001) Prediction of total genetic value using genome-wide dense marker maps. Genetics 157: 1819–1829.

Michiels, F., S. Morioka, T. Scheirlinck and T. Komari (1997) Stamen-specific promoters from rice. U.S. Patent No. 5,639,948.

Nagamura, Y., B.A. Antonio, Y. Sato, A. Miyao, N. Namiki, J. Yonemaru, H. Minami, K. Kamatsuki, K. Shimura, Y. Shimizu *et al.* (2011) Rice TOGO Browser: a platform to retrieve integrated information on rice functional and applied genomics. Plant Cell Physiol. 52: 230–237.

Ni, F., J. Qi, Q. Hao, B. Lyu, M. Luo, Y. Wang, F. Chen, S. Wang, C. Zhang, L. Epstein *et al.* (2017) Wheat *Ms2* encodes for an orphan

protein that confers male sterility in grass species. Nat. Commun. 8: 15121.

Okada, R., Y. Nemoto, N. Endo-Higashi and T. Izawa (2017) Synthetic control of flowering in rice independent of the cultivation environment. Nat. Plants 3: 17039.

Ozawa, K. and F. Takaiwa (2010) Highly efficient *Agrobacterium*-mediated transformation of suspension-cultured cell clusters of rice (*Oryza sativa* L.). Plant Sci. 179: 333–337.

Paddon, C.J. and R.W. Hartley (1985) Cloning, sequencing and transcription of an inactivated copy of *Bacillus amyloliquefaciens* extracellular ribonuclease (barnase). Gene 40: 231–239.

Sakai, H., S.S. Lee, T. Tanaka, H. Numa, J. Kim, T. Kawahara, H. Wakimoto, C. Yang, M. Iwamoto, T. Abe *et al.* (2013) Rice annotation project database (RAP-DB): an integrative and interactive database for rice genomics. Plant Cell Physiol. 54: e6.

Sato, Y., B.A. Antonio, N. Namiki, H. Takehisa, H. Minami, K. Kamatsuki, K. Sugimoto, Y. Shimizu, H. Hirochika and Y. Nagamura (2011) RiceXPro: a platform for monitoring gene expression in *japonica* rice grown under natural field conditions. Nucleic Acids Res. 39: D1141–D1148.

Sato, Y., H. Takehisa, K. Kamatsuki, H. Minami, N. Namiki, H. Ikawa, H. Ohyanagi, K. Sugimoto, B.A. Antonio and Y. Nagamura (2013) RiceXPro Version 3.0: expanding the informatics resource for rice transcriptome. Nucleic Acids Res. 41: D1206–D1213.

Song, Z.P., B.R. Lu and J.K. Chen (2001) A study of pollen viability and longevity in *Oryza rufipogon*, *O. sativa*, and their hybrids. Int. Rice Res. Notes 26: 31–32.

Sun, Q., Y. Zhang, T. Rong, S. Dong, D. Ma and C. Zhang (2008) Establishment of transgenic acceptor and transformation of *barnase* gene by particle gun in maize inbred line 18-599 (white). Front. Agric. China 2: 37–43.

Tamaru, N. (1994) Breeding studies on genetic male sterility and hybrid sterility in rice (*Oryza sativa* L.). Mem. Fac. Agr. Hokkaido Univ. 19: 203–256.

Tanaka, J. (2010) Transgenic male sterility permits efficient recurrent selection in autogamous crops. Crop Sci. 50: 1124–1127.

Tanaka, J. and S. Ikeda (2002) Rapid and efficient DNA extraction method from various plant species using diatomaceous earth and a spin filter. Breed. Sci. 52: 151–155.

Tanaka, J. and Y. Tabei (2014) Effort to increase breeding efficiency by reproduction control using NBT-SPT (seed production technology) process, reverse breeding, early flowering in fruit trees, and TMS recurrent selection in autogamous crops. Seibutsu-no-Kagaku Iden 68: 117–124.

Tanaka, J., T. Hayashi and H. Iwata (2016) A practical, rapid generation-advancement system for rice breeding using simplified biotron breeding system. Breed. Sci. 66: 542–551.

Tsuchiya, T., K. Toriyama, M.E. Nasrallah and S. Ejiri (1992) Isolation of genes abundantly expressed in rice anthers at the microspore stage. Plant Mol. Biol. 20: 1189–1193.

Yang, K., Y. Chen, M. Shi, R. Converse, X. Chen, B. Zhao, Y. Zhang and J. Lv (2017) A novel dominant rice male sterility mutant, *OsDMS-1*, simultaneously controlled by independent loci on chromosomes 1, 2, and 3. Mol Breed. 37: 25.

Yonemaru, J., T. Yamamoto, S. Fukuoka, Y. Uga, K. Hori and M. Yano (2010) Q-TARO: QTL Annotation Rice Online database. Rice 3: 194–203.

Zhang, C., K.H. Norris-Caneda, W.H. Rottmann, J.E. Gulledge, S. Chan, B.Y. Kwan, A.M. Thomas, L.C. Mandel, R.T. Kothera, A.D. Victor *et al.* (2012) Control of pollen-mediated gene flow in transgenic trees. Plant Physiol. 159: 1319–1334.