ORIGINAL PAPER

A gene controlling the number of primary rachis branches also controls the vascular bundle formation and hence is responsible to increase the harvest index and grain yield in rice

Tomio Terao · Kenji Nagata · Kazuko Morino · Tatsuro Hirose

Abstract The quantitative trait locus controlling the number of primary rachis branches (PRBs) in rice was identified using backcrossed inbred lines of Sasanishiki/Habataki// Sasanishiki///Sasanishiki. The resultant gene was *ABERRANT PANICLE ORGANIZATION 1* (*APO1*). Habataki-genotype segregated reciprocal recombinant lines for the *APO1* locus increased both the number of PRB (12–13%) and the number of grains per panicle (9–12%), which increased the grain yield per plant (5–7%). Further recombination dividing this region revealed that different alleles regulated the number of PRB and the number of grains per panicle. The *PRB1* allele, which includes the *APO1* open reading frame (ORF) and the proximal promoter region, controlled only the number of PRB but not the number of grains per panicle.

In contrast, the *HI1* allele, which includes only the distal promoter region, increased the grain yield and harvest index in Habataki-genotype plants, nevertheless, the ORF expressed was Sasanishiki type. It also increased the number of large vascular bundles in the peduncle. *APO1* expression occurred not only in developing panicles but also in the developing vascular bundle systems. In addition, Habataki plants displayed increased *APO1* expression in comparison to Sasanishiki plants. It suggests that *APO1* enhances the formation of vascular bundle systems which, consequently, promote carbohydrate translocation to panicles. The *HI1* allele is suggested to regulate the amount of *APO1* expression, and thereby control the development of vascular bundle systems. These findings may be useful to improve grain yield as well as quality through the improvement of translocation efficiency.

T. Terao (✉) · T. Hirose
Rice Physiology Research Sub-Team,
Hokuriku Research Center,
National Agricultural Research Center,
National Agriculture and Food Research Organization,
1-2-1, Inada, Joetsu, Niigata 943-0193, Japan
e-mail: terao@affrc.go.jp

K. Nagata
Rice Physiology Research Sub-Team,
National Agricultural Research Center for Western Region,
National Agriculture and Food Research Organization,
6-12-1 Nishi-fukatsucho, Fukuyama,
Hiroshima 721-8514, Japan

K. Morino
Rice Genetic Engineering Research Sub-Team,
Hokuriku Research Center,
National Agricultural Research Center,
National Agriculture and Food Research Organization,
1-2-1, Inada, Joetsu, Niigata 943-0193, Japan

Introduction

Increased rice yield will play an important role in meeting the demands of an ever-increasing global population, because approximately half of the world's population subsists primarily on rice. Researchers have recently intensified their genetic analyses of rice yield, through quantitative trait locus (QTL) analysis and map-based cloning of the genes related to rice yield. One of the most important factors is the number of grains per panicle. Many different gene/QTLs have been reported to determine the number of grains per panicle (Zhuang et al. 1997; Xiao et al. 1998; Yagi et al. 2001; Nagata et al. 2002; Mei et al. 2003; Kobayashi et al. 2004; Tian et al. 2006; Yoon et al. 2006). One of the most important QTLs is located on chromosome 1 (*Gn1*) and is identified as cytokinin oxidase (Ashikari et al. 2005). Another gene, *DEP1,* which increases the

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number of rachis branches, thereby increasing grain yield, has also been identified (Huang et al. 2009). A gene that controls grain kernel size has been identified as RING type E3 ubiquitin ligase (Song et al. 2007); a gene controlling grain kernel width has been identified in indica and japonica rice varieties (Shomura et al. 2008); a gene related to grain-filling has also been identified as cell wall invertase (Wang et al. 2008). Researchers have also begun to pyramid some of these QTLs into one strain (Ashikari and Matsuoka 2006; Ando et al. 2008).

Another approach to determine the genes that control the panicle architecture is to identify them through the analysis of mutants that have changed the panicle structure. The loss-of-function *lax-1* mutation abrogating the axillary meristem initiation results in reduced branch formation in the panicle (Komatsu et al. 2003a). Conversely, the mutation of *FZP* promotes the formation of supernumerary axillary meristems (Komatsu et al. 2003b), while the overexpression of *RCN1* also enhances the secondary branch numbers through the delayed phase change from the branch shoot to floral meristem (Nakagawa et al. 2002). Previous studies have reported that *ABERRANT PANICLE ORGANIZATION 1* (*APO1*) was able to control spikelet numbers in rice (Ikeda et al. 2007; Ikeda-Kawakatsu et al. 2009). A loss-of-function *apo1* mutation has been shown to lead to the precocious transition of rachis meristem and branch meristem into spikelet meristem resulting in a reduced number of spikelets (Ikeda et al. 2001, 2007), whereas gain-of-function *Apo1-D* mutants delayed these transitions for rice panicle architecture and increased the number of grains (Ikeda-Kawakatsu et al. 2009).

Important factors in improving rice yield include yield-determining components, such as the number of panicles per unit area, the number of spikelets per panicle, the percentage of fully ripened grains, and the weight of 1,000 mature kernels (Matsushima 1957). These factors are interrelated, so that increases in one component may lead to decreases in others. The plant, with finite metabolic resources, may compensate for increases in the number of grains per panicle by decreasing the ripening percentage. Therefore, scientists aim to identify genes/QTLs that increase one aspect of grain maturation without affecting others, in order to allow for easy manipulation of the potential yield. The goal is to prevent compensation effects, which may occur when the sink size is increased without a corresponding increase in the source supply. Simultaneous increases in sink size and source supply can be obtained by increasing the source supply and/or improving translocation efficiency. It is important to ensure that ripening traits and grain quality are not negatively impacted by increases in the number or size of grain kernels.

Using recombinant inbred lines, Nagata et al. (2002) identified two QTLs, on chromosomes 1 and 6 that deter-

mine the number of grains per panicle. The QTL on chromosome 6, *PBN6* (Ando et al. 2008), increased the number of grains per panicle without reducing ripening percentage (Nagata et al. 2002). Ando et al. (2008) narrowed down the physical distance of this QTL into 390 kb between molecular markers S1306 and RM3430. This QTL increased the number of grains by increasing the number of primary rachis branches (PRBs) while keeping the number of grains per rachis unchanged (Nagata et al. 2002). Hence, increasing the number of PRBs may increase the translocation route from culm to grain. In addition, competition among grains within a rachis is reduced in comparison to the competition among secondary rachis branches. Therefore, increasing the number of PRBs is an important way to increase the number of grains per panicle without reducing ripening percentage, while minimizing the negative impact on grain quality. In contrast, *Gn1* (Ashikari et al. 2005), located on chromosome 1, drastically increased the number of grain kernels (Yagi et al. 2001) but also negatively impacted ripening traits (Nagata et al. 2002). This QTL increased the number of grains mainly by increasing the number of secondary rachis branches, which may cause severe competition among the grains within a rachis and consequently cause a shortage in translocated carbohydrates (Nagata et al. 2002).

In this report, we identified a gene at the *PBN6* QTL that determines the number of PRBs. We also analyzed the effects of this gene on yield and yield-determining components. Using rice lines that are recombinant at this locus, we further investigated how this locus controls each of the yield-determining components, such as harvest index, number of PRBs and number of grains per plant. This research should illuminate the factors that ultimately determine rice grain yield.

Materials and methods

Map-based cloning was performed to identify the gene responsible for the number of PRBs. We selected SHA48, which includes a heterogeneous region, in chromosome 6 as well as chromosome 1, between S10581EH and C112 markers (118.9–122.9 cM), from the BC_2F_5 mapping population of Sasanishiki/Habataki//Sasanishiki///Sasanishiki (Nagata et al. 2002). This line also includes Habataki-genotype region in chromosome 1 between S11122EH and R210 (28.5–33.1 cM) and chromosome 5 between R372EH and C1268E (26.1–33.1 cM). Reciprocally recombined progenies were identified continuously up to BC_2F_{10} to narrow the heterogeneous region in chromosome 6, with the chromosome 1 heterogeneous region fixed to the Sasanishiki type. Around 10,000 plants were used to determine the genotype. The genotype was determined according to the

method described in Nagata et al. (2002). The specific primers that were used for each marker are shown in Table 1. Rice plants were grown in the paddy field at Hokuriku Research Center, National Agricultural Research Center, Joetsu, Japan. We measured yield-determining components according to the method described in Nagata et al. (2002). After the plants were harvested and air-dried, we measured the number of panicles per plant, total plant length, stem length, total shoot weight, and head weight. Stems and leaves were dried in the drying oven at 80°C and dry weights were measured. For the measurement of panicle traits, five medium-sized panicles were obtained from each plant. We measured the panicle length, the number of PRBs, the number of secondary rachis branches, the number of grains per panicle, and the number of sterile grains. After the grains were detached, we measured the weight of the grain kernels with hulls attached by measuring the water content using a "Riceter J" grain moisture tester (Kett Electric Laboratory, Tokyo, Japan). To determine ripening percentage, the grains were water selected and the percentage of sunk grains with a relative density of >1.0 was measured.

Further research on reciprocal recombination within the *PBN6* locus was carried out using the 04SHA422-12-8.8-18.31 line. Yield-determining elements were identified in progeny grown in 2004 and 2005 (Table 2). The 2005 experimental design included a split plot with three replicates using five segregated lines that each included ten plants. The reciprocal recombinants identified were grown in the same field in 2006. Yield-determining elements were measured using segregated progeny. In the 2006 experiment on SHA-HI lines, the head alone was harvested for the measurement of yield components. Shoot biomass factors were measured during another experiment that included three replicates carried out at the same field.

Statistical analyses were carried out using the GLM procedure on SAS ver. 9.1 (SAS Institute, Cary, NC, USA).

Gene expression

We studied the gene expression of young panicles with RT-PCR. Young Sasanishiki and Habataki panicles were collected on July 4, 7, 10, and 13, with panicle sizes ranging from 0.2 to 2 mm. As the heading dates of the Sasanishiki plants and Habataki plants during the sampled year were July 29 and 30, respectively, these sampling dates correspond to 25, 22, 19 and 16 days before the heading of the Sasanishiki plants and 26, 23, 20 and 17 days before the heading of the Habataki plants.

Table 1 PCR primers used for mapping, expression analysis, in situ hybridization and transformation

Marker name	Marker type	Restriction enzyme	Forward PCR primer 5′–3′	Reverse PCR primer 5′–3′	Remarks
3628-28	STS		cactccgcccgacatc	atggacatgcaacggaatg	
3628-17	CAPS	*Dra*I	acatggacttcgctctcagc	gaacaccaagatgcccaagt	
3628-D1	CAPS	*Xho*I	atgcgacaagtgttttggac	gaacaccaagatgcccaagt	Same reverse primer as 3628-17
3628-D22	CAPS	*Sac*I	acgccttcaacagctactgg	gctgctactggctcatcctt	
3628-54	STS		ccaaaactgcacctggaga	gtcgtcgttgctggc	
3628-52	CAPS	*Hae*III	ttcgtttcatgtaaaattccttga	tctccaggtgcagttttgg	
3628-43	CAPS	*Spe*I	ccattggcctcctcgac	tcaaggaattttacatgaaacgaa	
3628-56	SNPs		cttctggccagcatgacttt	atccaaactgagaaactcta	For Habataki type
			cttctggccagcatgacttt	ctcacaagaaagatgcacct	For Sasanishiki type
3628-60	SSR		gtcggccgtcgagaagag	aactgcgcgtgcacctc	
3628-55	SSR		atgaacactgtccaacaaattgttt	ccggtttggtttgtctcag	
3628-41	CAPS	*Dra*I	tttgagatagtcgccgaggt	tcatcagcctttctcttccaa	
3628-7	CAPS	*Dra*I	actgcacgaaaaggagagga	gtacgacgtgcctcgatttc	
3628-50	STS		ctgcatcacgccatcaatac	tgcatcgagtttgttgctaga	
3628-24	CAPS	*Pst*I	gatcgacgagggacggtact	tgctttattcgattgaggcata	
3628-21	STS		cccgtgaacatgtccaaact	tatcagaaggtgggccacaa	
3628-67L1			ctctcctccctcgatccc		
3628-UFL1			cagcggacgacatggac		
3628-U2R1				gagcagcagcgtcttgtg	
3628-U2R2				ccttgctccatacgttctcc	
3628-51L3			caggtaagggctccgttgga		
3628-53R3				tgcgtagcatgttttgcagt	

Table 2 Yield-determining components in the 2004 experiment segregated progeny of 03SHA422-12-8.8-18.31, which has a heterogeneous region between 3628-52 and 3628-55

Genotype	Traits												
	n	SL (cm)	SDW (g)	HDW (g)	HI	NPP	NGP	1RB	2ry/1ry	GW (mg)	%STG	%SG	SGW (g)
2004													
Sasanishiki	44	97.5 b	50.4	28.8	0.571 c	15.3	89.7 c	9.5 c	1.52 a	23.1 a	5.40	92.6	28.7 b
Habataki	46	99.2 a	51.2	30.4	0.595 a	15.3	98.1 a	10.7 a	1.41 b	22.5 c	5.66	90.8	30.1 a
Hetero	82	99.1 a	51.8	30.3	0.586 b	15.1	95.4 b	10.3 b	1.45 ab	22.8 b	5.60	92.0	29.6 a
H/S ratio		1.02	1.02	1.06	1.04	1.00	1.09	1.13	0.93	0.98	1.05	0.98	1.05
Significance		**	NS	NS	***	NS	***	***	*	**	NS	NS	*
2005													
Sasanishiki	150	96.8	48.4	25.3	0.521	13.8	80.4	8.44	1.45	26.4	3.08	94.8	27.7
Habataki	150	98.0	47.4	26.6	0.559	13.5	90.8	9.47	1.47	25.6	3.24	93.0	29.2
H/S ratio		1.01	0.98	1.05	1.07	0.98	1.13	1.12	1.01	0.97	1.05	0.98	1.07
Significance		**	NS	*	***	NS	***	***	NS	***	NS	**	***

Six rows of 30 segregated plants were used in the 2004 experiment. Three replicates of two genotypes, each including five rows of ten plants, were used in the 2005 experiment

Same letter represents no significant difference in the Waller–Duncan k ratio t test

n Number of plants used, *SL* shoot length, *SDW* shoot dry weights, *HDW* head dry weights, *HI* harvest indexes, *NPP* number of panicles per plant, *NGP* number of grains per panicle, *1RB* number of primary rachis branches per panicle, *2ry/1ry* ratio of secondary to primary rachis branches, *GW* grain dry weight with hull, *%STG* percentage of sterile grains, *%SG* percentage of sunk grains with water selection, *SGW* sunk grain weights per plant, *H/S ratio* the ratio of Habataki to Sasanishik, *NS* not significant

*, ** and ***significant at the 5, 1 and 0.1% levels, respectively

RNA was prepared using the CTAB method (Chang et al. 1993). After treatment with RNase-free DNase (DNase I, amplification grade Invitrogen, Carlsbad, CA, USA) to remove DNA contamination, cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen), following the protocol described in Hirose and Terao (2004). Specific 3628-67L1 and 3628-U2R1 primers were used for PCR to amplify the regions, including the intron (Table 1). Actin was used as the internal standard (Yamanouchi et al. 2002). Also the quantitative real-time PCR analysis was carried out using the Smart Cycler System (Cepheid Co., Sunnyvale, CA, USA), according to the method described in Hirose et al. (2006). Aliquots of the same cDNA prepared from young panicles of Habataki plants and Sasanishiki plants corresponding to 66 ng of total RNA, and the same primer set described above was used. Ubiquitin was used for the internal standard in this experiment. Since there was no difference in efficiency between *APO1* and ubiquitin mRNA in the validation experiment, the DDCT method was used for relative quantification (Pfaffl 2001).

Counting the number of vascular bundles in the peduncle

Dried pieces of peduncles that had been cut about 1 cm below the panicle base node were used to count the number of large vascular bundles and measure their diameters. One piece was obtained from each of the five selected panicles for which yield components were measured. Peduncles were swelled with water, filled with epoxy resin, and sectioned along the transverse axis. Then the peduncle diameter was measured, and the number of large vascular bundles was counted.

In situ hybridization

In situ hybridization was carried out according to the method described in Hirose et al. (2002). Young panicles from Habataki and Sasanishiki plants were collected on July 5 and 9, about 24 and 20 days before heading, respectively. The hybridization probes were made using PCR primer sets 3628-67L1 and 3628-U2R2 (Table 1).

Transgenic plants

To suppress the expression of *APO1*, RNAi-*APO1* was constructed using a p2K1[+] binary vector, as described in Moritoh et al. (2005). A 373 bp Habataki *APO1* fragment was PCR-amplified using the 3628-UFL1 and 3628-U2R1 primer sets (Table 1). As this fragment included a *Sac*II restriction enzyme digestion site that would be digested in a later step, this site was corrupted by substituting CCGCGG into TCGCGG. This mutated fragment was sub-cloned into pGEM-Teasy (Promega Corporation, Madison, WI, USA)

and its sequence was verified. A *Sac*II–*Spe*I fragment and a *Sal*I–*Apa*I fragment were inserted into the pGUS27 vector (Miki and Shimamoto 2004) in the inverted direction upstream and downstream of the gus linker fragment. Then a *Sac*I–*Kpn*I fragment was cloned into the p2K1$^+$ binary vector. The construct was introduced into Sasanishiki using the *R. radiobacter*-mediated method described in Toki et al. (2006).

To investigate the differences of the functions between the Habataki-type *APO1* and the Sasanishiki-type *APO1*, we introduced Habataki *APO1* into a Sasanishiki plant with a native promoter of Habataki alleles. Using primer sets 3628-51L3 and 3628-53R3 (Table 1) and LA *Taq* polymerase (Takara Bio Inc., Shiga, Japan), we obtained a *Hin*dIII-digested PCR-amplified fragment from Habataki plants. This fragment included the *APO1* open reading frame (ORF), with the promoter region up to −1,731 and a 789-bp 3′ region. This fragment was inserted into the *Hin*dIII restriction enzyme site in the pTN1 vector (Fukuoka et al. 2000). After checking the insertion direction, the construct was introduced into Sasanishiki using the *R. radiobacter*-mediated method as above.

Results

Map-based cloning of a gene that regulates the number of PRBs

The *PBN6* QTL, which controls the number of PRBs, is located on chromosome 6, between R2549EH and G329. These loci are 69.3 and 107.3 cM, respectively, from the end of the short-arm side of chromosome 6. The region between these markers was mapped at a high resolution (Fig. 1a) and included an AP003628 PAC clone. Further fine mapping was carried out using CAPs, STS and SSR markers that were generated based on the genome sequence information from the japonica rice cultivar, Nipponbare (Fig. 1b) (http://rgp.dna.affrc.go.jp/). The line was heterogeneous in this region; eight reciprocal recombinant lines were found. We carried out progeny testing to determine the average number of PRBs. The number of PRBs segregated similarly to the genotype markers 3628-60, 3628-55 and 3628-41 (Fig. 1c). Accordingly, the QTL for the number of PRBs was determined to be located between markers 3628-41 and 3628-56, which contain about 11 kb. This result is much narrower than the region decided in the preceding report that included 390 kb (Ando et al. 2008). Only one predicted gene, Os06g0665400, existed at the 28357085–28358390 position on chromosome 6 (Fig. 1b). This gene was identical to *APO1*, a gene that has been cloned previously, during the analysis of a mutant plant called *apo-1*, and that is thought to regulate the transforma-

tion of the meristem into the panicle primordia (Ikeda et al. 2001, 2007). Notably, the *apo-1* mutant displayed abnormally shaped panicles with reduced numbers of grains (Ikeda et al. 2001). It was found that these serious defects in the *apo-1* mutant are due to the insertion of a stop codon in the *APO1* gene (Ikeda et al. 2007). While this gene was found to be functional in Habataki and Sasanishiki plants, slight differences of the sequence may have affected the number of PRBs.

Further reciprocal recombination occurred in the heterogeneous region of the *PBN6* locus in the 03SHA422-12-8-18.31 line (Fig. 2). This region was divided into two sub-regions between markers 3628-55 and 3628-41. The SHA-PRB line had a heterogeneous region that included markers 3628-55 and 3628-60, which corresponded to the ORF region of the *APO1* gene and the proximal promoter region up to −2,287 bp, respectively. The SHA-HI line had a heterogeneous region including markers 3628-41, which corresponded to the distal promoter region past −2,832 bp (see below; Fig. 3).

DNA sequencing

The DNA sequence was established to identify differences in this region between Sasanishiki and Habataki plants, as well as to find the position of the reciprocal recombination. These sequences were compared to the published Nipponbare sequence (Sasaki et al. 2002, http://rgp.dna.affrc.go.jp/). Figure 3 shows the DNA sequences that differ among Habataki, Sasanishiki and Nipponbare plants in this region. The base number, counted from the start of the ORF, uses Nipponbare as a reference. Notably, 216 bp differed between Habataki and Sasanishiki plants out of a total of 11,086 bp between the 5′ side recombination site in SHA422-12-8-18.31 (a, −9,070 bp) and the 3628-56 marker (2,015 bp). The difference between Sasanishiki and Nipponbare plants was 14 bp, which is much smaller than the 202 bp difference between Habataki and Nipponbare plants. However, a higher frequency of base differences between Sasanishiki and Nipponbare plants occurred proximal promoter to the ORF region of *APO1*. Eight base pair differences occurred between Sasanishiki and Nipponbare, from −350 bp at the start of the ORF to the end of the ORF. This suggests that the origin of the Sasanishiki *APO1* ORF region may differ from the origin of the Nipponbare region. Figure 3 also shows the site of recombination between SHA-HI and SHA-PRB (b and c, respectively). SHA-PRB recombination occurred between −2,687 and −2,287 bp, while SHA-HI recombination occurred between −2,827 and −2,798 bp.

The amino acid sequences deduced for the ORF in Nipponbare, Habataki and Sasanishiki *APO1* are shown in Fig. 4. There were five sites of amino acid difference

Fig. 1 Map-based cloning of the QTL for the number of primary rachis branches. **a** Positioning of the QTL on PAC clone AP003628. **b** Predicted genes are marked with *arrows* between 40,000 and 95,000 bp on the AP003628 clone. *Solid boxes* represent the primer positions of CAPS, STS or SSR markers. *Dotted boxes* represent the predicted region of the QTL according to the results shown in *C*. The marker name is shown in *C*. **c** Graphical genotypes of the segregated progeny for which reciprocal recombination occurred within the above mentioned region; *bar graphs* of the number of primary rachis branches. *Open boxes* in the graphical genotype, Sasanishiki type; *closed boxes* Habataki type. *Bar graphs* represent the number of primary rachis branches in the segregated progeny shown in the graphical genotype as well as their parent cultivars Habataki (*filled bars*) and Sasanishiki (*open bars*). Determination of phenotype segregation is shown beside the *bar graph*. Possible location of the graphical genotype is shown in the *dotted box*; the genotype region of the markers and the phenotype of primary rachis branches matched. *Error bars* standard deviations, *broken and dotted lines* represent the mean and standard deviations of parent cultivars Habataki and Sasanishiki, respectively, for the comparison of values in segregated progeny
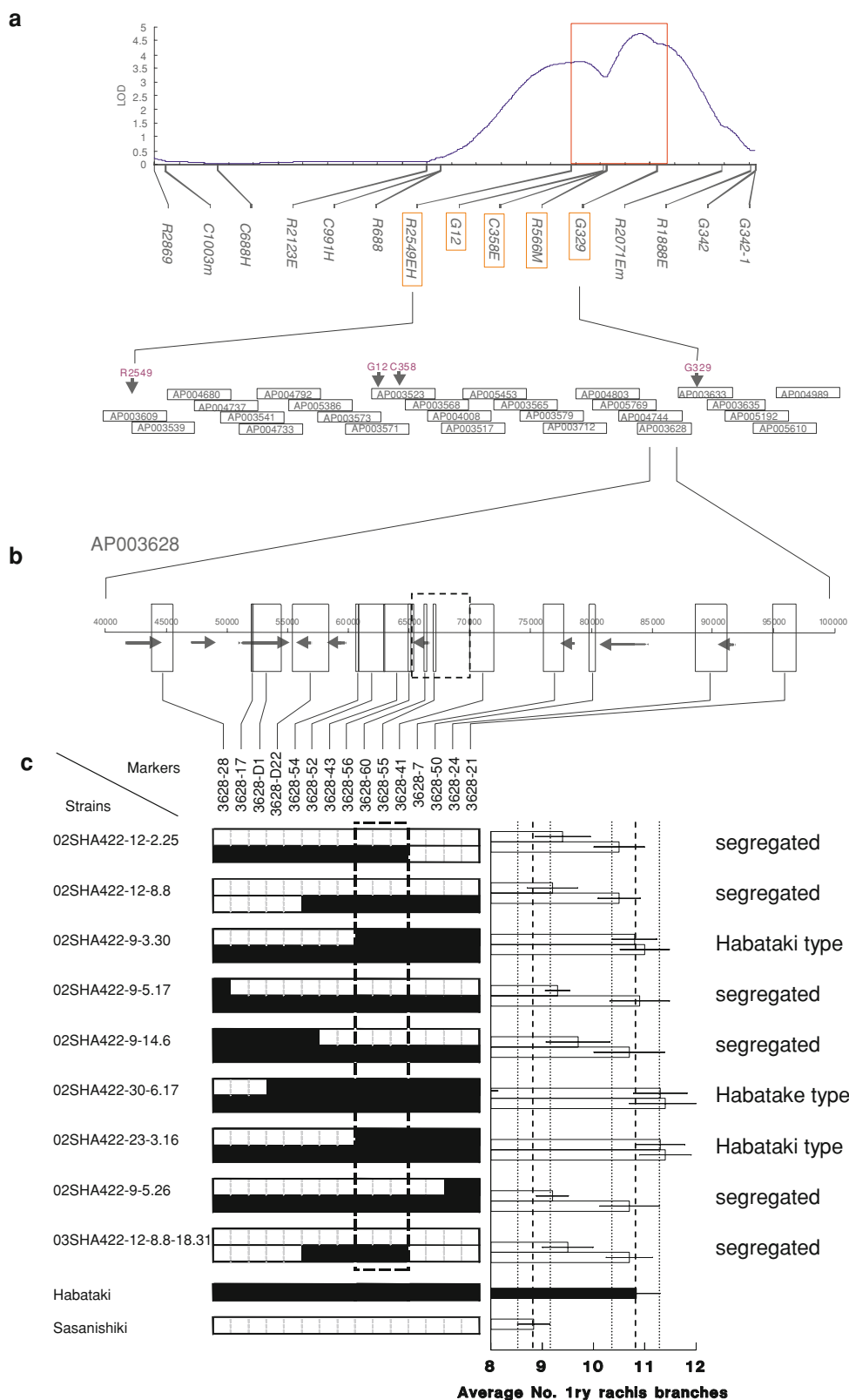
between Nipponbare and Habataki plants; there were four sites of difference between Nipponbare and Sasanishiki plants. As mentioned above, the sequence differences between Sasanishiki and Nipponbare plants seemed fre-quent in the ORF region, even though both varieties were japonica-type cultivars. One of the differences, a shift from isoleucine in Nipponbare and Sasanishiki plants to valine in Habataki plants, was located in the F-box protein motif.

Fig. 2 The position at which the reciprocal recombination occurred within the heterogeneous region of the line 03SHA422-12-8-18.31. *Thick arrow* represents *APO1* ORF and *narrow arrows* represent other predicted genes. The base numbers in AP003628 were shown to denote the position of recombination sites. Recombination site positions are *a* 61978–62124, *b* 68306–68904, *c* 69410–69439 and *d* 75540–75683
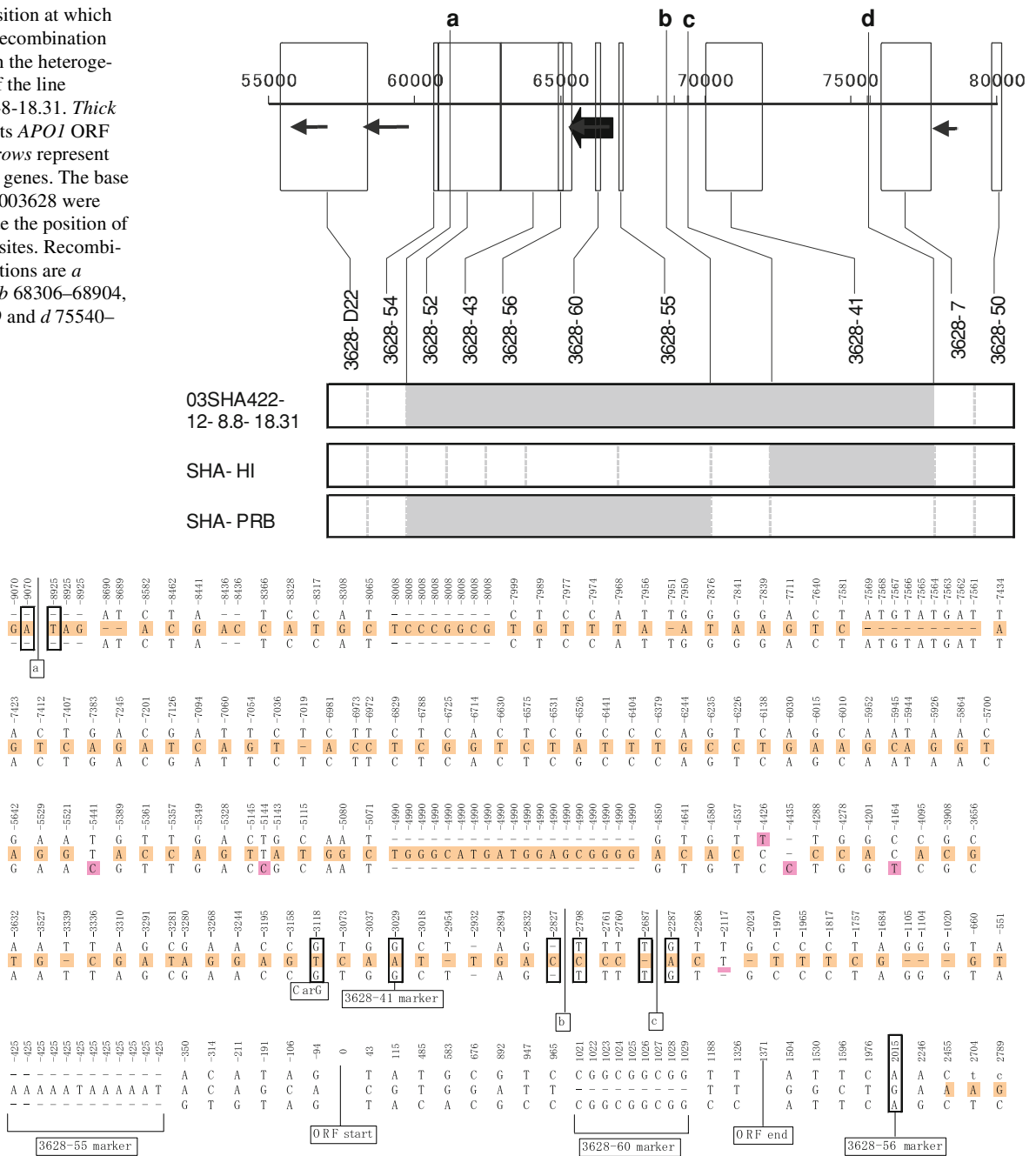

Fig. 3 DNA differences at the *PBN6* QTL: the *PRB1* allele, which is responsible for the number of primary rachis branches; and the *HI1* allele, which affects the number of grains per panicle and harvest index in Nipponbare, Habataki and Sasanishiki. The base count shown on the sequences represents Nipponbare base pair number, based on the ORF starting position. **a–c** Recombination sites shown in Fig. 2

This motif may bind substrates for ubiquitin-mediated proteolysis.

To identify promoter region differences between Habataki and Sasanishiki plants that may cause the difference in *APO1* gene expression, we performed a PLACE search (Higo et al. 1999). This search revealed that one of the CarG8 motifs was missing in Habataki plants due to substitution of a G with a T at the −3,118 position in Nipponbare and Sasanishiki types (CarG in Fig. 3).

Yield and yield-determining components

The yield-determining elements were measured using the segregated progeny from the 03SHA422-12-8.8-18.31 line obtained in 2004 and 2005 (Table 2). These plants segregated the region including the *APO1* gene, but both the 3′ and 5′ sides of this region were fixed in the Sasanishiki genotype (Fig. 1). Also, the other heterogeneous region in chromosome 1 was fixed into the Sasanishiki genotype.
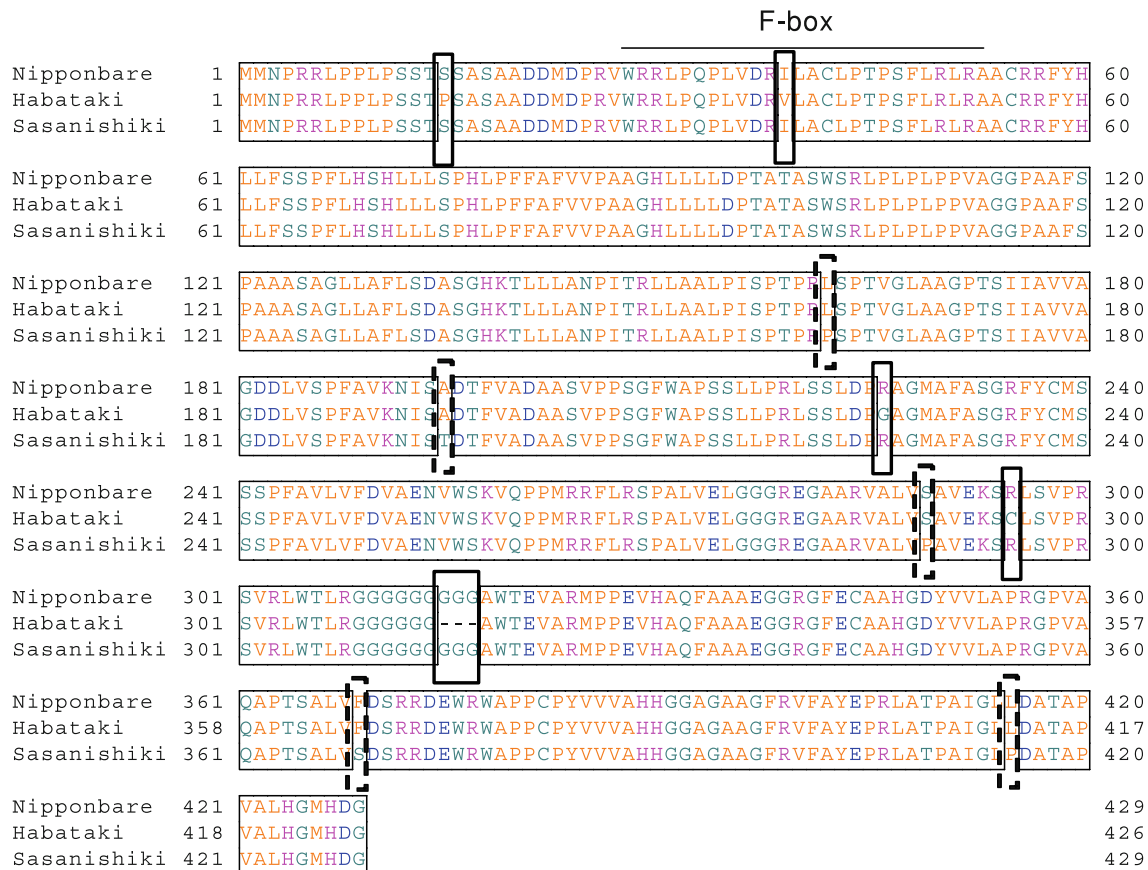
F-box

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Nipponbare      1 MMNPRRLPPLPSSTSSASAADDMDPRVWRRLPQPLVDRILACLPTPSFLRLRAACRRFYH 60
Habataki        1 MMNPRRLPPLPSSTPSASAADDMDPRVWRRLPQPLVDRVLACLPTPSFLRLRAACRRFYH 60
Sasanishiki     1 MMNPRRLPPLPSSTSSASAADDMDPRVWRRLPQPLVDRILACLPTPSFLRLRAACRRFYH 60

Nipponbare     61 LLFSSPFLHSHLLLSPHLPFFAFVVPAAGHLLLLDPTATASWSRLPLPLPPVAGGPAAFS 120
Habataki       61 LLFSSPFLHSHLLLSPHLPFFAFVVPAAGHLLLLDPTATASWSRLPLPLPPVAGGPAAFS 120
Sasanishiki    61 LLFSSPFLHSHLLLSPHLPFFAFVVPAAGHLLLLDPTATASWSRLPLPLPPVAGGPAAFS 120

Nipponbare    121 PAAASAGLLAFLSDASGHKTLLLANPITRLLAALPISPTPRLSPTVGLAAGPTSIIAVVA 180
Habataki      121 PAAASAGLLAFLSDASGHKTLLLANPITRLLAALPISPTPPLSPTVGLAAGPTSIIAVVA 180
Sasanishiki   121 PAAASAGLLAFLSDASGHKTLLLANPITRLLAALPISPTPPPSPTVGLAAGPTSIIAVVA 180

Nipponbare    181 GDDLVSPFAVKNISADTFVADAASVPPSGFWAPSSLLPRLSSLDPRAGMAFASGRFYCMS 240
Habataki      181 GDDLVSPFAVKNISADTFVADAASVPPSGFWAPSSLLPRLSSLDPGAGMAFASGRFYCMS 240
Sasanishiki   181 GDDLVSPFAVKNISTDTFVADAASVPPSGFWAPSSLLPRLSSLDPRAGMAFASGRFYCMS 240

Nipponbare    241 SSPFAVLVFDVAENVWSKVQPPMRRFLRSPALVELGGGREGAARVALYSAVEKSRLSVPR 300
Habataki      241 SSPFAVLVFDVAENVWSKVQPPMRRFLRSPALVELGGGREGAARVALYSAVEKSCLSVPR 300
Sasanishiki   241 SSPFAVLVFDVAENVWSKVQPPMRRFLRSPALVELGGGREGAARVALPAVEKSRLSVPR 300

Nipponbare    301 SVRLWTLRGGGGGGGGGAWTEVARMPPEVHAQFAAAEGGRGFECAAHGDYVVLAPRGPVA 360
Habataki      301 SVRLWTLRGGGGGG---AWTEVARMPPEVHAQFAAAEGGRGFECAAHGDYVVLAPRGPVA 357
Sasanishiki   301 SVRLWTLRGGGGGGGGGAWTEVARMPPEVHAQFAAAEGGRGFECAAHGDYVVLAPRGPVA 360

Nipponbare    361 QAPTSALVHDSRRDEWRWAPPCPYVVVAHHGGAGAAGFRVFAYEPRLATPAIGLLDATAP 420
Habataki      358 QAPTSALVHDSRRDEWRWAPPCPYVVVAHHGGAGAAGFRVFAYEPRLATPAIGLLDATAP 417
Sasanishiki   361 QAPTSALVSDSRRDEWRWAPPCPYVVVAHHGGAGAAGFRVFAYEPRLATPAIGLPDATAP 420

Nipponbare    421 VALHGMHDG 429
Habataki      418 VALHGMHDG 426
Sasanishiki   421 VALHGMHDG 429
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Fig. 4 Differences in the deduced amino acid sequence among Nipponbare, Habataki and Sasanishiki. The *solid box* represents the difference between Nipponbare and Habataki, while the *broken box* represents the difference between Nipponbare and Sasanishiki. *F-box* F-box motif

Thus, these segregated plants were mutually near-isogenic; they might be called isogenic, because the segregated region included only 1 gene, although they are not isogenic when compared to Sasanishiki plants. In both years, the traits showing significant increases in the Habataki genotype were shoot length (by 2% (2004) and 1% (2005)), head length (3% in both years), the number of PRBs per panicle (by 13%; 12%), the number of spikelets per panicle (by 9%; 13%), harvest indexes (by 4%; 7%), and the number of sunk grains (with hull) (by 7%; 11%). The number of secondary rachis branches per panicle increased in the Habataki plants, but the ratio of secondary to PRBs did not change in 2005 and even decreased in 2004. This suggests that the genotype difference in this region does not affect the number of secondary rachis branches. In contrast, the grain weight decreased in Habataki plants (by 2% (2004) and 3% (2005)). However, the magnitude of the reduction in grain weight (2–3%) was smaller than the magnitude of the increase in the number of grains per panicle (9–13%). Ripening percentage, as represented by the ratio of sunk grains to total grains, decreased significantly in the 2005 experiment. This parameter also decreased in the 2004 experiment, but the change was not significant. Notably, the

magnitude of the decrease in the ripening percentage was small (2%) compared to the increase in the number of grains, even in the 2005 experiment. In Habataki-genotype plants, the weight of sunk grains per plant increased 5 and 7% in 2004 and 2005, respectively.

The yield-determining components of the segregated progeny of the SHA-PRB and SHA-HI lines, which have different heterogeneous regions within the *PBN6* locus, were evaluated in 2006. The measurements of the SHA-PRB and SHA-HI lines are shown in Tables 3 and 4, respectively. The yield-determining components of the SHA-PRB progeny that segregated the ORF and proximal promoter region showed a difference in the number of PRBs. Habataki-type progeny (SHA-PRB-B) had more PRBs than Sasanishiki-type progeny (SHA-PRB-A). However, this increase in the number of PRBs did not directly increase the number of grains per panicle. Instead, the number of grains per primary rachis branch was reduced, and therefore the number of grains per panicle was not altered. This decrease in the number of grains per primary rachis branch occurred because the number of secondary rachis branches per primary rachis branch was reduced. Other yield-determining factors, such as, harvest index, grain

Table 3 Yield-determining components for segregated progeny of the SHA-PRB line, which has a heterogeneous region between 3628-52 and 3628-55

Genotype	Traits													
	n	SL (cm)	SDW (g)	HDW (g)	HI	NPP	NGP	1RB	NGR	2ry/1ry	GW (mg)	%STG	%SG	SGW (g)
SHA-PRB-A (Sasanishiki)	68	99.0	50.7	30.0	0.596	11.8	104	9.43 c	11.1 a	1.92 a	21.4	2.44	95.2	25.0
SHA-PRB-B (Habataki)	60	98.3	48.4	28.9	0.604	11.5	106	10.42 a	10.1 c	1.65 c	21.2	2.54	93.8	24.0
SHA-PRB-H (hetero)	141	98.2	48.8	29.0	0.598	11.5	104	9.86 b	10.6 b	1.78 b	21.3	2.67	94.0	24.2
H/S ratio		0.99	0.95	0.97	1.01	0.98	1.01	1.10	0.92	0.86	0.99	1.04	0.99	0.96
Significance		NS	NS	NS	NS	NS	NS	***	***	***	NS	NS	NS	NS

NGR Number of grains per primary rachis branch, other abbreviations are the same as in Table 2

Table 4 Yield-determining components for segregated progeny of the SHA-HI line, which has a heterogeneous region including 3628-41

Genotype	Traits								
	n	NPP	HDW (g)	NGP	1RB	2RB	GW (mg)	%STG	%SG	SGW (g)
A[a]									
SHA-HI-A (Sasanishiki)	83	11.4	24.4 b	104 b	9.17 b	18.1 b	21.6	2.44	92.6	23.6 b
SHA-HI-B (Habataki)	58	12.0	26.7 a	111 a	9.38 a	20.3 a	21.0	2.98	90.5	25.3 ab
SHA-HI-H (hetero)	138	12.0	26.5 a	109 a	9.29 ab	19.7 a	21.4	2.51	92.0	25.6 a
H/S ratio		1.05	1.10	1.07	1.02	1.12	0.97	1.22	0.98	1.07
Significance		NS	**	**	*	***	NS	NS	NS	**

Genotype	Traits			
	n	SL (cm)	SDW (g)	HDW (g)	HI
B[b]					
SHA-HI-A (Sasanishiki)	179	93.2	40.2	22.9	0.568
SHA-HI-B (Habataki)	180	93.8	40.1	23.4	0.584
H/S ratio		1.01	1.00	1.02	1.03
Significance		NS	NS	NS	*

2RB Number of secondary rachis branches per panicle, other abbreviations are the same as in Table 2

[a] Panicle traits were measured in an experiment with ten rows of 30 segregated plants

[b] Shoot characteristics and the harvest index were measured in an experiment with three replicates of two segregated genotypes, including 12 rows of five plants

weight, the number of panicles per plant and the percentage of fertile spikelets, did not change. Since these lines segregated at the ORF and proximal promoter region, this segregated region is considered to control the number of PRBs. The number of grains per panicle seems to be regulated by a different region. We designated this region the *PRB1* allele because it is a part of *PBN6* locus, which only acts to change the number of Primary Rachis Branches.

The yield-determining components of the segregated progeny of SHA-HI showed that the panicle weight, the number of grains per plant, and the number of grains per panicle were increased in Habataki-genotype progeny (SHA-HI-B). The number of secondary rachis branches increased ~12% among the Habataki genotype; this difference was highly significant. The number of PRBs increased only 2% among the Habataki genotype. This 2% increase was significant at $P < 0.05$, but the extent of the increase was reduced compared to that of secondary rachis branches. Accordingly, the increase in the number of spikelets per panicle in SHA-HI-B resulted from an increased number of secondary rachis branches. The difference in the grain weights between SHA-HI-A and SHA-HI-B was not significant. The increased number of grains per panicle did not reduce the grain weights but increased the sunk grain weight per plant and the harvest index. Hence, we designated this region as the *HI1* allele because it is a part of the

PBN6 locus that can control the *Harvest Index*. This allele included the distal promoter region of *APO1* beyond −2,832 bp; thus, the grain yield per panicle seems to be regulated in this region.

These results indicate that two important factors in panicle architecture, the number of PRBs per panicle and the number of spikelets per panicle, are controlled independently. This is demonstrated by the fact that the increased number of PRBs per panicle did not directly increase the grain yield per panicle. These components are controlled by different alleles on the same gene. Moreover, the effects of these two alleles were additive. The line with a Habataki genotype *PBN6* that includes Habataki genotypes at both *PRB1* and *HI1* alleles exhibited a higher number of PRBs, demonstrating an effect at the *PRB1* allele. This line also exhibited increases in the number of spikelets per panicle, harvest index, the number of vascular bundles in each peduncle and peduncle diameter, demonstrating an effect at the *HI1* allele.

Gene expression

The expression of *APO1* during young panicle development (Fig. 5a) was studied using semi-quantitative RT-PCR (Fig. 5). Because the amplified fragment included an intron, we amplified a shorter band when the template was cDNA (Fig. 5, lane cDNA) as compared to genomic DNA (Fig. 5, lane genome DNA). Both PCR product sizes corresponded to the sizes expected without and with the intron, respectively. *APO1* expression in the Habataki plants was consistently higher than that in the Sasanishiki plants. After 30 cycles of PCR amplification, *APO1* expression was evident only in Habataki cDNA sampled on July 7, corresponding to ~23 days before heading. When the number of cycles was increased to 32, the Habataki samples showed obvious *APO1* expression on July 4, and showed the highest expression on July 7, while reduced but still obvious expression was seen on July 10

Fig. 5 *APO1* expression at the young panicle of Habataki (*H*) and Sasanishiki (*S*) plants, sampled on July 4, July, 7 July 10 and July 13, measured by semi-quantitative RT-PCR. The size of the amplified cDNA was 82 bp smaller than that of genomic DNA because an intron was included between primer sets 67L1 and U2R1. Data for 30, 32 and 35 PCR cycles are shown. Actin was used for the internal standard. **a** Sampled part of panicle. **b** Quantification of *APO1* expression level in the same sample measured by real-time PCR
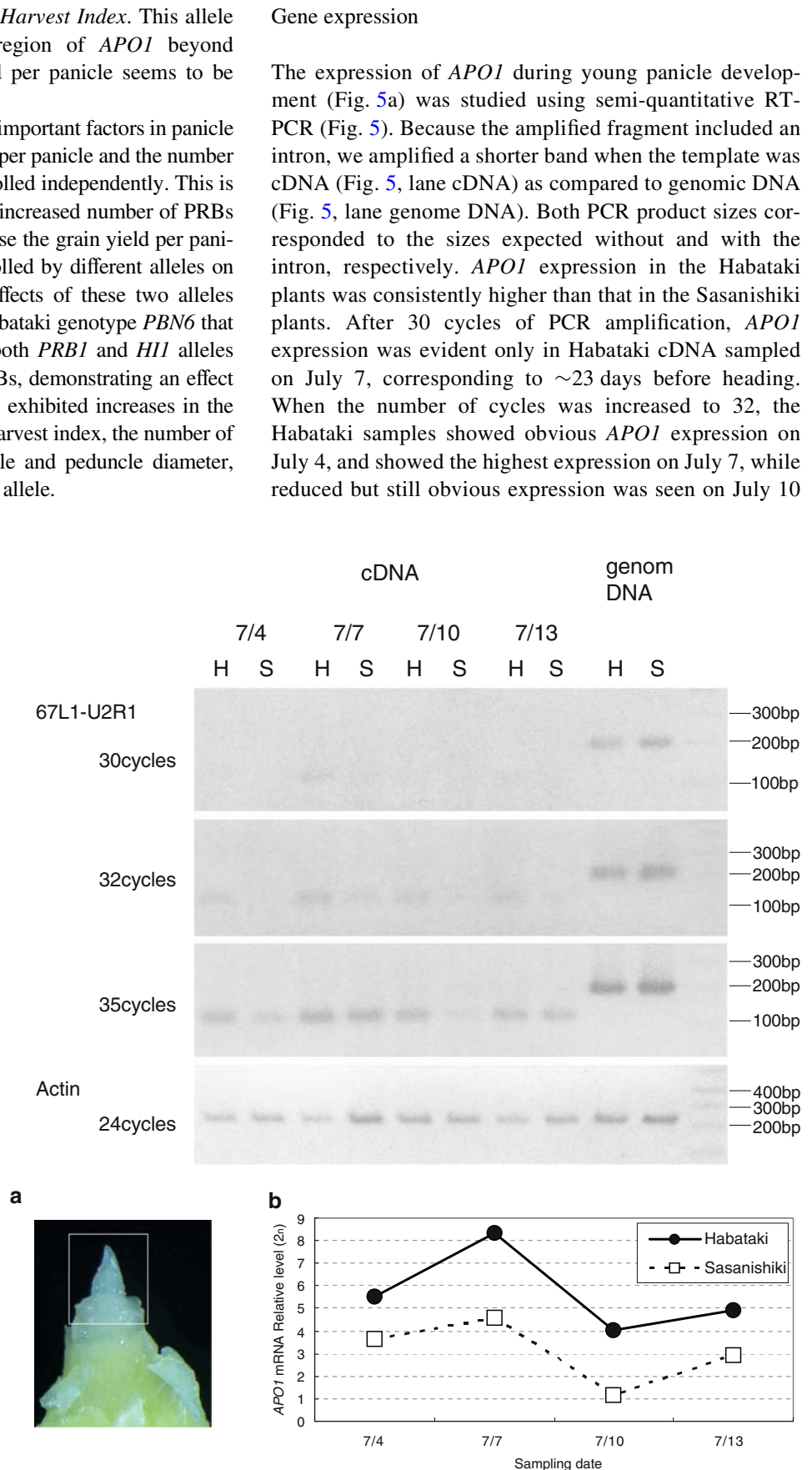
and 13. In the Sasanishiki plants, *APO1* expression was not detectable in samples obtained on July 4, 10 or 13 and was only detectable in the sample obtained on July 7, although the expression level was still lower than in Habataki plants. After 35 cycles, the Sasanishiki plants expressed *APO1* at all stages, with the lowest levels observed in samples obtained on July 4 and 10. To determine by how much the expression levels of *APO1* differed between Habataki and Sasanishiki plants, quantitative real-time PCR analyses were performed using the same samples of Habataki and Sasanishiki plants (Fig. 5b). The expression levels in Habataki plants were 3.5, 13, 7 and 4 times ($2^{1.8}$, $2^{3.8}$, $2^{2.9}$ and $2^{2.0}$ times) higher than that of Sasanishiki plants on July 4, 7, 10 and 13, respectively. Thus, the peak strength of *APO1* expression in Habataki

plants was estimated to be around ten times higher than that of Sasanishiki plants.

As presented in Fig. 6, we also measured expression of *APO1* at the region beneath the developing panicle, the next block below the samples used for the analysis presented in Fig. 5 (Fig. 6a). In this case, samples obtained on July 7, July 10 and July 13 were used. The *APO1* expression levels were almost the same as the young panicle. The expression in Habataki plants was higher than that in Sasanishiki plants, and the expression was highest in the July 7 samples. Quantitative real-time PCR analyses revealed that *APO1* expression in Habataki plants was 6, 9 and 9 times ($2^{2.6}$, $2^{3.2}$ and $2^{3.2}$) higher than that of Sasanishiki plants on July 7, 10 and 13, respectively (Fig. 6b). Previous studies reported that *APO1* is expressed exclusively in the panicle

Fig. 6 *APO1* expression at the region beneath the developing panicle in Habataki (*H*) and Sasanishiki (*S*) plants, sampled on July 7, July 10 and July 13. The other conditions were the same as those described in Fig. 5
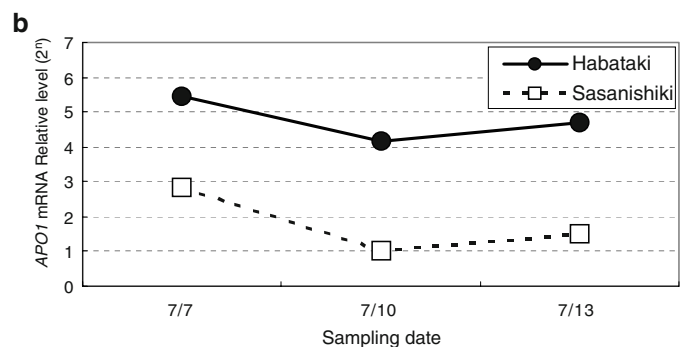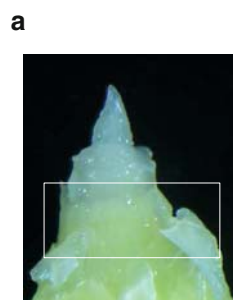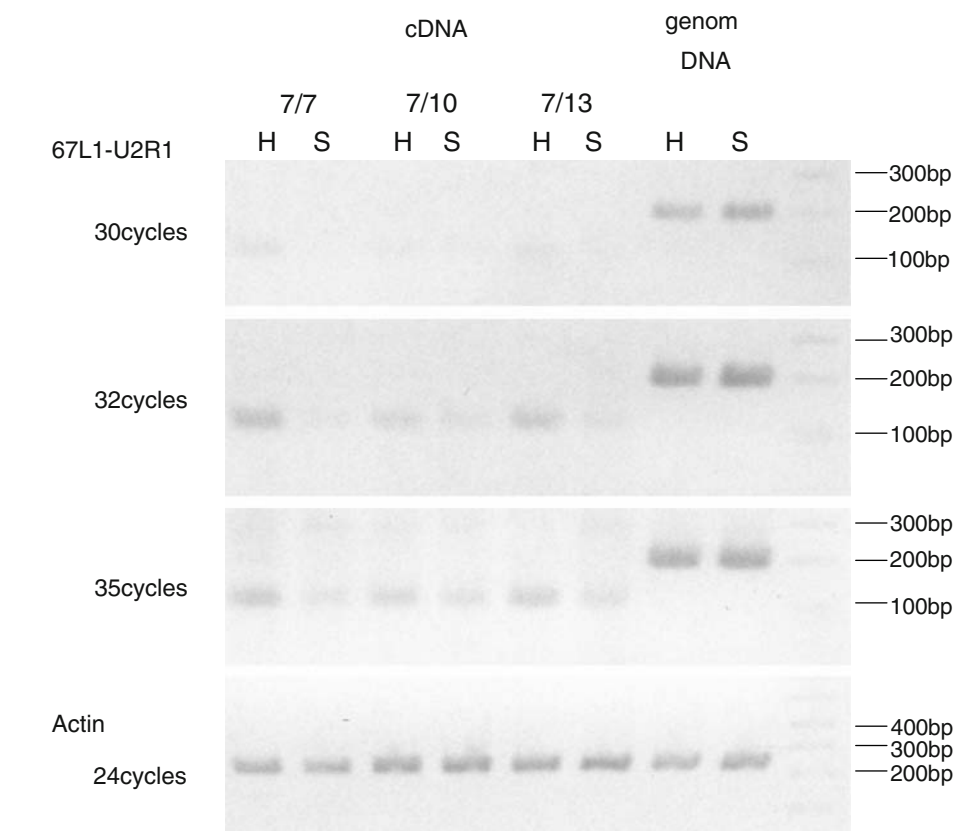
primodia and young panicles, particularly in the developing rachis branches (Ikeda et al. 2007). However, our results suggest that this gene is expressed in other parts of developing panicles, and that it affects processes other than the developmental change from meristem to panicle primodia. Figure 6b also shows that *APO1* expression in the region beneath the young panicle was much slowly decreased compared to that in the panicle. Also the expression levels in this region in Habataki plants on July 10 and 13 was about nine times higher than that in Sasanishiki plants, whereas the difference in developing panicle reduced to seven and four times. This suggests that *APO1* expression in Habataki plants is not only higher than that in Sasanishiki plants but also keeps higher expression to the later stage particularly in the region other than the growing panicle.

Transgenic plants

We cultivated T0 and T1 generations of RNAi-*APO1* plants, and counted the numbers of PRBs as well as grains per panicle. The averages of the number of PRBs in the two differently transformed T0 generation plants were 6.22 ($n = 21$) and 5.71 ($n = 17$), which are slightly lower than the 7.57 ($n = 5$) observed in the non-transgenic Sasanishiki plants grown in the same conditions. In the T1 generation segregated plants, each ten segregated T1 plants from six independent T0 plants were grown and the number of PRBs per panicle as well as the number of grains per panicle were measured. The average number of PRBs in the plants, either heterogeneous or homogeneous RNAi-*APO1*, was 7.44, which was significantly lower than that of RNAi-null plants with an average of 8.52 (Table 6). Also, the average number of grains per panicle in the RNAi-*APO1* plants was 58.01, significantly lower than the 71.07 of the RNAi-null plants (Table 6). We could not measure the expression level of *APO1* in the RNAi-*APO1* plants, because the destructive sampling of young panicles required for the measurement of mRNA would prevent the measurements of the number of PRBs and of the grains per panicle thereafter. However, significant differences were detected both in the number of PRBs and of grains per panicle between the segregated plants, both with RNAi-*APO1* and without. This strongly suggests that the suppression of *APO1* expression in RNAi plants caused the reduction of both the numbers of PRBs and of grains per panicle.

In the experiment that introduced Habataki *APO1* into Sasanishiki plants, we cultivated a T3 generation of transgenic plants by introducing Habataki *APO1* with a 1,731-bp promoter region and a 789-bp 3′ region into Sasanishiki plants. We compared T3-segregated progeny of null plants and two-copy introduced plants, which might be considered homo-introduced plants derived from single-copy intro-

duced plants. Each of the 12, 10 and 8 T3 plants from three independent lines was analyzed. Null plants had an average of 8.4 PRBs, and two-copy plants (homo) had an average of 8.8; these values are not significantly different. The number of grains per panicle in the homo plants was 98.1, compared to 91.1 in the null plants; this difference is also not significant. These data suggest that the exogenous Habataki *APO1* gene was not functional in the transgenic plants. Interestingly, Ikeda et al. (2007) reported that transformation with the *APO1* gene from wild-type plants could not rescue mutant plants when the introduced gene contained a 2.8-kb promoter region, but the same gene could rescue mutant plants when introduced with a 5-kb promoter region. Durfee et al. (2003) showed that an *Arabidopsis* T-DNA insertion mutant occurred at the −4,000-bp position in the promoter region of the *UNUSUAL FLORAL ORGAN* (*UFO*) gene. We therefore concluded that the length of the promoter region is not sufficient in the introduced Habataki gene, which prevents its effective function in transgenic plants.

Number of large vascular bundles in the peduncle

We studied the effects of the *PRB1* and *HI1* alleles on the formation of vascular bundles in the peduncle by counting the number of large vascular bundles in Habataki-type and Sasanishiki-type segregated progeny of the SHA-PRB and SHA-HI lines (Table 5). Habataki-type segregated progeny with the *HI1* allele (SHA-HI-B) had 10.5 large vascular bundles on average, compared to 9.1 among Sasanishiki-type progeny (SHA-HI-A). This 15% increase is statistically significant. The effect of the *PRB1* allele on the number of large vascular bundles was much smaller but still significant. Habataki-type progeny (SHA-PRB-B) averaged 9.8 vascular bundles, compared to 9.3 among Sasanishiki type (SHA-PRB-A), an increase of ~5%. Habataki-type progeny that had been segregated with the *HI1* allele (SHA-HI-B) displayed increased peduncle diameter. SHA-HI-B peduncles were about 9% thicker than those of the SHA-HI-A plants (1.34 vs. 1.23 mm, respectively). No difference in the diameter of the peduncle was observed between SHA-PRB-A- and SHA-PRB-B-type progenies. Accordingly, we concluded that the *HI1* allele affected vascular bundle formation in the peduncle. In the Habataki genotype, the *HI1* allele increased the number of large vascular bundles, and consequently increased peduncle diameter. Since the *HI1* allele corresponds to a promoter region further than 2.8 kb past the start of *APO1*, we hypothesize that differential expression of the *APO1* gene affected vascular bundle formation as well as peduncle formation.

The relationship between the number of vascular bundles and the number of PRBs in segregated progeny carrying the *HI1* or *PRB1* allele is shown in Figs. 7 and 8,

Table 5 *t* Test of the number of vascular bundles at the peduncle and of peduncle diameter in Sasanishiki-type (A) and Habataki-type (B) segregated progeny of SHA-HI and SHA-PRB lines

Line	Genotype	Statistics					Equality of variances				*t* Tests			
		n	Mean	SD	Minimum	Maximum	NumDF	Den DF	*F* value	Pr > *F*	Variances	DF	*t* Value	Significance
Number of vascular bundles in the peduncle														
SHA-HI	A	17	9.11	0.44	8.4	9.8	16	18	1.21	0.69	Equal	34	−10	***
	B	19	10.50	0.40	9.8	11.6								
	Difference		−1.39											
SHA-PRB	A	21	9.31	0.54	8.25	10.4	20	22	1.55	0.32	Equal	42	−3.17	**
	B	23	9.77	0.43	9.2	10.8								
	Difference		−0.46											
Diameter of peduncles														
SHA-HI	A	17	1.23	0.05	1.11	1.32	18	16	1.29	0.62	Equal	34	−5.9	***
	B	19	1.34	0.06	1.23	1.44								
	Difference		−0.11											
SHA-PRB	A	21	1.27	0.06	1.15	1.46	20	22	1.04	0.93	Equal	42	−1.01	NS
	B	23	1.29	0.06	1.21	1.45								
	Difference		−0.02											

SD Standard deviation, *NumDF*, *DenDF* numerator and denominator degree of freedom, respectively, *Pr > F* the probability of a greater *F′* value, *DF* degree of freedom, other abbreviations are the same as in Table 2
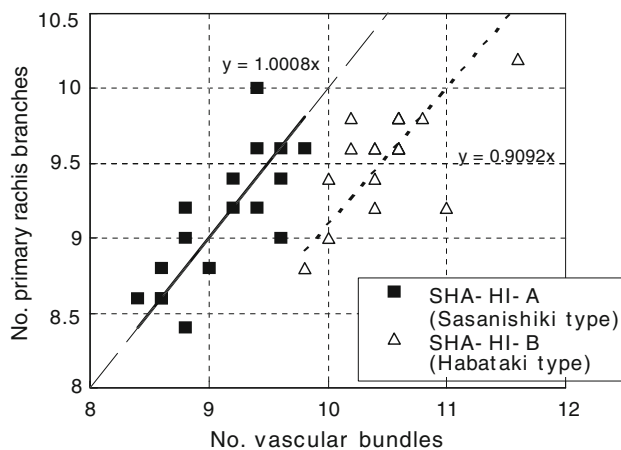
Fig. 7 Correlation of the number of vascular bundles and the number of primary rachis branches in the segregated progeny of SHA-HI. *Closed square* and *solid line* represent segregated progeny carrying the *HI1-Sasanishiki* allele; associated regression line passes through the origin (SHA-HI-A). *Open triangles* and *dotted line* represent segregated progeny carrying the *HI1-Habataki* allele (SHA-HI-B). Equations for regression lines are also shown. *Thin broken line* shows a 1:1 correlation

Fig. 8 Correlation of the number of vascular bundles and the number of primary rachis branches in the segregated progeny of SHA-PRB. *Closed squares* and *solid line* represent segregated progeny carrying the *PRB1-Sasanishiki* allele; associated regression line passes through the origin (SHA-PRB-A). *Open triangles* and *dotted line* represent segregated progeny carrying the *PRB1-Habataki* allele (SHA-PRB-B). Other markings are the same as in Fig. 7

respectively. For both SHA-HI-A and SHA-PRB-A, i.e., the Sasanishiki-type progeny of *HI1* and *PRB1* alleles, the ratio of the number of vascular bundles to the number of PRBs was about 1 (1.0008 in SHA-HI-A and 1.0129 in SHA-PRB-A). This indicates that the number of vascular bundles corresponded to the number of PRBs. SHA-HI-B,
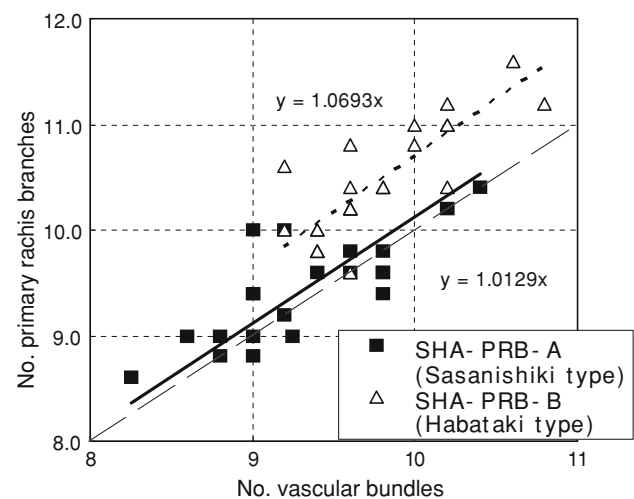
the Habataki-type progeny carrying the *HI1* allele, displayed significantly increased numbers of vascular bundles. Hence, this ratio was reduced to 0.9, although we also observed a slight but significant increase in the number of PRBs (Table 4A). This means that the increased number of large vascular bundles in SHA-HI-B did not directly cause

the proportional increase in the number of PRBs. Rather, the ratio in SHA-PRB-B, Habataki-type progeny carrying the *PRB1* allele, increased to 1.1. This was due to the dramatic increase in the number of PRBs, which outweighed the slight but significant increase in the number of vascular bundles (Table 5). This also indicates that the increase of PRBs in SHA-PRB-B was not caused by the increased number of large vascular bundles.

In situ hybridization

We performed in situ hybridization, to localize *APO1* expression in developing Habataki panicles. Figure 9 shows a transverse section of a young panicle from a Habataki plant sampled on July 9. The *APO1* gene was expressed in the developing PRB, as reported by Ikeda et al. (2007). The *APO1* gene also appeared to be expressed in the vascular bundles of developing leaves. We sought to determine whether this vascular bundle staining represented true *APO1* gene expression in vascular bundle cells, or artifact staining due to the higher density of small developing cells. Therefore, we used a sense probe as a control (Fig. 10). While the antisense probe clearly deposited color in the developing vascular bundles, the sense probe did not stain vascular bundles. We therefore concluded that the *APO1* gene is expressed not only in the developing panicle but also in developing vascular bundles.
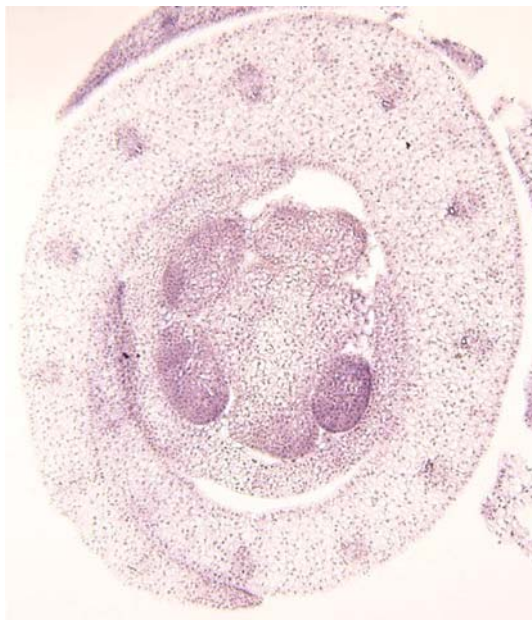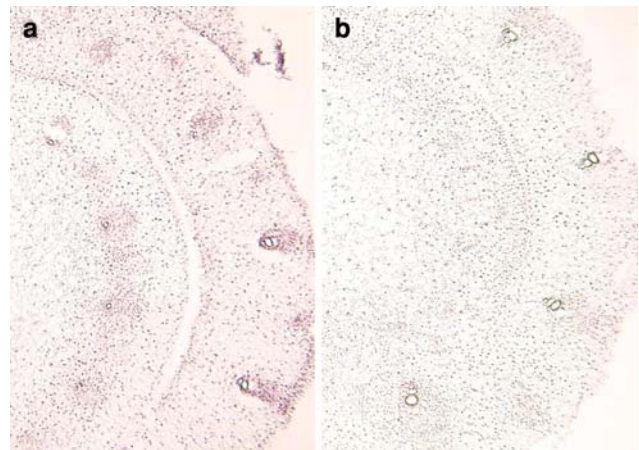

Fig. 10 In situ hybridization of the transverse section of the basal part of a young panicle from a Habataki plant with developing vascular bundles. **a** Anti-sense probe, **b** sense probe

Discussion

Identification of the *PBN6* gene and its effects on yield and yield components

We identified the gene containing the *PBN6* QTL, which determines the number of PRBs, to be *APO1*. We also showed that the Habataki allele, in comparison to the Sasanishiki allele, of this QTL/*APO1* increases the number of PRBs, the number of grains per panicle, and the harvest index, thereby increasing grain yield. Mutating this gene results in aberrant panicle organization (Ikeda et al. 2007; Ikeda-Kawakatsu et al. 2009), similar to *UFO* in *Arabidopsis thaliana*, *PROLIFERATING FLORAL ORGANS* in *Lotus japonicus*, *STAMINA PISTILLOIDA* in *Pisum sativum*, and *FIMBRIATA* in *Antirrhinum* (Levin and Meyerowits 1995; Wilkinson and Haughn 1995; Simon et al. 1994; Pouteau et al. 1998; Taylor et al. 2001; Zhang et al. 2003). Previous mutational analysis and the complementation experiment showed that this gene controls the production of spikelets and inflorescence structures. The present study also confirmed that RNAi-*APO1* plants reduced the number of PRB and the number of grains per panicle (Table 6). Accordingly, it is important to improve the Sasanishiki grain yield as well as that of other japonica rice cultivars. Notably, our findings suggest that this gene has the potential to contribute higher grain yield in widely grown rice cultivars, both in indica rice cultivars as well as in the newly developed indica–japonica crossed cultivars. Recently, a gain-of-function mutant of *APO1* gene *apo1-D*, which increased the expression level of *APO1* and hence increased the number of PRB as well as the number of grains per panicle, was reported (Ikeda-Kawakatsu et al. 2009). Ikeda-Kawakatsu et al. (2009) also reported that an

Fig. 9 In situ hybridization of a transverse section of young panicle obtained from a Habataki plant using the *APO1* anti-sense probe

Table 6 *t* Test of the number of primary rachis branches and of grains per panicle in RNAi-*APO1* and RNAi-null segregated T1 progeny of transgenic Sasanishiki

Genotype	*n*	Statistics				Equality of variances				*t* Tests			
		Mean	SD	Minimum	Maximum	Num DF	Den DF	*F* value	Pr > *F*	Variances	DF	*t* Value	Significance
Number of primary rachis branches													
RNAi-*APO1*	28	7.45	1.12	5	9	6	27	1.86	0.249	Equal	33	−2.10	*
RNAi-null	7	8.52	1.53	6	9.6								
Difference		−1.07	1.21										
Number of grains per panicle													
RNAi-*APO1*	28	58.01	13.24	31.5	78	6	27	2.64	0.077	Equal	33	−2.05	*
RNAi-null	7	71.07	21.49	33.75	87.5								
Difference		−13.06	15.08										

Abbreviations are the same as in Table 5

Undulate rachis 1 (*Ur1*) mutant (Nagao et al. 1958) occurred in the same mechanism: the insertion of *nDart1* transposon (Tsugane et al. 2006). Those gain-of-function mutants increased the number of PRBs and secondary rachis branches and hence the number of grains per panicle. Although it has not been proven that *apo1-D* mutant itself is directly usable for breeding high yielding cultivar, the mutation on this gene is usable for breeding of high yielding cultivars (Murai et al. 2005).

Two alleles of *PBN6* differentially control yield-determining component

The ability of *APO1* to alter the number of PRBs follows from the reported role of *APO1* in regulating the initiation of panicle and rachis primodia (Ikeda et al. 2007). As expected from this *APO1* ability, the Habataki allele of the *PBN6* locus clearly increased the number of PRBs as well as the number of grains per panicle, hence increasing grain yield per plant (Table 2). Because of this, we first considered that the increase of the number of PRBs should be the key factor of increasing the grain yield per plant, since increasing the number of PRBs may increase the potential number of grains per panicle. However, during later experiments, we divided the locus into two sub-regions; *PRB1* and *HI1* alleles, and obtained results that did not support the original hypothesis. The correlation that we observed between an increased number of grains per panicle and an increased number of PRBs was an additive effect of activity at those two alleles. The *PRB1-Habataki* allele increased the number of PRBs but decreased the number of grains per rachis and hence caused no net increase of the number of grains per panicle. The *HI1-Habataki* allele is less effective in increasing the number of PRBs but did increase the number of grains per primary rachis branch, and hence increased the number of grains per panicle. At both alleles, the positive and negative effects canceled out, with regard to the number of grains per rachis. What is left is the increase in the number of PRBs, caused by the *PRB1-Habataki* allele, and the increase in the number of grains per panicle, caused by the effect of *HI1-Habataki* allele, which seem correlated but that are regulated separately. The fact that the *PRB1-Habataki* allele had no effects on increasing the number of grains per panicle suggests that an increase in the potential sink size may not increase grain yield unless there is a sufficient source supply. Contrarily, the *HI1-Habataki* allele had the effect of increase the number of grains per panicle. This might be caused through the improvement of translocation efficiency (see below).

The *PRB1* allele corresponds to the ORF and proximal promoter region of the *APO1* gene. On the other hand, the *HI1* allele corresponds to the distal promoter region of the same gene. The *HI1* allele controls the number of grains, number of secondary rachis branches per panicle, the harvest index, the number of large vascular bundles, and the peduncle diameters, and limits the increase in the number of PRBs to 2%. These findings demonstrate that the distal promoter region, but not the ORF and proximal promoter region, regulates the yield. Interestingly, differences in this region affect yield but not the translated product. Distal promoter region activity was also able to rescue *apo1* mutation in rice (Ikeda et al. 2007) and *Arabidopsis* (Durfee et al. 2003). Thus, promoter region variation may control each part of the panicle structure in different ways, even when the same gene is expressed. This might be caused through the difference of special distribution of expression such as rachis and vascular bundle primordia as well as the difference of expression strength.

Effect of increasing harvest index on the grain yield

In addition to increase the number of PRBs and grain yield, *APO1* has two more important functions that we identified. These are the ability to change the harvest index and the

ability to alter the number of vascular bundles. Increasing the harvest index is one of the most effective ways to increase grain yield (Snyder and Carlson 1984; Zhang and Kokubun 2004). This study has shown that the *PBN6* locus and *HI1* allele represent the QTL for harvest index and that these QTLs are regulated by *APO1*. Few reports in the literature have examined QTLs in rice for their harvest index. Mao et al. (2003) reported four main-effect QTLs for the harvest index (on chromosomes 1, 4, 8 and 11) and one epistatic QTL on chromosome 5. Lanceras et al. (2004) described harvest index QTLs on chromosomes 1 and 3, but did not report the harvest index QTL on chromosome 6. Notably, increasing the harvest index may be the most effective way to increase the yield per unit area because high-yielding bigger plants may require larger areas or lower optimum planting density. Therefore, increasing the harvest index by improving yield without altering plant size allows farmer to increase grain yield per unit area. A higher harvest index also increases yield per unit input of solar radiation or soil nutrients. Our research indicates that breeding cultivars of high harvest index requires Habataki alleles at *PBN6* locus and *HI1-Habataki* allele. The improvement in the harvest index and possibly translocation efficiency may be important for researchers working to pyramid genes that improve grain yield with genes that increase sink size. It may be necessary to collect genes with compensatory roles, such as those for sink size and source potential, in addition to collecting genes with similar roles, such as those that control the number of grains and grain size, which was now being examined (Ashikari and Matsuoka 2006; Ando et al. 2008).

Effect of *HI1* allele on vascular bundle system formation

How does the promoter region of *APO1* affect the harvest index as well as grain yield? The underlying mechanism may be increased translocation efficiency. Sasahara et al. (1999) showed that one of the QTLs for the number of vascular bundles in the peduncle was located near the C962 marker on chromosome 6. This location is very close to the *PBN6* locus, as well as to *APO1*. We therefore considered the possibility that the number of vascular bundles in the peduncle was also controlled by *APO1*. Our results clearly showed that the *HI1* allele controls the number of vascular bundles in the peduncle as well as peduncle diameter. In contrast, the *PRB1* allele has no effect on peduncle diameter and a limited effect on the number of large vascular bundles. Thus, attaching the Habataki-type promoter to *APO1* increased the number of vascular bundles, which might be favorable to enhance the translocation of carbohydrate from leaves to panicles. Ikeda-Kawakatsu et al. (2009) also showed that the number of vascular bundles was increased in the gain-of-function mutant *apo1-D1*, in which the

APO1 transcription level increased because of the insertion of *nDart1-0* transposon into the negative regulation region of *APO1* promoter. The *apo1-D1* mutant increased the number of PRBs and the number of vascular bundles, and constitutive overexpressed *APO1* plants showed a much higher number of PRBs and vascular bundles. In that same study, Ikeda-Kwakatsu et al. suggested that the increase of meristem size and subsequent increase of panicle branching occurred according to the increase of the vascular bundles. However, our results clearly revealed that the number of vascular bundles and the number of rachis branches are regulated independently. The apparently synchronized increase in the number of PRBs and the number of vascular bundles shown in Ikeda-Kawakatsu et al. (2009) was considered to have occurred because of the somehow constitutive expression of *APO1*, not only in the constitutive *APO1* overexpressing transgenic plants but also in the *apo1-D1* mutant. Instead, it is suggested that *APO1* expression in the panicle primordia and vascular bundle primordia are regulated independently, and this consequently caused the different development of PRBs and vascular bundles.

Published research has established that, in japonica-type cultivars, each of the large vascular bundles is connected to a primary rachis branch. However, in indica-type cultivars (e.g., Habataki types), the number of large vascular bundles in the peduncle is much higher than the number of PRBs; therefore, some of the large vascular bundles are directly connected to the secondary rachis branches (Fukushima and Akita 1997). Increased numbers of large vascular bundles without accompanying increases in the number of PRBs result in more large vascular bundles that are directly connected to secondary rachis branches. Alternatively, having too few vascular bundles for a given number of PRBs may cause the branching of vascular bundles, as they struggle to connect to two PRBs. This may reduce translocation efficiency.

If the expression site, duration, and strength of the *APO1* gene differed while the expressed gene remained the same, and expression still resulted in the formation of vascular bundle systems, one would expect that the expression strength and site should differ between Habataki and Sasanishiki plants. We therefore investigated the expression of the *APO1* gene at the panicle initiation stage. We observed a higher expression of *APO1* in Habataki plants compared to Sasanishiki plants throughout panicle development (Fig. 5). Furthermore, *APO1* was expressed not only in the panicle, but also in the region beneath the developing panicle (Fig. 6). In this region, only the vegetative stem and leaf sheath was included, but this region is also the place where vascular bundles develop. The expression of *APO1* in the developing vascular bundles was also confirmed using in situ hybridization (Figs. 9, 10). Moreover, *APO1* expression in this region beneath the

panicle was kept higher in Habataki plants than in Sasanishiki plants in the later developing stage. This might be consistent with the higher number of vascular bundles developed in this region in Habataki plants. Therefore, it appears that higher *APO1* expression is necessary to increase the number of vascular bundles that develop in the peduncle, which may improve translocation efficiency as well as harvest index.

Effects of gene structure on panicle formation

The number of PRBs was regulated by the *PRB1* allele, which includes the ORF and promoter region up to −2,686 bp. Several differences were observed between Habataki and Sasanishiki alleles in this region. One difference that may be of functional significance is a substitution of valine (in Habataki type) for isoleucine (in Nipponbare and Sasanishiki types) in the F-box protein motif, which may bind substrates for ubiquitin-mediated proteolysis. This variation may produce different numbers of PRBs. However, this switch may not affect the number of PRBs because both valine and isoleucine were observed in several dicot *UFO* homologs. Ikeda et al. (2007) suggested that the F-box motif in *UFO* homologs carrying *APO1* is nonfunctional because of the lack of a protein–protein interaction motif at the C-terminal.

Furthermore, the region containing the ORF varied more between Sasanishiki and Nipponbare plants, which suggests the possibility of functional differentiation among japonica cultivars. These differences in *PRB1* are considered to be non-critical for the fundamental function of this gene because both of the expressed genes result in the development of normal panicles. However, the number of PRBs was ∼10% higher in the Habataki genotype.

There must be some variations in the distal promoter region beyond −2,827 bp that differentially affect the number of spikelets per panicle as well as the harvest index. One of the candidate sources of this variation is the CArG box. Durfee et al. (2003) suggested that the CArG box might be the recognition site for the MADS box DNA-binding protein. There are five CArGCW8AT motifs in the Nipponbare and Sasanishiki rice cultivar, at −720, −1,122, −2,707, −3,118, and −4,273 in the promoter region of *APO1*. The Habataki plants lack a CArGCW8AT motif at −3,118 bp, which was instead included in the *HI1* allele. Ikeda et al. (2007) showed that a version of the *APO1* gene with a 5-kb promoter region rescued the *apo-1* mutant, while the version with a 2,924-bp promoter region did not. This CArG motif at −3,126 bp was placed within the functional promoter region. Therefore, this CArG motif may regulate *APO1* gene expression and increase the number of spikelets per panicle, and the harvest index. This CArG motif may act as a repressor or negatively regulate *APO1*

gene expression because the lack of this motif in Habataki plants increased the expression of *APO1*. When Ikeda et al. (2007) introduced *APO1* with a 2,924-bp promoter and longer 3′ flanking region to mutant plants, these plants overexpressed the *APO1* gene and displayed abnormal shaped panicles with a higher numbers of spikelets per panicle. These authors hypothesized that there is a repressor region in the 3′ flanking region. However, it is also possible that the repressor is located in the promoter region, between −2,924 and −5,000 bp. Ikeda-Kawakatsu et al. (2009) showed that insertion of *nDart1-0* 3.5 kb upstream region of the *APO1* enhance *APO1* expression, which supports the regulatory role of distal promoter region of this gene.

Higher expression of *APO1* in Habataki increased grain number

APO1 mRNA expression in the young developing panicle was about ten times higher in Habataki plants as in Sasanishiki plants, at the peak expressing stage. Also, the RNAi-*APO1* reduced both the number of PRBs and the number of grains per panicle. Thus, higher *APO1* expression in Habataki may increase the number of PRBs and the number of grains per panicle. However, Ikeda et al. (2007) showed that the overexpression of *APO1*, produced by the introduction of the 3-kb promoter *APO1* gene fragment into *apo1* mutants, resulted in the development of abnormal panicles that had too many PRBs and sterile spikelets. They consider that a moderate increase of *APO1* expression is necessary to increase the panicle size in the mutants as well as the transformed plants although expression levels were not quantified (Ikeda-Kawakatsu et al. 2009). It is not clear whether this ten times higher *APO1* expression in Habataki plants to be called moderate; this level of increase in *APO1* expression observed in Habataki plants in concert with non-constitutive expression may increase the number of grains per panicle while keeping normal panicle architecture.

Conclusions

The *APO1* gene is responsible for the increase of the number of PRBs, the harvest index, and the number of grains per panicle, thereby increasing grain yield. It also controls not only the development of panicle primordia but also the development of vascular bundle systems. Vascular bundle system development was regulated by the *HI1* allele, which may correspond to the distal promoter region of the *APO1* gene. This effect on developing vascular bundle systems increased grain yield through the increase of harvest index, with only a limited reduction in grain quality or ripening percentage.

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