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Original Paper

Genetic studies of transgenic rice plants overproducing an antibacterial peptide show that a high level of transgene expression did not cause inferior effects on host plants

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Abstract Overexpression of an oat thionin gene (*Asthi1*) confers enhanced disease resistance of rice (a cultivar "Chiyohonami") against seed-transmitted phytopathogenic bacteria. The isolated paddy field test for evaluation of agronomic traits using T4 transgenic rice plants which are homozygous with a high level of Asthi1 protein has shown that transgenic rice possesses, in addition to enhanced anti-bacterial resistance, slight different characteristics compared with the original cultivar such as slight decrease in plant height, grain yield and simultaneity in heading behavior in addition to 2 days earlier in heading date. To address whether this difference was caused by ectopic high level expression of the transgene, T4 plants were back-crossed with wild-type and selfed, and the progenies were analyzed. Almost all characteristics observed in the field were found to be genetically segregated. While only a limited number of plants were used, the present findings indicate that the altered characteristics found in transgenic plants were not attributable to integration of *Asthi1* transgene or its high expression itself, but instead to possible spontaneous mutations which may have occurred during *in vitro* culture for *Agrobacterium*-mediated transformation.

Key words: Back-cross, ectopic expression, inheritance, oat thionin gene, transgenic rice plant.

Many kinds of transgenic plants with useful foreign genes have been generated so far, and a large number of genetically engineered plants such as herbicide resistant plants (Owen and Zelaya 2005) and insect resistant plants (Bates et al. 2005) are practically used for agricultural production. To obtain the plants with a high level of transgene expression for useful characteristics, strong promoters have been preferentially used. However, a high level of ectopic foreign gene expression is thought to cause inferior effects such as disturbance of basal metabolism and altered expression of endogenous genes similar to transgene in host plants. Because the information on the effect of high level of transgene expression is limited, studies on the altered characteristics in transgenic plant and its genetic inheritance are absolutely needed.

Using *Agrobacterium*-mediated transformation method, we have previously generated hygromycin-resistant 41 individual transgenic rice plants (T0) in which an oat cell wall-bound thionin gene (*Asthi1*) was expressed under the control of a strong promoter E7ΩI: seven tandem repeats of the 0.2 kb *Califlower mosaic virus* (CaMV) 35S enhancer (E7), the omega sequence from *Tobacco mosaic virus* and the 1st intron of phaseolin gene in pE7133 (Iwai et al. 2002). The promoter conferred 70-fold higher expression compared with the *35S* promoter in pBI221 in rice protoplasts (Mitsuhara et al. 1996). In all T0 plants, high levels of *Asthi1* transcripts were found by RNA gel blot analysis. Among the progenies prepared by self-pollination of 41 T0 plants, the number of the plants with both hygomycin-resistance gene (*hph*) and high levels of Asthi1 protein (20～100 μg/g fresh leaf) appeared to be 19, and plants lacking both hygromycin resistance (Hyg^r) and accumulation of Asthi1 were numbered to be 22, indicating the introduced *Asthi1* and *hph* genes were silenced in more than a half of T1 plants. Among the 19 T1 plants, three plants were selected as Asthi1 overproducers with a single copy of transgene as T2. T3 plants which were prepared by self-pollination exhibited enhanced resistance to seed-transmitted bacterial diseases such as *Burkholderia* (*B.*) *plantarii* and *B.*

Abbreviations: CaMV; *Califlower mosaic virus*, Asthil; oat thionin
This article can be found at http://www.jspcmb.jp/

glumae in the experiments under laboratory growth chamber and isolated green house conditions (Iwai et al. 2002). One of T3 plants (designated CT2-1-4-6, homozygous) was selected, and its seeds (T4) were sowed and grown in an isolated paddy field to evaluate agronomic traits and environmental risk (Yatou et al. 2003). In the field, we found the T4 plants (progenies of CT2-1-4-6) exhibited almost similar phenotypes compared with wild-type cv. Chiyomonami. However several characteristics differed from those of the original cultivar were found such as 2.9 cm lower in plant height, 10% lower in grain yield, 2 days earlier in heading date, decrease in simultaneousness in heading and fine necrosis of leaf tip in later ripening period.

In this study, we therefore used the line CT2 and its progenies and addressed whether ectopic foreign gene expression at high level causes inferior effects on the host plant. Altered phenotypes of back-crossed and selfed progenies of T4 plants were analyzed. We indicate here that the possible reason of the different characters was not induced by integration and expression of transgene, but by spontaneous mutations during tissue culture for transformation of plants.

Materials and methods

Rice materials

The progenies of the later generations (T1 to T4) were prepared by successive selfing of T0 plants of the transgenic rice plant (*Oryza sativa* cv. Chiyohonami) line CT2, which expressed *Asthi1* (an oat thionin gene) and *hph* (a hygromycn resistance gene) by a strong constitutive promoter E7ΩI and by improved CaMV *35S* promoter, respectively (Mochizuki et al. 1999; Iwai et al. 2002). The seedlings of T4, homozygous on *Asthi1* and *hph*, were grown in isolated paddy field in 2001 at Joetsu city, Japan (Yatou et al. 2003) and selfed to obtain T5. T4 plants separately grown in an isolated green house were back-crossed with wild-type plants to obtain back-crossed seeds (BC; wild-type×T4, and RBC; T4×wild-type), respectively. On April 27 in the next year 2002, these seeds (T5, BC0 and RBC0) were sowed and grown in an isolated green house (20–30°C) in Tsukuba city, Japan. The resulting seeds [T6, BC1 (B-1 to B-5)] prepared by selfing were used for further analysis. On May 2nd in 2003, 24 selfed seeds (B-1-1 to -24) originated from B-1 plant were sowed and grown in the green house to study segregation of the altered characteristics.

Analysis of hygromycin-resistance

Rice seeds were sowed on the agar-medium containing 50 μg ml^{-1} hygromycin B (Sigma Co. USA). Green seedlings 4 to 6 cm in height and those close to1.5 cm in height were counted at 2 weeks after imbibition. They were considered to be hygromycin-resistant and semi-resistant, respectively. Non-transformed wild-type of this age under the same conditions was faded green and smaller than 1.0 cm.

Detection of transgene products

Total proteins were extracted from leaves and precipitated with acetone, and separated by SDS-PAGE according to Epple et al. (1995), after which western blot was performed using anti-Asthi1 polyclonal antibodies with synthesized Asthi1 as the standard to quantify the Asthi1 protein according to Iwai et al. (2002).

Analysis of inserted site of transgene

Genomic DNA was prepared from a T5 transgenic rice plant by Isoplant DNA extraction kit (Nippon Gene Co. Japan) according to manufacture's instructions. To amplify adjacent region of rice genome and the left border of inserted T-DNA, genomic DNA was digested by *Eco*RI, extracted by phenol, self-ligated and used as template for the inverse PCR (Jonathan S 1991). First inverse PCR was preformed by using primers 5′-cttgctgcaactctctcagg-3′ and 5′-tttcccagtcacgacgttgt-3′, then aliquot of the PCR product was used as the template for second nested PCR by using primers 5′-gcggtgaagggcaatcagc-3′ and 5′-gcgattaagttgggtaacgc-3′. The PCR product was cloned into a plasmid vector and sequenced by ABI310 capillary sequencer (Applied Biosystems). The sequence was analyzed by Rice BLAST (Rice Genome Research Program, http://riceblast.dna.affrc.go.jp/). Adjacent region of rice genome and the left border of T-DNA were amplified using genomic DNA as a template and primers 5′-cactctacatgctttgcaaatc -3′ and 5′-cgctcatgatcagattgtcg-3′. All PCR was performed by ExTaq DNA polymerase (Takara Co. Japan) according to manufacture's instructions.

Result

Inheritance of foreign gene expression in the progenies of transgenic CT2 rice plant

Figure 1 depicts the inheritance of Asthi1 protein production and hygromycin resistance in T1 to T6 generation of transgenic rice line CT-2. Plants of T2 to T4 generation contained over 100 μg Asthil per g fresh leaf, but level of this protein appeared to decrease in later generations. T5 plants showed 5 μg or lower Asthil per g fresh leaf, and T6 plants showed even lower or no detectable level of the protein (Figure 1A). This result was confirmed by separate experiments; the Asthil protein levels were varied but far lower than 10 μg protein per g fresh leaf in almost all T5 plants tested (Figure 1B).

The change of hygromycin resistance in these

A

Expression of transgene in the progenies of CT2

Generation of tested plant	Parent plant	Level of Asthi1	Hyg^r
T1	CT2 (T0)	++	2/3
T2	CT2-1 (T1)	++	26/30
T3	CT2-1-4 (T2)	++	27/30
T4	CT2-1-4-6 (T3)	++	30/30
T5	CT2-1-4-6-1 (T4)	±	25*/30
T6	CT2-1-4-6-A-1 (T5)	± or -	25*/30

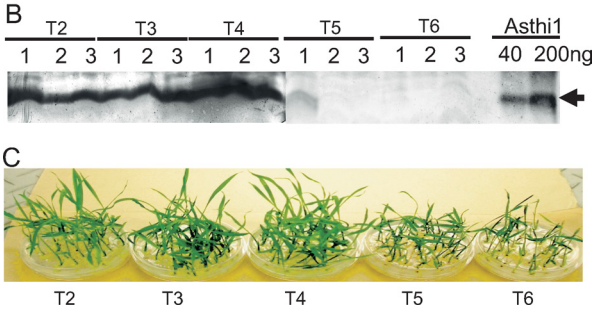

Figure 1. Inheritance of transgene expression in the progenies of CT2. A, Asthi1 protein level and hygromycin resistance. Five to six individuals for each generation was analyzed for Asthi1 protein. ++: over 100 μg Asthil/g fresh leaf, ±: ca. 5 μg Asthil/g fresh leaf, —: not detectable. A 30 seeds were tested for hygromycin resistance. Numbers with or without asterisks represent hygromycin resistant and semi-resistant plants, respectively. B, Immunoblot analysis on Asthi1 in the upper leaf of 14 day-old rice seedling. Each lane contained protein equivalent to one mg fresh leaves taken from three different individuals of T2 to T6 generations. Rightmost two lanes correspond to 40 and 200 ng synthesized Asthi1 protein. C, Phenotypes of plants of T2 to T6 generations in the agar medium containing hygromycin. Two weeks after imbibition. The upper cover of plastic dish was removed and photographed. See text for details.

A

Characteristics of the back-crossed progenies of CT2-1-4-6-A (T4) lines

Plant	Hyg^r	Level of Asthi1		Plant height	Heading date	Total weight of panicle	Delayed heading	Hyg^r in the next progeny
		Juvenile	Adult					
WT - 1	-	-	nd	110 cm	8/2	144 g	-	-
- 2	-	-	nd	110	8/2	135	-	nd
- 3	-	-	nd	110	8/2	123	-	nd
BC0: B-1	nd	++	++	113	8/4	134	-	24/29
B-2	nd	++	++	110	8/4	120	-	22/28
B-3	nd	+	±	110	8/2	133	-	4*/26
B-4	nd	++	++	115	8/2	114	-	26/29
B-5	nd	++	nd	nd	nd	nd	nd	nd
RBC0: RB-1,2,3,4,5		++	nd	nd	nd	nd	nd	nd
T5:								
CT2-1-4-6-A-1	+	±	-	108	8/2	148	+	22*/23
-2	+	±	-	110	8/3	141	+	17*/21
-3	+	+	-	110	8/2	137	+	22*/24

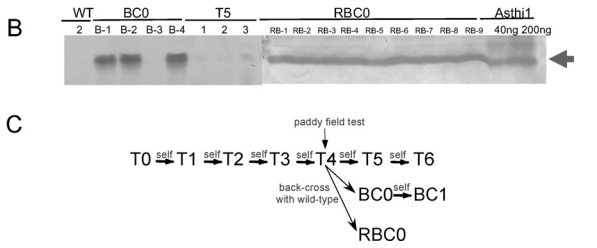

Figure 2. Characteristics of back-crossed progenies of T4 (CT2-1-4-6-A). A, BC0: back-crossed plants (wild-type×T4), RBC0: reciprocally back-crossed plants (T4×wild-type). Juvenile: young plant (14 day-old), Adult: adult plant (3.0–3.5 month-old). ++: over 100 μg Asthil/g fresh leaf, +: 5<, ≥100 μg Asthil/g fresh leaf, ±: ca 5 μg Asthil/g fresh leaf. —: not detectable. nd: not determined. The letter A in the line number (e.g., CT2-1-4-6-A-1) means that the sample does not represent individuals from a line but may represent individuals from different lines. See legend of Figure 1 for asterisked numbers. B, Immunoblot analysis on Asthi1 in the 4th leaf of rice seeding. Each lane contained protein equivalent to one mg fresh leaves taken from three different individuals. Rightmost two lanes correspond to 40 and 200 ng synthesized Asthi1 protein. C, Schematic representation of pedigree of CT2 transgenic and its back-crossed line. Paddy field test was done using T4 (Yatou et al. 2003).

generations was somewhat resembling to that of Asthil production (Figure 1C). T2 to T4 seedlings grown on the agar medium containing 50 μg ml^{-1} hygromycin for 2 weeks after imbibition were 4 to 6 cm high and green (hygromycin resistant), while that in T5 and T6 seedlings were about 1.5 cm high and green. Under the same conditions, the wild-type was lower than 1.0 cm and faded green. Therefore, it is concluded that T5 and T6 generations keep decreased but distinct level of hygromycin resistance (hygromycin semi-resistant).

Back-crossing with wild-type recovered a high level of Asthi1 protein expression

Generally, phenotypes of CT2 (T0) and its progenies (T1 to T3) were likely similar to those of wild-type plants when limited number of plants were examined. However, through inspection on a paddy field test on 2001 showed that T4 plants exhibited several characteristics different from the original wild-type cultivar Chiyohonami such as 2.9 cm lower in plant height, 10% lower in grain yield, 2 days earlier in heading date, decrease in simultaneousness in heading and fine necrosis of leaf tip in later ripening period. To address whether the altered characteristics found in T4 generation could be segregated from *Asthi1* transgene by back-crossing in the later generations, plants of back-crossed lines (BC0 and RBC0 generations) were first inspected in comparison with those of wild-type and T5 plants. The seeds were sowed and grown in an isolated green house as described in **Materials and methods**. Results are summarized in Figure 2.

The level of hygromycin resistance of three independent back-cross lines such as B-1, -2 and -4 (BC0 generation) was high but that of B-3 and T5 lines was low (shown in the rightmost column in Figure 2A). The level of Asthi1 was extremely high in the three back-cross lines, B-1, -2 and -4 lines and all RB plants (RBC0 generation) tested, but it was low in B-3 and T5 lines (Figure 2B). The result of the reduced level of Asthi1 in T5 plants agrees with that of Figure 1. The fact that the levels of both proteins derived from the introduced genes (*Asthi1* and *hph*) were high in B-1, -2 and -4 plants but were low in B-3 and T5 plants indicates that gene silencing of both genes may have occurred in a nearly synchronous manner in these plants.

The B-1, -2, -3, -4 and -5 seeds were sowed on April 27, 2002 and grown in an isolated green house. The

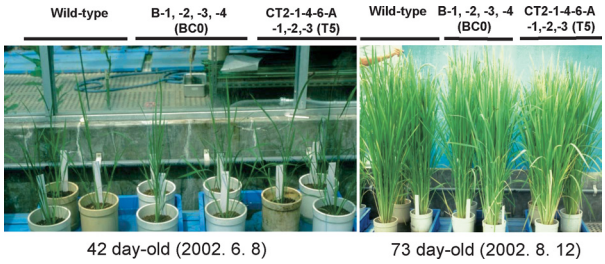

Figure 3. Phenotypes of BC0, T5 and wild-type plants grown in an isolated green house. Left; 42 day-old plants, and right; 73 day-old plants.

seedling grew normally and exhibited almost similar phenotypes compared to those of wild-type and the T5 plants as depicted in Figure 3. The phenotype "fine necrosis of leaf tip in later ripening period", which was found in T4 plants in the paddy field, was not detected in B-1 to B-5 (BC0) and T5 plants (Figure 3). The 30 selfed seeds (BC1) originated from B-1 were directly sowed in soil on May 2nd, 2003, and 24 among 30 seedlings were grown in an isolated green house for further analysis.

Some plants in the next generation of B-1 maintained the high level of Asthi1 and exhibited normal phenotype

The phenotypes of the 24 lines of BC1 plants (B-1-1 to -24) were compared with those of wild-type and T5 plants. Data are compiled in Figure 4, and the typical pictures of these plants are depicted in Figure 5. Generally, the phenotypes in the isolated green house were almost similar to those of wild-type and also to T5 plants, yet there were slight but distinct differences among these plants. Since B-1 plant is of BC0 generation, B-1 should be heterozygous on transgene. Theoretically, BC-1 plants obtained by selfing B-1 must segregate to null, heterozygous and homozygous genotype.

The content of Asthi1

Of the 24 BC1 plants, 19 of them constitutively overproduced Asthi1 protein at high level very similar to that observed in T2 to T4 generation and in BC0 plants in all growth stages tested. The remaining 5 plants showed much reduced (B-1-19 and -21) or non-detectable (B-1-1, -2 and -23) level of Asthil protein even 27 days after sowing (a young stage) as depicted in Figure 4B. Asthi1 protein level in B-1-19 and -21 was further decreased at 42 and 66 days, indicating proceeding of transgene silencing. Judged from the level of Asthi1 in the progenies and segregation rate of hygromycin resistance (see below), B-1, -2 and -23 were considered to contain no transgene. Absence of *Asthi1* gene in B-1, -2 and -23 was confirmed by genomic PCR (data not shown).

A

generation	Plant	Level of AsThi1	Hyg^r in the next progeny	Genotype	Height (cm)	Heading date	Heading type	Total weight of panicles (g)	Grain yield (g)
WT	WT-1	–	0/30		110	8/13	W	112.0	
	WT-2	–			107	8/14	W	111.6	
	WT-3	–			105	8/13	W	117.2	
	WT-4	–			113	8/13	W	103.9	20.4
	WT-5	–			114	8/13	W	130.1	21.0
	Mean of WT				110±3.8			115±9.7	
BC1	B-1-5	++	30/30	+/+	115	8/11	D	117.0	21.4
	B-1-6	++	30/30	+/+	115	8/12	4	115.8	21.0
	B-1-8	++	30/30	+/+	109	8/11	1	110.8	
	B-1-17	++	30/30	+/+	110	8/13	W	#	
	B-1-22	++	30/30	+/+	107	8/15	2	#	
	B-1-24	++	30/30	+/+	108	8/17	D	#	
	B-1-3	++	29/29	+/+	115	8/11	1	116.9	
	B-1-20	++	29/29	+/+	110	8/12	3	#	
	B-1-16	++	29/30	+/+	110	8/11	4	101.2	
	B-1-15	++	29/29	+/+	108	8/11	1	101.2	
	B-1-12	++	30/30	+/+	115	8/11	4	113.3	20.8
	B-1-7	++	23/30	+/–	108	8/11	D	#	
	B-1-9	++	23/30	+/–	110	8/11	1	115.1	21.0
	B-1-11	++	23/30	+/–	113	8/11	D	118.0	
	B-1-10	++	21/30	+/–	113	8/11	4	120.5	
	B-1-13	++	21/30	+/–	110	8/12	D	#	
	B-1-18	++	21/30	+/–	113	8/12	4	#	
	B-1-4	++	20/30	+/–	110	8/11	1	100.3	
	B-1-14	++	20/30	+/–	110	8/12	D	112.7	20.8
	B-1-21	±	14±/30	+/?	105	8/11	3	#	
	B-1-19	±		+/?	108	8/14	D	#	
	B-1-1	–	0/30	–/–	110	8/12	D	95.2	20.6
	B-1-2	–	0/30	–/–	113	8/12	4	111.4	21.0
	B-1-23	–		–/–	110	8/12	D	#	
	Mean of BC1				110±2.8			111±7.9	20.9±0.25
T5	CT2-1-4-6-A-1	+	22±/30	+/+	106	8/11	1	102.3	20.6
	CT2-1-4-6-A-2	±	22±/30	+/+	110	8/15	2	105.0	19.6
	CT2-1-4-6-A-3	±	16±/30	+/+	105	8/12	3	83.1	20.7
	CT2-1-4-6-A-4	+	25±/30	+/+	105	8/12	4	103.9	20.4
	CT2-1-4-6-A-5	±	25±/30	+/+	105	8/11	1	102.9	21.9
	Mean of T5				106±2.2			99.4±9.2	20.6±0.83
					≦106	≦8/12	D, 1, 2	≦105	

B

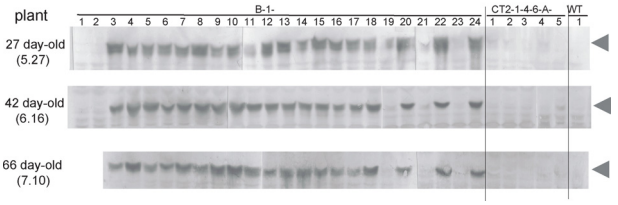

Figure 4. Characteristics of BC1 plants as compared with those of wild-type (WT) and T5 plants. A, see the legend of Figure 1 for levels of Asthi1 and Hyg^r. Height of 3.5 month-old plants was determined. Type of heading day in each plants was classified into 5 types (see text and also Figure 6). The types different from those were indicated as D. Red numbers represent the values distinct from the standard values of wild-type shown in blue numbers. B, Immunoblot analysis for Asthi1 in the upper fully developed leaf of 27 to 66 day-old plants. Each lane contained protein equivalent to one mg fresh leaves. Twenty four progeny plants of BC1 (BC1-1 to -24), and five independent T5 plants, and five independent wild-type plants were analyzed. #; Plants were in a water-deficient condition in the last ripening period after heading, and their respect values were not listed.

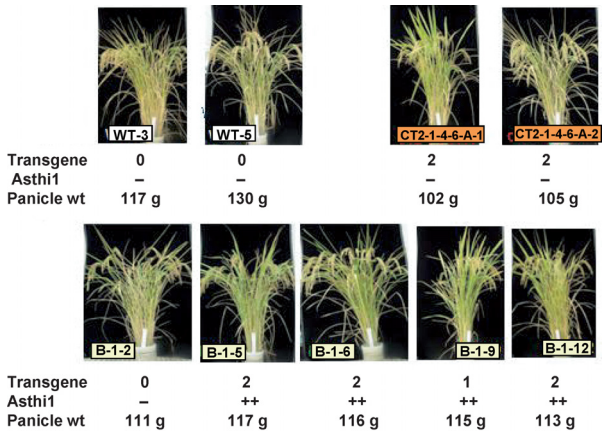

Figure 5. Phenotypes of 4 month-old BC1 plants (bottom) as compared with those of wild-type (top left) and T5 (top right) plants. Transgene; number of transgene copies in the genome, Asthi 1; content of Asthi1, and Panicle wt; total weight of panicles in a plant.

Hygromycin resistance

Judged from the segregation ratio of hygromycin resistance (4th column of Figure 4A), 11 and 8 of the 24 plants were concluded to have homozygous and heterozygous genotype of the transgene respectively. B-1-21 showed abnormal behavior in hygromycin resistance and segregation ratio; 14 and 16 of 30 plants tested were semi-resistant and resistant, respectively. Silencing of hygromycin resisntace gene would be a possible reason.

Total weight of panicles

Total weight of panicles in a plant in wild-type plants was 115±9.7 g, while that of T5 plants was 99.4±9.2 g (Figure 4A). Values distant from mean value below standard deviation of wild-type plants were designated minimum standard value (105.3 g) and values lower than this value were shown in red in Figure 4A. Four lines, B-1-1, -4, -15 and -16, exhibited total weight of panicles lower than the standard value. The genotype of them was null, heterozygous, homozygous and homozygous, respectively. The Asthi1 protein level was null, high, high and high, respectively. These results indicate that genotype of transgene or expression level of *Asthi1* transgene did not directly correlate with the yield of total panicles.

Plant height

Height of 3.5 month-old plants was 110±3.8 cm in wild-type plants while that of T5 plants was 106±2.2 cm. Plant height values of BC1 plants that were lower than the minimum standard value of height (106.2 cm) were shown in red in Figure 4A. Regardless of *Asthi1* genotype nor its expression, the plant height of all BC1 plants but B-1-21 was normal or greater than the minimal standard value. Because different genotypes and different levels of Asthi1 protein are included in the 24 plants, the result indicates altered height of plant is not attributable to with the presence or expression, or both, of *Asthi1* transgene.

Date and behavior of heading

The heading date, which is defined as the day of first heading, of T5 plants was slightly different from that of wild-type. It was August 13 in four out of five wild-type plants, but that of four T5 plants was August 11 or 12. When August 13 is designated the standard date in wild-type, 20 of the 24 showed heading date earlier than Aug 12 as shown in red in Figure 4A. Clearly, these 20 plants are different in *Asthi1* transgene genotype and its expression. These results indicate phenotype "slightly earlier heading day" does not correlate with presence or expression, or both, of *Asthi1* transgene.

The heading behavior was further investigated by counting the number of tillers with heading panicle in a plant, and the results are shown in Figure 6. All wild-type plants exhibited similar heading profiles, but the simultaneity in heading behavior of T5 was varied by individuals.

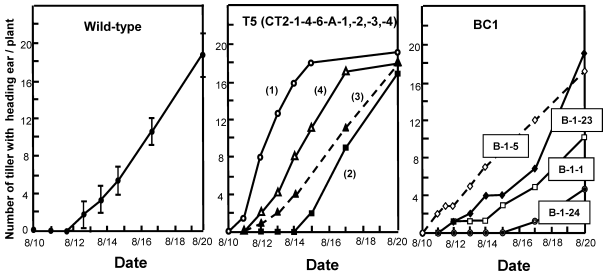

Figure 6. Comparison of the heading behaviors among wild-type (left), T5 (center) and BC1 (right) plants. The number of tiller with heading panicle was counted on August 10 to 20, 2003. Data represent mean of five independent wild-type plants±S.D. In T5, (1); CT2-1-4-6-A-1, (2); CT2-1-4-6-A-2, (3); CT2-1-4-6-A-3, (4); CT2-1-4-6-A-4.

The heading behavior of T5 was classified into 4 types; type 1 (CT2-1-4-6-A-1), type-2 (CT2-1-4-6-A-2), type 3 (CT2-1-4-6-A-3), and type 4 (CT2-1-4-6-A-4). The CT2-1-4-6-A-5 also showed type 1 behavior (data not shown). The heading behavior of BC1 plants (B-1-1 to -24) was classified into 6 types; type W resembling that of the wild-type, type 1 to 4 resembling respective type of T5, and type D which does not resemble any of the type W or type 1 to 4. B-1-17 belonged to type W. B-1-3, -4, -8, -9 and -15 were grouped in type 1, B-1-22 was in type 2, B-1-20 and -21 were in type 3, and B-1-2, -6, -10, -12, -16 and -18 were in type 4 (see 8th column in Figure 4A). The remaining 9 of the 24 were grouped in type D. Four type D lines (B-1-1, -5, -23 and -24) are depicted in Figure 6. The simultaneity of heading behavior of B-1-1 and B-1-24 (both belong to type D) appeared to be very low. Types D, 1, and 2, which are distinct from type W, were regarded as altered types, and they were shown in red in Figure 4A. Clearly, these BC1 plants include plants with various *Asthi1* genotype and various levels of Asthi1 protein. From these results, it was suggested that difference in the simultaneity in heading behavior does not correlated to the presence of *Asthi1* transgene or its expression, or both.

Others

The phenotype "fine necrosis of leaf tip in later ripening period", which was found in T4 plants at the paddy field in 2001, was not clearly found under the green house conditions in BC1 plants in 2003, as well as in BC0 plants (B-1 to B-4) in 2002.

The results described above showed the altered characteristics found in T4 plants, such as slight decrease in plant height, slightly reduced total weight of panicles and less simultaneous heading profile in addition to 2 days earlier heading, were not co-segregated with *Asthi1* and *hph* transgenes in BC1 plants. χ^2 test of "plant

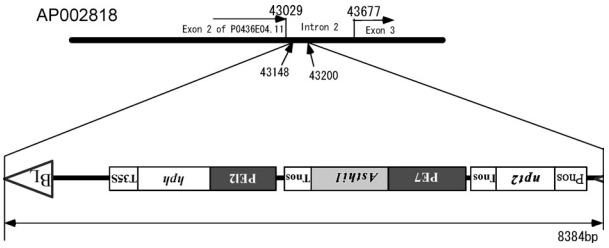

Figure 7. Schematic diagram of integration site of transgene. Exon2, intron2, exon3 of P0436E04.11 are shown. BL; left border of T-DNA of *Agrobacterium* Ti plasmid, Pnos; the 5′-upstream sequence of *Agrobacterium* nopalin synthase gene, *npt2*; coding sequence of the neomycin phosphtransferase II gene, Tnos; the 3′-terminator sequence of *Agrobacterium* nopaline synthase gene, PE7; the promoter of artificial promoter cassette pE7133 (Mitsuhara et al. 1996), *Asthi1*; coding region of *Asthi1* gene (Iwai et al.), PEl2; the promoter of artificial promoter cassette pE2113 (Mitsuhara et al. 1996), *hph*; hygromycin phosphotransferase gene, T35S; 3′-terminator sequence of CaMV 35S transcript.

height" and "total weight of panicles" supports this interpretation. Further, it was suggested that such different phenotypes themselves can be segregated each other (Figure 4A). Conversely, the integration and/or expression of transgene cannot account for such altered phenotypes. But, instead, possible mutations at different loci in the genome during transformation steps may cause such alterations.

The Asthi1 gene was inserted in the upper arm of chromosome 1

To analyze the integration site of the *Asthi1* fusion gene in rice genome, genomic DNA was prepared from a T5 seedling, and sequences adjacent to the left border of T-DNA integration was amplified by the inverse PCR. After subcloning, the PCR product was sequenced. The sequence contains the left border of the T-DNA with 4 nucleotide deletion and a rice genomic sequence which corresponds to 42985–43147 of PAC clone AP002818 at 0.3 cM of chromosome 1. Then, sequence adjacent to the right border of transgene was amplified using the primers complement to T-DNA sequence next to the right border and rice genomic sequence which may exist opposite side of the integration, and the PCR product was cloned and sequenced. These results indicated almost complete T-DNA region was integrated into the 2nd intron of P0436E04.11 gene with about 55 nt deletion in inverse orientation relative to the transcript (Figure 7). The P0436E04.11 corresponds to full length cDNA clone AK069975 which encodes a protein with unknown function. No locus affecting flowering time has been found around P0436E04.11 gene based on the search using Gramene database (http://acorn.cshl.org/qtl/help.html) and a recent report on flowering time (Nakagawa et al. 2005).

Discussion

Using a model rice plant line which overproduces an extremely high level of transgene product, we indicate here that accumulation of foreign Asthi1 protein at a high level did not induce any undesirable characteristics in the host plants. By back-crossing and subsequent selfing, several altered characteristics observed in T4 generation in paddy field (Yatou et al. 2003) segregated each other in the progenies. This segregation pattern did not correlate to that of the *Asthi1* transgene genotype or expression of the transgene. Those altered characters might be already included in the original transgenic rice plant CT2 by possible genetic and/or epigenetic mutations which may have been induced during tissue culture for transformation. By the stresses during *in vitro* culture of cells, somaclonal variation is known to be induced (Bajaj YPS 1990, Kaeppler et al. 2000), accompanying hyper-methylation of DNA (Schmitt et al. 1997), activation of transposons (Hirochika 1993) and genome rearrangements (Benzion et al. 1986). Our findings presented here provide a line of evidence that back-crossing is a promising way to exclude such unexpected mutations. The results on the inheritance of *Asthi1* and *hph* expression suggested that back-crossing suppresses progression of transgene silencing. Because the experiments were done using a limited number of plants, the data presented here are preliminary. However, we hope that the evidences provide here would help through understanding the effects of transgene expression in plant characteristics.

The foreign gene product Asthi1 protein is a basic protein with pI 9.13 which is constitutively produced in oat coleoptile and its overproduction in rice induced clear resistance to soil-born bacterial infection (Iwai et al. 2002). We found that the protein is secreted into the apoplast and tightly binds cell wall, and treatment with 1M NaCl is necessary for extraction of Asthi1 from leaf tissue of transgenic rice plant. Growing body of evidence indicates that overproduced Asthi1 protein is localized in cell walls as a wall-bound form but not as a free form (Iwai et al. 2002). Such characteristics of Asthi1 would contribute on normal phenotypes of transgenic rice in which such a high level of Asthi1 is produced.

From the analysis of transgene expression, silencing of transgene was found to be induced with generations. Both *Asthi1* accumulation and hygromycin resistance were greatly suppressed in T5 and the later generations. The mechanism of transgene silencing in this system is interesting, and remains to be analyzed further in future studies.

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