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Research Paper

Pyramiding and evaluation of both a foreign *Bacillus thuringiensis* and a *Lysine-rich protein* gene in the elite *indica* rice 9311

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Gene pyramiding is an efficient approach for the genetic improvement of multiple agronomic traits simultaneously. In this study, we pyramided two foreign genes, *cry1Ac* driven by the rice *Actin I* promoter, and *lysine-rich protein* (*LRP*), driven by the endosperm-specific *GLUTELIN1* (*GT1*) promoter, into the elite *indica* cultivar 9311. These two genes were chosen in an attempt to enhance insect-resistance and Lysine (Lys) content. In the pyramided line, the foreign gene *cry1Ac* was efficiently expressed in the leaves and stems, and exhibited highly efficient resistance to striped stem borer (SSB, *Chilo suppressalis* Walker) in the laboratory and rice leaf folder (RLF, *Cnaphalocrocis medinalis* Guenee) in the field. Furthermore, the *LRP* gene was highly expressed in the endosperm and produced a remarkable increase of Lys content in the seeds of the pyramided line. The data from field trials demonstrated that most of the agronomic traits including yield were well maintained in the pyramided line compared to the parental control. These results strongly suggest that the foreign *cry1Ac* and *LRP* genes have remarkable application potential in rice, and the resultant pyramided line serves as an ideal bridge material for the improvement of insect-resistance and high Lys rice in the future.

Key Words: *cry1Ac*, insect resistance, *lysine-rich protein* (*LRP*), high lysine, gene pyramiding, rice (*Oryza sativa* L.).

Introduction

Rice is widely cultivated worldwide, and serves as a significant staple food for more than 50% of the world's population (Sautter *et al.* 2006). However, rice yield suffers considerable damages by Lepidopteran rice pest species in most rice producing areas, especially in recent years (Chen *et al.* 2008). Though the spray of poisonous chemical insecticides has partially decreased loss of yield, it has caused serious contamination to the environment and food products, and has likely resulted in human health problems (Frutos *et al.* 1999, Tu *et al.* 2000). The recent research, which produces a crystal toxin protein against Lepidopteran insects, reports that Bt is an efficient and environmentally friendly biological insecticide against the target insects (Perlak *et al.* 1990). Furthermore, recent advances in plant biotechnology show great progress against the target insects through expression of the Bt protein in plants (Alam *et al.* 1999, Barton *et al.* 1987, Tu *et al.* 2000). The *Bt* genes *cry1Ab*, *cry1Ac*, *cry1C*, and *cry2A* were widely used in rice and showed outstanding performance against the target insects (Chen *et al.* 2005, 2008, Tang *et al.* 2006, Ye *et al.* 2009). In addition, these genes were successfully introduced into elite cultivars with different genetic background by sexual crossing, and the improved lines also exhibited excellent efficacy against the target insects in field evaluations (Liu *et al.* 2015, Riaz *et al.* 2006, Yang *et al.* 2011). Therefore, the expression of foreign *Bt* genes in plants is an efficient method to improve resistance to Lepidopteran insects, and has great value for commercial application.

Rice is a major source of protein for animals. However, the protein in rice has an incomplete amino acid profile because of a deficiency in essential amino acids for humans and livestock (Sautter *et al.* 2006). Lysine (Lys) abundance in rice seeds is at an extremely low level compared with other crops (WHO 2007) and was deemed to be the first limiting essential amino acid in rice. Though many efforts to breed rice with high Lys content by traditional genetic approaches have been made, no desired results have been achieved. With the development of plant biotechnology, a

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genetic engineering approach has been comprehensively applied to improving the Lys content of rice. For example, the expression of a lysine-rich binding protein (BiP), in which Lys accounts for 9.4% of the total amino acids, led to a significant increase of the Lys content in transgenic rice seeds (Kawakatsu *et al.* 2010). Transgenic rice by over-expressing two endogenous rice lysine-rich histone proteins had a 35% Lys increase in rice seeds compared to wild-type, and no obvious unfolded protein response was observed (Wong *et al.* 2015).

Gene pyramiding is an efficient and useful approach to utilize the gene resources for the improvement of crops. To date, the genetic improvement of multiple agronomic traits simultaneously through gene pyramiding has been widely adopted and shows great application potential in staple crop breeding programs. Pyramiding a fused *cry1Ab/1Ac* gene conferring resistance to Lepidopteran insects and a *Xa21* gene providing resistance to bacterial blight disease resulted in desirable target phenotypes in rice, and the pyramiding genes exhibited a yield-stabilizing effect on the recipient line and its hybrids (Jiang *et al.* 2004). In an effort to breed resistance to both bacterial blight and sheath blight diseases, the rice *RC7* and *Xa21* genes were introduced into an elite rice line Minghui 63 by means of marker-assisted selection (MAS), and the resulting pyramided line displayed high resistance levels to both diseases without adverse effects (Datta *et al.* 2002). Ramalingam *et al.* (2002) pyramided the *Xa21* and *wx* genes by MAS into rice, and successfully improved the bacterial blight resistance and the waxiness trait of wild-type. Additionally, no obviously deleterious effects from the two genes were observed in the pyramided line (Ramalingam *et al.* 2002). In another study, a series of agronomic trait-related genes (*sd1*, *Sub1A*, *Pi9*, *Xa21* and *Xa27*) was pyramided into a rice line by MAS breeding scheme (Luo and Yin 2013). The improved rice line not only produced satisfactory agronomic traits but also adapted to different environments and climates compared to the control variety. Additionally, the result demonstrated that pyramiding with multiple genes was feasible and practicable.

In this study, we pyramided two foreign genes, *cry1Ac* and *LRP*, into the elite *indica* rice cultivar 9311. The pyramided line, 9311*LRP/cry1Ac* showed excellent resistance to target insects in both the laboratory and the field, and displayed remarkable increases of Lys content in seeds compared with the parent line 9311. Furthermore, most agronomic traits in the 9311*LRP/cry1Ac* line had no significant differences from the 9311 line. These results indicated that the pyramided rice line has great application potential for the improvement of insect resistance and grain quality in the future.

Materials and Methods

Cultivars

The transgenic rice line, which contains a foreign *LRP* gene (patent number: US6184437), was used as the donor parental material of foreign gene (unpublished results). The elite rice cultivar *Oryza sativa* ssp. *Indica* cv. 9311, from the Agricultural Research Institute of Lixiahe, China, was used as recurrent parent to cross with donor transgenic rice line to generate the 9311*LRP* line. The 9311*cry1Ac* line (Liu *et al.* 2010), kindly supplied by the National Key Laboratory of Genetic Improvement, China, was used as the donor of the *cry1Ac* gene to cross with 9311*LRP* to create the pyramided line 9311*LRP/cry1Ac*.

Insecticidal activity assay in the laboratory and field

To assay insect resistance, the pyramided line with the *cry1Ac* and *LRP* genes was evaluated by artificial infestation using first-instar larvae of striped stem borers (SSB, *Chilo suppressalis* Walker) in the laboratory. The *cry1Ac* donor 9311*cry1Ac* was used as a positive control, and 9311 and 9311*LRP* were used as negative controls. Fresh flag leaves and stems at the late filling stage were collected from the experimental paddy field (Hangzhou 2015) and cut into 5–7 cm long pieces. Three pieces of flag leaves and stems were placed into a culture vessel (90 mm in diameter), and 15 first-instar larvae of SSB were added. Then, the petri dish was sealed tightly with parafilm, and incubated at 28°C in an 80% relative humidity environment. Three independent biological replicates were performed for each treatment. The larval mortality was recorded after incubation for 6 days.

To evaluate insecticidal activity in the field, the previously described rice lines were planted in experimental paddy fields in 2015. No insecticides were applied in the experimental fields during the entire growing season. Insecticidal activity was evaluated through natural infestation using rice leaf folders (RLF, *Cnaphalocrocis medinalis* Guenee). The number of leaves damaged by the RLF was investigated at the tillering stage, when the insect-caused damage had ceased.

Collection of agronomic field traits

Plants were grown in the experimental paddy field for the evaluation of agronomic traits. The parental line 9311 was used as a control. A randomized block design was adopted with three replicates. Each plot consisted of 8 rows with 10 plants per row, with 15.0 cm spacing between plants and 24.0 cm between rows. The middle 6 plants in each row were used for measurements of agronomic traits, including heading date, plant height, number of tillers per plant, number of grains per panicle, self-fertility rate, 1000-grain weight, and single-plant yield.

Molecular identification through MAS

DNA samples were isolated from fresh leaves and prepared using the CTAB method (Murray and Thompson 1980). Two pairs of specific primers were designed for the identification and pyramiding of the *cry1Ac* and *LRP* genes (**Supplemental Table 1**). Amplification by PCR was performed in 20 μl of reaction volume containing 40 ng of

sample DNA, 0.2 µL of 10 µM primers, 1.4 µL of 2 mM dNTPs, 2 µL 10 × Mg^{2+}, and 1.0 U rTaq DNA polymerase (Takara Bio Inc. Japan). The PCR cycle was 94°C for 4 min, then 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 40 sec, followed by 72°C for 10 min. The amplified products were detected by agarose gel electrophoresis (1%, 120 V for 30 min).

Extraction of total RNA and gene expression analysis by qRT-PCR

Total RNA was extracted from the leaves, stems and mature endosperms using the Plant RNA Kit (OMEGA, USA). Each sample consisted of mixed RNA collected from more than 5 plants. First-strand cDNA was generated using the Perfect Real Time Primescript RT reagent (TaKaRa, Japan) in 20 µL of reaction mixture containing 5 µg of total RNA following the manufacturer's instructions. qRT-PCR was performed in triplicate on a Roche LightCycler® 96 system as described (Zhang *et al.* 2012). Data analyses with the 2^{-DDCT} method were performed as previously described (Livak and Schmittgen 2001). The actin gene was used as an internal reference to assay the relative expression levels of the *LRP* gene in the tested lines, and the primers used in the study are listed in **Supplemental Table 1**.

Amino acid analysis

Amino acids were analyzed in seeds harvested at the mature stage. The seeds were ground into powder for amino acid analysis. Each sample consisted of a mixed powder of more than 200 grains. The Acid hydrolysis method was used to treat the samples for the total amino acid assay, which measures the amounts of aspartate (Asp), threonine (Thr), serine (Ser), glutamate (Glu), glycine (Gly), alanine (Ala), cysteine (Cys), valine (Val), methionine (Met), isoleucine (Ile), leucine (Leu), tyrosine (Tyr), phenylalanine (Phe), lysine (Lys), histidine (His), proline (Pro), and arginine (Arg). Approximately 0.2 g seed flour was hydrolyzed in 5 mL, 6 M HCl under nitrogen conditions and then heated at 110°C for 24 hours. The hydrolyzed samples with 5 mL were evaporated in a rotary evaporator at 60°C for 15 min. The residues were dissolved in 10 mL 0.02 M HCl, and the supernatants were filtered with a 0.45 µm Nylon Acrodise filter. Prepared samples were separated into three parts for technical replicates and assayed by the automatic amino acid analyzer L-8900 (Hitachi, Japan).

Quantification assays of the insecticidal Cry1Ac protein

The enzyme-linked immunosorbent assay (ELISA) kit AP003 CRBS (EnviroLogix, Portland) was used for Cry1Ac protein quantification assays. Fresh samples from the leaf, stem and endosperm at the late filling stage were placed in liquid nitrogen and ground into a powder. Approximately 20 mg of powder was suspended in 500 µL of the extraction/dilution buffer, and then diluted to appropriate concentrations (diluted 300 times for leaf tissue and 50 times for stem and endosperm tissue). The assay was carried out following the manufacturer's instructions (EnviroLogix). Optical density values of the samples were measured at the 450 nm wavelength using a multi-mode microplate reader (Synergy H1, USA), and the values were used to calculate the content of Cry1Ac protein.

Results

Breeding of the pyramided line with the cry1Ac and LRP genes

There were two steps for the breeding of the *LRP* and *cry1Ac* pyramided line. The first step was to introduce the *LRP* gene from donor transgenic rice line into recurrent parental 9311 line, via recurrent backcrossing for three generations. Marker-assisted selection was used in each generation for target-gene selection, and the derived line was named 9311*LRP*. The second step was to pyramid the *cry1Ac* and *LRP* genes by crossing the 9311*LRP* line with the 9311*cry1Ac* line followed by two rounds of selfing plus foreground selection by MAS. A homozygous line containing the *cry1Ac* and *LRP* genes was obtained, named 9311*LRP/cry1Ac*, and confirmed by PCR detection (**Fig. 1**).

Cry1Ac protein quantification in pyramided plants

Cry1Ac protein content in fresh leaves, stems at the late filling stage and endosperms at the grain maturing stage was measured in the 9311*cry1Ac* line and the pyramided 9311*LRP/cry1Ac* line. The results showed that the content of Cry1Ac protein varied considerably among different tissues (**Fig. 2**) and that the concentration of Cry1Ac protein was significantly lower in the leaf and stem than in the endosperm. In the 9311*cry1Ac* line, the Cry1Ac protein contents ranged from 0.13 µg g^{-1} in the leaves to 0.31 µg g^{-1} in the stems, and reached 1.47 µg g^{-1} in the endosperm. In the pyramided 9311*LRP/cry1Ac* line, the Cry1Ac protein concentrations ranged from 0.29 µg g^{-1} in the leaves to 0.18 µg g^{-1} in the stems, and to 0.91 µg g^{-1} in the endosperm (**Fig. 2**). These results corresponded with the original intention that the pyramided 9311*LRP/cry1Ac* line would

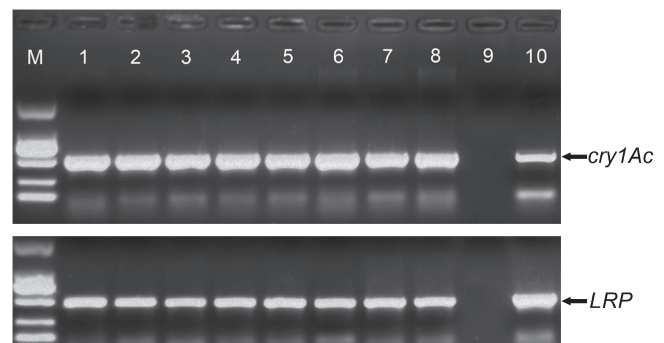

Fig. 1. PCR identification of the *LRP* and *cry1Ac* genes in the homozygous line. Lanes 1–8 are 8 plants from the homozygous line, and lanes 9 and 10 are negative control 9311 and positive controls 9311*cry1Ac* and 9311*LRP*, respectively. M: molecular weight marker.

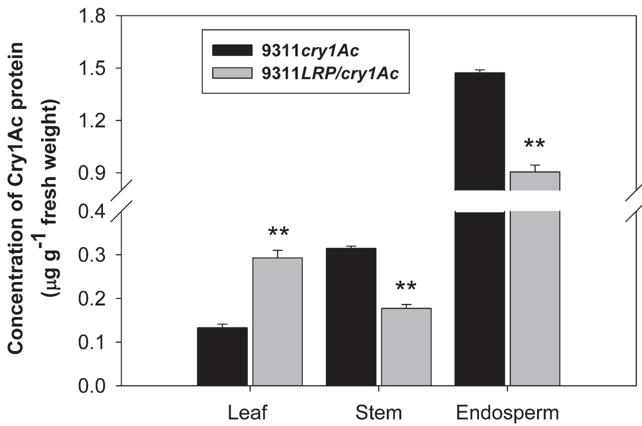

Fig. 2. The Cry1Ac protein concentrations in the 9311*cry1Ac* and 9311*LRP/cry1Ac* lines. Error bars indicate ± SD (n = 3). *P* values were generated by the two-tailed *t* test, and ** $P < 0.01$ denotes statistical significance.

efficiently express Cry1Ac protein in the leaf, stem and endosperm tissues.

Evaluation of insecticidal activity

To evaluate the insect resistance in the pyramided line, the larvae of SSB were used in the laboratory. The results showed that the larval mortalities were extremely low in the negative controls, 9311 and 9311*LRP*, and serious damage was observed in both leaves and stems (**Fig. 3A**, **3B**). However, the positive control 9311*cry1Ac* and the pyramided line 9311*LRP/cry1Ac* showed significant resistance to SSB, and no obvious damage occurred in the leaves or stems (**Fig. 3A**, **3B**). In the leaves and stems of the donor line 9311*cry1Ac*, the larval mortality was 95.6% and 80.8%, respectively (**Fig. 3C**). The pyramided line, 9311*LRP/cry1Ac*, showed a similar level of larval mortality, which reached 93.3% in leaves and 75.6% in stems (**Fig. 3C**). These results indicated that the *cry1Ac* gene had a significant effect

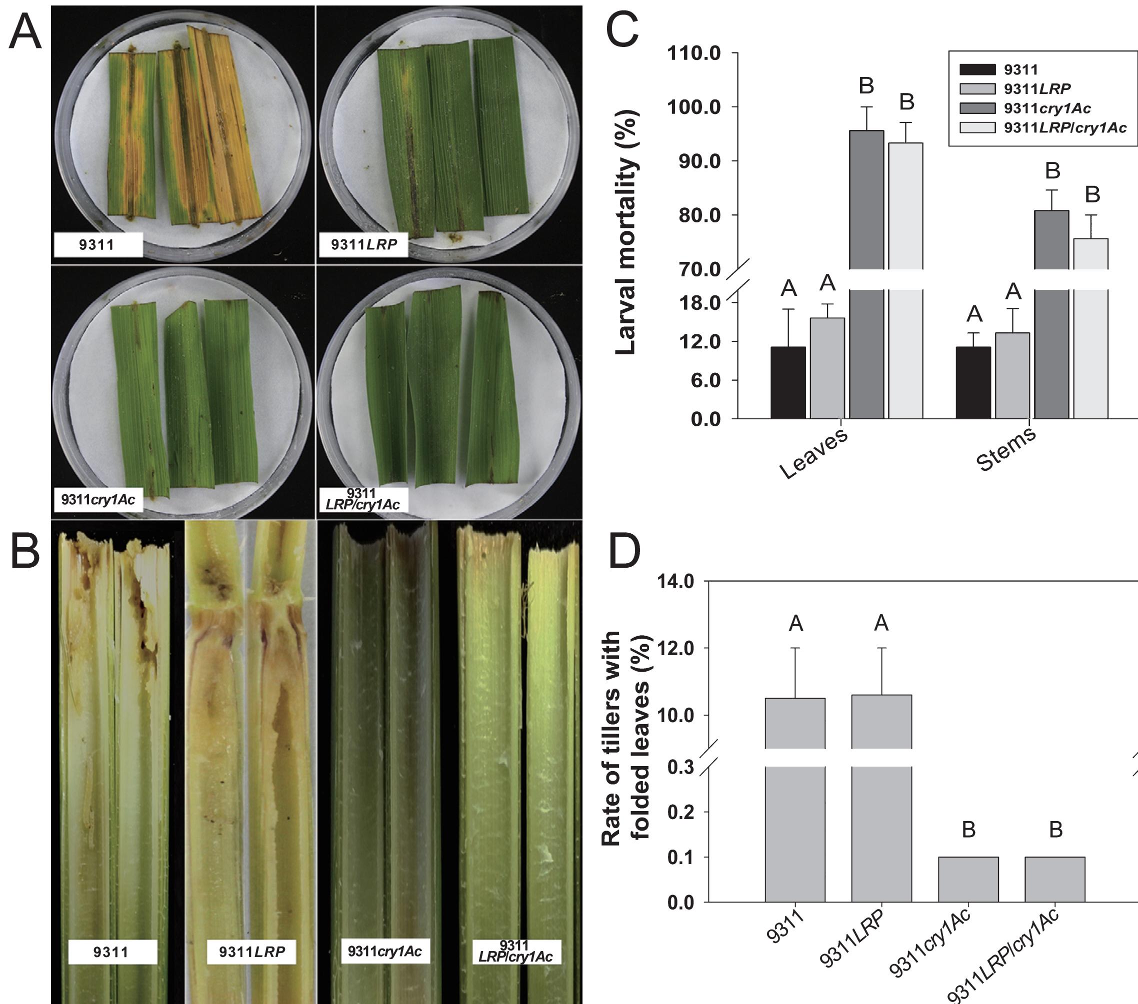

Fig. 3. Insecticidal performance of the pyramided line in the laboratory and field. (A–B) Insecticidal performance against SSB larvae in leaves and stems in the laboratory assay, respectively. (C) Larval mortality in corresponding lines measured after a manual infestation in the laboratory. (D) Rate of tillers with folded leaves after a natural infestation in the field. Error bars indicate ± SD (n = 3). Columns with the same letters are not significantly different at $P < 0.01$.

against SSB in the laboratory and that the resistance was stably introduced into the pyramided line.

Additionally, we investigated the RLF resistance in the pyramided line through natural infestation in the field in 2015. The rate of tillers with folded leaves was approximately 0.1% in both the positive control 9311*cry1Ac* and the pyramided line 9311*LRP/cry1Ac*, which were much lower than the rates in the negative controls 9311 (10.5%) and 9311*LRP* (10.6%) (**Fig. 3D**). This result further suggested that the *cry1Ac* gene in the pyramided line showed an outstanding resistance to RLF in the field.

The expression pattern of the LRP gene

In the original donor line, the foreign *LRP* gene was driven by an endosperm-specific *GT1* promoter (unpublished results). Therefore, we examined the expression levels of the *LRP* gene in different tissues of the pyramided line to determine the tissue specificity of *LRP* expression. The results showed that the *LRP* transcripts were in low abundance in both the leaves and stems of the 9311*LRP* line (**Fig. 4**). Similarly, only a small amount of *LRP* transcripts were detected in both leaves and stems in 9311*LRP/cry1Ac* (**Fig. 4**). However, the expression levels of the *LRP* gene dramatically increased in the endosperm of both the 9311*LRP* and 9311*LRP/cry1Ac* lines (**Fig. 4**). These results illustrated that the expression patterns of *LRP* gene in the donor line of 9311*LRP* and pyramided line of 9311*LRP/cry1Ac* behaved as expected when using an endosperm-specific promoter and no obvious changes in the *LRP* expression pattern occurred in the different genetic backgrounds.

Lys concentration in the seeds

We compared the content of 17 amino acids in brown rice between 9311*LRP* and 9311*LRP/cry1Ac*. As shown in **Table 1**, the Lys content was 30.34 μmol/g in 9311*LRP* line, which was a 19.09% increase compared with the parent line 9311. It is worth noting that the proportion of Lys to total amino acids increased to 4.35% in the 9311*LRP* line, while the proportion was only 3.95% in 9311. In addition to Lys, most of the other essential amino acids also increased compared to 9311, such as Thr (12.58%), Val (9.81%), Ile (7.48%), Leu (10.86%) and His (13.75%). Moreover, most of other amino acids had moderate increases except for Cys, Met, and Phe, such that the total amount of amino acids increased by 8.16% in the 9311*LRP* line compared to the parent line 9311 (**Table 1**).

The Lys content in the pyramided line 9311*LRP/cry1Ac*

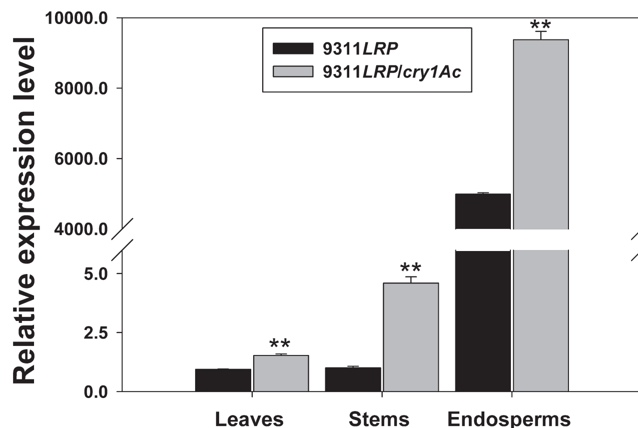

Fig. 4. qRT-PCR analysis of the *LRP* transcripts in the leaves, stems and endosperms of the pyramided line. Error bars indicate the s.e.m. calculated from three technical replicates. *P* values were generated by the two-tailed *t* test, and ** $P < 0.01$ denotes statistical significance.

Table 1. The amino acid content in corresponding lines (Hangzhou 2015)

Amino acids	Total content of amino acids in brown rice (μmol/g)					The proportions in total amino acids (%)		
	9311	9311*LRP*	± %	9311*LRP/cry1Ac*	± %	9311	9311*LRP*	9311*LRP/cry1Ac*
Asx	60.52 ± 0.73	65.50 ± 1.96	8.22	53.96 ± 0.47**	–10.84	9.38	9.39	8.66
Thr	29.41 ± 0.70	33.11 ± 0.65*	12.58	30.13 ± 0.30	2.46	4.56	4.74	4.83
Ser	44.62 ± 1.24	47.21 ± 1.17	5.81	42.97 ± 0.44	–3.69	6.92	6.76	6.89
Glx	111.05 ± 2.61	114.89 ± 2.83	3.46	103.99 ± 0.92	–6.35	17.21	16.46	16.69
Gly	58.73 ± 1.40	64.29 ± 1.80	9.47	57.71 ± 0.59	–1.73	9.10	9.21	9.26
Ala	59.14 ± 1.78	66.82 ± 1.66	12.97	61.30 ± 0.63	3.65	9.17	9.57	9.84
Cys	4.56 ± 0.30	2.95 ± 0.11*	–35.28	3.06 ± 0.05*	–32.87	0.71	0.42	0.49
Val	44.36 ± 1.74	48.71 ± 1.46	9.81	43.51 ± 0.38	–1.92	6.88	6.98	6.98
Met	13.71 ± 0.19	11.53 ± 0.27**	–15.9	12.13 ± 0.17**	–11.55	2.13	1.65	1.95
Ile	23.67 ± 1.10	25.44 ± 0.70	7.48	22.30 ± 0.17	–5.79	3.67	3.65	3.58
Leu	50.10 ± 1.79	55.55 ± 1.21	10.86	48.68 ± 0.46	–2.84	7.77	7.96	7.81
Tyr	14.54 ± 0.55	15.67 ± 0.18	7.75	13.38 ± 0.08	–7.98	2.25	2.25	2.15
Phe	27.20 ± 1.04	27.11 ± 0.79	–0.33	25.68 ± 0.26	–5.57	4.22	3.88	4.12
Lys	**25.48 ± 0.68**	**30.34 ± 0.84***	**19.09**	**26.93 ± 0.29**	**5.71**	**3.95**	**4.35**	**4.32**
His	16.68 ± 0.56	18.98 ± 0.34*	13.75	17.35 ± 0.13	4.03	2.59	2.72	2.78
Arg	40.21 ± 0.72	41.95 ± 1.23	4.33	37.09 ± 0.47*	–7.77	6.23	6.01	5.95
Pro	21.21 ± 2.68	27.82 ± 1.34	31.13	23.04 ± 0.22	8.60	3.29	3.99	3.70
Total	645.21 ± 13.86	697.86 ± 16.46	8.16	623.23 ± 6.02	–3.41			

[a] Glutamine (Gln) and asparagine (Asn) are hydrolyzed to glutamate (Glu) and aspartate (Asp) under acidic conditions. The final content of Glx indicates the sum of the Gln and Glu content and the final content of Asx is the sum of the Asp and Asn content. Data are given as means ± s.e.m. (n = 3). Data on the difference between the samples were examined by two-tailed *t* test. Significant differences at the levels of * $P < 0.05$ and ** $P < 0.01$.

Table 2. Agronomic performance of the pyramided lines (Hangzhou 2015)

Line	Heading date (days)	Plant height (cm)	No. of tillers per plant	No. of grains per panicle	1000-grain weight (g)	Self-fertility rate (%)	Single-plant yield (g)
9311	100	121.2 ± 1.0	5.4 ± 0.2	209.8 ± 4.9	28.2 ± 0.2	71.0 ± 1.5	25.2 ± 0.4
9311*LRP*	101	123.6 ± 0.9	5.6 ± 0.5	234.3 ± 5.0*	26.3 ± 0.1**	75.0 ± 1.2	24.8 ± 1.2
9311*cry1Ac*	101	120.4 ± 0.9	5.6 ± 0.2	218.6 ± 7.3	28.3 ± 0.1	73.2 ± 1.1	25.3 ± 0.6
9311*LRP/cry1Ac*	100	120.2 ± 1.4	5.4 ± 0.2	224.2 ± 1.8*	26.0 ± 0.1**	72.8 ± 1.7	24.9 ± 1.0

[a] Significantly different from the performance of the parent 9311 line at levels of * $P < 0.05$ and ** $P < 0.01$.

was 26.93 μmol/g. This was only a 5.71% increase compared with 9311 and a remarkable decrease compared to the Lys content of the 9311*LRP* line (30.34 μmol/g) (**Table 1**). Interestingly, although the amino acid content differed, the *LRP* gene was efficiently expressed in both endosperms of 9311*LRP* and 9311*LRP/cry1Ac* (**Fig. 4**). Furthermore, the content of some amino acids in 9311*LRP/cry1Ac* showed a small increase, such as Thr, Ala, His and Pro, and others significantly decreased, such as Asx, Met and Arg. The total amount of amino acids decreased by 3.41% compared to 9311. The Lys proportion of the total amino acids reached 4.32% in 9311*LRP/cry1Ac*, which was similar to the proportion of Lys seen in 9311*LRP* (4.35%) but significantly higher than the proportion in 9311 (3.95%) (**Table 1**). These results highlighted the obviously different effect on Lys content improvement that the *LRP* gene had in the pyramided 9311*LRP/cry1Ac* line versus the 9311*LRP* line. This was probably an indication of some interference by the *cry1Ac* gene in the genetic background of the 9311 line.

Agronomic performance of the improved lines

In addition to the tests of insect resistance and Lys content, we also evaluated important agronomic traits in the pyramided lines. As shown in **Table 2**, the *LRP* donor line 9311*LRP* and the pyramided line 9311*LRP/cry1Ac* had similar agronomic performance levels and showed no significant differences from the parental line of 9311 in heading date, plant height, numbers of tillers per plant, self-fertility rate and single-plant yield. The 1000-grain weight of the 9311*cry1Ac* line was 28.3 g, which was identical to 1000-grain weight of 9311. However, the 1000-grain weights of both the 9311*LRP* and 9311*LRP/cry1Ac* (26.3 g and 26.0 g, respectively) were significantly lower than 9311 (**Table 2**).

Discussion

Multi-gene pyramiding is an efficient method of improving different target traits in plants simultaneously, and many previous studies have shown desirable results in rice (Maruthasalam *et al.* 2007, Ni *et al.* 2015, Wan *et al.* 2014). However, there have been few efforts to pyramid foreign genes in rice. In this study, we performed MAS pyramiding of the foreign *cry1Ac* and *LRP* genes into the elite *indica* 9311 line simultaneously. In the pyramided line, the *cry1Ac* gene was efficiently translated into Cry1Ac protein, which was confirmed by ELISA (**Fig. 2**). Previous studies suggested that the Bt content in leaves and stems had a downward trend throughout plant growth and development, and showed higher concentrations at the tillering stage than in the endosperm at the filling stage (Liu *et al.* 2015, Yang *et al.* 2011). In our study, the Cry1Ac content in the leaves and stems of the pyramided line was 0.29 μg g^{-1} and 0.18 μg g^{-1}, respectively, and was significantly lower than in the endosperm at the late filling stage (**Fig. 2**). This result indicates that the Cry1Ac protein content was still expressed in chlorophyllous tissues at the late growth stages of plants, though the content was low in leaves and stems.

We also assayed target-insect resistance of the 9311*LRP/cry1Ac* line at the late filling stage in the laboratory. Larval mortality rates were 93.3% in leaves and 75.6% in stems, and these were significantly higher than those in the parental line, 9311 (**Fig. 3**). Furthermore, the surviving larvae were much smaller after feeding on the pyramided line, and showed a more sluggish response than in the parental line (data not shown). These results illustrated that the relatively low concentration of the Cry1Ac protein in the pyramided line at the late filling stage was sufficient for resistance to target insects. This was probably in favor of the energy translocation from source to sink, which in turn maintained the agronomic performance. These results indicate that application of the *cry1Ac* gene is a feasible and practical means for the improvement of targeted-insect resistance in rice.

qRT-PCR results (**Fig. 4**) showed that the *LRP* expression patterns in both the *LRP*-introduced line and the pyramided line conformed to the expression manner of *GT1* promoter in rice. In this study, the Lys content in seeds of the 9311*LRP* line increased by approximately 20%, which was significantly higher than the Lys content in the 9311 line (**Table 1**). This result suggested that the different genetic background probably had a minor effect on the ability of the foreign *LRP* gene to increase the Lys content. However, the Lys content in the pyramided 9311*LRP/cry1Ac* line was 26.93 μmol/g, which is a mere increase of 5.71% when compared with the 9311 line and a much lower Lys content than seen in the 9311*LRP* line (30.34 μmol/g) (**Table 1**). The result indicates that the foreign *LRP* gene effect was most likely influenced by the simultaneous expression of the *cry1Ac* gene in the 9311 genetic background. However, no obvious negative effects on insect-resistance by the introduction of the foreign *LRP* gene were observed in the 9311*LRP/cry1Ac* line (**Fig. 3**). Based on these results, we proposed that simultaneously pyramiding multiple foreign

genes of different types probably faces challenges such as unforeseen gene interactions, though each gene functioned well alone in the plants, and breeders have to pay more attention to the possible interference among target genes before pyramiding. In this study, we pyramided the foreign Bt gene and LRP gene in an elite line 9311 by crossing. Comparing to the approach for simultaneously transforming both *cry1Ac* and *LRP* into a recipient plant using a single T-DNA vector containing these two genes, the strategy used in this study is easier than transforming two genes together into a recipient plant whenever in terms of transforming efficiency or vector construction. Moreover, the strategy is suitable to evaluate the interaction between the pyramided foreign genes and to test the effect of individual foreign genes on rice agronomic traits.

There were no obvious changes in agronomic traits observed between the parental 9311 and the 9311*cry1Ac*, indicating that the *cry1Ac* gene had no negative effects on agronomic traits in the 9311 genetic background (**Table 2**). Previous studies showed that the significant increase in Lys in seeds of transgenic plants had negative effects on seeds development and plant development (Falco *et al.* 1995, Kawakatsu *et al.* 2010, Lee *et al.* 2001, Shaul and Galili 1992). Furthermore, in our other experiment, we also observed that the *LRP* gene donor line (transgenic line PA110) had 15.7 g of 1000-grain weight and 131.2 grain per panicle on average, which were significantly different from those 18.6 g of 1000-grain weight and 124.4 grain per panicle in wild-type (the data submitted elsewhere). In this study, we also observed significant differences in grain per panicle and grain weight in lines of 9311*LRP/cry1Ac* and 9311*LRP*, whereas in 9311*cry1Ac*, there were no significant differences from parent line 9311. These findings suggested that the expression of the *LRP* gene caused the fortification of Lys, and then lead to negative effects on some agronomic traits such as grain weight and grain per panicle. However, the single-plant yield in both 9311/*LRP* and 9311*LRP/cry1Ac* lines showed no significant differences from the parental 9311 line because of the increased number of grains per panicle (**Table 2**). These results suggest that the pyramided 9311*LRP/cry1Ac* line has potential commercial value for the improvement of rice insect-resistance and Lys fortification.

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Literature Cited

Alam, M.F., K. Datta, E. Abrigo, N. Oliva, J. Tu, S.S. Virmani and S.K. Datta (1999) Transgenic insect-resistant maintainer line (IR68899B) for improvement of hybrid rice. Plant Cell Rep. 18: 572–575.

Barton, K.A., H.R. Whiteley and N.-S. Yang (1987) *Bacillus thuringiensis* δ-endotoxin expressed in transgenic *Nicotiana tabacum* provides resistance to Lepidopteran insects. Plant Physiol. 85: 1103–1109.

Chen, H., W. Tang, C. Xu, X. Li, Y. Lin and Q. Zhang (2005) Transgenic indica rice plants harboring a synthetic *cry2A** gene of *Bacillus thuringiensis* exhibit enhanced resistance against lepidopteran rice pests. Theor. Appl. Genet. 111: 1330–1337.

Chen, H., G. Zhang, Q. Zhang and Y. Lin (2008) Effect of transgenic *Bacillus thuringiensis* rice lines on mortality and feeding behavior of rice stem borers (Lepidoptera: Crambidae). J. Econ. Entomol. 101: 182–189.

Datta, K., N. Baisakh, K.M. Thet, J. Tu and S.K. Datta (2002) Pyramiding transgenes for multiple resistance in rice against bacterial blight, yellow stem borer and sheath blight. Theor. Appl. Genet. 106: 1–8.

Falco, S.C., T. Guida, M. Locke, J. Mauvais, C. Sanders, R.T. Ward and P. Webber (1995) Transgenic canola and soybean seeds with increased lysine. Nat. Biotechnol. 13: 577–582.

Frutos, R., C. Rang and M. Royer (1999) Managing insect resistance to plants producing *Bacillus thuringiensis* toxins. Crit. Rev. Biotechnol. 19: 227–276.

Jiang, G.H., C.G. Xu, J.M. Tu, X.H. Li, Y.Q. He and Q.F. Zhang (2004) Pyramiding of insect- and disease-resistance genes into an elite *indica*, cytoplasm male sterile restorer line of rice, ‘Minghui 63’. Plant Breed. 123: 112–116.

Kawakatsu, T., S. Wang, Y. Wakasa and F. Takaiwa (2010) Increased lysine content in rice grains by over-accumulation of BiP in the endosperm. Biosci. Biotechnol. Biochem. 74: 2529–2531.

Lee, S.I., H.U. Kim, Y.H. Lee, S.C. Suh, Y.P. Lim, H.Y. Lee and H.I. Kim (2001) Constitutive and seed-specific expression of a maize lysine-feedback-insensitive dihydrodipicolinate synthase gene leads to increased free lysine levels in rice seeds. Mol. Breed. 8: 75–84.

Liu, X., Z. Yang, G.J. Gao, Y.J. Lin, X.P. Zhu, J.Y. Yu and Y.Q. He (2010) Development of *Bt* rice by molecular marker-assisted selection and assays for insect-resistance. Mol. Plant Breed. 1. doi: 10.5376/mpb.2010.01.0002.

Liu, X., J. Zhang, C. Zhang, L. Wang, H. Chen, Z. Zhu and J. Tu (2015) Development of photoperiod- and thermo-sensitive male sterility rice expressing transgene *Bacillus thuringiensis*. Breed. Sci. 65: 333–339.

Livak, K.J. and T.D. Schmittgen (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods 25: 402–408.

Luo, Y. and Z. Yin (2013) Marker-assisted breeding of Thai fragrance rice for semi-dwarf phenotype, submergence tolerance and disease resistance to rice blast and bacterial blight. Mol. Breed. 32: 709–721.

Maruthasalam, S., K. Kalpana, K.K. Kumar, M. Loganathan, K. Poovannan, J.A.J. Raja, E. Kokiladevi, R. Samiyappan, D. Sudhakar and P. Balasubramanian (2007) Pyramiding transgenic resistance in elite indica rice cultivars against the sheath blight and bacterial blight. Plant Cell Rep. 26: 791–804.

Murray, M.G. and W.F. Thompson (1980) Rapid isolation of high

molecular weight plant DNA. Nucleic Acids Res. 8: 4321–4325.

Ni, D., F. Song, J. Ni, A. Zhang, C. Wang, K. Zhao, Y. Yang, P. Wei, J. Yang and L. Li (2015) Marker-assisted selection of two-line hybrid rice for disease resistance to rice blast and bacterial blight. Field Crops Res. 184: 1–8.

Perlak, F.J., R.W. Deaton, T.A. Armstrong, R.L. Fuchs, S.R. Sims, J.T. Greenplate and D.A. Fischhoff (1990) Insect resistant cotton plants. Biotechnology (N.Y.) 8: 939–943.

Ramalingam, J., H.S. Basharat and G. Zhang (2002) STS and microsatellite marker-assisted selection for bacterial blight resistance and waxy genes in rice, *Oryza sativa* L. Euphytica 127: 255–260.

Riaz, N., T. Husnain, T. Fatima, R. Makhdoom, K. Bashir, L. Masson, I. Altosaar and S. Riazuddin (2006) Development of *Indica* Basmati rice harboring two insecticidal genes for sustainable resistance against lepidopteran insects. S. Afr. J. Bot. 72: 217–223.

Sautter, C., S. Poletti, P. Zhang and W. Gruissem (2006) Biofortification of essential nutritional compounds and trace elements in rice and cassava. Proc. Nutr. Soc. 65: 153–159.

Shaul, O. and G. Galili (1992) Increased lysine synthesis in tobacco plants that express high levels of bacterial dihydrodipicolinate synthase in their chloroplasts. Plant J. 2: 203–209.

Tang, W., H. Chen, C. Xu, X. Li, Y. Lin and Q. Zhang (2006) Development of insect-resistant transgenic *indica* rice with a synthetic *cry1C** gene. Mol. Breed. 18: 1–10.

Tu, J., G. Zhang, K. Datta, C. Xu, Y. He, Q. Zhang, G.S. Khush and S.K. Datta (2000) Field performance of transgenic elite commercial hybrid rice expressing *Bacillus thuringiensis* δ-endotoxin. Nat. Biotechnol. 18: 1101–1104.

Wan, B., Z. Zha, J. Li, M. Xia, X. Du, Y. Lin and D. Yin (2014) Development of elite rice restorer lines in the genetic background of R022 possessing tolerance to brown planthopper, stem borer, leaf folder and herbicide through marker-assisted breeding. Euphytica 195: 129–142.

WHO (2007) Protein and amino acid requirements in human nutrition. World Health Organization technical report series, pp. 1–265.

Wong, H.W., Q. Liu and S.S.M. Sun (2015) Biofortification of rice with lysine using endogenous histones. Plant Mol. Biol. 87: 235–248.

Yang, Z., H. Chen, W. Tang, H. Hua and Y. Lin (2011) Development and characterisation of transgenic rice expressing two *Bacillus thuringiensis* genes. Pest Manag. Sci. 67: 414–422.

Ye, R., H. Huang, Z. Yang, T. Chen, L. Liu, X. Li, H. Chen and Y. Lin (2009) Development of insect-resistant transgenic rice with Cry1C*-free endosperm. Pest Manag. Sci. 65: 1015–1020.

Zhang, C.C., W.Y. Yuan and Q.F. Zhang (2012) *RPL1*, a gene involved in epigenetic processes regulates phenotypic plasticity in rice. Mol. Plant 5: 482–493.