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
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, 9311LRP/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 9311LRP/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 9311LRP line. The 9311cry1Ac 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 9311LRP to create the pyramided line 9311LRP/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 9311cry1Ac was used as a positive control, and 9311 and 9311LRP 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 × Mg2+, 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 Primerscript 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−ΔΔCT 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 9311LRP. The second step was to pyramid the cry1Ac and LRP genes by crossing the 9311LRP line with the 9311cry1Ac line followed by two rounds of selfing plus foreground selection by MAS. A homozygous line containing the cry1Ac and LRP genes was obtained, named 9311LRP/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 9311cry1Ac line and the pyramided 9311LRP/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 9311cry1Ac 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 9311LRP/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 9311LRP/cry1Ac line would
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 9311LRP, and serious damage was observed in both leaves and stems (Fig. 3A, 3B). However, the positive control 9311cry1Ac and the pyramided line 9311LRP/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 9311cry1Ac, the larval mortality was 95.6% and 80.8%, respectively (Fig. 3C). The pyramided line, 9311LRP/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...
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 9311cry1Ac and the pyramided line 9311LRP/cry1Ac, which were much lower than the rates in the negative controls 9311 (10.5%) and 9311LRP (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 9311LRP line (Fig. 4). Similarly, only a small amount of LRP transcripts were detected in both leaves and stems in 9311LRP/cry1Ac (Fig. 4). However, the expression levels of the LRP gene dramatically increased in the endosperm of both the 9311LRP and 9311LRP/cry1Ac lines (Fig. 4). These results illustrated that the expression patterns of LRP gene in the donor line of 9311LRP and pyramided line of 9311LRP/cry1Ac behaved as expected when using an endosperm-specific promoter and no obvious changes in the expression patterns of 9311LRP/cry1Ac occurred in the different genetic backgrounds.

Lys concentration in the seeds

We compared the content of 17 amino acids in brown rice between 9311LRP and 9311LRP/cry1Ac. As shown in Table 1, the Lys content was 30.34 μmol/g in 9311LRP 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 9311LRP 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 9311LRP line compared to the parent line 9311 (Table 1).

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

| Amino acids | 9311 Total | 9311LRP Total | ± % | 9311LRP/cry1Ac Total | ± % | 9311LRP/cry1Ac ± % |
|-------------|-----------|--------------|----|---------------------|----|-------------------|
| Pro         | 21.21 ± 2.68 | 27.82 ± 1.34 | 31.13 | 23.04 ± 0.22 | 8.60 | 3.70               |
| Lys         | 25.48 ± 0.68 | 30.34 ± 0.84* | 19.09 | 26.93 ± 0.29 | 5.71 | 4.32               |
| Met         | 13.71 ± 0.19 | 11.53 ± 0.27** | –15.9 | 12.13 ± 0.17** | –11.55 | 2.13               |
| His         | 16.68 ± 0.56 | 18.98 ± 0.34* | 13.75 | 17.35 ± 0.13 | 4.03 | 2.59               |
| Arg         | 40.21 ± 0.72 | 41.95 ± 1.23 | 4.33 | 37.09 ± 0.47* | –7.77 | 6.23               |
| Pro         | 21.21 ± 2.68 | 27.82 ± 1.34 | 31.13 | 23.04 ± 0.22 | 8.60 | 3.70               |
| Total       | 645.21 ± 13.86 | 697.86 ± 16.46 | 8.16 | 623.23 ± 6.02 | –3.41 | –3.12               |

*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             |
| 9311LRP                 | 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             |
| 9311cry1Ac              | 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             |
| 9311LRP/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             |

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

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 9311LRP/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 9311LRP 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 9311LRP/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 9311LRP 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 9311LRP/cry1Ac line (Fig. 3). Based on these results, we proposed that simultaneously pyramiding multiple foreign
Pyramiding Bacillus thuringiensis and Lysine-rich protein genes in rice

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 9311cry1Ac, 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 9311LRP/cry1Ac and 9311LRP, whereas in 9311cry1Ac, 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 9311LRP and 9311LRP/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 9311LRP/cry1Ac line has potential commercial value for the improvement of rice insect-resistance and Lys fortification.

Acknowledgements

We thank Professor Yu-qing He (College of Life Science and Technology, Huazhong Agricultural University) for kindly supplying the parental line, 9311cry1Ac. This work was financially supported by the National Program on Research and Development of Transgenic Plants of China (2011ZX08001-001, 2012ZX08001-001) and the Special Fund for Introduction of the International Advanced Agricultural Science and Technology (“948” program) by the Chinese Ministry of Agriculture (2006-Z14).

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