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Generation and DNA Characterization of High-lysine Mutants by Biochemical Selection from Callus Culture of "Hwayeongbyeo"

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ABSTRACT

Lysine is the first essential amino acid for optimal nutrient quality in rice grain. For the narrow genetic diversities of lysine contents in rice, somaclonal variation was the source of mutation in our breeding program. Biochemical selection was conducted using 1 mM *S*-(2-aminoethyl) cysteine followed by two passages of 5 mM lysine plus threonine in the callus subculture medium. The lysine contents in endosperm of all progenies recovered from the biochemical selection were higher than those of their donor cultivar "Hwayeongbyeo". These elevated lysine levels of mutants were successfully transmitted to M_4 generation. The lysine contents in endosperm varied 3.85 to 4.80% compare to their donor cultivar "Hwayeongbyeo" was 3.85%. Three of high-lysine germplasms, Lys-1, Lys-2 and Lys-7 were selected by biochemical selection and rapid screening methods. DNA analysis showed that a new insertion of *Tos* 17 which mapped to rice chromosome 11 on the high-lysine mutant, Lys-2.

Key words : Rice, callus, high-lysine, biochemical selection, *Tos* 17

INTRODUCTION

In the rice market, grain quality is assessed in terms of appearance, taste, nutritional value and processing suitability. Previous quality studies gave greater emphasis to physiochemical improvement than nutritional value. However, rice has the lowest protein contents among the cereals and is also low in fiber, rice protein is unique among the cereal proteins in being richest in glutelin and lowest in prolamin (Mossé, 1968). Because of its low prolamin contents, rice protein, together with oat protein, has higher lysine content than the other cereal proteins (Juliano *et al.*, 1973). But rice has relatively low levels of lysine contents in the endosperms and poor genetic resources compared to the other cereals. Lysine is the first limiting essential amino acid for optimal nutritional quality in rice grain. The high-lysine mutants have been subsequently screened and discovered in many crops

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including maize (Mertz *et al.*, 1964), barley (Munck *et al.*, 1970) and sorghum (Singh and Axtell, 1973). In rice, International Rice Research Institute (IRRI) reported only 2 high-lysine germplasms could be confirm after the screening of 10,493 entries from world germplasm collection (Juliano *et al.*, 1973). Because of narrow genetic diversity of lysine contents in rice biochemical selection is suggested to recover high-lysine mutants by in vitro selection (Schaeffer and Sharpe, 1987). Since five high-lysine rice germplasms have been released by in vitro selection, no further systematic approaches were made into the practical rice breeding field to enhance lysine contents of endosperm (Schaeffer *et al.*, 1994).

The variants regenerated via a tissue culture cycle have been reported in many crops (Larkin and Scowcroft, 1981). Both desirable and deleterious variants have been reported including plant height, panicle shape, days to heading, sterility, grain shape & quality, disease resistance and chemical compositions of endosperm in rice, (Cao *et al.*, 1991; Oono, 1985; Xie, 1990). Larkin *et al.* (1989) proposed several factors that could be a possible cause of somaclonal variation; chromosome number & structure, amplification of genes, single gene mutation, mobilization of transposable elements and DNA methylation. Recently, a type of transposable elements, *Tos* 17, which is activated during tissue culture, was reported as potential causes of somaclonal variation in rice (Hirochika *et al.*, 1996). The publication of draft sequences of japonica and indica rice, another types of DNA transposon, miniature inverted-repeat transposable element (MITE) called *m-Ping*, was reported (Jiang *et al.*, 2003). The activation of MITE element, *m-Ping*, occur with high frequency during anther culture (Kikuchi *et al.*, 2003).

In this paper, we report generation of high-lysine rice mutants by biochemical selection from embryo derived callus and DNA characterization of selected high-lysine mutant Lys-2.

MATERIALS AND METHODS

Biochemical selection and progeny evaluation

The calli were induced from the seed culture of "Hwayeongbyeo" in N_6 medium supplemented with 2 ppm of 2,4-D for one month, then transferred to media supplemented with 1 mM of *S*-(2-aminoethyl)-cysteine (AEC) for 2 weeks. Well-grown calli were transferred to a medium supplemented with 5 mM of lysine plus threonine after two weeks and repeat one more passage for the third selection. The plants were regenerated from the calli (M_0) survived inhibitory level of selecting medium. Up to five generations were cultivated in the breeding field and major agronomic traits and amino acid compositions were evaluated each year.

Amino acid analysis and ninhydrin color test

To analyze for constituent amino acids, brown rice samples were ground in a cyclon miller (Udy) and hydrolyzed in 6 N-HCl at 110℃ for 24 hours. The hydrolysate was analyzed with an automated amino acid analyzer (Biochrom 20, Pharmacia Co.). For rapid screening of lysine contents in seed, 10 kernels of brown rice were split lengthwise with a sharp knife and placed in the test tubes. The kernels were covered with 10 ml of water and 300±50 mg of dry ninhydrin buffer mixture (16% ninhydrin, 58% sodium citrate, and 26% citric acid, pH 5.0) was added. The contents were heated just to the boiling point and the tubes were allowed to stand for 5 min. After cooling, the solution was filtered with Whatman No. 2 filter paper and optical density (OD) value at 570 nm was read by spectrophotometer.

DNA analysis

Total genomic DNAs were extracted from young leaves, following the protocol described by Chen and Ronald (1999). Approximately 10 ug of rice genomic

DNA was digested with restriction enzyme *EcoR* I and separated by electrophoresis on a 0.8% agarose gel. DNA gel-blot analysis was carried out according to standard procedures under high-stringency hybridization conditions with *Tos* 17 specific DNA probe (Sambrook *et at.*, 1989). To analyze flanking DNA of *Tos* 17 insertion, we carried out a TAIL-PCR and suppression PCR (Miyao *et al.*, 1998) using GeneAmp System 9700 (Applied Biosystem). The amplified fragments were separated by electrophoresis, isolated and directly sequenced with "*Tos* 17-tail 4" primer (5'-ATC CAC CTT GAG TTT GAA GGG-3'). Database searches were performed with programs BLAST (http://www.gramene.org/Multi/blastview).

RESULTS AND DISCUSSION

Biochemical selection and progeny analysis

The scheme of our experiments to recover enhanced lysine rice is shown in Figure 1. For the narrow genetic diversities, we employed somaclonal variation as mutation initiation. More than 500 plants were

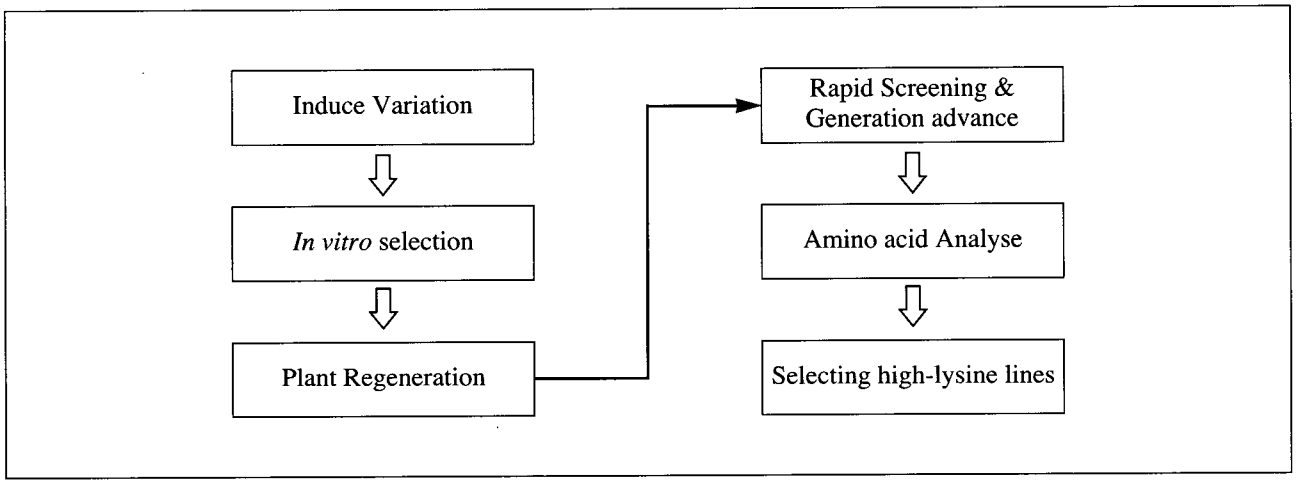

Fig. 1. Scheme for breeding high-lysine rice.

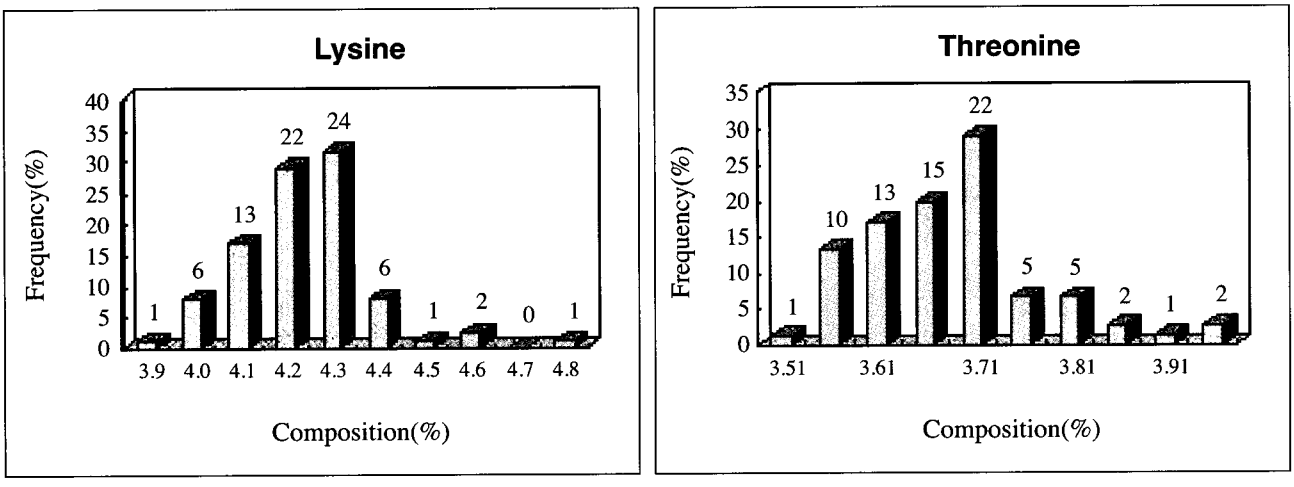

Fig. 2. Frequency distributions of amino acid composition among the mutants: n=69, "Hwayeongbyeo" embryo culture derived plants at M_3 generation (M_4 seed).

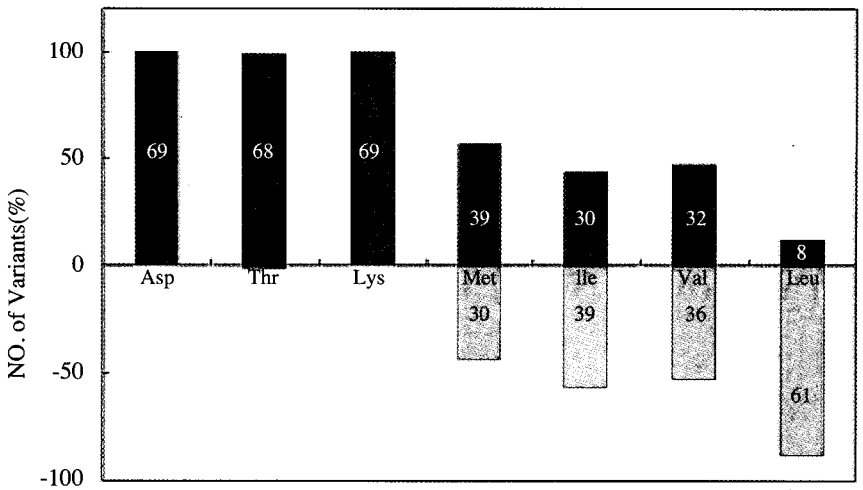

Fig. 3. Percentage of variants in 7 major amino acid of 69 lines of biochemically selected mutants of "Hwayeongbyeo" (Number in bars indicate that the no. of plants aberrant range of amino acids in parental plant).

regenerated and the seeds were collected from regenerated plant (M_0). In earlier generation, Rapid screening method (Nam *et al.*, 2000) was used for screening M_1 seeds of M_0 plants and M_2 seeds of M_1 plants. The mutant screen of M_3 and M_4 seeds was conducted both Rapid screen methods and analyzing constituent amino acids of brown rice using amino acid analyzer. Finally, 69 of high-lysine candidate lines were selected in M_3 generation. The amino acid analysis showed that most of candidate lines were higher levels of lysine and threonine contents than their donor cultivar "Hwayeongbyeo". The frequency distributions of lysine and threonine composition showed normal distribution among the mutant lines (Fig. 2). Most of the recovered plants from biochemical selection showed higher contents of aspartic acid, lysine and threonine in endosperm than those of the donor cultivar (Fig. 3). This is because the plants are regenerated from resistance calli in the presence of inhibitory levels of lysine, threonine and S-AEC on the selection medium.

Table 1. Ranges of amino acid composition in 69 lines of biochemically selected mutants of "Hwayeongbyeo"

Amino acid	Range (%)	Difference (%)	Amino acid	Range (%)	Difference (%)
Asp	10.05~10.97	0.92	Met*	0.83~2.11	1.28
Thr*	3.53~3.98	0.45	Ile*	3.97~4.34	0.37
Ser	4.49~4.83	0.34	Leu*	7.51~8.22	0.71
Glu	16.60~18.29	1.69	Tyr	3.49~4.19	0.70
Pro	4.31~4.82	0.51	Phe*	4.69~5.20	0.51
Gly	4.69~5.32	0.63	His	2.32~2.76	0.44
Ala	5.23~6.45	1.22	Lys*	3.86~4.80	0.96
Cys	1.10~3.04	1.94	Arg	8.24~9.03	0.79
Val*	5.96~6.37	0.41	Amm	2.04~2.97	0.93

* indicates essential amino acids.

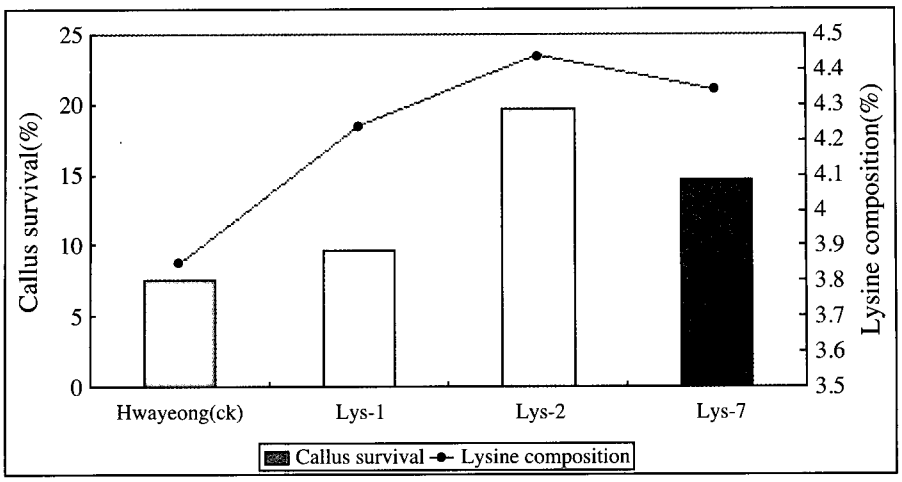

Fig. 4. Comparisons between callus survival rates and lysine contents of high-lysine mutant lines, Lys-1, Lys-2 and Lys-7.

The lysine composition of brown rice varied 3.86 to 4.80% and the threonine composition varied 3.53 to 3.98% compare to their donor cultivar "Hwayeongbyeo" was 3.85 and 3.54%, respectively (Table 1). Among the 69 progeny lines of biochemically selected, the three lines, Lys-1, Lys-2 and Lys-7 were selected considering other agronomic traits and lysine contents. The major agronomic traits of these three lines were not significantly different from their donor cultivar "Hwayeongbyeo" except for lysine contents (data not shown). To clarify the regulation of biosynthetic pathway of lysine, the calli were induced from the seed of Lys-1, Lys-2 and Lys-7. The callus survival rates of selected mutants tend to accord with the composition of

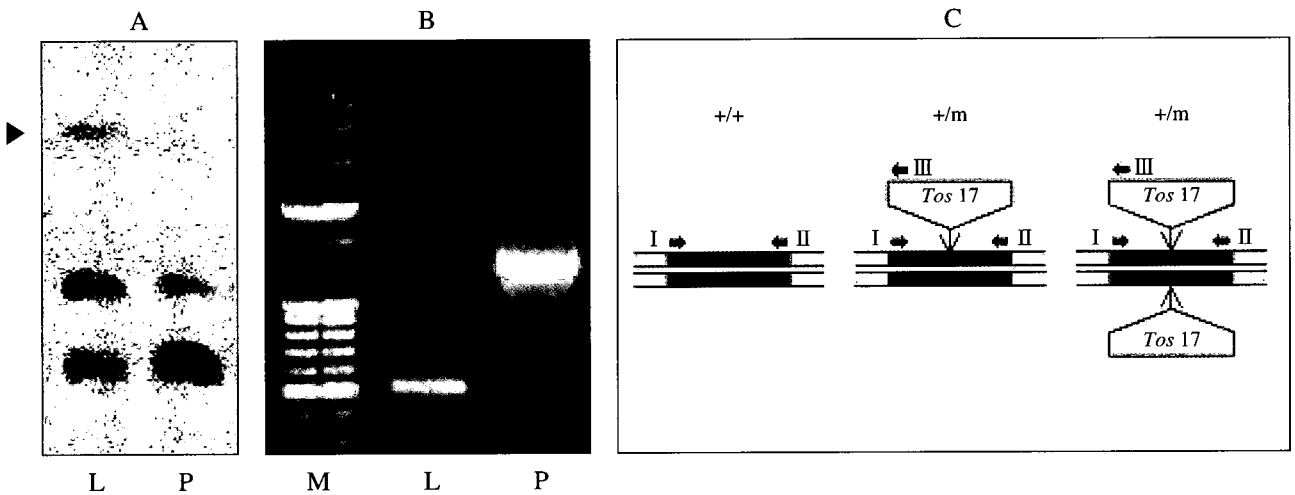

Fig. 5. A; Southern blot analysis of high-lysine mutant line "Lys-2" (L; Lys-2, P; parent, arrow indicate newly identified band), B; genotyping by PCR conformation (M; DNA marker, L; Lys-2, P; parent). C; The scheme of 3 way PCR to confirm genotype of *Tos* 17 insertion (I , II ; Genomic DNA primers, III; *Tos* 17 specific primer).

lysine. The lysine composition of "Hwayeongbyeo", Lys-1, Lys-2 and Lys-7 was 3.85, 4.24, 4.44 and 4.34% and callus survival rates were 7.6, 9.7, 19.7, and 14.7%, respectively (Fig. 4). However, even in high-lysine rice, selection was difficult because the target traits are too complicated to analyze and the population is large. The absorbance at 570 nm in the ninhydrin color test was highly significantly correlated with lysine in the rice endosperm. This color test could be used for rapid and mass screening of lysine content in rice endosperm, paving the way for breeding high-lysine rice. The high-lysine rice could be bred with little effort by the aid of biochemical selection and rapid screening method "Ninhydrin color test".

DNA analysis

Since the initiation of mutations was based on the somaclonal variation, we investigated the activity of retrotransposon, *Tos* 17. A new copy of *Tos* 17 was identified on the high-lysine line, Lys-2, by Southern blot (Fig. 5, A). Polymerase chain reaction (PCR) characterization indicated that a new insertion of *Tos* 17 was mapped to rice chromosome 11 corresponding to BAC clone OSJNBa0074E19 (Genebank Acc No. AC120307). Three sets of PCR primers were performed to confirm the presence of a new insertion of *Tos* 17 for the genotyping (Fig. 5, B, C). PCR results indicate that Lys-2 is homozygous to the flanking region of new *Tos* 17 insertion. Southern blot analysis showed a new positively-hybridizing band and flanking sequence was rescued from high-lysine mutant Lys-2 using TAIL- and suppression PCR. GeneBank (NCBI) database search showed that Tos 17 insertion is intergenic region on chromosome 11. More extensive studied will be need clarifying whether a new *Tos* 17 insertion affects recovering high-lysine trait. Whether *Tos* 17 may cause of high-lysine mutants, in a sense of broadening genetic diversity, somaclonal variation will provides good genetic resources.

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