J. Plant Biotechnology(2000) *Vol. 2(1). pp.35~41*

Journal of Plant Biotechnology

Herbicide Resistant Cabbage (*Brassica oleracea* ssp. *capitata*) Plants by *Agrobacterium*-mediated Transformation

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Key words: cabbage, transformation, Basta, herbicide resistance

Abstract

Transgenic cabbage (*Brassica oleracea* ssp. *capitata*) plants resistant to the commercial herbicide Basta[R] were obtained by *Agrobacterium tumefaciens* - mediated transformation.

Hypocotyl segments of *in vitro* grown plants were infected with *Agrobacterium tumefaciens* LBA 4404 harboring plasmid pMOG6-Bar which contains *hpt* and *bar* genes. Explants were cultured on callus induction medium (MS basal medium + 1 mg/L NAA + 2 mg/L BA + 2 mg/L $AgNO_3$ + 100 mg/L carbenicillin + 250 mg/L cefotaxime) supplemented with 15 mg/L hygromycin. Hygromycin resistant calluses were transferred to shoot regeneration medium (MS basal medium + 0.1 mg/L NAA + 2 mg/L BA + 3% sucrose + 2 mg/L $AgNO_3$ + 15 mg/L hygromycin + 250 mg/L cefotaxime + 100 mg/L carbenicillin). In order to induce roots, elongated shoots were placed on the MS medium without plant growth regulators and hygromycin.

Southern blot analysis of several putative transgenic plants indicated that one to five intact copies of *hpt* and *bar* genes were incorporated into the genome. Expression of *bar* gene was confirmed by Northern blot analysis and by herbicide resistant phenotype. Seed progeny from self-pollinated transformants expressed the herbicide resistance and showed Mendelian segregation of the introduced gene.

Introduction

Conventional plant breeding techniques together with genetic engineering approaches have been widely used to improve crop quality and yield.

Estimates of worldwide losses caused by weeds to several crops vary considerably; some reaching 20 % of total yields. Therefore, the production of transgenic crops with herbicide resistance is an important issue to address.

Phosphinothricin (glufosinate), the active principle of the herbicide Basta[R], is a glutamate analogue that inhibits glutamine synthetase. The inhibition of glutamine synthetase by phosphinothricin (PPT) in plants results in an accumulation of ammonium, which is mainly derived from photorespiration. High concentrations of ammonium interfere with the electron-transport systems of both chloroplasts and mitochondria, resulting in the production of free radicals and ultimately leading to cell death. The enzyme phosphinothricin acetyltransferase (PAT), encoded by the bar gene, inactivates PPT through a-cetylation (Thompson et al., 1987).

Several crops have been transformed with the *bar* gene, including tobacco, tomato, and potato showing tolerance to the common spray of glufosinate used to eradicate weeds (De Greef et al., 1989). The *bar* gene was also introduced and expressed in alfalfa, sugar beet (D'Hallulin et al., 1990, 1992), *Atropa* (Saito et al., 1992), wheat (Vasil et al., 1992), rice (Datta et al., 1992), peanut (Brar et al., 1994), turfgrass (Hartman et al., 1994), *Arabidopsis* (Akama et al., 1995), and sugarcane (Enriquez-Obregon et al., 1998). Canola with resistance to PPT was commercialized by AgrEvo (1995). Plant Genetic Systems

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Received Oct. 22, 1999; accepted Dec. 28, 1999

also produced canola with both herbicide resistance and pollination control (1995).

Cabbage, which belongs to *Brassica* vegetables, has been transformed both with *A. tumefaciens* (Yu and Shao, 1988; Christey et al., 1988; Berthomieu et al., 1994) and *A. rhizogenes* (He et al., 1991; Berthomieu et al., 1992). These studies were focused on the development of efficient transformation methods. Recently, transgenic cabbage plants with insect resistant genes (Bt genes) were produced and showed resistance to diamondback moth (Bai et al., 1993; Metz et al., 1995). Although herbicide resistance has been introduced into other *Brassica* species such as cauliflower (De Block et al., 1989; Mukhopadhyay et al., 1991), cabbage transformation with the *bar* gene has not yet been reported.

The objective of this study was to develop transgenic cabbage plants resistant to herbicide as a breeding material. The transformation protocol was based on the use of hypocotyl segments of *in vitro*-grown seedlings and *A. tumefaciens* LBA 4404 containing plasmid pMOG6-Bar which harbors the *bar* gene.

Materials and Methods

Plant materials

Cabbage (*Brassica oleracea* ssp. *capitata*) seeds were soaked in 70% ethanol for 1 min, surface sterilized in 50% (v/v) chlorox for 20 minutes and rinsed five times in sterile distilled water. The seeds were placed on MSO medium (MS basal medium + 3% sucrose) and incubated in a culture room (18h light/6h dark) for 6-7 days. Hypocotyls were cut into 0.5cm segments and precultured on callus induction medium (MS basal medium + 1 mg/L NAA + 2mg/L BA + 2 mg/L $AgNO_3$ + 3% sucrose) for 1 day before *Agrobacterium* inoculation.

Vector construction and bacterial strain

Plasmid pBCUBNK-Bar containing the *bar* gene under 35S promoter of cauliflower mosaic virus was digested with *Hin*dIII. A 2.0kb fragment containing P35S - *bar* - Ti7 was inserted into the *Hin*dIII site of pMOG6 that contains *hpt* (hygromycin phosphotransferase) gene, to generate pMOG6-Bar (Figure 1). Plasmid pMOG6-Bar was subsequently introduced into LBA 4404 by using the freeze and thaw method.

Transformation, selection and regeneration

The transformation protocol was based on the method described by Metz et al. (1995). Efficient shoot regeneration from *in vitro*-grown cabbage hypocotyls and as well as the optimization of parameters which affect transformation, such as preculture period, bacterial concentration, cocultivation period, effect of tobacco feeder layer, and $AgNO_3$, have been investigated previously by using *A. tumefaciens* LBA 4404 harboring pIG121 (Ohta et al., 1990) which contains *npt*II, *hpt*, and intron-GUS genes (Lee, 1997).

A. tumefaciens containing pMOG6-Bar was grown for 2 days in liquid YEP (10g/L bacto peptone + 10g/L yeast extract + 5g/L NaCl) supplemented with 50 mg/L kanamycin. The bacteria were pel-

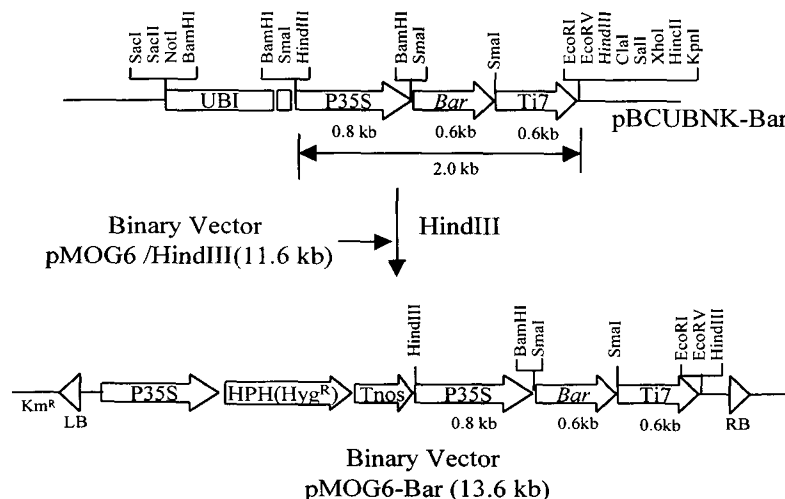

Figure 1. Construction of pMOG6-Bar. LB: Left border, RB: Right border, P35S: CaMV 35S promoter, HPH: hygromycin phosphotransferase, Tnos: 3'signal of nopaline synthase, *Bar*: phosphinothricin acetyltransferase, Ti7: terminator.

leted (2,100 × g) and resuspended in liquid callus induction medium. The bacterial suspension was adjusted to about 5×10^7 cells/mL prior to coculture.

Precultured hypocotyls were incubated in the bacterial suspension for 15-20 minutes. Inoculated hypocotyls without blotting to remove excess liquid were placed horizontally on sterile 7cm filter paper (Whatman #1) over 2mL of 3-4 day old tobacco suspension cells plated on callus induction medium. Explants were co-cultivated for 2 days in the dark at 25℃. In order to eliminate bacteria, explants were then washed three times in liquid callus induction medium supplemented with 250 mg/L cefotaxime and 100 mg/L carbencillin. The excess liquid was removed by blotting on sterile filter paper and explants were transferred to selective medium (callus induction medium + 15 mg/L hygromycin + 250 mg/L cefotaxime + 100 mg/L carbenicillin + 2 mg/L $AgNO_3$). Subcultures to fresh selective medium were performed every 2 weeks. After 4-6 weeks, calluses with resistance to hygromycin were transferred to shoot regeneration medium (MS basal medium + 0.1mg/L NAA + 2 mg/L BA + 3% sucrose + 2 mg/L $AgNO_3$ + 15 mg/L hygromycin + 250 mg/L cefotaxime + 100 mg/L carbenicillin). Transformed plants were transferred to soil and grown to maturity.

Southern blot hybridization

Total genomic DNA was isolated from leaf tissue by the procedure described by Shure et al. (1983). The procedures for restriction enzyme digestion, electrophoresis, and Southern blot analysis were carried out according to Sambrook et al. (1989). DNA (10μg) was digested with *Hin*dIII or *Bam*HI. The probe for *hpt* was 1.1 kb fragment from pCH. The *bar* probe was 0.6kb fragment from pBar.

Northern blot analysis

The isolation of total RNA was performed as previously described by Verwoerd et al. (1989). Northern blot analysis was carried out in accordance with the instruction manual from Hybond™ membrane (Amersham Life Science).

PCR analysis

PCR amplification was performed in volumes of 50μL containing 500ng template DNA (total genomic DNA), 0.2mM each of dNTP, 100pM primers (5'-AGCCTGACCTATTGCATCTCC-3' and 5'-TGTCCGTCAGGACATTGTTGG-3', for *hpt*; 5'-GGTCTGCAATCGTCAACC-3' and 5'-TCAGATCTCGGTGACGGGCA-3' for *bar*), 2.5 unit Taq DNA polymerase (Gibco BRL), 5μL 10 x buffer (Gibco BRL), 1.5mM $MgCl_2$. Following an initial denaturation step at 95℃ for 5 min, the amplification program was set 35 cycles of 94℃ for 1 min, 58℃ for 1 min and 72℃ for 2 min. PCR products were separated by electrophoresis on 1% agarose gels containing ethidium bromide in 1xTBE, and visualized under UV light.

Evaluation of herbicide tolerance and progeny analysis

An aqueous 0.6% solution of commercial Basta^R (PPT ammonium glufosinate content, 18%) was applied to the leaves as recommended by the suppliers. Herbicide tolerance was evaluated after 10-20 days.

To obtain the T_1 progeny seeds, transgenic plants were self-pollinated by bud pollination. T_1 plants with 2-3 leaves were evaluated for Basta^R tolerance as above. Stable inheritance of *bar* gene was confirmed by PCR and Southern blot analysis.

Results and Discussion

Transformed cabbage plants were obtained via *A. tumefaciens*-mediated transformation. In our previously established *in vitro* regeneration protocol, calluses and shoots were formed from the cut end of explants when hypocotyl segments were cultured on MS medium supplemented with 1 mg/L NAA + 2 mg/L BA + 2 mg/L $AgNO_3$ (Lee, 1997). However, only calluses were produced from these explants after *Agrobacterium* cocultivation and culture on the same medium supplemented with 15 mg/L hygromycin. Shoot regeneration from hygromycin-resistant calluses was only achieved upon subculture on shoot induction medium, in which NAA concentration was decreased to 0.1 mg/L. Similar intermediary callusing phases were described in transformation protocols for *B. napus* (De Block et al., 1989), *B. campestris* (Mukhopadhyay et al., 1992), and *B. oleracea* hypocotyl explants (Metz et al., 1995). Maintaining selection throughout the shoot regeneration was essential to get true transformants.

Two kinds of transgenic calluses were recovered; compact green calluses, which gave rise to shoots, and friable calluses, which proliferated without shoot formation. A total of seventy five hygromycin resistant calluses were formed from about 1,500 cocultivated hypocotyl segments. The transformation efficiency based on the rate of hygromycin resistant calluses produced was 5%. This rate was lower than the 10% efficiency reported by Metz et al. (1995). The total forty transformed plants obtained from this work were successfully cultivated to maturity in

greenhouse conditions.

In order to evaluate the phenotypic expression of the *bar* gene, commercial formulations of Basta[R] were applied to the leaves of forty transformed and control cabbage plants. All transformants tested showed resistance to Basta[R]. In contrast, control plants completely bleached within 10-20 days after spraying (Figure 2). All transformants were vernalized and self pollinated by bud pollination to obtain T_1 progeny.

Southern blot analysis was performed on the total genomic DNA of eleven transformants. When the genomic DNA was digested with *Hin*dIII and hybridized with the *bar* probe, a band of the expected 2. 0kb length was observed whereas no hybridization signals were detected in DNA from control plants (Figure 3A). When the genomic DNA digested with *Hin*dIII was allowed to hybridize with the *hpt* probe, the number of hybridizing bands reflected the number of copies of integrated genes in the transformants (Figure 3B). The copy number of integrated genes varied from one to five, in contrast to the 1-2 copies insertion pattern described in *Arabidopsis* (Akama et al., 1995) and sugarcane (Enriquez-Obregon et al., 1998).

In order to detect the expression of the *bar* gene, total RNA from transformants was hybridized with the *bar* probe. All transformants gave signals with the expected 0.58kb band (Figure 4).

Five out of forty transformants were not able to grow normally and showed severely twisted leaves with heavy incision. This abnormal phenotype was also observed in protoclones produced from hypocotyl protoplast culture of non-transformed cabbage (Lee et al., 1997). Therefore, in addition to the presence of the transgenes, it is possible that the phenotypic alteration observed in the present work is a consequence of the callus phase involved in the regeneration process. Although these five plants received cold treatment for vernalization, bolting did not occur.

To obtain T_1 progeny, transformants with normal

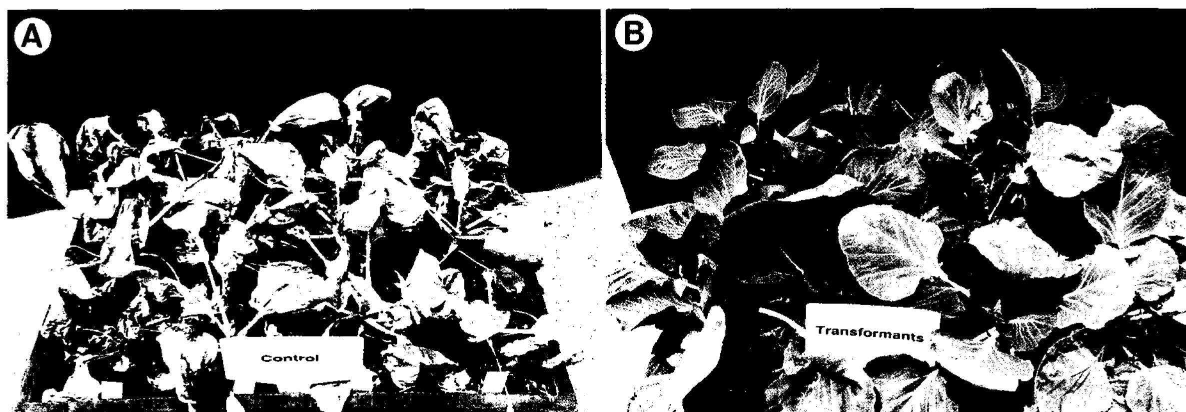

Figure 2. Response of cabbage untransformed (A) and transformed (B) cabbage after Basta[R] application to leaves

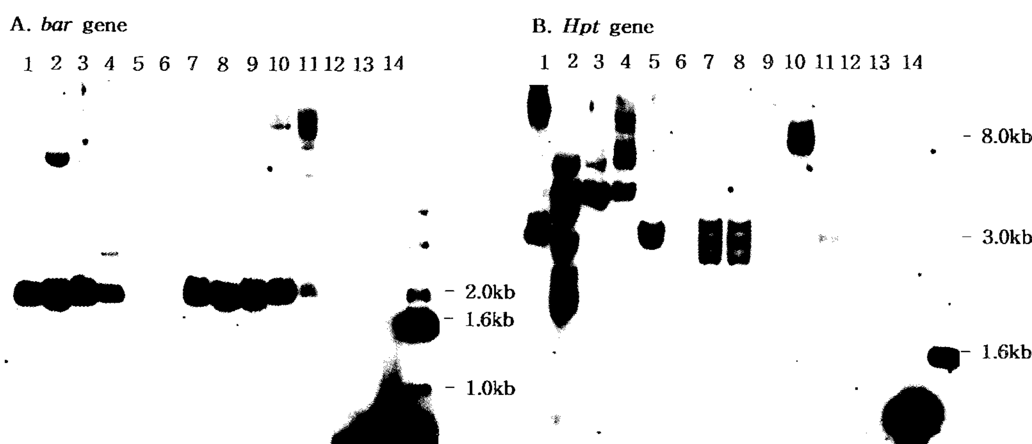

Figure 3. Genomic Southern blot analysis of T_0 plant transformed with *A. tumefaciens*. Total genomic DNA was digested with *Hin* dIII. The *bar* (A) and *hpt* (B) genes were used as probes. Lanes 1-11: transformed plants (Tb1-Tb11), lane 12: untransformed plant, lane 14: plasmid DNA of *bar* (A) and *hpt* (B) gene.

Table 1. Segregation of herbicide resistance in T_1 progeny of selfed transformants of cabbage.

Plant line	Number of T_1 progeny plants analysed	Number of Basta^R resistant plants	Number of Basta^R susceptible plants	$X^2(3:1)^a$
Tb5	31	24	7	0.01
Tb6	74	60	14	1.47
Tb25	40	30	10	0.00

[a]The difference from the expected 3:1 segregation is not significant at $P<0.05$

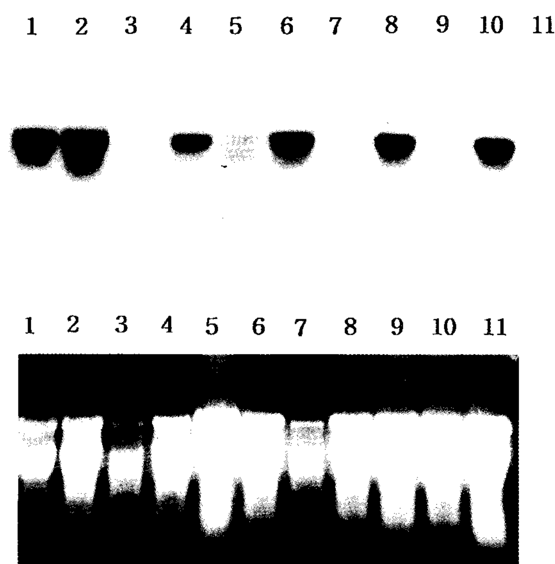

Figure 4. Northern blot analysis of T_0 plant transformed with *A. tumefaciens*. The *bar* gene was used as probe. Lanes 1-10: transformed cabbage (Tb1-Tb10), lane 11: untransformed cabbage.

growth were selfed by bud pollination. Twenty eight plants out of thirty five transformants set seed and the amount of T_1 seeds varied with each transgenic plant. Metz et al. (1995) reported that recovery of plants from seedling explants are more likely to be tetraploid, because both diploid and tetraploid plants can develop from the same callus. Thus, the inability to produce seeds may also be partially explained by intervening callus phase during the regeneration process.

In order to examine the segregation ratio in T_1 transformants, seven plants with more than 60 seeds among twenty eight T_1 transformants were tested for the herbicide resistance. Two-month-old greenhouse plants were sprayed with aqueous 0.6% solution of Basta. Three transformants (Tb5, Tb6, and Tb25) gave a progeny segregation ratio of 3:1 for resistance, which is the expected Mendelian inheritance of one independent locus (Table 1). The T_1 progeny of these three transformants showed normal growth and phenotype. The remaining transformants exhibited other segregation ratios (data not shown).

In order to evaluate the inheritance of the *bar* gene, PCR analysis was performed on the genomic DNA of T_1 progeny. The PCR products of *hpt* and *bar* genes showed the expected size (*hpt* gene: 367bp, *bar* gene: 490bp) in Tb5 and Tb6 T_1 progeny (Figure 5). In order to confirm the copy number of the *bar* gene, Southern blot analysis was performed on T_1 progeny genomic DNA of Tb5 and Tb6 transformants. A single band was observed in Tb6 line and two bands were observed in Tb5 line, suggesting

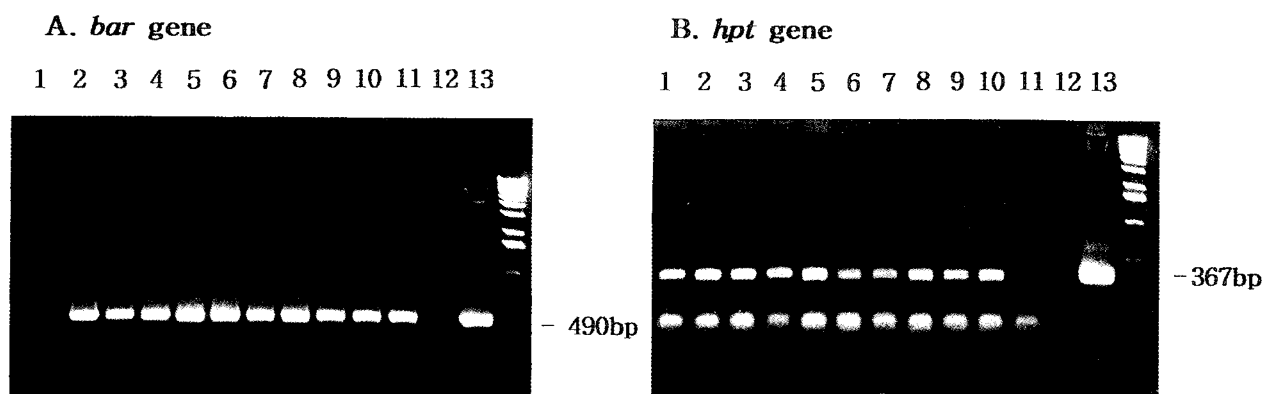

Figure 5. PCR analysis of T_1 progeny of Tb5 and Tb6 transgenic cabbage plants. A: lanes 1-11: transformants, lane 12: control, lane 13: plasmid DNA containing the *bar* gene. B: lanes 1-10: transformants, lane 11: control, lane 13: plasmid DNA containing the *hpt* gene.

that Tb6 and Tb5 lines contained one and two intact *bar* genes, respectively (Figure 6). However, the segregation of T_1 progeny in Tb5 line conformed to the Mendelian inheritance. This could be explained by the integration of the *bar* gene into the same locus.

In conclusion, transgenic cabbage plants resistant

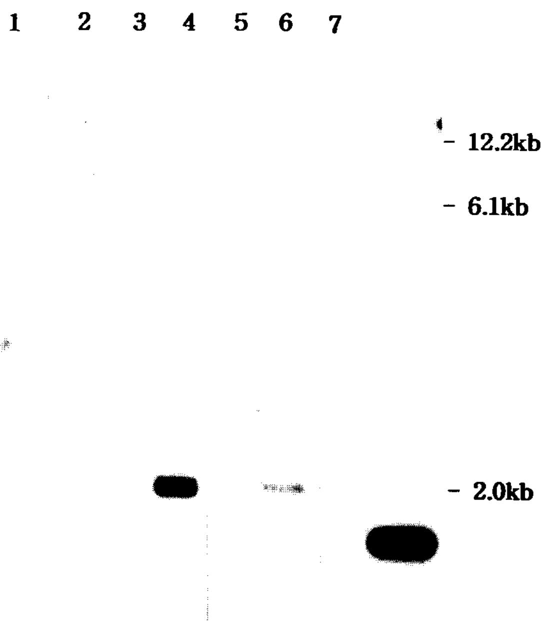

Figure 6. Genomic Southern blot analysis of T_1 progeny of Tb5 and Tb6 transgenic cabbage plants. The *bar* gene was used as probe. Lanes 1 and 2: Tb6 and Tb5 genomic DNA digested with *Bam*HI, lanes 3 and 4: Tb6 and Tb5 genomic DNA digested with *Hind*III, lane 5: untransformed cabbage, lanes 6 and 7: two copies of pMOG6-Bar and one copy of MOG6-Bar.

to herbicide were obtained from hypocotyl segments inoculated with *A. tumefaciens* LBA 4404 harboring pMOG6-Bar which contains *hpt* and *bar* genes. T_1 progeny showed strong herbicide resistance, Mendelian segregation ratio (3:1), and normal phenotype. In order to get homozygous plants with the *bar* gene, these plants were vernalized and are being selfed by bud pollination.

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