

Application of the acetolactate synthase gene as a cisgenic selectable marker for *Agrobacterium*-mediated transformation in Chinese cabbage (*Brassica rapa* ssp. *pekinensis*)

Ken-ichi Konagaya^a, Mai Tsuda, Ayako Okuzaki, Sugihiko Ando^b, Yutaka Tabei*

National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8602, Japan

*E-mail: tabei@affrc.go.jp Tel & Fax: +81-29-838-8372

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Abstract Heading Chinese cabbage (*Brassica rapa* ssp. *pekinensis*) has been recalcitrant to regeneration and transformation. In particular, there are few reports concerning Japanese cultivars. We evaluated the factors that inhibit *Agrobacterium*-mediated transformation in heading Chinese cabbage. Then, we investigated the effects of selectable markers using heading Chinese cabbage cv. Chihiri 70 and compared the effects with those in broccoli cv. Ryokurei (*B. oleracea* var. *italica*) as a control for an easily transformable *Brassica* species. To utilize a selectable marker derived from a host plant cisgene, we cloned a genomic DNA fragment containing regulatory and coding sequences of the acetolactate synthase (ALS) gene from Chinese cabbage and mutagenized it to a herbicide resistant form. After transformation of Chinese cabbage and broccoli with this construct, transgenic plants were efficiently selected with the herbicide bispyribac sodium salt and screened by DNA gel blot analysis. The average transformation frequency of Chinese cabbage was $1.2 \pm 0.2\%$, which was similar to those in reports using antibiotic selectable markers and was lower than for broccoli ($13.9 \pm 2.0\%$). Furthermore, the escape rate was restricted at a low level (about 35–50% lower than hygromycin selection), which is an advantage on practical transformation. We confirmed transgene inheritance and herbicide resistance of potted plants in the T₁ generation. This report is the first to describe a selection system for the transformation of a *Brassica* crop that uses a herbicide-tolerant selectable marker derived from a cisgene.

Key words: Acetolactate synthase, *Brassica oleracea*, *Brassica rapa*, cisgenic selectable marker, transformation.

The genus *Brassica* includes highly diverse crops that are grown and bred worldwide. In particular, *B. rapa* includes various vegetables such as Chinese cabbage, pak choi, turnip, and others. Chinese cabbage (*B. rapa* ssp. *pekinensis*) is one of the most important vegetables in Asia and is widely used for traditional fermented pickles and as a cooked vegetable. Although Chinese cabbages are classified as heading, semi-heading, and non-heading types based on the leaf morphology, the heading type accounts for the majority of Japan's Chinese cabbage market.

In August 2011, the *Brassica rapa* Genome Sequencing Project published a draft genome sequence of Chinese cabbage (Wang et al. 2011). The research group in Japan published 5476 independent full-length cDNA

sequences from approximately 10,000 clones (Abe et al. 2011). Since the gene sequences of Chinese cabbage have high similarity (ca. 90%) with those of the model plant *Arabidopsis thaliana* (Wang et al. 2011), information obtained using *A. thaliana* can be applied to studies of other plants in the family Brassicaceae. Genetic transformation is an essential tool for using these resources for reverse-genetic studies, molecular breeding, etc. Successful *Agrobacterium*-mediated transformation of *Brassica* crops has been reported in *B. oleracea*, *B. rapa*, *B. napus*, *B. juncea*, and *B. carinata* (reviewed in: Cardoza and Stewart 2004; Poulsen 1996; Sparrow et al. 2011). In particular, there are many reports of transformation for broccoli (*B. oleracea* var. *italica*) using *Agrobacterium tumefaciens* (Chen et al.

Abbreviations: 2,4-D, 2,4-dichlorophenoxy acetic acid; ALS, acetolactate synthase; BA, 6-benzylamino purine; BrALS3, *Brassica rapa* acetolactate synthase; BS, bispyribac sodium; GM, genetically modified; GUS, beta-glucuronidase; MS, Murashige and Skoog; NAA, Naphthaleneacetic acid; PCR, Polymerase chain reaction; RFLP, Restriction fragment length polymorphism; TAIL-PCR, thermal asymmetric interlaced PCR; T-DNA, transfer-DNA.

^a Present address: Forest Bio-Research Center, Forestry and Forest Products Research Institute, Hitachi, Ibaraki 319-1301, Japan.

^b Present address: Department of Life Science, Graduate School of Agricultural Science, Tohoku University, Sendai, Miyagi 981-8555, Japan.

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2001; Kuginuki et al. 2001; Metz et al. 1995; Suri et al. 2005), and transgenic broccoli has been subjected to field trials (Waterer et al. 2000). However, *B. rapa*, especially heading Chinese cabbage, is known as one of the most recalcitrant members of *Brassica* genus to transformation (Bagheri 2009; Kuginuki et al. 2001; Narasimhulu and Chopra 1988). Thus, it is essential to develop an efficient transformation method to overcome genotype dependency. The recalcitrance of Chinese cabbage to transformation could be due to the low efficiency of *Agrobacterium* infection, low regeneration efficiency of the infected tissues, and low selection efficiency for transformed cells (Lee et al. 2004; Takasaki et al. 1997). Bacterial antibiotic resistance genes such as *nptII* and *hpt* (Cho et al. 2001; Jun et al. 1995; Kuginuki et al. 2001; Lee et al. 2004; Park et al. 2005; Shin et al. 2003; Vanjildorj et al. 2009; Zhang et al. 2000), herbicide resistance genes such as *bar* (Lim et al. 2007), and a mannose-6-phosphate isomerase gene from *Escherichia coli* (Min et al. 2007) have been used as selectable markers for Chinese cabbage transformation. However, to date, no plant-derived gene has been used as a selectable marker for Chinese cabbage transformation.

Acetolactate synthase (ALS) catalyzes the first step in the biosynthesis of the essential branched chain amino acids, and it is a target enzyme for a number of herbicides. An amino acid substitution at a particular position in ALS has been shown to confer resistance to herbicides such as sulfonylureas and imidazolinones and to pyrimidinyl carboxy herbicides. Mutant forms of the *ALS* gene have been reported to be useful as a selectable marker in many plant species, such as potato (Andersson et al. 2003), oilseed mustard (Ray et al. 2004), rice (Okuzaki et al. 2007; Osakabe et al. 2005), maize (Zhang et al. 2005), cotton (Rawat et al. 2008), wheat (Ogawa et al. 2008), and soybean (Tougou et al. 2009). The use of an *ALS* gene derived from the host crop itself would be expected to result in a more publicly acceptable genetically modified (GM) crop compared with the use of a conventional antibiotic selectable marker.

In the present study, we examined differences in *Agrobacterium* infectivity and regeneration ability using the Japanese cultivar of heading Chinese cabbage with broccoli as a comparison control representing an easily transformable *Brassica* species. In addition, we successfully tested the use of a mutated *ALS* gene derived from Chinese cabbage as an effective *in vitro* selection marker for heading Chinese cabbage and for broccoli.

Materials and methods

Plant material

Chinese cabbage (*B. rapa* ssp. *pekinensis* cv. Chihiri 70) and broccoli (*B. oleracea* var. *italica* cv. Ryokurei) were used in this study. Hypocotyls were cultured according to Takasaki

et al. (1997) and Kuginuki and Tsukazaki (2001) with some modifications. The seeds (Takii Seed Co., Japan) were surface sterilized with sodium hypochlorite solution (0.5% active chlorite) plus 0.1% Tween-20 for 15 min and then rinsed three times with sterile distilled water. Plant boxes (60 mm×60 mm×100 mm; AGC Techno Glass Co., Japan) containing 0.8% agar-solidified MS medium (Murashige and Skoog 1962) were inoculated with 20–30 seeds each. The hypocotyls were excised from 1-week-old seedlings, cut into 7–10 mm segments, and cultured in 20×90 mm Petri dishes containing callus induction medium (BCC2 or BCC3 in the section mentioned below). All cultures were incubated at 22°C under a 16-h light/8-h dark photoperiod, except for the cocultivation in the dark. The plates were sealed with surgical tape (Nichiban, Japan).

Isolation of Chinese cabbage *ALS* gene and vector construction

Genomic DNA from the leaves of the Chinese cabbage cv. Muso (a common Japanese cultivar, Takii Seed Co.) was extracted using the DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The orthologous fragment of the *ALS* gene was isolated by polymerase chain reaction (PCR) using KOD-Plus-DNA polymerase (Toyobo, Japan) and the BnALS1F5 and BnALS1(WL)R primers (Table 1) derived from the *B. napus* gene (GenBank accession number Z11526) in the sequence database. The PCR product was cloned into the pCR-Blunt II-TOPO vector (Invitrogen, USA) and sequenced with an automated DNA sequencer (ABI PRISM 3100; Applied Biosystems, USA) using the BigDye Terminator Cycle Sequencing Kit (v.3.1; Applied Biosystems). The upstream and downstream regions of the Chinese cabbage *ALS* (*BrALS3*; GenBank accession number AB618066) fragment were isolated by thermal asymmetric interlaced PCR (TAIL-PCR; Liu et al. 1995) using specific primers (Table 1). The flanking region of the open reading frame (ORF) containing the promoter and terminator was amplified by PCR using specific primers (*BrALS-F-Pac* and *BrALS-R-Sbf*) with added restriction sites (Table 1). Double point mutations (Trp556Leu and Ser635Ile) of *BrALS3* for herbicide resistance were introduced by site-directed mutagenesis (Sambrook and Russell 2001). These mutations were matched to the amino acid substitutions in *Arabidopsis* or rice *ALS* genes that confer resistance to pyrimidinyl carboxy herbicides including bispyribac sodium (BS) (Kawai et al. 2010; Osakabe et al. 2005), since the positions of the amino acid substitutions involved in herbicide resistance are conserved among plants (Mazur and Falco 1989). The pSMAH-35S:GUS vector (Figure 1A, Konagaya et al. 2008) was constructed by the replacement of the beta-glucuronidase (*GUS*) gene from the binary vector pSMAH628Ω-M2GUS (H. Nakamura et al., in preparation) with the cauliflower mosaic virus (CaMV) 35S promoter and *Intron-GUS* from pIG121-Hm (Ohta et al. 1990). The pSMAA-35S:GUS vector (Figure 1B) was constructed by the replacement of the *HPT* cassette from pSMAH-35S:GUS with the mutated *ALS* (*BrmALS3*) cassette.

Table 1. Primers used.

Name	Sequence 5'-3' ^a	Application
BnALS1F5	TTACGCTCGTTTCCTCCGGCAAA	PCR for coding region of <i>BrALS3</i>
BnALS1F6	CTTGGCATGCACGGGACTGTGT	Specific primer 1 for TAIL-PCR for 5' upstream region of <i>BrALS3</i> ORF
AD20	NGTCGASWGANAWGAA	Arbitrary primer for TAIL-PCR for 5' upstream region of <i>BrALS3</i> ORF
BrALS-R3	GGCGTCTCTTGAAGGCGTCAGT	Specific primer 1 for TAIL-PCR for 3' downstream region of <i>BrALS3</i> ORF
BnALS1R2	ATCCGGCGAGGGACCTGTCTCTG	Specific primer 2 for TAIL-PCR for 3' downstream region of <i>BrALS3</i> ORF
BrALS-R4	GGGACCCGAAGTGGCAATGCAGA	Specific primer 3 for TAIL-PCR for 3' downstream region of <i>BrALS3</i> ORF
AD2	TCTTICGNACITNGGA	Arbitrary primer for TAIL-PCR for 3' downstream region of <i>BrALS3</i> ORF
BnALS1(WL)F	TGGTCATGCAATTGGAAGATCGGTT	Specific primer 2 for TAIL-PCR for 5' upstream region of <i>BrALS3</i> ORF
BnALS1(WL)R	AACCGATCTTCCAATTGCATGACCA	Site-directed mutagenesis for <i>BrmALS3</i> PCR for coding region of <i>BrALS3</i> Specific primer 3 for TAIL-PCR for 5' upstream region of <i>BrALS3</i> ORF Site-directed mutagenesis for <i>BrmALS3</i>
BnALS1(SI)F	TTACCGATGATCCCAATTGGTGGCA	Site-directed mutagenesis for <i>BrmALS3</i>
BnALS1(SI)R	TGCCACCAATTGGGATCATCGGTAA	Site-directed mutagenesis for <i>BrmALS3</i>
BrALS-F-Pac	<u>GT</u> TAA <u>TT</u> AACTGAAACAATGAGTGTGAAAG	PCR for <i>BrmALS3</i> cassette (<i>PacI</i> site are underlined)
BrALS-R-Sbf	<u>CCTGCAGGATGTTT</u> ATTTCAACACAGATGT	PCR for <i>BrmALS3</i> cassette (<i>SbfI</i> site are underlined)
mGUS-31	CCCAACCCGTGAAATCAAAAAACTC	<i>GUS</i> probe for Southern hybridization
759-L	ATGTTTGAACGATCGGGGAAAT	<i>GUS</i> probe for Southern hybridization
Bogai-F10	ATTACTATTTCGACACTGTTTGATTC	<i>Bogai</i> probe for Southern hybridization
Bogai-R10	ATACCACCACCAACCTAGTCTAC	<i>Bogai</i> probe for Southern hybridization
BoA3F11oku	GGTGAGAAGTAGAAACCCAACG	PCR for the <i>Bogai</i> gene
BoGAlut-R01	AGAGTCCACTGATTTTGAAACTCAAACAACC	PCR for the <i>Bogai</i> gene
BrALS9oku	GCTTTGCAAGGGATGAACAAGG	PCR for the <i>BrmALS3</i> gene
BrALS-R1866	GATGACATCCAACAGGTACGG	PCR for the <i>BrmALS3</i> gene
Actin-F	GTGACAATGGAAGTGAATGG	PCR for the <i>BrACT1</i> gene
Actin-R	AGACGGAGGATAGCGTGAGG	PCR for the <i>BrACT1</i> gene

^aI, Inosine; N, A or C or G or T; S, G or C; W, A or T.

The pSMAA-BoA3:Bogai vector (Figure 1C) was constructed by replacement of the *GUS* cassette from pSMAA-35S:GUS with the *Bogai* (Konagaya et al. 2008) cassette derived from a broccoli genome sequence.

Bacterial strains

Binary vectors were transferred to *A. tumefaciens* strain GV3101 (Koncz and Schell 1986) by the freeze-thaw method (An et al. 1988). The *Agrobacterium* cells were cultured overnight at 28°C in 50 ml tubes containing liquid Luria broth (pH 5.2) supplemented with 25 mg l⁻¹ gentamicin, 25 mg l⁻¹ rifampicin, 100 mg l⁻¹ spectinomycin, 10 mM MES, and 20 μM acetosyringone. The *Agrobacterium* cells were then centrifuged at 13,000×g for 1 min and resuspended to OD₆₀₀=0.1 with liquid cocultivation medium MS-1 (MS salts, 100 mg l⁻¹ myo-inositol, 1.3 mg l⁻¹ thiamine-HCl, 200 mg l⁻¹ KH₂PO₄, 1 mg l⁻¹ 2,4-D, 10 mM MES, 3% sucrose, pH 5.2) supplemented with 200 μM acetosyringone. The suspended cells were kept for 2 h at room temperature to ensure efficient induction of *vir* genes (Nanasato et al. 2011, 2012).

Transformation

Hypocotyl explants from the Chinese cabbage cv. Chihiri 70 and the broccoli cv. Ryokurei were precultured on BCC3 [B5 salts and vitamins (Gamborg et al. 1968), 1% sucrose, 0.4% gelrite, pH 5.8, 10 mg l⁻¹ 1-naphthaleneacetic acid (NAA)] and BCC2 media (B5 salts and vitamins, 1% sucrose, 0.4% gelrite, pH 5.8, 3 mg l⁻¹ NAA), respectively, for 3 days. For co-cultivation, hypocotyls were immersed in the bacterial suspension for 30 min, blotted on sterile paper to remove excess *Agrobacterium*, transferred onto 0.4% gelrite-solidified MS-1 medium, and cultured for 3 days in the dark. After co-cultivation, the explants from the cultivars were rinsed three times with sterile distilled water and cultured on BCC3 and BCC2 media, respectively, supplemented with 200 mg l⁻¹ carbenicillin for 7 days to eliminate *Agrobacterium* and induce calli. *Agrobacterium* infection efficiency was determined by performing a histochemical assay of transient GUS activity after decolonization and callus induction for 7 days. The cultured explants were transferred to BCR medium (BCR: B5 salts and vitamins, 3 mg l⁻¹ 6-benzylamino purine, 1 mg l⁻¹ zeatin, 10 mg l⁻¹ AgNO₃, 1% sucrose, 0.4% gelrite, pH 5.8)

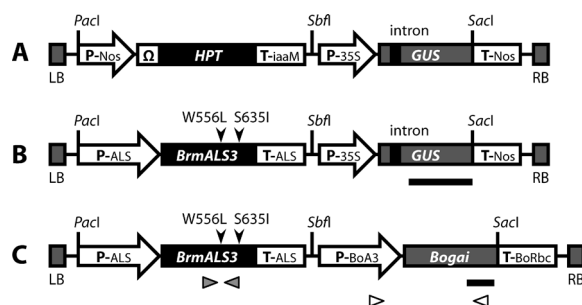


Figure 1. Schematic of the T-DNA region (not to scale) of transformation vector constructs. (A) pSMAH-35S:GUS, (B) pSMAA-35S:GUS, and (C) pSMAA-BoA3:Bogai. The vector backbone was pSMAH628 Ω -M2GUS (H. Nakamura et al. in preparation), which contains the spectinomycin resistance gene as a bacterial selectable marker. P-Nos, nopaline synthase promoter; Ω , omega sequence from *Tobacco mosaic virus*; HPT, hygromycin phosphotransferase gene; T-iaaM, polyadenylation signal from the *Agrobacterium iaaM* gene; P-35S, CaMV 35S promoter; GUS, beta-glucuronidase gene containing a castor bean intron; T-Nos, nopaline synthase terminator; *BrmALS3*, mutated acetolactate synthase gene derived from the *Brassica rapa* ssp. *pekinensis* cv. Muso gene *BrALS3*; P-ALS, *BrALS3* promoter; T-ALS, *BrALS3* terminator; P-BoA3, *B. oleracea* BoA3 promoter; *Bogai*, *B. oleracea* GAI mutant (Konagaya et al. 2008); T-BoRbc, *B. oleracea Rubisco* terminator (GenBank accession number AB745449); RB, right border; LB, left border. Black arrowheads represent the two mutation sites in *BrmALS3*: tryptophan to leucine at codon 556 (W556L) and serine to isoleucine at codon 635 (S635I). Open arrowheads and gray arrowheads represent the primer sets for *Bogai* and *BrmALS3* detection, respectively. Black bars indicate the GUS and *Bogai* probe regions used for DNA gel blot analysis.

supplemented with 200 mg l⁻¹ carbenicillin and selective agents (20 mg l⁻¹ hygromycin or 0.25 mg l⁻¹ BS; Wako, Japan) and subsequently subcultured every 2 weeks. After 3–4 weeks, calli with green shoots or green calli were transferred to a shoot maturation medium (B5 salts and vitamins, 1% sucrose, 0.4% gelrite, pH 5.8) supplemented with 200 mg l⁻¹ carbenicillin and the selective agents. When they reached ca. 2 cm in height, green shoots were excised from calli and placed on a root induction medium (MS half-salts and vitamins, 1 mg l⁻¹ NAA, 1% sucrose, 0.4% gelrite, pH 5.8) supplemented with 200 mg l⁻¹ carbenicillin and the selective agents. After 3 days, the green plantlets were transferred to plant boxes containing 0.4% gelrite-solidified MS medium supplemented with 200 mg l⁻¹ carbenicillin and the selective agents. The leaf segments of green shoots transformed with pSMAA-35S:GUS or pSMAH-35S:GUS were tested for GUS expression.

Histochemical GUS assay

GUS assays were conducted by a modification of a method originally described by Jefferson (1987). Hypocotyls or leaf segments were incubated for 12 h at 37°C in a GUS assay solution containing 100 mM sodium phosphate (pH 7.0), 0.3% Triton X-100, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 20% methanol, and 1 mM 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-gluc). The stained tissues were bleached by several changes of 70% ethanol.

DNA gel blot and PCR analyses

Genomic DNA from Chinese cabbage and broccoli were isolated as described by Ando et al. (2005). DNA (5 μ g) was digested with *Hind*III or *Xba*I. The digest was fractionated on a 0.9% agarose gel and transferred to a GeneScreen Plus membrane (PerkinElmer Life and Analytical Sciences) according to the manufacturer's instructions. The GUS and *Bogai* probes were prepared by PCR (amplified regions in Figure 1) using the primer sets mGUS-31/759-L and *Bogai*-F10/*Bogai*-R10, respectively (Table 1). Primer labeling, hybridization, and successive washings were performed as described by Ando et al. (2005). PCR was performed with KOD-Plus-DNA polymerase and gene specific primers. The PCR primer pairs (Table 1, Figure 1) were as follows: BoA3F11oku and BoGAIut-R01 for the *Bogai* gene, BrALS9oku and BrALS-R1866 for the *BrALS3* and *BrmALS3* genes, and Actin-F and Actin-R for the actin gene (*BrACT1*; GenBank accession number AJ242531). Cycling conditions were 94°C for 2 min; 35 cycles of 94°C for 30 s, 62°C for 15 s, and 68°C for 2 min; and a 10-min final extension at 68°C. To confirm the *BrmALS3* gene at the molecular level, we performed PCR restriction fragment length polymorphism (PCR-RFLP) analysis with *Mfe*I digestion (Osakabe et al. 2005), since the introduction of the mutations produced two new *Mfe*I sites in the *BrALS3* gene (Supplemental Figure S2).

BS herbicide treatment of T₁ progeny

The primary transgenic plants were vernalized at 4°C for approximately 1 month. Selfed progeny were then recovered by bud pollination. T₁ seeds were sown in petri dishes on water-saturated filter paper and incubated under continuous light at 27°C for 2 days. Germinated seeds were transferred into pots (6 cm diameter) with standard soil and cultivated in a growth chamber at 21°C under a 16-h light/8-h dark photoperiod. Seedlings were screened by PCR for the inheritance of the transgene. After 12 days of incubation, 25 g a.i. ha⁻¹ BS solution were sprayed over the leaves of the transgenic Chinese cabbage every other day for two weeks, and the growth of the herbicide-treated plants was assessed.

Results and discussion

Infectivity of *Agrobacterium* in Chinese cabbage and broccoli

Regeneration, as well as transformation, is difficult with *B. rapa* compared with *B. oleracea*, as described by Kuginuki et al. (2001). The difficulty in transforming *B. rapa* has been considered to be due to the low *Agrobacterium* infection efficiency and low regeneration efficiency of the infected tissues (Takasaki et al. 1997). To identify differences in *Agrobacterium* infectivity between Chinese cabbage (*B. rapa*) and broccoli (*B. oleracea*) we investigated infection efficiency by transient GUS expression in hypocotyls. Broccoli hypocotyls exhibited an infection efficiency of 61.2%. In contrast,

Chinese cabbage showed significantly higher infection frequencies of 99.2% (Figure 2, Table 2). Moreover, both the size and the number of blue spots on the cut ends of hypocotyls were considerably larger than in broccoli (Figure 2). The same result was obtained with the *B. rapa* variants Ryokusai No. 1 and Marubakomatsuna (data not shown). These results indicate that *Agrobacterium* infection efficiency of *B. rapa* is higher than that of broccoli, at least among the cultivars examined in this study. Consequently, difficulties in transforming *B. rapa* is thought to be because of low shoot regeneration

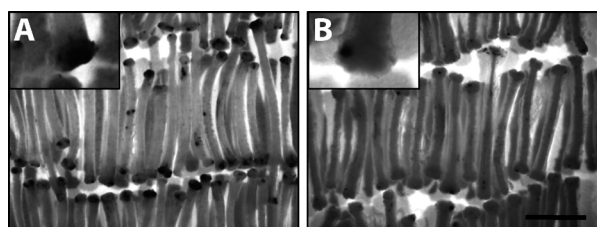


Figure 2. Histochemical detection of GUS activity in hypocotyls. (A) Chinese cabbage cv. Chihiri 70; (B) broccoli cv. Ryokurei. Hypocotyl segments of 7-day-old seedlings were cocultivated for 3 days with *Agrobacterium* harboring pSMAA-35S:GUS. Chihiri 70 was then cultured on BCC3 medium and Ryokurei was cultured on BCC2 medium for one week. The number of GUS-positive spots was significantly higher in Chihiri 70 explants (A) compared with Ryokurei explants (B). The inset shows a 3× magnified image of GUS-positive spots observed in the cut end of a hypocotyl segment. Bar, 5 mm.

Table 2. *Agrobacterium* infection frequency of Chinese cabbage cv. Chihiri 70 and broccoli cv. Ryokurei.

	Chihiri 70	Ryokurei
No. of infected explants	122	533
No. of GUS-positive explants	121	326
Infection frequency (%) ^a	99.2	61.2

^aInfection frequency (%)=(no. of GUS-positive explants/no. of infected explants)×100. A significant difference between Chihiri 70 and Ryokurei was found by Tukey's multiple comparison test ($p<0.001$).

Table 3. Transformation frequencies for Chinese cabbage cv. Chihiri 70 and broccoli cv. Ryokurei with 0.25 mg l⁻¹ bispyribac sodium (BS) and 20 mg l⁻¹ hygromycin as the selection agents. BCC3 and BCC2 were used for callus induction in Chihiri 70 and Ryokurei, respectively.

	Chihiri 70		Ryokurei	
	BS	Hygromycin	BS	Hygromycin
Total no. of infected explants	1320	1020	180	180
Total no. of green calli ^a	425	583	175	352
Total no. of green shoots ^b	22	21	29	39
Total no. of elongated plants ^c	15	11	25	29
Total no. of transformed plants ^d	15	11	25	27
Total no. of escape shoots ^e	7	10	4	12
Transformation frequency (%) ^{f,h}	1.2±0.2	1.1±0.1	13.9±2.0	15.0±1.7
Escape shoot inducing frequency (%) ^{g,h}	27.7±15.2	42.9±14.3	14.4±7.5	29.8±6.8

^aGreen callus produced on BCR medium containing the selective agent. ^bGreen shoots produced on shoot maturation medium containing the selective agent; each shoot was from an independent transformation event. ^cShoots elongated and rooted on hormone-free MS medium containing the selective agent. ^dDetermined by detection of *GUS* gene by DNA gel blot analysis. ^eNo. of escape shoots=no. of green shoots–no. of transformed plants. ^fTransformation frequency (%)=(no. of transformed plants/no. of infected explants)×100. ^gEscape shoot induction frequency (%)=(no. of escape shoot/no. of green shoots)×100. ^hData are mean values±standard errors from three independent experiments.

efficiency or insufficient selection efficiency by antibiotics (e.g., kanamycin or hygromycin).

Comparisons of selectable marker genes

From our results described above, regeneration efficiency from explants should be important for efficient transformation of Chinese cabbage. Chihiri 70 was used in our experiment because Chihiri 70 has been reported as a cultivar with high regeneration ability from cotyledonary explants (Zhang et al. 1998) and the ability was also confirmed by our preliminary regeneration test (Supplemental Figure S1) using hypocotyl explants. In addition, the shoot regeneration efficiency of Chihiri 70 in the optimal callus induction medium was comparable to that of broccoli (Supplemental Figure S1). On the other hand, improvement of selection efficiency should be also crucial points to examine the efficient transformation of *B. rapa*. For transformant selection, we tested the *BrmALS3* (binary vector pSMAA-35S:GUS) and *hpt* (binary vector pSMAH-35S:GUS) genes conferring resistance to BS and hygromycin, respectively. Transformants were obtained from all combinations of *Brassica* species and selection agents, and transformation frequency showed same efficiency between BS and hygromycin in both *Brassica* species (Table 3).

In Chihiri 70, *Agrobacterium*-infected hypocotyl explants showed green calli and shoot buds on cut ends of hypocotyls within 5 weeks when incubated in BCR medium containing BS or hygromycin for selection (Figure 3A). Each of the green calli or shoot buds regenerated 2–4 multiple shoots when cultured on shoot maturation medium for 30–45 days (Figure 3B). After cultivation on root induction medium for 3 days, regenerated shoots were transferred to hormone-free solid MS medium led to the formation of rooted plantlets within 20–30 days (Figure 3C). Histochemical GUS assays of the leaves from transformed lines exhibited characteristic blue color after incubation in X-gluc

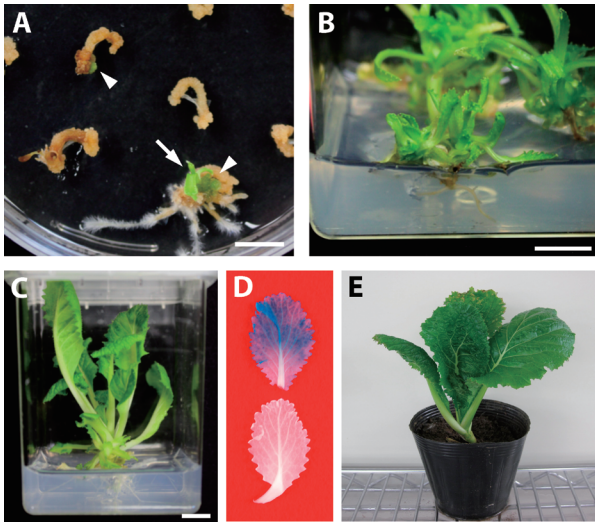


Figure 3. *Agrobacterium*-mediated transformation of Chinese cabbage cv. Chihiri 70 at different stages of *in vitro* regeneration and screening of transformed plants. (A) Appearance of green shoots (arrow) and green calli (arrow head) from hypocotyl segments on BCR medium containing 0.25 mg l^{-1} bispyribac sodium (BS). (B) Regeneration of multiple shoots from green callus on shoot maturation medium containing 0.25 mg l^{-1} BS. (C) Individual shoot in hormone-free MS media for elongation and rooting. (D) Histochemical GUS assay of transgenic (upper) and nontransformed (lower) leaves. (E) Potted plant in the greenhouse. Bars, 1 cm.

solution unlike leaves of untransformed plants (Figure 3D). Full-grown rooted plantlets were transferred to pots in the greenhouse (Figure 3E). The *GUS* gene was detected by DNA gel blot analysis in all elongated plants (Figure 4A, Table 3). The transformation frequencies of 1.2% and 1.1% were nearly the same with the BS and hygromycin selections, respectively. These frequencies with both the mutated *ALS* gene and *hpt* gene were approximately 1.6–2.7% (Zhang et al. 2000) and 1.1% (Kuginuki et al. 2001) reported using conventional methods with the *nptII* gene in the Japanese cultivar of heading Chinese cabbage. Ours is the first report to show that the mutated *ALS* and *hpt* genes are available as selectable markers for the Japanese cultivar of Chinese cabbage.

When Ryokurei explants were transferred to BCR media containing selection agents, most of the explants showed green callus or adventitious shoot formation in one or both of the cut ends of hypocotyls within 4 weeks. Resistant plantlets were obtained after 2 months on shoot maturation medium. Transformation frequencies, based on DNA gel blot analysis, were 13.9% for BS selection and 15.0% for hygromycin selection. These frequencies were higher than the previously reported 10% transformation frequency based on acquisition of kanamycin-resistant shoots (Kuginuki et al. 2001) and 4.3% transformation frequency using *GUS* (Mets et al. 1995).

With both Chihiri 70 and Ryokurei, the proportions

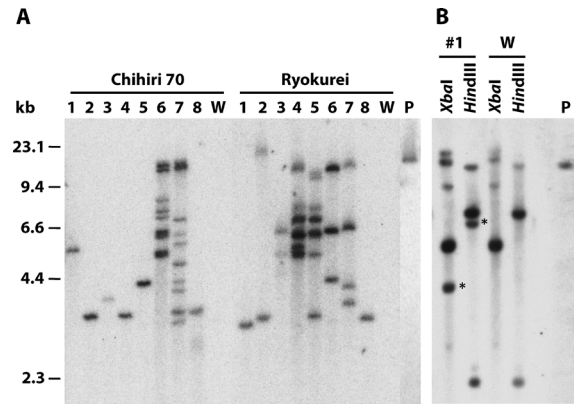


Figure 4. DNA gel blot analysis of transgenic Chinese cabbage cv. Chihiri 70 and broccoli cv. Ryokurei plants using BS as the selection agent. (A) *HindIII*-digested genomic DNA from a wild-type plant (W), eight primary transgenic Chihiri 70 plants, and eight primary transgenic Ryokurei plants transformed with pSMAA-35S:*GUS* were hybridized with a ^{32}P -labeled *GUS*-specific probe. Lane P, *SacI*-digested pSMAA-35S:*GUS* (50 pg). (B) *XbaI*- or *HindIII*-digested genomic DNAs from a primary transgenic Chihiri 70 plant transformed with pSMAA-BoA3:*Bogai* (#1) and from a wild-type plant (W) were hybridized with a ^{32}P -labeled *Bogai*-specific probe. Lane P, *SacI*-digested pSMAA-BoA3:*Bogai* (50 pg). Asterisks indicate bands derived from the transgene.

of escapes (no elongated shoots on selection medium or *GUS*-negative shoots by DNA gel blot analysis) in the BS selection were lower (27.7% and 14.4%, respectively) than in the hygromycin selection (42.9% and 29.8%, respectively). Furthermore, the number of green calli in the Ryokurei BS selection was more than half of the hygromycin selection, despite equal transformation frequencies. These results indicated that selection on BS reduces escape, thereby contributing to transformation efficiency.

Genetic analysis of transgenic plants

To confirm the stable integration of the transgenes into the plant genomes, DNA gel blot analysis was performed on all elongated plants on MS medium containing BS using a *GUS* probe. Figure 4A shows DNA gel blot analysis of representative transgenic plants transformed with pSMAA-35S:*GUS*. The *GUS* probe hybridized to digested DNA from all BS-resistant plants, but not to digested DNA from the wild-type plant (Figure 4A, Table 3). These results indicate that the transgene is almost certainly incorporated into the plant genome in the elongated plants obtained by BS selection. The copy number of the transgene varied from 1 to 10, although there were no relationships between the *Brassica* species or selective agent and the copy number.

We attempted to introduce a useful trait, such as male sterility, into Chinese cabbage using the *BrmALS3* selectable marker system. pSMAA-BoA3:*Bogai* (Figure 1C) contained the stamen-specific promoter (BoA3) driving the gibberellin insensitive (*Bogai*) gene and the

Rubisco gene terminator. This *Bogai* cassette, derived from the broccoli cv. Ryokurei, confers male sterility on *A. thaliana* (Konagaya et al. 2008) and broccoli (Konagaya et al. in preparation). Chihiri 70 hypocotyl explants were inoculated with *Agrobacterium* harboring pSMAA-BoA3:*Bogai* and selected on BS-supplemented medium as described above. The transformation frequency was 1.0%, approximately equal to the transformation frequency with pSMAA-35S:*GUS* (Table 3). DNA gel blot analysis of line #1 transformed with pSMAA-BoA3:*Bogai* is shown in Figure 4B. Although several bands were detected in the wild type because of hybridization of the *Bogai* probe to the endogenous *GAI* gene, a new band was detected in line #1 (Figure 4B). As a result, we considered the transgene in line #1 to be a single copy. However the pollen of the vernalized transformant (line #1) was observed (data not shown). Since the recovery of male sterile lines from *BoA3:Bogai* transformants was low in *Arabidopsis* and broccoli (Konagaya et al. 2008; Konagaya et al. in preparation), obtaining a male sterile line using *BoA3:Bogai* may require production of many independent transformants and selection of them. Transgene inheritance was confirmed in T_1 -generation Chinese cabbage plants using genomic PCR analysis and PCR-RFLP. Amplification of *Bogai* was observed in the T_1 generation plants (Figure 5A), indicating successful transgene inheritance in the next generation. In addition, PCR-RFLP showed that the *BrmALS3* gene was also inherited in *Bogai* T_1 progeny (Supplemental Figure S2). The positive:negative segregation ratios fitted the expected ratios of 3:1 at a probability level of 5% ($\chi^2=0.22$, $p=0.64$) by the chi-squared test, suggesting that the transgenes were inherited as a single locus and corresponding to the DNA gel blot analysis (Figure 4B).

Herbicide tolerance in T_1 progeny

To confirm herbicide resistance in T_1 progeny with the *BrmALS3* gene, we treated T_1 Chihiri 70 plantlets with a BS dilution series (0, 0.5, 1.0, 6.3, 25.0, and 50.0 g a.i. ha⁻¹) in preliminary examination. We found that 25 g a.i. ha⁻¹ was an appropriate concentration that completely killed non-transgenic Chihiri 70. After 2 weeks, the treated leaves of the *Bogai* intact progeny (Figure 5B, #1-1) were alive and green, whereas the leaves of the control wild-type plants and the *Bogai*-deleted progeny (Figure 5B, #1-4) turned brown and gradually died. These results suggested that the *BrmALS3* gene could be introduced into Chinese cabbage as a selectable marker gene and that transgenic Chinese cabbage could regenerate through BS selective culturing.

Conclusions

The transformation efficiency of Chinese cabbage

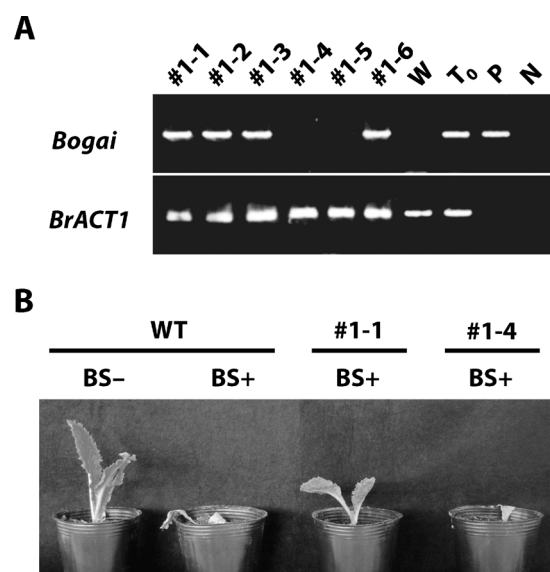


Figure 5. Genomic PCR analysis (A) and BS resistance (B) of T_1 plants from pSMAA-BoA3:*Bogai* transformants. (A) Upper and lower panels show the PCR results obtained using *Bogai*-specific primers (Figure 1; 1.9 kb) and the actin gene (*BrACT1*, 0.5 kb), respectively. The *BrACT1*-specific primer was used to verify the quality of the genomic DNA samples. Lanes #1-1–#1-6, independent T_1 progeny from line #1; Lane W, wild-type plant; Lane T_0 , line #1 primary transformant; Lane P, pSMAA-BoA3:*Bogai* (positive control); Lane N, without template DNA (negative control). (B) BS sensitivities after two weeks of treatment (25 g a.i. ha⁻¹). The T_1 progeny that inherited the *Bogai* gene (#1-1) exhibited vigorous growth under BS treatment, whereas the WT and the T_1 progeny that had lost the insertion of pSMAA-BoA3:*Bogai* (#1-4) were killed. BS+ and BS– indicate treatment with or without BS, respectively.

was lower than that of broccoli, despite a higher *Agrobacterium* infection efficiency (Figure 2). This result indicates that improvement of regeneration and selection efficiency should be required for developing more effective transformation system in Chinese cabbage. Reliable and efficient transformation system should contribute to produce novel varieties and to promote basic research with utilizing information of genome sequence of Chinese cabbage (Wang et al. 2011). Here, we have shown that the *BrmALS3* gene can be used as an effective selectable marker in Chinese cabbage and broccoli. Cisgenesis is new plant breeding techniques (Lusser et al. 2012) based on the use of natural genes (cisgenes) from the target plant itself or from a cross compatible species. This report demonstrates for the first time that a mutated *ALS* gene under the control of a promoter and terminator derived from the cisgene is available as a selectable marker for *Agrobacterium*-mediated transformation in *Brassica* species. Selectable markers derived from familiar food plant genes may help promote public acceptance of GM crops. Furthermore, use of cisgenes may allow for exceptions or mitigations of food safety regulations if the European Food Safety Authority (EFSA) Panel concludes that any hazards

associated with cisgenic plants are similar to those associated with conventionally bred plants (EFSA Journal 2012; 10(2): 2561; <http://www.efsa.europa.eu/en/efsajournal/pub/2561.htm>). The transformation techniques based on cisgenesis in this study will be informative to address both environmental and consumer concerns for GM agriculture.

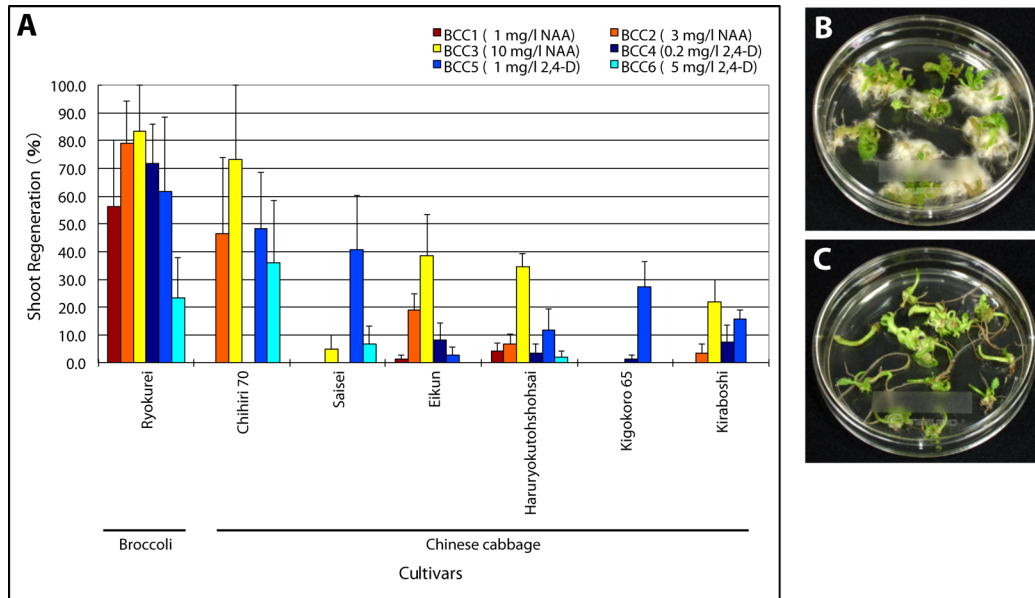
Acknowledgments

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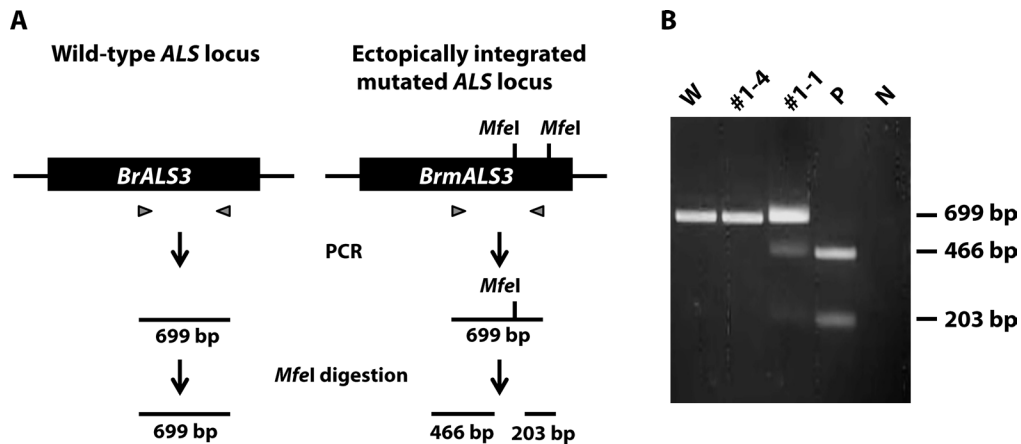
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Supplemental Figure S1. (A) Shoot regeneration from hypocotyl explants of Chinese cabbage (*B. rapa* ssp. *pekinensis*) and broccoli (*B. oleracea* var. *italica*). Hypocotyl segments of 7-day-old seedlings were cultured on six different callus induction medium [B5 salts and vitamins (Gamborg et al. 1968), 1% sucrose, 0.4% gelrite, pH 5.8, 1 mg l⁻¹, 3 mg l⁻¹, or 10 mg l⁻¹ 1-naphthaleneacetic acid (NAA), or 0.2 mg l⁻¹, 1 mg l⁻¹, or 5 mg l⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D), corresponding to BCC1–6, respectively] for 2 weeks. The explants were transferred to shoot regeneration medium (BCR: B5 salts and vitamins, 3 mg l⁻¹ 6-benzylamino purine, 1 mg l⁻¹ zeatin, 10 mg l⁻¹ AgNO₃, 1% sucrose, 0.4% gelrite, pH 5.8) and subsequently subcultured every 2 weeks. After 5 weeks, the numbers of explants forming adventitious shoots were counted. Data are from three replicates with 10 hypocotyl explants for each replicate. Bars represent standard errors of the means. (B, C) Shoots developing from hypocotyl explants of Chinese cabbage cv. Chihiri 70 (B) and broccoli cv. Ryokurei (C). Hypocotyl segments of Chihiri 70 and Ryokurei were cultured on BCC3 and BCC2 media, respectively, for 2 weeks and then cultured on BCR medium for 5 weeks.



Supplemental Figure S2. (A) Schematic of the PCR-RFLP assay. Black boxes represent the ORFs of the ALS genes. Arrowheads represent the primer sets for *BrALS3* and *BrmALS3* detection. (B) PCR-RFLP assay of T1 progeny. *MfeI*-digested genomic PCR products were separated by 2% agarose gel electrophoresis. Lane W, wild type; Lanes #1-4 and #1-1, independent T1 progeny [#1-4 and #1-1 correspond to *Bogai*-lost/BS-sensitive and *Bogai*-inherited/BS-resistant progeny, respectively, in Figure 6]; Lane P, pSMAA-BoA3:*Bogai* (positive control); Lane N, without template DNA (negative control).