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Introduction of resistance to herbicide *Basta*® in Savoy cabbage

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Abstract

Resistance to herbicide *Basta*® was introduced into pure inbred lines of Savoy cabbage (*Brassica oleracea* L. var. *sabauda*) by cocultivation of cotyledon and hypocotyl explants with *Agrobacterium tumefaciens* strains AGL1/pDM805 and LBA4404/pGKB5 (LB5-1). Shoot regeneration occurred on Murashige and Skoog medium supplemented with 1 mg dm^{-3} 6-benzyladenine and 0.5 mg dm^{-3} indole-3-butyric acid at 42.3 % and 71.4 % of hypocotyl explants treated with AGL1/pDM805 and LB5-1, respectively. Putative transformants that survived selection on 10 mg dm^{-3} phosphinothricin (L-PPT) supplemented medium were confirmed by GUS assay and PCR analysis. The transformation rate was 58 % with AGL1/ pDM805 and 25 % with LB5-1. Rooted plantlets were acclimated and then again screened for *Basta*®-resistance by spraying with 15 - 60 mg dm^{-3} L-PPT. Surviving plants were selfed and *Basta*®-resistance was demonstrated in T_1 progeny.

Additional key words: *Agrobacterium*, *bar* gene, *Brassica oleracea* var. *sabauda*, transformation.

Introduction

Brassica oleracea with its numerous varieties comprise some of the most important vegetable species of the Balkan region. One of them, *Brassica oleracea* var. *sabauda*, is native to south-east Europe. Constant improvement of various agronomical traits is a permanent task of cabbage breeders for which methods of genetic engineering have been adopted recently. Most of the research on transgenic plants in the *Brassica* group has been dedicated to *B. napus* (Poulsen 1996). Among *B. oleracea* species most of transformation research was done on cauliflower and broccoli (Puddephat *et al*. 1996). There is no data for transformation of Savoy cabbage.

Basta® is a commerial name of herbicide phosphinothricin (L-PPT) and for successful application of this non-selective herbicide breeding plants with specific resistance is necessary.

We report here the results of our study on the genetic transformation of *B. oleracea* var. *sabauda* L. using two different *A. tumefaciens* strains both carrying *bar* gene which provides *Basta*® resistance. A highly efficient system of transformation and regeneration was established.

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Abbreviations: BA - 6-benzyladenine, GUS - β-glucuronidase; IBA - indole-3-butyric acid; L-PPT - phosphinothricin; MS medium - Murashige and Skoog medium; PCR - polymerase chain reaction.
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Materials and methods

Plants: Transformation studies were performed on leading inbred line (Gg-1) of Savoy cabbage (*B. oleracea* L. var. *sabauda*), obtained and used in conventional breeding programs of the Centre for Vegetable Crops, Smederevska Palanka. Seeds were rinsed 1 min in 70 % ethanol, surface sterilized for 20 min in 15 % commercial bleach (4 - 6 % NaOCl) and then germinated on hormone-free basal Murashige and Skoog (1962; MS) medium, containing inorganics, vitamins and cofactors according to MS with 0.64 % agar and 2.0 % sucrose .

Bacterial strains: Two *Agrobacterium tumefaciens* strains used in experiments were: AGL1/pDM805 and LBA4404/pGKB5 (LB5-1). Plasmid pDM805 (Tingay *et al*. 1997) contains a chimeric *Streptomyces hygroscopicus* phosphinothricin acetyl transferase gene (*bar*) under the control of the promoter from the maize ubiquitin 1 gene (*Ubi*1) and a chimeric *E. coli* β-glucuronidase (GUS) gene *uidA* (Jefferson *et al*. 1987) under the control of the promoter from the rice actin 1 (*Act*1) gene. Plasmid pGKB5 (Bouchez *et al.* 1993) has the *uidA* gene without promoter, the *npt*II gene under control of nopaline synthase promoter and *bar* gene with the 35S promoter.

The *Agrobacterium* strains were maintained on YEB medium (Van Larebeke *et al*. 1977) supplemented with 20 mg dm^{-3} rifampicin and 5 mg dm^{-3} tetracycline for *A. tumefaciens* AGL1/pDM805 and 100 mg dm^{-3} kanamycin and 50 mg dm^{-3} rifampicin for *A. tumefaciens* LB5-1. Bacterial suspensions were incubated at 28 °C without shaking for 24 h prior to inoculation.

Transformation and plant regeneration: Hypocotyl and cotyledon segments were excised from 10-d-old seedlings and pre-cultured for 48 h on the regeneration medium (BM medium supplemented with 1.0 mg dm^{-3} BA and 0.5 mg dm^{-3} IBA). The preconditioned explants were immersed for 10 to 15 min in the *Agrobacterium* suspension, washed in 500 mg dm^{-3} *Tolycar* (cefotaxime, *Jugoremedija*, Zrenjanin, Serbia and Montenegro) for 20 min, briefly dried on filter paper and placed on the same media. After 2 d of co-cultivation AGL1/pDM805 explants were transferred to the regeneration medium supplemented with 500 mg dm^{-3} *Tolycar* and 2.0 mg dm^{-3} $AgNO_3$ or without $AgNO_3$. For cultures cocultivated with *A. tumefaciens* LB5-1, 25 mg dm^{-3} kanamycin was added to the regeneration medium.

Shoots which appeared on hypocotyl and cotyledon explants cultured on regeneration medium after 5 weeks were further transferred to the selection medium with 10 mg dm^{-3} L-PPT and 200 mg dm^{-3} *Tolycar*. Antibiotic concentration was gradually reduced in every subculture (5 weeks) in order 200 → 100 → 50 → 0 mg dm^{-3}. Surviving shoots were multiplied on MS medium with 0.5 mg dm^{-3} BA and 0.1 mg dm^{-3} IBA, rooted for 5 weeks on MS medium with 4 % sucrose and 4.0 mg dm^{-3} IBA and then acclimated in a greenhouse. Plants were sprayed with L-PPT at 15 - 60 mg dm^{-3}. Growth room conditions were: temperature 22 ± 2 °C, 16-h photoperiod with irradiance of 33.5 - 46.5 μmol m^{-2} s^{-1}. After acclimatization, vernalization was performed for 8 - 12 weeks at temperature of 4 - 8 °C.

Histochemical localization of GUS expression was done according to Jefferson *et al*. (1987). The putative transformed plants and the T_1 transgenic plants were analyzed by the polymerase chain reaction. Plant DNA was isolated according to Xiaomei *et al*. (1994). Polymerase chain reaction (PCR) was performed to amplify a 366-bp fragment of GUS gene. The primers used were: 5'-TAGCGGGACTTTGCAAGTG-3´ and 5´-GTTTTTGCAGCAGAAAAGCC-3´. Before amplification, samples were denaturated at 95 °C for 4 min. PCR reaction comprised 39 cycles. Each of these cycles consisted of a denaturation step at 95 °C for 1 min, followed by annealing at 55 °C for 2 min and a polymerisation reaction at 72 °C for 3 min. PCR products were visualised after electrophoresis on a 1 % agarose gel stained with ethidium bromide under UV light. PCR was carried out using the PE Applied Biosystems kits (*Roche*, New Jersey, USA).

Statistical analysis: For statistical evaluation of differences between the growth parameters of transformed and control plants Student *t*-test was used. Data are means of three independent experiments.

Results

Shoot regeneration: Hypocotyl and cotyledon explants of Savoy cabbage Gg-1 transformed with two *A. tumefaciens* strains manifested high potential for shoot regeneration. After 35 d on the regeneration medium 42.3 % of hypocotyl explants inoculated with AGL1/pDM805, 71.4 % of hypocotyl explants inoculated with LB5-1 and 60 % of control explants regenerated shoots (Table 1). Percentage of shoot regenerated cotyledon explants was lower, 31.2, 42.9, and 36.2 %, respectively. Shoot regeneration was accompanied with callus proliferation which did not affect it. Average number of shoots regenerated per explants was also high, especially in hypocotyl explants, but lower than in control (Table 1).

Table 1. Shoot regeneration from explants (C - cotyledon, H - hypocotyl) inoculated with two *A. tumefaciens* strains after 35 d on the regeneration medium (MS medium supplemented with 1.0 mg dm^{-3} BA and 0.5 mg dm^{-3} IBA) with or without 25 mg dm^{-3} kanamycin (Kn) or 2.0 mg dm^{-3} $AgNO_3$ (Ag). Means ± SE.

Bacterial strain	Number of explants		Kn	Ag	Explants with shoots [%]		Number of shoots [explant^{-1}]	
	C	H			H	C	H	C
LB5-1	320	350	-	-	71.4	42.9	8.70 ± 0.67	3.00 ± 0.68
	336	320	+	-	61.5	35.1	6.90 ± 0.95	1.60 ± 0.66
AGL1/pDM805	316	378	-	-	42.3	31.2	3.70 ± 0.32	13.00 ± 0.11
	295	317	-	+	34.6	27.4	2.90 ± 0.95	1.50 ± 0.05
Control	36	42	-	-	60.0	36.2	11.70 ± 0.96	3.15 ± 0.10

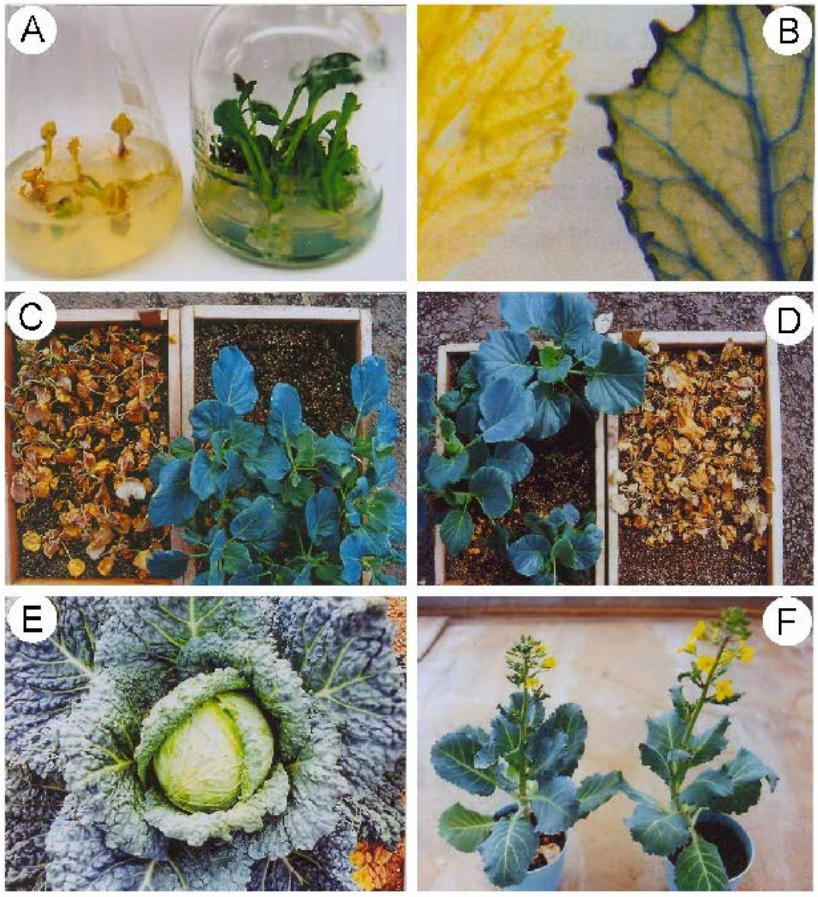

Fig. 1. *A. tumefaciens* mediated transformation of *B. oleracea* var. *sabauda* L. (Gg-1 line). *A* - Shoot culture transformed with *A. tumefaciens* AGL1/pDM805 (*right*) and the control culture (*left*) on the selective media containing 10 mg dm^{-3} L-PPT. *B* - Leaves of control (*left*) and AGL1/pDM805 transformed plants (*right*) after GUS staining. *C* - *A. tumefaciens* AGL1/pDM805 transformed plants (*right*) and untransformed control (*left*) after spraying with 10 cm^3 dm^{-3} *Basta*®. *D* - Plants transformed by *A. tumefaciens* LB5-1 (*left*) and untransformed control (*right*) after spraying with 10 cm^3 dm^{-3} *Basta*®. *E* - Acclimated Savoy cabbage plant transformed with *A. tumefaciens* AGL1/pDM805. *F* - Flowering AGL1/pDM805 transformed plants.

Addition of silver nitrate at 2.0 mg dm^{-3} did not improve explants growth and their shoot regeneration capacity, on the contrary, it significantly decreased it. Kanamycin used as a selective agens in LB5-1 treated explants also decreased callus proliferation and shoot regeneration (Table 1).

Efficiency of transformation: The passive selection pressure induced by addition of 10.0 mg dm^{-3} L-PPT in the medium enabled high survival and regeneration rates of *Agrobacterium* treated explants (Fig. 1*A*). It was 58 % for AGL1/pDM805 inoculated explants and 25 % for LB5-1 inoculated explants. A small percentage of shoots,

up to 5 %, survived the selective pressure imposed by 100 mg dm^{-3} kanamycin applied to LB5-1 treated explants.

First test used for evaluation of transformed shoots was the GUS histological test. AGL1/pDM805 regenerants had 82 % percentage of GUS positive shoots. No GUS positive shoots observed in LB5-1 transformants is expectable result since in this construct GUS gene is without promoter. It should be noted that shoots with positive GUS reaction were not found in control (Fig. 1*B*). PCR analysis confirmed our presumption that GUS positive shoots regenerated on selective media were transformed (Fig. 2*A*). PCR positive plants were further multiplied on MS medium with 0.5 mg dm^{-3} BA and 0.1 mg dm^{-3} IBA on which some plants already formed roots (Table 2). 100 % of rooting for both strains was achieved on MS medium with 40 g dm^{-3} sucrose and 4.0 mg dm^{-3} IBA. All the cultures showed no hyperhydricity and little differences in comparison to cultures of untreated control plants. Differences were mainly in values registered for standard culture parameters like shoot length or root length (Table 3).

Table 2. The growth parameters of transformed and control shoots after 35 d on multiplication medium (MS medium suplemented with 0.5 mg dm^{-3} BA and 0.1 mg dm^{-3} IBA). Means ± SE, * - statistically significant at $P < 0.05$.

Strain	Number of shoots	Shoot length [mm]	Number of leaves	Number of axillary buds	Rooted shoots [%]	Number of roots [$plant^{-1}$]
AGL1	30	39.00 ± 7.29*	25.00 ± 8.08*	5.30 ± 1.58*	80.0	7.17 ± 4.56*
LB5-1	32	35.59 ± 8.37	15.72 ± 2.07*	3.25 ± 1.57*	31.2	2.12 ± 1.66*
Control	37	33.97 ± 4.75	8.16 ± 1.07	7.59 ± 1.36	37.8	0.95 ± 1.27

Table 3. Comparing analysis of transformed and control clones after 35 d on rooting medium (MS medium with 4 % sucrose and 4.0 mg dm^{-3} IBA). Means ± SE, *,** - statistically significant at $P < 0.05$ and < 0.01, respectively.

Strain	Number of shoots	Shoot length [mm]	Root length [mm]	Vitrified plants [%]
AGL1	119	49.23 ± 3.57*	71.50 ± 7.76**	0.00
LB5-1	114	54.21 ± 16.86*	70.89 ± 14.19	1.96
Control	59	34.29 ± 1.04	55.44 ± 6.25	0.00

Regenerated plants were transferred to greenhouse condition. Acclimation efficiency was 100 % for shoots transformed with AGL1/pDM805, 85.7 % for shoots transformed with LB5-1, and 90 % for control plants.

Final test comprised of spraying acclimated plants with *Basta*® at concentration twice higher than in standard weed protection scheme (10 cm^3 dm^{-3} *Basta*® = 30 mg L-PTT). PCR positive Savoy cabbage plants survived spraying with 30 and 60 mg dm^{-3} L-PPT which was lethal for control nontransformed plants (Fig. 1*C*,*D*). PCR positive plants had a transient leaf discoloration few days after spraying but they quickly recovered and successfully survived another spraying with the same concentration of *Basta*® one month later. Plants that survived *Basta*® spraying were planted in the open field where they continued growing and showed unchanged phenotype (Fig. 1*E*).

Part of PCR positive and L-PPT resistant plants were brought to maturity and selfed (Fig. 1*F*). Not many seeds were produced, but plants from T_1 generation were also PCR positive (Fig. 2*B,C*) and resistant to *Basta*® sprays. This evidence indicates stable integration and expression of the *bar* gene in transformed Savoy cabbage plants.

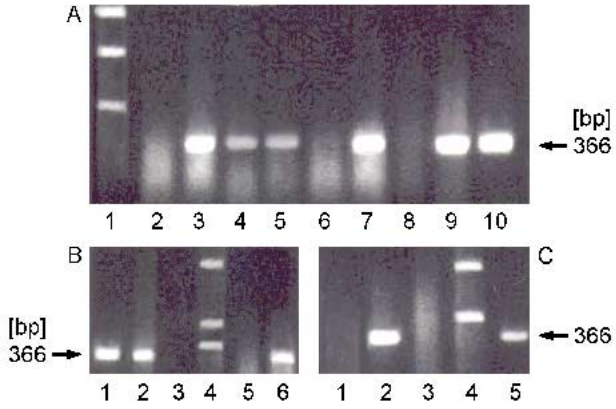

Fig. 2. PCR analysis of Savoy cabbage Gg-1 line transformed T_0 plants (*A*) and their T_1 progeny (*B,C*). *A*: *lane1* - 1 kb DNA ladder; *lane 2* - untransformed control plant; *lanes 3, 4* and *5* - plants transformed with AGL1/pDM805; *lane 6* - untransformed control plant; *lane 7* - plant transformed with LB5-1; *lane 8* - blank; *lane 9* - vector AGL1/pDM 805; *lane 10* - vector LB5-1. *B: lanes 1* and *2* - AGL1/pDM805 transgenic plants; *lane 3* - blank; *lane 4* - 1 kb DNA ladder; *lane 5* - untransformed control plant; *lane 6* - vector AGL1/pDM805. *C: lane 1* - blank; *lane 2* - LB5-1 transgenic plant; *lane 3* - untransformed control plant; *lane 4* -1 kb DNA ladder; *lane 5* - vector LB5-1.

Discussion

The primary aim of our work to develop reliable and high-frequency system for introducing *Basta*® resistance in pure inbred lines of Savoy cabbage was successfully achieved. Two different strains of *A. tumefaciens* both carrying the *bar* gene provided regenerants which could survive spraying with *Basta*® at concentrations up to four times higher than those recommended. The AGL1/pDM805 vector in general was a better choice than LB5-1, observed mainly from higher survival percentages (58 % vs. 25 %) and the overall ease of operation. Kanamycin resistance is the most frequently used marker in *Brassica* transformation (Passelegue and Kerlan 1996, Babić *et al*. 1998, Radčuk *et al*. 2000). However, it is known that kanamycin can inhibit shoot regeneration in explants (Pua *et al*. 1987, Thomzik 1993). The similar effect was seen in the present study.

Segments of hypocotyls and cotyledons are the most frequently used explants for *Brassica* transformation. Both hypocotyl and cotyledon explants in our case provided high regeneration capacity and shoot cultures established from these two sources did not differ. Some authors consider silver nitrate as an absolute prerequisite for obtaining transformed *Brassica* shoots (De Block *et al.* 1989, Pental *et al*. 1993). Schröder *et al*. (1994) showed a ten times improved shoot regeneration efficiency of non-transformed hypocotyl explants treated with silver nitrate but in transformants of *B. oleracea* var. *capitata* such effect could not be observed (Radčuk *et al*. 2000). In our study silver nitrate was detrimental since it reduced regeneration efficiency of shoots and callus in applied concentration.

The expression of the GUS-intron gene is a reliable indicator of plant transformation, since the intron-containing *uid*A gene can express efficiently in plant cells but not in *Agrobacterium* (Hu *et al.* 2002). Procedure which we developed enabled very high transformation efficiency, 82 %. In other *Brassicaceae,* transformation efficiency based on GUS assay was 30 - 50 % in *B. carinata* (Babić *et al*. 1998), 30 % in *B. napus* and *B. oleracea* var. *botrytis* (De Block *et al*. 1989, Passelegue and Kerlan 1996) and 2 - 3 % in *B. oleracea* var. *capitata* (Radčuk *et al*. 2000). The high transformation efficiency can be result of adequate preconditioning (48 h) and cocultivation time (48 h) as was reported in *Brasica napus* transformation (Cardosa and Stewart 2003).

It is interesting to note that the same vectors had much lower transformation efficiencies in other species. Thus in *Triticum aestivum* L. efficiency of transformation with AGL1/pDM805 was 0.12 % (Mitić *et al*. 2004) and 3.3 - 6.0 % for *Lotus corniculatus* L. (Nikolić 2000, Nikolić *et al*. 2003/4).

After spraying with four times increased concentration of *Basta*® herbicide transformed plants survived the treatment and control plants died. This was confirmation of *bar* gene activity. Evidence collected from GUS assay, PCR analysis and *bar* gene expression provided proof of integrative transformation of Savoy cabbage by *A. tumefaciens*. An efficient protocol for the production of transgenic Savoy cabbage plants was developed. Advantage of our system is that obtained transgenic plants are normal with respect to morphology.

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