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Agrobacterium rhizogenes-mediated transformation of *Brassica oleracea* var. *sabauda* and *B. oleracea* var. *capitata*

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Abstract

Agrobacterium rhizogenes A4M70GUS-mediated transformation of Savoy cabbage (*Brassica oleracea* L. var. *sabauda*) and two local lines of cabbage (*B. oleracea* L. var. *capitata*) was obtained using hypocotyl and cotyledon explants. The percentage of explants which formed roots was very high in all genotypes: 92.3 % in Savoy Gg-1, 64.4 % in cabbage $P_{22}I_{5,}$ and 87.2 % in $P_{34}I_{5.}$ Spontaneous shoot regeneration of excised root cultures grown on the hormone-free medium occurred in all three genotypes. In cabbage lines $P_{22}I_5$ and $P_{34}I_5$ shoot regeneration was higher (9.3 and 2.6 % respectively) than in Savoy cabbage Gg-1 (1.3 %). Transgenic nature of hairy root-derived plants was evaluated by GUS histological test and PCR analysis. All the tested cabbage shoots were GUS positive whilst in a Savoy cabbage GUS expression was registered only in 55 % of tested clones. PCR analysis demonstrated the presence of the GUS gene in regenerated shoot clones and in T_1 progeny.

Additional key words: cabbage, *GUS* gene, hairy roots, plant regeneration, Savoy cabbage, T_1 progeny.

Introduction

It is well known that *Brasica oleracea* species are susceptible to *Agrobacterium rhizogenes* which enables fast and successfull production of transgenic plants. There are papers reporting transformation of kale (Christey and Sinclair 1992, Hosoki *et al.* 1994), cauliflower (Petit *et al.* 1983, David and Tempe 1988, Puddephat *et al.* 2001), Brussels sprouts (Hamada *et al.* 1989, Hosoki and Kigo 1994), broccoli (Hosoki *et al.* 1991, Henzi *et al.* 1999, 2000) and cabbage (Berthomieu and Jouanin 1992, Christey *et al.* 1997, 1999). According to Puddephat *et al.* (1996) and Christey (2001), there are substantial differences in the transformation response between various species and genotypes.

In this paper we present *A. rhizogenes*-mediated transformation and regeneration efficiency of Savoy cabbage (*B. oleracea* var. *sabauda*) and compare it with transgenic cabbage (*B. oleracea* var. *capitata*) obtained by the same method. Savoy cabbage is a variety native to south-east Europe. Although not distant Savoy and plain cabbage have marked differences in most agronomic traits. *A. tumefaciens*-mediated transformation of Savoy cabbage was published recently (Sretenović-Rajičić *et al.* 2004). This is the first report on *A. rhizogenes*-mediated transformation and hairy root-derived plants of Savoy cabbage.

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Abbreviations: BA - 6-benzyladenine; GUS - β-glucuronidase; IBA - indole-3-butyric acid; KIN - kinetin (6-phurphuryl aminopurine); MS medium - Murashige and Skoog medium; PCR - polymerase chain reaction; TDZ - thidiazuron.
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Materials and methods

Plants: Experiments were performed using Savoy cabbage (*Brassica oleracea* L. var. *sabauda*) inbred line Gg-1 and inbred lines $P_{34}I_5$ and $P_{22}I_5$ of common cabbage (*B. oleracea* L. var. *capitata*). These are the leading inbred lines produced by conventional breeding programs of the Centre for Vegetable Crops, Smederevska Palanka. Seeds were surface sterilized and germinated *in vitro* as described previously (Sretenović-Rajičić *et al.* 2004).

Bacterial strain: *Agrobacterium rhizogenes* strain A4M70GUS (Tepfer and Casse Delbart 1987) was used in all transformation experiments. Strain contains a cointegrative plasmid with a GUS construct integrated into the TL region of the pRiA4. GUS construct contains *uidA* sequence under the 70S promoter (enhanced-doubled 35S CaMV promoter), followed by *nos* poly-adenylation sequence.

Agrobacterium culture was maintained on the YEB media (Van Larebeke *et al.* 1977) supplemented with 50 mg dm^{-3} neomycin. Prior to inoculation, bacteria were grown over night in the liquid YEB media at 28 °C temperature.

Transformation procedure: Transformation procedure was similar to the one previously described (Sretenović-Rajičić *et al.* 2004). Briefly, hypocotyls and cotyledon segments excised from 10-d-old aseptically germinated seedlings, pre-cultured for 48 h on the Murashige and Skoog (1962; MS) medium supplemented with 1.0 mg dm^{-3} BA and 0.5 mg dm^{-3} IBA, were dipped into the suspension of AM70GUS *A. rhizogenes.* After 10 - 15 min explants were dried on filter paper and placed on hormone-free MS medium. After 2 d of co-cultivation explants were first rinsed in 500 mg dm^{-3} *Tolycar* solution and then placed on medium supplemented with 200 mg dm^{-3} *Tolycar* to suppress bacterial growth. Roots which appeared on hypocotyls and cotyledon explants were excised and transferred to medium with reduced concentration of *Tolycar* (100 and 50 mg dm^{-3}). After two subcultures *Tolycar* was completely omitted.

Plant regeneration: Shoots which spontaneously regenerated from hairy-root cultures were transferred to MS medium containing 0.5 mg dm^{-3} 6-benzyladenine (BA) and 0.1 mg dm^{-3} indole-3-butyric acid (IBA). Multiplied shoots were rooted for 5 weeks on MS medium supplemented with 4 % sucrose and 4.0 mg dm^{-3} IBA. Plantlets were acclimated and grown in a greenhouse. Vernalization was performed for 8 - 12 weeks at 4 - 8 °C. All *in vitro* cultures were maintained under constant conditions including: temperature 22 ± 2 °C, 16-h photoperiod, irradiance 33.5 - 46.5 µmol m^{-2} s^{-1}.

Evidence of transformation: Plant DNA was isolated according to Zhou *et al.* (1994). PCR reaction was performed to confirm the presence of the GUS gene. The following primers were used:
5'-TAGCGGGACTTTGCAAGTG-3´ and
5´-GTTTTTGCAGCAGAAAAGCC-3´. PCR reaction comprised 30 cycles. Each of PCR cycles consisted of denaturation step at 94 °C for 30 s, followed by annealing at 60 °C for 30 s and a plymerisation reaction at 72 °C for 30 s. PCR products were visualised after electrophoresis on a 1 % agarose gel stained with ethidium bromide under UV irradiation.

Histochemical localization of GUS expression was done according to Jefferson *et al.* (1987).

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

Results

Roots appeared on cotyledon and hypocotyl explants 7 - 10 d after inoculation with *A. rhizogenes* A4M70GUS. After 35 d the percentage of explants which formed roots was very high in all genotypes: 92.3 % in Savoy Gg-1, 64.4 % in cabbage $P_{22}I_5$, and 87.2 % in $P_{34}I_5$ (Table 1). Percentage of explants producing roots depended on the genotype and not on the type of explants (hypocotyl/ cotyledon). Mean number of roots per explants depended both on genotype and type of explants. Thus in Savoy more roots were produced by cotyledon explants and in cabbage by hypocotyl explants (Table 1).

On the hormone-free medium excised roots showed typical hairy root phenotype characteristics – intensive root branching and plagiotropic growth (Fig. 1*A*).

Table 1. Hairy roots production on hypocotyl (H) and cotyledon (C) explants cultured 35 d on hormone free medium. Means ± SE, * - differences among genotypes statistically significant at $P < 0.05$, $n = 200$ - 400.

	Number of explants		Explants with roots [%]		Number of roots [explant^{-1}]	
	H	C	H	C	H	C
Gg-1	367	246	91.4	92.3	12.4 ± 0.07*	16.9 ± 0.98*
P_{34} I_5	403	236	87.1	87.3	6.5 ± 0.86	3.8 ± 0.76
P_{22} I_5	301	264	72.1	64.1	3.8 ± 0.75	2.3 ± 0.12

Spontaneous shoot regeneration of excised root cultures on the hormone-free medium occurred in all three genotypes (Fig. 1*B*). In cabbage lines $P_{22}I_5$ and $P_{34}I_5$ it was higher (9.3 and 2.6 % respectively) than in Savoy Gg-1 (1.3 %), (Table 2).

On media supplemented with growth regulators excised root cultures produced calli in all hormone combination and in all three genotypes. Shoot regeneration was registered only in cabbage line $P_{22}I_5$ and it was the highest on medium containing 1.0 mg dm^{-3} BA and 0.5 mg dm^{-3} IBA (Table 2). The average number of shoots regenerated per explant were still lower then on the hormone-free media.

Transgenic nature of hairy root-derived plants was evaluated by GUS histological test. All the tested cabbage shoots (12 $P_{22}I_5$ and 24 $P_{34}I_5$) were GUS positive whilst in a Savoy cabbage GUS expression was registered only in 55 % of tested clones (11 out 21). False positive GUS shoots were detected only in $P_{34}I_5$ control line. PCR analysis of GUS positive shoot clones showed the presence of the GUS gene and confirmed the transformation (Fig. 2*A*)

GUS positive clones were further multiplied and maintained. Growth characteristics of transformed and control non-transformed plants were studied on shoot multiplication medium with 1.0 mg dm^{-3} BA and 0.5 mg dm^{-3} IBA (Table 3) and rooting media with 4.0 mg dm^{-3} IBA and 4 % sucrose (Table 4). On shoot multiplication

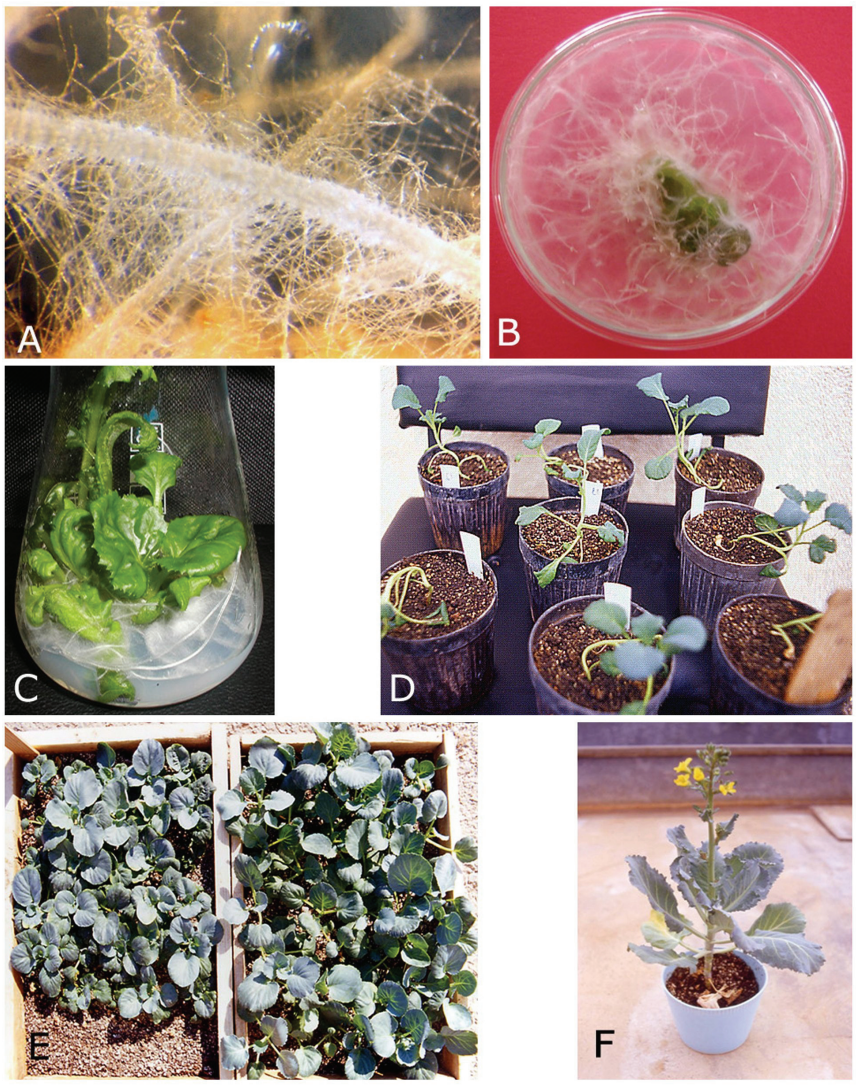

Fig. 1. *A. rhizogenes* A4M70GUS-mediated transformation of Savoy cabbage Gg-1 line and cabbage $P_{34}I_5$ and $P_{22}I_5$ lines. *A* - Hairy root culture of Savoy cabbage transformed with *A.rhizogenes* A4M70GUS. *B* - Spontaneus shoot regeneration from hairy root culture of Savoy cabbage Gg-1 on the media with no growth regulators. *C* - Savoy cabbage transformed shoot culture. *D* - Acclimated transformed Savoy cabbage Gg-1 plants 18 d after transfer to soil. *E* - Acclimated transformed cabbage $P_{34}I_5$ plants 18 d after transfer to soil (*right*) and untransformed control 12 d after transfer to soil (*left*). *F* - $P_{34}I_5$ plant transformed with A4M70GUS, flowering in the greenhouse.

Table 2. Effect of growth regulators on shoot regeneration from transformed root explants. Means ± SE, n = 250 - 300. GRF - growth regulator-free medium.

Growth regulators [mg dm^{-3}]	Explants with callus [%]			Shoot regeneration [%]			Number of shoots [explant^{-1}]		
	$P_{34}I_5$	$P_{22}I_5$	Gg-1	$P_{34}I_5$	$P_{22}I_5$	Gg-1	$P_{34}I_5$	$P_{22}I_5$	Gg-1
GRF	60.0	25.0	18.2	9.3	2.6	1.3	2.1 ± 0.8	3.8 ± 0.2	3.1 ± 0.5
BA 0.5 + IBA 0.1	96.4	50.0	72.6	0	0	0	0	0	0
BA 1.0 + IBA 0.5	95.0	93.6	100.0	0	50.0	0	0	1.5 ± 0.1	0
BA 0.5 + NAA 0.1	68.2	67.9	82.0	0	32.6	0	0	1.5 ± 0.1	0
BA 1.0 + NAA 0.2	100.0	100.0	100.0	0	18.8	0	0	1.5 ± 0.1	0
KIN 0.5 + IBA 0.2	50.0	0	25.0	0	0	0	0	0	0
KIN 2.0 + IBA 1.0	55.6	62.5	58.3	0	25.0	0	0	1.5 ± 0.5	0

Table 3. The growth parameters of transformed and non-transformed (control) shoots on MS medium suplemented with 1.0 mg dm^{-3} BA and 0.5 mg dm^{-3} IBA. Subculture duration 5 weeks. Means ± SE, * - differences among transformed and control plants are statisticaly significant at $P < 0.05$.

Genotype	Plasmid	Number of shoots	Shoot length [mm]	Rooted shoots [%]	Number of leaves	Number of lateral shoots
$P_{34}I_5$	A4M70GUS	69	21.3 ± 5.5*	33.3	15.6 ± 2.7*	3.9 ± 1.8*
	cotrol	28	13.5 ± 4.9	14.3	9.8 ± 1.4	1.1 ± 0.6
P_{22} I_5	A4M70GUS	30	18.9 ± 2.9	23.3	11.4 ± 1.3*	2.8 ± 0.9*
	cntrol	30	19.8 ± 2.9	30.0	7.6 ± 1.2	11.1 ± 0.3
Gg-1	A4M70GUS	32	32.6 ± 6.4	100.0	4.9 ± 1.0*	1.3 ± 0.5*
	control	37	24.3 ± 9.1	100.0	11.5 ± 2.1	2.2 ± 1.0

Table 4. The growth parameters of transformed and non-transformed (control) shoots on MS medium suplemented with 4.0 mg dm^{-3} IBA and 4 % sucrose. Subculture duration 5 weeks. Means ± SE, * - differences among transformed and control plants are statisticaly significant at $P < 0.05$.

Genotype	Plasmid	Number of shoots	Number of roots [plant^{-1}]	Root length [mm]	Shoot length [mm]	Number of leaves
$P_{34}I_5$	A4M70GUS	119	9.1 ± 1.6	41.8 ± 6.9	32.4 ± 9.7*	66.5 ± 1.9*
	control	66	6.0 ± 0.4	35.7 ± 4.4	27.8 ± 6.7	10.4 ± 1.8
$P_{22}I_5$	A4M70GUS	117	5.4 ± 0.4	44.8 ± 2.4*	55.5 ± 2.1*	4.9 ± 0.6
	control	66	7.3 ± 1.6	25.6 ± 1.8	37.8 ± 2.9	6.9 ± 1.0
Gg-1	A4M70GUS	142	24.5 ± 2.4*	72.1 ± 15.2	56.3 ± 6.6*	12.6 ± 2.9
	control	58	11.0 ± 1.8	51.6 ± 3.2	43.5 ± 9.8	10.0 ± 2.6

medium transformed plants had lower shoot multiplication (Gg-1 and $P_{22}I_5$), higher shoot elongation (Gg-1 and $P_{34}I_5$) and better leaf production ($P_{22}I_5$ and $P_{34}I_5$). Control plants of $P_{22}I_5$ had higher shoot multiplication and elongation than transformed plants. Also in Savoy Gg-1 control had better leaf production than transformed plants. It is interesting that both transformed and control plants of Savoy Gg-1 had 100 % rooting on this cytokinin supplemented medium.

Rooting medium (Table 4) enabled 100 % rooting of all three cabbage lines. It also increased shoot and root length of transformed plants in all three lines. Root production was significantly higher in Savoy Gg-1 (Fig. 1*C*) and leaf production in $P_{34}I_5$. Only in $P_{22}I_5$ control plants had somewhat higher production of roots and leaves than transformed plants. It is interesting to note that transformed plants of all three genotypes were longer and better developed than the control plants. None of them exhibited typical hairy-root phenotype characteristics including wrinkled leaves and short internodes.

Acclimated plantlets grown in the greenhouse appeared to be much more elongated than the control non-transformed plants (Fig. 1*D*,*E*). Their roots remained plagiotropic. Transformed plantlets were brought to flowering (Fig. 1*F*). They set seeds which germinated enabling PCR analysis to be performed in the progeny. It showed stable integration of *uid*A gene in the F1 plants of three investigated cabbage lines (Fig. 2*B*).

Discussion

In the present study we performed *Agrobacterium rhizogenes* A4M70GUS-mediated transformation of two local lines of cabbage and for the first time obtained succesful *A. rhizogenes*-mediated transformation of Savoy cabbage. *Agrobacterium rhizogenes* mediated transformation of *Brassica oleracea* var. capitata has been already reported by Berthomieu and Jouanin (1992), Christey *et al.* (1992, 1997, 1999). In all reports 5 - 7 d are necessary until the roots appear on the inoculated explant. These data correspond with our results.

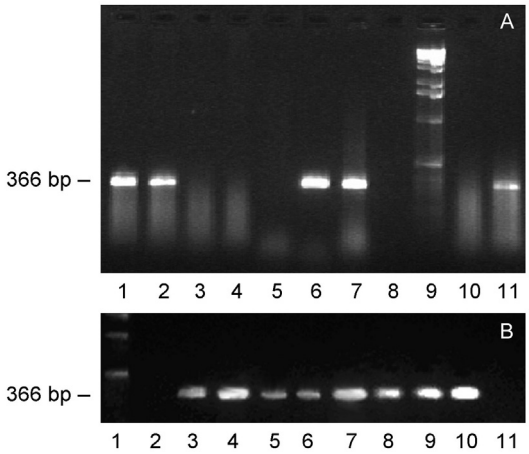

Fig. 2. PCR analysis of *A. rhizogenes* A4M70GUS transformed plants of Savoy cabbage and cabbage (*A*) and their progeny (*B*). *A*: *lane 1* - transformed cabbage $P_{22}I_5$ plant, *lane 2* - transformed Savoy cabbage Gg-1 plant, *lanes 3, 4* and *5* - untransformed control plants of cabbage $P_{22}I_5$, Savoy cabbage Gg-1 and cabbage $P_{34}I_5$, *lane 6* - vector A4M70GUS, *lane 7* - transformed cabbage $P_{34}I_5$ plant, *lane 8* - blank, *lane 9* - 1 kb DNA ladder, *lane 10* - GUS positive control cabbage $P_{34}I_5$ plant, *lane 11* - GUS positive transformed cabbage $P_{34}I_5$ plant. *B*: *lane 1* - 1 kb DNA ladder, *lane 2* - blank, *lanes 3, 4* and *5* - Savoy cabbage Gg-1 transgenic plants, *lanes 6* and *7* - cabbage $P_{34}I_5$ transgenic plants, *lanes 8* and *9* - cabbage $P_{22}I_5$ transgenic plants, *lane 10* - vector A4M70GUS, *lane 11* - untransformed control.

According to Christey (2001) transformation frequency between different species or cultivars are variable. There were also differences in the percentage of explants developing hairy roots depending on the type of explants, bacterial strain used and transformation conditions. The transgenic root production of our domestic inbred cabbage lines was twice higher than in other reports (Christey *et al.* 1997). It is noteworthy that the transformation rate of Savoy cabbage was very high (90 %), but only 50 % of regenerated plants were GUS positive. Similar results were reported in Brussels sprouts transformation (Hosoki and Kigo 1994). Hairy roots-derived cabbage plants were 100 % GUS positive. High percent of GUS posititive clones has been already reported for some other species transformed with the same *A. rhizogenes* A4M70GUS strain (potato - Miljuš-Đukić *et al.* 1996, *Lotus corniculatus* - Nikolić *et al.* 2003/4, *Aesculus hippocastanum* - Zdravković-Korać *et al*. 2004).

Plant regeneration of our varietes was rather succesful. Shoots regenerated from the transformed roots on the hormone-free media. Shoot regeneration from hairy roots often occurs spontaneously (Bijelović *et al.* 2004). In *Brassica* spontaneous shoot regeneration from hairy root cultures on hormone-free medium was occasionally noted in some cultivars (cauliflower and rapid-cycling *B. oleracea*) but was never observed in cabbage lines. Only the use of TDZ promoted shoot regeneration in cabbage (Christey *et al.* 1997).

Altered phenotype of transformed plants is characteristic of *A. rhizogenes* mediated transformation. In *Brassica* transformants abnormalities have been reported in cauliflower (David and Tempe 1988), *Brasica napus* (Boulter *et al.* 1990), kale (Christey and Sinclair 1992) and some other species reviewed in Poulsen (1996). In some *Brassica oleracea* varieties the differences between transformed and control plants are incospicious (Christey *et al.* 1997, Christey and Braun 2001). Berthomieu and Jouanin (1992) obtained rapid-cycling cabbage plants with normal appearance but reduced male fertility. Although the transformed shoots of cabbage lines which we investigated manifested some phenotypic changes – shoot and root elongation and plagiotropic root growth, this plants were fertile and produced T1 transgenic generation plants.

Comparing the two *B. oleracea* varieties, it seems that hairy root regeneration was better in Savoy cabbage plants, but plant regeneration from the transformed roots was better in cabbage genotypes. Similar observation was reported for *Brasica oleracea vs. Brasica campestris* (Christey 2001). Our results confirm finding that *A. rhizogenes*-mediated transformation of *B. oleracea* is genotype specific, which seems to be a general observation for the whole *Brassica* genus.

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