FACTORS AFFECTING *Agrobacterium tumefaciens*-MEDIATED TRANSFORMATION OF BROCCOLI

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SUMMARY

Transgenic broccoli plants were obtained by transformation with *Agrobacterium tumefaciens* carrying endochitinase, *gus* and *nptII* genes. The effects of the type and age of explant, *Agrobacterium* strain, and silver nitrate on transformation efficiency were examined. Cotyledonary-petioles from 5-day-old seedlings were successfully transformed with octopine strain LBA4404 or nopaline strain GV3101. No transformed shoots were obtained from 15-day-old cotyledonary-petioles or from hypocotyls at any developmental stage. Higher transformation rates were obtained with GV3101 (11-16%), compared to LBA4404 (1-6%). Improvement in transformation rates was also observed when explants were exposed to silver nitrate during the first 7 days of selection, but only when the LBA4404 strain was used. Use of silver nitrate on explants inoculated with GV3101 did not increase shoot differentiation. Selection for kanamycin resistance allowed differentiation of many escapes, but transgenic shoots were also recovered. Transgenic status of green regenerated plants was confirmed by NptII and PCR assays which showed a high correlation with root formation on selective medium. The efficient protocol developed for broccoli transformation is suggested for use with other *Brassica oleracea* varieties.

KEY WORDS: *Brassica oleracea* var. *italica*, cotyledonary-petioles, nopaline, octopine, silver nitrate, transformation efficiency

FACTORES QUE AFECTAN LA TRANSFORMACIÓN DE BRÓCOLI MEDIADA POR *Agrobacterium fumefaciens*

RESUMEN

Plantas transgénicas de brócoli fueron obtenidas por medio de *Agrobacterium tumefaciens* conteniendo los genes endoquitinasa, *gus* y *nptII*. Se analizaron los efectos del tipo y edad del explante, cepa de *Agrobacterium* y utilización de nitrato de plata en la eficiencia de transformación. Pecíolos- cotiledonares de plántulas de 5 días-post-germinación fueron exitosamente transformados con la cepa octopina LBA4404 o la cepa nopalina GV3101. No se obtuvieron brotes transformados de explantes provenientes de plántulas de 15 días post-germinación, ni de hipocotilos en ningún estado de desarrollo. Mayores tasas de transformación fueron obtenidas con GV3101 (11-16%), comparado con LBA4404 (1-6%). El aumento en las tasas de transformación también fue observado cuando los explantes fueron expuestos a nitrato de plata durante los primeros 7 días de selección, pero solamente cuando la cepa LBA4404 fue utilizada. El uso del nitrato de plata en explantes inoculados con GV3101 no incrementó la diferenciación de brotes. La selección por resistencia a kanamicina permitió la diferenciación de muchos escapes, pero brotes transgénicos también fueron obtenidos. La condición transgénica fue confirmada por análisis NptII y de PCR los cuales mostraron una fuerte correlación con la formación de raíz en medio de selección.

PALABRAS CLAVE: *Brassica oleracea* var. *italica*, eficiencia de transformación, nitrato de plata, nopalina, octopina, pecíolos-cotiledonares

INTRODUCTION

Brassica oleracea is a polymorphic species, which includes several vegetable crops, such as cabbage (var. *capitata*), Brussels sprouts (var. *gemmifera*), cauliflower (var. *botrytis*), kohlrabi (var. *gongyloides*), kale (vars. *medullosa*, *ramosa*, and *acephala)*, and broccoli (var. *italica*) (Puddephat *et al.*, 1996). The constant attack from pests and diseases, together with limited resistance sources among non-cultivated plants, has led to search for alternative sources of resistance genes that may be utilized by means of gene transfer. The primary method of gene transfer for *Brassica* is *Agrobacterium*-mediated transformation.

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Most of the studies have used diverse genotypes, bacterial strains, and transgenes, so common procedures for the transformation of *B. oleracea* have not emerged. The principal difficulties relate to efficient combination of plant regeneration with gene transfer. The type and age of explants used are important factors to consider. De Block *et al.* (1989) inoculated hypocotyl explants from 14 day-old rapeseed and cauliflower seedlings. Hypocotyls formed calli under selective conditions; these gave rise to shoot formation and a high transformation efficiency (10-30%). However, Metz *et al.* (1995) were unable to reproduce these results. Instead, they used flowering stalk explants from mature plants (based on Fry *et al.*, 1987) or hypocotyl and petiole explants from *in vitro*-grown broccoli and cabbage seedlings. Transformation efficiency varied depending on the explants used (Metz *et al.*, 1995).

According to Bhalla and Smith (1998) younger seedlings (5 day-old) of *B. oleracea* var. *botrytis* regenerate better than older seedlings (12-20 day-old). Other reports indicated that older explants regenerated well after transformation of broccoli and cabbage (Metz *et al.*, 1995), cauliflower and *B. napus* (De Block *et al.*, 1989), and rapid cycling cabbage (Berthomieu *et al.*, 1994).

Transgenes used range from reporter genes such as *gus* (Hosoki and Kigo, 1994; Christey and Sinclair, 1992); selectable marker genes like *nptII* (Passelegue and Kerlan, 1996; De Block *et al.*, 1989; Bhalla and Smith, 1998), *hpt* (Passelegue and Kerlan, 1996), and *bar* (De Block *et al.*, 1989), and genes with agronomical effects such as Bt genes (Metz *et al.*, 1995; Christey *et al.*, 1997; Cao *et al.*, 1999), and antisense orientation of ethylene-forming enzyme (Christey *et al.*, 1997). Different *A. tumefaciens* strains have been used to transform *Brassicas.* Octopine or nopaline strains are commonly used; however, their efficiency has been reported to be genotype dependent (Puddephat *et al.*, 1996).

The goal of the experiments described below was to improve broccoli transformation efficiency by optimizing some of the variables in *Agrobacterium tumefaciens*-mediated transformation of this plant. These include type and age of explants, *A. tumefaciens* strain, and use of silver nitrate for regeneration improvement. Large numbers of transgenic broccoli plants were produced by combining the methods reported for *B. napus* by Moloney *et al.* (1989) and the John Innes Center, UK; and for *B. oleracea* by Metz *et al.* (1995).

MATERIALS AND METHODS

Plant material. Broccoli (*Brassica oleracea* var. *italica* F1 hybrid 'Green Comet') (Harris Seeds, Rochester, NY), were used as plant material.

Seed sterilization. Seeds were surface sterilized with 70% ethanol (v:v) for 3 minutes. Alcohol was removed, and 20% Clorox plus 0.1% Tween-20 (v:v) (Sigma) was added for 20 minutes. Seeds were washed three times with double distilled sterile water.

Seed germination *in vitro*. Seeds were germinated in hormone-free Murashige-Skoog (MS) medium (Murashige and Skoog, 1962) with 3% sucrose (w:v), pH 5.8 and 2.2 g·liter^{-1} Phytagel (Sigma) at 25 °C, 16 h photoperiod, and light intensity of 45-70 μmol·m^{2}·s^{-1}.

Explants used. Explants were taken from 5-15 day-old *in vitro* grown seedlings. Explants consisted of 8-10 mm long hypocotyl segments or cotyledonary-petiole explants that included the entire cotyledon plus 2-3 mm of petiole.

Constructs used. The pBin19ESR construct contains the endochitinase cDNA gene (*ThEn42*) from the biocontrol fungus *Trichoderma harzianum,* which has antifungal effects (Hayes *et al.*, 1994). The *ThEn42* gene was regulated by a double cauliflower mosaic virus (CaMV) 35S-promoter and a leader sequence from alfalfa mosaic virus (AMV) that enhances expression (Datla *et al*., 1993). The pBI121 construct contains the β-glucuronidase gene (*gus*) and is flanked by a single CaMV promoter and *nos* terminator (Jefferson, 1987; Clontech). Both pBin19ESR and pBI121 constructs have the *nptII* gene that provides kanamycin resistance under control of the *nos* promoter and *nos* terminator (Figure 1).

Agrobacterium tumefaciens. Two different *A. tumefaciens* strains were used: LBA4404 (pTiAch5), an octopine strain, and GV3101 (pTiC58), a nopaline strain. *Agrobacterium* was grown overnight at 30 °C on liquid LB medium with 50 mg·liter^{-1} kanamycin under shaking conditions. *Agrobacterium* was used at OD_{600}= 1.2 to 1.6.

Transformation of *Brassica oleracea* and *B. napus*

Inoculation. Cotyledonary-petioles and hypocotyls were dissected from 5 or 15 day-old seedlings and immediately inoculated with *A. tumefaciens* (either LBA4404 or GV3101) containing pBin19ESR or pBI121. The petiole of cotyledons were inoculated by slightly dipping them for approximately 3 seconds into a 1:10 dilution of *Agrobacterium* and MS liquid hormone-free medium with 1% sucrose (w:v). Hypocotyls were submerged in the same MS medium-*Agrobacterium* solution and incubated for 3 minutes. Liquid was then discarded, and hypocotyls blotted.

Cocultivation. Inoculated explants were transferred to Cocultivation Medium, which contained MS salts, 3.7 mg·liter^{-1} benzylaminopurine, 3% sucrose (w:v), and 0.7% Phytagar (w:v) (Gibco BRL), pH 5.8. Petioles were placed into the medium with the cotyledon up; hypocotyls were placed horizontally on the medium. Explants were incubated for 72 h under the same conditions as seed germination.

Elimination of *A. tumefaciens*. Explants were then transferred to *Agrobacterium* Elimination Medium. This

medium was the Cocultivation Medium with addition of 300 mg·liter^{-1} timentin (290 mg·liter^{-1} sterile ticarcillin disodium and 10 mg·liter^{-1} clavunalate potassium) (Smith Kline Beecham).

Selection. After 7 to 10 days in *Agrobacterium* Elimination Medium explants were transferred to Selective Medium, same as Elimination Medium plus 25 mg·liter^{-1} kanamycin. Explants were transferred to fresh medium every two to three weeks.

Silver nitrate. Some Explants were exposed to 5 mg·liter^{-1} of silver nitrate either during the first seven days of selection or continuously during a period of eight weeks.

Kanamycin resistance. Putative transgenic plants were screened for kanamycin resistance. The NptII expression assay consisted on placing small leaf segments (~1 cm^2) cut from putative transformants and placed on Petri dishes containing the NptII expression medium (Fry *et al.*, 1987).

PCR assays. PCR analysis was done using endochitinase primers for pBin19ESR-derived plants or *nptII* primers for pBI121-derived plants.

Endochitinase 5' primer: ATG TTG GGC TTC CTC GG

Endochitinase 3' primer: CGC TCC CTG CAT AAT CG

nptII 5' primer: TCG GCT ATG ACT GGG CAC AAC AGA

nptII 3' primer: AAG AAG GCG ATA GAA GGC GAT GCG

Amplification conditions for both sets of primers consisted of 3 min 94°C for one cycle, 1 min 94°C, 1 min 55°C, and 2 min 72°C for 35 cycles. DNA was isolated according to Hu and Quiros (1991).

GUS assay. For early transformation screening histochemical GUS assays were carried out with small leaf tissue (~1-2 cm^2), from the putative transgenic plants. Leaf samples were incubated overnight at 37°C in a 50 nM $NaPO_4$, pH 7.0 buffer containing 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-glucoronide) (Jefferson, 1987). Samples were scored based on presence or absence of blue coloration in the tissue.

RESULTS

Effect of explant type and age on shoot regeneration

More than 90% of hypocotyls and cotyledonary-petioles from 5- and 15-day-old seedlings initiated shoot formation after seven to ten days in culture. No differences in shoot regeneration were observed between ages or between explants. Shoot development was achieved after three to four weeks.

Effect of explant type and age on transformation efficiency

Cotyledonary-petioles and hypocotyls from 5 or 15 day-old seedlings were inoculated with *A. tumefaciens* LBA4404 containing the pBI121 plasmid. Transformation efficiency was based on the number of inoculated explants that differentiated at least one green shoot that was positive in the GUS assay, divided by the total of inoculated explants. For 5 day-old cotyledonary-petioles the transformation efficiency was 5.2%. Five day-old hypocotyls and both types of explants at 15 day-old regenerated no green shoots (Table 1A).

Shoot differentiation occurred in 80% of the inoculated 5-day-old cotyledonary-petioles but only a few of these shoots remained green. All shoots differentiated from hypocotyls turned white. Callus formation at the cut edge of inoculated cotyledonary-petioles was an intermediate phase in regeneration of green shoots. Shoots regenerated directly from the cut surface were less likely to remain green. No green shoots or escapes were obtained from 15 day-old explants.

Green shoot formation was also obtained using 5 day-old cotyledonary-petioles inoculated with LBA4404-pBin19ESR. Transformation efficiency was variable, ranging from 1.0 to 4.5% in three experiments (data not shown).

Effect of *A. tumefaciens* strain on transformation efficiency

Initial experiments using cotyledonary-petiole and hypocotyl explants inoculated with LBA4404 showed a low and inconsistent transformation efficiency (Tables 1A).

Transformation efficiency was 3-fold higher when 5 day-old cotyledonary-petiole explants were inoculated with *Agrobacterium* strain GV3101 (GV3101-pBI121) rather than LBA4404 (Table 1A and 1B). However, 15 day-old cotyledonary-petioles still did not form green shoots after inoculation with GV3101-pBI121 (Table 1B). On the other hand, hypocotyls, which were recalcitrant to transformation using LBA4404-pBI121 (Table 1A), regenerated transformed shoots at a rather high efficiency (8.3%) using GV3101-pBI121 (Table 1B).

Transformation efficiency with pBin19ESR was notably improved when cotyledonary-petioles were inoculated with GV3101 (Table 2). Transformation of 'Green Comet' broccoli increased more than 5 fold (11.4-15.8% vs. 1.0-2.5%) with GV3101, compared to LBA4404.

TABLE 1. Effect of explant type and age on transformation of broccoli inoculated with *A. tumefaciens* strains carrying pBI121.

A. LBA4404

		Explant age			
		5 days			15 days
	Explants	Green shoots	Positive GUS assay	Transformation efficiency (%)[z]	Green shoots
Cotyl. Petioles	96	6	5	5.2	0
Hypocotyls	48	0	-	-	0
Total		6	5		0

[z] Transformation efficiency is defined as the percentage of explants producing at least one kanamycin-resistant and GUS positive shoot divided by the number of inoculated explants x 100

B. GV3101

		Explant age			
		5 days			15 days
	Explants	Green shoots	Positive GUS assay	Transformation efficiency (%)[z]	Green shoots
Cotyl. Petioles	96	32	18	18.7	0
Hypocotyls	48	8	4	8.3	0
Total		40	29		0

[z] Transformation efficiency is defined as the percentage of explants producing at least one kanamycin-resistant and GUS positive shoot divided by the number of inoculated explants x 100

TABLE 2. Transformation of *Brassica* spp. with LBA4404 or GV3101 *A. tumefaciens* strains containing pBin19ESR. The explants were 5 day-old cotyledonary-petioles.

	LBA4404			GV3101		
	Explants	Kan^R shoots[z]	Transform. efficiency[y]	Explants	Kan^R shoots[z]	Transform. efficiency[y]
B. oleracea	200	5	2.5	1000	114	11.4
'Green Comet'	1044	11	1.1	450	71	15.8
	1080	11	1.0	-	-	-

[z] Number of shoots that rooted on selective medium containing kanamycin (25 mg·liter^{-1}) and were positive in the NptII expression assay
[y] Transformation efficiency is defined as the percentage of explants producing at least one kanamycin-resistant shoot divided by the number of inoculated explants x 100

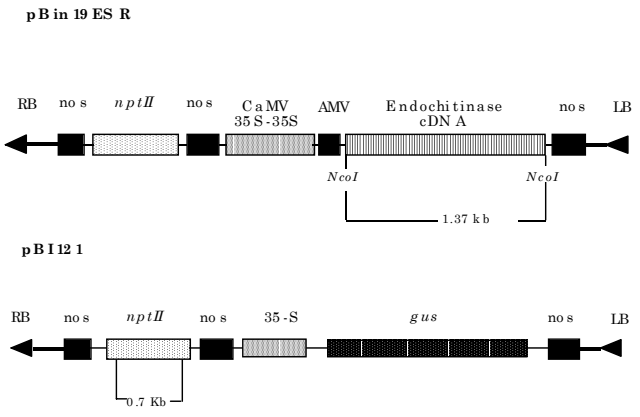

Figure 1. Constructs used in *Brassica oleracea* transformation. A. pBin19ESR construct containing *T. harzianum* endochitinase gene; B. pBI121 construct containing *gus* gene.

Effect of silver nitrate on shoot differentiation and transformation efficiency.

Non-inoculated explants in non-selective medium containing silver nitrate had a reduced rate of regeneration. Both one week and continuous exposure to silver nitrate reduced shoot formation in hypocotyls and cotyledonary-petioles (data not shown).

The effect of silver nitrate on transformation was analyzed on broccoli cotyledonary-petioles inoculated with LBA4404- or GV3101-pBin19ESR (Table 3). Explants inoculated with GV3101-pBin19ESR did not show a substantial difference in transformation efficiency without silver nitrate (5.0%) or with silver nitrate (6.4%). However, cotyledonary-petioles inoculated with LBA4404-

pBin19ESR showed an increase in number of shoots regenerated in selective medium with silver nitrate. Explants in medium with no silver nitrate regenerated either a low number of shoots or none at all (Table 3).

TABLE 3. Effect of one-week exposure to silver nitrate on transformation of broccoli cotyledonary-petioles with different *A. tumefaciens* strains containing pBin19ESR .

	Explants	-$AgNO_3$	Explants	+$AgNO_3$
GV3101	**960**	**5.0**	**1000**	**6.4[z]**
	190	2.1	383	4.7
LBA4404	200	0	200	2.5
	980	0	1080	1.0
	1200	0	1044	1.1

[z] Percentage of explants that generated at least one transgenic shoot. Each comparison represents an independent transformation experiment.

Screening of putative transformant plants.

Leaves from most putative transgenic shoots remained green and developed calli and roots at the cut edges in the NptII expression assay (data not shown). Controls and non-transgenic explants remained the same size and bleached completely after one week.

PCR assays were done on 69 plants recovered from 2 transformation experiments to confirm the presence of the transgenes and compare to the results from the NptII assay. All kanamycin resistant plants amplified the endochitinase gene (Figure 2) and nptII gene fragment (data not shown). Results from the NptII expression assay on plants transformed with the endochitinase gene were highly correlated with the results from PCR assays and with the rooting of shoots in selective medium with no hormones. In most cases, lines remained consistent among the screening assays (data not shown).

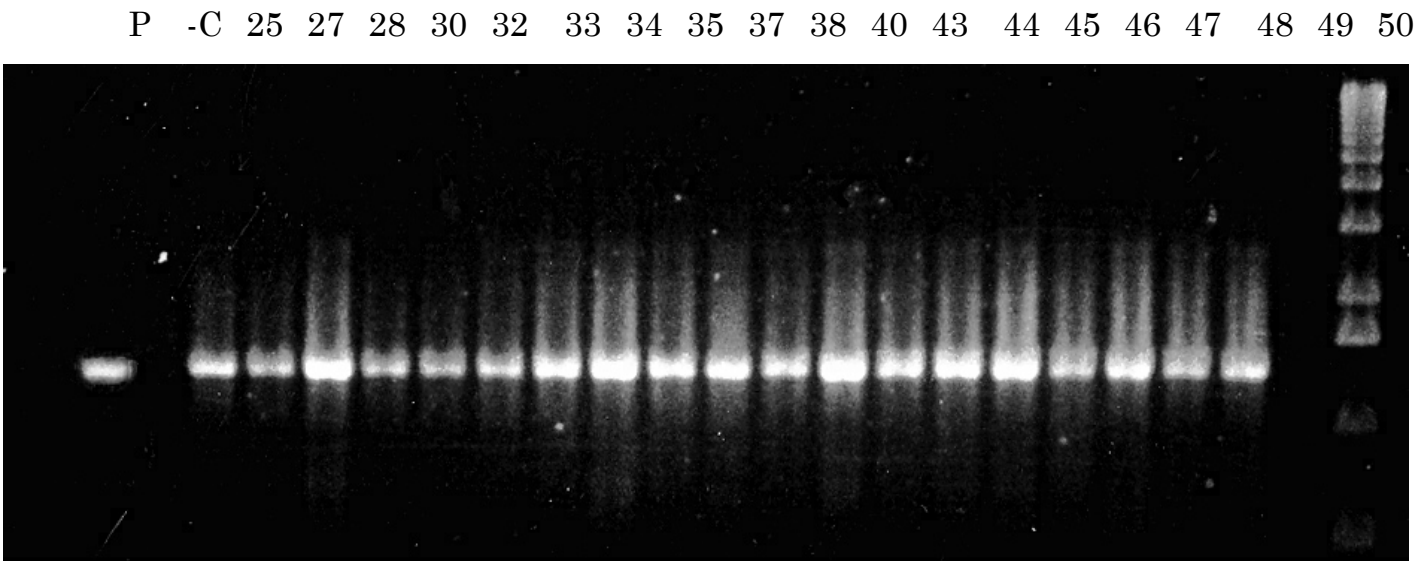

Figure 2. PCR amplification of the *T. harzianum* endochitinase gene in broccoli plants. Lane P: plasmid positive control; Lane –C: Non-transgenic seed-grown broccoli; Lanes 25-50: putative transgenic plants. Arrow indicates 1.366 Kb endochitinase fragment.

DISCUSSION

In our experiments origin of shoot formation played an important role in transformation efficiency rates. Shoots that developed directly from the cut edge were less likely to remain green in kanamycin selective medium, whereas shoots forming from an intermediate layer of callus had a better chance to remain green. Mukhopadhyay *et al.* (1992) reported that *de novo* shoots originated from callus tissue were more likely to be transformed compared to shoots coming directly from the vascular parenchyma cells at the cut edge of the hypocotyl and cotyledonary-petioles of *B. campestris*. Similar observations were made in *B. napus* hypocotyls (De Block *et al.*, 1989) and *A. thaliana* cotyledonary-petioles (Schmidt and Willmitzer, 1988).

Failure of shoot regeneration from inoculated hypocotyls disagrees with a number of reports on *Brassica* transformation. Hypocotyls have been the most commonly used explant for *Agrobacterium*-mediated transformation of cauliflower (De Block *et al.*, 1989; Ding *et al.*, 1998), broccoli (Metz *et al.* 1995; Cao *et al.*, 1999); cabbage (Metz *et al.*, 1995; Berthomieu *et al.*, 1994), *B. juncea* (Pental *et al.*, 1993), *B. campestris* (Mukhopadhyay *et al.*, 1992), *B. carinata* (Babic *et al.,* 1998), *B. nigra* (Gupta *et al.*, 1993), and *B. rapa* (Radke *et al.,* 1992). A possible reason for lack of shoot regeneration from 'Green Comet'

hypocotyls in our results compared to Cao *et al.* (1999) may be that they used a different *Agrobacterium* strain (ABI) and included a feeder layer during cocultivation. On the other hand differences from Metz *et al.* (1995) involved the use of the 35S-promoter for the *nptII* gene in their construct. A better expression of the *nptII* gene permits a more efficient detoxification of kanamycin and therefore a higher shoot differentiation.

In our experience 5-6-day-old cotyledonary-petioles were highly efficient in regeneration of transgenic broccoli shoots. Similar results regarding cotyledonary-petioles were observed in *B. napus* (Moloney *et al.*, 1989) and more recently in other *Brassica* species such as *B. campestris* (Mukhopadhyay *et al.*, 1992; Jun *et al.*, 1995; Christey *et al.*, 1997), *B. oleracea* var. *botrytis* (Bhalla and Smith, 1998), *B. oleracea* (various genotypes) (Christey *et al.*, 1997), and *B. carinata* (Babic *et al.*, 1998).

Other reports have indicated that explants from older seedlings (12-20 days) of broccoli, cabbage, cauliflower and *B. napus* had good regeneration (De Block *et al.*, 1989; Berthomieu *et al.*, 1994; Metz *et al.*, 1995); however, none of these reports compared explants from younger or older seedlings.

Silver nitrate has been reported to be an inhibitor of ethylene action, and it is known to induce morphogenesis of various species *in vitro*. The stimulating effect of silver nitrate on shoot regeneration from wheat was described by Purnhauser *et al.* (1987). They suggested that ethylene plays a role in suppressing morphogenesis in plant tissue cultures and that silver nitrate interferes with the incorporation of ethylene at its receptor site. Beneficial effects of silver nitrate on *Brassica* species were reported by Chi and Pua (1989), De Block *et al.* (1989), Chi *et al.* (1990), Sethi *et al.* (1990), and Mukhopadhyay *et al.* (1992).

In this study, addition of silver nitrate increased the shoot formation on hypocotyls and cotyledonary-petioles inoculated with *Agrobacterium* (pBI121-LBA4404). Interestingly, we did not find a major improvement on shoot formation from cotyledonary-petioles inoculated with *Agrobacterium* strain GV3101; however, contrary to our results with GV3101, De Block *et al.* (1989) reported that silver nitrate (2-5 $mg \cdot liter^{-1}$) was a prerequisite for efficient shoot regeneration under selective conditions for cauliflower hypocotyls and cotyledonary-petioles inoculated with nopaline strain C58C1Rif. Similarly, Pental *et al.* (1993) found that inclusion of silver nitrate in the medium was essential for high frequency of regeneration of hypocotyl explants of *Brassica juncea* using nopaline strain GV2260. Radke *et al.* (1992) also reported that regeneration frequencies of *Brassica rapa* were increased by using silver nitrate and the succinamopine strain EHA101. Similarly, Mukhopadhyay *et al.* (1992), found that silver nitrate enhanced shoot regeneration of *B. campestris* cotyledonary-petiole and hypocotyl explants.

The use of a nopaline strain (GV3101) instead an octopine strain (LBA4404) greatly improved transformation efficiency. Similarly, Damgaard *et al.* (1997) reported that nopaline strains GV3850 and MP90 gave 6-16% transformation efficiency in *B. napus* compared to octopine strains LBA4404, which gave only 0-1% efficiency. Mauro *et al.* (1995) observed a strong and significant interaction between genotypes and strains of *A. tumefaciens* upon inoculation of twenty-six soybean genotypes with three strains of *A. tumefaciens*. The strains of *A. tumefaciens* used included C-58 (nopaline), Bo-542 (agropine), and Ach-5 (octopine). The genotypes showed differential susceptibility to the three *Agrobacterium* strains.

However, Raharjo *et al.* (1996) did not find a major difference between nopaline and octopine strains in pickling cucumber transformation, both yielding a maximum of 5%. Instead a supervirulent leucinopine strain increased this rate up to 12%. Similarly Beclin *et al.* (1993) reported no difference in the efficiency of transformation obtained by disarmed LBA4404 and GV3101 used with rapid cycling cabbage.

Van Wordragen and Dons (1992) and Winans (1992) reported that in addition to transferring DNA to plants, some strains release other signal molecules of low molecular weight. Genetic organization of the *vir* region of the octopine Ti plasmid varies slightly to nopaline Ti plasmid. Virulence of the nopaline strain may be in part related to presence of a non-transferred cytokinin biosynthetic gene called trans-zeatin synthase (*tzs*) gene, which is absent in octopine strain, resulting in the synthesis of cytokinins at the time T-DNA is transferred. Cytokinin stimulates dedifferentiation and cell division; therefore, weakening of the plant cell wall could facilitate T-DNA transfer at the site of infection. Similarly, Regensburg and Hooykaas (1993) indicated that other *vir* regions from different Ti plasmids may vary slightly in the genes they contain: for instance, the *virF,*and *pinF* genes, which are present in the *vir*-region of octopine Ti plasmids, are absent from nopaline Ti plasmids. Whether or not *pinF*, *virF*, and *tzs* genes have an essential role in virulence and T-DNA transfer and integration remains to be studied.

Transformed shoots were obtained from explants after 4 to 6 weeks in selective medium with kanamycin. Although kanamycin did not inhibit shoot differentiation from untransformed cells, the shoots turned white soon after emergence. Metz *et al.* (1995) suggested that regeneration of untransformed shoots from *B. oleracea* hypocotyls was due to detoxification of kanamycin in localized areas of the callus. We observed a similar regeneration pattern; however, after several transfers to fresh selective medium, it was possible to select kanamycin-resistant shoots from escapes. Transgenic plants remained green after several transfers to Selective Medium and no bleaching patterns were observed.

Cao *et al.* (1999) used hygromycin rather than kanamycin as a selective agent for broccoli transformation. According to them selection with hygromycin (10 mg·liter^{-1}) was more efficient than kanamycin since it eliminated differentiation of non-transgenic shoots and no escapes were detected in further molecular analysis. Similarly, Pental *et al.* (1993) reported that transformation efficiency using the *hpt* gene was higher (11-36%) than reported by Barfield and Pua (1991) with *nptII* gene (7.1%). Kuvshinov *et al.* (1999) also found a 90% formation of escapes with kanamycin selection compared to only 8% with hygromycin.

Despite the time invested in the selection phase (6-8 weeks), we were able to obtain transgenic shoots, whose status was confirmed by the NptII expression assay. The correlation between kanamycin selection, GUS assay, and PCR analysis was very high (>95%) (data not shown).

CONCLUSIONS

From this study we conclude that young cotyledonary-petioles of broccoli have *de novo* regeneration ability suitable for transformation. The effect of silver nitrate will depend on the plant genotype and its recalcitrance for transformation, *Agrobacterium* strain, and level of stress or ethylene production by the explant. Inclusion of silver nitrate to improve shoot differentiation may be advantageous in some *Brassica* genotypes, and it is recommended to analyze the effect of this component in each case. Explants inoculated with *Agrobacterium* strain GV3101 regenerated more transgenic compared to LBA4404. The nopaline strain is more virulent than the octopine strain and induces a more efficient T-DNA transfer into plant cells. The transformation protocol would be suitable for other *B. oleracea* genotypes and may result in higher transformation rates than previously reported.

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