Short Communication Plant Biotechnology, **21** (3), 225-228 (2004)

Effect of Silver Nitrate on Shoot Regeneration and *Agrobacterium*-mediated Transformation of Turnip (*Brassica rapa* L. var. *rapifera*)

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Received 8 March 2004; accepted 7 May 2004

Abstract

Six turnip cultivars (*Brassica rapa* var. *rapifera*) exhibited shoot regeneration ability of 0-44.0% from their hypocotyl sections. Shoot regeneration from hypocotyl sections of 5 turnip cultivars was markedly enhanced by adding $AgNO_3$ into a shoot regeneration medium. Transgenic turnip plants were obtained by the *Agrobacterium*-mediated transformation procedure incorporating $AgNO_3$ in the shoot regeneration medium. Transformation efficiencies (percentage of stable transformants per total sections infected) were 1.0% and 0.5% for 'Hinonakabu' and 'Honbenidaimarukabu'.

Key words: *Agrobacterium*-mediated transformation, *Brassica rapa*, $AgNO_3$, Shoot regeneration, Turnip.

Abbreviations

$AgNO_3$, silver nitrate; B5, B5 medium (Gamborg *et al.*, 1987); BA, 6-Benzylamino purine; Cb, Carbenicillin; 2, 4-D, 2, 4-Dichorophenoxyacetic acid; GUS, β-Glucuronidase; Km, Kanamycin; Km^R, Kanamycin-resistant.

Introduction

Brassica rapa L. contained various subspecies and varieties, oil seed (ssp. *oleifera*), Chinese cabbage (ssp. *pekinensis*), leaf vegetable (ssp. *chinensis*) and turnip (var. *rapifera*). Genetic transformation is an important technique for crop improvement. Using the *Agrobacterium*-mediated transformation, transgenic plants have been successfully obtained from hypocotyl sections of oil seed (Radke *et al.*, 1992) and leaf vegetable (Takasaki *et al.*, 1997), and from cotyledon explants of Chinese cabbage (Jun *et al.*, 1995). However, *Agrobacterium*-mediated transformation of turnip has not been successful yet.

B. rapa is recalcitrant to shoot regeneration compared to other Brassica species (Narashimhulu and Chopra, 1988). Ethylene is involved in recalcitrant in vitro shoot differentiation of Chinese cabbage, in which shoot regeneration is enhanced on medium containing inhibitors of ethylene biosynthesis, aminoethoxyvinylglycine (AVG), aminooxyacetic acid (AOA), and an ethylene antagonist, $AgNO_3$ (Chi and Pua, 1989; Chi *et al.*, 1991).

In this paper, we investigated the optimal concentration of $AgNO_3$ for increasing shoot regeneration from hypocotyl sections of turnip cultivars, and applied *Agrobacterium*-mediated transformation procedure to three turnip cultivars.

Materials and Methods

Six Japanese turnip cultivars, 'Hinonakabu', 'Honbenidaimarukabu', 'Syogoinkabu', 'Tsudakabu', 'Tamayuki' and 'Takane' (provided by Watanabe Seed. Co., Miyagi, Takii Seed Co., Kyoto and Sakata Seed Co., Kanagawa) were used as experimental plant materials. Shoot regeneration and plant transformation experiments were carried out according to the procedure described by Takasaki *et al.* (1997). Hypocotyl sections were placed on a callus induction medium [B5 salts and vitamins, 1 mg l^{-1} 2, 4-D, 3% sucrose, 0.6% phytoagar, pH 5.8] for 7 days, then transferred to a shoot regeneration medium [B5 salts and vitamins, 3 mg l^{-1} BA, 1 mg l^{-1} zeatin, 1% sucrose, 0.6% phytoagar, pH 5.8] with 0, 5, 10, 15 and 20 mg l^{-1} $AgNO_3$, respectively. After one week, the sections were transferred to a fresh shoot regeneration medium every two weeks.

Two hundred hypocotyl sections of each cultivar were infected with *Agrobacterium tumefaciens* strain EHA101 (Hood *et al.*, 1986) harboring a binary vector pIG121Hm (Hiei *et al.*, 1994). After cocultivation on tobacco feeder cells for 3 days at 25℃ in the dark, the sections were cultured on the callus induction medium containing 500 mg l^{-1} Cb for 7 days, then transferred to the shoot regeneration medium with or without 10 mg l^{-1} $AgNO_3$ containing 500 mg l^{-1} Cb and 10 mg l^{-1} Km. The sections were subsequently transferred to fresh shoot regeneration medium without $AgNO_3$ every two weeks. Green shoots were excised from calli and placed on a shoot maturation medium [B5 salts and vitamins, 1% sucrose, 0.6% phytoagar, pH 5.8] containing 500 mg l^{-1} Cb and 50 mg l^{-1} Km. Three weeks later, Km^R shoots were transferred to a root induction medium [B5 salts and vitamins, 2 mg l^{-1} indol-3-butyric acid, 1% sucrose, 0.6% phytoagar, pH 5.8] containing 250 mg l^{-1} Cb and 50 mg l^{-1} Km. After 3-4 weeks, histochemical GUS expression was assayed on the leaf segment of rooted plantlets as described by Jefferson (1987).

Results and Discussion

Shoot regeneration abilities were evaluated from hypocopyl sections of six Japanese turnip cultivars. 'Honbenidaimarukabu', 'Hinonakabu' and 'Tsudakabu' exhibited shoot regeneration frequency (No. of the regenerated sections / total sections x100) at 4.0%, 44.0% and 28.0%, respectively, but 'Syogoinkabu', 'Takane' and 'Tamayuki' were never regenerated (**Table 1** and **Table 2**). Among ethylene inhibitors (AVG and AOA) and ethylene antagonist ($AgNO_3$), $AgNO_3$ showed the greatest enhancement on shoot regeneration of Chinese cabbege (Chi and Pua, 1989; Chi *et al.*, 1991). To increase shoot formation, hypocotyl sections of 'Honbenidaimarukabu' were cultured on shoot regeneration media containing 5-20 mg l^{-1} $AgNO_3$ for one week. The presence of $AgNO_3$ markedly enhanced shoot formation and increased the incidence of shoot regeneration to 36.0-48.0% (**Table 1**).

Shoot regeneration medium with 10 mg l^{-1} $AgNO_3$ gave 'Honbenidaimarukabu' 46.0% accompanied by a maximum of 4.3 shoots per hypocotyl. We chose this medium and evaluated another five turnip cultivars for shoot regeneration. The presence of $AgNO_3$ also enhanced shoot formation from these sections of 'Hinonakabu', 'Syogoinkabu', 'Tamayuki' and 'Tsudakabu', exhibiting an incidence of shoot regeneration of 72.0%, 30.0%, 48.0% and 44.0%, respectively (**Table 2**), which indicated that ethylene evolution *in vitro* is probably involved in the recalcitrance of turnips. No shoot was obtained from the sections of 'Takane' under the presence of $AgNO_3$. Chinese cabbages without shoot regeneration ability produce higher level of ethylene in the presence of $AgNO_3$ than those with shoot regen-

Table 1 Effect of $AgNO_3$ on shoot regeneration from 50 hypocotyl sections in turnip cv. Honbenidaimarukabu.

$AgNO_3$ (mg l^{-1})	Shoot regenaration frequency (%)	No. of shoot per hypocotyl
0	4.0	2.4
5	42.0	3.7
10	46.0	4.3
15	48.0	3.8
20	36.0	3.4

Table 2 Effect of $AgNO_3$ on shoot regeneration from 50 hypocoryl sections in five tutnip cultivars.

Cultivar	Shoot regeneration frequency (%)	
	-	+
Hinonakabu	44.0 (2.3)	72.0 (2.1)
Syogoinkabu	0.0	30.0
Takane	0.0	0.0
Tamayuki	0.0	48.0 (1.6)
Tudakabu	28.0 (2.2)	44.0 (1.7)

Shoot regeneration frequency was investigated on shoot regeneration medium with (+) or without (-) 10 mg l^{-1} $AgNO_3$. Average number of regenerated shoots per hypocotyl section is shown in parenthesis.

Table 3 Transformation efficiencies ofthree turnip cultivars.

Cultivar	$AgNO_3$[1]	No. of sections	Calli[2]	Shoots[3]	Plants[4]	GUS+ plants[5]	Transformation efficiency (%)[6]
Honbenidaimarukabu	-	200	126	3	0	0	0.0
	+	200	135	34	2	1	0.5
Hinonakabu	+	200	137	8	2	2	1.0
Tsudakabu	+	200	131	6	1	0	0.0

[1] Shoot regeneration medium with (+) or without (-) 10 mg l^{-1} $AgNO_3$.

[2] Number of calli praduced on shoot regeneration medium containing 10 mg l^{-1} Km.

[3] Number of shoots produced on shoot regeneration medium containing 10 mg l^{-1}Km.

[4] Number of shoots produced on root induction medium containing 50 mg l^{-1}Km.

[5] Number of GUS positive plantlets.

[6] Transformation frequencey (%)=(Number of GUS+ plant)/(total munber of hypocotyl sections)

eration ability (Zhang *et al.*, 1998). Different shoot regeneration ability among six turnip cultivars may be due to some differences in ethylene production or sensitivity.

Using a leaf vegetable cultivar 'Osome' previously, we searched for factors (bacterial strains, infection time, cocultivation temperature and period, Km or hygromycin selection) influencing *Agrobacterium*-mediated transformation, and obtained transgenic 'Osome' plants with a high transformation efficiency of 5% under the following conditions, cocultivation with EHA101 (pIG121Hm) at 25℃ for 3 days and Km selection (Takasaki *et al.*, 1997). Under the same transformation condition, the hypocotyl sections of 'Honbenidaimarukabu', Hinonakabu' and 'Tsudakabu' were transformed with *A. tumefaciens* strain EHA101 harboring a binary vector pIG121Hm, and cultured on the shoot regeneration medium without or with 10 mg l^{-1} $AgNO_3$. One hundred thirty-five Km^R calli, 34 Km^R shoots and a GUS positive plant were obtained from the hypocotyl sections of 'Honbenidaimarukabu' on the medium with $AgNO_3$, but 126 Km^R calli, 3 Km^R shoots and no GUS positive plant from those on the medium without $AgNO_3$ (**Table 3**). The transformation efficiency depends mainly upon the infection frequency of *Agrobacterium* and the shoot regeneration frequency. The medium with and without $AgNO_3$ produced the almost same number of Km^R callus from the hypocotyl sections, but the medium with $AgNO_3$ exhibited the shoot regeneration frequency 11 times as high as the medium without $AgNO_3$. This indicated that addition of $AgNO_3$ to shoot regeneration medium enhanced shoot formation from Km^R calli, leading to the production of GUS positive plant.

One hundred thirty-seven and 131 Km^R calli were formed, and 8 and 6 Km^R shoots were regenerated from the hypocotyl sections of 'Hinonakabu' and 'Tsudakabu' on the medium with $AgNO_3$, respectively. Two GUS positive plants were obtained from 'Hinonakabu', but not any from 'Tsudakabu' (**Table 3**). 'Hinonakabu' exhibited the highest shoot regeneration ability among three turnip cultivars, but produced Km^R shoots less than 'Honbenidaimarukabu'. 'Hinonakabu' had half hypocotyl thickness of 'Honbenidaimarukabu'. The Km^R calli formed from 'Hinonakabu' were small and damaged by *Agrobacterium* infection, which is due to the lower shoot regeneration from Km^R calli.

We used $AgNO_3$ for increasing shoot regeneration from hypocotyl sections of turnip cultivars, and were successful to produce the transgenic 'Hinonakabu' and 'Honbenidaimarukabu' plants with the transformation efficiencies of 1.0% and 0.5%, respectively. The present procedure will be applicable to some other turnip cultivars showing high shoot regeneration frequency on the medium with $AgNO_3$.

Acknowledgment

We thank Watanabe Seed. Co., Miyagi, Takii Seed Co., Kyoto and Sakata Seed Co., Kanagawa for providing us with the seeds of Japanese turnip cultivars.

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