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Effect of Antibiotics and Herbicide on Shoot Regeneration from Cotyledon and Hypocotyl Explants of Chinese Cabbage

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ABSTRACT To develop a selection system for regenerating plants from transformed tissues, effects of four antibiotics (kanamycin, hygromycin, carbenicillin, cefotaxime) and herbicide (phosphinotricin) on shoot regeneration from cotyledon and hypocotyl explants of Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*) were studied. For cotyledon, shoot induction was not significantly affected by kanamycin at 1 mg · L^{-1}, but the number of shoots formed was significantly reduced at 2 mg · L^{-1}, and no shoots were regenerated from any explants at 6 mg · L^{-1} or higher. Hypocotyl explants showed similar result as cotyledon. Kanamycin at 7 mg · L^{-1} may be adequate for selecting Chinese cabbage transformants. Hygromycin at 4 mg · L^{-1} or higher completely inhibited the growth and shoot regeneration of Chinese cabbage explants. Therefore, resistance gene to hygromycin may also be used as a selective marker for Chinese cabbage transformation. Carbenicillin and cefotaxime, the cephalosporin type of antibiotics, had little effect on shoot regeneration of Chinese cabbage explants. Since carbenicillin and cefotaxime have low toxicity to Chinese cabbage, they are suitable for use in tissue culture to eliminate *Agrobacterium* in transformation experiments after co-cultivation. Shoot regeneration from cotyledon and hypocotyl explants was significantly reduced in presence of 1 mg · L^{-1} phosphinotricin (PPT) and completely inhibited by 2 mg · L^{-1} or higher. PPT, same as antibiotics, may also be used to select transformed cells. Since Chinese cabbage is known to be recalcitrant to in vitro shoot regeneration compared to other Brassica species, even though lower levels of selectable markers result in more transformants but simultaneously allow more untransformed escapes to develop, lower levels of antibiotics and herbicides could be successfully used as a selectable marker to reduce selection pressure.

Additional key words: *Agrobacterium*, *Brassica campestris* ssp. *pekinensis*, carbenicillin, cefotaxime, hygromycin, kanamycin, phosphinotricin

Introduction

Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*), a member of the genus *Brassica*, is one of the most important vegetables for agricultural production and is widely cultivated in Asia, especially in Korea, Japan, and China.

Genetic engineering techniques, such as *Agrobacterium*-mediated DNA transfer, have great potential to improve established cultivars by introducing genes of interest while maintaining commercially desirable phenotypes. Gene transfer is not limited by relatedness; genes from any species, including nonplant genes, can be inserted into the Chinese cabbage. A requisite for exploiting genetic engineering techniques is the availability of plant regeneration including selection system.

Selectable marker genes are essential for the introduction of agronomically useful genes into important crop plants. Since the incorporation of genes into plant chromosomes during genetic modification processes occurs at an extremely low frequency, selectable marker genes are joined to the agronomically useful genes. Transformation of plant cells has been achieved using chimeric genes that confer resistance to toxic drugs such as antibiotics and herbicides. In the presence of a selective agent, only those cells that are transformed and express the selectable marker gene will divide and regenerate. Plants regenerated from the surviving cells will contain the selectable marker gene joined to the agronomic gene of interest.

The objective of this research was to determine the effect of antibiotics, commonly used in *Agrobacterium* transformation, on shoot regeneration of Chinese cabbage. Various antibiotic resistance genes are used as selectable marker genes in the production of transgenic plants (Yoder and Goldbrough, 1994). In *Brassica* transformation, the most commonly used is neomycin phosphotransferase gene (*npt*II) from transposon Tn5 (Beven et al., 1983) which confers resistance towards some aminoglyco-

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sides such as kanamycin, neomycin, gentamicin and paromycin. Another effective selection marker for *Brassica* transformation is the hygromycin phosphotransferase gene (*hpt*) from *E. coli* providing resistance to hygromycin (van den Elzen et al., 1985; Waldron et al., 1985). Antibiotics used for elimination of *Agrobacterium*, particularly cefotaxime and carbenicillin may also influence plant cell growth and regeneration. Cefotaxime has been reported to enhance callus growth of wheat (Mathias and Boyd, 1986) and plant regeneration from a number of species, including birch (Valobra and James, 1990) barley (Mathias and Musaka, 1987), and apple (James et al., 1988).

Other types of selectable marker are obtained by using herbicide resistance genes. The predominant gene in this group used for *Brassica* transformation is the *bar* gene (Thompson et al., 1987) which confers resistance to the herbicides bialaphos and phosphinotricin (de Block et al., 1989). However, a detailed examination of these antibiotics and herbicide on Chinese cabbage tissue has not been reported. The objective of this research was to develop a selection system for regenerating plants from transformed tissues of Chinese cabbage, which will be used for applying genetic engineering technology to improve this vegetable crop.

In this paper, we report the effects of antibiotics and herbicide on shoot induction from cotyledon and hypocotyl explants of Chinese cabbage (*Brassica campestris* ssp. *pekinensis* cv. 'Seoul').

Materials and Methods

The effects of antibiotics and herbicide on shoot regeneration were studied on shoot induction media which were determined for cotyledon and hypocotyl, respectively. Cotyledon and hypocotyl explants were collected from seedlings of Chinese cabbage (*Brassica campestris* ssp. *pekinensis* cv. 'Seoul'). Seed were first dipped in 70% EtOH for 1 min. Then the seeds were surface-sterilized in 0.8 % sodium hypochlorite solution for 20 min and rinsed sterile distilled water five times. The seeds were germinated in 70 × 170 mm bottle containing MS basal medium (Murashige and Skoog 1962) with vitamins, 3 % sucrose, and 0.8 % agar. The pH was adjusted to 5.8 before autoclaving.

Cotyledons including 1−2mm petioles and hypocotyl were excised from 4−6 day-old seedlings right before the emergence of the first leaf. The excised explants were placed into each 87 × 15 mm plastic petri plates containing shoot induction medium with various concentrations of antibiotics and herbicide. Shoot induction media were MS medium with 2.0 mg · L^{-1} napthalene acetic acid (NAA) and 1.0 mg · L^{-1} benzyl adenine (BA) for cotyledon and with 1.0 mg · L^{-1} NAA and 5.0 mg · L^{-1} BA for hypocotyl, respectively. Antibiotic treatments for each experiment were kanamycin B at 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 mg · L^{-1}; hygromycin at 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 mg · L^{-1}; cefotaxime at 0, 200 or 400 mg · L^{-1}; and carbenicillin at 0, 500 or 1000 mg · L^{-1}. Herbicide, phosphinothricin (PPT), was tested at 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 mg · L^{-1}. All antibiotics and PPT were filter sterilized and added to cooled medium after autoclaving. Five plates (replicates) containing five cotyledons or five hypocotyls for each treatment, were placed in a completely randomized design. The petri plates were cultured and kept in a light thermostat with a 18/6 h light period and 25±2 ℃ temperature. Data were recorded after 4 weeks for the number of shoots exceeding 5 mm in length. All experiments described above were repeated three times, and data were combined and analyzed with the statistical program SAS (SAS Insitute 1985). F-tests were used to determine the significance of treatments and mean separations were based on least significant difference (LSD).

Results and discussion

Kanamycin had a significant effect on callus formation and shoot regeneration of Chinese cabbage. Callus formation was not affected by kanamycin at 5 mg · L^{-1}, but dramatically inhibited when increased to 7 mg · L^{-1}, and was completely inhibited at 10 mg · L^{-1} (data not shown). For shoot induction from cotyledon was not affected by kanamycin at 1 mg · L^{-1}, but the numbers of shoots formed were significantly reduced at 2 mg · L^{-1}, and no shoots were reduced from any explants at 6 mg · L^{-1} or higher (Table 1). Hypocotyl explants showed similar result as cotyledons. Kanamycin-treated cotyledon and hypocotyl explants bleached and turned white with toxic concentration of kanamycin. Callus formation and shoot regeneration of Chinese cabbage can be inhibited at 6 mg · L^{-1} and 10 mg · L^{-1}, kanamycin respectively indicating that Chinese cabbage is a kanamycin-sensitive species. The high concentration of kanamycin may reduce transformation efficiency in Chinese cabbage transformation system. Some papers reported the inhibition of regeneration in *B. napus* by kanamycin (Thomzik and Hain, 1990) and of embryogenesis and root formation in B. nigra (Gupta et al., 1993). Most procedures have used 50−100 mg · L^{-1} kanamycin, however, these concentrations may not be optimal for Chinese cabbage. Kanamycin at 7 mg · L^{-1} may be adequate for selecting Chinese cabbage transformants, instead of 50−100 mg · L^{-1} used for other crops (de Block, 1988; Horsch et al., 1985).

Callus formation of all explants was not affected by hygromycin at 1 or 2 mg · L^{-1}, but was significantly reduced in presence of 3 mg · L^{-1} and completely inhibited by 5 mg · L^{-1} or higher (data not shown). Hygromycin at 4 mg · L^{-1} or higher completely inhibited the growth and shoot regeneration from Chinese cabbage explants. The sensitive tissues turned black and died, in contrast with kanamycin-treated tissues that turned white. Hygromycin had stimulatory effects on potato regeneration (Park et al., 1995), but this study did not show such an

Table 1. Effect of kanamycin and hygromycin on shoot regeneration from cotyledon and hypocotyl explants of Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*).

Antibiotics ($mg \cdot L^{-1}$)	Number of shoots regenerated per explant[z]	
	Cotyledon	Hypocotyl
Kanamycin[y]		
0	1.73a	1.47a
1	1.43a	1.07b
2	0.87b	0.57c
3	0.33c	0.27cd
4	0.13c	0.07d
5	0.07c	0.00d
6	0.00c	0.00d
7	0.00c	0.00d
8	0.00c	0.00d
9	0.00c	0.00d
10	0.00c	0.00d
Hygromycin[y]		
0	1.57a	1.40a
1	0.93b	0.77b
2	0.53c	0.37c
3	0.20cd	0.10c
4	0.00d	0.00c
5	0.00d	0.00c
Carbenicillin		
0	1.57a	1.30a
500	1.47a	1.30a
1000	1.43a	1.10a
Cefotaxime		
0	1.27a	1.30a
200	1.23a	1.17a
400	1.07a	1.10a

[z]Each value represents a mean of five replications of five explants each from three experiments. The means followed by the same letter are not significantly different at the 0.05 probability level according to LSD.

[y]Data on kanamycin of 10, 20, 30, 40 50, and 100 $mg \cdot L^{-1}$, and hygromycin of 6, 7, 8, 9, 10, 20, 30, 40, 50, and 100 $mg \cdot L^{-1}$ were not shown, where no single shoot regenerated in all media with cotyledon and hypocotyl explants.

effect. Hygromycin has inhibition activity against both prokaryotic and eukaryotic cells by interfering with protein synthesis (Cabanas et al., 1978; Gonzalez et al., 1978). Therefore, the gene encoding resistance to hygromycin may also be used as a suitable selective marker for Chinese cabbage transformation. Carbenicillin and cefotaxime, the cephalosporin type of antibiotics, had little effect on shoot regeneration from Chinese cabbage explants (Table 1). Fortunately, the tested concentrations were much higher than those required to eliminate *Agrobacterium*. Antibiotics for removing *Agrobacteriumium* from the explants after cocultivation must ideally be lethal to the bacteria while at the same time being harmless to the plant material. Since carbenicillin and cefotaxime have low toxicity to Chinese cabbage, they are suitable for use in Chinese cabbage tissue culture to eliminate *Agrobacterium* in transformation experiments after co-cultivation. However, the disadvantages of cefotaxime are that it can enhance vitrification and necrosis of shoots and decrease shoot regeneration while carbenicillin has been reported to reduce the negative effects of silver nitrate (de Block et al., 1989; Schroder et al., 1994). Carbenicillin has also proved to be superior to vancomycin, which also used as selective agent, regarding shoot regeneration (Schroder et al., 1994).

Callus formation of Chinese cabbage explants was not affected by PPT at 1 $mg \cdot L^{-1}$, but was significantly reduced in presence of 2 $mg \cdot L^{-1}$ and completely inhibited by 3 $mg \cdot L^{-1}$ or higher (data not shown). However, shoot regeneration from cotyledon and hypocotyl explants was significantly reduced in presence of 1 $mg \cdot L^{-1}$ phosphinotricin and completely inhibited by 2 $mg \cdot L^{-1}$ or higher (Table 2). The phospinothricin sensitive tissues turned white and died, in contrast with hygromycin-treated tissues that turned black and died. PPT is a non-selective herbicide that interferes with the conversion of glutamic acid and ammonia to glutamine, resulting in accumulation of ammonia ions for cells to cause death (Lacuesta et al., 1989). The predominant gene used for *Brassica* transformation is the bar gene (Thompson et al., 1987) which confers resistance to the herbicide bialaphos and PPT (de Block et al., 1989). Regeneration of transformed plants with phosphinotricin has been obtained with several transformation systems and this selection marker offers the additional possibility of selection of transformed cells.

In summary, the ability of cotyledon and hypocotyl tissues to regenerate shoots was significantly reduced by infection with *Agrobacterium* and the antibiotic selection. Furthermore certain selectable markers are detrimental to plant regeneration. The level of selective agent is dependent on the type of explant and genotype applied. For Chinese cabbage, kanamycin at 7 $mg \cdot L^{-1}$

Table 2. Effect of phosphinotricin (PPT) on shoot regeneration from cotyledon and hypocotyl explants of Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*).

Phosphinotricin[y] ($mg \cdot L^{-1}$)	Number of shoots regenerated per explant[z]	
	Cotyledon	Hypocotyl
0	1.63a	1.37a
1	0.63b	0.43b
2	0.00c	0.00c
3	0.00c	0.00c
4	0.00c	0.00c
5	0.00c	0.00c

[z]Each value represents a mean of five replications of five explants each from three experiments. The means followed by the same letter are not significantly different at the 0.05 probability level according to LSD.

[y]Data on phosphinotricin of 6, 7, 8, 9, 10, 20, 30, 40, 50, and 100 $mg \cdot L^{-1}$ were not shown, where no single shoot regenerated in all media with cotyledon and hypocotyl explants.

and hygromycin at 4 mg · L^{-1} may be adequate for selecting transformed tissues, instead of 50 − 100 mg · L^{-1} used in other crops for selection. PPT at 2 mg · L^{-1} may also be used to select transformed cells. Therefore, the gene encoding resistance to herbicide may also be used as a suitable selective marker for Chinese cabbage transformation. This experiment showed that kanamycin, hygromycin and PPT resistance are all useful selection markers for regeneration from cotyledon and hypocotyl tissues and may be appropriate for transformation experiments at lower concentrations than those concentrations used for other vegetable crops. Since Chinese cabbage is known to be recalcitrant to in vitro shoot regeneration compared to other *Brassica* species (Murata and Orton 1987. Jain et al. 1988, Narashimhulu and Chopra 1988), even though lower levels of antibiotics result in more transformants but simultaneously allow more untransformed escapes to develop (Fry et al., 1987; Radke et al., 1988), lower levels of antibiotics and herbicides could be successfully used as selectable marker to reduce selection pressure. Carbenicillin and cefotaxime, which showed low toxicity to Chinese cabbage tissue, are suitable for use in tissue culture to eliminate *Agrobacterium* in transformation experiments after co-cultivation. Kanamycin, hygromycin and PPT, at concentrations found to inhibit callus and shoot formation, will be used successfully in subsequent genetic transformation studies to select transformed cells and shoots.

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항생제와 제초제가 배추 자엽 및 배축 절편체로부터의 신초 형성에 미치는 영향

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배추(*Brassica campestris* L. ssp. *pekinensis*) 형질전환 세포로부터의 식물체 재분화를 위한 선발체계를 확립하기 위하여 kanamycin, hygromycin, carbenicillin, cefotaxime등 4가지 항생제와 제초제 phosphinotricin가 자엽 및 배축 절편체로부터의 신초형성에 미치는 영향을 조사하였다. 자엽절편체는 kanamycin 1 mg · L^{-1}처리에서 신초형성에 아무 영향을 받지 않았으나 2 mg · L^{-1} 처리부터 형성수가 감소하여 6 mg · L^{-1} 이상 처리구부터는 신초가 전혀 형성되지 않았다. 배축 절편체도 자엽과 비슷한 결과를 나타내어 kanamycin의 경우 7 mg · L^{-1}의 농도가 배추 형질전환체의 선발에 적당하다고 생각되었다. Hygromycin은 4 mg · L^{-1} 처리부터 신초형성을 억제하여 배추형질전환을 위하여 낮은 농도로 사용할 수 있는 선발 마커로 생각된다. Cephalosporin type의 항생제인 carbenicillin과 cefotaxime은 신초형성에 아무 영향을 주지 않아 배추 절편체에 독성을 주지 않는 결과를 보여 주어, *Agrobacterium*과의 공동배양 후 이를 제거하는데 적당한 항생제라 할 수 있었다. 제초제인 phosphinotricin의 경우 1 mg · L^{-1} 처리부터 신초 형성이 감소하기 시작하여 2 mg · L^{-1} 이상부터는 완전히 억제되어 항생제와 더불어 형질전환 세포의 선발에 이용될 수 있다고 생각된다. 배추는 다른 배추속의 작물과 비교할 때 신초 재분화가 어렵기 때문에 비록 escape율이 높아진다고 해도 낮은 농도의 항생제나 제초제를 사용하여 선발압을 낮추어 주는 것이 형질전환에 효과적이라 생각된다.

추가 주요어 : *Agrobacterium*, *Brassica campestris* ssp. *pekinensis*, carbenicillin, cefotaxime, hygromycin, kanamycin, phosphinotricin