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Transformation of *Brassica napus* with Glutathione Reductase Gene

Hyo Shin Lee, Min Sup Chung and Jinki Jo

Glutathione reductase 유전자 도입에 의한 유채(油菜)의 형질전환

이효신 · 정민섭 · 조진기

摘 要

오존을 포함한 광화학 산화물에 대한 저항성을 가지는 목초를 구축하기 위하여 배추의 cytosolic glutathione reductase 유전자를 분리하여 *Agrobacterium tumefaciens*를 매개로 한 유채 (*Brassica napus* L. cv. Chungpoong)로의 형질전환에 관한 실험을 하여 다음과 같은 결과를 얻었다.

배추로부터 분리한 glutathione reductase 유전자는 총 길이 1,763bp로서 502개의 아미노산으로 구성된 분자량 54.4kDa의 단백질을 암호화하며, 오존 및 paraquat 처리에 의해 그 발현이 급격히 증가하는 것으로 밝혀졌다. 이 유전자를 식물체 형질전환용 벡터인 pBKS1-1의 CaMV 35S promoter에 subcloning하여 식물체 내에서 항상적으로 발현가능한 binary vector, pBKS-GR1을 구축하였다. 구축된 pBKS-GR1을 *Agrobacterium tumefaciens* LBA4404에 도입한 다음, 엽병이 부착된 유채의 자엽과 공배양하여 형질전환을 유도하였다. 유도된 shoots를 kanamycin이 첨가된 재분화배지에서 선발한 다음, 뿌리를 유도하고 완전한 정상식물체로의 발달을 유도하였다. 이때 배지중의 $AgNO_3$ 첨가는 shoots의 형성을 크게 증가시키는 것으로 나타났다. Kanamycin이 첨가된 배지에서 선발된 재분화 식물체에 대한 Southern blot analysis 결과, 유채의 genome상에 배추의 glutathione reductase 유전자의 coding sequence (1.8kb)가 정상적으로 도입되었음을 확인하였다.

I. INTRODUCTION

Ozone (O_3) is one of constituents of photochemical air pollutants and is considered to have serious effects on vegetation. Ozone is shown to be phytotoxic at high concentrations ($>0.2\mu l/\ell$) given for short time periods. Ozone applied at low levels ($\leq 0.15\mu l/\ell$) is also known to affect plant growth and development when the exposing period lasts more than weeks. The effects include a decline in net photosynthesis, foliar injury, altered patterns of flower formation, reduction in shoot and root growth, accelerated

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senescence of organs, and reduction in crop yield (Langebartels et al., 1991). These ozone damages are thought to be resulted from the action of free radicals and other oxidants produced by the interaction of O_3 and its degradation products with plant tissue constituents (Heath, 1980). Cellular antioxidant system is a front-line defense against oxygen free radicals. The activity of glutathione reductase (GR), one of enzymatic antioxidants, has been shown to increase in response to Ozone.

Glutathione reductase catalyzes the conversion of GSSG to GSH using NADPH as an electron donor in active oxygen scanvenge system. GR is widely distributed in all organisms, and has been purified and characterized from a variety of bacteria, animal and plant species. Properties such as molecular weight, subcellular localization, different isozymes, and optimum pH have all been reported (Halliwell and Foyer, 1978; Mahan and Burke, 1987; Anderson et al., 1990; Edwards et al., 1990; Foyer et al., 1991; Hausladen and Alscher, 1994). It has also been demonstrated about enhanced activity in response to a number of stress conditions such as ozone fumigation, paraquat treatment, greening, cold treatment, and the combination of magnesium deficiency with high intensity light (Guy et al., 1984; Mehlhorn et al., 1987; Tanaka et al., 1988; Cakmak and Marschner, 1992; Edwards et al., 1994).

We have been investigating the role of GR in several oxidative stresses including ozone fumigation and paraquat treatment. To elucidate the relationship between O_3 tolerance and enhanced expression level of GR, we have made transgenic *Brassica napus* (*B. napus*) plants with *Brassica campestris* (*B. campestris*) cytosolic GR cDNA. We use the transformed *B. napus* plants to test whether the plants with elevated levels of cytosolic GR are tolerant to O_3 at dosages that normally injure plants.

Ⅱ. MATERIALS AND METHODS

1. Bacteria and Plant cultivar

E. coli strain HB101 was used as host cell of comstructed expression vector, pBKS-GR1. *Agrobacterium tumefaciens* LBA4404 was used in *B. napus* transformation. *B. napus* L. cv. Chungpoong was used in *Agrobacterium*-mediated transformation, and Both of the transformed and untransformed plants were grown in a greenhouse at approximately 28℃ with 16-hour light and an 8-hour dark cycle.

2. Standard DNA techniques

Preparation of phage and plasmid DNA, restriction enzyme digestion, agarose gel electrophoresis, and bacterial transformation were carried out using standard procedures (Sambrook et al., 1989). DNA probes were labelled with [α-^{32}P] dCTP by means of the random primer labeling procedure of Feinberg and Vogelstein (1984) using a kit (Amersham), and purified using a Sephadex G-50 Quick Spin Column (Boehringer Mannheim).

3. Construction of expression vector, pBKS-GR1

The full-length cDNA encoding *B. campestris* cytosolic glutathione reductase (GenBank accession number AF008441) was used in transformation of *B. napus*. The plant binary vector, pBKS1-1 had a cauliflower mosaic virus (CaMV) 35S promoter expressing constitutively in plants and a NPT-Ⅱ gene for kanamycin selection. pBKS1-1 derived from pBI121 was digested with *Sma* Ⅰ and ligated with *B. campestris* GR cDNA, resulting in pBKS-GR1.

4. Transformation of plant tissues

The plant binary vector containing *B. campestris* GR cDNA was transferred to *Agrobacterium tumefaciens* strain LBA4404 by two cycles of freeze-thaw method. Transformation and regeneration of *B. napus* were performed according to Lee et al. (1997). The sterilized *B. napus* cotyledonary petioles were cocultivated with *Agrobatetium* cells (10^8 cells/ml) harboring pBKS-GR1 for 3 days. Shoot formation were done on solid MS media (Murashige and Skoog, 1962) containing 0.5mg/ℓ of NAA, 2.0mg/ℓ of BAP, 3mg/ℓ of $AgNO_3$, 30mg/ℓ of sucrose, 2mg/ℓ of gelrite, 100mg/ℓ of cefotaxime, and 50mg/ℓ of kanamycin sulfate. For rooting, shoots were transferred to hormone-free MS media containing 50mg/ℓ of kanamycin sulfate. After root development, these plantlets were transferred to potting soil and grown in a greenhouse.

5. Southern blot analysis

Genomic DNA was isolated from leaves of tobacco seedlings grown in MS media supplemented with 50mg/ℓ of kanamycin sulfate as described by Murray and Thompson (1980). The isolated genomic DNA was digested with *Xba* I and *Pst* I, and fractionated by 0.8% agarose gel electrophoresis. The fractionated DNA was denatured twice with 1.5M NaCl and 0.5M NaOH, and neutralized with 1.5M NaCl and 0.5M Tris-HCl (pH 7.2). The denatured DNA was blotted onto Hybond-N^+ nylon membrane (Amersham). The blot was prehybridized for 3h at 42℃ in a buffer containing 50% formamide, 5× SSC, 5× Denhardt's solution, 50mM Na-Pi (pH 6.5), 0.1% SDS and 100mg/ml of denatured salmon sperm DNA. Hybridization was performed for 18h at 42℃ in the same solution supplemented with [α-^{32}P] labeled *B. campestris* GR cDNA.

III. RESULTS AND DISCUSSION

1. Construction of expression vector, pBKS-GR1

In order to construct transgenic plants which are resistant to oxidative stresses including ozone, *B. campestris* cytosolic glutathione reductase cDNA (BcGR1) was isolated and characterized (GenBank accession number AF008441). The expression of *B. campestris* GR was strongly induced by paraquat treatment or by ozone fumigation at the onset of stress. The cDNA clone consists of 1763 nucleotides which contain a full open reading frame of 502 amino acid residues with a molecular mass of 54.5kDa, 62 nucleotides of 5' untranslated region, 192 nucleotides of 3' untranslated region, and a poly(A)$^+$ tail. The putative polyadenylation signal occurs 78 nucleotides upstream from the poly(A)$^+$ tract located at the 3' end of the cDNA.

The cDNA was subcloned into the unique *Sma* I site of the plant transformation vector pBKS1-1, downstream of the constitutive CaMV 35S promoter and upstream of the *nos* termination sequence, in place of the *uid*A (GUS) reporter gene (Fig. 1A). *E. coli* strain HB101 was transformed with pBKS-GR1 and the trans formented *E. coli* was grown in the LB medium containing 50μg/ml of kanamycin sulfate. Transformed colonies were selected and the correct positioning of *B. campestris* GR cDNA in pBKS-GR1 between CaMV 35S promoter and *nos* terminator was confirmed with restriction mapping (Fig. 1B). This constructed plasmid pBKS-GR1 was used for making transgenic *B. napus* plants. *A. tumefaciens* LBA4404 was transformed with the pBKS-GR1 by two cycles of freeze-thaw method. The transformed colonies were selected on YEP medium containing 50μg/ml of kanamycin sulfate and 100μg/ml of rifampicin.

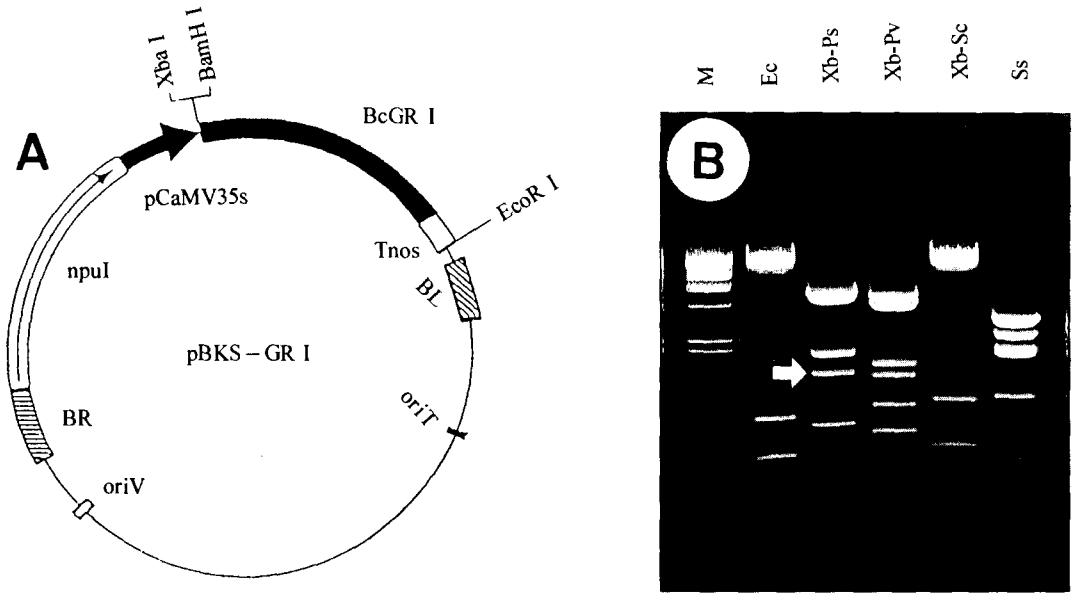

Fig. 1. Construction of an expression vector, pBKS-GR1.
[A] The structure of pBKS-GR1. nptⅡ, neomycin phosphotransferase typeⅡ gene; Tnos, nopaline synthase terminator; BR, T-DNA right border; BL, T-DNA left border.
[B] Identification of pBKS-GR1. M: DNA molecular marker (λ DNA, HindⅢ cut). Ec, *Eco*RⅠ; Xb, *Xba*Ⅰ; Ps, *Pst*Ⅰ; Pv, *Pvu*Ⅱ; Sc, *Sca*Ⅰ; Ss, *Ssp*Ⅰ. Arrow indicates 1.8kb GR cDNA fragment.

2. Transformation of plant tissues

In order to transform *B. napus* plants with GR gene, *B. napus* cotyledonary petioles were cocultivated with *Agrobaterium* cells harboring pBKS-GR1 for 3 days. Ten days after transfer to the MS solid medium with kanamycin, organ differentiation was observed in some cotyledonary petioles maintaining green while other petiloes were etiolated with time in the same medium. Fifteen days after transfer to the solid medium, induction of chloroplasts were also observed at the cut ends of petioles. Green shoots were induced vigorously in the cocultivated cotyledonary petioles while green shoots were not formed in the uncocultivated cotyledonary petioles (Fig. 2A). The addition of 3mg/ℓ of $AgNO_3$ into media stimulated approximately two or three times in shoot formation (data not shown). The transformed shoots were selected on kanamycin-containing MS-n/B medium. The induced green shoots were transferred to hormone-free MS medium containing 50mg/ℓ of kanamycin sulfate for root induction (Fig. 2B). Shoots and roots were induced about 30 and 20 days after cocultivation, respectively. After root development, these plantlets were transferred to potting soil and grown in a greenhouse condition for sampling for the confirmation of transformation.

3. Confirmation of transgenic *Brassica napus* plants

Agrobacterium-mediated *B. napus* transformation resulted in transgenic plants harboring pBKS-GR1. The plants were selected on MS medium containing 50mg/ℓ of kanamycin, and then analyzed for the presence

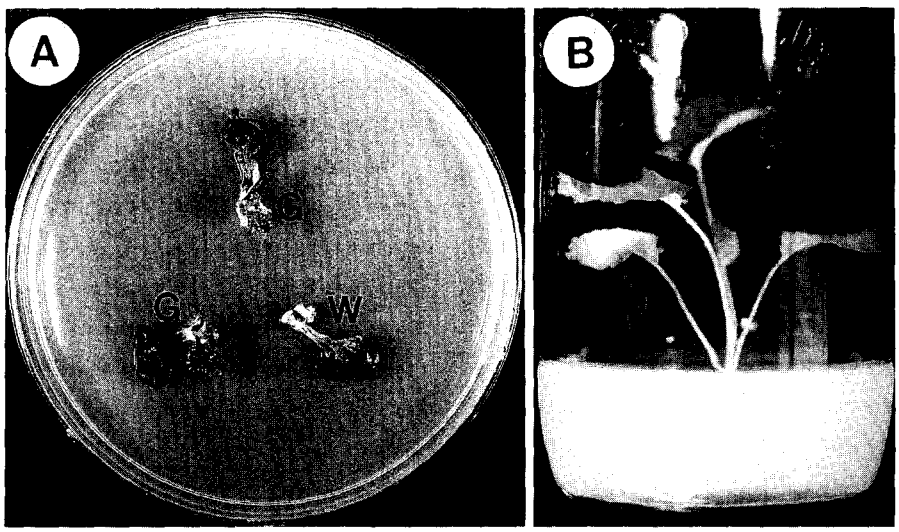

Fig. 2. Production of transgenic *Brassica napus* plants.

[A] Shoot formation from cotyledonary petioles in the selection medium (W: white, G: green).

[B] Normal green plant selected from the medium with kanamycin sulfate.

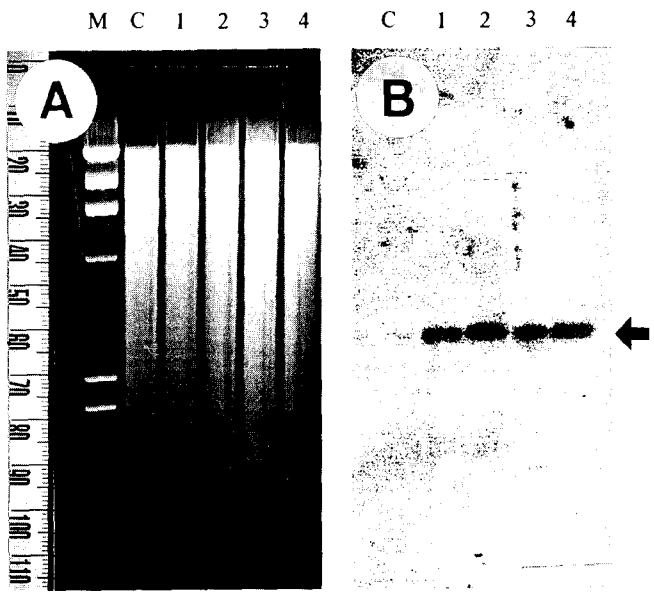

Fig. 3. Southern blot analysis of transformed *B. napus* plants.

Ten μg of *B. napus* genomic DNA was digested with *Xba* I and *Pst* I. The blot was hybridized with the [α-^{32}P] labeled *B. campestris* GR cDNA.

[A] Agarose gel electrophoresis. Lane M: DNA molecular marker (λ DNA, HindIII cut). Lane C: wild type plant. Lane 1-4: transgenic plants.

[B] Autoradiogram. In lane 1, 2, 3 and 4, a 1.5kb (arrow) band was sho-wed as *B. campestris* GR gene.

of the GR gene by Southern blot analysis. Transgenic plants were normal in appearance and showed no abnormal growth defects when compared to nontransgenic plants. Genomic DNA was extracted from leaves of the transgenic and nontransgenic *B. napus* plants. Both DNAs were digested to completion with *Xba* I and *Pst* I, and fractionated by agarose gel electrophoresis (Fig. 3A), and transferred onto nylon membrane. The blot was hybridized with [α-^{32}P] labeled *B. campestris* GR cDNA as a probe. As shown in Fig. 3B, presence of a 1.5kb fragment hybridizing to the BcGR1 probe in transgenic plants verified the introduction of the full length coding sequence of the BcGR1 cDNA (Fig. 3). Any hybridization signal was not obtained from untransformed *B. napus* plants.

It is not clear at present whether the plant cells acquire tolerance to oxidative stresses only by the cytosolic form of GR, but we can infer that there are some relations between the induction of cytosolic GR and the acquirement of tolerance to ozone by the fact that the GR gene is strongly expressed by ozone fumigation or by paraquat treatment. In Cu/Zn-SOD, an enzyme related to active oxygen scavenging system like GR, Pitcher et al. (1991 and 1996) explained that the transformed plants with cytosolic Cu/Zn-SOD gene did show tolerance to ozone while those with chloroplastic Cu/Zn-SOD gene did not show tolerance to ozone even though the expression was increased more than 15 times. We, therefore, have constructed the plant transformed with cytosolic form of GR gene derived from *Brassica campestris* to confirm this deduction, and using this gene we are studying the expression mechanism and the tolerance to oxidative stresses.

IV. SUMMARY

This study was conducted to construct of the transgenic plants which are resistant to oxidative stresses including ozone with *B. campestris* cytosolic glutathione reductase cDNA using the binary vector system of *Agrobacterium tumefaciens*.

The 1.8kb *B. campestris* cytosolic GR cDNA was subcloned into the unique *Sma* I site of the plant transformation vector pBKS1-1, downstream of the constitutive CaMV 35S promoter and upstream of the nos termination sequence, in place of the *uid*A (GUS) reporter gene. The resulting plant transformation vector, pBKS-GR1, was introduced into *A. tumefaciens* LBA4404 by two cycles of freeze-thaw method. The *B. napus* cotyledonary petioles were transformed by the *Agrobaterium* harboring pBKS-GR1. Transformed shoots were induced and selected on regeneration medium supplemented with kanamycin. The shoot formation was increased remarkably by addition of $AgNO_3$ in MS media. The transgenic plants were analyzed for the presence of the *B. campestris* GR gene by Southern blot analysis and it was confirmed that a foregin gene was stably integrated into the genomes of *B. napus plants*.

V. LITERATURE CITED

1. An, G., B.D. Watson, and C.C. Chiang. 1986. Transformation of tobacco, tomato, potato and *Arabidopsis thaliana* using a binary Ti vector system. Plant Physiol. 81:301-305.
2. Anderson, J.V., J.L. Hess, and B.I. Chevone. 1990. Purification, characterization, and immunological properties for two isoforms of glutathione reductase from eastern white pine. Plant Physiol. 94:1402-1409.
3. Cakmak, I., and H. Marschner. 1992. Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase, and glutathione reductase in bean leaves. Plant Physiol. 98:1222-1227.

4. Creissen, G., E.A. Edwards, C. Enard, A. Wellburn, and P. Mullineaux. 1992 Molecular characterization of glutathione reductase cDNA from pea. The Plant Journal. 2:129-131.
5. Edwards, E.A., S. Rawsthorne, and P. Mullineaux. 1990. Subcellular distribution of multiple isoforms of glutathione reductase in leaves of pea. Planta. 180:278-284.
6. Edwards, E.A., G.P. Creissen, and P. Mullineaux. 1994. Synthesis and properties of glutathione reductase in stressed peas. Planta. 192:137-143.
7. Feinberg, A.P., and B. Vogelstein. 1984. A technique for radiolabelling DNA fragments to high specific activity. Anal. Biochem. 137:266-267.
8. Foyer, C.H., M. Lelandais, C. Galap, and K.J. Kunert. 1991. Effects of elevated cytosolic glutathione reductase activity on the cellular glutathione pool and photosynthesis in leaves under normal and stress conditions. Plant Physiol. 97:863-872.
9. Guy, C.L., J.V. Carter, G. Telenosky, and C.T. Guy. 1984. Characterization of partially purified gluta-thione reductase from cold-hardened and nonhar-dened spinach leaf tissue. Cryobiology. 21:443-453.
10. Halliwell, B., and C.H. Foyer. 1978. Properties and physiological function of a glutathione reductase purified from spinach leaves by affinity chromato-graphy. Planta. 139:9-17.
11. Hausladen, A., and R.G. Alscher. 1994. Purification and characterization of glutathione reductase isozymes specific for the state of cold hardiness of red spruce. Plant Physiol. 105:205-213.
12. Heath, R.L. 1980. Initial events in injury to plants by air pollutants. Annu. Rev. Plant Physiol. 31:396-431.
13. Langebartels C., K. Kerner, S. Leonardi, M. Schraudner, M. Trost, W. Heller, and H. Sandermann. 1991. Biochemical Plant Responses to Ozone. I. Differential Induction of polyamine and ethylene biosynthesis in tobacco. Plant Physiol. 95:882-889.
14. Lee, H.S., Son, D.Y., and J.K. Jo. 1997. Transformation of *Brassica napus* with Acid Phosphatase Gene. J. Korean Grassl. Sci. 17(3):285-292.
15. Madamanchi, N.R., J.V. Anderson, R.G. Alscher, C.L. Cramer, and J.L. Hess. 1992. Purification of multiple forms of glutathione reductase from pea seedlings and enzyme levels in ozone-fumigated pea leaves. Plant Physiol. 100:138-145.
16. Mahan, J.R., and J.J. Burke. 1987. Purification and characterization of glutathione reductase from corn mesophyll chloroplasts. Physiol. Plant. 71:352-358.
17. Mehlhorn, H., D.A. Cottam, P.W. Lucas, and A.R. Wellburn. 1987. Induction of ascorbate peroxide and glutathione reductase actvities by interactions of air pollutants. Free Rad. Res. Commun. 3:1-5.
18. Murray, M.G., and W.F. Thompson. 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 8:4321-4325.
19. Pitcher, L.H., E. Brennan, A. Hurley, P. Dunsmuir, J.M. Tepperman, and B.A. Zilinskas. 1991. Overproduction of petunia chloroplastic copper/zinc superoxide dismutase does not confer ozone tolerance in transgenic tobacco. Plant Physiol. 97:452-455.
20. Pitcher, L.H., and B.A. Zilinskas. 1996. Overexpression of copper/zinc superoxide dismutase in the cytosol of transgenic tobacco confers partial resistance to ozone-induced foliar necrosis. Plant Physiol. 110:583-588.
21. Sambrook, J., Fritsch, E.F., and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual, 2nd Ed, Cold Spring Harbor Laboratory Press, New York.
22. Tanaka, K., H. Saji, and N. Kondo. 1988. Immunological properties of spinach glutathione reductase and inductive biosynthesis of the enzyme with ozone. Plant Cell Physiol. 29:637-642.

Correction

Transformation of *Brassica napus* with Acid Phosphatase Gene

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Due to an error by the authors, Fig. 4 is changed. Corrected Fig. 4 is shown here. The correction does not affect the interpretation of the result or the conclusion drawn in the paper.

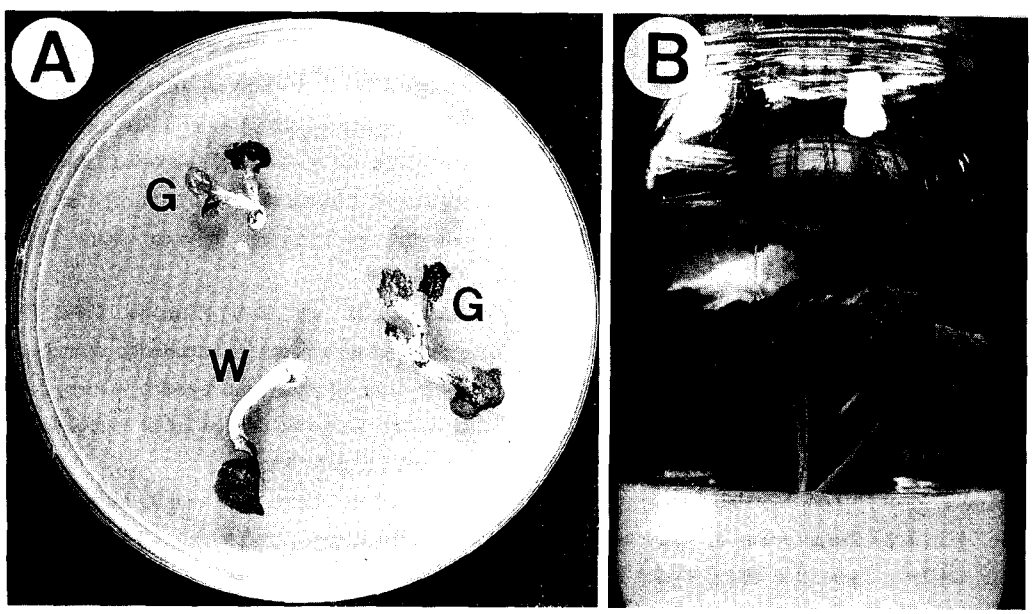

Fig. 4. Production of transgenc *Brassica napus* plants.
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