EXPRESSION OF CHITINASE GENE IN TRANSGENIC RAPE PLANTS

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Abstract :The hypocotyl and cotyledon of *Brassica napus* L. H165 and *Brassica juncea* DB3 were transformed with chitinase gene and herbicide-resistance gene by co-culture with *Agrobacterium tumefacients* LBA4404, and rape plants were obtained which could grow on the medium containing herbicide. The PCR result showed that exotic genes were integrated in the genome of the rape. Further study was performed to determine the impact of temperature on the transgenic rate and the differentiation of explants.

INTRODUCTION

Rape is one of the most important oil crops. The fungal disease, such as *Pyrenopeoziza brassica* Raw, *Leptosphaeria maculans Cylindrosporium concentricum*, and especially *Rhizoctonia solani*, can largely reduce the production and quality of the oil seed [1,2]. In the past, selection of new resistant variety by traditional breeding and using chemical pesticide is the major measure to prevent fungal disease, but traditional breeding is not always effective, and the pesticide can cause large pollution to the environment. The advent of plant transgenic technology offered a new approach to obtain resistant crop variety.

Chitinase hydrolyses chitin existing in hyphae of fungal and prevents fungi from infecting plants and propagating inside plant tissues [3]. Furthermore, there are not any chitinase target in plant [4]. Hence, the chitinase gene was successfully transformed into tobacco [5], rice [6], and strawberry [7].Some transgenic plants highly resistant to *Phoma lingam*, *Cylindrosporicum concentricum*[8],*Rhizoctonia solani*[5] were also obtained. In this study, the chitinase gene was successfully introduced into rape plant, which may result in selection of plant lines resistant to fungal disease.

MATERIAL AND METHODS

MATERIAL

TYPE OF RAPE

B.napus winter rape H165 and *B.juncea* spring rape DB_3 were used as donor material.

MEDIUM

Bacteria-free seeding medium, tissue induction and differentiation medium used were based on GAO et al[9].

STRAINS AND PLASMID

The common virulent *Agrobacterium tumefaciens* LBA4404 carrying chintinase gene and bar gene, controlled by CaMV35s and Ubiquitin promoters, respectively.

METHODS

CULTURE OF AGROBACTERIUM TUMEFACIENS

A. Tumefaciens was grown from glycerol stock in MG/L liquid medium[10] supplemented with 100 mg/l kanamycin and 50mg/L streptecin. The culture was incubated overnight at 27~29°C with shaking (250 rpm) until log phase, then the cells were pelleted by centrifugation at 4.5 g for 10 min followed by resuspensiom in inoculation medium [9]. The inoculation density of *Agrobacterium* (OD600) ranged from 1.0 to 2.0.

EXPLANT PREPARATION AND AGROBACTERIUM INFECTION

Seed sterilization, germination and *Agrobacterium* infection were based on GAO et al[9].

OBSERCATION BY SEM

Explants from hypocotyls and cotyledon were cultured on induction medium for 5, 9 and 11 days with light all day and night at 24±1°C, individually, and then they were proceed to produce as described previously[11] and then observed by SEM. Those hypocotyls and cotyledon cultured on medium without inducing substances were as control.

EFFECT OF TEMPERATURE ON THE TRANSFORMATIONAL RATE OF RAPE

DB_3 explants were dipped in solution containing *Agrobacterium tumfacious* for 5 min, and then were cultured in co-cultured medium at 19, 22, 25, 28 or 31°C under dark for 2 days before being cultured on selecting medium with 15mg/L PPT selectional pressure. Each treatment was composed of 100 explants and repeated for 3 times.

ROOTS AND PLANTS OF HERBICIDE –RESISTANT BUDS

The herbicide-resistant buds were sliced down and transferred to rooting medium after they had grown to 2-3cm, and then planted on bacteria-free soil in flower-basin at room temperature when 4-5 leaves appeared.

PCR assay

Genomic DNA samples were isolated from leaf tissues of putative transgenic plants as described by Dellaporta[12] and amplified by polymerase chain reaction. A left primer 5'-GCTCCACCTCCGATTA CTGC-3'and a right primer 5'-GCGTTGCCGTTGTTCTCCTC-3'were used to amplify an 438bp fragment of the chitinase gene. The PCR reactions were subjected to 30 cycles of: 4min pre-denatured at 94°C, 45s at 94°C, 45s at 45°C, and 105s prolonged at 72°C.

RESULTS AND DISCUSSIONS

Effect of temperature on transformation rate of rape

Highest fastness callus rate was found in rape co-cultured at 25°C. Transformation rate at 19°C,22°C,or33°C was apparently lower than that at 22°C and 25°C. Transformation rate of rape was affected significantly by temperature, and 22°C to 25°C were the most suitable transformation temperature range. Previously we had found 25°C was the most suitable transformation temperature to Maize, although Dillion[13] found there was no obvious difference on the transformation rate of tabacco between 19°C and 22°C. Hence, it might be concluded that both temperature and species have relationship with transformation rate.

Table1 Influence of temperature on *Agrobacterium tumefaciens* transformation

Material	temperature	explant	resistant callus				resistant callus	DUNCAN's
	(^{0}C)	NO.	I	II	III	mean	frequency(%	test
	19	300	11	13	8	10.67	10.67	B
	22	290	17	22	22	20.33	21.03	A
DB3	25	290	23	27	18	22.67	23.44	A
	28	300	12	16	11	13.00	13.00	B
	31	290	9	13	8	10.00	10.34	B

INDUCTION OF CALLUS ON COTYLEDON

Puffs appeared in the nick on hypocotyls after it was cultured on inducing medium for 5 days, and dark green trude appeared on day 9. By SEM, rape explant in slit was composed of cuticle and cortex. No obvious changes in morphorlogy and cell biology of blade after the cotyledon cultured on inducing medium for 2 days. However, on day 5 puffs appeared in the nick on the cotyledon, and attached medium turned brown and thicken, and cell turned much bigger in the nick of cotyledon, which showed cell dedifferentiation started. On day 9, active cell aggumulated into a cell mass. On day 11, mitotic cells spreaded all through to form separated cell mass, and scattered callus were visible (Figure3).

INDUCTION OF CALLUS ON HYPOCOTYLS

After culture for 5 days, puffs appeared on hypocotyls explant. On day 11 hypocotyls began to be thick, cutide slit, and light green, and tumor-shape tissue began to form in the nick. By SEM ,buldge appeared in the nick which means the dedifferentiation started. Active cell mitosis happened after 9 days of culture, and on day 11 dedifferentiation cell underwent mitosis rapidly to produce even-sized cells with long shape, and then cells turned to uneven-sized round or oval forms. Obvious changes of cells in the surface of the nick in hypocotyls were observed and

showed in figure 3-6. It is suggested that hypocotyls was dedifferentiated from the cells in folium tissue on the surface, and 400-600um or so from that of the nick (Figure 3).

TRANSGENIC PLANTSt

As shown in Table 2, 22 fastness plants were screened out on PPT medium, 11 were positive assayed by PCR. Of these 11 ones, 6 were H165, 5 were DB_3(Figure 3). All of 22 plants came from hypocotyls explant.

Table 2 Result s of transformation

Variable	hypocotyls					cotyledon			
	1	2	3	4	5	1	2	3	4
NO. of explants	380	400	400	350	400	400	400	390	400
Forming shoots	18	25	11	20	18	8	5	6	2
transgenic plant	4	6	1	7	4	0	2	0	0
transformation frequency		1.14%						0.01%	

PCR ASSAY

Six of H165, and 5 of DB_3 were positive as in Figure 6. The size of the fragment amplified was the same with that of control, which suggested that target gene had been integrined in genome of rape (Figure 1).

EFFECT OF EXPLANT ON TRANSFORMATION OF RAPE

The development of reliable transformation protocols for recalcitrant species depends on the ability to deliver intact DNA molecules into the nuclear genome of regenerable cells and to recover fertile adult plants from tissue culture. The choice of starting material (explant) has proved to be crucial in successful *Agrobacterium*-mediated rape transformation. The regenerative way of bud differentiation of rape explant is very important to *Agrobacterium* infected and regeneration of transformation plant. It has been identified that transformation induced by *Agrobacterium* occurred mainly in cutide cells of explant. It will be very difficult to get nice transgenic plant when the regenerative cells were from inner cells. Hypocotyl of *Brassica juncea* DB_3 has been found to be one of the nicest explant[14 15 16].

EFFECT OF TEMPERATURE ON TRANSFORMATION RATE

Temperature is believed to be a very important factor to affect. The transformation rate has an obvious relationship with the transportant talent of T-DNA . Fullner et al[17] ever suggested that 190C was helpful to T-DNA complex dissemble, as well as the most suitable temperature of the transportation of T-DNA . But the induction of Vir gene and the integration and steady expression of foreign gene fragment needed to be at 250C[18]. On the other hand, it was suggested that there was a most suitable temperature to be transformed by *Agrobaterium*, because cells in mitosis were liable to absorb exotic gene fragment[19]. Above 32°C,formative changes of Vir gene led to its unabled expression. Higher temperature also inhabited $VirD_4$, VirG, $VirB_1$ and Vir11, which were essential genes for the formation of T-DNA complex[20]. M.G.Salas et

al[18] suggested that at 25°C, exotic gene were much more liable to insert and integrant in plant cell and more effective in transformation.

REFERENCE

1. Rawlinson CJ，Muthyalu G，1979，Disease of winter oilseed rape：occurrence，effects and control，*Journal of Agricultural Science*，93：593-606

2. Evans EJ，Gladers P，Davies JLM，Ellerton DR，Hardwick NV，et al，1984，Current status of diseases and disease control of winter oilseed rape in England，*Aspects of Applied Biology*，6：323-324

3. Molano J, Polacheck I, Duran A, et al. 1979,An endochitinase from wheat germ. *The Journal of Biochemical Chemistry*,10: 4901-4907.

4. Bell AA. 1981, Biochemical mechanisms of disease resistance. *Ann Rev. Plant Physiol*, 32: 21-81.

5. Brogile.K.,Chet.I.,Holliday.M.,et al,1991,Transgenic plants with enhanced resistance to the fungal pathogen *Rhizotonia solani,Science*,254:1194~1197

6. Lin.W.,Anuratha.C.S.,Datla.K.,et al,1995,Genetic engineering of rice for resistance to sheath blight, *Bio/ Technology*, 13:686~691

7. ASAO.H.,Nishizawa.Y.,Arai.S.,et al,1997,Enhanced resistance against a fungal pathogen Sphaerotheca humuli in transgenic strawberry expression a rice chitinase gene,*Plant Biotechnology*,14(3):145~149

8. Rene Grison，Bruno GB，Michel S，Nicole L，et al，1996，Field tolerance to fungal pathogens of *Brassica napus* constitutively expressing a chimeric chitinase gene，*Nature Biotechnology*，14：643-646

9. GAO Wu-jun, WANG Jing-xue, LU Long-dou, SUN Yi, DUAN Hong-ying, 2002，Study on effects role of AgNO3 in rape gene transformation, *Chinese Agricultural Science Bulletin*, 18（4）：43-45

10. Tingay S, McElroy D, Kalla R, Fieg S, Wang MB, Thornton S,Brettell R (1997) *Agrobacterium tumefaciens*-mediated barley transformation. *Plant J* 11:1369-1376

11. S. Jayasankar • B.R. Bondada • Z. Li • D.J. Gray 2002, A unique morphotype of grapevine somatic embryos exhibits accelerated germination and early plant development *Plant Cell Rep*,20:907–911

12. Dellaporta SL，Wood J，Hicks JB，1983，A plant molecular DNA minipreparation version：*J.Plant Mol Biol Reporter* 1：19-21

13. Dillen W, DeClercq J, Kapila J, Zambre M, Van Montagu M, Angenon G 1997，The effect of temperature on *Agrobacterium tumefaciens*-mediated gene transfer to plants. *Plant J* 12:1459–1463

14. Fazeks GA, Sedmach PA, Palmer MV. 1986, Genetic and environmental effects on in vitro shoot regeneration from cotyledon explants of *Brassica juncea*. *Plant Cell Tissue Org.Cult*., 6: 177-180.

15. Sharma KK, Bhojwaniss, Thorpe TA. 1990, Factors affecting high frequency differentiation of shoots and roots from cotyledon explants of *Brassica jncea*(L.)*Czern. Plant Sci*, 66: 247-253.

16. Mukhopadhyay.A， Arumugam.N， Nandkumar.PBA， Pradhan.AK， Gupta.V， Pental.D, 1992，*Agrobacterium tumefaciens*-Mediated genetic Transformation of oilseed *Brassica campestris*：transformation frequency is strongly influenced by the mode of shoot regeneration，*Plant Cell Reports*， 11：506-513

17. Fullner KJ, Lara JC, Nester EW . 1996, Pilus assembly by *Agrobacterium* T-DNA transfer genes. *Science* , 273:1107–1109

18. M. G. Salas, S. H. Park, M. Srivatanakul and R. H. Smith. 2001,Temperature influence on stable T-DNA integration in plant cells. 20(8):701–705

19. Kudirka DT, Colburn SM, Hinchee MA, Wright MS . 1986, Interactions of Agrobacterium tumefaciens with soybean [*Glycine max* (L.) Merr.] leaf explants in tissue culture. *Can J Genet Cytol* , 28:808–817

20. Ishida,Y., Saito,H., Ohta,S. et al. 1996,High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens* . *Natural Biotechnol*, 14(6):745~750

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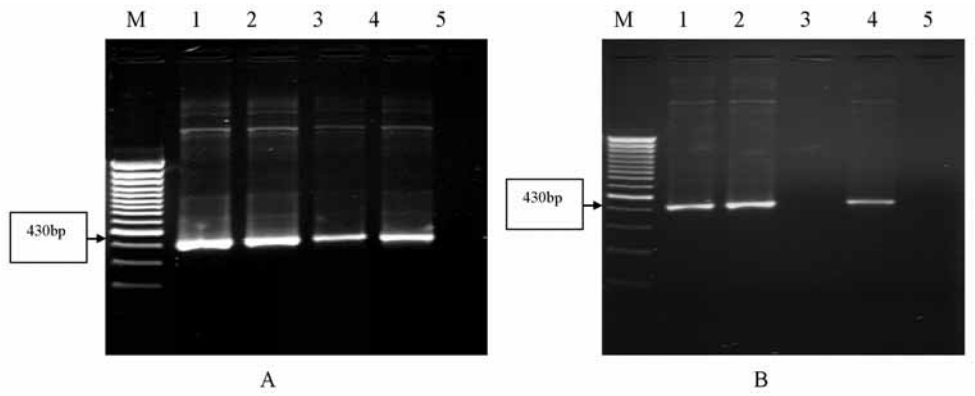

Fig.1 PCR assay of transgenic rape（A ：DB_3 ，B：H165）

M、DNA Marker；1、positive control；2～4、transgenic plants ；5、negative control

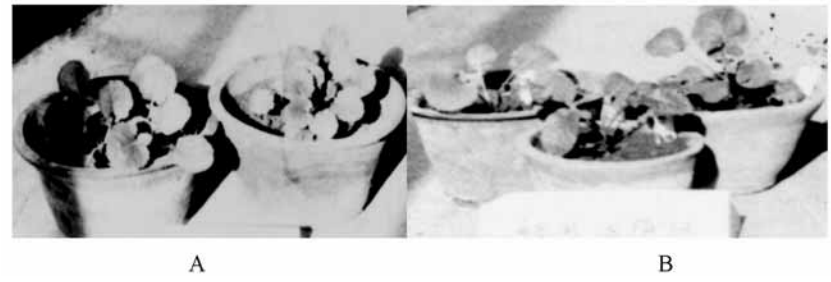

Fig.2 transgenic plants（A ：DB_3 ，B：H165）

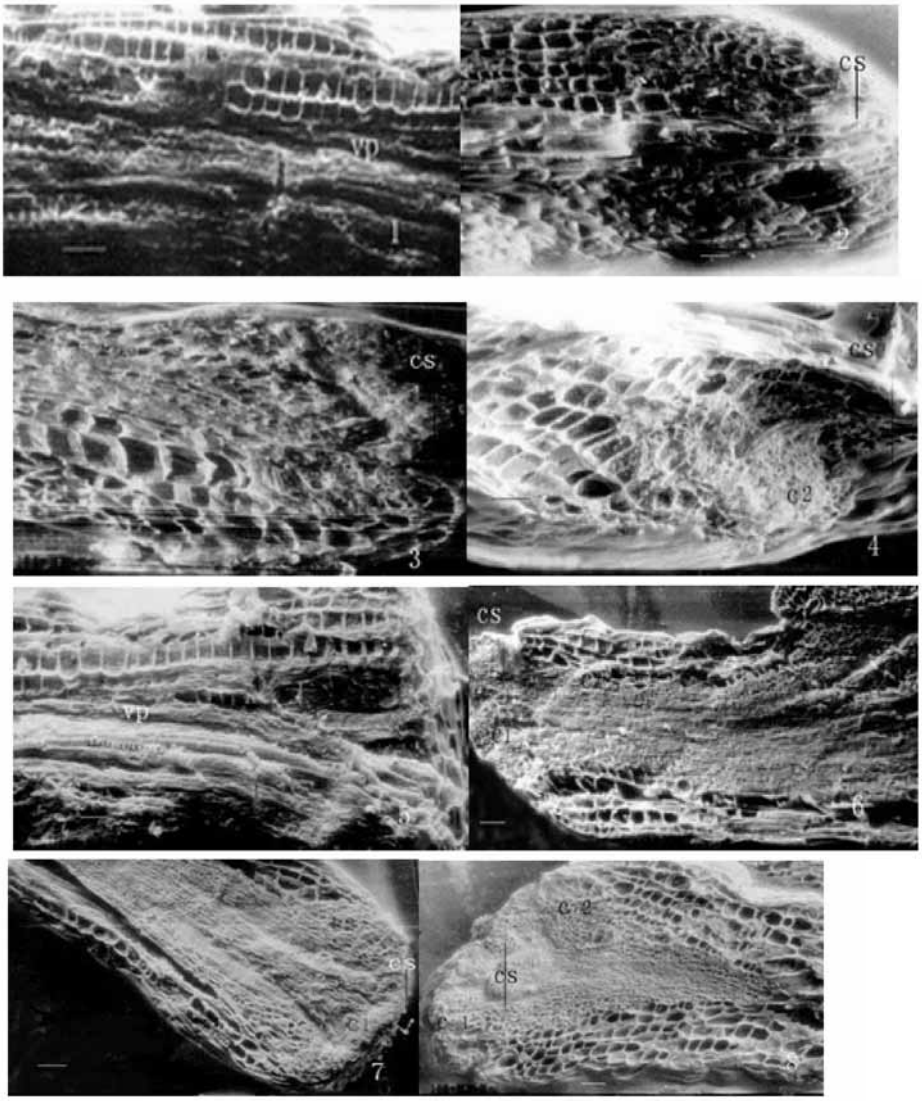

fig. 2 Histology of shoot bud differentiation from rape cotyledon and hypocotyl explants

1. cotyledon control
2. cotyledon 5d culture on induce callus medium
3. cotyledon 9d culture on induce callus medium
4. cotyledon 11d culture on induce callus medium
5. hypocotyl control
6. hypocotyl 5d culture on induce callus medium
7. hypocotyl 9d culture on induce callus medium
8. hypocotyl 11d culture on induce callus medium

note：Bar 1and 3. 200μm； 2 and 5. 200μm；4. 150μm；6，7 and 8. 300μm

vp. vascular parenchvma；cs.surface of cut；c1. The firest way of bud regeneration；c2. The second way of bud regeneration