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Segregation patterns for integration and expression of *Coniothyrium minitans* xylanase gene in *Arabidopsis thaliana* transformants

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Abstract. A xylanase gene *cxy1*, isolated from the mycoparasitic fungus *Coniothyrium minitans*, has been transferred into *Arabidopsis thaliana* by *Agrobacterium*-mediated transformation. Ten *A. thaliana* transformants with herbicide resistance were selected, and the transformation efficiency of the modified "Floral Dip" method was about 0.08 %. Most transformants had one or two copies of T-DNA inserts with 3R:1S or 15R:1S segregation ratios while high proportions of susceptible progenies were also observed in some transgenic plants. The pMB4-2 transformant segregated as 1R:2S ratio in its T_2 and T_3 generations, suggesting that only one copy of T-DNA was integrated into this line's genome and the insertion likely interrupted a gene important or essential to its gametophytic development or alternatively, affected the transmission of such gene. The genetic analysis of the pMB4/*cxy1*-6 transformant indicated that two copies of T-DNAs were inserted independently into this line's genome, in which the resistant homozygotes on one locus were non-viable. The RBB-Xylan assay for six pMB4/*cxy1* transformants indicated that two lines (pMB4/*cxy1*-3 and pMB4/*cxy1*-6) expressed the *cxy1* gene, and variations for the xylanase activity were observed among T_2 progenies of pMB4/*cxy1*-6 transformant.

Keywords: *Agrobacterium*-mediated transformation; *Arabidopsis thaliana*; *Coniothyrium minitans*; Xylanase gene.

Introduction

Xylan is a complex polysaccharide which consists of a backbone of xylose residues linked by β-1,4-glycosidic bonds. Xylan fibers constitute a significant portion of plant hemi-cellulose and contribute to the strength of plant secondary walls (Thomson, 1993). The hydrolysis of xylan by xylanases (E.C. 3.2.1.8) produced by fibrolytic bacteria and fungi is necessary in the degradation of plant tissue. Xylanolytic enzymes have numerous applications in industry and biotechnology, such as in woody biopulping processes (Bieley, 1985), forage fibre digestions (Gilbert and Hazelwood, 1991), and agricultural waste degradation.

Agrobacterium-mediated gene transfer is the most commonly used technique for plant transformation (Zambryski, 1992; Zupan and Zambryski, 1995). The '*Agrobacterium* Vacuum Infiltration', a non-tissue culture approach for *in planta* transformation, was first achieved in *Arabidopsis thaliana* (L.) Heynh. (Feldmann and Marks, 1987; Bechtold et al., 1993; Chang et al., 1994; Katavic et al., 1994). Later, a relatively simplified protocol called the "Floral Dip" was described by Clough and Bent (1998). However, it still requires sterile conditions for seed germination and transformant selection. Recently, we successfully modified this method by germinating *A. thaliana* seeds and selecting transgenic seedlings in Cornell Peat-Lite Mix in plastic pots under greenhouse conditions.

Coniothyrium minitans Campbell is a mycoparasite which attacks the sclerotia (Huang, 1977; Huang and Kokko, 1987) and hyphae (Huang and Hoes, 1976; Huang and Kokko, 1988) of *Sclerotinia sclerotiorum* (Lib.) de Bary, an important fungal pathogen of higher plants (Purdy, 1979). A novel xylanase gene *cxy1* has been isolated from *C. minitans* (Laroche et al., 2000) and expressed in *Pichia pastoris* (Guilliermond) Phaff (Lu et al., 1999). The objective of our study is to explore the impacts of *cxy1* gene for resistance to *S. sclerotiorum* in plants and for improvement of forage utilization in animal production. This report describes the experimental results for *cxy1* integration and expression in *A. thaliana*.

Materials and Methods

Construction of Ti Plasmid

The *Agrobacterium tumefaciens* (Smith & Townsend) Conn strain EHA105 was provided by Dr. F. Eudes (Lethbridge Research Centre, Agriculture and Agri-Food Canada), and the binary vector pMB4 was obtained from "The Baker Lab" at the Gene Expression Center (Berkeley, CA). Strain EHA105 already contains the helper vector,

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which encodes the gene conferring rifampicin resistance (Hood et al., 1993). The pMB4 vector, a large Ti plasmid (12,547 bp) with a 4,000 bp T-DNA, was originally maintained in *Escherichia coli* Migula strain DH5α on Luria and Broth (LB) medium (Difco Laboratories) with kanamycin (50 µg/ml). The *bar* gene conferring herbicide resistance (De Block et al., 1987; Thompson et al., 1987) was inserted after the first 35S promoter, and the second 35S promoter was used for the expression of the xylanase gene *cxy1*.

Both pMB4 vector and the *cxy1* cDNA in pBluescript (λZAP cDNA Cloning Kit, Stratagene) were digested with two enzymes (*Xho* I and *Xma* I), and ligated to form Ti plasmid (Figure 1). The constructed pMB4 Ti plasmid was transformed into *E. coli* strain DH5α, and positive clones were selected on LB medium for kanamycin (50 µg/ml) resistance. The plasmids were extracted from the positive DH5α clones, and transformed into *A. tumefaciens* strain EHA105 by electroporation. The positive EHA105 clones were selected on LB medium for resistance to both kanamycin and rifampicin (50 µg/ml each). An EHA105 culture (250 ml) was prepared overnight (16 h) in LB medium, collected and resuspended (D_{600} = 1.0) in 5% sucrose solution with 0.2% surfactant Citowet-plus (BASF Canada, Inc.) for the floral dip procedure.

Floral Dip Transformation

The transformation protocol performed in this study was adopted from the method of Clough and Bent (1998), with the modification of using surfactant Citowet-plus instead of Silwet L-77 and growing *A. thaliana* seedlings in Cornell Peat-Like Mix (Boodley and Sheldrake, 1977) in 12-cm-diameter plastic pots instead of growing the seedlings in Petri dishes with 1/2 Murashige and Skoog (MS) medium (Sigma M-5519).

Seedlings of *A. thaliana* (ecotype Columbia) were grown in Cornell Peat-Like Mix in plastic pots and kept in a growth cabinet (16 h light with 120 µE/m^2/s at 24ºC and 8 h dark at 20ºC) until they reached the flowering stage (about 5 weeks). No fertilizer was supplied during the experiment, and plants were watered when needed.

Two *A. tumefaciens* treatments, (1) EHA105 strain transformed with pMB4 T-DNA only and (2) EHA105 strain transformed with pMB4 T-DNA plus *cxy1* gene, were applied to *A. thaliana* seedlings independently. The aboveground tissues of eight seedlings were dipped into EHA105 suspension for 3 to 5 s, and the dipped seedlings were covered with clear plastic film (Saran Wrap, Dow Chemical, Inc., Canada) to maintain high humidity for 24 h. The floral dip was repeated again 5 days later. The

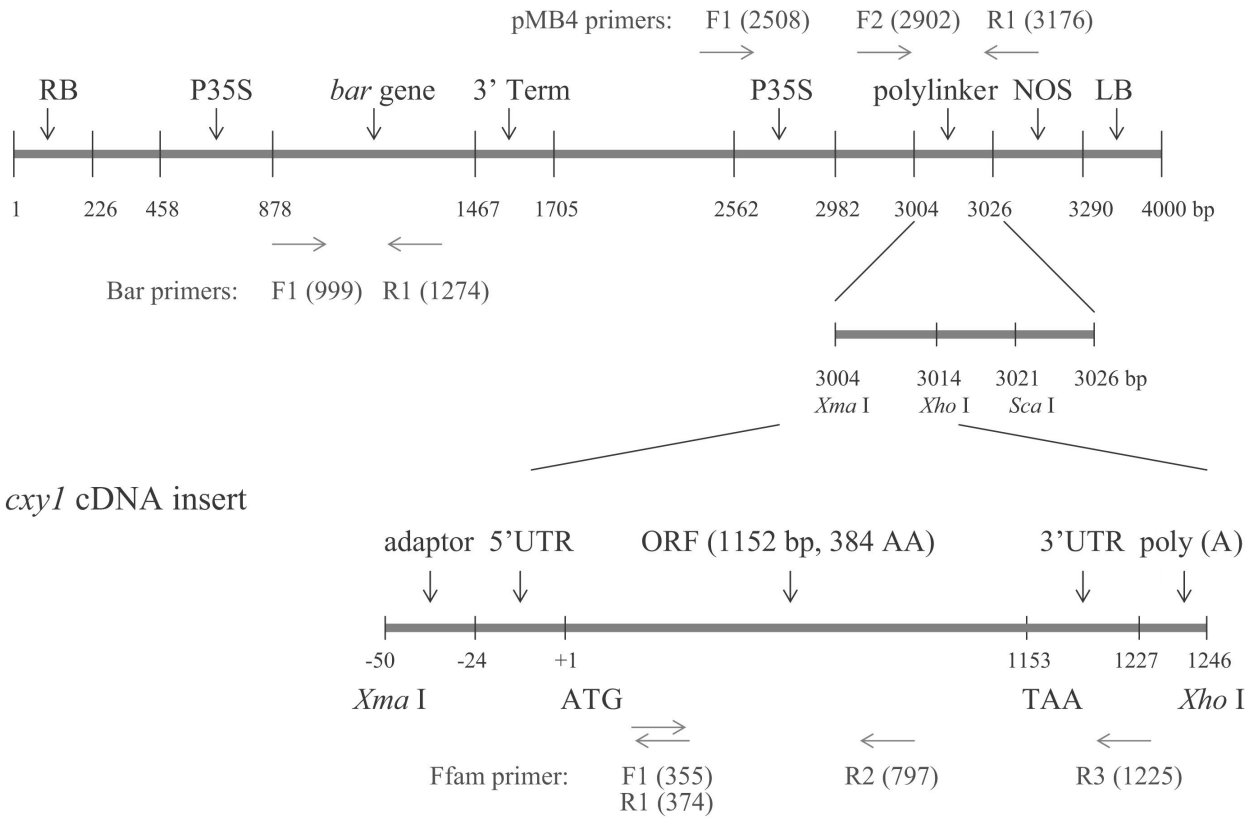

Figure 1. pMB4 T-DNA construction of *A. tumefaciens* with *C. minitans* xylanase gene (*cxy1*) for transformation of *A. thaliana* and locations of the degenerated primers. RB, right border; LB, left border; P35S, 35S promoter of the Cauliflower Mosaic Virus; NOS, terminator of the nopaline systhase gene; UTR, untranslated region; ORF, open reading frame. PCR primer positions are indicated in parentheses and primer sequences are listed in Table 1.

EHA105-treated seedlings were kept in the growth cabinet until maturity.

Evaluation of Transgenic Seedlings

All T_1 seeds, collected from the EHA105-treated *A. thaliana* plants, were sown in Cornell Peat-Like Mix in 52 × 26 cm plastic trays and stratified at 4ºC for two days in darkness to improve their germination. The T_1 seedlings were grown under the same conditions as mentioned above.

The transgenic seedlings were screened and selected by spraying 0.1% Liberty (Registered mix: Venture 25DG, 15% glufosinate ammonium, AgrEvo) directly onto aboveground tissues of 5-day old T_1 seedlings. The spray was repeated twice at 5-day intervals. The T_1 transformants were transplanted into Cornell Peat-Like Mix in 12-cm-diameter plastic pots (one plant per pot) to produce T_2 progenies. The same procedure was followed to obtain T_3 populations from the T_2 generation.

Genetic analysis for herbicide resistance was conducted on T_2 segregating populations of each pMB4 or pMB4/*cxy1* transformant independently. Further genetic studies on T_3 progenies selected from four T_2 families were conducted. The segregation ratios for resistance to the herbicide Liberty were determined for each transformant and statistically analyzed by χ^2 test for the hypotheses of Mendelian segregation.

Genomic DNA was isolated from fresh leaf tissues of T_1, T_2, and T_3 transgenic seedlings using the FastDNA Kit (BIO 101, Inc., Cat. 6540-400). The transformants were further confirmed by PCR amplifications with specific primers (Figure 1; Table 1). Primers pMB4-F1, pMB4-F2, and pMB4-R1 were generated from the pMB4 sequence to detect the *cxy1* insert after the second 35S promoter region. Primers Bar-F1 and Bar-R1 were generated from the *bar* gene sequence to determine the T-DNA fragment after the first 35S promoter. Primers Ffam-F1, Ffam-R1, Ffam-R2, and Ffam-R3 were generated from the *cxy1* sequence to verify the *cxy1* inserts in *A. thaliana* pMB4/*cxy1* transformants.

Xylanase Expression in Transformants

Enzymatic solutions of *A. thaliana* T_2 and T_3 seedlings were extracted from 0.5 mg leaf tissues with 1 ml of 100 mM $NaPO_4$ buffer (pH 6.5), in the presence of protease inhibitor at the recommended concentration of 10 ml/tablet (Roche Molecular Biochemicals, Complete, Mini, EDTA-free, Cat. No. 1836170).

Xylanase activity was analyzed by the RBB-Xylan assay (Lu et al., 1999). The substrate solution was prepared as 10 mg/ml Remazol Brillian Blue R-D-Xylan (RBB-Xylan, Sigma M-5019) in 100 mM $NaPO_4$ buffer (pH 6.5). The mixture of sample supernatant and substrate solution (100 µl each) was incubated at 37ºC for 60 min, and the enzymatic reaction was terminated with 800 µl 95% ethanol. The supernatant for each reaction was collected to measure the xylanase activity at 570 nm. Xylanase (E.C. 3.2.1.8) (Sigma X-4001) was used as the positive and standard control. One unit of xylanase activity was defined as liberating 1 µmole of the reducing sugar measured as xylose equivalents from xylan per minute at pH 6.5 at 37°C.

Results

Efficiency of Floral Dip Transformation

Transformations of the xylanase gene *cxy1* into *A. thaliana* genome using *A. tumefaciens* strain EHA105 were successful in this study, and the efficiency of the modified Floral Dip method was consistent in two independent experiments (Table 2). When the results of two experiments were combined, herbicide resistance was detected in 4 out of 5269 T_1 seedlings in the pMB4 T-DNA treatment while 6 out of 7477 T_1 seedlings displayed this resistance in the pMB4 T-DNA plus *cxy1* insert treatment. The overall transformation efficiency was approximately 0.08%, higher than the efficiency of *in planta* transformation reported by Feldmann and Marks (1987) and similar to the results of the Floral Dip transformation reported by Clough and Bent (1998).

Table 1. PCR primers for amplification of pMB4 T-DNA in *A. thaliana* transformants.[a]

Name	Sequence (5' → 3')	Length (nt)
pMB4-F1	GGC GAA CAG TTC ATA CAG AGT	21
pMB4-F2	ACG TAA GGG ATG ACG CAC AAT	21
pMB4-R1	CCC ATC TCA TAA ATA ACG TCA	21
Bar-F1	CAT CGA GAC AAG CAC GGT CAA CTT C	25
Bar-R1	CTC TTG AAG CCC TGT GCC TCC AG	23
Ffam-F1	GGT CAG GTC ACA TGC GAG AAC	21
Ffam-R1	TTC TCG CAT GTG ACC TGA CC	20
Ffam-R2	ATG CCT TTG GTC ACC TTT GC	20
Ffam-R3	GGC TTT ATT ACC TGC TGG CA	20

[a]The expected T-DNA fragments can be amplified from *A. thaliana* transgenic plants with different primer combinations: pMB4-F1 and pMB4-R1 (without insert, 669 bp; with *cxy1* insert, 1953 bp); pMB4-F2 and pMB4-R1 (without insert, 275 bp; with *cxy1* insert, 1559 bp); Bar-F1 and Bar-R1 (276 bp); pMB4-F1 and FfamR1 (920 bp); pMB4F2 and FfamR2 (949 bp); pMB4F1 and FfamR2 (1343 bp); pMB4F2 and FfamR1 (526 bp).

Table 2. Transformation efficiency of *Agrobacterium* T-DNA into *A. thaliana* using the modified "Floral Dip" method[a].

Treatment	pMB4 T-DNA			pMB4 T-DNA + *cxy1*		
T_1 plants	Total No.	Herb.^{R} No.	Efficiency	Total No.	Herb.^{R} No.	Efficiency
Exper. I	2695	2	0.074%	2681	2	0.075%
Exper. II	2574	2	0.078%	4796	4	0.083%

[a]*Agrobacterium tumefaciens* strain EHA105 was used in Floral Dip for the transformation of pMB4-T DNA only and pMB4 T-DNA plus *cxy1* gene.

Molecular Analysis of Transformants

PCR amplification of the pMB4 T_1 and T_2 DNAs indicated that the *bar* gene was detected in *A. thaliana* transformant genome. The predicted DNA fragments of 276 bp and 669 bp were verified in T_1 pMB4 transformants with the (1) Bar-F1 and Bar-R1 primers and (2) pMB4-F1 and pMB4-R1 primers, respectively. When further tested on T_2 populations of the pMB4-2 transformant, these two fragments were also detected in T_2 herbicide resistant plants while no PCR amplification was observed in T_2 susceptible plants (Figure 2).

PCR amplification of the pMB4/*cxy1* T_1 and T_2 DNAs indicated that the *cxy1* gene was integrated into *A. thaliana* transformants selected with herbicide resistance. With the pMB4-F2 and pMB4-R1 primers, the 1,559 bp fragment was detected from pMB4/*cxy1* T_1 transformants while the 275 bp fragment was amplified from transformants with pMB4 (data not shown). In addition, the predicted fragment sizes of 920 bp, 949 bp, 1343 bp, and 526 bp were verified in the four primer combinations: (I) pMB4-F1 and Ffam-R1, (II) pMB4-F2 and Ffam-R2, (III) pMB4-F1 and Ffam-R2, and (IV) pMB4-F2 and Ffam-R1, respectively (Figure 3).

Genetic Analysis of T_2 Populations

Four T_2 *A. thaliana* populations, derived independently from four pMB4 transformants (pMB4-1, pMB4-2, pMB4-3, and pMB4-4), were analyzed for segregation of herbicide resistance (Table 3). For each transformant, we used the χ^2 test to analyse the segregation fitness with different segregation ratios (such as 1R:1S, 1R:2S, 1R:3S, 2R:1S, 3R:1S, 11R:1S, 15R:1S) then selected the ratio with the highest P value and listed it in our tables. Two transformants exhibited the inheritance of herbicide resistance as a single T-DNA insert with a 3R:1S ratio, and one transformant (pMB4-1) behaved as one copy of a T-DNA insert with non-viable resistant homozygotes (2R:1S). The pMB4-2 transformant was observed to have 162 resistant and 303 susceptible individuals in its T_2 population fitting a 1R:2S segregation ratio.

Six T_2 *A. thaliana* populations, developed independently from six pMB4/*cxy1* transformants, were analyzed for segregation of herbicide resistance (Table 3). Four transformants (pMB4/*cxy1*-2, pMB4/*cxy1*-3, pMB4/*cxy1*-4, pMB4/*cxy-1*-5) inherited as a single T-DNA insert with a 3R:1S ratio. One transformant (pMB4/*cxy1*-1) behaved as two copies of T-DNA inserts with a 15R:1S ratio. The pMB4/*cxy1*-6 transformant exhibited 424 resistant and 41 susceptible individuals in its T_2 population fitting a 11R:1S segregation ratio.

Genetic analysis of T_3 Populations

In order to explore the nature of segregation distortion in the pMB4-2 transformant, 16 T_3 populations were derived independently from 16 resistant T_2 plants, and their

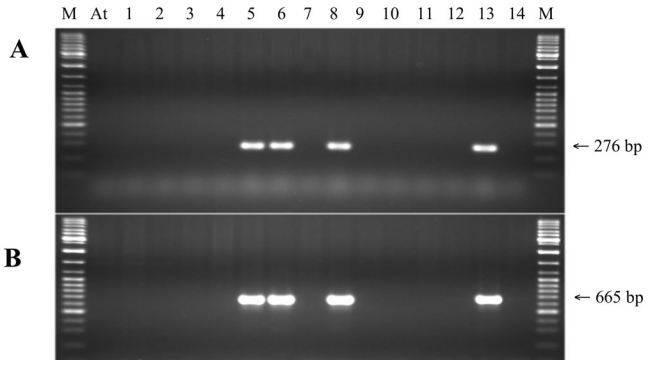

Figure 2. PCR detection of T-DNA inserts in T_2 progenies of *A. thaliana* pMB4-2 transformant. A refers to PCR with Bar-F1/R1 primers; B refers to PCR with pMB4-F1/R1 primers. M, DNA size marker (100 bp ladder, Life Technology); At, wild *A. thaliana* (negative control). Fourteen T_2 individuals were analyzed, only four (No. 5, 6, 8, 13) exhibited herbicide resistance.

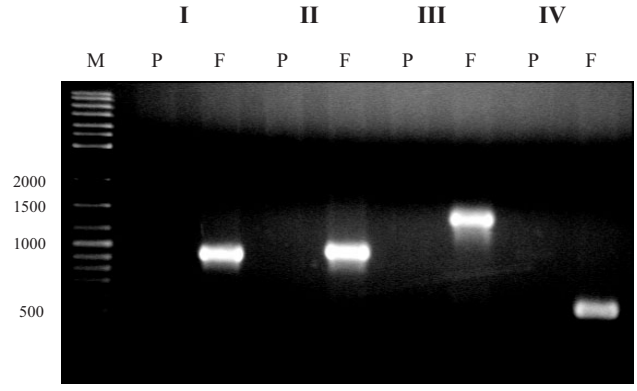

Figure 3. PCR detection of *C. minitans* xylanase gene (*cxy1*) in *A. thaliana* T_1 transformants. (I) pMB4-F1 and Ffam-R1 primers; (II) pMB4-F2 and Ffam-R2 primers; (III) pMB4-F1 and Ffam-R2 primers; (IV) pMB4-F2 and Ffam-R1 primers. M, DNA size marker (100 bp ladder, Life Technology); P, *A. thaliana* pMB4-3 transformant; and F, *A. thaliana* pMB4/*cxy1*-3 transformant.

Table 3. Segregation for the herbicide resistance in T_2 progenies of *A. thaliana* transgenic plants[a].

Transformant	Total T_2 No.	Herbicide^R	Herbicide^S	χ^2 test	P value
pMB4-1	416	275	141	2R : 1S	0.60 – 0.90
pMB4-2	465	162	303	1R : 2S	0.40 - 0.60
pMB4-3	316	227	89	3R : 1S	0.10 - 0.20
pMB4-4	256	184	72	3R : 1S	0.20 - 0.50
pMB4/*cxy1*-1	413	385	28	15R : 1S	0.50 - 0.70
pMB4/*cxy1*-2	526	387	139	3R : 1S	0.20 - 0.50
pMB4/*cxy1*-3[b]	433	337	96	3R : 1S	0.10 - 0.20
pMB4/*cxy1*-4	424	326	98	3R : 1S	0.20 - 0.50
pMB4/*cxy1*-5	347	266	81	3R : 1S	0.20 - 0.50
pMB4/*cxy1*-6[b]	465	424	41	11R : 1S	0.20 - 0.50

[a] The null hypothesis for χ^2 test was rejected at the 5% level ($P < 0.05$), and the P values were used to determine the fitness of T_2 segregation. Segregation ratios presented indicated that the null hypothesis was not rejected, indicating that the frequencies conformed acceptably well to those predicted by the null hypothesis. The 3R:1S segregation suggests that one copy of T-DNA was inserted into *A. thaliana* genome; the 2R:1S segregation suggests that one copy of T-DNA was inserted in a locus that led to nonviable resistant homozygotes; the 11R:1S segregation suggests that two copies of T-DNA were inserted in one locus that led to nonviable resistant homozygotes; the 15R:1S segregation suggests that two copies of T-DNAs were independently inserted into *A. thaliana* genome; and, the 1R:2S segregation suggests that one copy of T-DNA was inserted with partial deficiency of T-DNA transmission.

[b] These transformants exhibited the xylanase activity.

segregation for herbicide resistance was analyzed (Table 4). All 16 populations were observed to segregate for the herbicide resistance, 15 populations with the 1R:2S ratio and 1 population with the1R:3S ratio.

Nineteen T_3 pMB4/*cxy1*-1 populations, derived independently from 19 T_2 resistant plants of pMB4/*cxy1*-1, were analyzed for segregation of herbicide resistance (data not shown). Six out of 19 T_3 populations were observed to have all resistant progenies. The other 13 populations exhibited both resistant and susceptible progenies, 7 populations with the 15R:1S ratio, 4 with the 3R:1S ratio, and 2 with the 2R:1S ratio.

Eight T_3 pMB4/*cxy1*-3 populations were derived independently from 8 T_2 resistant plants of pMB4/*cxy1*-3, and their segregations for herbicide resistance were analyzed (data not shown). In these 8 T_3 populations, 4 populations were observed to have all resistant progenies, 3 populations were segregated with the 3R: 1S ratio and 1 population with the 2R:1S ratio.

Twenty T_3 pMB4/*cxy1*-6 populations, derived independently from 20 resistant T_2 plants of pMB4/*cxy1*-6, were analyzed for segregation of herbicide resistance (Table 5). Seven out of 20 T_3 populations were observed to have all resistant progenies. The other populations exhibited both resistant and susceptible progenies, 5 populations with the 11R:1S ratio, 5 with the 3R:1S ratio, and 3 with the 2R:1S ratio.

Xylanase Activity in A. thaliana Transformants

The RBB-Xylan assay for six pMB4/*cxy1* transformants of *A. thaliana* indicated that only pMB4/*cxy1*-3 and pMB4/*cxy1*-6 expressed xylanase activity (Table 3; Figure 4) although the full length of *cxy1* gene was integrated into all pMB4/*cxy1* transformants. The pMB4/*cxy1*-3 was determined to produce xylanase activity of 186 mU, and similar activities were detected in its T_2 and T_3 progenies (data not shown). Both DNA evidence and xylanase activity verified that the *cxy1* gene was integrated in *A. thaliana*

Table 4. Segregation for the herbicide resistance in T_3 progenies of *A. thaliana* pMB4-2 transformant[a].

T_2 plant	Herb.^{R}	Herb.^{S}	χ^2 test	T_2 plant	Herb.^{R}	Herb.^{S}	χ^2 test
P2-1	23	47	1R : 2S	P2-9	49	91	1R : 2S
P2-2	39	61	1R : 2S	P2-10	36	88	1R : 2S
P2-3	22	45	1R : 2S	P2-11	44	93	1R : 2S
P2-4	35	56	1R : 2S	P2-12	45	92	1R : 2S
P2-5	30	64	1R : 2S	P2-13	78	151	1R : 2S
P2-6	41	94	1R : 2S	P2-14	54	104	1R : 2S
P2-7	60	147	1R : 2S	P2-15	49	101	1R : 2S
P2-8	67	117	1R : 2S	P2-16[b]	27	82	1R : 3S

[a]The null hypothesis for χ^2 test was rejected at the 5% level ($P < 0.05$), and the P values were used to determine the fitness of T_3 segregation. Segregation ratios presented indicated that the null hypothesis was not rejected, indicating that the frequencies conformed acceptably well to those predicted by the null hypothesis.

[b]1R:3S segregation was detected in this population.

Table 5. Segregation for the herbicide resistance in T_3 progenies of *A. thaliana* pMB4/*cxy1*-6 transformant[a].

T_2 plant	Herb.^{R}	Herb.^{S}	χ^2 test	T_2 plant	Herb.^{R}	Herb.^{S}	χ^2 test
F6-1	52	24	2R : 1S	F6-11	60	18	3R : 1S
F6-2[b]	49	0	all R	F6-12	93	0	all R
F6-3	75	5	11R : 1S	F6-13	80	8	11R : 1S
F6-4	85	4	11R : 1S	F6-14	74	0	all R
F6-5	64	0	all R	F6-15	46	5	11R : 1S
F6-6	34	26	2R : 1S	F6-16	98	0	all R
F6-7	66	19	3R : 1S	F6-17	88	7	11R : 1S
F6-8[b]	48	0	all R	F6-18	86	45	2R : 1S
F6-9	82	48	2R : 1S	F6-19	73	21	3R : 1S
F6-10	56	16	3R : 1S	F6-20	33	0	all R

[a]The null hypothesis for χ^2 test was rejected at the 5% level ($P < 0.05$), and the P values were used to determine the fitness of T_3 segregation. Segregation ratios presented indicated that the null hypothesis was not rejected, indicating that the frequencies conformed acceptably well to those predicted by the null hypothesis. The 3R:1S segregation suggests that one copy of T-DNA was inserted into *A. thaliana* genome; the 2R:1S segregation suggests that one copy of T-DNA was inserted in a locus that led to non-viable resistant homozygotes; the 11R:1S segregation suggests that two copies of T-DNA were inserted in one locus that led to non-viable resistant homozygotes.

[b]These T_2 plants and their T_3 progenies exhibited the xylanase activity.

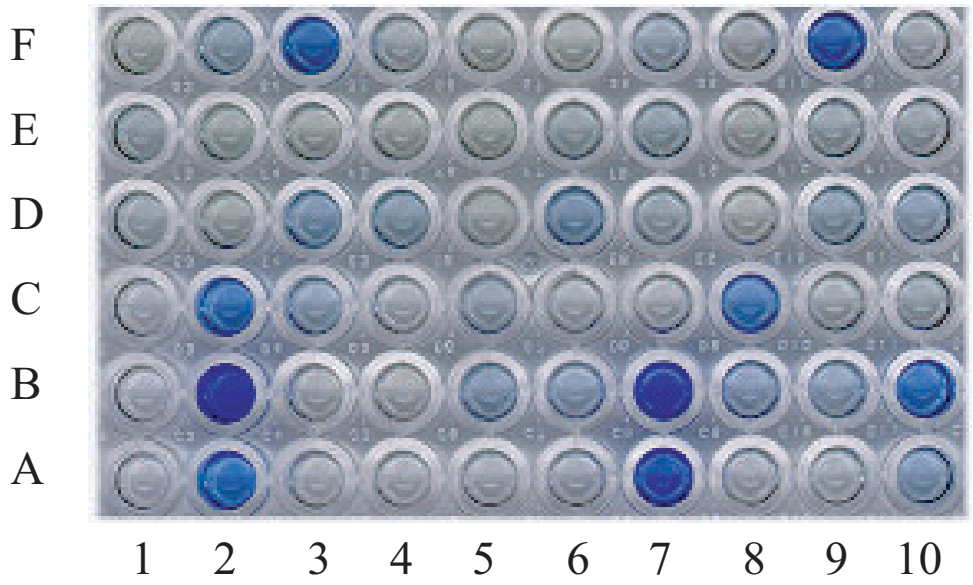

Figure 4. RBB-Xylan assay for *A. thaliana* transformants. A1, H_2O; A2, standard xylanase (Sigma X-4001, 120 mU); A3, *A. thaliana* only; A4, *A. thaliana* pMB4-1 transformant; A5-A10, *A. thaliana* pMB4/*cxy1* transformant 1 to 6, in which A7 and A10 exhibited xylanase activity; the same sample order in Row B as in Row A; C1 to F10, 40 T_2 progenies of pMB4/*cxy1*-6, in which C2, C8, F3, and F9 exhibited the xylanase activity. The reactions were 1 h for Row A and 12 h for Row B to Row F.

pMB4/*cxy1*-3 transformant and could be normally expressed through successive generations.

In contrast, the pMB4/*cxy1*-6 transformant of *A. thaliana* had weaker xylanase activity (80 mU), and the segregation was observed in its T_2 generation (Figure 4). Of total 40 T_2 plants tested, only four were verified to have activity similar to their T_1 transformants, and the T_3 progenies from these 4 T_2 plants were also observed to have activity similar to their parents (data not shown). In addition, nine more were detected to express the minimal xylanase activity after 12 h of incubation, versus 1 h for the standard RBB-Xylan assay.

Discussion

This study demonstrated that the xylanase gene *cxy1* can be efficiently transformed into *A. thaliana* by *Agrobacterium* infection with our modified Floral Dip method. Most of our transformants had one or two copies of T-DNA inserts with the 3R:1S or 15R:1S segregation ratios, exhibiting Mendelian inheritance in their T_2 and T_3 populations. For example, pMB4/*cxy1*-2 transformant was shown to have one copy of the T-DNA insert in the *A. thaliana* genome. However, the 2R:1S and 11R:1S segregation ratios were also observed in the T_2 and T_3 progenies of some pMB4 or pMB4/*cxy1* transformants (Table

3). Such an exceptional proportion of herbicide susceptible progenies in transgenic plants has been reported by other investigators (Feldmann and Marks, 1987; Valvekens et al., 1988; Errampalli et al., 1991; Katavic et al., 1994; Feldmann et al., 1997; Bonhomme et al., 1998; Howden et al., 1998; Harbord et al., 2000), and may be due to the instability of the inserts, the loss of expression of the resistance gene, or the induction of a recessive mutation for gamete or embryo lethality. In our study, the insertions of T-DNA likely interrupted a gene that is important or essential in plant development; therefore, the homozygous resistant genotypes couldn't survive, resulting in the 2R:1S or 11R:1S distortion ratios.

The 1R:1S ratio is more common for gametophytic lethal in either male or female gametes of *A. thaliana* transformants (Howden et al., 1998); however, other distorted ratios were also reported in previous studies (Feldmann and Marks, 1987; Katavic et al., 1994; Bonhomme et al., 1998). In this study, segregation of the 1R:2S ratio was unexpected in the T_2 and T_3 progenies of pMB4-2 transformant (Table 3, 4). Since the herbicide resistance can be inherited in its T_2 and T_3 generations, the pMB4-2 transformant seems to behave as a stable insertion. In addition, as resistant individuals had the T-DNA insert and susceptible ones didn't in their T_3 progenies (Figure 2), loss of *bar* gene expression is an unlikely cause. Based on genetic and molecular studies, we propose that only one copy of T-DNA was integrated into *A. thaliana* pMB4-2 transformant, and the insertion likely interrupted a gene important or essential to its gametophytic development. This insertion event could have resulted in a reduction of transmission (Bonhomme et al., 1998; Howden et al., 1998). It is possible that the homozygous resistant genotypes in its T_2 and T_3 progenies couldn't survive, and the gametophytic development in this transformant may favor the gametes without the T-DNA insert, resulting in the 1R:2S segregation distortion. Further study through reciprocal backcrosses with wild *A. thaliana* plants can confirm this unequal gamete transmission through both micro- or megagametophytes.

The genetic analysis of the pMB4/*cxy1*-6 transformant indicated that two copies of T-DNAs were inserted into the *A. thaliana* genome independently, in which the resistant homozygote on one locus was non-viable. If X refers to one T-DNA insertion and Y refers to another independent insertion, and the X insertion is lethal to *A. thaliana* growth, the T_1, T_2, and T_3 genotypes of pMB4/*cxy1*-6 can be predicted as follows:

T_1	T_2		T_3
	2 R XxYY	→→→	All R
	4 R XxYy	→→→	11R:1S
XxYy	2 R Xxyy	→→→	2R:1S
	1 R xxYY	→→→	All R
	2 R xxYy	→→→	3R:1S
	1 S xxyy	→→→	All S

Theoretically, a total of 11 resistant genotypes in the T_2 population can produce 3/11 T_3 populations with all resistant progenies, 4/11 T_3 populations with an 11R:1S ratio, 2/11 T_3 populations with an 3R:1S ratio and 2/11 T_3 populations with an 2R:1S ratio. The observed segregation of the pMB4/*cxy1*-6 T_2 progenies was observed to fit the 11R:1S ratio (Table 3). In 20 pMB4/*cxy1*-6 T_3 populations derived independently from 20 T_2 progenies, 7 were observed to have all resistant progenies, 5 were segregated with an 11R:1S ratio, 4 with a 3R:1S ratio, and 4 with a 2R:1S ratio (Table 5). Therefore, observed segregation of the pMB4/*cxy1*-6 T_2 and T_3 progenies support the hypothesis for the above genotypic model.

It is a common observation that not all T-DNA insertions are expressed in plants, and in most cases only one copy of the multiple inserted T-DNAs seems to be functional (Hobbs et al., 1990; Chang et al., 1994; McCabe et al., 1999). Herbicide resistance was used to screen and select the pMB4 or pMB4/*cxy1* transgenic genotypes in this study; therefore, the *bar* gene should have been functionally expressed in all our transformants. However, xylanase activity was only detected in two out of six pMB4/*cxy1* transformants, and *cxy1* expression obviously varied among T_2 progenies of pMB4/*cxy1*-6 transformant (Figure 4) though PCR amplification confirmed the presence of *cxy1* gene in all pMB4/*cxy1* transformants (Figure 3). The genetic analyses in this study indicated that pMB4/*cxy1*-6 had two copies of T-DNA inserts with one locus non-viable for resistant homozygotes; therefore, the segregation for xylanase activity in the T_2 generation suggested that the *cxy1* gene may be expressed from only one T-DNA insert and that homozygous resistance on this locus was non-viable. The lack of expression of the *cxy1* gene, isolated from the fungus *C. minitans*, in *A. thaliana* may be due to a difference in codon usage. The *cxy1* silencing in most of our transformants is likely due to the gene hypermethylation (Matzke and Matzke, 1990) or to T-DNA rearrangement, deletion, or mutation (Errampalli et al., 1991) in the *A. thaliana* genome. Further studies to identify the insertion patterns of pMB4/*cxy1* transformants are warranted in order to establish the inheritance of the *cxy1* expression in *A. thaliana* genome.

Pathogenesis-related (PR) proteins are reported to be effective sources for plant defenses under both biotic and abiotic stresses (Yun et al., 1997). As a PR-like protein, our xylanase gene *cxy1* was isolated from a special mycoparasite *C. minitans* (Laroche et al., 2000) and has now been expressed in a higher plant genome, providing a basis for investigating transgenic *Arabidopsis* plants for antifungal resistance to *S. sclerotiorum* and other plant pathogens. In addition, our *Arabidopsis* transformants will be useful in the analysis of *cxy1* function and xylan hydrolysis in higher plants since little xylanase activity was detected in the original *Arabidopsis* plants (ecotype Columbia). The results reported in this study make it possible to use the transgenic *Arabidopsis* as a model plant to further study the degradation of xylan in higher plants for improvement of forage utilization in animal production.

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Literature Cited

Bechtold, N., J. Ellis, and G. Pelletier. 1993. *In planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C. R. Acad. Sci. Paris. **316:** 1194-1199.

Bieley, P. 1985. Microbial xylanolytic systems. Trends Biotechnol. **3:** 286-290.

Bonhomme, S., C. Horlow, D. Vezon, S.de Laissardiere, A. Guyon, M. Ferault, M. Marchand, N. Bechtold, and G. Pelletier. 1998. T-DNA mediated disruption of essential gametophytic genes in Arabidopsis is unexpectedly rare and cannot be inferred from segregation distortion alone. Mol. Gen. Genet. **260:** 444-452.

Boodley, J.W. and R. Sheldrake, Jr. 1977. Cornell peat-like mixes for commercial plant growing. N. Y. State Coll. Agric. and Life Sci. Inform. Bull. No. 43, pp. 8.

Chang, S.S., S.K. Park, B.C. Kim, B.J. Kang, D.U. Kim, and H. G. Nam. 1994. Stable genetic transformation of *Arabidopsis thaliana* by *Agrobacterium* inoculation *in planta*. The Plant J. **5:** 551-558.

Clough, S.J. and A.F. Bent. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. The Plant J. **16:** 735-743.

De Block, M., J. Botterman, M. Vandewiele, J. Dockx, C. Thoen, V. Gossele, N.R. Movva, C. Thompson, M. Van Montagu, and J. Leemans. 1987. Engineering herbicide resistance in plants by expression of a detoxifying enzyme. The EMBO J. **6:** 2513-2518.

Errampalli, D., D. Patton, L. Castle, L. Mickelson, K. Hansen, J. Schnall, K. Feldmann, and D. Meinke. 1991. Embryonic lethals and T-DNA insertional mutagenesis in *Arabidopsis*. The Plant Cell **3:** 149-157.

Feldmann, K.A. and M.D. Marks. 1987. *Agrobacterium*-mediated transformation of germinating seeds of *Arabidopsis thaliana*: a non-tissue culture approach. Mol. Gen. Genet. **208:** 1-9.

Feldmann, K.A., D.A. Coury, and M.L. Christianson. 1997. Exceptional segregation of a selectable marker (KanR) in *Arabidopsis* identifies gene important for gametophytic growth and development. Genetics **147:** 1411-1422.

Gilbert, H.J. and G.P. Hazelwood. 1991. Genetic modification of fibre digestion. Proc. Nutrition Soc. **50:** 173-186.

Harbord, R.H., C.A. Napoli, and T.P. Robbins. 2000. Segregation distortion of T-DNA markers linked to the self-incompatibility (S) locus in *Petunia hybrida*. Genetics **154:** 1323-1333.

Hobbs, S.L., P. Kpodar, and C.M.O. DeLong. 1990. The effect of T-DNA copy number, position and methylation on reporter gene expression in tobacco transformants. Plant Mol. Biol. **15:** 851-864.

Hood, E.E., S.B. Gelvin, L.S. Melchers, and A. Hoekema. 1993. New *Agrobacterium* helper plasmids for gene transfer to plants. Transgen. Res. **2:** 208-218.

Howden, R., S.K. Park, J.M. Moore, J. Orme, U. Grossniklaus, and D. Twell. 1998. Selection of T-DNA-tagged male and female gametophytic mutants by segregation distortion in *Arabidopsis*. Genetics **149:** 621-631.

Huang, H.C. 1977. Importance of *Coniothyrium minitans* in survival of sclerotia of *Sclerotinia sclerotiorum* in white sunflower. Can. J. Bot. **55:** 289-295.

Huang, H.C. and J.A. Hoes. 1976. Penetration and infection of *Sclerotina sclerotiorum* by *Coniothyrium minitans*. Can. J. Bot. **54:** 406-410.

Huang, H.C. and E.G. Kokko. 1987. Ultrastructure of hyperparasitism of *Coniothyrium minitans* on sclerotia of *Sclerotina sclerotiorum*. Can. J. Bot. **65:** 2483-2489.

Huang, H.C. and E.G. Kokko. 1988. Penetration of hyphae of *Sclerotina sclerotiorum* by *Coniothyrium minitans* without the formation of appressoria. Phytopath. Zeit. **123:** 133-139.

Katavic, V., G.W. Haughn, D. Reed, M. Martin, and L. Kunst. 1994. *In planta* transformation of *Arabidopsis thaliana*. Mol. Gen. Genet. **245:** 363-370.

Laroche, A.J., T.Y. Huang, M.M. Frick, Z.-X. Lu, H.C. Huang, and K.J. Cheng. 2000. *Coniothyrium minitans* Xylanase Gene *cxy1*. United States Patent No. 6, 121, 034.

Li, G.Q., H.C. Huang, E.G. Kokko, and S.N. Acharya. 2002. Ultrastructural study of mycoparasitism of *Gliocladium roseum* on *Botrytis cinerea*. Bot. Bull. Acad. Sin. **43:** 211-218.

Lu, Z.-X., T.Y. Huang, M.M. Frick, H.C. Huang, K.J. Cheng, and A. Laroche. 1999. Expression of the xylanase gene *cxy1* from *Coniothyrium minitans* in *Pichia pastoris*. The 2nd International Molecular Farming Conference, London, Ontario, Canada, Abstr. pp. 60.

Matzke, M.A. and A.J.M. Matzke. 1990. Gene interactions and epigenetic variation in transgenic plants. Dev. Genet. **11:** 214-223.

McCabe, M.S., F. Schepers, A. van der Arend, U. Mohapatra, A.M.M. de Laat, J.B. Power, and M.R. Davey. 1999. Increased stable inheritance of herbicide resistance in transgenic lettuce carrying a *petE* promoter-*bar* gene compared with a CaMV 35S-*bar* gene. Theor. Appl. Genet. **99:** 587-592.

Purdy, L.H. 1979. *Sclerotinia sclerotiorum*: history, diseases, symptomatology, host range, geographic distribution, and impact. Phytopathology **69:** 875-880.

Thomson, J.A. 1993. Molecular biology of xylan degradation. FEMS Microbiol. Rev. **104:** 65-82.

Thompson, C.J., N.R. Movva, R. Tizard, R. Crameri, J.E. Davies, M. Lauwereys, and J. Botterman. 1987. Characterization of the herbicide resistance gene *bar* from *Streptomyces hygroscopicus*. The EMBO J. **6:** 2519-2523.

Tu, J.C. 1997. An integrated control of white mold (*Sclerotinia sclerotiorum*) of beans, with emphasis on recent advances in biological control. Bot. Bull. Acad. Sin. **38:** 73-76.

Valvekens, D., M.V. Montagu, and M.V. Lijsebettens. 1988. *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. Proc. Natl. Acad. Sci. USA **85:** 5536-5540.

Yun, D.J., R.A. Bressan, and P.M. Hasegawa. 1997. Plant antifungal proteins. Plant Breeding Reviews **14:** 39-88.

Zambryski, P. 1992. Chronicle from *Agrobacterium*-plant cell DNA transfer story. Ann. Rev. Plant Physiol. Plant Mol. Biol. **43:** 465-490.

Zupan, J.R. and P. Zambryski. 1995. Transfer of T-DNA from *Agrobacterium* to the plant cell. Plant Physiol. **107:** 1041-104.

Coniothyrium minitans 木質素酵素 (xylanase) 基因在 *Arabidopsis thaliana* 轉化植株中整合和表達的分離模式

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Cxy1 是由重寄生性真菌 *Coniothyrium minitans* 分離出的木質素酵素 (xylanase) 基因。通過農桿菌轉化 (*Agrobacterium*-mediated transformation) 途徑，作者已將 cxy1 基因轉入阿拉伯芥 (*Arabidopsis thaliana*) 基因組。利用除草劑抗性已鑑定和篩選出 10 株轉化植株，其轉化率為 0.08%。大多數轉化植株有一套或二套 T-DNA，其後代的分離比例為 3:1 或 15:1。然而也發現一些轉化植株呈現高比例的除草劑感性後代。pMB4-2 轉化植株的自交二代及自交三代的分離比例均為 1:2，表明只有一套 T-DNA 被轉入該植株基因組，而且其整合位點干擾了一個與植株配子體發育有關的基因。對 pMB4/*cxy1*-6 轉化植株的遺傳分析表明二套 T-DNA 被分別轉入該植株基因組，其後代中的同質純合轉化植株無生活力。應用 RBB-Xylan 分析法對 6 個 pMB4/*cxy1* 轉化植株及其後代進行木質素酵素活性測定後表明 2 個轉化植株 (pMB4/*cxy1*-3 和 pMB4/*cxy1*-6) 能正常表述 *cxy1* 基因，其中 pMB4/*cxy1*-6 轉化植株後代木質素酵素活性有很大的變異。

關鍵詞：農桿菌轉化；阿拉伯芥；*Coniothyrium minitans*；木質素酵素基因。