Plant Pathology (2006) **55**, 187–193 Doi: 10.1111/j.1365-3059.2005.01342.x

Expression of an oxalate decarboxylase gene from *Flammulina* sp. in transgenic lettuce (*Lactuca sativa*) plants and resistance to *Sclerotinia sclerotiorum*

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Sclerotinia sclerotiorum causes rot in a broad range of crops including lettuce, soybean, dry bean and tomato. Pathogenesis of *Sclerotinia* has been associated with the copious production of oxalic acid. Enzymes capable of degrading oxalic acid have been utilized to produce transgenic resistant plants. Transgenic lettuce lines containing the decarboxylase gene (*oxdc*) isolated from a *Flammulina* sp. were produced by *Agrobacterium*-mediated transformation. Out of 80 regenerated plants, PCR analysis revealed the presence of the *oxdc* gene in 34 lines. Except for eight lines, the primary transformants transferred the foreign gene to the first generation in a Mendelian fashion. In a detached-leaf assay inoculated with agar plugs of a 2-day-old *S. sclerotiorum* culture, two lines (P100 and P43) were symptomless, while line P57 showed a delay in symptom development when compared with a nontransgenic control line. RT-PCR analysis carried out with the resistant lines showed the expression of *oxdc* gene transcripts.

Keywords: *Flammulina* sp., oxalate decarboxylase, oxalic acid, *Sclerotinia sclerotiorum*, transgenic lettuce

Introduction

Sclerotinia sclerotiorum is a broad host-range pathogenic ascomycete, which can cause diseases in more than 400 plant species including many agronomically important crops such as tomato, soybean, dry bean and lettuce (Boland & Hall, 1994). In lettuce (*Lactuca sativa*), diseased plants are unmarketable as they rot once infected with the fungus. The disease is difficult to control when the fungus is established in the soil because the sclerotia of *S. sclerotiorum* are long-lived (Tu, 1997). Although some breeding lines of *L. sativa* and *L. serriola* have shown some degree of resistance against *S. sclerotiorum*, there are no resistant commercial cultivars of lettuce, and disease control is based on the use of fungicides, with ascospores as the target (Whipps *et al*., 2002; Young *et al*., 2004).

Pathogenesis of *S. sclerotiorum*, *Sclerotium cepivorum* and *Sclerotium rolfsii* has been associated with the production of oxalic acid by these fungi (Bateman & Beer, 1965; Magro *et al*., 1984; Godoy *et al*., 1990). Previous studies provided evidence for the involvement of oxalic acid in fungal pathogenesis. Treatment of healthy sunflower and common bean plants with oxalic acid or fungal culture filtrates resulted in foliar symptoms identical to those found in diseased plants (Noyes & Hancock, 1981; Marciano *et al*., 1983; Godoy *et al*., 1990). In addition, plants that showed *in vitro* resistance to oxalic acid were more tolerant to *S. sclerotiorum* (Noyes & Hancock, 1981; Rowe, 1993; Kolkman & Kelly, 2000). Marciano *et al*. (1983) detected high concentrations of oxalic acid in sunflower tissues infected by *S. sclerotiorum*. Moreover, prototrophic mutants of *S. sclerotiorum* deficient in oxalic acid production were nonpathogenic to common bean plants. In contrast, the oxalic acid-producing wild type was pathogenic (Godoy *et al*., 1990). The exact role of oxalic acid during infection is not well understood. However, several cellulolytic and pectolytic enzymes secreted by the fungi during invasion of plant tissues have maximal activities at low pH, and oxalate might increase enzymatic activity by shifting the apoplastic pH (Bateman & Beer, 1965; Lumsden, 1976; Magro *et al*., 1984). Oxalic acid may chelate calcium, compromising the function of calcium-dependent defence responses and weakening the cell wall (Bateman & Beer, 1965; Marciano *et al*., 1983; Ferrar & Walker, 1993). In addition, oxalate suppresses oxidant biosynthesis,

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Accepted 1 September 2005

disabling the earliest resistance response in plant cells (Cessna *et al*., 2000).

With a view to the degradation of oxalic acid, genes for enzymes such as oxalate oxidase and oxalate decarboxylase have been isolated and characterized. Oxalate oxidase catalyses the oxygen-dependent oxidation of oxalate to CO_2 and hydrogen peroxide (Kotsira & Clonis, 1997). Oxalate decarboxylases break down oxalate to generate formate and carbon dioxide (Reinhardt *et al*., 2003). The introduction of these genes in transgenic plants has been proposed in order to produce *Sclerotinia*-resistant lines. The barley oxalate oxidase gene was introduced into canola (Thompson *et al*., 1995) and peanut (Livingstone *et al*., 2005). The wheat oxalate oxidase gene was introduced into poplar (Liang *et al*., 2001), tobacco (Berna & Bernier, 1997), soybean (Donaldson *et al*., 2001) and sunflower (Burke & Rieseberg, 2003). The oxalate decarboxylase gene from *Collybia velutipes* (syn. *Flammulina velutipes*) was introduced into tomato and tobacco (Kesarwani *et al*., 2000).

As *S. sclerotiorum* is one of the most important pathogens of lettuce, the main goal of this research was to isolate and to introduce an oxalate decarboxylase gene (*oxdc*) from a *Flammulina* sp. into lettuce plants.

Materials and methods

Isolation of the oxalate decarboxylase gene

An oxalate decarboxylase gene from the edible mushroom *Flammulina* sp. was isolated using RT-PCR. The mycelium of the fungus (isolate IJF 140502, Embrapa Germplasm Bank) was cultivated on static liquid medium (5% dextrose, 1% peptone, 0·1% KH_2PO_4, 0·05% $MgSO_4{\cdot}7H_2O$ and 1% Difco malt extract, pH 5·2) (Mehta & Datta, 1991) for 25 days at 25°C. Oxalic acid was added to the culture at 12·5 mm. After 12 h, total RNA was isolated from 250 mg of mycelium of *Flammulina* sp. using the Micro-to-Midi Total RNA kit (Invitrogen). Total RNA was used to produce cDNA using the reverse transcriptase Superscript II (Invitrogen), according to the protocol suggested by the manufacturer. PCR reaction was carried out in a PTC-100 thermocycler (MJ Research) in 50 μL solution containing 40 ng cDNA, 60 mm Tris–SO_4 pH 8·9, 18 mm $(NH_4)_2SO_4$, 2 mm $MgSO_4$, 250 nm of each dNTP, 200 nm of each primer and 5 U Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen). The mixture was overlaid with mineral oil, denatured at 94°C (2 min), and subjected to 35 cycles of amplification (94°C for 30 s, 55°C for 30 s, 68°C for 2 min) with a final elongation step of 10 min at 68°C. The primers 5′-TCTAGATGTTCAACAACTTCCAACG-3′ and 5′-GGATCCTCAGTTCACAGGACCAAC-3′ were used to amplify a sequence of 1344 bp, corresponding to the coding sequence from the oxalate decarboxylase (*oxdc*) gene. The sequence was cloned into pCR2·1TOPO (Invitrogen) to generate the vector pTOPOOxDc, sequenced by using universal M13 and T7 primers on an automatic sequencer (Applied Biosystems) and deposited in GenBank (accession no. AY238332).

Plasmid vector

The *oxdc* coding sequence was removed from pTOPOOxDc and cloned into the vector pUC19-35SAMVNOS, between the *Xba*I and *Bam*HI sites, under the control of the doubled 35S promoter from *Cauliflower mosaic virus* and a sequence enhancer from *Alfalfa mosaic virus*. The pUC19-35SAMVNOS vector was derived from the pBI426 (Datla *et al*., 1991) in which the *gus–nptII* gene fusion was removed with *Nco*I and *Sac*I, and the *Nco*I site was deleted by partial digestion of nuclease S1. The *oxdc* expression cassette was removed from the pUC19-35SAMVNOS vector with *Pvu*II and cloned into the binary vector pCAMBIA1390 (CAMBIA) in the *Sma*I site, resulting in the transformation vector pCambOxDc. The vector pCambOxDc carries the selectable marker gene *htp* to confer hygromycin resistance.

Agrobacterium strain EHA 105, containing the vector pCambiaOxDc, was used to transform lettuce plants.

Lettuce transformation

The transformation of lettuce cv. Verônica was performed according to Curtis *et al*. (1994). Cotyledons from 48-h-germinated seeds were excised, then cocultured with bacterial suspension for 15 min. Explants were transferred to MS medium (Murashige & Skoog, 1962) supplemented with 0·1 mg L^{-1} 6-benzylaminopurine (BAP), 0·1 mg indolylbutyric acid (IBA), 10 mg hygromycin and 250 mg cefotaxime L^{-1}. Calluses were transferred to MS medium containing 0·1 mg BAP L^{-1} for shoot regeneration.

PCR screening of transformed plants

Plantlets were screened by PCR for the presence of the *oxdc* gene. Genomic DNA was isolated from young leaf discs according to Doyle & Doyle (1987). PCR reactions were carried out according Aragão *et al*. (2002). The primers OXDC 873 (5′-TGGGCTCGACAGAGGAGAAG-3′) and OXDC 371 (5′-CTCGGCAGCAGAATGAGGTC-3′) within the gene *oxdc* coding sequence were used to amplify a 502-bp sequence.

Progeny analysis

Seeds of the first generation (R_1) of self-pollinated plants were germinated on half-strength MS medium containing 10 mg hygromycin and 250 mg cefotaxime L^{-1}. χ^2 analyses, using the correction factor of Yates (Steel & Torrie, 1980), were performed to determine whether or not the observed segregation ratio was consistent with a Mendelian ratio (3 : 1 or 15 : 1), with a 95% level of confidence.

Inoculation of transgenic plants with *S. sclerotiorum*

An isolate of *S. sclerotiorum* obtained from tomato plants grown in Guaíra, SP, Brazil and kept at Embrapa Hortaliças (Brasília, DF, Brazil) was used in the experiments. The fungus was grown on PDA medium (20% potato,

2% dextrose, 1·5% agar). Inoculation was carried out according to Dickson & Hunter (1983). A mycelial agar plug 2 mm in diameter was cut from the growing margins of a 2-day-old *S. sclerotiorum* culture and applied to the adaxial surface of a leaf detached from a 9-week-old plant (10 leaves from each transgenic and nontransgenic line). Symptoms were observed every 12 h and lesion length recorded. Leaves were kept in a plant growth chamber (Conviron) at 20°C, 90–100% relative humidity and a photoperiod (50 μmol m^{-2} s^{-1}) of 12 h.

RT-PCR expression analysis

Lines P20, P43, P57 and P100 were analysed for the presence of *oxdc* gene transcripts. Total RNA was extracted from leaves using the Micro-to-Midi Total RNA kit. The remaining genomic DNA was eliminated by DNase digestion of the RNA samples. Total RNA was used to produce cDNA using the reverse transcriptase Superscript II, according to the protocol suggested by the manufacturer. PCR reactions were carried out as described above, except for the following changes: 25 ng cDNA, 1·5 U *Taq* DNA polymerase, and amplification cycles of 95°C for 1 min, 57°C for 1 min and 72°C for 1 min, with a final elongation step of 5 min at 72°C. Primers OXDC 873 and OXDC 371 were used to amplify a sequence within the *oxdc* gene. As an internal control, primers rRNA1 (5′-AACGGCTACCACATCCAAGG-3′) and rRNA2C (5′-TCATTACTCCGATCCCGAAG-3′) were used to amplify a sequence from the *18S rRNA* gene.

Results

The oxalate decarboxylase gene (*oxdc*) from *Flammulina* sp. isolate IJF 140502 showed 99·26% identity when compared with the GenBank sequence from *F. velutipes* (accession AF2000683), revealing differences in only 10 bp. No differences were found in the protein amino acid sequence.

Out of 80 regenerated plants, 34 lines (42·5%) revealed the presence of the *oxdc* gene in the PCR analysis. All transgenic lines were rooted and acclimatized. Seven lines showed an abnormal phenotype with smaller leaves and thinner stems.

All acclimatized transgenic lines (R_0 generation) were allowed to produce seeds. The progeny (R_1 generation) of 34 self-fertilized transgenic lines were screened by PCR analysis for the presence of the *oxdc* gene (Fig. 1). With the exception of eight lines (P11, P37, P39, P41, P61, P81, P82, P88), the R_0 plants transferred the foreign gene to the R_1 generation in a Mendelian fashion (Table 1). Twenty-three lines presented a segregation ratio of 3 : 1 and three lines a segregation ratio of 15 : 1.

Detached leaves were inoculated with 2-mm-diameter agar plugs from growing margins of 2-day-old *S. sclerotiorum* cultures and lesion length was recorded. Lines P100 and P43 did not show any symptoms (Fig. 2). Although the mycelium of *S. sclerotiorum* was able to initiate growth from the plug and attach to the leaf surface at inoculation sites of resistant transgenic plants, no colonization of the foliar tissue was observed (Fig. 2c). In contrast, tissue colonization was extensive on nontransgenic plants (Fig. 2d). Line P57 showed a delay in symptom

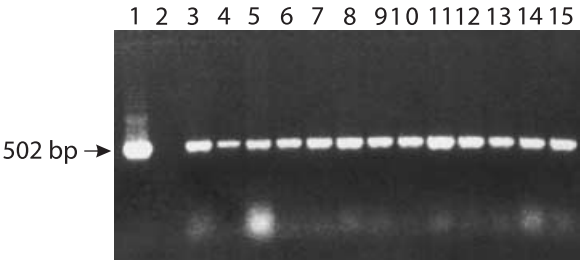

Figure 1 PCR analysis of putative transformed lettuce lines for detection of the *oxdc* gene. Lane 1, positive control (plasmid pCambiaOxDc); lane 2, nontransgenic plant; lanes 3–15, transgenic lines.

Table 1 Segregation analysis in 34 lines of transgenic lettuce

Line R_0	R_1 generation[a]		Segregation ratio tested	χ^2	P[b]
	Positive	Negative			
P1	42	8	3 : 1	2·16	0·14
P2	35	15	3 : 1	0·66	0·41
P3	42	8	3 : 1	2·16	0·14
P5	40	10	3 : 1	0·66	0·41
P6	45	4	15 : 1	0·30	0·58
P11	20	25	3 : 1	22·41	0·00
P14	39	11	3 : 1	0·24	0·62
P17	41	6	15 : 1	3·40	0·07
P19	35	15	3 : 1	0·66	0·41
P20	39	6	3 : 1	3·26	0·07
P21	39	11	3 : 1	0·24	0·62
P22	41	4	15 : 1	0·56	0·46
P23	36	13	3 : 1	0·06	0·80
P37	24	25	3 : 1	17·69	0·00
P39	44	6	3 : 1	4·51	0·03
P40	40	10	3 : 1	0·67	0·41
P41	27	22	3 : 1	10·35	0·01
P42	41	9	3 : 1	1·31	0·25
P43	37	11	3 : 1	0·11	0·73
P48	40	8	3 : 1	1·77	0·18
P54	37	12	3 : 1	0·01	0·93
P57	39	11	3 : 1	0·24	0·62
P58	37	12	3 : 1	0·01	0·93
P59	37	13	3 : 1	0·03	0·87
P61	29	19	3 : 1	5·44	0·02
P63	33	17	3 : 1	2·16	0·14
P81	5	43	3 : 1	106·77	0·00
P82	29	18	3 : 1	4·43	0·03
P88	7	41	3 : 1	93·44	0·00
P99	33	17	3 : 1	2·16	0·14
P100	36	12	3 : 1	0·00	1·00
P101	41	9	3 : 1	1·31	0·25
P102	32	18	3 : 1	3·22	0·07
P103	36	12	3 : 1	0·00	1·00

[a]Data are based on *in vitro* tolerance to hygromycin.
[b]*P*, probability that observed ratios reflect expected segregation ratio of 3 : 1 or 15 : 1.

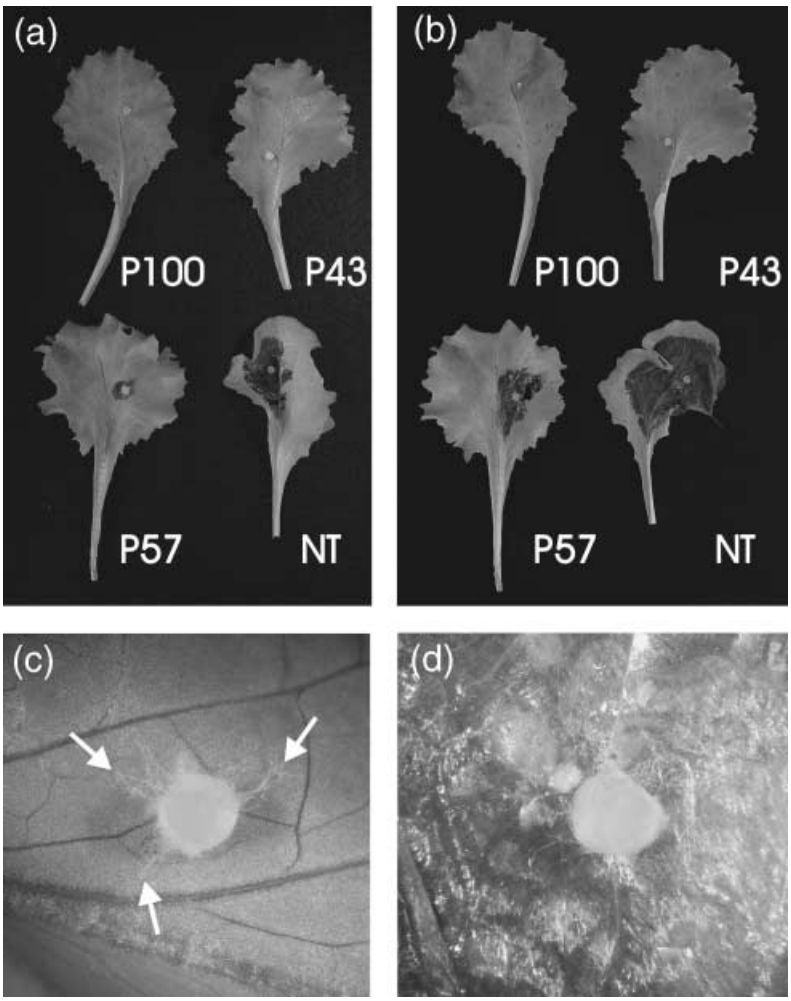

Figure 2 Pathogenesis assay of transgenic lines P100, P43 and P57 for resistance to *Sclerotinia sclerotiorum*. Symptoms were observed on detached leaves (a) 12; (b) 24 h after inoculation with 2-mm mycelial agar plugs. (c) Detail of the mycelium of *S. sclerotiorum* initiating growth from the plug and attaching to the leaf surface at the inoculation site of a resistant transgenic plant (arrows); (d) line P43 and damaged susceptible nontransgenic plants. NT, nontransgenic line.

development compared with the nontransgenic line (Fig. 2a,b). The progression of disease development in 33 transgenic lettuce lines over a 36-h period is illustrated (Fig. 3). Except for transgenic lines P57, P43 and P100 (Fig. 3a,d,h, respectively), there were no significant differences in lesion length compared with the controls (nontransgenic plants). Symptoms were more severe for line P2 at 36 h than for the other lines from its group (Fig. 3c). As observed in the control plants, in all transgenic susceptible lines the lesions extended across the whole leaf within 48 h (data not shown).

RT-PCR analysis for detection of *oxdc* gene transcripts was carried out with three resistant lines (P43, P57, P100) and one susceptible transgenic line (P20). The resistant lines showed *oxdc* gene expression, while line P20 and the nontransgenic plant did not produce *oxdc* gene transcripts (Fig. 4). All plants tested displayed similar levels of expression of the *18S rRNA* gene.

Discussion

The expression of the oxalate decarboxylase gene (*oxdc*) from *Flammulina* sp. in lettuce has allowed the production of transgenic lines resistant to the necrotrophic fungus *S. sclerotiorum*.

With the exception of seven lines, the transgenic plants produced presented a normal phenotype. Seven transgenic lines showed an abnormal phenotype, with smaller leaves and narrow stems. Similar results were observed by Pileggi *et al.* (2001). These phenotypes may be attributable to somaclonal variation caused by exposure to growth regulators during callus growth and plant regeneration. Insertional gene mutation could also be involved. Further experiments with plasmid rescue should be considered in order to evaluate this possibility.

The progenies (R_1 generation) of self-pollinated transgenic lines were tested for the presence of the *oxdc* gene. The analyses indicated that most of the transgenic plants contained the transgene(s) integrated in a single locus, as indicated by the 3 : 1 segregation ratio. This is a desirable property for introducing transgenic plants into a breeding programme aimed at the development of new cultivars.

Several studies support the concept that most of the foreign genes introduced by *Agrobacterium* are normally transmitted to the progeny (Gelvin, 1998). In this study, eight lines did not transfer the introduced foreign gene to

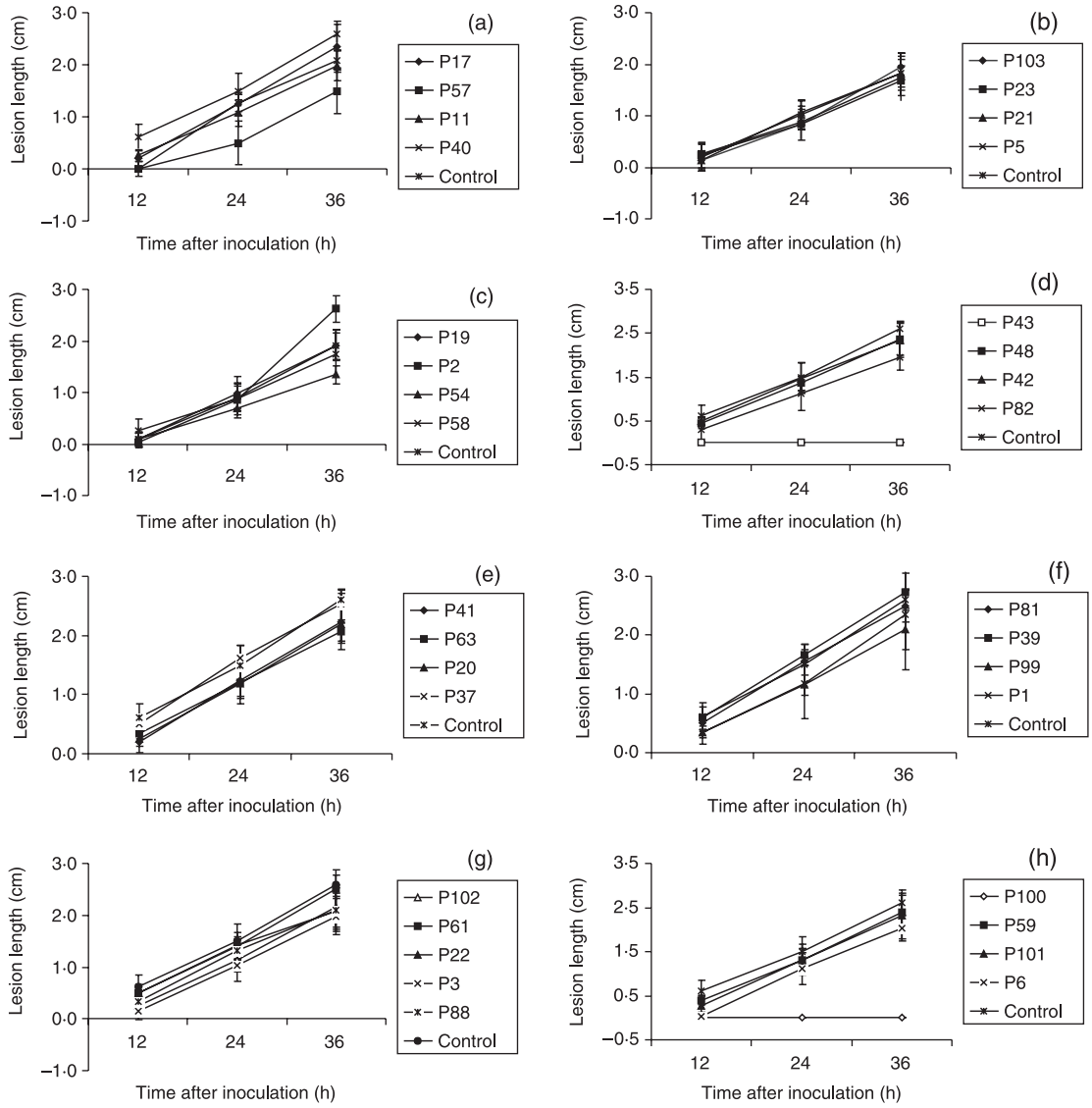

Figure 3 Lesion-length progression following inoculation of detached leaves of 33 transgenic lettuce lines and control with mycelial agar plugs containing *Sclerotinia sclerotiorum*. Transgenic lines and control indicated on right border of each block. Bars, SD (n = 10).

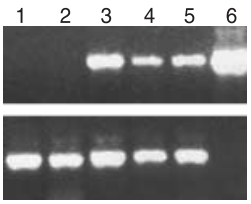

Figure 4 RT-PCR for detection of expression of *oxdc* and 18S rRNA genes in transgenic lettuce lines. Upper lanes: (1) nontransgenic plant; (2) P20; (3) P43; (4) P57; (5) P100; (6) plasmid vector used in the PCR reaction. Lower lanes: lettuce *18S rRNA* gene as internal control.

the R_1 generation in the Mendelian segregation ratio expected for self-pollinated plants. An insertional mutation of an essential gene required for ovule fecundation or development might account for this aberrant inheritance. In addition, physical transgene elimination has been reported in several plants and has been attributed to intrachromosomal recombination (Fladung, 1999); genetic instability resulting from the conditions of tissue culture (Risseeuw *et al*., 1997; Joersbo *et al*., 1999); or a genomic defence process (Srivastava *et al*., 1996; Romano *et al*., 2005).

From the total of 34 putative transgenic lines produced, only three lines presented resistance to *S. sclerotiorum*. The expression of a transgene located in active chromatin or heterochromatin is highly variable, even among lines independently transformed with the same construct. Many factors may be responsible for variable transgene expression, including the tendency for exogenous DNA to undergo rearrangement prior to integration, effects related to the transgene copy number and integration position, and effects of DNA hypermethylation (Meyer, 1998; Abranches *et al*., 2000).

The introduction of genes coding for enzymes able to degrade oxalic acid in transgenic plants has been successful in generating *Sclerotinia*-resistant canola (Thompson *et al*., 1995); poplar (Liang *et al*., 2001); tobacco (Berna & Bernier, 1997; Kesarwani *et al*., 2000); soybean (Donaldson *et al*., 2001); sunflower (Burke & Riesenberg, 2003); tomato (Kesarwani *et al*., 2000); and peanut (Livingstone *et al*., 2005). Apparently, only transgenic soybean and sunflower plants with partial resistance have so far been tested under field conditions (Burke & Rieseberg, 2003; Cober *et al*., 2003). Correlation of *Sclerotinia* disease ratings between glasshouse, laboratory and field evaluations has been reported (Kim *et al*., 2000). Although this correlation was inconsistent in some cases (Vuong *et al*., 2004), *in vivo* resistance assays with both oxalic acid and *Sclerotinia* mycelium plug inoculation have consistently been correlated with resistance in the field (Kesarwani *et al*., 2000; Kolkman & Kelly, 2000; Chipps *et al*., 2005). In soybean, Wegulo *et al*. (1998) demonstrated that both mycelial inoculation of detached leaves and the response of detached stems to oxalic acid correlated with disease resistance in the field.

RT-PCR analyses revealed the expression of the *oxdc* gene in the three resistant lines (P43, P57, P100), while no *oxdc* gene transcripts were observed in the susceptible transgenic and nontransgenic lines analysed. These results suggest that expression of the *oxdc* gene is associated with resistance to *Sclerotinia*.

The results show that expression of the oxalate decarboxylase gene in a highly susceptible species is efficient in generating resistance to *S. sclerotiorum*. The interaction of *Sclerotinia* with epidermal cells of transgenic and nontransgenic lettuce is currently being studied at the ultrastructural level. This may help to elucidate the cellular basis of this plant–pathogen interaction. In addition, studies are being carried out to evaluate the resistance of lettuce lines under field conditions.

Acknowledgements

We would like to thank Dr Arailde Fontes Urben (Embrapa Recursos Genéticos e Biotecnologia) and Dr Gilmar Paulo Henz (Embrapa Hortaliças) for providing the *Flammulina* sp. and *Sclerotinia* isolates, respectively, and Warley Almeida for his technical assistance. This work was supported by CNPq (grant number 305441/2004-3).

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