High resistance to *Sclerotinia sclerotiorum* in transgenic soybean plants transformed to express an oxalate decarboxylase gene

W. G. Cunha\(^ab\), M. L. P. Tinoco\(^ab\), H. L. Pancoti\(^a\), R. E. Ribeiro\(^a\) and F. J. L. Aragão\(^a\)*

\(^a\)Embrapa Recursos Genéticos e Biotecnologia, PqEB W5 Norte, 70770-916, Brasília; and \(^b\)Departamento de Biologia Celular, Universidade de Brasília, Campus Universitário, 70910-900 Brasília, DF, Brazil

Pathogenesis of *Sclerotinia sclerotiorum* (white mould) has been associated with fungal secretion of oxalic acid (OA). Enzymes capable of degrading OA have been utilized to produce transgenic resistant plants. Transgenic soybean lines containing the decarboxylase gene (oxdc) isolated from *Flammulina* sp. were produced by the biolistic process. Molecular analysis revealed successful incorporation of the gene into the plant genome and showed that the oxdc gene was transferred to the progeny plants. Sixteen T2 transgenic events were screened for *S. sclerotiorum* resistance using a detached leaf assay. The white mould disease progress curve displayed a significant delay in symptom development in all oxdc events compared with the nontransgenic genotype. Area under the disease progress curve (AUDPC) showed reduction in severity ranging from 61% to 96% comparing the oxdc events with the nontransgenic control. One event (OXDC.9.21) presented some plants that did not show any symptoms after 92 h. RT-PCR analysis for detection of oxdc gene transcripts suggested that expression of the oxdc gene is associated with resistance to *S. sclerotiorum*.

**Keywords**: oxalate decarboxylase, oxalic acid, transgenic soybean, white mould

Introduction

White mould, caused by the necrotrophic ascomycete fungus *Sclerotinia sclerotiorum*, is a worldwide disease. The fungus infects more than 400 plant species, including important crops such as cotton, tomato, sunflower, dry bean and soybean (Boland & Hall, 1994). In soybean, the disease is also called sclerotinia stem rot (SSR) and its incidence causes significant damage to yield and grain quality. For an increase of 10% in disease incidence the yield losses range from 83 to 335 kg ha\(^{-1}\) (Danielson et al., 2004). SSR is one of the most important soybean diseases in the USA and Brazil and these countries are responsible for more than 60% of total world soybean production. In the USA, SSR is generally found in farms in the states of Iowa, Illinois, Minnesota, Nebraska and Pennsylvania (Wrather & Koenning, 2009). Historically, these states produce about 53% of the total soybean produced in the USA (http://www.nass.usda.gov/Statistics_by_Subject/index.asp). In 2004, when climatic conditions were favourable to the pathogen’s development, the soybean yield suppression caused by SSR was more than 60 million bushels (Wrather & Koenning, 2009). In Brazil, the disease usually occurs in the southern states and in highland areas of the Central Region. In these regions, yield losses of up to 60% have been verified (L.H.C. da Silva, FESURV, Brazil, personal communication).

Disease control management is concentrated on agronomic practices, such as planting under tillage, use of lodging resistant varieties, wide-row planting and rotation with non-host crops. However, in favourable environmental conditions for pathogen development, these practices are not enough. Recommended chemical control has poor efficiency due to low penetration and uneven distribution of fungicides, caused by the already formed plant canopy, since the infection starts at the plant’s reproductive stage. Additionally, the use of fungicides for SSR control increases production costs. Consequently, resistant varieties would be the best alternative for SSR management. However, such a disease is difficult to control genetically where breeding programmes have had limited success. Genetic resistance to *S. sclerotiorum* is complex, has low heritability and is restricted to a few lines that have shown only partial resistance (Kim & Diers, 2000; Vuong et al., 2008). In addition, the plant escape mechanisms (flowering date, plant height, lodging, canopy architecture and maturity) and environmental conditions make SSR resistance evaluations difficult in the field.

*E-mail: aragao@cenargen.embrapa.br

Published online 18 March 2010
Recent studies showed that some of the identified quantitative trait loci (QTL) for resistance to SSR were associated with escape mechanisms (Kim & Diers, 2000). For these reasons, soybean breeding has not made available a SSR-resistant variety on the market.

Previous studies provided evidence for the involvement of oxalic acid (OA) in S. sclerotiorum pathogenesis and OA tolerance has been correlated to disease tolerance (Bateman & Beer, 1965; Maxwell & Lumsden, 1970; Noyes & Hancock, 1981; Marciano et al., 1983; Magro et al., 1984; Tu, 1985; Godoy et al., 1990; Wegulo et al., 1998; Cessna et al., 2000; Kolkman & Kelly, 2000; Favaron et al., 2004; Guimarães & Stotz, 2004; Kim et al., 2008; Walz et al., 2008b). Treatment of healthy plants with OA or fungal culture filtrates resulted in foliar symptoms identical to those found in diseased plants (Bateman & Beer, 1965; Noyes & Hancock, 1981; Marciano et al., 1983). In addition, plants that showed in vitro and field tolerance to OA were more tolerant to S. sclerotiorum (Noyes & Hancock, 1981; Tu, 1985; Wegulo et al., 1998; Kolkman & Kelly, 2000) and mutants of S. sclerotiorum unable to synthesize OA were less pathogenic when compared with a wild type (Godoy et al., 1990). A potential alternative to developing an SSR-resistant soybean variety is the introduction of genes that generate enzymes capable of degrading the OA. Several transgenic plants expressing genes encoding enzymes that can metabolize OA, such as oxalate oxidases (OXO) and oxalate decarboxylases (OXDC), have shown an enhanced resistance to S. sclerotiorum (Kesarwani et al., 2000; Donaldson et al., 2001; Cober et al., 2003; Hu et al., 2003; Livingstone et al., 2005; Dias et al., 2006; Dong et al., 2008; Walz et al., 2008a). OXO catalyses the oxygen-dependent oxidation of oxalate to carbon dioxide and hydrogen peroxide. OXDC breaks down oxalate to generate carbon dioxide and formate in the absence of any cofactor requirement. In addition, OXDC is specific to OA and has high activity at acidic or neutral pH (Kesarwani et al., 2000).

The main goal of this research was to introduce an oxalate decarboxylase gene (oxdc) from a Flammulina sp. into the soybean genome in order to generate transgenic lines resistant to S. sclerotiorum.

Materials and methods

Plasmid vector and soybean transformation

The oxdc coding sequence was removed from pTO-POOxdc and cloned into the vector pUC19-35SdAMV-NOS, between the XbaI and BamHI sites, under the control of the doubled 35S promoter from Cauliflower mosaic virus and a sequence enhancer from Alfalfa mosaic virus (Dias et al., 2006). The oxdc expression cassette was excised with EcoRI and HindIII from the vector pOXDC (Dias et al., 2006) and inserted into the pBluKSP (Fermentas) to generate the pBluKSPOXDC vector. The oxdc cassette is composed of the oxdc coding sequence under control of the doubled 35S promoter from Cauliflower mosaic virus and a sequence enhancer from Alfalfa mosaic virus, and nos gene terminator. The 5718-bp fragment containing the mutated Arabidopsis ahas gene (selectable marker that confers imidazolinone-specific resistance) was removed with XbaI from the vector pAC321 (Rech et al., 2008) and cloned into the SpeI site of the vector pBluKSPOXDCAHAS, resulting in the transformation vector pBluKSPOXDCAHAS (Fig. 1).

Soybean transformation was carried out according to Rech et al. (2008) and Aragão et al. (2000). Mature seeds of a commercial variety (BR-16) were surface-sterilized and soaked in distilled water for 18–20 h. The embryonic axes were excised from seeds, and the apical meristems were exposed by removing the primary leaves and bombarded as previously described by Aragão et al. (2000). Multiple shooting was induced by cultivating the embryonic axes derived shoots 2–3 cm in length, they were individually transferred to a plastic pot containing a mixture of fertilized soil and vermiculite and acclimatized in a greenhouse to produce seeds. A total of 1152 explants were bombarded with the vector pBluKSPOXDCAHAS.

PCR screening of transformed plants

The recovered plants and progenies were screened by PCR to detect the presence of the oxdc gene. Genomic DNA from young leaves was extracted by the CTAB method (Dole & Doyle, 1987). The PCR analyses were carried out according to Aragão et al. (2002). The primers OXDC 371 (5′-CTCGGCGGATGAGGACG-3′) and OXDC 873 (5′-TGCGGAGACAGAGAAGAG-3′) were used to amplify a 522-bp sequence within the oxdc coding sequence.

Detached leaf assays

The detached leaf assay was carried out using plants from the T3 generation. Sclerotia were collected from a soybean infected field located in Distrito Federal (Brazil) and

![Figure 1 Diagram representing the pBluKSPOXDCAHAS transformation vector containing the ahas gene (ahas5’: ahas gene promoter; ahasCDS: Arabidopsis thaliana AHAS coding sequence; ahas3’: ahas gene terminator) and oxdc expression cassettes (d35S: doubled 35S promoter from Cauliflower mosaic virus; AMV: non-translation sequence enhancer from Alfalfa mosaic virus; nos3’: nopaline synthase terminator). Black bar represents the probe used in the Southern analysis.](image-url)
cultured on potato dextrose agar (PDA) medium at room temperature to produce mycelia. To produce the inoculum for the detached leaf assays, a single 5 mm diameter mycelial plug was placed at the centre of a new PDA plate. The fresh culture was incubated at 20°C for 2 days in the dark. Mycelial plugs with 5 mm diameter were cut from the growing margins and applied to the adaxial surface of the middle leaflet of the youngest fully-expanded trifoliate leaves detached from plants (at V3 stage, according to Fehr et al., 1971). Leaves were immediately placed on a Petri dish containing three moist filter papers and incubated at 20°C in the dark. Leaves were photographed at three different times (42, 66 and 90 h) and images were used to measure the infected area using the QUANT v1.0.1 software (Vale et al., 2003). The area under the disease progress curve (AUDPC) was used to summarize the progress of disease severity according to Shaner & Finney (1977).

Southern blot analysis

Genomic DNA was isolated according to Dellaporta et al. (1983). Southern blotting and hybridization were carried out as previously described (Sambrook et al., 1989). Genomic DNA (15 μg), digested with EcoRI, was separated on a 1% agarose gel, blotted onto a nylon membrane (Hybond) and hybridized with the probe labelled with α[32P]-dCTP (3000 Ci mol⁻¹) using a random primer DNA labelling kit (Pharmacia Biotech) according to the manufacturer’s instructions. The probe was obtained by digesting the pBluKSPOXDCAHAS with BamHI and XbaI to generate the 1350-bp fragment (corresponding to oxdc coding sequence) that was isolated from a 1% agarose gel and purified using the Wizard SV Gel Clean-Up System (Promega).

RT-PCR expression analysis

RT-PCR were carried out as described by Dias et al. (2006). The relative intensity of the bands was determined by scanning the PCR gel and analyzing the band intensity using the Quantity One™ program v4.6.3. Relative quantification was done by comparing intensities of the oxdc and 18s rRNA bands.

Results

PCR analyses revealed the presence of the oxdc gene in 10 generated plantlets (Fig. 2). Each of the 10 oxdc plants was considered a distinct T0 event (named OXDC.1 to OXDC.10). All seeds from the T0 events were planted in a greenhouse and the T1 plants were screened by PCR to detect the presence of the oxdc gene. Transformants presented phenology (plant height, site of insertion of first pod, number of branches, internode length, foliar area, total number of flowers and pods) similar to the non-transformed plants. Seeds from 16 T1 events (OXDC.1.01; OXDC.1.02; OXDC.2.03; OXDC.2.04; OXDC.3.05; OXDC.3.06; OXDC.4.07; OXDC.4.08; OXDC.5.09; OXDC.5.10; OXDC.6.11; OXDC.6.12; OXDC.7.16; OXDC.7.17; OXDC.8.18 and OXDC.9.21) were harvested to plant the T2 generation.

About 10 PCR-positive plants of each oxdc event and 10 plants of the nontransgenic variety BR-16, which showed similar growing development in the greenhouse, were screened for S. sclerotiorum resistance using the detached leaf assay. The disease progress curve displayed a significant delay in the symptom development in all oxdc events compared with the nontransgenic genotype (Fig. 3). Some plants of the event OXDC.9.21 did not show any symptoms after 92 h. Extensive mycelia penetration was not observed using a stereomicroscope over a period of 90 h or even after 114 h (Fig. 4j–k). In the control plants, the lesions expanded across the whole leaf after 90 h (Fig. 4). The average of the infected area 90 h after inoculation ranged from 0.45 cm² (OXDC.9.21) to 7.19 cm² (OXDC.2.03) in the transgenic events, while in the control it was 17.60 cm². The AUDPC showed a severity reduction of 96% (OXDC.9.21) to 61% (OXDC.2.03) comparing the oxdc events with the nontransgenic control.

RT-PCR analysis for detection of oxdc gene transcripts was carried out with one plant from event OXDC.9.21 (that exhibited the highest resistance to S. sclerotiorum), one plant of OXDC.6.11 (that presented partial resistance) and one of the nontransgenic genotypes. The transgenic events showed oxdc gene expression, while in the nontransgenic plant oxdc transcripts were not observed (Fig. 5).

Since the event OXDC.9.21 revealed the highest S. sclerotiorum resistance, it was selected for further characterization by Southern blot. Results revealed the presence of two integrated copies of the oxdc gene and that all transgenic plants analyzed from this event presented the same integration pattern (Fig. 6). DNA isolated from a nontransgenic plant did not hybridize with the oxdc coding sequence probe.
Figure 3. Disease progress curve of T2 oxdc soybean transgenic events and the nontransgenic control (BR-16) obtained by the detached leaf assay using Sclerotinia sclerotiorum mycelial agar plug. The infected area measured in each time was an average of 10 different plant repetitions except for the OXDC.1.02, OXDC.2.04, OXDC.5.10, OXDC.7.16, OXDC.7.17 and OXDC.8.18 that were 9, 6, 4, 8, 6 and 9 repetitions, respectively. Bars = standard deviation.

Figure 4. Resistance response of transgenic plants to Sclerotinia sclerotiorum inoculation. Symptoms were observed on detached leaves 42 h (a, d and g), 66 h (b, e and h) and 90 h (c, f and i) after inoculation of transgenic plants OXDC.9.21.2 (a–c), OXDC.6.11.7, (d–f) and nontransgenic control (g–i) with 5-mm mycelial agar plugs. Detail of soybean line OXDC.9.21.2 response to the mycelium of S. sclerotiorum growth 90 h (j) and 114 h (k) after inoculation compared to a nontransgenic plant 90 h after inoculation (l).
the OXDC.6.11.7), while no transcripts corresponding to gene in the resistant lines tested (OXDC.9.21.2, 18S rRNA et al. 2008a). Results from the detached leaf assays showed that plants transformed with the oxdc gene presented different levels of resistance to S. sclerotiorum. Compared to the non-transformed control, all transgenic events demonstrated a significant delay in lesion development. Similar results were previously reported in tobacco, tomato and lettuce (Thompson et al., 1995; Berna & Bernier, 1997; Donaldson et al., 2001; Cober et al., 2003; Hu et al., 2003; Livingstone et al., 2005; Dong et al., 2008) and the oxalate decarboxylase gene has been incorporated into tobacco, tomato and lettuce (Kesarwani et al., 2000; Dias et al., 2006; Walz et al., 2008a). In contrast to oxalate oxidase enzyme activity, which converts OA into carbon dioxide and hydrogen peroxide, oxalate decarboxylase promotes OA conversion into carbon dioxide and formate and does not lead to a development of additional hydrogen peroxide (Kesarwani et al., 2000; Walz et al., 2008a). The production of oxygen radical synthesis interactions seems to be important, as they weaken the host tissue at early stages of infection and consequently enhance the growth and development of the pathogen (Govrin & Levine, 2000). Kim et al. (2008) concluded that S. sclerotiorum-secreted oxalate induces increased levels of reactive oxygen species (ROS) such as hydrogen peroxide and superoxide in the plant in the early stages of the infection, which appears to trigger a programmed cell death (PCD) pathway, resulting in plant cell death and the generation of a suitable environmental niche for fungal pathogenic development, nutrient acquisition, and the establishment of a necrotrophic relationship. Walz et al. (2008b) observed moderate hydrogen peroxide accumulation in inoculated tobacco plants in the first 24 h after infection and a subsequent decrease. These studies suggested that in the early stages of pathogenesis at relatively high pH (>5) the oxalate produced by the fungus induces increased reactive oxygen species.

Discussion

The progenies (T2 generation) of self-pollinated transgenic lines were tested for resistance to S. sclerotiorum. Results from the detached leaf assays showed that plants transformed with the oxdc gene presented different levels of resistance to S. sclerotiorum. Compared to the non-transformed control, all transgenic events demonstrated a significant delay in lesion development. Similar results were previously reported in tobacco, tomato and lettuce transgenic plants transformed to express an oxdc coding gene (Kesarwani et al., 2000; Dias et al., 2006; Walz et al., 2008a).

RT-PCR analysis revealed the expression of the oxdc gene in the resistant lines tested (OXDC.9.21.2, OXDC.6.11.7), while no transcripts corresponding to the oxdc gene were observed in the susceptible nontransgenic lines analyzed. Results suggested that line OXDC.6.11.7, exhibiting only moderate resistance to S. sclerotiorum, revealed a presence of oxdc gene transcripts at a much lower level (Fig. 5b) than observed in the line OXDC.9.21.2, which exhibited a high resistance level to the pathogen. These results suggest that expression of the oxdc gene is associated with resistance to the fungus and that the resistance level is dependent on the transgene expression level. The association of the presence of the gene and resistance is also supported by the fact that all transgenic lines presented a level of resistance to the pathogen while all nontransgenic genotypes tested were susceptible to infection. The results have shown that the generation of highly S. sclerotiorum-resistant soybean lines is feasible by the expression of the oxalate decarboxylase gene. The interaction of Sclerotinia with epidermal cells of transgenic and nontransgenic soybean plants is currently being studied at the ultra-structural level. This may help to elucidate the cellular basis of this plant-pathogen interaction. In addition, studies will be carried out to evaluate the resistance of soybean lines under field conditions. To confirm the enhanced resistance shown by the detached leaf assay, transgenic event OXDC.9.21 will be further tested under field conditions. Although the values were inconsistent in some situations, a significant correlation of SSR disease ratings between the detached leaf assay and field evaluations has been reported (Wegulo et al., 1998; Kim et al., 2000; Hoffman et al., 2002). To ensure the consistency of the detached leaf assay, this study used uniform leaf size, fixed the inoculum placement and provided adequate moisture during the test, as discussed by Wegulo et al. (1998).

Expression of oxalate-detoxifying enzymes in transgenic plants has been successful in generating resistant lines. Wheat oxalate oxidase has been constitutively expressed in canola, tobacco, soybean, poplar, sunflower, peanut and tomato (Thompson et al., 1995; Berna & Bernier, 1997; Donaldson et al., 2001; Cober et al., 2003; Hu et al., 2003; Livingstone et al., 2005; Dong et al., 2008) and the oxalate decarboxylase gene has been incorporated into tobacco, tomato and lettuce (Kesarwani et al., 2000; Dias et al., 2006; Walz et al., 2008a). In contrast to oxalate oxidase enzyme activity, which converts OA into carbon dioxide and hydrogen peroxide, oxalate decarboxylase promotes OA conversion into carbon dioxide and formate and does not lead to a development of additional hydrogen peroxide (Kesarwani et al., 2000; Walz et al., 2008a). The production of oxygen radical synthesis interactions seems to be important, as they weaken the host tissue at early stages of infection and consequently enhance the growth and development of the pathogen (Govrin & Levine, 2000). Kim et al. (2008) concluded that S. sclerotiorum-secreted oxalate induces increased levels of reactive oxygen species (ROS) such as hydrogen peroxide and superoxide in the plant in the early stages of the infection, which appears to trigger a programmed cell death (PCD) pathway, resulting in plant cell death and the generation of a suitable environmental niche for fungal pathogenic development, nutrient acquisition, and the establishment of a necrotrophic relationship. Walz et al. (2008b) observed moderate hydrogen peroxide accumulation in inoculated tobacco plants in the first 24 h after infection and a subsequent decrease. These studies suggested that in the early stages of pathogenesis at relatively high pH (>5) the oxalate produced by the fungus induces increased reactive oxygen species.
(hydrogen peroxide and superoxide) levels and a programmed cell death response in plant tissue that is required for disease development (Kim et al., 2008). As OA accumulates, the pH decreases and it suppresses the reactive oxygen species and programmed cell death, enabling the pathogen’s further ingress into plant tissue (Kim et al., 2008). OXDC transgenic plants would be a better model in which to study the plant pathogen interaction, considering that there is no additional hydrogen peroxide production, unlike in OXO plants. In practical terms, more studies are needed to determine which oxalate-detoxifying strategy would be more appropriate.

Acknowledgements

We gratefully acknowledge the financial support of FINEP and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, grant number 500028/2006-0). W. Cunha was supported by a fellowship from CNPq.

References

Donaldson PA, Anderson T, Lane BG, Davidson AL, Simmonds DH, 2001. Soybean plants expressing an active oligomeric oxalate oxidase from the wheat gf-2.8 (germin) gene are resistant to the oxalate-secreting pathogen Sclerotinia sclerotiorum. Physiological and Molecular Plant Pathology 59, 297–307.


