SCLEROTINIA HEAD ROT RESISTANCE CONFERRED BY WHEAT OXALATE OXIDASE GENE IN TRANSGENIC SUNFLOWER

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Summary :

A wheat oxalate oxidase gene has been successfully transferred to sunflower via transformation. The objective of this work was to test the ability of this gene to enhance *Sclerotinia* resistance in field trials employing artificial inoculation techniques.In several field trials, transgenic inbred lines containing the wheat oxalate oxidase gene were tested alone and in hybrid combination with non-transgenic lines having varying degrees of ``natural" genetic resistance to head rot. The field screening results provided strong evidence of enhanced *Sclerotinia* resistance associated with the transgenic events. Inbreds and hybrids carrying the oxalate oxidase events were more resistant to *Sclerotinia* than non-transgenic isolines and corresponding isogenic hybrids. Significant increases in resistance were observed independent of the genotype, even in those possessing higher levels of natural resistance.

INTRODUCTION

Head rot caused by the fungus *Sclerotinia sclerotiorum* is a very important sunflower disease in Argentina, the United States of America, and several European countries. It can produce severe yield losses, especially when humid or wet conditions occur in combination with cool temperatures during the beginning of flowering stage. Ascospores of the fungus germinate on the flowers and invade floral and receptacle tissues producing head rot. When conditions are favorable for fungal growth the head can be completely destroyed (Gulya *et al*., 1997).

Significant genetic variability for *Sclerotinia* resistance/tolerance exists within cultivated sunflower germplasm, but complete resistance has not been obtained. Oxalic acid (OA), a major *S. sclerotiorum* toxin, plays an important role during pathogenesis by acidifying host tissues and chelating calcium from host cell walls (Dutton and Evans, 1996). The reduced quantity of calcium linked to cell wall pectates weakens the wall, allowing fungal enzymes to degrade them easily. OA production appears to be an essential pathogenic determinant and provides a target for interfering with pathogenicity (Magro *et al*., 1984, Godoy *et al*., 1990). Dickman and Chet (1998) working with OA-degrading bacteria found that they were useful as a bio-control strategy against *Sclerotium rolfsii* and *S. sclerotiorum*. They also suggest that molecular manipulation of OA-degrading enzyme genes could be an alternative strategy for the control of such diseases. Oxalate oxidase enzymes degrade OA (Lane *et al*., 1993). The wheat oxalate oxidase gene has been successfully transferred to sunflower by transformation. Advanta and Pioneer are collaborating to characterize the resulting transgenic events. Greenhouse and field experiments were conducted in several locations. The objective of the present work was to determine the ability of this gene to increase sunflower resistance to *Sclerotinia* head rot.

MATERIALS AND METHODS

The wheat oxalate oxidase gene was incorporated into a transformation cassette driven by the ``SCP1" promoter (Lu *et al.*, 2000), and containing an omega prime (o') enhancer and PINII terminator. This cassette (SCP1::o'::oxox::PINII) was used to transform `SMF3', as described by Scelonge et al. (2000). SMF3 is a single headed restorer line with moderately low *Sclerotinia* head rot resistance. In previous experiments, several transgenic events were evaluated. Among them, two SCP1::oxox:o'::PINII (oxox) events, TF28 and TF34, were selected for field experimentation under artificial *Sclerotinia* inoculation. Three field trials were conducted at Balcarce, Argentina during the summer 98/99. Experiments were planted in several 250 m2 protective cages. Transgenic inbred lines carrying the oxox transgenic events were tested alone and in hybrid combination with non-transgenic lines possessing varying degrees of natural genetic resistance to head rot. The evaluation plot comprised a single row with approximately 13-15 plants. Additional transgenic events, not described here, were included in the experiments.

Table 1: Description of the SCP1::oxox::o'::PINII transgenic sunflower germplasm evaluated

Event	**Promotor::Gene**	**GMO Lines [number of rows]**
TF34	SCP1::o'::oxox::PINII	Y9820LM [2],Y9854LM [2]
TF28	SCP1::o'::oxox::PINII	Y9821LM [2]

Inbred Line Experiment

A total of 40 lines, 7 check lines (including SMF3) and 33 transgenic inbred lines were tested in an incomplete block design. The check line SMF3 was sown in five rows (5 replications) and each of the remaining check lines in two rows (2 replications). An indication of the oxox transgenic inbred material and the number of rows sown is presented in Table 1.

Hybrid Experiments

Experiment 1. A total of 9 transgenic hybrids and 6 non-transgenic hybrids were planted in an incomplete block design. Transgenic hybrids were obtained by crossing the SMF3 transgenic inbred lines with six non-transgenic inbreds. The non-transgenic hybrids were obtained by crossing the SMF3 line with the same six non-transgenic inbreds. All resulting hybrids were single headed. The oxox transgenic and non-transgenic check hybrids tested are shown in Table 2. In each cell of the table, the number of rows sown for each hybrid combination is indicated.

Table 2: SCP1::o'::oxox::PINII transgenic and check hybrids

Event	Promotor::Gene	Inbred1	Inbred2	Inbred3	Inbred4	Inbred5	Inbred6
Check	None	2	2	2	2	2	2
TF34	SCP1::oxox::o'::PINII	3	3	2	2	2	2
TF28	SCP1::oxox::o'::PINII	2			2	2	

Experiment 2. A selected oxox event, TF34, was evaluated in hybrid combination with four inbred lines possessing varying degrees of head rot resistance. Hybrids constructed as for Experiment 1 were arranged in a Youden Square design (Montgomery, 1976) with 5 replications.

Inoculation and Disease Evaluation Plants were artificially inoculated with *Sclerotinia* ascospores (standard methodology) at the beginning of flowering (Tourvieille and Vear, 1984). Head rot evaluations were made 40 days later using a 1 to 9 scale. A score of 1 corresponded to 90-100% head area affected and a score of 9 was assigned to entries having no indication of head infection. Each 1-point increment corresponded to a 12.5% change in the affected head area. Statistical analyses are based on the weighted average disease severity score (weighted DSI). The weighting factor is the number of plants in the evaluation row. Analysis of variance using Type III Sum of Squares and multiple comparison tests are applied (SAS/STAT User´s Guide, 1989).

RESULTS AND DISCUSSION

Strong evidence of transgenic effects was observed. Materials carrying the oxox events were more resistant to *Sclerotinia* than the SMF3 isoline and isogenic hybrids using SMF3.

Inbred Line Experiment. Figure 1 and Table 3 summarize the results from the inbred experiment. Figure 1 shows a box plot for each transgenic event in the SMF3 inbred and several non-transgenic checks. Within each box, the dot (·) indicates the average response value (DSI),

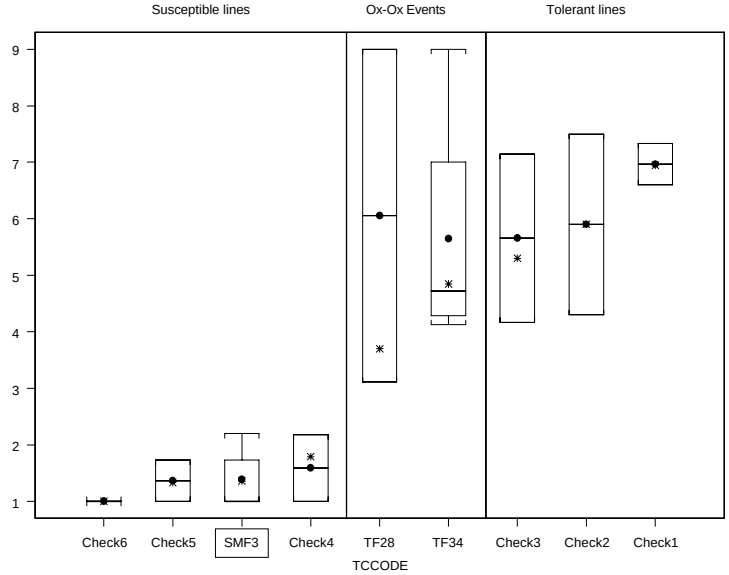

Figure 1. Box-plots of the disease severity index (DSI) at 40 days from inoculation.

Table 3: Event means, check line means and 5% Dunnet´s multiple comparison test

Material	Check 6	Check 5	SMF 3	Check 4	TF28	TF3 4	Check 3	Check 2	Check 1
Rows	2	2	5	2	2	4	2	2	2
DSI (Weighted)	1	1.33	1.36	1.79	3.7	4.85	5.3	5.9	6.95
Dunnet´s 5%						***	***	***	***

Note: Lines and events different from SMF3 are indicated with ***

the asterisk (*) the weighted DSI, and the segment line the median value. The difference between the weighted and non-weighted average score in some of the inbred events is due to the variability in the number of plants. Transgenic oxox inbred lines showed resistance levels similar to the non-transgenic moderately resistant check lines. Table 3 shows the weighted DSI ordered according to the level head rot resistance. Events and check lines are compared against SMF3 using Dunnet´s 5% significance test. The high incidence of the disease on the susceptible check lines indicated a successful inoculation with few or no escapes. Severe expression of the disease on the susceptible checks.showed that environmental conditions were appropriate.

Hybrid Experiments

Experiment 1. Table 4 shows the ANOVA for the Hybrid Experiment 1, where 9 transgenic hybrids and six check hybrids were tested. The analysis is structured as a factorial experiment with two factors, where each factor corresponds to the type of parent involved in the hybrid construction: 1) Non-transgenic parent and 2) Transgenic event parent (including the no-gene event SMF3). Since not all levels of the first factor (Non-transgenic parent) are crossed with TF28 the experiment is considered as an incomplete factorial. The analysis indicates: a) gene effects: at least one transgenic event has a DSI value different from SMF3, b) difference in the resistance to head rot among the non-transgenic parents; and c) no interaction between these two

factors. The lack of non-transgenic x transgenic interaction indicates that the event (in an SMF3 background), had similar effects when combined with the different non-transgenic inbreds. It is shown in Figure 2 where the difference in the hybrid responses between SMF3 and its isogenic transgenic lines with various events is almost constant for each non-transgenic parent.

Table 4. Analysis of variance and t-test contrasts for the hybrid experiment 1.

Source	DF	SS (Type III)	F	Pr>F
Non-transgenic parent	5	219.8809	5.34	0.0051
Transgenic parent (& SMF3)	2	199.9413	12.15	0.0007
Non-transgenic x Transgenic	7	28.8947	0.50	0.8191
Block	2	1.8855	0.11	0.8925
Error	15	123.4115		

Hybrids carrying TF34 show a significant ($p < .01$) average DSI increase of 2.3 with respect to hybrids carrying SMF3. There is no statistical evidence of differences between hybrids carrying the two-transgenic events TF28 and TF34.

Experiment 2. Table 5 shows the ANOVA for the hybrid experiment using the selected event TF34. The analysis is structure as a complete factorial experiment with two factors. The first corresponds to four non-transgenic parental check lines and the second to the isogenic lines SMF3 and TF34. Each non-transgenic check line was crossed with the two isogenic lines, SMF3 and TF34. The analysis shows evidence for differences in resistance to head rot, both among the non-transgenic lines and between SMF3 and TF34. There is no evidence of interaction between these two factors indicating similar gene effects in each of the transgenic hybrids. Figures 2 shows the adjusted DSI mean value and its standard error for each hybrid. The parallelism in the response of the isogenic hybrids indicates an additive effect of the oxox gene to the natural genetic resistance of the hybrid.

CONCLUSIONS

Evidence was obtained that the inclusion of transgenic oxox events increased *Sclerotinia* head rot resistance. Inbreds and hybrids carrying the events showed significantly higher resistance to head rot than the non-transgenic isoline SMF3 and corresponding isogenic hybrids. The TF34 events frequently showed the best resistance both in the inbredsand in the hybrids. The resistance level of the best transgenic inbred lines was close to the levels of the moderately resistant checks. This implies an increase on the DSI score, of the transgenic inbred line in relationship to SMF3, of almost 4 points in the 1 to 9 scale used to measure susceptibility. The transgenic hybrids, particularly those from TF34, raised resistance levels more than 2 points compared with the isogenic hybrids, independently of the non-transgenic line genotype. This shows that it is possible to increase *Sclerotinia* head rot resistance by using the transgenic events described here, even with genotypes possessing high levels of natural resistance. Thus, it should be possible to combine these transgenic events with natural resistance to provide a higher level of *Sclerotinia* protection than is currently available in commercial hybrids.

Table 5. Analysis of variance for hybrid experiment 2.

Source	DF	SS (Type III)	F	Pr>F
Column	4	153.78	4.94	0.0058
Row	7	66.61	1.22	0.3346
Non-transgenic parent	3	417.63	17.87	0.0000
Transgenic parent (SMF3 vs. TF34)	1	812.64	104.35	0.0000
Non-transgenic x Transgenic	3	1.68	0.07	0.9743
Error	21	163.55		

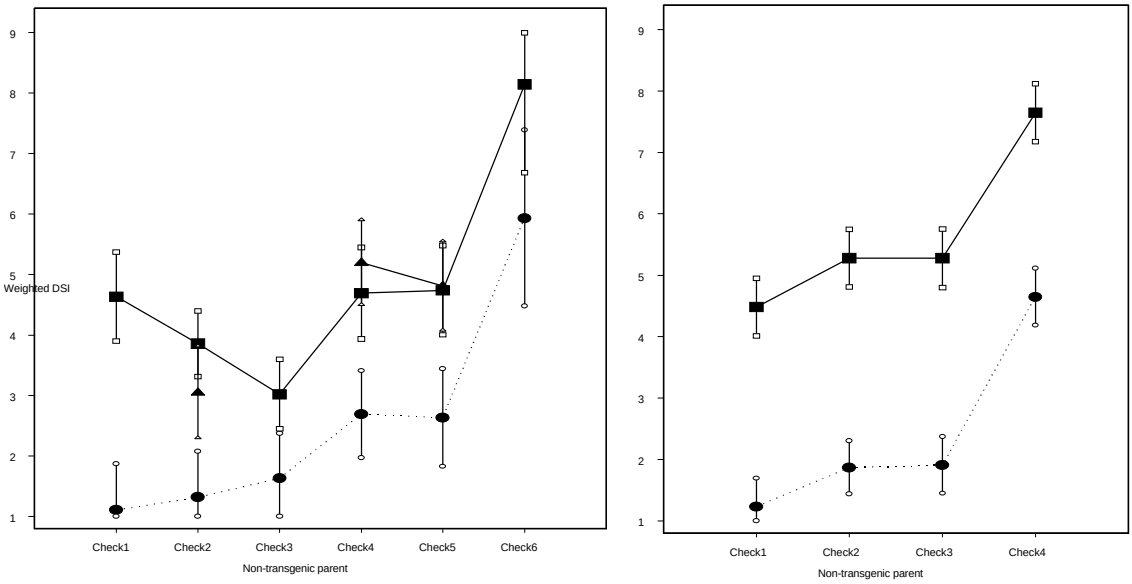

Figure 2. Hybrids weighted DSI and standard error – Experiments 1 (left) and 2 (right)

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