TRANSGENIC *SCLEROTINIA* RESISTANCE IN SUNFLOWER (*Helianthus annuus*, L.)

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Abstract

The wheat oxalate oxidase gene was isolated from a Pioneer line and incorporated into a transformation cassette for constitutive plant expression consisting of a proprietary promoter (SCP1) and the potato proteinase inhibitor (PINII) 3' region. Binary plasmid, pPHP10335, containing this version of the gene (oxox) was used for sunflower transformation. An intact meristem transformation system was used to produce transgenic sunflower and no selectable marker gene was necessary. The enzymatic assay for the oxox gene product served to allow the identification and recovery of T0 transgenic plants. Enzyme assays, ELISA analysis, and Southern analysis confirmed the presence of oxox in T1 transgenic plants. *Sclerotinia* bioassays developed for all three phases of the disease in sunflower, root rot, mid-stalk rot, and head rot, were used to test PHP10335 transgenic events. Presence of the oxox gene reduced the reproductive success of Sclerotinia in some transgenic lines as measured in the mid-stalk bioassay by quantitation of sclerotial bodies. Some transgenic lines that showed resistance in the mid-stalk bioassay also showed reduced infection of the floral surface in the head rot bioassay and increased numbers of wilt resistant individuals in the root rot bioassay.

Introduction

Sclerotinia sclerotiorum is one of the most successful fungal plant pathogens in the world, infecting primarily dicotyledonous plant species. No sunflower germplasm has complete genetic resistance to *Sclerotinia* and chemical control of the disease is not practical. Oxalic acid (OA) has been identified as a key component of the infection process. A strategy for resistance is to obtain plants that are resistant to free OA by engineering them to degrade it. We isolated genes of this type and worked to express them in transgenic sunflower. Numerous transgenic lines were produced for evaluation in *Sclerotinia* bioassays. Greenhouse and field trials of OA degrading plants show that effective resistance can be obtained in this way. Our goal is to produce transgenic lines in the numbers needed to rigorously evaluate constructs and to determine the potential for integration into hybrids. Evaluation of some transgenic lines is presented.

Methods

Disarmed *Agrobacterium tumefaciens* strain EHA105 was used in all transformation experiments. Binary vector pPHP10335, based on pBin19 was introduced into EHA105 using a freeze-thaw trans-formation method. Bacteria for plant transformation experiments were grown overnight in liquid suspension cultures using YEP medium with the appropriate antibiotics required for binary plasmid maintenance. Binary plasmid PHP10335 (Figure 1) contains the wheat oxalate oxidase gene (described in WO 99/04013, 1998) isolated from Pioneer wheat variety 2548 (PVP# 8900112). The gene has one amino acid substitution (data not shown) relative to the predicted sequence of wheat germin sequence gf2.8. The oxalate oxidase gene was combined with a Pioneer proprietary constitutive promoter called SCP1, the omega prime mRNA leader sequence, and the potato proteinase inhibitor (PINII) 3' region. It was inserted between the T-DNA borders of pBin19 and introduced into *Agrobacterium* for transformation.

Sunflower transformation was done using sunflower line SMF3 and a modified meristem culture protocol. The modifications involve secondary culture of nodal meristems excised from transformation sectors (WO 98/ 51806). Transgenic shoots (T0) were identified by oxalate oxidase enzyme assay (Suigura, *et al*., 1979) or by ELISA analysis. No antibiotic selectable marker gene was used. T1 seed were germinated and the resulting greenhouse-grown plants screened by these same assays. In addition, samples from T1 plants were collected for Southern analysis. T2 seed from oxalate oxidase positive T1 plants were used for the initial *Sclerotinia* bioassay. *Sclerotinia* bioassays used artificial inoculations. Protocols were developed for simulation of the basal-stalk or root, mid-stalk, and head phases of the disease. Transgenic sunflower populations inoculated for the basal-stalk rot assay were scored for wilt symptoms. The mid-stalk bioassay was scored by observing lesion size and type during infection, and then by the number and mass of sclerotia in the stalk at maturity. The head rot bioassay was scored as the percent of the head diameter showing damage or covered by fungal mycelium.

Results

Transformation- Transgenic event production for 10 experiments is given in Table 1. Many transformation experiments were initiated to recover a population of transgenic lines. This facilitated determination of the range of gene activity that could be obtained using both protein measures and bioassay performance. Early work with the sunflower meristem transformation system showed that transgenic sunflower shoots could be obtained in the absence of selectable marker genes such as NPTII. Instead, a visual marker or any robust molecular assay for a gene of interest can be used to identify positive T0 shoots. The oxalate oxidase enzyme assay or ELISA was used to identify PHP10335 transformants. Plant meristem transformation methods yield chimeric T0 plants that may have zero, a few, or many T1 transgenic seed. Table 1 shows

the number of T1 transgenic lines that were recovered from these experiments. Some of the T1 losses were due to germination problems and recovery and not chimeric plants with no progeny.

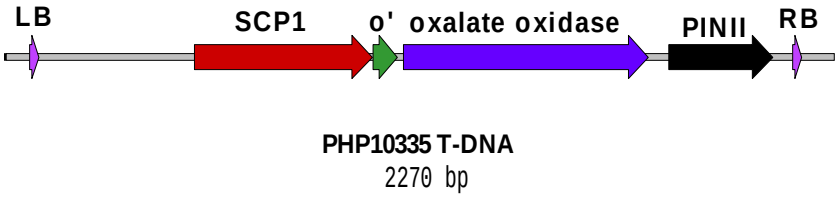

Figure 1. Map of the T-DNA region of binary plasmid PHP10335 based on pBin 19. LB, left T-DNA border; SCP1, constitutive promoter; o', omega prime; PINII, 3' region; RB, right T-DNA border.

Table 1. Results of sunflower transformation experiments using meristem explants and *Agrobacterium tumefaciens* strain EHA105/pPHP10335.

Experiment	Total Explants (No.)	T0 Events (No.)	T1 Events (No.)	Event Codes	Bioassay Events
1	126	1	0	none	None
2	162	4	0	none	None
3	90	4	3	A, B, C	A, C
4	70	4	1	D	D
5	77	8	5	E, F, G, H, I	F, G, H, I
6	120	5	3	J, K, L	J, K, L
7	96	3	1	M	M
8	515	5	1	N	N
9	392	3	2	O, P	P
10	424	7	5	Q, R, S, T, U	Q
Total	2072	46	21	-	-
Frequency	NA	(46/2072) 2.2%	(21/46) 46%	NA	NA

T1 seed populations were germinated and leaves of young plants sampled for oxalate oxidase enzyme assay and ELISA. Final confirmation of stable transgene integration in enzyme active, ELISA positive-T1 plants was done by sampling for Southern analysis at a later stage of development. ELISA analysis was used as an indication of the total activity of the SCP1 promoter-driven wheat gene and the enzyme assay was used to measure the accumulated enzyme activity. Enzyme activity and ELISA results generally correlated. This was not always the case between enzyme activity and bioassay performance, however, probably due to the variable expression phenotypes between transgenic lines. In order to produce seed for *Sclerotinia* bioassay, the confirmed transgenic T1 plants were grown to flowering and selfed.

Bioassay - Events were first tested in Woodland, CA by using the *Sclerotinia* mid-stalk bioassay. Plants were grown to flowering and three petioles of each plant were inoculated with fungal mycelium. The spread of the disease as it progressed down the petiole to the stem and then into the stem was recorded. Lesion size and type were scored on live plants. Following dry down, stems of inoculated plants were split to collect and count sclerotia (Figure 2). Disease lesions spread rapidly on the petiole of all infected lines but differences were pronounced when the lesion reached the stem. The stem lesions of non-transgenic and control lines elongated steadily and girdled the stem, often killing the plant. *Sclerotinia* resistant transgenic lines stopped the progression of the stem lesions quite quickly and showed a dark ring at the lesion border. Many sclerotia were formed in the stalk of plants that did not have resistance. Table 2 shows the results of 3 separate mid-stalk bioassays run to characterize resistance levels of some PHP10335 transgenic events.

Table 2. *Sclerotinia* mid-stalk bioassay of T2 PHP10335 transgenic sunflower. The first assays were performed in greenhouse at Woodland, CA.

Bioassay	Event/Controls (genetic status)	OxOx ELISA	OxOx ELISA Range (ppm)	Sclerotial Body Number (no. Ave.)	Sclerotial BodyWeight (g ave.)	Lesion Type[1] (ave.)
Summer, 1997	C (T2 heterozy.)	7/17 positive	30 – 600	77	3.0 g	2
		10/17 negative	0	80	2.9	2
	J (T2 homozy.)	15/15 positive	>1000	16	0.3	4
	SCL susceptible	5/5 negative	0	120	3.3	1
	SMF3	12/12 negative	0	64	2.0	3
Spring, 1998	A (T2 heterozy.)	19/12 positive	290 - 640	142	5.4	2
		3/12 negative	0	101	5.5	2
	D (T2 homozy.)	5/5 positive	8100 - 17600	78	2.1	2
	F (T2 homozy.)	6/6 positive	280 - 1000	101	5.3	2
	G (T2 homozy.)	6/6 positive	2500 - 14400	55	1.7	2
	G (T2 heterozy)	5/6 positive	850 - 1250	99	4.3	2
		1/6 negative	0	119	6.5	2
	H (T2 heteroz.)	12/13 positive	280 - 6800	85	3.4	2
		1/13 negative	0	122	6.5	2
	I (T2 homozy.)	10/10 positive	690 - 3600	122	4.1	2
	K (T2 heteroz.)	5/7 positive	5000 - 8000	108	3.5	2
		2/7 negative	0	104	3.1	2
	L (T2 heterozy.)	18/21 positive	1300 - 27300	67	1.9	2
		3/21 negative	0	137	5.7	1
	M (T2 heteroz.)	4/5	11700 – 18600	37	1.1	2
		1/5	0	69	2.1	1
	SMF3	11/11 negative	0	105	6.0	2
Summer, 1999	J6 (T4 homozy.)	8/8 positive	nd	0	0	7
	N (T2 mixed)	23/29 positive	nd	6	0.2	7
		6/29 negative	nd	86	1.6	3
	P (T2 heterozy.)	26/32 positive	nd	2	0.1	7
		6/32 negative	nd	19	0.6	6
	Q (T2 mixed)	32/40 positive	nd	1	0	7
		8/40 negative	nd	50	1.0	5
	SMF3	16/16 negative	nd	41	1.0	4

Lesion type 1: susceptible to Sclerotinia, 9 : resistant. na - not applicable; nd - not determined.

T1 transgenic plants can be heterozygous or homozygous and can show variable resistance depending on the transgene copy numbers. An example is event G in Table 2 that showed higher oxalate oxidase protein levels and better bioassay performance in the homozygous state. The lesion type data of the Spring 1998 mid-stalk bioassay did not show differences between any of the lines tested due to infection severity. The mid-stalk bioassay is a severe disease test relative to natural infection and can be variable. Check lines were used to monitor severity, as well as to provide a comparison for transgenic performance. The sclerotia data of the spring 1998 assay showed that some transgenic lines have reduced fungal infection.

Events that showed strong performance in the mid-stalk bioassay were tested in the root-rot and head-rot bioassays. The head-rot bioassay was made with ascospores from cultured *Sclerotinia* apothecia which were suspended in liquid medium at a defined concentration. A uniform volume of the suspension was then sprayed on to sunflower heads in the field. The humidity level on plants in the field was maintained so that disease progression was not hindered. Disease scores for head rot tests in Argentina and France are shown in Figure 3. Transgenic event J performed better than the SMF3 non-transgenic control lines in each

bioassay. Resistance to head-rot was also significant for events D, G, and K. The SMF3 line exhibited Sclerotinia tolerance, especially for mid-stalk and head-rot. The Argentine assay was so severe that Sclerotinia tolerant checks (HT1, 2) were nearly completely destroyed. When there was not 100% disease, transgenic lines ranked similarly to controls. Root-rot bioassays were made by mixing actively growing *Sclerotinia* mycelium into the soil at the base of sunflower plants at stage R3 to R4. Plants were then scored for wilt symptoms. Plants that did not wilt developed normal flowers and set normal quantities of seed. Figure 4 shows the performance of populations of transgenic and non-transgenic control plants. Transgenic lines D and J showed relatively strong resistance for mid-stalk and head-rot and were the most resistant to root-rot.

Conclusions

Constitutive expression of the wheat oxalate oxidase gene in sunflower produced an easily assayable enzymatic activity in leaves. The oxalate oxidase assay in combination with the sunflower meristem transformation system can be used to produce transgenic plants that do not contain antibiotic-resistance selectable marker genes. Twenty-one independent PHP10335 transgenic lines were produced from 10 experiments as determined by molecular analyses of T1 plants including enzyme assays, ELISA analysis, and Southern analysis. The lines contained from 1 to >5 molecular integrations. They showed varying levels of protein accumulation and *Sclerotinia* resistance measured by mid-stalk bioassay. A subset of the events showed significantly enhanced resistance in the mid-stalk bioassay relative to the non-transgenic sunflower line used for transformation and to susceptible and tolerant check lines. A greenhouse mid-stalk bioassay can be used year round to select transgenic lines that should be advanced into field root-rot and head-rot bioassays. The lines with the best mid-stalk resistance showed good resistance to root-rot and head-rot in the field tests. Capability to produce many transgenic lines and bioassays for all 3 phases of *Sclerotinia* disease allow the selection of the lines with whole plant resistance. These can then be introgressed into elite lines and hybrids.

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References

Bevan, M. 1984. Binary *Agrobacterium* vectors for plant transformation. Nucl. Acids Res. 12, 8711-8721.

Holsters M., de Waele D., Depicker A., Messeus E., Van Montagu M., Schell J. 1978. Transfection and transformation of Agrobacterium tumefaciens. Mol. Gen. Genet. 163: 181-187.

Altier D., Bidney D., Coughlan S., Falak I., Yalpani N., Lu G., Mancl M., Nazarian K., Scelonge C. 1998. The induction of stress related factors in plants. WO 99/04013.

Lane B., Dunwell J., Ray J., Schmitt M., Cuming A. 1993. Germin, a protein marker of early plant development, is an oxalate oxidase. J. Biol. Chem. 268: 12239-12242.

Bowen B., Bruce W., Lu G., Sims L., Tagliani L. 1999. Synthetic promoters. WO 99/43838.

Gallie R., Sleat D., Watts J., Turner P., Wilson T. 1987. The 5' leader sequence of tobacco mosaic virus RNA enhances the expression of foreign gene transcripts *in vitro* and *in vivo*. Nucleic Acids Res. 15: 3257 - 3273.

An G., Mitra A., Choi H., Costa M., An K., Thornburg R., Ryan C. 1989. Functional analysis of the 3' control region of the potato wound-inducible proteinase inhibitor II gene. Plant Cell 1: 115-122.

Burrus, M., Chanabe C., Alibert, G., Bidney D. 1991. Regeneration of fertile plants from protoplasts of sunflower (*Helianthus annuus* L.). Plant Cell Rep. 10: 161-166.

Bidney D., Scelonge C., Martich J., Burrus M., Sims L., Huffman G. 1992. Microprojectile bombardment of plant tissues increases transformation frequency by *Agrobacterium tumefaciens*. Plant Mol. Biol. 18: 301-313.

Bidney D., Scelonge C., Wang L. 1998. Recovery of transformed plants without selectable markers by nodal culture and enrichment of transgenic sectors. WO 98/51806.

Suigura, et al., Chem. Pharm. Bull., 27(9): 2003-2007 (1979)