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GENETIC TRANSFORMATION AND HYBRIDIZATION

Transformation and segregation of GFP fluorescence and glyphosate resistance in horseweed (*Conyza canadensis*) hybrids

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Abstract The goal of this research was to generate a breeding population of horseweed segregating for glyphosate resistance. In order to generate a marker to select between hybrids of glyphosate resistant (GR) and glyphosate susceptible (GS) horseweed, a GR horseweed accession from western Tennessee was transformed with a green fluorescent protein (GFP) transgene. The GFP marker allowed for the simple and accurate determination of GR hybrid plants by visual observation. GR plants were shown to be transgenic via the green fluorescence under UV light, and resistant to glyphosate when sprayed with the field-use-rate 0.84 kg acid equivalent ha^{-1} of glyphosate (i.e. Roundup^{TM}) herbicide. An in vitro screen for glyphosate resistance in seedlings was developed, and a 5 μM glyphosate concentration was found to reduce dry weight in GS seedlings but not in GR seedlings. The GR plants containing GFP were then hand-crossed with GS plants from eastern Tennessee under greenhouse conditions, with GS plants acting as the pollen acceptor. Resulting seed was collected and germinated for GFP fluorescence screening. Seedlings that exhibited the transgenic GFP phenotype were selected as F_1 hybrids between GR and GS horseweed. Thirty GS × GR hybrids were produced on the basis of a green-fluorescent GFP phenotype of GR plants. GS × GFP/GR F_1 hybrids produced F_2 seeds, and F_2 plants were shown to segregate for GFP fluorescence and glyphosate resistance independently. Both traits segregated at a Mendelian 3:1 ratio, indicating a single gene is responsible for each phenotype.

Keywords Herbicide resistance · Hybridization · Weeds · Transgenic · Tissue culture · In vitro assay

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Introduction

Herbicide resistant transgenic crops have been widely adopted in the United States during the past 10 years. The United States Department of Agriculture estimated that herbicide-resistant acreage (alone or in insect-resistant stacks represented 87% of all transgenic soybean grown in the United States, 81% of cotton and 26% of corn in 2005 (NASS 2005). The most widely utilized herbicide resistant trait is the Roundup Ready^{TM} trait for glyphosate tolerance. It is not unusual for farmers to follow glyphosate-tolerant soybean with glyphosate-tolerant cotton or corn and to use glyphosate as the only herbicide for weed control. The multi-year, multi-season use of glyphosate has been associated with the occurrence of glyphosate resistant weed species (Koger et al. 2004; Lee and Ngim 2000; Perez and Kogan 2003). Horseweed (*Conyza canadensis*) has become a more problematic weed in the United States because of the emergence

of glyphosate resistant populations, its adaptability to no-till production practices (Brown and Whitwell 1988), fecundity (Vencill and Banks 1994; Weaver 2001), and plasticity in growth and reproduction (Regeher and Bazzaz 1979).

The evolution of herbicide resistance might be the most important trait for increased weediness. That said, after 30 years of extensive glyphosate use throughout the world, only four species have been confirmed to have evolved resistance to this herbicide. The first species was rigid ryegrass (*Lolium rigidum*) in Australia (Powles et al. 1998; Pratley et al. 1999), followed by goosegrass (*Eleusine indica*) in Malaysia (Lee and Ngim 2000), horseweed in the United States (VanGessel 2001), and finally Italian ryegrass in Chile (Perez and Kogan 2003) and elsewhere. There are reports of other important weeds recently evolving glyphosate tolerance, but data has not yet appeared in peer-reviewed literature (see www.weedscience.org for listings). The evidence points to glyposphate tolerance as a growing problem. Large numbers (half million acres) of glyphosate tolerant horseweed were found on cropland in western Tennessee for the first time in 2002, and subsequent research has documented further spread into surrounding states (Main et al. 2004). In 2004 there was an estimated 2 million acres of cropland with glyphosate-resistant horseweed in Tennessee alone.

Growing herbicide-tolerant crops have environmental benefits, namely the increased practice of no-till and reduced tillage agriculture (Carpenter et al. 2002). For example, no- and reduced-tillage farming practices reduced erosion of western Tennessee loess soil deposits by up to 90% (NRCS 2000). Conservation tillage could be the largest environmental benefit of Roundup Ready™ crops, one not to be taken lightly when considering the risks of the development and spread of glyphosate-resistant weeds. The large benefits associated with glyphosate use actually compel us to understand risks associated with overuse of glyphosate. Elucidation of the molecular mechanisms conferring resistance to glyphosate in weedy species would aid in the more accurate assessment of risk of glyphosate use.

The overall goal of our research is to develop a model to describe and elucidate non-target site glyphosate resistance. As first steps, here we describe the genetic transformation of resistant horseweed with a green fluorescent protein marker, the development of an in vitro screen for glyphosate resistance and the generation of a breeding population segregating for glyphosate resistance.

Materials and methods

Plants

Two single plant-decent horseweed accessions from Tennessee were used in this study. A resistant accession from western Tennessee (35.65N latitude 89.51W longitude) and a susceptible accession from eastern Tennessee (35.97N latitude 83.85W longitude) were used as the starting parental lines for all research (Mueller et al. 2003). The resistant accession was shown to have high levels of resistance (84% and 69% survivorship) to one to four times field rate of glyphosate (0.84 and 3.36 kg ae/ha) (Mueller et al. 2003; Main et al. 2004). The susceptible accession was shown to be highly susceptible (1% survivorship) at both rates (Main et al. 2004).

Transformation and plant regeneration

Seeds from glyphosate-resistant horseweed were surface sterilized (10% commercial chlorine bleach and 0.001% Tween 20 solution) for 2.5 min. Seeds were washed with 70% ethanol for 1 min, then rinsed with sterile water three times and allowed to air dry. Sterilized seeds were germinated on Murashige and Skoog (MS) basal media (Murashige and Skoog 1962). All plant media used 0.2% Gelrite gellan gum (Sigma, St. Louis, MO) as a solidifying agent. All components were autoclaved prior to pouring the media, with the exception of antibiotics that were filter-sterilized and added post-autoclaving. Leaves from GR horseweed at the 8- to 12-leaf stage were cut into 1 cm^2 sections, which served as the explant material for transformation. Explants were co-incubated with *Agrobacterium tumefaciens* strain GV3850 harboring the pBIN–mGFP5–ER plasmid (Haseloff et al. 1997; Harper et al. 1999) that featured a cauliflower mosaic virus 35S promoter 5′ to a green fluorescence protein (mGFP5–ER) reporter gene and nos terminator. A kanamycin selection cassette was also present within the T-DNA borders. mGFP5-ER has dual excitation peaks in the UV and blue spectra and is accumulated in the endoplasmic reticulum. Explants were placed on DBI medium (DeVerna and Collins 1984) containing 1 mg/L of indole acetic acid and 2 mg/L kinetin in 100 mm Petri dishes for 3 days to initiate shoot organogenesis. Explants were moved to DBI with antibiotics (200 mg/L kanamycin (Sigma) and 400 mg/L timentin (GlaxoSmithKline, Research Triangle Park, NC) to select for transformed horseweed cells and against *Agrobacterium* growth. GFP fluorescence was monitored in callus tissue, and GFP callus was preferentially selected during tissue culture using either a 150 W UV spotlight or epifluorescence microscopy (Stewart 2001). A subset of the horseweed explants were not co-incubated with *Agrobacterium*, and went through the same tissue culture and regeneration system without any antibiotics for selection. GFP expression in plant tissue was visualized by an epifluorescence microscope (SZX12 Research Stereo Microscope, Olympus, Tokyo, Japan) under blue light from a FITC filter set. Explants were photographed on Petri dishes using a digital camera (Olympus Q color 3 with Q Capture software).

Explants were transferred to fresh media every 14 days until shoots were excised. Developing shoots were excised from green-fluorescent callus and placed on MS medium plus antibiotics supplemented with 2 mg/L IAA to induce rooting. Multiple shoots cut from the same explant were considered to be clones of the same transgenic event, and were labeled with the same event designation (example: two shoots excised from Event 1 were designated shoots Event 1-1 and Event 1-2). Rooted shoots were transferred to soil-less potting media, hardened off, and moved to the greenhouse and allowed to set seed.

GFP fluorescence and glyphosate resistance screening of T_1 plants

Transgenic (T_1 generation) horseweed plants (Events 1 and 2) were grown in greenhouse. Sixty-eight individuals were randomly selected from lines (T_1 generation) 1-1, 1-3, 1-4, 1-5, 1-6, 2-1, 2-2, 2-3, 2-4, 2-6, and non-transgenic-resistant and non-transgenic-susceptible plants for growth and analysis. All plants were grown under an 8/16 h photoperiod and 26/20°C thermoperiod.

Fluorescence spectrophotometry

A Fluoromax-2 fluorescence spectrophotometer (Jobin Yvon & Glen Spectra, Edison, NJ) using DataMax and GRAMS/386 software (Galactic Industries Corporation, Salem, NH) was used to quantify GFP fluorescence in leaf tissue of all T_1 plants. Fluorescence measurements were taken on 10 non-transgenic plants as negative controls. The oldest non-senescing leaf at the six- to eight-leaf stage was excited at 385 nm, and emission spectra was scanned and recorded from 420 to 600 nm. A fiber optic cable provided the excitation light to leaf tissue in vivo, and then collected the emission spectra (fluorescence) with no damage to the leaves. Intensity was measured at 508 nm (green light) in counts per second (cps). The 450 nm wavelength, outside the GFP fluorescence spectrum, was set as the anchor for each sample for signal standardization controlling for probe angle (Richards et al. 2003).

Glyphosate assay on plants

T_1 horseweed plants at the 8- to 12-leaf stage were sprayed with Roundup Ultra Max™ (Monsanto Corporation, St Louis, MO, 480 g ae/L of the isopropylamine salt containing surfactants) at the typical field rate (0.84 kg ae/ha). Two weeks post-application, mortality was recorded by visual assay. Plants with green apical meristems were categorized as alive, and plants with necrotic meristems were determined to be dead. The resistance ratio (alive/dead) was calculated per plant line.

In vitro assay for glyphosate resistance

Horseweed seeds from confirmed resistant and susceptible horseweed accessions from Tennessee were grown on media containing a range of glyphosate concentrations in order to develop an in vitro resistance screen for seedlings. MS was used as the basal media in 100 mm Petri plates, and glyphosate was filter sterilized and added to the media post-autoclaving. Accord™ (Dow AgroSciences Corporation, glyphosate, 360 g ae/L of the isopropylamine salt containing no surfactants) was used as the glyphosate source since preliminary studies implicated that surfactants could confound results. Glyphosate was added to the media at an experimental range of 0–10 μM (at 2.5 μM intervals). For each experiment, seeds from both resistant and susceptible plants were surface sterilized as above. For each concentration, three replicate plates were used with 16 seeds per plate (48 total individuals per concentration $\times$ 2 plant types). Effect of glyphosate on plants was determined by analysis of dry weight after germination and growth on glyphosate media for 14 days. Dry weight was determined by removing the plants from each plate and drying them in a 55°C oven for 48 h. Dry weight per plant was calculated and significant differences ($p<0.05$) between resistant and susceptible plants at different glyphosate concentrations were reported on the basis of rank transformed data (SAS, Cary, NC).

Hybridization between glyphosate susceptible and resistant GFP-transgenic horseweed

The GFP fluorescent lines (Events 2 and 3) were each crossed with GS plants, and GFP fluorescence was used to select for F_1 hybrids. Flowering greenhouse-grown horseweed plants were used for hybridization experiments. T_0 GFP plants (Event 2-2, Event 2-3, and Event 3-1) were used as pollen donors, and GS plants were used as the pollen acceptors. Hybridization was performed by removing a flower from the paternal plant line and rubbing its anthesizing disk flowers against the female ray flowers (Yamasue et al. 1992). The seeds (achenes) were germinated, screened for GFP expression (expected for hybrids) at the two-leaf stage with a hand-held UV light in the dark (Stewart 2001). GFP expressing F_1 hybrids were grown in the greenhouse, and allowed to set seed. F_2 seeds were harvested, and screened for segregation of both GFP fluorescence and glyphosate resistance.

Segregation of GFP fluorescence and glyphosate resistance in F_2 hybrids

F_2 hybrid seeds were germinated from a subset of seeds from each cross, and grown under greenhouse conditions in 18 cell (8 cm^2 per cell) flats. A total of 100 F_2 hybrids were grown from six original F_1 plants from the same population

(GS1 × Event 2-3, GS2 × Event 2-2, GS3 × Event 3-1; GS1, GS2 and GS3 represent three different glyphosate susceptible horseweed lines). Ninety 8 cm × 8 cm cells containing an average of 159 ± 49 plants were screened, totaling 14,310 plants. A total of 32 hybrids were selected on the basis of green-fluorescence. Each F_2 hybrid was screened for GFP fluorescence at the four- to eight-leaf stage, and sprayed at the 8- to 12-leaf stage with Roundup Ultra Max at the field rate. Glyphosate survivorship was determined as above.

Southern blot analysis of F_2 hybrids

Southern blot analysis was performed to assay for transgenicity. Genomic DNA was extracted from young tissue of four horseweed samples: non-transgenic GR, non-transgenic GS, GS1 × Event 2-3 F_2 hybrid, and GS2 × Event 2-2 F_2 hybrid using a CTAB-based procedure (Dellaporta et al. 1983). After digestion with *Hin*dIII, which restricts once within the T-DNA, genomic fragments were purified with QIAquick PCR purification columns (QIAGEN, Valencia, CA) prior to electrophoretic separation on a 1% agarose gel. Fragments were transferred to Zeta-Probe GT membrane (Bio-Rad, Hercules, CA) and probed with PCR products containing the full-length open reading frame of mGFP5er or *C. canadensis* EPSPS1. PCR products were radiolabeled with α-^{32}P dCTP using Prime-It II Random Primers Labeling Kit (Stratagene, La Jolla, CA). Labeled probe was purified using mini Quick Spin DNA columns (Roche Diagnostics, Indianapolis, IN). Southern blots were hybridized with labeled probe overnight at 42°C in ULTRAhyb hybridization buffer (Ambion, Austin, TX) and washed according to manufacturer's protocol. Blots were visualized by exposure to phosphor-imaging screens (Storage Phosphor Screen GP, Eastman Kodak, Rochester, NY) and scanned using Personal FX phosphoimager (Bio-Rad). Image analysis was performed using Quantity One software (Bio-Rad).

Results

Transformation efficiency and plant regeneration

GFP seemed to reliably yield a scorable green-fluorescent trait enabling selection of transgenic tissue and plants (Fig. 1). A total of 242 leaf segment explants were co-incubated with *Agrobacterium*. At 45-day post-incubation 219 explants contained GFP callus sectors, demonstrating 90% transformation efficiency, whereas non-transgenic callus and regenerated plants appeared red under epifluorescence microscopy. Calli were heterogenous for green fluorescence, but segments of callus were homogeneously green-fluorescent, i.e. it appeared that GFP transgenic cells were growing from definite loci (Fig. 1D). Shoot production occurred 45–90 days post-incubation, and six explants produced GFP shoots. The 1.2% transgenic plant efficiency rate was certainly limited by low plant-regeneration rates from transgenic calli. The GFP expressing shoots appeared morphologically normal when visualized under white light (Fig. 1E) and fluoresced green when visualized under epifluorescent conditions (Fig. 1F), in contrast with non-transgenic plants (Fig. 1G & H). The roots of the transgenic plants exhibited the same pattern of green-fluorescence (data not shown). Meristematic regions of intact transgenic plants in pots were green-fluorescent (Fig. 1J), as were flowers (Fig. 1M).

Explants produced numerous shoots from the same GFP callus sectors. Multiple shoot clones per transgenic event were excised, and moved to rooting media. Three transgenic T_0 events produced shoots with roots (Event 1-3). In Events 1 and 2, five clones were rooted from each original callus mass. Plants were grown under greenhouse conditions, and were allowed to produce seeds. Events 1 and 2 produced viable T_1 seeds, whereas Event 3 never produced selfed seeds but were able to produce seeds in a cross.

GFP fluorescence and glyphosate resistance screening

All T_1 lines of Events 1 and 2 and the controls (GS and GR) were sprayed with field-level applications of glyphosate. Approximately 82% plants of Event 1 and 85% plants of Event 2 were found to be resistant to glyphosate injury (Table 1). Eighty-five percent of non-transgenic GR plants were resistant to glyphosate and only 6% of GS plants survived glyphosate treatments.

The average green-fluorescence (at 508 nm) of 340 individuals (mixture of homozygous and hemizygous individuals) of transgenic T_1 horseweed line Event 1 was $433 \pm 33 \times 10^3$ counts of photons per second (cps), whereas the average fluorescence (at 508 nm) of 340 individuals of transgenic T_1 horseweed line Event 2 was $369 \pm 28 \times 10^3$ (Table 1). Non-transgenic, including null segregant plants, had $257 \pm 41 \times 10^3$ cps fluorescence, which represents background fluorescence (Fig. 2).

Hybridization between glyphosate susceptible and tolerant GFP horseweed

Horseweed flowers are composite in nature with yellow disk florets surrounded by ray florets and they produce copious quantities of seeds (ca. 10^5 per plant). The flowers are approximately 5 mm in diameter. Lack of any published crossing technique in horseweed and the extremely small size of the flowers were the greatest impediments in our earlier attempts to produce hybrids, necessitating the development of a facile GFP-marking technique. The result of handcrosses

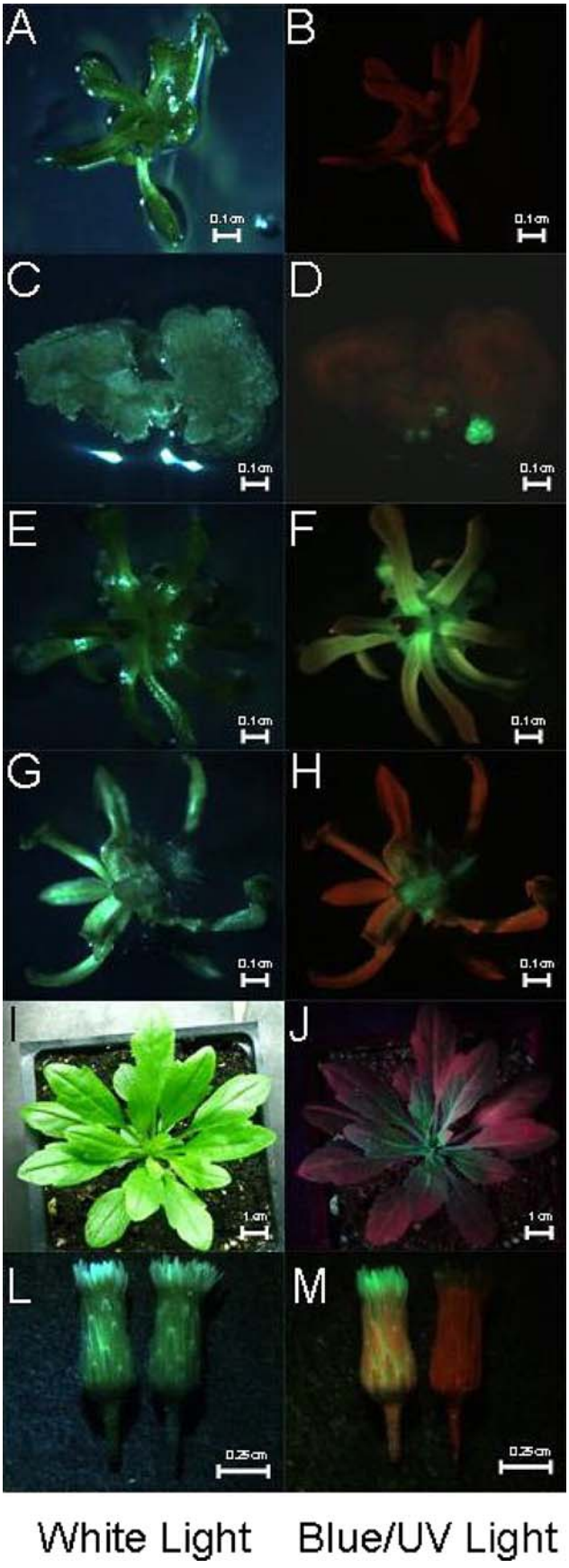

Table 1 Glyphosate resistance in transgenic GFP lines Event 1 and Event 2

	Total plants sprayed	Survived	Resistant (%)
TN-resistant parent	34	29	85.3
TN-susceptible parent	34	2	5.9
Event 1			
1-1	58	53	91.4
1-2	58	45	77.6
1-3	58	53	91.4
1-4	58	27	46.6
1-5	58	53	91.4
1-6	58	53	91.4
Average			81.6 ± 18.0
Event 2			
2-1	58	42	72.4
2-2	58	53	91.4
2-3	58	56	96.6
2-4	58	57	98.3
2-5	58	38	65.5
Average			84.8 ± 14.9

was the production of 34 F_1 hybrids expressing GFP out of thousands of non-transgenic progeny. Hybrids appeared orange under UV light whereas the non-transgenic plants appeared bright red (Fig. 3).

In vitro screen for glyphosate resistance

When grown from seed on media, resistant and sensitive horseweed biotypes responded differently to glyphosate (Fig. 4). At 5 and 7.5 μM glyphosate treatments, difference in mean dry weight between resistant and sensitive plants was significantly different ($p<0.05$), indicating that either of these concentrations are effective in an in vitro screen for resistance at early growth stages.

Segregation of GFP fluorescence and glyphosate resistance in F_2 hybrids

The F_2 population and GS and GR parents (as controls) were sprayed with glyphosate and screened for GFP fluorescence.

◀ **Fig. 1** GFP expression in transgenic horseweed at different stages during tissue culture. **A** and **B** Wild-type meristem demonstrating red fluorescence under blue light; **C** and **D** horseweed callus with a small transformed GFP sector visible under blue light; **E** and **F** transgenic horseweed meristem; **G** and **H** production of roots from a transgenic meristem with the characteristic GFP fluorescence visible in the roots under blue light; **I** and **J** recovered transgenic plant growing in soil-less media under UV light; **L** and **M** transgenic flower (*left*) with a wild-type flower (*right*) with the visible GFP fluorescence in the petals of the transgenic flower. Panels **A**–**H**, and **L** and **M** were produced using an epifluorescence dissecting microscope under white and blue light, and Panels **I** and **H** were taken with a Sony digital camera (macroscopic) under white and UV light

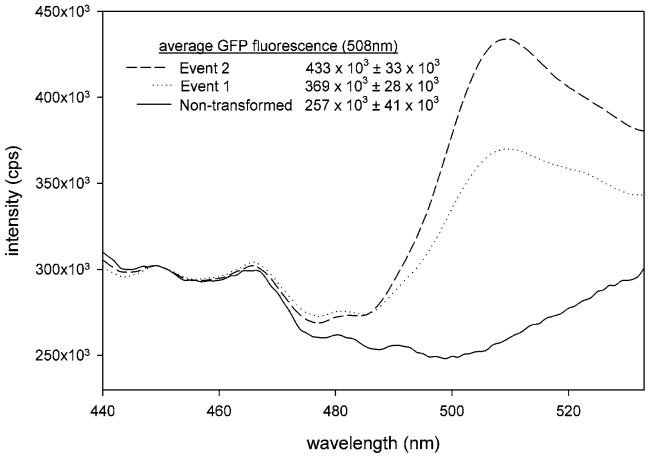

Fig. 2 Standardized fluorescence spectrometrogram of transgenic T_1 horseweed leaves and leaves from a control plant. Excitation wavelength was 385 nm. Means are presented ± standard error

None of the parents showed any GFP fluorescence, as expected (Table 2). All GR plants survived glyphosate spray with many fewer of the GS plants surviving (Table 2). The population developed from the cross GS1 × 2-3 segregated independently for glyphosate resistance following a 3:1 segregation ratios (Table 2). Two lines of this population did not segregate for GFP at the 3:1 ratio, however when all plants in the line were evaluated for GFP segregation, no significant difference from the expected 3:1 ratio was observed. The population developed from the cross GS2 × 2-3 also segregated independently for glyphosate tolerance and GFP traits following 3:1 segregation ratios (Table 2). We observed that the population developed from the cross GS3 × 2-2 segregated at a ratio of 3:1 for the glyphosate resistance trait but two lines of this population did not segregate at a ratio of 3:1 for the GFP fluorescence trait, possibly suggesting a sampling error. Also, the GS2 × 2-3 had a higher than expected survivorship. This could be the result of sampling error or a confounding effect of the 10% survivorship of susceptible individuals in our glyphosate-spraying regime (Table 2).

Table 2 Segregation of F_2 population from the cross TN-susceptible × TN-resistant for GFP and glyphosate resistance traits

	Total plants sprayed	Survived	GFP
TN resistant	119	119	0
TN susceptible	119	16	0
GS1 × 2-3			
Plant 1	119	94, ns	74, s
Plant 2	118	84, ns	89, ns
Plant 3	119	88, ns	74, s
Average		89 ± 5.03, ns	79 ± 8.66, ns
GS2 × 2-3			
Plant 1	119	100, s	96, ns
GS3 × 2-2			
Plant 1	119	96, ns	83, s
Plant 2	119	95, ns	89, ns
Plant 3	119	83, ns	85, s
Average		91 ± 7, ns	86 ± 3, ns

χ^2 test (1 df, $p > 0.05$) was performed on the basis of observed and expected phenotypes. The expected phenotype of the F_2 population is 3:1 Mendelian segregation ratio. Means ± standard error are presented. s: significantly different from the expected observation; ns: not significantly different from the expected observation; GS1, GS2 and GS 3 represent three different susceptible lines of horseweed plants.

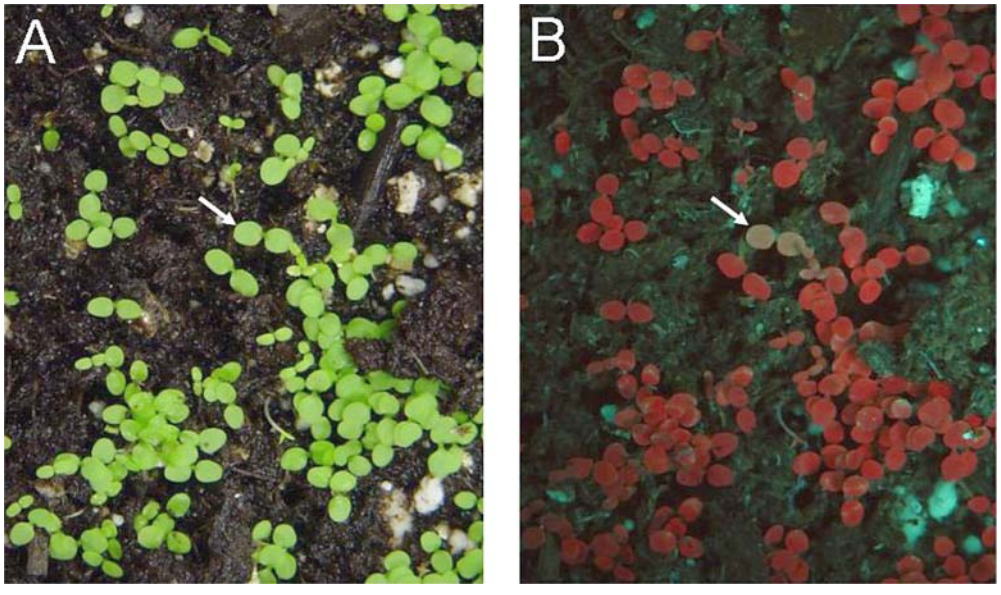

Fig. 3 Screening of F_1 horseweed hybrids for GFP fluorescence. F_1 hybrid horseweed illuminated plantlets with white light **A** or a 150 W U V spotlight **B** to screen for fluorescence. *Arrow* indicates a green-fluorescent plant in the midst of non-transgenic plants

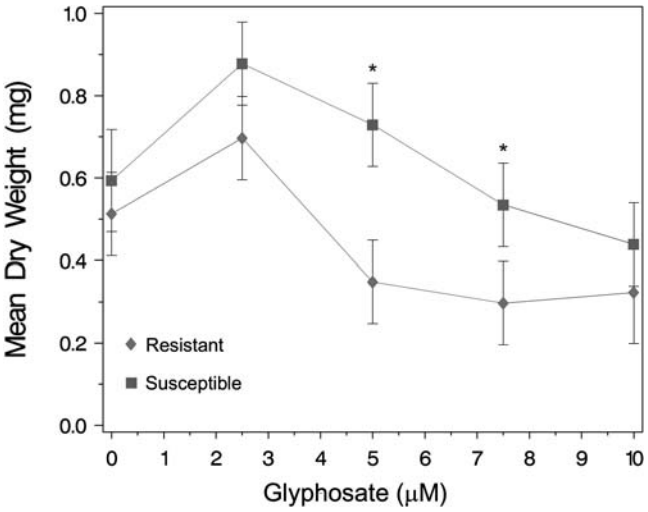

Fig. 4 In vitro screen of glyphosate resistance in sensitive and resistant horseweed at the two-leaf stage. Mean dry weight ± SE of horseweed plants grown on MS media containing glyphosate (0–10 μM), $^{*}p<0.05$

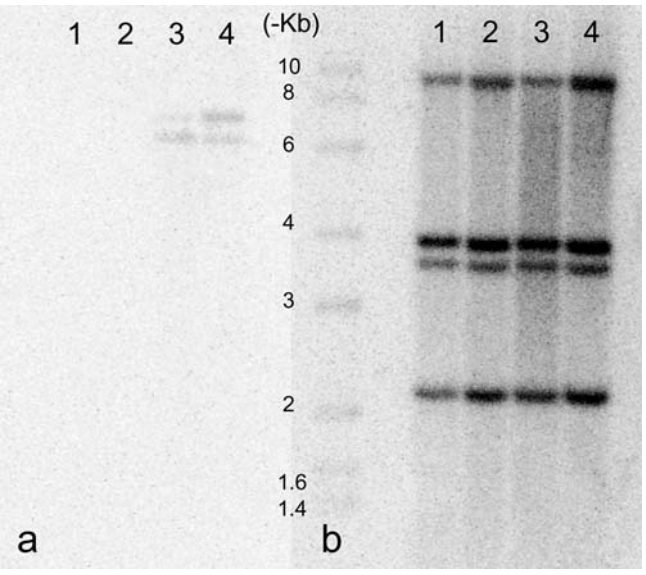

Fig. 5 Southern analysis of horseweed. Southern blot analysis of *Hin*dIII digested genomic DNA hybridized to GFP probe (**a**) or EPSPS1 cDNA probe (**b**). Genomic samples were loaded into the following lanes: 1, non- transgenic GR; 2, non-transgenic GS; 3, GS1 × Event 2-3 F_2 hybrid; 4, GS2 × Event 2-2 F_2 hybrid

Southern blot analysis

Southern analysis of genomic DNA from two F_2 populations derived from the same transgenic event (Event 2) showed identical banding patterns when hybridized with a GFP probe (Fig. 5a). The GFP fragment did not hybridize to untransformed genomic DNA from either susceptible or resistant horseweed parents. Digestion with the enzyme *Hin*dIII restricts the GFP T-DNA fragment 5′ of the 35S promoter; each insertion of the T-DNA into the genome should result in a single hybridizing band. Dual bands observed in GFP F_2 hybrid populations suggest two copies of the transgene were integrated into closely linked loci in the genome. The single locus inference is derived from segregation data noted above.

Multiple hybridizing bands were observed when EPSPS1 (5-enolpyruvylshikimate-3-phosphate synthase) cDNA was used to probe horseweed genomic DNA (Fig. 5b). Southern blot analysis of the EPSPS family in sensitive and resistant biotypes and transgenic hybrids showed no variability when *Hin*dIII was used for restriction analysis (similar to Heck et al. in press). Analysis of publicly available genomic sequence of *Conyza canadensis* EPSPS1 (Genbank accession AY545666) predicts three hybridizing bands, of which one fragment size is known (2.2 kb). The four hybridizing bands observed in Fig. 5b are estimated to be approximately 2.2, 3.6, 3.9 and 9 kb in size. The smallest hybridizing band corresponds to the predicted size of an internal *Hin*dIII fragment of EPSPS1, the source of the fourth hybridizing band is not known. The high stringency of hybridization in the Southern blot analysis reduces but does not rule out DNA hybridization of other EPSPS gene family members to the EPSPS1 probe, especially EPSPS2 (Genbank accession AY545667) (Heck et al. in press).

Discussion

Weed resistance to glyphosate is considered weak relative to evolved resistance to other classes of herbicides, such as those that target photosystem II, tubulin, ALS (acetolactate synthase) or ACCase (acetyl CoA carboxylase). In some instances, resistance to these have been 100-fold greater than the prevalent sensitive biotypes (Gressel 2002). However, the relative weakness of glyphosate resistance could result in major agriculture problems for two reasons. First, glyphosate is widely used, therefore the selection pressure for weed resistance is widespread and sizable (Baucom and Mauricio 2004). Second, the resistance shown (4- to 13-fold normal doses) might allow resistant weeds to survive and reproduce in agricultural fields, leading to a feedback loop of advanced evolution for resistance (Main et al. 2004; Koger et al. 2004; Mueller et al. 2003; VanGessel 2001).

Numerous studies have investigated the physiological mechanisms of glyphosate resistance and the transmission genetics of its inheritance. Goosegrass resistance to glyphosate was caused by a mutation of proline to serine at position 106 in the EPSPS target site (Baerson et al. 2002; Ng et al. 2003). In all other cases, target site mutations have been ruled out as a causal factor. In rigid ryegrass, the observed resistance reduced translocation of glyphosate, suggesting that altered cellular transport may contribute to the potential resistance mechanism (Lorraine-Colwill et al. 2003). In this case, resistance was inherited as an incompletely dominant nuclear-encoded trait with most of the resistance ascribed to a single gene (Lorraine-Colwill et al. 2001). Horseweed

seems to be similar in these regards. Apparent altered cellular distribution that impaired phloem loading and plastidic import of glyphosate was observed in GR horseweed (Feng et al. 2004). EPSPS enzymes from sensitive and resistant horseweed appear to have similar inhibition by glyphosate (Mueller et al. 2003; Feng et al. 2004). Since resistance appears to be at the cellular level and is a non-target mechanism, a genomic approach could be fruitful in elucidating the molecular resistance mechanism (Basu et al. 2004). In addition, data suggest that GR in horseweed, like ryegrass, is the result of a single-locus dominant or semi-dominant trait (Zelaya et al. 2004). Our segregation results are congruent with these findings.

Only one other study has described a tissue culture and transformation system for *C. canadenisis* (Scheiber et al. 2006). In those experiments, transgenic plants were never recovered, but transient transformation was demonstrated as was the establishment of callus cultures and regenerated plants from non-transgenic callus. They used callus as an explant for transformation experiments and reported 13% transformation efficiency (Scheiber et al. 2006), whereas we used leaf disks as explants and observed a 90% transformation efficiency for producing transgenic callus. Different media were used between the two papers (salts, auxins and cytokinins) and Scheiber et al. (2006) included 50 mg/L kanamycin in the media, whereas we used 200 mg/L. We have performed little optimization for selection agents or hormones, thus there is room for improvements in efficiency for the system. While it is difficult to determine the basis of the increased transformation efficiency, in both cases, it was apparent that callus was easy to culture. We experienced very low shooting rates from callus, but did not use giberellic acid in media as did Scheiber et al. (2006), who apparently (rates not reported) had better success recovering plants from callus. Future transformation experiments should focus on optimizing media for recovery of shoots and roots.

Both GFP and GR can be described as semi-dominant traits that can be assayed in a dominant fashion. GFP fluorescence is additive from the hemizygous to homozygous state (Richards et al. 2003) and GR is phenotypically equivalent (Zelaya et al. 2004). Thus, the appearance of green-fluorescence co-segregating with GR in the F_1 hybrids, and independently segregating in the F_2 hybrids is congruent with published findings and lends credence to the scheme of using transgenics to test if candidate GR genes are causal agents for resistance.

From theory and empirical evidence (Baucom and Mauricio 2004; Owen and Zelaya 2005), it is clear that non-target GR is expected to become more prevalent in weed species. As genomic resources are developed for weeds, it will become tractable to perform genome-wide scans to identify non-target resistance mechanisms (Basu et al. 2004). Unlike rational target site mutations, genome-wide scans will only identify candidate genes that must be subsequently overexpressed in a GS plant to demonstrate that it indeed confers resistance. The most appropriate plant would be a GS conspecific that is transgenic for the single gene addition: the rationale for developing *C. canadensis* transformation. It is clear that including a putative candidate GR gene construct genetically linked with GFP should be an appropriate strategy for confirming resistance.

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