Theor Appl Genet (2002) 105:674–679
DOI 10.1007/s00122-002-0931-3

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Transmission of herbicide resistance from a monoecious to a dioecious weedy *Amaranthus* species

Received: 8 August 2001 / Accepted: 27 December 2001 / Published online: 18 May 2002

Abstract The genus *Amaranthus* includes several important monoecious and dioecious weed species, and several populations of these species have developed resistance to herbicides. These species are closely related and two or more species often coexist in agricultural settings. Collectively, these attributes raise the concern that herbicide resistance might transfer from one weedy *Amaranthus* species to another. We performed research to determine if a dominant allele encoding a herbicide-insensitive form of acetolactate synthase (ALS) could be transferred from a monoecious species, *A. hybridus*, to a dioecious species, *A. rudis*. Numerous F_1 hybrids were obtained from controlled crosses in a greenhouse between *A. rudis* and herbicide-resistant *A. hybridus*, and most (85%) of these hybrids were herbicide-resistant. Molecular analysis of the *ALS* gene was used to verify that herbicide-resistant hybrids contained both an *A. rudis* and an *A. hybridus ALS* allele. Although hybrids had greatly reduced fertility, 42 BC_1 plants were obtained by backcrossing 33 hybrids with male *A. rudis*. Fertility was greatly restored in BC_1 progeny, and numerous BC_2 progeny were obtained from a second backcross to *A. rudis*. The herbicide-resistance allele from *A. hybridus* was transmitted to 50% of the BC_1 progeny. The resistance allele was subsequently transmitted to and conferred herbicide resistance in 39 of 110 plants analyzed from four BC_2 families. Parental species, hybrids, and BC_2 progeny were compared for 2C nuclear DNA contents. The mean hybrid 2C nuclear DNA content, 1.27 pg, was equal to the average between *A. rudis* and *A. hybridus*, which had 2C DNA contents of 1.42 and 1.12 pg, respectively. The mean 2C DNA content of BC_2 plants, 1.40 pg, was significantly ($\alpha < 0.01$) less than that of the recurring *A. rudis* parent and indicated that BC_2 plants were not polyploid. This report demonstrates that herbicide resistance can be acquired by *A. rudis* through a hybridization event with *A. hybridus*.

Keywords Acetolactate synthase · *Amaranthus* · Herbicide resistance · Hybridization · Introgression

Communicated by J. Dvorak

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Introduction

Repeated use of a herbicide often results in the development of weed populations resistant to that herbicide. This is a natural-selection process and occurs simply because herbicide-susceptible individuals are controlled, whereas plants with a gene conferring resistance to the herbicide survive and reproduce, thereby increasing the frequency of the resistance gene.

For herbicide-resistance evolution to occur, however, there must be variation for resistance within the population. Typically, this variation arises from spontaneous genetic mutations (Jasieniuk et al. 1996). For example, point mutations in the gene encoding acetolactate synthase (ALS), the target site of sulfonylurea and imidazolinone herbicides, can result in an enzyme with greatly reduced sensitivity to these herbicides (Guttieri et al. 1996). In addition to mutation, a second source of resistance genes in a given population is migration. That is, intraspecific gene flow from a resistant population can initiate resistance development in a population consisting entirely of sensitive genotypes (Jasieniuk et al. 1996). A third source of resistance genes is interspecific gene flow. The recent commercialization of herbicide-resistant crops has heightened awareness of crop-to-weed gene flow (Ellstrand 2001). Interspecific weed-to-weed gene flow is also a potential avenue of herbicide-resistance evolution, in particular for weed species that co-exist with one or more closely related weed species. The significance of weed-to-weed gene flow in herbicide-resistance evolution is virtually unknown, however, as there have been few investigations in this area (Darmency 1996).

The genus *Amaranthus* includes several important weed species, often referred to as pigweeds. Two of these species, *A. hybridus* L. and *A. spinosus* L., have been ranked among the world's 18 worst weeds (Holm et al. 1991), and two others, *A. retroflexus* L. and *A. viridis* L., are among the most widely distributed weeds of the world (Holm et al. 1997). Many pigweed species are capable of producing 100,000 or more seeds per plant (Weaver and McWilliams 1980), and several populations of these species have developed herbicide resistance (Heap 2001). High seed production may be an important factor in herbicide-resistance development in these species, as it provides abundant material on which herbicide selection can act.

Another factor that might contribute to the development of herbicide resistance in weedy *Amaranthus* populations is interspecific gene flow. As early as 1940, Murray reported that pigweed species could cross under experimental conditions to produce hybrids with varying levels of fertility. More recently, Wetzel et al. (1999) obtained fertile progeny from crosses between *A. rudis* Sauer and *A. palmeri* S. Wats. and demonstrated the transfer of a herbicide-resistance gene from the former to the latter. Thus, it is possible that herbicide resistance could evolve in an *A. palmeri* population via hybridization with herbicide-resistant *A. rudis*. The likelihood that these two species would hybridize in nature is relatively high because both are dioecious and, therefore, obligate outcrossing species.

Amaranthus rudis often coexists with monoecious pigweeds. For example, in Illinois and other areas of the midwestern United States, many agronomic fields contain both *A. rudis* and *A. hybridus*. We therefore were interested in determining if transfer of a herbicide-resistance gene could occur between these two species. Herein we present evidence that herbicide resistance could evolve in *A. rudis* via hybridization with herbicide-resistant *A. hybridus*.

Materials and methods

Plant material

Amaranthus rudis and *A. hybridus* were used as the female and male parents, respectively, for the initial cross. *Amaranthus rudis* used in the study came from two separate field collections made in 1992 from Champaign and Fayette counties in the Illinois, USA. Results from preliminary experiments indicated that less than 1% of plants in these two populations contained resistance to ALS-inhibiting herbicides. *Amaranthus hybridus* was obtained from a field collection in 1997 from Edgar County, Illinois. This population had developed resistance to imidazolinone herbicides (Hager et al. 1998) due to a dominant mutation in the gene encoding ALS (unpublished data). The 2*n* chromosome number for both species is typically 32, although 2*n* = 34 is occasionally observed in *A. hybridus* (Murray 1940; Pal et al. 1982).

Crosses

Plants were grown in greenhouses at Urbana, Illinois at day and night temperatures of approximately 28 °C and 22 °C respectively. Natural light was supplemented with sodium vapor lamps to provide a 16/8-h(day/night) photoperiod. Seeds were planted directly in soil mix [1 part each of Drummer silty clay loam (fine-silty, mixed, mesic Typic Haplaquoll), perlite, and peat] or after stratification in moist paper towels at 4 °C for 10–30 days. Plants were started in flats and transplanted into 1.4-l pots when approximately 10–15 cm tall.

When *A. rudis* plants became identifiable as males or females, female plants were moved to a different greenhouse room to be pollinated by *A. hybridus*. Over a period of about 1 week, controlled pollinations were repeatedly performed by positioning the inflorescences of the two species together and releasing pollen by flicking and shaking the *A. hybridus* stems. The female *A. rudis* plants were kept isolated from male *A. rudis* plants until seed harvest. Male *A. rudis* plants were used as the recurring parent for subsequent backcrosses. These were performed by simply isolating female hybrid or BC_1 plants with the recurring parent and allowing pollination to occur.

Evaluation of progeny

Recovered seeds were stratified and planted, and plants were grown as described above. Three procedures (described below) were used to determine if plants obtained from the initial cross and from subsequent backcrosses were hybrids (or of hybrid origin) and whether they inherited herbicide resistance. Pressed and dried samples of confirmed F_1 hybrids and BC_2 plants, as well as plants from the parental populations, were deposited with the Illinois Natural History Survey Herbarium (ILLS).

Herbicide response evaluation

Imazethapyr (Pursuit, BASF) was applied to plants using a compressed air, moving nozzle, laboratory spray chamber as described previously (Foes et al. 1998). Putative hybrid plants were treated with imazethapyr at 210 g acid equivalent ha^{-1} (3× typical field use rate) when 5–10 cm tall and evaluated 2 weeks later. BC_2 progeny were treated with two sequential applications of 140 g imazathpyr ha^{-1}. The first application was applied when plants were 5–10 cm tall, the second application occurred 1 week later, and plants were evaluated 1 week after the second application. Relatively few BC_1 progeny were recovered; therefore, these were not subjected to herbicide treatment. Tissue samples were taken from some plants prior to herbicide treatment to be used for DNA analysis.

Analysis of *ALS* gene

Polymerase chain reactions (PCRs) were performed with alsf1 and alsr1 primers to amplify region A of *ALS* essentially as described by Foes et al. (1998). Samples for the PCRs consisted of a total volume of 25 ul with 20 m*M* Tris-Cl (pH 8), 50 m*M* KCl, 2 m*M* $MgCl_2$, 0.2 m*M* each dNTP, 0.4 u*M* each primer, 1.0 U *Taq* DNA polymerase (Gibco BRL), and 40 ng genomic DNA. The thermoprofile began with 1 min at 95 °C; then 35 cycles of 1 min at 94 °C, 1 min at 57 °C, and 1 min at 72 °C; followed by 5 min at 72 °C. Ten microliters of each resultant PCR was subjected to digestion with 5 U *Eco*RV restriction enzyme (Gibco BRL) with the manufacturer's buffer at 1× in 30-ul reactions. Products were fractionated by electrophoresis on 1.2% agarose gels containing ethidium bromide and photographed atop a UV light box.

DNA content analysis

Nuclear DNA content analysis was performed using flow cytometry and 2-week-old seedlings of maize (*Zea mays* L.) inbred line W22 (5.35 pg/2C nucleus) as a standard (Biradar and Rayburn 1993). Immediately prior to nuclei extraction, 1–2 cm^2 of leaf tis-

sue from *A. hybridus*, *A. rudis*, hybrid, or BC_2 plants were chopped and combined with chopped tissue from a 1-cm-long maize stem segment. Nuclei were isolated and stained essentially as described previously (Rayburn et al. 1989; 1992). Briefly, the chopped tissue was homogenized in extraction buffer and then filtered through 250-µm and 53-µm nylon meshes. Nuclei in the filtrate were pelletized by centrifugation and then resuspended in propidium iodide (PI) stain. After a 20-min incubation at 37 °C, PI salt solution was added, and nuclei were incubated on ice at least 1 h before flow cytometry. Flow cytometry was conducted on a Coulter EPICS XL-MCL 4-color flow cytometer (Beckman Coulter) equipped with an argon ion laser operated at 15 mW, with an excitation wavelength of 488 nm. At least 8,000 nuclei per sample were analyzed.

Results and discussion

Most female *A. rudis* plants, when pollinated by *A. hybridus*, produced several hundred seeds, and the germination of these seeds was comparable to that of parental seeds. When seedlings obtained from the *A. rudis* × *A. hybridus* cross were sprayed with a threefold field rate of imazethapyr, approximately 85% survived. Plants that did not survive may have inherited a herbicide-sensitive *ALS* allele from *A. hybridus* (as this population was still segregating for resistance); they may have been derived from "escape" pollinations by herbicide-sensitive waterhemp; or they may have inherited the *A. hybridus* resistance gene, but it was not expressed. Regardless of why a minority of the progeny obtained from the *A. rudis* × *A. hybridus* cross did not survive herbicide treatment, herbicide resistance in the majority suggested that they were hybrids and that the herbicide-resistance gene from *A. hybridus* could function in the hybrid background.

Further confirmation that the herbicide-resistant progeny were hybrids came from molecular analysis of the *ALS* gene. The portion of *ALS* amplified by PCR (region A, Foes et al. 1998) was polymorphic for an *Eco*RV restriction site between the *A. rudis* and *A. hybridus* parental populations used in this study (Fig. 1). Thus, amplification of *ALS* region A by PCR followed by *Eco*RV digestion allowed us to determine the parental origin of *ALS* alleles in hybrids (Fig. 2). Of the putative hybrid plants (those that survived herbicide treatment) tested, most (more than 95%) contained both *A. rudis* and *A. hybridus ALS* alleles as determined by the *Eco*RV polymorphism and thus were confirmed hybrids.

While most confirmed hybrids were vigorous and appeared healthy, a small percentage had inferior phenotypes and reduced vigor. The vigorous plants had phenotypes that were intermediate between the two parental species. Generally, the hybrids had more branches and leaves than typical of *A. rudis*, and the leaves tended to be thicker and less glossy than those observed for *A. rudis* but narrower than those typical of *A. hybridus*. Also, stems of several hybrids were sparsely pubescent, whereas stems of *A. rudis* and *A. hybridus* are glabrous and densely pubescent, respectively. As observed by Murray (1940) for crosses between dioecious and monoecious species, all of the hybrid plants were dioecious. Hybrids consisted of similar numbers of male and female plants. Males produced few anthers, and those that were observed did not dehisce and appeared to be completely infertile. Female plants produced *A. rudis*-like flowers with visible styles.

Female confirmed hybrids were isolated in a greenhouse with herbicide-sensitive male *A. rudis* for pollination. A total of 242 seeds were obtained from 33 female hybrids. After stratification and planting, 42 of these seeds (17%) germinated and grew. In contrast, germination percentages of similarly aged and stratified seed from the *A. rudis* parent were typically greater than 50%.

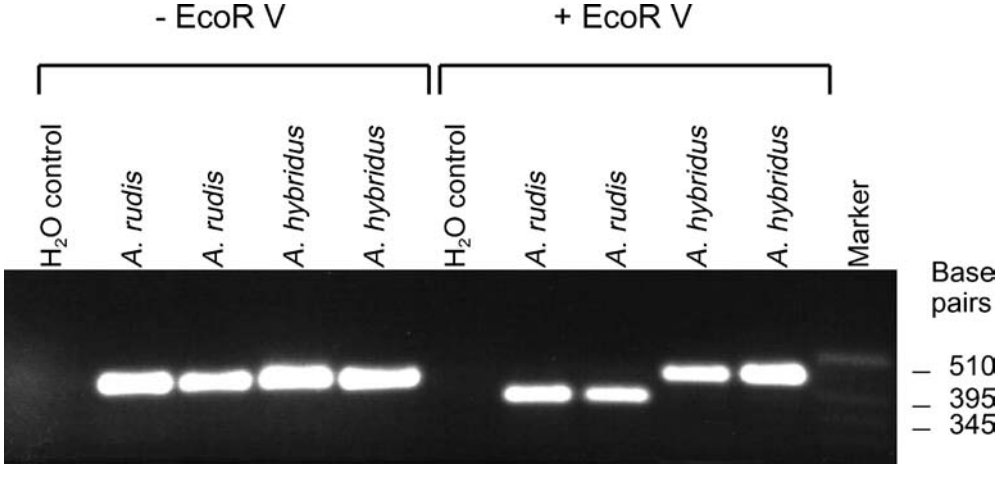

Fig. 1 Distinguishing *Amaranthus rudis* and *A. hybridus ALS* alleles. DNA preparations from two individual plants of each species were used as templates for PCR reactions in which a region spanning 444 bp of *ALS* was amplified. An aliquot of each PCR was digested with *Eco*RV restriction enzyme. Products from both nondigested (*–EcoRV*) and digested (*+EcoRV*) aliquots were fractionated through a 1.2% agarose gel containing ethidium bromide and photographed atop UV light. The amplified region of *ALS* from *A. rudis* contained an *Eco*RV site such that digestion with this enzyme resulted in fragments of 374 bp and 70 bp (the latter not visible). The *A. hybridus ALS* fragment did not contain an *Eco*RV site. Template DNA was not added to the water control PCR reaction

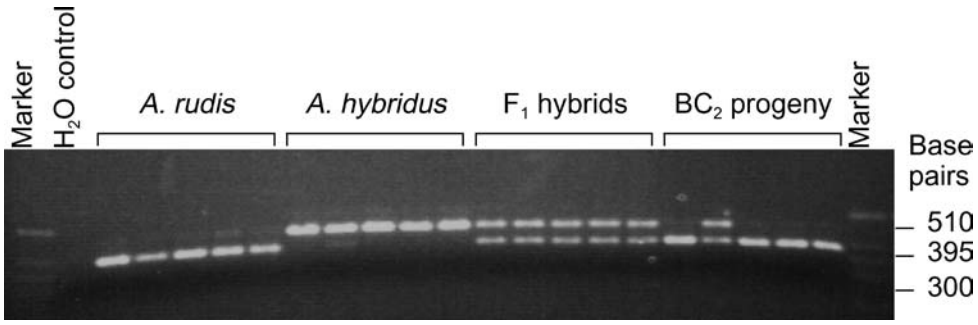

Fig. 2 Identification of the parental origin of *ALS* alleles in hybrids and BC_2 progeny. *ALS* alleles were identified in DNA preparations from five plants each of *A. rudis*, *A. hybridus*, F_1 hybrids, and BC_2 progeny as described in Fig. 1. Only products after *Eco*RV digestion are shown. Of the hybrids and BC_2 plants represented in the figure, all of the hybrids and only the BC_2 plant with the *A. hybridus ALS* allele survived subsequent treatment with imazethapyr

Table 1 Inheritance of an *Amaranthus hybridus* herbicide-resistance gene in BC_2 progeny. BC_1 progeny were obtained from crossing F_1 hybrids (*Amaranthus rudis* × resistant *A. hybridus*) with *A. rudis*. BC_2 progeny were obtained by a second round of *A. rudis* backcrossing using BC_1 plants that inherited the *A. hybridus* resistance gene from the hybrid parent. The resistance gene encoded a herbicide-insensitive form of acetolactate synthase

BC_2 family	Number of BC_2 plants analyzed	BC_2 plants with *A. hybridus* resistance gene	
		Number	Percentage
1	30	13	43
2	30	3	10
3	30	11	37
4	20	12	60
Total	110	39	35

Because few BC_1 plants were obtained, they were not sprayed to test for herbicide resistance. However, these plants were evaluated using the PCR/*Eco*RV molecular marker for *ALS*. Segregation of the *A. hybridus ALS* allele in the BC_1 progeny was difficult to accurately predict without knowing how chromosomes paired during meiosis in the hybrids. However, assuming that polyploids are not produced and independent chromosomal assortment, 50% of the progeny should contain the *A. hybridus* allele. Consistent with this, 21 of the 42 BC_1 plants had both *A. rudis* and *A. hybridus ALS* alleles.

Compared to the F_1 hybrids, phenotypic variability was more apparent among BC_1 plants. Generally, however, BC_1 plants had phenotypes that were similar to the recurring *A. rudis* parent and, like the F_1s, all of the BC_1s were dioecious. Most plants were vigorous, although several clearly had reduced vigor. Male plants produced much less pollen than *A. rudis*, but they did produce some. Female plants produced normal-looking, *A. rudis*-like flowers.

Female BC_1 parents that inherited the *A. hybridus ALS* allele were backcrossed with herbicide-sensitive male *A. rudis* as the recurrent parent. The production of BC_2 seed was much greater than in the previous generation, but was variable among BC_1 parents. Some plants produced fewer than 100 seeds, and some produced 1,000 seeds or more. At the level of gross anatomy, BC_2 plants were indistinguishable from *A. rudis*.

Table 1 presents inheritance data of the *A. hybridus ALS* allele for four BC_2 families. Among families, the number of plants that inherited the resistance gene ranged from 10% to 60% and, overall, 35% of the BC_2 progeny inherited the resistance gene (Table 1). All of the 110 plants included in Table 1 were analyzed for the presence of the *A. hybridus* allele by both molecular analysis (as shown in Fig. 2) and by herbicide-response evaluation. All of the plants that contained the *A. hybridus ALS* allele survived two sequential 2X field-rate applications of imazethapyr whereas the other plants did not. Although less than the 50% inheritance that would be expected for a similar intraspecific cross, the *A. hybridus* herbicide-resistance allele was inherited, and functional, in a high percentage of BC_2 progeny.

Results from preliminary experiments indicated that 2C DNA contents varied between *A. rudis* and *A. hybridus*. We therefore took advantage of this finding to provide further confirmation that progeny obtained from the crossing experiments were indeed hybrids or, for BC_2 progeny, were of hybrid origin. (BC_1 plants were no longer available when DNA content analysis was performed). As shown in Fig. 3, DNA content analysis by flow cytometry clearly distinguished ($\alpha < 0.01$) the two parental species, with mean values of 1.42 and 1.12 pg among five plants each of *A. rudis* and *A. hybridus*, respectively. DNA content for each of five hybrid plants was intermediate to parental values, and the mean value (1.27 pg) obtained from these five plants was exactly equal to the expected value based on 50% *A. rudis*/50% *A. hybridus* genome composition.

DNA content values were similar among five BC_2 plants regardless of whether they inherited the *A. hybridus ALS* allele and ranged from 1.39 to 1.41 pg (Fig. 3). The mean for these five plants, 1.40 pg, was significantly ($\alpha < 0.01$) less than the *A. rudis* mean, thereby providing further evidence that these plants were in fact derived from hybrids. If one assumed BC_2 progeny had 87.5% *A. rudis*/12.5% *A. hybridus* genome composition, the expected 2C DNA content was 1.39 pg, which was close to the observed value. Murray (1940) reported that BC_1 plants obtained from F_1 (*A. rudis* × *A. quitensis*

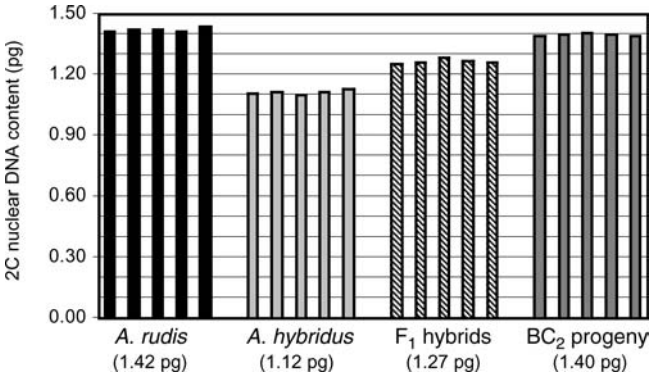

Fig. 3 DNA content analysis of parental and progeny plants. Flow cytometry was used to determine 2C nuclear DNA contents in five plants each of parental species (*A. rudis* and *A. hybridus*), F_1 hybrids, and BC_2 progeny. Mean DNA content for each set of five plants is indicated in *parenthesis*. The second BC_2 plant from the left inherited the *A. hybridus ALS* allele, whereas the other BC_2 plants shown did not

Kunth) × *A. rudis* were triploid.[1] Although we did not perform DNA content analysis on BC_1 plants, our DNA content data from BC_2 plants are inconsistent with polyploid formation.

Numerous hybrid plants were obtained from the initial *A. rudis* × *A. hybridus* cross, and BC_1 plants produced numerous progeny. Thus, the bottleneck in gene movement from *A. hybridus* to *A. rudis* would appear to be in the low fertility of the hybrid (F_1) generation. Others have also observed a relatively high production of hybrids but rare production of BC_1 progeny in many interspecific crosses involving weedy as well as cultivated *Amaranthus* species (Gupta and Gudu 1991; Pal and Khoshoo 1973; Wetzel et al. 1999). Low fertility of interspecific hybrids is a common phenomenon and generally attributed to meiotic abnormalities related to a lack of complete pairing between chromosomes of the two parent species (Appels et al. 1998).

In interspecific hybrids obtained from crosses between certain weedy and cultivated *Amaranthus* species (all monoecious and with $2n = 34$), Pandey (1999) observed primarily bivalent chromosome associations in metaphase I but also observed univalent, trivalent, and quadrivalent associations. Relationships among these monoecious species are presumably closer than the relationship between the monoecious and dioecious species used in our study. It seems likely, therefore, that there would also be meiotic abnormalities in *A. rudis* × *A. hybridus* progeny. Results of AFLP analysis of hybrids obtained between *A. rudis* and another dioecious *Amaranthus* species, *A. palmeri*, suggested that the hybrids contained multiple chromosomal rearrangements (Wetzel et al. 1999).

[1] Murray (1940) described this cross as (*Acnida tamariscina* × *Amaranthus hybridus*) × *Acnida tamariscina*. Upon reexamination of the specimens used by Murray, Sauer (1953) determined that the *A. hybridus* was actually *A. quitensis*. Also, *Acnida tamariscina* has since been renamed to *Amaranthus rudis* (Sauer 1972)

Despite the meiotic abnormalities and chromosomal rearrangements that likely accompanied hybridization between *A. rudis* and *A. hybridus*, the herbicide-resistance gene from *A. hybridus* was inherited through two backcrosses with *A. rudis* and remained functional. Sauer (1957) argued against the importance of hybridization between dioecious and monoecious *Amaranthus* species, stating that such crosses "run into a blind alley of sterility". The very low fitness we observed in the hybrids – we recovered an average of 1.3 seedlings per female hybrid – is consistent with Sauer's notion. However, low fitness of hybrid plants could be offset in the presence of herbicide selection if the hybrids acquired herbicide resistance from one of the parents, as occurred in our controlled crosses. To take this a step further, the existence of herbicide resistance in several populations of pigweed species, coupled with repeated herbicide selection pressure, could promote gene transfer among these species by counteracting low hybrid fitness.

Interspecific transferal of a herbicide-resistance gene from controlled crosses has now been demonstrated between dioecious *Amaranthus* species (Wetzel et al. 1999) and between a dioecious and a monoecious *Amaranthus* species (this report). It is unknown how frequently gene transferal among pigweed species occurs in nature. Recently obtained data from isozyme analysis, however, suggest gene transfer has occurred naturally between *A. rudis* and both *A. hybridus* and *A. retroflexus* (D. B. Pratt, Iowa State University, personal communication). Collectively, these reports indicate a significant potential for the evolution of herbicide resistance in weedy *Amaranthus* species by interspecific gene transfer. Such potential should be considered in the development of weed management strategies targeted at these species.

Acknowledgements We thank Loyd Wax and Aaron Hager for providing *A. rudis* and *A. hybridus* seed, respectively, and Ken Robertson for preparing herbarium samples. This material is based on work supported by a grant from the Illinois Soybean Program Operating Board.

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