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Interspecific and intraspecific transference of metabolism-based mesotrione resistance in dioecious weedy *Amaranthus*

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SUMMARY

Pollen-mediated gene flow (PMGF) might play an important role in dispersing herbicide resistance alleles in dioecious weedy *Amaranthus* species. Field experiments in a concentric donor–receptor design were conducted to quantify two sets of PMGF studies, an interspecific (*Amaranthus tuberculatus* × *Amaranthus palmeri*) and an intraspecific (*A. tuberculatus* × *A. tuberculatus*). In both studies, PMGF was evaluated using a resistant *A. tuberculatus* phenotype with enhanced mesotrione detoxification via P450 enzymes as a source of resistance alleles. For interspecific hybridization, more than 104 000 putative hybrid seedlings were screened with three markers, one phenotypic and two molecular. The two molecular markers used, including 2-bp polymorphisms in the internal transcribed spacer region, distinguished *A. palmeri*, *A. tuberculatus* and their hybrids. Results showed that 0.1% hybridization between *A. tuberculatus* × *A. palmeri* occurred under field research conditions. For intraspecific hybridization, 22 582 seedlings were screened to assess the frequency of gene flow. The frequency of gene flow (F_{GF}) varied with distance, direction and year of the study. The farthest distance for 90% reduction of F_{GF} was at 69 m in 2015 however, after averaging across directions it was 13.1 and 26.1 m in 2014 and 2015, respectively. This study highlights the transfer of metabolism-based mesotrione resistance from *A. tuberculatus* to *A. palmeri* under field research conditions. The results presented here might aid in the rapid detection of *A. palmeri* among other *Amaranthus* species and show that PMFG could be expediting the increase of herbicide resistance in *A. palmeri* and *A. tuberculatus* across US crop production areas.

Keywords: *Amaranthus palmeri*, *Amaranthus tuberculatus*, gene flow, herbicide resistance evolution, HPPD-inhibitor herbicide, hybridization, Palmer amaranth, waterhemp.

INTRODUCTION

The dioecious weedy *Amaranthus* species *Amaranthus palmeri* and *Amaranthus tuberculatus* are currently the most economically important weed species infesting row crop areas in the southern and north-central USA, respectively (Steckel, 2007; Webster and Nichols, 2012; Ward *et al.*, 2013). Various farm practices have contributed to the adaptation of *Amaranthus* species to modern cropping systems, particularly the reduction in the use of soil-applied herbicide and increased adoption of no-tillage row crop production systems (Culpepper, 2006; Owen, 2008). Additionally, these species have biological characteristics that increase their ability to be problem weeds, including dioecism and high fecundity (Hartzler *et al.*, 2004; Refsell and Hartzler, 2009; Ward *et al.*, 2013). The *Amaranthus* genus is notorious for a high rate of spontaneous intraspecific and interspecific hybridization (Grant, 1959; Greizerstein and Poggio, 1995; Ellstrand *et al.*, 1996). Dioecism in *Amaranthus* species is an important component of adaptation and evolution of these species, and may partially explain the high level of herbicide resistance existing across this genus. Populations of *A. palmeri* and *A. tuberculatus* have evolved resistance to six herbicide sites of action (SOA) throughout the USA (Tranel *et al.*, 2011; Bernards *et al.*, 2012; Ward *et al.*, 2013). The high frequency of herbicide resistance in this genus, dioecy, large population sizes and intense selection pressure make *Amaranthus* an excellent model for studying plant evolution at the landscape level.

Herbicide resistance alleles might be spread via pollen-mediated gene flow (PMGF) (Jasieniuk *et al.*, 1996). Liu *et al.* (2012) reported that pollen from an *A. tuberculatus* population has great longevity and long-distance dispersal. As a result, there is the potential for intraspecific (Sosnoskie *et al.*, 2012; Sarangi *et al.*, 2017) and interspecific hybridization in *Amaranthus* species (Tranel *et al.*, 2002; Trucco *et al.*, 2005, 2006), transferring herbicide resistance traits (Gaines *et al.*, 2012). Therefore, PMGF might be an important aspect of the transfer of herbicide resistance genes, and it is likely to increase the adaptation success of dioecious *Amaranthus* species in modern cropping-systems.

Several molecular markers have been developed to distinguish *Amaranthus* species and their interspecific hybrids (Wetzel *et al.*, 1999a; Tranel *et al.*, 2002; Wright *et al.*, 2016); however, most markers rely on restriction fragment length polymorphisms (RFLPs), which can be expensive and time-consuming when genotyping large number of plants. More recently, a quantitative marker has been developed to identify *A. palmeri* from other *Amaranthus* species in mixed seed collections (Murphy *et al.*, 2017). This assay is particularly useful as *A. palmeri* has recently been listed as a noxious weed in several states in the north-central USA. Molecular assays for rapid identification of *Amaranthus* species and their hybrids are necessary for the identification and management of *Amaranthus* species.

While molecular markers exist for determining certain hybrids, most intraspecific PMGF studies in weedy *Amaranthus* are based on phenotypic herbicide resistance traits (Liu *et al.*, 2017; Sarangi *et al.*, 2017). Target-site resistance (TSR) mechanisms are usually conferred by a single dominant gene and can be used as a robust marker for identifying intraspecific hybridization (Mallory-Smith *et al.*, 2015). However, non-target-site resistance (NTSR) mechanisms have also recently been reported in *A. tuberculatus* and *A. palmeri* populations (Evans *et al.*, 2017; Figueiredo *et al.*, 2017; Kaundun *et al.*, 2017; Nakka *et al.*, 2017; Küpper *et al.*, 2018); some of these have complex inheritance (Hausman *et al.*, 2011) or the resistance gene has not yet been determined (Délye, 2013a). Recently there has been an increased interest in deciphering the genetic basis of NTSR mechanisms (Délye *et al.*, 2013b), but our knowledge of this area is far from complete (Gaines *et al.*, 2014; Duhoux *et al.*, 2015; Pan *et al.*, 2016). Even though PMGF is one of the key components for understanding ecological and evolutionary dynamics of NSTR mechanisms, the role of expression of NTSR herbicide resistance alleles in interspecific and intraspecific hybrids or F_1 individuals under field research conditions remains to be explored.

Morphological similarities and potential hybridization between *Amaranthus* species are likely to increase the complexity of identification and management (Wassom and Tranel, 2005); therefore, it is critical to develop molecular markers that robustly, effectively and accurately detect *Amaranthus* species. We have developed two competitive allele-specific PCR (KASP) markers (He *et al.*, 2014) to distinguish *A. palmeri*, *A. tuberculatus* and their interspecific hybrid. We hypothesized that: (i) PMGF with herbicide resistance transfer would occur from mesotrione-resistant (R) *A. tuberculatus* to mesotrione-susceptible (S) *A. palmeri* populations (interspecific hybridization) and (ii) PMGF transferring herbicide resistance would occur from R *A. tuberculatus* to S *A. tuberculatus* populations (intraspecific hybridization) under field conditions.

RESULTS

Interspecific (R *A. tuberculatus* × S *A. palmeri*) hybridization

More than 104 000 putative hybrid (R *A. tuberculatus* × S *A. palmeri*) seedlings were screened with 185 g active ingredient (ai) ha^{-1} (1.75 times the mesotrione field rate), which was used as a cutoff rate expected to keep false positives low. Only 2.4% and 0.9% of putative hybrid seedlings survived mesotrione application in 2014 and 2015, respectively (Table 1).

Two KASP molecular marker assays were employed to test putative hybrids and eliminate false positive hybrids that survived the mesotrione screening test. The first marker was a single nucleotide polymorphism (SNP) in the acetolactate synthase (ALS) gene (ALS-SNP) and the second marker was a 2-bp polymorphism in the internal transcribed spacer (ITS) of the ribosomal coding region (TBP-ITS). The R *A. tuberculatus* and S *A. palmeri* parents clustered at their respective genotypes, except for a single S *A. palmeri* individual for the ALS-SNP that clustered in the no template control (NTC; Figure 1). The majority (79 out 86) of putative hybrids that survived mesotrione application clustered in the S *A. palmeri* region with only seven hybrids classified as heterozygotes for both SNPs (Figure 1). The rate of hybrids using KASP molecular assays was 0.042 and 0.128 for 2014 and 2015, respectively. These numbers were further used to calculate the frequency of interspecific hybridization.

In 2014, interspecific hybrids were found in the north-east (0.5 m) and north-west (5 m) block. In 2015, hybrids were found south (0.5 m), two east (0.5 m) and two at 15 m (north-east and north-west) of the R *A. tuberculatus* pollen-source block. The total estimated hybridization frequency was similar for both years (0.001), and power analysis confirmed the experiment's precision for detecting hybrids (Table 1).

Intraspecific (R *A. tuberculatus* × S *A. tuberculatus*) hybridization

The model predicted the frequency of gene flow (F_{GF}; F_1 individuals) to be highest near the pollen-source block

Table 1 Frequency of interspecific pollen-mediated gene flow (PMGF) between resistant *Amaranthus tuberculatus* and susceptible *Amaranthus palmeri* at different distances (pooled directions) under field research conditions at the Haskell Agricultural Laboratory of the University of Nebraska–Lincoln in 2014 and 2015.

		Mesotrione treatment[b]		KASP assays[c]		Hybridization[d]	
Year	Distance[a]	Emerged plants (*E*)	Living plants (*A*)	Plants analyzed	Hybrids	Estimated	Frequency
	m	#					
2014	0	4154	215	4	0	9	0.0022
	0.5	15 546	471	25	0	20	0.0013
	2	11 879	351	8	1	15	0.0013
	5	13 083	394	3	1	17	0.0013
	15	12 696	162	1	0	7	0.0005
	30	8351	86	3	0	4	0.0004
	45	8822	89	3	0	4	0.0004
	Total	74 531	1768	47	2	75	0.0010
2015	0	1044	25	1	0	3	0.0031
	0.5	5998	107	11	3	14	0.0023
	2	6050	47	5	0	6	0.0010
	5	6449	57	6	0	7	0.0011
	15	5297	49	6	2	6	0.0012
	30	8351	40	5	0	5	0.0006
	45	4889	13	5	0	2	0.0003
	Total	38 078	338	39	5	43	0.0011

[a]Distance of mesotrione-susceptible *A. palmeri* female plants from the pollen-source block of mesotrione-resistant *A. tuberculatus*.
[b]Number of emerged (*E*) and living (*A*) plants (putative hybrids, resistant *A. tuberculatus* × susceptible *A. palmeri*) treated with 185 g ai ha^{-1} (1.75 times the mesotrione field rate) in interspecific hybridization only.
[c]Subsamples of living plants (*A*) from mesotrione treatment. The frequency of hybridization detected in the KASP assays (*T*) was 0.042 (2/47) and 0.128 (5/39) for 2014 and 2015, respectively. Hybrids were detected using two molecular KASP assays.
[d]The frequency of hybridization was calculated by combining phenotypic and molecular markers as described in equation (3) (frequency of hybridization = *TA*/*E*). Estimation of the number of hybrids was calculated from frequency of hybridization × living plants (*A*). A power analysis using binomial probabilities was 0.99 of the theoretical frequency of 0.5% hybridization at $\alpha = 0.05$.

(0 m) and decrease exponentially with distance (Figure 2). The maximum F_{GF} at 0.5 m was 0.67 in 2015, which is below the expected random mating value (1) and just over twice the F_{GF} in 2014 (0.32) (Table 2). The modeling efficiency (ME) of predicted F_{GF} ranged from 0.12 to 0.71, and the ME averaged lower than 0.40.

Female S *A. tuberculatus* plants were not included inside the pollen-source block; therefore, F_{GF} at 0 m was not determined. However, the double-exponential model predicted F_{GF} at 0 m for each year and cardinal direction (Figure S1 in the online Supporting Information). The F_{GF} at 0 m aided in predicting distance at 50% and 90% F_{GF} reduction (Figure 3). The F_{GF} reduction values varied according to direction. In 2014, the distance for 90% F_{GF} reduction ranged from 1.5 to 19 m; however, in 2015, values varied from 4 to 69 m.

The average wind speed during the 6-week pollination period was lower than 6 m s^{-1} blowing from all directions, but with higher wind frequencies from the south in both years (Figure 4). Wind frequency did not correlate with F_{GF}, but the frequency of wind direction was variable across weeks in 2014 and 2015 (Figures S2 and S3). Therefore, the wind was blowing from different directions in different weeks, and typically varied within a day. The *Amaranthus* species in the present study are dioecious and flowering time varied within 6 weeks from the earliest to latest blooming plants, and even plants in the same distance/direction showed different flowering patterns.

DISCUSSION

Interspecific (R *A. tuberculatus* × S *A. palmeri*) hybridization

Amaranthus palmeri is described as the most economically damaging weed of southern US cropping-systems (Ward *et al.*, 2013). In recent years, *A. palmeri* has migrated into north-central states, overlapping in territory with *A. tuberculatus*, a major weed existing in north-central cropping systems (Kohrt *et al.*, 2017). Here, we failed to reject our hypotheses, demonstrating that transference of NTSR mechanisms of mesotrione resistance from R *A. tuberculatus* to S *A. palmeri* occurs under field research conditions.

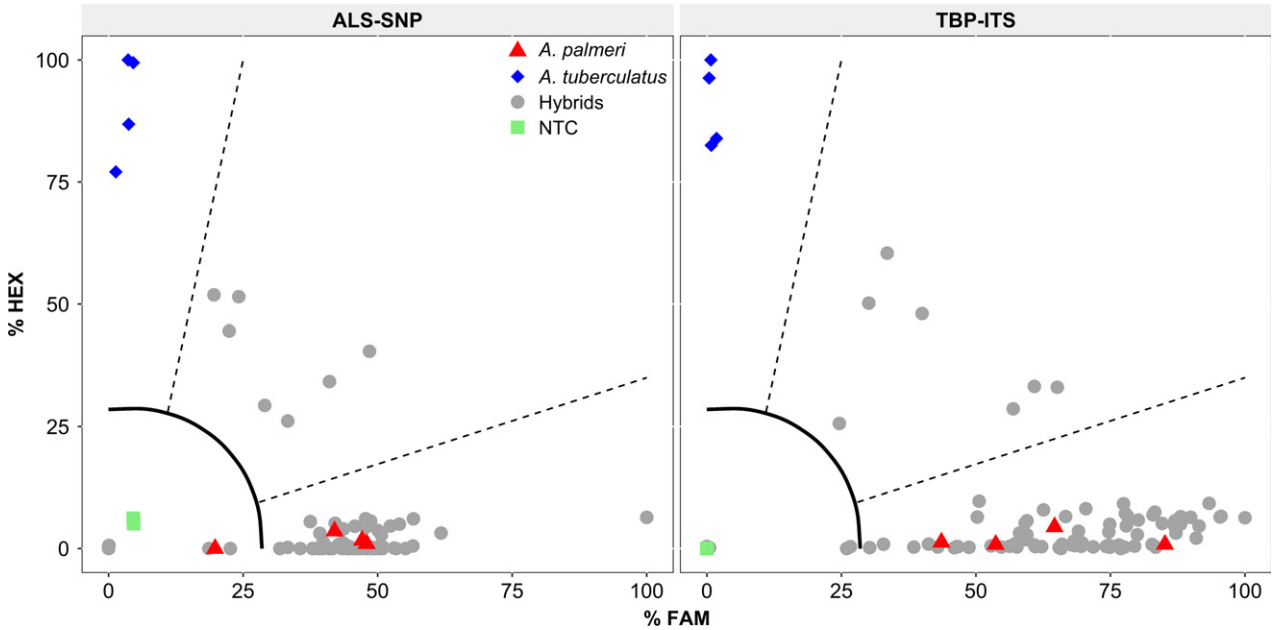

Figure 1. Cluster analysis of competitive allele-specific PCR (KASP) assays of suspected hybrids (*A. tuberculatus* × *A. palmeri*) with two molecular markers. An acetolactate synthase single nucleotide polymorphism (ALS-SNP) marker (left) and a 2-bp polymorphism in the internal transcribed spacer (TBP-ITS) marker (right). Parental *Amaranthus tuberculatus* and *Amaranthus palmeri* were used as a positive control and no template for the negative control (NTC). Dashed lines represent cutoffs for making genotyping calls. The solid quarter circle is the cutoff for no amplification. Cutoffs were determined based on the *K*-means cluster analysis. [Colour figure can be viewed at wileyonlinelibrary.com].

Therefore, the complexity of managing weeds of these dioecious *Amaranthus* species is likely to increase in row crops of the north-central USA due to species coexistence in this region and potential hybridization between *A. tuberculatus* and *A. palmeri*.

The fitness penalties of R *A. tuberculatus* and S *A. palmeri* hybrids were not the objective in our study. Nonetheless, transfer of *A. palmeri* herbicide resistance genes to *A. tuberculatus* has been previously documented, showing apparent normal hybrid growth and fertility (Wetzel *et al.*, 1999b; Franssen *et al.*, 2001a,b). However, other studies have demonstrated negative effects in the *A. tuberculatus* and *A. palmeri* (reciprocal cross) hybrids, including irregular crossing over in meiosis (Steinau *et al.*, 2003) and triploid apomictic seed (Trucco *et al.*, 2007). The majority of weedy *Amaranthus* species are diploid (2*n*) with their chromosome number ranging from 32 to 34 (Trucco *et al.*, 2007; Gaines *et al.*, 2012). Similar chromosome numbers could facilitate hybridization in *Amaranthus* species (Trucco *et al.*, 2005; Nandula *et al.*, 2014). While *A. tuberculatus* is shown to have 32 chromosomes, *A. palmeri* was documented with 34 chromosomes (Grant, 1959; Gaines *et al.*, 2012). In our study, R *A. tuberculatus* and S *A. palmeri* hybrid formation was low (0.001), which might be due to the differential chromosome number between these two species. Additional research to investigate the potential fitness trade-offs associated with *A. palmeri* and *A. tuberculatus* hybrids carrying NTSR mechanisms is necessary, including the possible balance of benefits of herbicide resistance from gene flow with fitness penalties due to hybridization.

The expansion of *A. palmeri* into the north-central USA means it is currently sharing the same habitat with several other weedy *Amaranthus* species, including *Amaranthus spinosus* (2*n* = 34), *Amaranthus powellii* (2*n* = 34), *Amaranthus retroflexus* (2*n* = 34) and *Amaranthus hybridus* (2*n* = 32) (Sauer, 1957, 1967; Grant, 1959; Wetzel *et al.*, 1999a). Additionally, 10 *Amaranthus* species are dioecious, which aids cross-pollination (Steckel, 2007). Pollen in *Amaranthus* can spread for long distances by wind dispersal, which can increase the chances of hybridization among species and spread herbicide resistance alleles (Franssen *et al.*, 2001b; Sosnoskie *et al.*, 2009; Liu *et al.*, 2012). In some cases, hybridization in *Amaranthus* species might be unidirectional. For example, Trucco *et al.* (2009) showed that *A. hybridus* can transfer herbicide resistance alleles to *A. tuberculatus* but the reciprocal genetic exchange was not possible. Nonetheless, transfer of glyphosate resistance genes was also documented from *A. palmeri* to *A. tuberculatus*, *A. hybridus* and *A. spinosus* (Gaines *et al.*, 2012; Nandula *et al.*, 2014). Many of the *Amaranthus* species have similar morphologies and coexist in the same location, and their ability to hybridize makes it difficult to clearly differentiate them on the basis of morphology. As we have demonstrated, robust molecular KASP assays (ALS-SNP and TBP-ITS) can be used to distinguish R *A. tuberculatus*, S *A. palmeri* and their hybrids.

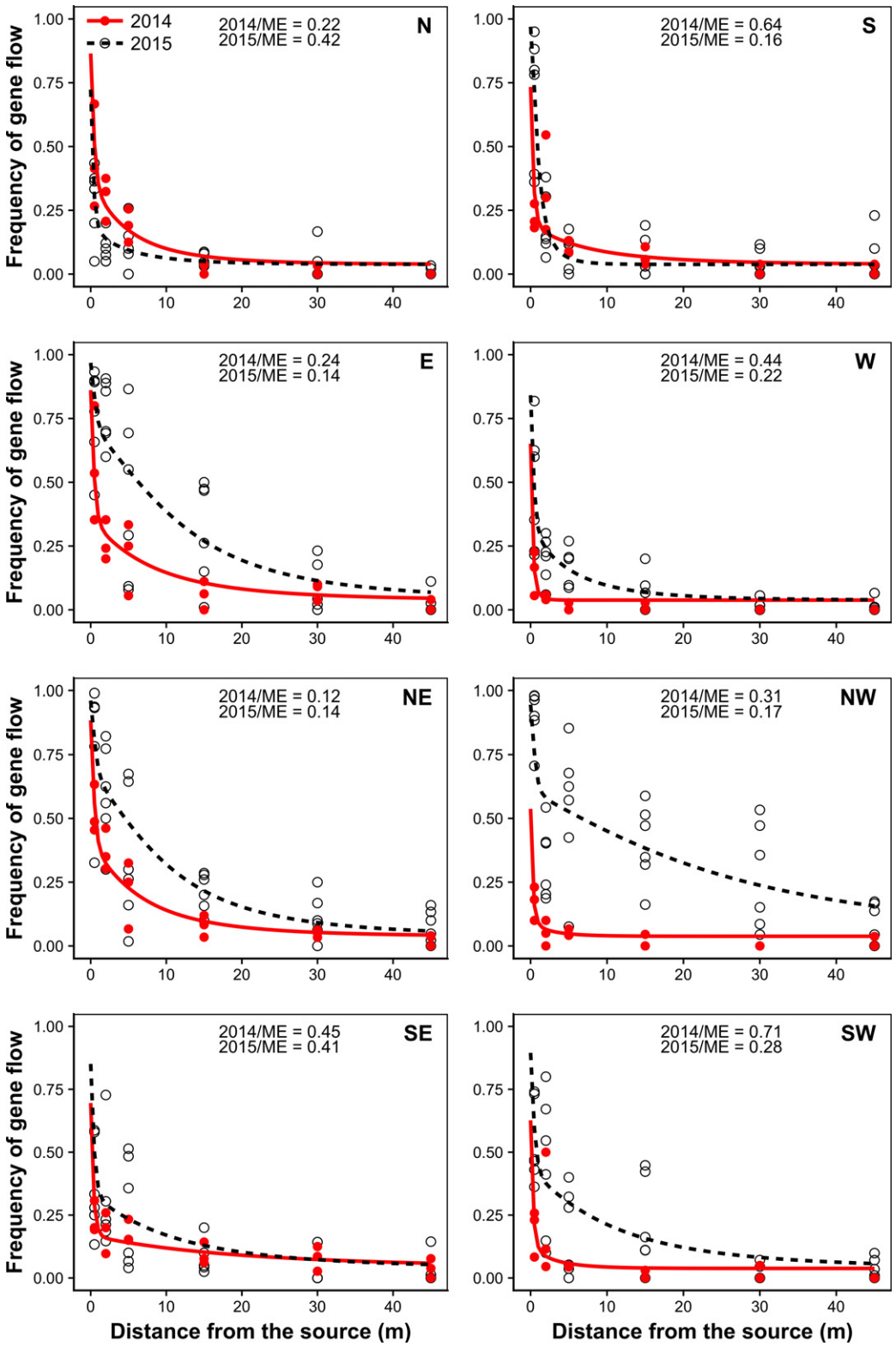

Figure 2. Intraspecific frequency of gene flow.
Intraspecific frequency of gene flow (mesotrione-resistant *Amaranthus tuberculatus* × mesotrione-susceptible *A. tuberculatus*) affected by distance and eight cardinal directions (N, NE, E, SE, S, SW, W, NW) in a field research experiment at the Haskell Laboratory of the University of Nebraska–Lincoln. Modeling efficiency (ME) for 2014 and 2015. [Colour figure can be viewed at wileyonlinelibrary.com].

Table 2 Frequency of intraspecific pollen-mediated gene flow (PMGF) in F_1 individuals (resistant *Amaranthus tuberculatus* × susceptible *A. tuberculatus*) under field research conditions at Haskell Agricultural Laboratory of the University of Nebraska-Lincoln in 2014 and 2015

		Mesotrione treatment[b]		F_1 individuals[c]	
Year	Distance[a]	Emerged plants	Living plants	Frequency	Power[d]
	m	#			
2014	0.5	574	185	0.32	0.96
	2	605	135	0.22	1.00
	5	725	103	0.14	1.00
	15	570	30	0.05	0.99
	30	611	24	0.04	0.97
	45	572	11	0.02	0.40
	Total	3657	488	0.13	1.00
2015	0.5	3832	2565	0.67	1.00
	2	3472	1399	0.40	1.00
	5	2774	912	0.33	1.00
	15	3187	654	0.21	1.00
	30	2566	287	0.11	1.00
	45	3364	174	0.05	1.00
	Total	19 195	5991	0.31	1.00

[a]Distance of mesotrione-susceptible *A. tuberculatus* female plants from the pollen-source block of mesotrione-resistant *A. tuberculatus*.
[b]Number of emerged and living plants (F_1 individuals, resistant *A. tuberculatus* × susceptible *A. tuberculatus*) treated with 210 g ai ha^{-1} (twice the mesotrione field rate) in intraspecific hybridization only.
[c]The frequency in F_1 individuals was calculated from: frequency = number of emerged plants/number of living plants.
[d]Power analyses calculated for $\alpha = 0.05$ using binomial probabilities.

While misdiagnosis of R *A. tuberculatus* and S *A. palmeri* hybrids with KASP assays may possibly be due to gene conversion, the chances of this happening at both loci is extremely low. Our results showed no evidence that allele conversion is happening at either locus, as the ALS-SNP and TBP-ITS (KASP assays) are in full agreement (Figure 1). The KASP assays greatly strengthen the case that heterozygous individuals are really hybrids and not artifacts of low levels of within-species polymorphism. Further hybridization studies with KASP markers, especially the TBP-ITS assay, in other *Amaranthus* species are necessary for testing their general usefulness. We believe that the TBP-ITS marker has great potential for weed management decisions because the TBP-ITS marker robustly detects R *A. tuberculatus*, S *A. palmeri* and their hybrid.

In the field research site and adjacent areas, *A. palmeri* was not present prior to this experiment. Thus it is unlikely that pollen from male S *A. palmeri* could have outcrossed the female plants. Post-emergence mesotrione treatment reduced the number of putative hybrids by killing a majority of screened seedlings; however, mesotrione by itself was not a robust marker for detecting interspecific hybridization. Several hypotheses were raised to explain the high number of putative hybrids produced that were not mesotrione resistant and the high number of living putative hybrid after mesotrione treatment. First, mesotrione efficacy on *A. palmeri* varies with temperature (Godar *et al.*, 2015). In this interspecific study, mesotrione screening was performed under field conditions in June until August in 2015 and 2016, and the variable temperature might have influenced mesotrione efficacy. Second, multiple plants were grown in a single experimental unit. Thus false hybrids may have survived mesotrione treatment due to low herbicide coverage and therefore low herbicide dose. Third, the high number of non-hybrid seeds that were produced could be explained by facultative apomixis in female *A. palmeri* plants (Ribeiro *et al.*, 2014). The molecular mechanisms of apomictic seed formation in *A. palmeri* need further exploration.

It is possible that more hybrids were produced but the individuals were susceptible to mesotrione (at 1.75 times the mesotrione field rate). Therefore, the herbicide treatment could have masked the true number of hybrids in this study. Mesotrione treatment was performed to test the hypothesis of transfer of herbicide resistance between *Amaranthus* species. Nonetheless, all putative hybrid seedlings screened using molecular markers were either true hybrids (heterozygous for the S *A. palmeri* marker) or

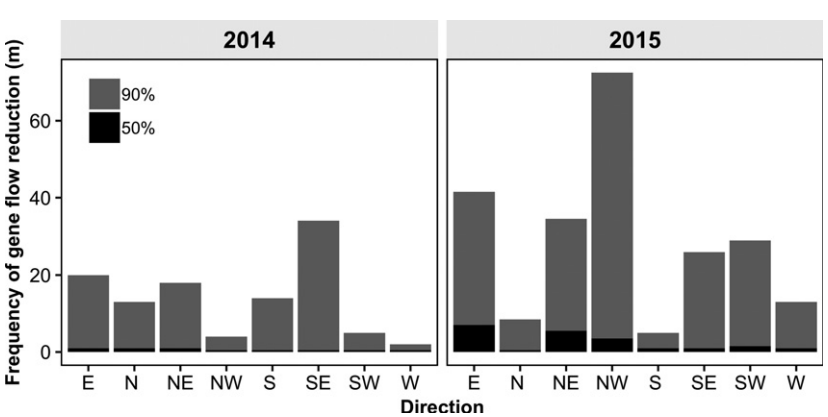

Figure 3. Distance estimation of gene flow reduction frequency.
Distance (m) estimation of 50% and 90% frequency of gene flow (F_{GF}) reduction in F_1 individuals in eight directions of the intraspecific hybridization (mesotrione-resistant *Amaranthus tuberculatus* × mesotrione susceptible *A. tuberculatus*) field research experiment in 2014 (left) and 2015 (right).

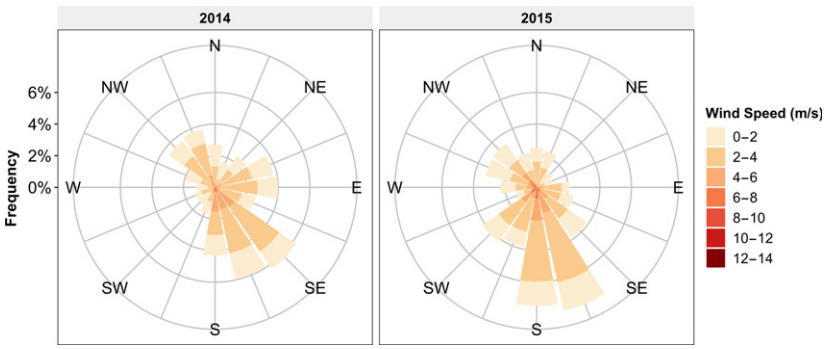

Figure 4. Wind rose plots.
Wind rose plots demonstrating the average hourly (07:00 to 15:00) wind frequency (%) and wind speed (m s^{-1}) grouped in 22.5° of direction (from which the wind is blowing) in a field experiment in 2014 (left) and 2015 (right). Wind data were recorded at 2 m above the soil surface and at the center of the pollen-source block of the field research experiment during the 6-week pollination period at the Haskell Agricultural Laboratory of the University of Nebraska–Lincoln. [Colour figure can be viewed at wileyonlinelibrary.com].

homozygous S *A. palmeri*. No homozygous R *A. tuberculatus* individuals were detected.

Intraspecific (R *A. tuberculatus* × S *A. tuberculatus*) hybridization

The transfer of herbicide resistance within populations of the same species is more likely to occur with high frequency (no genetic barrier) than between two different species (Sarangi *et al.*, 2017). Herbicide resistance traits are commonly used to measure hybridization as they can be easily screened for in F_1 seedlings if they are autosomal dominant (Mallory-Smith *et al.*, 2015). Here, we accept our hypothesis as it was shown that under field research conditions mesotrione resistance alleles were transferred from R *A. tuberculatus* to S *A. tuberculatus* progeny through pollen. The frequency of gene flow was rapidly reduced within 45 m of the pollen source and varied with distance and direction, which was also documented with PMGF studies that used ALS (Liu *et al.*, 2012) and glyphosate resistance genes (Sosnoskie *et al.*, 2012; Ganie and Jhala, 2017; Sarangi *et al.*, 2017) in *Amaranthus* species. The mechanism of mesotrione resistance in R *A. tuberculatus* is herbicide detoxification via cytochrome P450 alleles (Oliveira *et al.*, 2018c). Therefore, in this study it was not possible to use a molecular marker to detect mesotrione resistance in F_1 seedlings due to the complexity of this herbicide resistance mechanism. Nonetheless, the modeling approach demonstrated that mesotrione resistance is inherited by the next generation under field research conditions. Oliveira *et al.* (2018a) documented that mesotrione resistance alleles in R *A. tuberculatus* are polygenic, incompletely dominant and nuclear inherited, and this was also the case in other mesotrione-resistant *A. tuberculatus* populations from Illinois (Huffman *et al.*, 2015) and Iowa (Kohlhase *et al.*, 2018). The polygenic nature of mesotrione resistance traits suggests that resistance may evolve at a slower pace within a population than single-gene dominant traits (Jasieniuk *et al.*, 1996; Huffman *et al.*, 2015).

The variability in the modeling approach demonstrated by the ME is probably related to the species biology, the phenotypic marker used and the complex polygenic nature of mesotrione resistance in this population. Variability within an experimental unit was expected as seeds were harvested from at least three S *A. tuberculatus* plants in the receptor–donor block. Therefore, each F_1 individual is likely to have a slightly different level of resistance, and it is possible that plants with either a high or a low resistance level were by chance sown in a determined experimental unit. It is also possible that ME of F_{GF} was higher in 2014 than 2015 because fewer replicates were used in 2015.

The mortality in F_1 plants could be due to pollination of S *A. tuberculatus* from adjacent areas as pollen can travel up to 800 m (Liu *et al.*, 2012), unintentional presence of S *A. tuberculatus* in the pollen-donor block, an inability of heterozygous individuals to survive herbicide (twice the mesotrione field rate) or potential apomictic seed production by S *A. tuberculatus*; this needs further exploration. Also, these limitations of the study and the complexity of mesotrione resistance could be the reason for there being no correlation between F_{GF} and wind frequency. We hypothesized that this result is probably due to the inconsistency of wind direction within a day and week and the variable flowering window of the species. Other studies of PMGF have shown that wind frequency or speed did not correlate with frequency of PMGF in *Echinochloa crus-galli* (Bagavathiannan and Norsworthy, 2014), *A. palmeri* (Sosnoskie *et al.*, 2009) and *Linum usitatissimum* (Jhala *et al.*, 2011). In general, PMGF studies are conducted with bare soil as a background, thus differences in F_{GF} and wind frequency found in this study versus others are expected. In this study, a soybean canopy (65 cm tall) was present, which creates different wind flow dynamics that could

have affected PMGF. When a soybean canopy is present, the wind flow would tend to be slowed down due to friction (aerodynamic resistance) with the canopy (Baldocchi *et al.*, 1983). Aerodynamic resistance to wind flow is a function of canopy height and increases with increasing canopy height. Therefore, pollen would not be able to travel as far for a given wind speed as over bare soil ground (Ganie and Jhala, 2017; Sarangi *et al.*, 2017), probably resulting in weaker correlation between F_{GF} and wind frequency.

CONCLUSIONS

This research has demonstrated that PMGF may be a factor contributing to the evolution of herbicide resistance in *Amaranthus* species in the landscape. Even with low interspecific hybridization and rapid exponential decay in intraspecific hybridization with distance, PMGF carrying metabolism-based mesotrione resistance alleles occurs in weedy *Amaranthus* under field research conditions. This result is significant as *A. palmeri* and *A. tuberculatus* are prolific seed producers, obligate outcrossers, fast growing competitive, and NTSR can confer resistance to different herbicide SOA. Therefore, even at low frequencies, PMGF might have important evolutionary consequences in weedy *Amaranthus*.

EXPERIMENTAL PROCEDURES

Plant materials

The seeds from R and S *A. tuberculatus* populations were harvested from Platte County and Dixon County, NE, USA, respectively. The R *A. tuberculatus* has been previously characterized with 18-fold resistance to mesotrione compared with an S *A. tuberculatus* population (Oliveira *et al.*, 2017). The mechanism of herbicide resistance in the R *A. tuberculatus* population was described as incomplete dominance with multiple genes conferring enhanced mesotrione metabolism via cytochrome P450 enzymes (Kaundun *et al.*, 2017; Oliveira *et al.*, 2017, 2018a,b,c). The S *A. palmeri* population was collected from Lancaster County, NE, USA in 2001. In a preliminary study, this population was characterized as susceptible in the greenhouse with a 95% control value of 173 (±62) g ai ha^{-1} of mesotrione. Seeds were soaked in water for a day before being manually sown in the field experiments.

Field experiments

Field experiments were conducted in 2014 and 2015 in the same field at Haskell Agricultural Laboratory (42°23′1″ N, 96°59′18″ W) of the University of Nebraska–Lincoln at Concord, NE, USA. The soil type at the research site was loam with 2.7% organic matter and pH 7.6. The endemic weed species present at this site were *Setaria viridis*, *Chenopodium album*, *Abutilon theophrasti*, *A. tuberculatus* (mesotrione susceptible) and *A. retroflexus*. The soil was tilled, and glyphosate-resistant soybean was planted at 370 500 seeds ha^{-1} in rows spaced 50 cm apart on 24 May 2014 and 25 May 2015.

The field experiment was conducted in an adapted concentric donor–receptor design, where the pollen-donor block was surrounded in eight directions by pollen-receptor blocks (Mallory-Smith *et al.*, 2015). The pollen-donor block was a square (10 m × 10 m) and there were eight receptor blocks, each measuring 4 m wide and 45 m long (Figure 5). The total experimental area (pollen source and eight pollen-receptor blocks) was 1540 m^2. Glufosinate (Liberty® 280 SL, BASF Agriculture, https://agriculture.basf.com/) was applied at 594 g ai ha^{-1} to each section of the experimental area on soybean at cotyledon stage to provide weed- and crop-free areas prior to the onset of the experiment. Soybean was grown around the experimental area, which simulated the real field scenario and helped to suppress the endemic weeds in the experimental site (Figure 5). The 3-ha area external to the experimental site was sprayed with glyphosate (PowerMax®, Bayer Crop Science, https://www.cropscience.bayer.com/) at 1320 g ai ha^{-1} for weed control, further reducing potential pollen contamination from the endemic *A. tuberculatus and A. retroflexus* population. Also, hoe weeding was performed within the experimental area for controlling undesired weed species that escaped herbicide treatment during the experiment period.

The *Amaranthus* species were manually sown on 2 June 2014 and 3 June 2015. This time was chosen because it is the natural starting germination window of *Amaranthus* species in north-eastern Nebraska. In the pollen-donor block, approximately 100 R *A. tuberculatus* seeds were transplanted 30 cm apart and 1 cm deep in the soil. A week later, the R *A. tuberculatus* population was sown again at 30 cm apart; therefore, R *A. tuberculatus* plants were spaced at a distance of 15 cm. Similarly, in the receptor

Figure 5. Aerial view of the field experiment.
Aerial view of the field experiment to quantify interspecific and intraspecific pollen-mediated gene flow from mesotrione-resistant *Amaranthus tuberculatus* (R) to mesotrione-susceptible *Amaranthus palmeri* and *A. tuberculatus* (S). The R *A. tuberculatus* was planted in the center of the field in a 10 m × 10 m pollen-source block. The pollen-receptor blocks were divided into eight cardinal direction blocks (N, NE, E, SE, S, SW, W, NW, 4 m × 45 m each). The S *A. palmeri* and S *A. tuberculatus* were planted at each pollen-receptor block at 0.5, 2, 5, 15, 30 and 45 m from the pollen-source block. At each distance in the pollen-receptor block, *A. palmeri* and *A. tuberculatus* were planted in 3 and 1 linear meters, respectively. [Colour figure can be viewed at wileyonlinelibrary.com].

blocks, 100 seeds of S *A. tuberculatus* and S *A. palmeri* were also sown in at an interval of a week spaced 15 cm apart. The receptor block plants were sown at six distances (0.5, 2, 5, 15, 30 and 45 m) in eight different blocks, including the cardinal (north, south, east, west) and the ordinal (north-east, north-west, south-east, south-west) directions (Figure 5). At each distance and direction, S *A. tuberculatus* and S *A. palmeri* were sown 1 and 3 m wide (four linear meters), respectively. Inside the pollen-donor area, S *A. palmeri* was also sown in two linear meters to simulate a 'worst-case' scenario, where S *A. palmeri* was surrounded by a dense population of R *A. tuberculatus* plants. The week's interval between planting was allowed to increase synchrony of the flowering period and therefore the chance of hybridization between species.

After the *A. tuberculatus* and *A. palmeri* seedlings had germinated, thinning was performed to reduce plant competition, but dense populations of *A. tuberculatus* and *A. palmeri* were maintained in the study (approximately 30 plants per linear meter). Male S *A. tuberculatus* and S *A. palmeri* plants were visually detected on a daily-basis prior to pollen shedding and removed from the pollen-receptor and pollen-donor blocks (S *A. tuberculatus*). Also, in the pollen-donor block, female R *A. tuberculatus* plants were similarly screened and removed. This procedure was performed to maximize outcrossing between male R *A. tuberculatus* and female S *A. tuberculatus* and S *A. palmeri* plants (Figure S4). Otherwise, pollen competition may have occurred, reducing the chances for distant pollen dispersal in the study (Liu *et al.*, 2012).

Both *Amaranthus* species in these studies displayed long flowering periods from mid July to late August in both 2014 and 2015. The height of *Amaranthus* species at flowering varied from 1 to 2 m. A weather station (61622 Vantage Pro2 Plus, Davis Instruments, https://www.davisinstruments.com/) was placed at 2 m above the soil surface in the center of this experiment. Hourly meteorological data on temperature, humidity and precipitation were recorded (Table S1). The wind speed and direction data were recorded from 07:00 to 15:00 due to the likelihood of *Amaranthus* species shedding pollen during that period (Sosnoskie *et al.*, 2012).

At maturity (late September), inflorescences of ten S *A. palmeri* and three S *A. tuberculatus* plants were harvested from each distance and direction. Harvesting was performed moving inward, starting at 45 m and progressing to 0.5 m for each species to reduce seed contamination. Mature inflorescences of species at each direction and distance were cleaned, labeled and bagged individually, then stored at 4°C to disrupt potential seed dormancy until they could be used in the herbicide resistance screening. The harvested seeds were termed putative hybrids (R *A. tuberculatus* × S *A. palmeri*) and F_1 (R *A. tuberculatus* × S *A. tuberculatus*) individuals to describe interspecific and intraspecific hybridization, respectively.

Interspecific hybridization (*A. tuberculatus* × *A. palmeri*) species diagnostic marker

Three markers (one phenotypic and two genotypic) were combined to detect hybrids. First, mesotrione resistance was used as a phenotypic marker to increase the probability of finding hybrids. Second, plants that survived mesotrione treatment were genotyped using two KASP assays to determine whether each individual was a true hybrid between S *A. palmeri* and R *A. tuberculatus* (Figure S5).

Mesotrione resistance marker. Hybrid seeds collected from the field study were planted separately in plastic trays (51 cm × 38 cm × 10 cm) containing potting mix (Miracle-Gro®, *Scotts*Miracle-Gro, https://scottsmiraclegro.com/) and evaluated for mesotrione resistance.

The mesotrione screening on putative hybrids was performed outdoors in the summer period of 2015 and 2016 at the Haskell Agricultural Laboratory. There was a high variability in hybrid germination in trays. For example, in each tray, putative hybrids varied from 0 to 1296 seedlings with an average of 209 and 63 seedlings per tray in 2014 and 2015, respectively. There were eight to nine replications of each experimental unit (plastic trays) in 2014 and 11 replications from the 2015 study. A total of 104 492 putative hybrids seedlings were screened in all distances and directions for the study in 2014 and 2015. Putative hybrids were sprayed at 5–8 cm tall with 1.75 times (interspecific hybridization only) the mesotrione field rate (105 g ai ha^{-1}; Callisto®, Syngenta Crop Protection, http://www.syngentacropprotection.com/). Mesotrione was applied as described in Oliveira *et al.* (2017). Hybrids were assessed as dead or alive 21 days after mesotrione application, and the number of surviving plants was recorded. In general, surviving hybrid seedlings showed 40% to 85% injury to mesotrione, using a scale rate of 0% to 100% (no injury to plant death), as described by Oliveira *et al.* (2018a,b). Leaf tissue of surviving hybrid seedlings was collected and stored at −80°C to be used for KASP assay analysis.

Competitive allele-specific PCR (KASP) assays. DNA was extracted from four parental S *A. palmeri* seedlings, four parental R *A. tuberculatus* plants, and 86 putative hybrids that survived mesotrione treatment at 1.75 times mesotrione field rate (phenotypic marker). DNA was extracted using a modified cetyl trimethylammonium bromide method (Doyle, 1987) described in detail by Patterson *et al.* (2017). All DNA was then diluted to 5 ng μl^{-1} for the KASP assays.

Two KASP assays were used to determine whether individuals had S *A. palmeri*, R *A. tuberculatus* or hybrid (heterozygous) genotypes. The first SNP was located at base pair 678 in the ALS coding sequence and has been used previously as a RFLP marker (Tranel *et al.*, 2002) and as a KASP marker (Küpper *et al.*, 2017). The second marker is a 2-bp polymorphism in the ITS of the ribosomal coding region (TBP-ITS), designed to distinguish *A. palmeri* from eight other *Amaranthus* species (Table S4). This polymorphism is at base pairs 496 and 497 in the ITS sequence from *A. palmeri* accession KP318856.1 of the National Center for Biotechnology Information (NCBI) nucleotide database (Figure 6). At this locus, *A. palmeri* has an adenosine (A) followed by a guanine (G), while all other species in the *Amaranthus* genus have a cytosine (C) followed by an adenosine (A).

The protocol detailed in Küpper *et al.* (2017) was used for the ALS marker. In brief, the ALS-SNP assay used the following primers: *A. tuberculatus* forward primer, 5′-GAAGGTGACCAAGTTCATGCTAAAAAGAAAGCTTCCTTAACAATTCTAGGG-3′ (FAM Tag underlined); *A. palmeri* forward primer, 5′-GAAGGTCGGAGTCAACGGATTAAAAAGAAAGCTTCCTTAACAATTCTAGGA-3′ (HEX Tag underlined); universal reverse primer, 5′-GTTGAGGTAACTCGATCCATTACTA AGC-3′. For the TBP-ITS, two species-diagnostic forward primers were developed that were identical, except for the final two 3′ nucleotides, which pair with base pairs 496 and 497. Additionally, each forward primer was tagged at its 5′ end with nucleotides that are specific for either a HEX- or FAM-labeled oligo that comes pre-mixed in KASP Master Mix (LGC Genomics, https://www.lgcgroup.com/). For the TBP-ITS assay we used the following primers: *A. tuberculatus* forward primer, 5′-GAAGGTCGGAGTCAACGGATTCGGGCGTGGATGGCCTAAAACA-3′ (FAM Tag

Figure 6. Alignment of internal transcribed spacer (ITS) of the ribosomal coding region from nine *Amaranthus* species.
The alignment indicates (^^) the 2-bp polymorphisms that differentiate *A. palmeri* from eight common *Amaranthus* species. Source: National Center for Biotechnology Information (NCBI).

underlined); *A. palmeri* forward primer, 5′-GAAGGTGACCAAGTTCATGCTCGGGCGTGGATGGCCTAAAAAG-3′ (HEX Tag underlined); universal reverse primer, 5′-ACCAATCGCCGCAGCAGC-3′.

For each assay, a primer mix was first generated and then added to the LGC Genomics Master Mix at the proper concentration. For the initial primer mix, both forward primers and the universal reverse primer were mixed as follows: 18 μl of 100 μM *A. tuberculatus* forward primer, 18 μl of 100 μM *A. palmeri* forward primer, 45 μl of 100 μM universal reverse primer. The primer mix was then diluted to 150 μl using 10 mM TRIS-HCl (pH 8.3). The final reaction mix contained 11.8 μl of primer mix and 432 μl of LGC Genomics Master Mix. Final reactions were performed in a 96-well optically clear plate using 4 μl of *Amaranthus* spp. DNA at 5 ng μl^{-1} with 4 μl of LGC Genomics Master Mix plus primers.

The PCR for the ALS-SNP was performed on a CFX Connect® real-time thermocyler (Bio-Rad Laboratories, http://www.bio-rad.com/) with the following cycling protocol: 94°C for 15 min; followed by 10 cycles of 94°C for 20 sec, 61 decreasing to 55°C for 60 sec (0.6°C touchdown per cycle); followed by 26 cycles of 94°C for 20 sec and 55°C for 60 sec. The PCR for the TBP-ITS was performed using the following protocol: 94°C for 15 min; followed by 10 cycles of 94°C for 20 sec, 63°C decreasing to 56°C for 60 sec (0.6°C touchdown per cycle); followed by 24 cycles of 94°C for 20 sec and 57°C for 60 sec. An end-point fluorescence reading was taken for each plate by cooling the plate to 30°C for 30 sec and reading the plate in both the HEX and FAM fluorescent channels.

The HEX and FAM fluorescence for each individual data point were transformed to percentage of the maximum and minimum fluorescence for each fluorophore within a plate as

$$\%F = \frac{X - \min.F}{\max.F - \min.F} \quad (1)$$

where %*F* is the relative fluorescence value (RFU) of HEX or FAM fluorescence, *X* is the HEX or FAM fluorescence of an individual data point and min. *F* and max. *F* are the lowest and highest HEX or FAM fluorescence signal from any reaction in a 96-well plate.

Data analysis. A clustering algorithm (*k*-means) was performed to group the data based on their HEX and FAM similarity for ALS-SNP and TBP-ITS assays. The *k*-means algorithm randomly assigns each individual data point to a cluster (Hartigan and Wong, 1979). Four known clusters ($k = 4$) were specified, including *A. palmeri* and R *A. tuberculatus*, hybrids and NTC. The cluster goodness of fit (GOF) was calculated as

$$\text{GOF} = \frac{bSS}{tSS} \quad (2)$$

where *bSS* is the between sum of squares for the four clusters and *tSS* is the total sum of squares of the cluster analysis. *K*-means minimize the within-group dispersion and maximize the between-group dispersion (Hartigan and Wong, 1979). A GOF value of 1 indicates that the total variance in the data set is explained by the clustering.

The frequency and estimated number of hybrids combining phenotypic and molecular markers were calculated combing field and laboratory work as follows:

$$\text{Frequency Hybridization} = \frac{T * A}{E} \quad (3)$$

where *T* is the total frequency of hybrids detected in the 86 hybrid samples of KASP analysis for each year, *A* is the number of living putative hybrid seedlings after mesotrione application in each distance and year and *E* is the total number of emerged hybrid seedlings that were treated with mesotrione each year.

Intraspecific hybridization (*A. tuberculatus* × *A. tuberculatus*) diagnostic marker

Seeds of the F_1 generation collected from the field study were planted separately in plastic trays (51 cm × 38 cm × 10 cm) containing peat:soil:sand:vermiculite (4:2:2:2) potting mix and evaluated for mesotrione resistance.

The F_1 mesotrione screening was performed under greenhouse conditions in 2015 and 2016 at the University of Nebraska–Lincoln. The greenhouse conditions were similar to those described by Oliveira *et al.* (2017). Also, there was high variability of plant germination in the trays. For example, in each tray germination of F_1 individuals ranged from 0 to 152 seedlings, with an average of 52 plants per tray. There were three and six replications from the study in 2014 and 2015, respectively. The F_1 individuals were sprayed when they reached a height of 5–8 cm with 210 g ai ha^{-1}, which is twice (intraspecific hybridization only) the mesotrione field rate. Mesotrione treatment was applied as described in Oliveira *et al.* (2018a,b). In each plastic tray (experimental unit), seedlings were assessed as dead or alive 21 days after mesotrione application.

Data analysis. An exponential decay curve is the best for describing PMGF studies biologically. In such a scenario, the frequency of outcrossing events is high near to the pollen donor, decreases rapidly with increasing distances from the pollen source and is leptokurtic in distribution (Mallory-Smith *et al.*, 2015).

The frequency of PMGF ranged from 0 to 1, and followed a binomial distribution (dead or alive). The statistical analysis for PMGF was performed using the gnm function of the generalized

non-linear models (package gnm) in R statistical software (Turner and Firth, 2015). Fourteen models were tested for describing the intraspecific frequency of gene flow (Table S2). The Akaike information criterion (AIC) was used to select the top model to describe the frequency of intraspecific gene flow in *A. tuberculatus*. According to the AIC criterion, the top model has the lowest AIC value (Oliveira *et al.*, 2018a,b).

Based on the AIC, the top model was a double exponential decay (Tables S2 and S3), with the F_{GF} in F_1 varying with distance from the pollen-source block, the direction of the pollen-receptor block and the year of the experiment:

$$\text{logit}(p_i) = I_0 + \exp[I_1 + d_1 \times \text{Distance}] + \exp[I_2(\text{Direction:Year}) + d_2(\text{Direction:Year}) \times \text{Distance}] \quad (4)$$

where p_i is the F_{GF} in the ith observation, I_0 is the overall intercept, I_1 and I_2 are intercepts of the first and second exponential instances and d_1 and d_2 are the decay rates. In this model, I_2 and d_2 vary with direction and year.

The distances for 50% and 90% F_{GF} reduction were estimated from equation (4) in each of the eight directions of the pollen-receptor block. The ME was calculated to test the goodness of fit of the top model for each direction (Werle *et al.*, 2014):

$$\text{ME} = 1 - \left[\frac{\sum_{i=1}^{n}(Oi - Pi)^2}{\sum_{i=1}^{n}(Oi - \bar{O}i)^2}\right] \quad (5)$$

where n is the number of data points, Oi is the observed value, Pi is the predicted value and $\bar{O}i$ is the mean observed value. The ME values range from $-\infty$ to 1, with values closer to 1 indicating better predictions.

Power analysis

A power analysis for binomial probabilities was performed to determine the statistical precision of hybridization with the sample size used in this experiment. The theoretical values used were 0.5% and 1% at $\alpha = 0.05$ for interspecific and intraspecific hybridization, respectively. The theoretical hybridization frequencies were compared with the observed hybridization frequencies as described in Jhala *et al.* (2011).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Frequency of gene flow predicted with a double exponential model.

Figure S2. Wind rose of wind frequency (%) and wind speed (m s^{-1}) in 2014.

Figure S3. Wind rose of wind frequency (%) and wind speed (m s^{-1}) in 2015.

Figure S4. Inflorescences of *Amaranthus tuberculatus* (male and female) and *Amaranthus palmeri* (female).

Figure S5. Methods for detecting the interspecific hybrids.

Table S1. Weather data (30-year average) at the experimental site.

Table S2. Candidate models for describing intraspecific hybrids.

Table S3. Coefficient estimation from a double-exponential decay model.

Table S4. *Amaranthus* species accession from the alignment of the internal transcribed spacer region.

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