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Inheritance of 2,4-dichlorophenoxyacetic acid (2,4-D) resistance in *Amaranthus palmeri*

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In this study, the inheritance of 2,4-D resistance in a multiple herbicide-resistant Palmer amaranth (KCTR) was investigated. Direct and reciprocal crosses were performed using 2,4-D-resistant KCTR and susceptible KSS plants to generate F_1 progenies. 2,4-D dose–response assays were conducted to evaluate the response of progenies from each F_1 family along with KCTR and KSS plants in controlled environmental growth chambers. Additionally, 2,4-D-resistant male and female plants from each of the F_1 families were used in pairwise crosses to generate pseudo-F_2 families. Segregation (resistance or susceptibility) of progenies from the F_2 families in response to a discriminatory rate of 2,4-D (i.e., 560 g ae ha^{-1}) was evaluated. Dose–response analysis of F_1 progenies derived from direct and reciprocal crosses suggested that the 2,4-D resistance in KCTR is a nuclear trait. Chi-square analyses of F_2 segregation data implied that 2,4-D resistance in KCTR is controlled by multiple gene(s). Overall, our data suggest that the 2,4-D resistance in KCTR Palmer amaranth is a nuclear inherited trait controlled by multiple genes. Such resistance can spread both via pollen or seed-mediated gene flow. In future, efforts will be directed towards identifying genes mediating 2,4-D resistance in KCTR population.

The introduction of herbicides in the 1940s revolutionized weed management in global agriculture. Herbicides are the primary tools used by growers to manage weeds in different cropping systems as well as in non-crop areas and home lawns. However, over-reliance on herbicides has resulted in the evolution of herbicide resistant weeds in many agriculturally important crops and cropping systems. There are currently 509 unique cases of herbicide resistance across 266 weed species worldwide, including 153 dicots and 113 monocots[1]. A critical aspect of the evolution of herbicide resistance in weeds is understanding how the resistance is inherited and spread[2]. Genetic factors, such as the degree of dominance of the trait and the number of alleles controlling the trait can impact how the trait can spread, which in turn will influence the weed management strategies[3]. For instance, herbicide resistance conferred by dominant gene(s) is expressed in both homozygous and heterozygous states, and therefore, can spread fast. In contrast, recessive traits can only express in a homozygous state and are slower to spread than dominant trait(s). If herbicide resistance is conferred by a single gene, it can spread faster compared to polygenic resistance traits, which require multiple recombination events during meiosis to accumulate multiple alleles (unless they are linked and inherit together). Knowledge of the inheritance of herbicide resistance in weeds can help understand the evolution of resistance under selection pressure[4].

Herbicide resistance mechanisms in weeds/crops are broadly grouped into two categories, *i.e.*, target-site resistance (TSR) and non-target site resistance (NTSR). TSR mechanisms directly involve the modification to herbicide target and usually follow the Mendelian single gene inheritance model. In contrast, the NTSR mechanisms do not affect herbicide target, instead, alter the physiological processes involved in herbicide action[5]. Metabolism of herbicides, a common NTSR mechanism is reported to be conferred primarily by multiple genes; albeit in rare cases by a single gene[6–8].

2,4-dichlorophenoxyacetic acid (2,4-D) is a synthetic auxinic herbicide (SAH) commonly used for controlling dicot weeds in cereal crops such as corn (*Zea mays* L.), wheat (*Triticum aestivum* L.), and sorghum (*Sorghum bicolor* L.). Despite being in use in agriculture for more than seven decades, the evolution of resistance to 2,4-D has been low. However, in the past decade, over-reliance on 2,4-D for controlling acetolactate synthase (ALS)- and 5-enol pyruvyl shikimate phosphate synthase (EPSPS)-inhibitor-resistant weeds, has led to an increase in

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F$_1$-family/population	b (SE)	d (SE)	e (SE)	RI
F$_1$-1	0.76 (0.12)	99.60 (4.57)	837.31 (162.98)	4.3
KCTR	0.65 (0.13)	100.74 (5.32)	1800 (566.45)	9.3
KSS	0.81 (0.16)	99.68 (5.52)	194 (46.96)	–

Table 1. Regression parameters describing the response of F$_1$-1 progenies along with 2,4-D susceptible (KSS), resistant (KCTR) Palmer amaranth to 2, 4-D under growth chamber conditions (Eq. 2).

weed resistance to SAHs. As of 2021, globally, 42 weeds are resistant to SAHs, amongst, 25 weeds have evolved resistance to 2,4-D[1]. This limit options for managing dicot weeds in cereal cropping systems. Moreover, increased adoption of commercialized 2,4-D-tolerant crop technology poses the risk of increased 2,4-D selection pressure, in several weeds[9].

Inheritance of 2,4-D resistance has been studied in several economically important weeds around the globe, such as prickly lettuce (*Lactuca serriola* L.), wild radish (*Raphanus raphanistrum* L.), wild mustard (*Sinapis arvensis*), and oriental mustard (*Sisymbrium orientale* L.)[6,10–12]. So far, the inheritance pattern of SAHs such as 2,4-D resistance has varied depending on the resistance mechanism and the weed species involved. For instance, 2,4-D resistance in prickly lettuce was governed by a single co-dominant allele[12]. Similarly, in wild mustard (*Brassica kaber* L.) a single gene was found to mediate 2,4-D resistance[10]. However, polygenic inheritance was documented for MCPA resistance in hemp-nettle (*Galeopsis tetrahit* L.)[13].

Palmer amaranth (*Amaranthus palmeri* S. Watson), a native of Mexico and the Southern United States, is one of the most common and troublesome weeds in the United States[14]. So far, Palmer amaranth has evolved resistance to 8 herbicide sites of action (SOAs) [1]. The reproductive biology of Palmer amaranth plays a crucial role in its ability to evolve resistance to herbicides. For instance, Palmer amaranth is a dioecious plant, making cross-pollination a necessity to produce seeds[15,16]. Female Palmer amaranth plants are characterized by high fecundity resulting in the production of abundant seeds per plant[17,18].

We documented a multiple resistant population of Palmer amaranth (Kansas Conservation Tillage resistant; KCTR) from Kansas which has evolved resistance to six herbicides, including 2,4-D[19,20]. Furthermore, recently we reported enhanced metabolism of 2,4-D in KCTR compared to KSS and MSS indicating metabolic resistance, even though research on possible co-existence of TSR is warranted[19]. Although resistance to 2,4-D in Palmer amaranth has been documented in two populations in Kansas[19–21], the information on the inheritance of 2,4-D resistance is lacking. This study was conducted to investigate the inheritance of 2,4-D resistance in KCTR Palmer amaranth. The specific objectives were: i) generate F$_1$ progenies and evaluate their response to 2,4-D application, and ii) generate F$_2$ progenies and determine if 2,4-D resistance in KCTR is conferred by either single or multiple genes.

Results

2,4-D dose–response with KCTR, KSS, and F1 progenies.

Response of progenies from F$_1$ families and parental Palmer amaranth populations, KCTR and KSS to 2,4-D was assessed via dose-responses experiments. Our data indicates that 90% of KCTR and 15% of KSS plants survived the discriminatory dose (1120 g ae ha^{-1}) of 2,4-D. The KSS plants which survived 2,4-D treatment, showed severe epinasty (downward curling of plant parts, a typical symptom of auxinic herbicide injury) and stayed stunted and did not show any new growth up to 4WAT. Nonetheless, several KCTR Palmer amaranth plants survived even the highest dose used in the study (2240 g ae ha^{-1}). Compared to that, 87% of F$_1$-1, 92% of F$_1$-2, and 89% of F$_1$-3 survived application of 2,4-D at 1120 g ae ha^{-1}. The F$_1$ plants in all the three families, generated by both direct or reciprocal crosses survived 2,4-D application, implying that the resistance to 2,4-D in KCTR Palmer amaranth is a nuclear trait. In the F$_1$-1 dose–response experiments, the amount of 2,4-D required to cause 50% reduction in growth (GR$_{50}$) of KCTR, F$_1$-1 and KSS were 1800, 837, and 194 g ae ha^{-1}, respectively (Table 1; Fig. 1), suggesting that the KCTR and F$_1$-1 are 9- and fourfold resistant to 2,4-D compared to KSS (Table 1). Similar results were obtained from F$_1$-2 and F$_1$-3 dose–response experiments (Tables 2, 3; Figs. 2, 3). The GR$_{50}$ of KCTR, KSS, and F$_1$-2 plants were 1188, 501, and 200 g ae ha^{-1}, respectively (Table 2; Fig. 2), indicating that the KCTR and F$_1$-2 plants were 6- and threefold resistant, respectively, to 2,4-D treatment compared to KSS. Likewise, the GR$_{50}$ of KCTR, KSS, and F$_1$-3 were 1317, 500, and 167 g ae ha^{-1}, respectively (Table 3; Fig. 3), with 7 and 3 RI compared to KSS (Table 3). P-values from the t-test for each F$_1$ family are presented in supplementary material (Table S1). Overall, the results of 2,4-D dose–response experiments suggest an intermediate response of F$_1$ progenies compared to KCTR and KSS plants.

Further, cumulative dry weight data from the F$_1$ dose–response assays were plotted for KSS, KCTR, and F$_1$ plants at each 2,4-D dose (Fig. 2). The spread and distribution of dry weight of non-treated (NT; 0 g ae ha^{-1}) KSS, KCTR, and the F$_1$ plants were similar (Fig. 2a). At doses up to 2× (1120 g ae ha^{-1}), F$_1$ and KCTR plants had visually similar distribution (Fig. 2b–e), however, at 4× dose (4×; 2240 g ae ha^{-1}), KCTR plants had a wider spread than F$_1$ plants (Fig. 2f), indicating higher level of resistance compared to F$_1$ progenies.

Segregation (2,4-D resistance or susceptibility) of F2 progenies.

Response of progenies from F$_2$ families and KSS population (as control) were evaluated by treating with 2,4-D at the field recommended dose of 560 g ae ha^{-1}. Unfortunately, as mentioned in the methods, the KCTR plants were not used in this experiment along with KSS Palmer amaranth due to lack of enough seed and poor germination at the time of conducting this
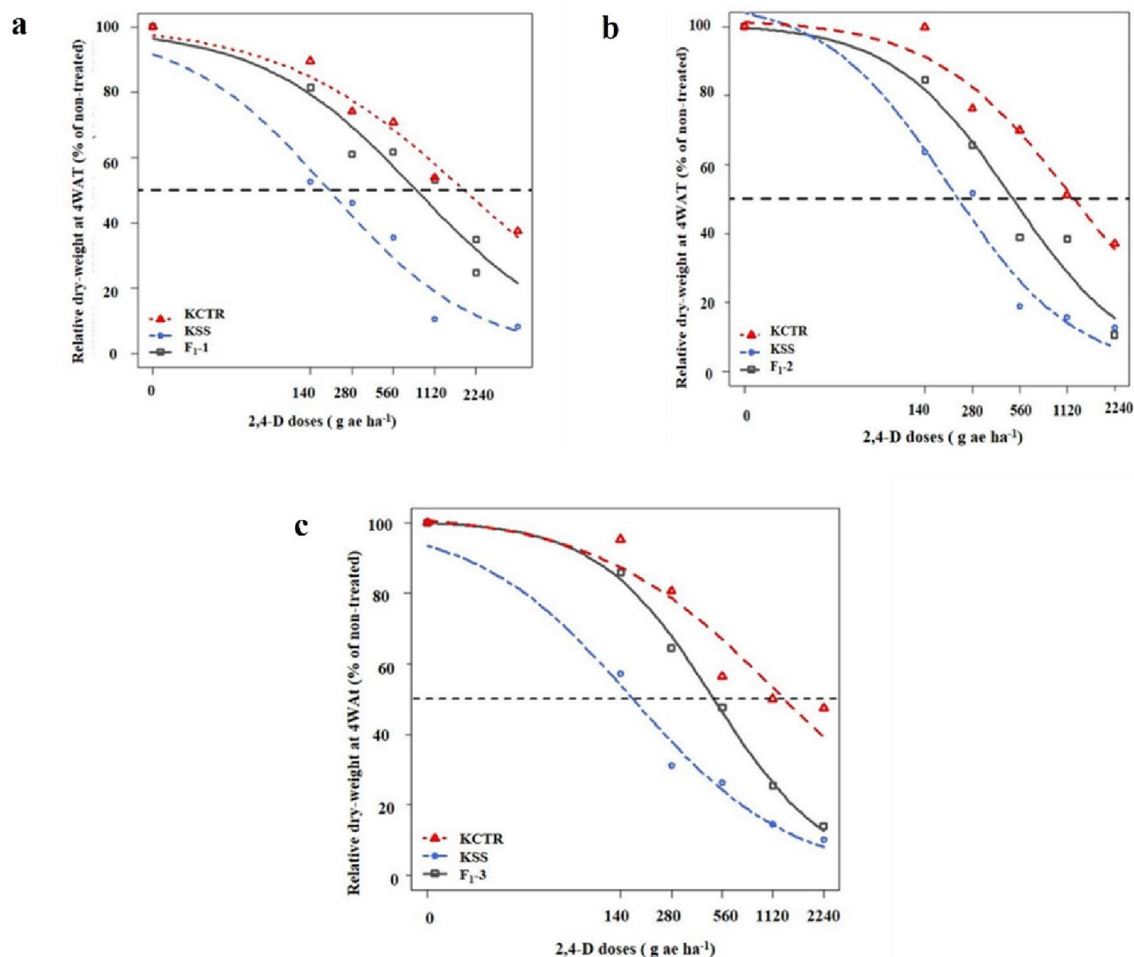
Figure 1. Dose–response curves representing the level of 2,4-D resistance in (**a**) F_1-1, (**b**) F_1-2, and (**c**) F_1-3 progenies compared to parental 2,4-D susceptible (KSS), and 2,4-D resistant (KCTR) Palmer amaranth. Relative shoot dry weight (% of non-treated) of seedlings were analyzed using the three-parameter log-logistic regression model (Eq. 2) at four weeks after treatment. Points in each plot indicate experimental response of the F_1 plants to respective rates of 2,4-D. Dotted arrow at the center of the plots represents the 50% of relative dry weight.

F_1-family/population	b (SE)	d (SE)	e (SE)	RI
F_1-2	1.13 (0.23)	100.80 (6.71)	501.18 (106.62)	2.5
KCTR	0.99 (0.23)	102.11 (6.69)	1188.02 (288.70)	5.9
KSS	1.09 (0.29)	107.87 (8.83)	200.02 (53.59)	–

Table 2. Regression parameters describing the response of F_1-2 progenies along with 2,4-D susceptible (KSS), resistant (KCTR) Palmer amaranth to 2, 4-D under growth chamber conditions (Eq. 2).
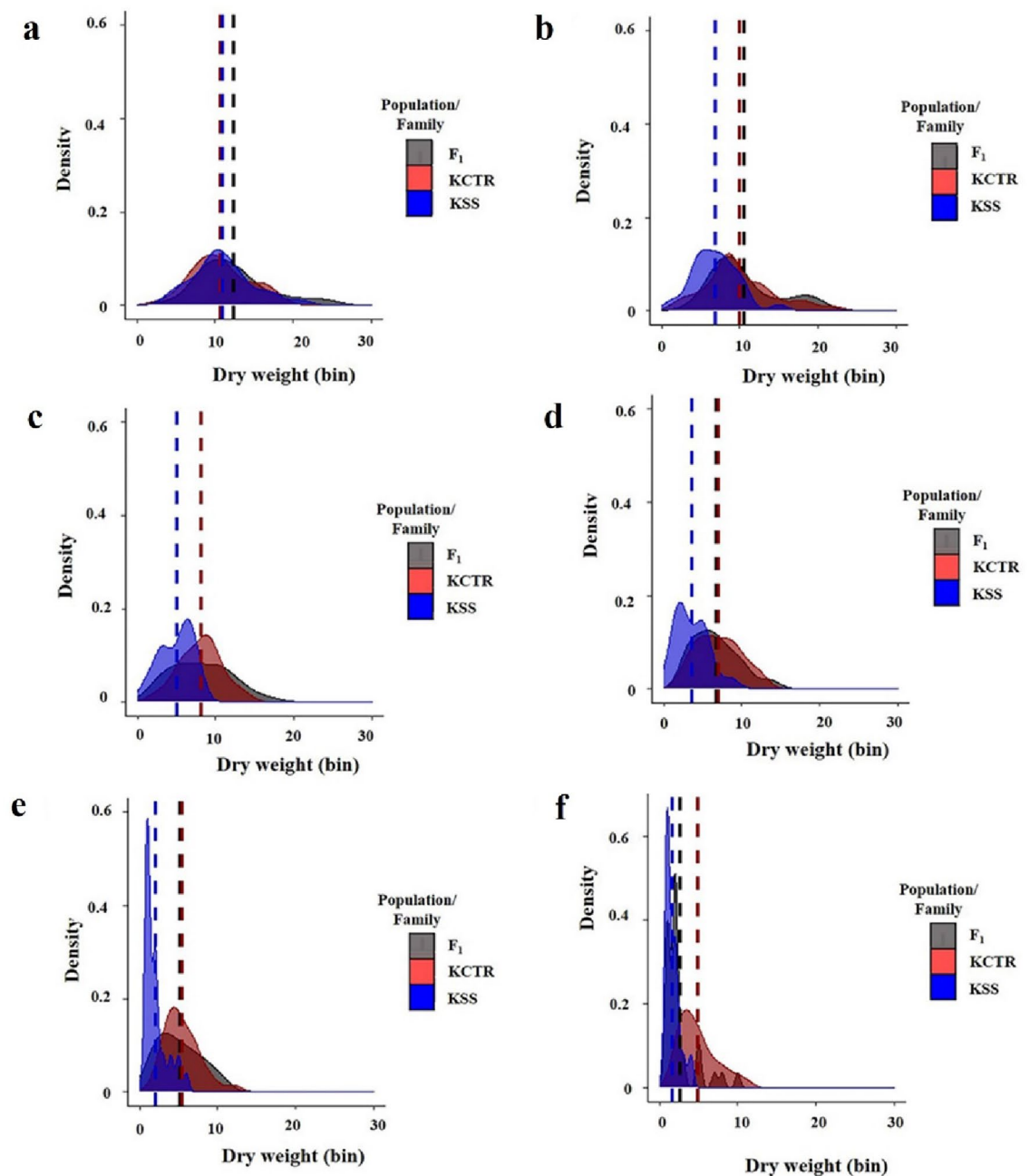
F_1-family/population	b (SE)	d (SE)	e (SE)	RI
F_1-3	1.27 (0.24)	100.61 (6.07)	500.2 (90.43)	3.0
KCTR	0.80 (0.17)	102.74 (5.61)	1316.97 (343.25)	7.9
KSS	0.93 (0.22)	100.31 (6.61)	166.50 (43.50)	–

Table 3. Regression parameters describing the response of F_1-3 progenies along with 2,4-D susceptible (KSS), resistant (KCTR) Palmer amaranth to 2, 4-D under growth chamber conditions (Eq. 2).

Figure 2. Cumulative dry weight distribution of 2,4-D susceptible (KSS), resistant (KCTR), Palmer amaranth and F_1 progenies in response to 2,4-D at (**a**) 0 (non-treated) (**b**) 140, (**c**) 280, (**d**) 560 (field recommended dose), (**e**) 1120, and (**f**) 2240 g ae ha^{-1} via density plots. Dry weight of samples was converted into bins and plotted in the X-axis. Y-axis was plotted by calculating density which is the proportion of total plants in each bin. Dotted lines represent mean dry weight of KSS, KCTR, and F_1 plants.

experiment. As expected, upon treatment with 560 g ae ha^{-1} of 2,4-D, all KSS plants (n = ~50), either completely died or had dried leaves and stunted growth at 4 WAT (Table 4). Interestingly, the percent survival of F_2 progenies highly varied among families. The phenotypes of F_2 progenies ranging from high resistance (plants showing low epinasty and high dry weight accumulation to very low-level resistance (plants showing high epinasty and low dry weight accumulation) were observed (Fig. 4). In the first run of the segregation assay, all F_2 families deviated from the expected 3:1 (resistance:susceptibility) segregation ratio (Table 4). In the second run of the segregation assay, except for the F_2-1 family, all other families did not follow the 3:1 (resistance and susceptible) segregation ratio (Table 4). Overall, when combined (run 1 and 2; Table 4) all F_2-families failed to segregate as 3:1 (resistance and susceptible) ratio, indicating that more than one gene(s) is involved in mediating 2,4-D resistance in KCTR.
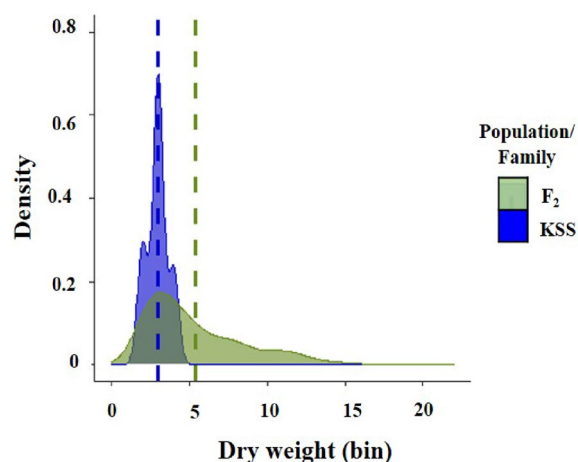
Figure 3. Cumulative dry weight data distribution of 2,4-D susceptible (KSS; n = 21) and F_2 progenies (n = 332) in response to 2,4-D at 560 g ae ha^{-1} (field recommended dose) via density plot. Dry weight of samples was converted into bins and plotted on the X-axis. Y-axis was plotted by calculating density which is the proportion of total plants in each bin. Dotted lines represent mean dry weight of 2,4-D susceptible (KSS) and F_2 plants.

Run	F_2 family/population	Plants treated	Observed survival		Expected survival		p-value
			Alive	Dead	Alive	Dead	
1	F_2-1	72	30	42	54	18	< 0.00001
	F_2-2	101	56	45	75.75	25.25	< 0.00001
	F_2-3-1	74	46	28	55.5	18.5	0.01076
	F_2-3-2	53	25	28	39.75	13.25	< 0.00001
	KSS	31	0	31	–	–	–
	Total	300	157	143	225	75	< 0.00001
2	F_2-1	92	63	29	69	23	0.14856
	F_2-2	20	9	11	15	5	0.00195
	F_2-3-1	124	79	45	93	31	0.00369
	F_2-3-2	96	46	50	72	24	< 0.00001
	KSS	20	0	21	–	–	–
	Total	332	197	135	249	83	< 0.00001
1 & 2 combined	F_2-1	164	93	71	123	41	< 0.00001
	F_2-2	121	65	56	90.75	30.25	< 0.00001
	F_2-3-1	198	125	73	148.5	49.5	0.00011
	F_2-3-2	149	71	78	111.75	37.25	< 0.00001
	KSS	51	0	51	–	–	–
	Total	632	354	278	474	158	< 0.00001

Table 4. Chi-square test for goodness of fit of the observed segregation of plants as resistance or susceptible (Eq. 4) to 2,4-D to the expected frequency for a single-locus model in pseudo-F_2 families of Palmer amaranth.

The distribution of shoot dry weight per plant (g) of the F_2 progenies (n = 332) and KSS plants (n = 21) was illustrated through a density plot (Fig. 3). The distribution showed a wide spread of dry weight bins for F_2 progenies representing high plant to plant variation in response to 2,4-D treatment (Fig. 3).

Discussion

The evolution of 2,4-D resistance in Palmer amaranth has been reported in two populations, from Kansas, USA, including, KCTR[19–21]. KCTR has been documented to have evolved predominantly metabolic resistance to at least five herbicide SOAs including ALS-, photosystem (PS) II-, protoporphyrinogen oxidase-, 4-hydroxyphenylpyruvate dioxygenase- inhibitors and SAHs[20]. Information on the genetic basis of 2,4-D resistance in Palmer amaranth is lacking. Therefore, in the current study, we investigated the inheritance of 2,4-D resistance in KCTR.

In the dose–response assays, KCTR plants, as expected showed a high level of 2,4-D resistance (6–ninefold) compared to KSS plants (Fig. 1a–c; Tables 1, 2, 3). Progenies generated by both direct and reciprocal crosses were resistant to 2,4-D, indicating that the 2,4-D resistance in KCTR is a nuclear trait (Fig. 1; Tables 1, 2, 3). In general,
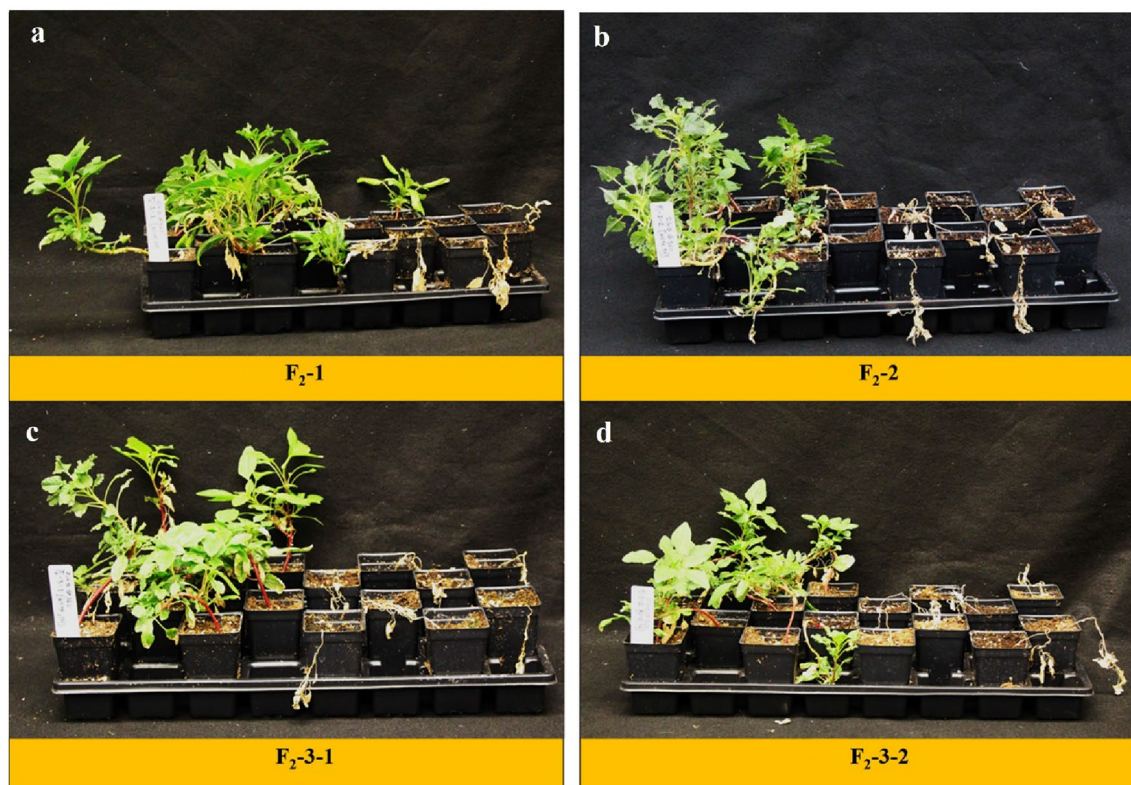
Figure 4. Diverse phenotype of F_2 progenies from family (**a**) F_2-1, (**b**) F_2-2, (**c**) F2-3-1, and (**d**) F_2-3-2 in response to 2,4-D at 560 g ae ha^{-1} (field recommended dose) at 4 weeks after treatment.

resistance to majority of herbicides is a nuclear trait. One exception is the TSR to PS II-inhibitors in weeds, which is inherited maternally/cytoplasmically[22]. Nuclear inheritance of 2,4-D inheritance has been reported in several weeds, including wild radish, wild mustard, oriental mustard, and prickly lettuce[6,10–12]. Nuclear traits are transferred by both seed and pollen of resistant plants. Previously, pollen-mediated intra- and interspecific transfer of TSR and NTSR traits have been documented in *Amaranthus* sp.[15,16,23–26]. Therefore, there is a potential of inter-and intra-specific transfer of 2,4-D resistance via pollen-mediated gene flow.

The results of F_1 dose–response assay showed an intermediate level of 2,4-D resistance in all F_1 families compared to the parental resistant (KCTR) and susceptible (KSS) plants (Figs. 1, 2; Tables 1, 2, 3). Analyses of data from both dose–response studies and distribution plotting of cumulative biomass of KCTR, KSS and F_1 progenies (Figs. 1, 2; Tables 1, 2, 3) confirmed that 2,4-D resistance in KCTR Palmer amaranth is a nuclear trait and transferred to progenies generated from both direct and reciprocal crosses. Most cases of herbicide resistance, including TSR or NTSR, have been reported to be controlled by either completely or incompletely dominant genes[6,11,26,27]. The 2,4-D resistance in wild radish was found to be incompletely dominant [6]. In contrast, inheritance of 2,4-D resistance in wild radish and dicamba resistance in kochia (*Bassia scoparia* L.) were found to be mediated by highly dominant alleles[6,28,29]. Both dominant and incompletely dominant traits can be expressed in homo- or heterozygosity, therefore, the spread of 2,4-D resistance will be faster than a recessive trait.

Segregation of F_2 progenies in KCTR Palmer amaranth at the field recommended dose of 2,4-D (560 g ae ha^{-1}) did not follow the Mendelian 3:1 (resistance: susceptible) ratio (expected for a single gene inheritance) (Table 4). This indicates that the 2,4-D resistance in KCTR Palmer amaranth is a polygenic trait. Furthermore, the chi-square goodness of fit test data suggests variability in the phenotypes among the F_2 resistant progenies, which cannot be explained by a single major gene-mediated resistance (Fig. 3). Although majority of TSR is controlled by a single gene[30,31], the inheritance of NTSR was reported to be conferred by both single and multiple genes[7,8,25–27]. For instance, monogenic metabolic resistance to chlorsulfuron and atrazine was documented in common waterhemp (*Amaranthus tuberculatus* Moq. Sauer.), Palmer amaranth, and sorghum[7,20,26,27,32]. Polygenic inheritance was reported for mesotrione resistance in common waterhemp and Palmer amaranth[7,26,27,32]. Similar to our findings, MCPA resistance in hemp-nettle was found to be polygenic[13]. On the other hand, single-gene inherited 2,4-D resistance was reported in wild mustard, oriental mustard, prickly lettuce[10–12]. Traits inherited by multiple genes spread slower than those with single alleles.

Our previous work indicates that KCTR Palmer amaranth evolved metabolic resistance, possibly mediated by cytochrome P450 activity[19,20]. In other 2,4-D, resistant weed species, such as common waterhemp metabolic resistance mediated by P450 activity has been reported[33,34]. Increased expression of a P450 cluster (*P450-81E8*) was recently reported in 2,4-D-resistant common waterhemp populations from both Nebraska and Illinois[35]. We have reported increased expression of this gene in KCTR plants with metabolic resistance to tembotrione[36]. Therefore, there is a need to check expression levels of this gene in 2,4-D resistant KCTR plants. Our findings

suggest more than one gene confers 2,4-D resistance in KCTR plants. Therefore, it is possible that a combination of P450s or other genes may be involved in metabolic resistance to 2,4-D in KCTR Palmer amaranth. Metabolic resistance to herbicides usually follows a three-step detoxification process, including several genes facilitating herbicide conversion, degradation, and transporting or compartmentalizing of degraded molecules into the vacuole of a plant cell. Metabolic resistance might also involve genes that help in plant recovery after herbicide treatment. More in-depth investigation is needed to decipher the genes involved in the metabolic resistance of 2,4-D in KCTR Palmer amaranth.

Materials and methods

Plant materials and growth conditions. 2,4-D-resistant (KCTR) and susceptible (KSS; Kansas Susceptible) Palmer amaranth populations were used in this study. KCTR population was originally collected in 2018 from a long-term conservation tilled field with continuous sorghum production in Riley County, Kansas[20]. Although the original field collected seed of KCTR was found resistant to multiple herbicides, including 2,4-D, we selected 2,4-D survivors by treating the plants with 2,4-D at 560 g ae ha^{-1}. These plants were kept in isolation under greenhouse conditions to produce next generation of seeds. This process was repeated for two generations so that the seed used in this study were more homogenous in nature with respect to 2,4-D resistance. KSS population is known to be susceptible to 2,4-D and was also collected from a nearby field in Riley County, Kansas and has been characterized previously[20,23,37,38]. Experiments to generate F_1 and F_2 progenies for this study were conducted either in greenhouse or controlled environmental growth chambers. The following greenhouse conditions were maintained: 30/24 °C (d/n) temperature and 16/8 h (d/n) photoperiod with natural light supplemented with 250 μmol m^{-2} s^{-1} illumination by sodium vapor lamps. Growth chambers were maintained at 32.5/22.5 °C (d/n) with a photoperiod of 15/9 h (d/n) with 60 ± 10% relative humidity and a light intensity of 750 μmol m^{-2} s^{-1} provided by incandescent and fluorescent bulbs[20]. Seeds of KCTR and KSS Palmer amaranth populations were sown in small plastic trays (21 × 6 × 4 cm) with commercial potting mix (Pro-Mix® premium potting mix, Premier Tech Home and Garden Inc., Ontario, CA) in the greenhouse and allowed to emerge. Palmer amaranth seedlings with 3–4 leaves were transplanted into small pots (6 × 6 × 6.5 cm) for herbicide treatment. Plants were watered as needed (procedure for herbicide treatment is given later).

Generation of F_1 and F_2 families of KCTR Palmer amaranth. Ten-12 cm tall KCTR seedlings (n = 20) were treated when 10–12 cm tall with 2,4-D (2,4-D 4L Amine, Winfield Solutions, LLC., St. Paul, Minnesota, USA) at 1120 g ae ha^{-1} and allowed to grow in the greenhouse. At 4 weeks after treatment (WAT) the survivors were identified and transferred to new pots (15 × 10 × 15 cm) to initiate flowering. Once flowered, healthy male and female KCTR plants were selected for performing direct and reciprocal crosses (Fig. 5). Direct crosses were performed by enclosing female (♀) KCTR (survivors of 2,4-D application) individually with male (♂) KSS (non-treated with 2,4-D) in a pollination bag (Fig. 5). Reciprocal crosses were performed with female (♀) KSS plants (non-treated with 2,4-D) paired with male (♂) KCTR (survivors of 2,4-D application) plants (Fig. 5). Before enclosing the plants, existing flowering parts were removed to ensure crossing between selected plants. Once both plants flowered, male Palmer amaranth plants were gently shaken to disperse pollen and pollinate the stigmas of female plants. At maturity, seeds were collected from each female plant separately, cleaned, and stored at 32 °C in greenhouses to allow over-ripening of the seeds necessary for good germination. In total three F_1 families were generated from these crosses, *i.e.*, F_1-1, F_1-2, and F_1-3 (Fig. 5). Plants from each F_1 family were further screened with 2,4-D (1120 g ae ha^{-1}) to identify female and male F_1 plants resistant to 2,4-D to be used to generate pseudo-F_2 families (Fig. 5). In total four F_2 families were generated from these crosses (F_2-1, F_2-2, F_2-3-1, F_2-3-2; Fig. 5). These progenies from the F_1 and F_2 families were used in further studies.

2,4-D dose–response study using KCTR, KSS, and F_1 progenies. Whole-plant dose–response experiments were conducted with parental KCTR, KSS Palmer amaranth plants, and F_1 progenies generated from three F_1 families. The experiments were conducted in a completely randomized design in growth chambers. KCTR, KSS, and progenies from individual F_1 families were germinated in small trays and transplanted into small pots as described previously. One week after transplanting, the seedlings were transferred to growth chambers (maintained at the same conditions as described in ('Plant Materials and Growth Conditions'). Palmer amaranth seedlings (10–12 cm tall) were treated with 2,4-D at the following doses i.e., 0 (non-treated), 140, 280, 560 (1×; field recommended dose), 1120, and 2240 (4×) g ae ha^{-1}. Thirty minutes after herbicide treatment, plants were returned to the growth chamber. 2,4-D was applied using a bench-track sprayer (Generation III, DeVries Manufacturing, Hollandale, Minnesota, USA) using a flat-fan nozzle tip (8002 Teejet, Spraying Systems Co., Wheaton, Illinois, USA) and was calibrated to spray equivalent to 187 L ha^{-1} (20 GPA). At 4 WAT, plant survival (dead or alive) was assessed at each dose of 2,4-D treatment. Palmer amaranth plants that survived 2,4-D dose (560 g ae ha^{-1} and above) and continued to grow were considered as resistant and those that showed severe epinasty (downward curling of plant parts, a typical symptom of synthetic auxin herbicides) and ceased to grow, were grouped as susceptible to 2,4-D. In response to 2,4-D treatment the plants that survived and Above-ground plant parts were harvested at 4 WAT and oven-dried at 65 °C for at least 72 h. Dose–response experiments with each F_1 family were repeated once, and 3–5 replications were maintained for each dose of 2,4-D. Dry weight was measured and converted into relative dry weight (% of non-treated) as described below (Eq. 1):

$$\text{Relative dry weight (\% of non-treated control)} = \frac{(\text{Dry weight of the sample}) \times 100}{(\text{Average dry weight of non-treated control})} \quad (1)$$
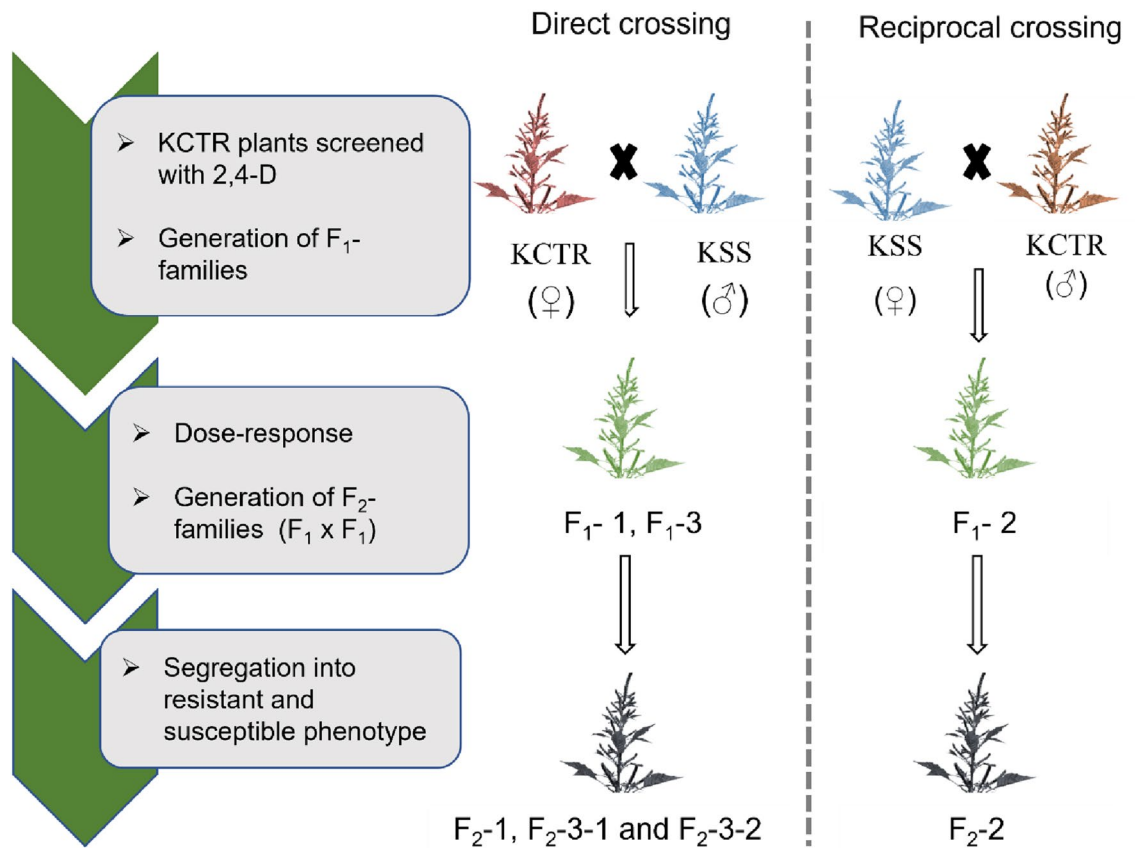
Figure 5. Schematic diagram showing the generation of F_1 and F_2 families of 2,4-D susceptible (KSS) and resistant (KCTR) Palmer amaranth and the experimental steps in the study (created with BioRender.com).

Evaluation of segregation (2,4-D resistance or susceptibility) of F_2 progenies. Seed of F_2-families (F_2-1, F_2-2, F_2-3-1, F_2-3-2) were planted and the seedlings were grown in the same greenhouse as described above. Based on the response of parental populations i.e., KCTR and KSS in greenhouse conditions, the field recommended dose of 2,4-D (560 g ae ha^{-1}) was identified to discriminate resistant (alive) or susceptible (dead) plants. The same dose was used to determine the segregation of F_2 progenies into resistant or susceptible plants. About 20–120 progenies (10–12 cm tall; Table 4) representing each F_2 family were treated with 560 g ae ha^{-1} of 2,4-D. About 20–30 plants of KSS Palmer amaranth were also treated along with the F_2 progenies as a negative control. Unfortunately, the KCTR plants were not included in this assay due to lack of enough KCTR seed and poor germination at the time of conducting this experiment. At 4 WAT, live or dead (resistance or susceptible) phenotype of the 2,4-D-treated plants was documented (Fig. 5). The experiment was repeated. For the second run of the experiment, at 4 WAT, the above-ground dry weight of F_2 plants was harvested, dried, and measured (as described above).

Data analysis. Relative dry weight data from two runs of 2,4-D dose–response experiments of each F_1 family was compared using Levene's test ($\alpha = 0.05$) in R (version 4.0.3, R Core Team, 2020) in an R-studio interface. Since it was non-significant, dry weight data were pooled across two runs. Dry weight datasets for each F_1 family were analyzed using non-linear log-logistic three-parameter '*drc*' models in R[39,40]. The three-parameter regression model used the following formula (Eq. 2):

$$Y = d + \exp\left[b\left(\log x - \log e\right)\right] \tag{2}$$

where Y is the response variable, x is the herbicide dose, d is the upper limit, b is the relative slope around e and e is GR_{50} (amount of 2,4-D required to reduce plant dry weight by 50%). The GR_{50} values of different F_1 families and populations were further tested via t-test using the function '*compParm*' in '*drc*' package to determine differences across the doses. In-built '*plot*' function in the '*drc*' package was used to visualize the dose–response curves. Resistance index (RI) was calculated as a ratio of GR_{50} of KCTR or F_1 families and GR_{50} of KSS population. Cumulative dry weight distribution of the F_1 progenies along with the parental populations (KSS and KCTR) at each dose of 2,4-D was illustrated (as density plot) using '*ggplot2*' package in R[41]. For the plot, dry biomass was represented as bins (each bin represents 0.25 g plant biomass) and proportion of total plants having biomass in that bin was plotted as density.

The number of F_2 progenies observed to have survived after 2,4-D treatment was compared with the expected number of survivors using chi-square analyses. The expected segregation of 2,4-D resistant (alive) or susceptible (dead) phenotypes was determined based on the Mendelian single locus segregation model, which suggests that the 2,4-D resistance in KCTR is controlled by a single gene. The following Eq. (3) was used to test this assumption.

$$\text{Exp. } F_2 = (1 \times \text{Obs. KCTR} + 2 \times \text{Obs. } F_1 + 1 \times \text{Obs. KSS}) \tag{3}$$

In this equation Exp. F_2 is the expected segregation frequency of the F_2 progenies and Obs. is the observed segregation frequency of KCTR, F_1, and KSS phenotypes. Based on previous experiments conducted in greenhouse conditions, the KCTR plants and F_1 progenies were found resistant to 560 g ae ha^{-1} of 2,4-D, while KSS plants were susceptible. This dose was used to determine resistant or susceptible plants among F_2 progenies. Chi-square goodness of fit test (χ^2) was used to analyze the data of the plant segregation as resistance or susceptible to 2,4-D by comparing the observed segregation frequency with the expected. The null-hypothesis (H_0) for the test was that segregation of 2,4-D resistance in F_2 progenies will follow the 3:1 ratio (resistant: susceptible) and p-value from each F_2 family was compared with the significance level of $\alpha = 0.05$. The formula used for the test is (Eq. 4):

$$\chi^2 = \left[(E - O)^2 / E \right] \tag{4}$$

where χ^2 is the Chi-square goodness of fit-value, O and E are the observed and expected segregation frequencies, respectively, of progenies in each F_2 family. H_0 was rejected if the obtained p-value was < 0.05. Dry weight data of the F_2 progenies were also visualized as density plot (each bin represents 0.1 g plant biomass).

Permissions required.
All the seed used was collected by the authors from the experimental field maintained by Dr. Peterson, one of the authors of this manuscript.

Guidelines required.
The collection of Palmer amaranth seed used in this study complies with the institutional guidelines.

Conclusion
Results of this study suggest that 2,4-D resistance in KCTR Palmer amaranth is a nuclear trait, governed by multiple genes. The possible presence of TSR to 2,4-D in KCTR has not been investigated yet and will be interesting future line of work. Future research will also be aimed at discovering metabolic genes that mediate 2,4-D resistance in KCTR. Availability of Palmer amaranth genome[42] and application of transcriptomic approach can enable identifying specific P450s involved in metabolic resistance in weeds. For example, a P450 gene (*P450-81A10v7*) was documented to confer metabolic resistance to at least 5 SOAs in rigid ryegrass (*Lolium rigidum* L.)[43]. Studies directed to investigate if metabolic resistance to 2,4-D is transferred via pollens to other *Amaranthus* sp. can help in formulating management practices specific to *Amaranthus* sp. Overall, 2,4-D has been a viable option to manage ALS- and EPSPS-inhibitor resistant Palmer amaranth; moreover, 2,4-D resistant crop technology has been shown to provide growers with options to effectively manage glyphosate-resistant Palmer amaranth[44]. However, the evolution of resistance to 2,4-D limits the use of this important herbicide by growers. Therefore, the incorporation of integrated weed management techniques will be necessary for reducing the evolution and spread of 2,4-D resistance.

Data availability
The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

References
1. Heap, I. *The International Herbicide-Resistant Weed Database*. Online. https://www.weedscience.org. Accessed 5 Jan 2022 (2022).
2. Preston, C. & Mallory-Smith, C. A. Biochemical mechanisms, inheritance, and molecular genetics of herbicide resistance in weeds. in *Herbicide Resistance and World Grains* (CRC Press, 2001).
3. Maxwell, B. D., Roush, M. L. & Radosevich, S. R. Predicting the evolution and dynamics of herbicide resistance in weed populations. *Weed Technol.* **4**, 2–13 (1990).
4. Menalled, F. *et al.* The eco-evolutionary imperative: Revisiting weed management in the midst of an herbicide resistance crisis. *Sustainability* **8**, 12 (2016).
5. Jugulam, M. & Shyam, C. Non-target-site resistance to herbicides: Recent developments. *Plants* **8**, 417 (2019).
6. Busi, R. & Powles, S. B. Inheritance of 2,4-D resistance traits in multiple herbicide-resistant *Raphanus raphanistrum* populations. *Plant Sci. Int. J. Exp. Plant Biol.* **257**, 1–8 (2017).
7. Kohlhase, D. R., Edwards, J. W. & Owen, M. D. K. Inheritance of 4-hydroxyphenylpyruvate dioxygenase inhibitor herbicide resistance in an *Amaranthus tuberculatus* population from Iowa, USA. *Plant Sci. Int. J. Exp. Plant Biol.* **274**, 360–368 (2018).
8. Pandian, B. A., Sathishraj, R., Prasad, P. V. V. & Jugulam, M. A single gene inherited trait confers metabolic resistance to chlorsulfuron in grain sorghum (*Sorghum bicolor*). *Planta* **253**, 48 (2021).
9. Egan, J. F., Maxwell, B. D., Mortensen, D. A., Ryan, M. R. & Smith, R. G. 2,4-dichlorophenoxyacetic acid (2,4-D)–resistant crops and the potential for evolution of 2,4-D–resistant weeds. *Proc. Natl. Acad. Sci. USA* **108**, E37 (2011).
10. Jugulam, M., McLean, M. D. & Hall, J. C. Inheritance of picloram and 2,4-D resistance in wild mustard (*Brassica kaber*). *Weed Sci.* **53**, 417–423 (2005).
11. Preston, C. & Malone, J. M. Inheritance of resistance to 2,4-D and chlorsulfuron in a multiple-resistant population of *Sisymbrium orientale*. *Pest Manag. Sci.* **71**, 1523–1528 (2015).

12. Riar, D. S., Burke, I. C., Yenish, J. P., Bell, J. & Gill, K. Inheritance and physiological basis for 2,4-D resistance in Prickly lettuce (*Lactuca serriola* L.). *J. Agric. Food Chem.* **59**, 9417–9423 (2011).
13. Weinberg, T., Stephenson, G. R., McLean, M. D. & Hall, J. C. MCPA (4-chloro-2-ethylphenoxyacetate) resistance in hemp-nettle (*Galeopsis tetrahit* L.). *J. Agric. Food Chem.* **54**, 9126–9134 (2006).
14. Van Wychen, L. *Survey of the Most Common and Troublesome Weeds in Grass Crops, Pasture, and Turf in the United States and Canada.* https://wssa.net/wp-content/uploads/2020-Weed-Survey_grass-crops.xlsx (Weed Science Society of America National Weed Survey Dataset, 2020).
15. Gaines, T. A. *et al.* Interspecific hybridization transfers a previously unknown glyphosate resistance mechanism in *Amaranthus* species. *Evol. Appl.* **5**, 29–38 (2012).
16. Sosnoskie, L. *et al.* Pollen-mediated dispersal of glyphosate-resistance in Palmer amaranth under field conditions. *Weed Sci.* **60**, 366–373 (2012).
17. Keeley, P. E., Carter, C. H. & Thullen, R. J. Influence of planting date on growth of Palmer amaranth (*Amaranthus palmeri*). *Weed Sci.* **35**, 199–204 (1987).
18. Webster, T. M. & Grey, T. L. Glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*) morphology, growth, and seed production in Georgia. *Weed Sci.* **63**, 264–272 (2015).
19. Shyam, C., Peterson, D. E. & Jugulam, M. Resistance to 2,4-D in Palmer amaranth (*Amaranthus palmeri*) from Kansas is mediated by enhanced metabolism. *Weed Sci.* **70**, 390–400 (2022).
20. Shyam, C., Borgato, E. A., Peterson, D. E., Dille, J. A. & Jugulam, M. Predominance of metabolic resistance in a six-way-resistant Palmer amaranth (*Amaranthus palmeri*) population. *Front. Plant Sci.* **11**, 614618 (2021).
21. Kumar, V., Liu, R., Boyer, G. & Stahlman, P. W. Confirmation of 2,4-D resistance and identification of multiple resistance in a Kansas Palmer amaranth (*Amaranthus palmeri*) population. *Pest Manag. Sci.* **75**, 2925–2933 (2019).
22. MacHado, V. S. & Bandeen, J. D. Genetic analysis of chloroplast atrazine resistance in *Brassica campestris*: Cytoplasmic inheritance. *Weed Sci.* **30**, 281–285 (1982).
23. Nakka, S., Godar, A. S., Thompson, C. R., Peterson, D. E. & Jugulam, M. Rapid detoxification via glutathione *S*-transferase (GST) conjugation confers a high level of atrazine resistance in Palmer amaranth (*Amaranthus palmeri*). *Pest Manag. Sci.* **73**, 2236–2243 (2017).
24. Nandula, V. K. *et al.* EPSPS amplification in glyphosate-resistant spiny amaranth (*Amaranthus spinosus*): A case of gene transfer *via* interspecific hybridization from glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*). *Pest Manag. Sci.* **70**, 1902–1909 (2014).
25. Oliveira, M. C. *et al.* Interspecific and intraspecific transference of metabolism-based mesotrione resistance in dioecious weedy *Amaranthus*. *Plant J. Cell Mol. Biol.* **96**, 1051–1063 (2018).
26. Shyam, C. *et al.* Genetic basis of chlorsulfuron, atrazine, and mesotrione resistance in a Palmer amaranth (*Amaranthus palmeri*) population. *ACS Agric. Sci. Technol.* https://doi.org/10.1021/acsagscitech.1c00005 (2021).
27. Huffman, J., Hausman, N. E., Hager, A. G., Riechers, D. E. & Tranel, P. J. Genetics and inheritance of nontarget-site resistances to atrazine and mesotrione in a waterhemp (*Amaranthus tuberculatus*) population from Illinois. *Weed Sci.* **63**, 799–809 (2015).
28. Jasieniuk, M., Morrison, I. N. & Brûlé-Babel, A. L. Inheritance of dicamba resistance in wild mustard (*Brassica kaber*). *Weed Sci.* **43**, 192–195 (1995).
29. Preston, C., Belles, D. S., Westra, P. H., Nissen, S. J. & Ward, S. M. Inheritance of resistance to the auxinic herbicide dicamba in kochia (*Kochia scoparia*). *Weed Sci.* **57**, 43–47 (2009).
30. Patzoldt, W. L., Dixon, B. S. & Tranel, P. J. Triazine resistance in *Amaranthus tuberculatus* (Moq) Sauer that is not site-of-action mediated. *Pest Manag. Sci.* **59**, 1134–1142 (2003).
31. Tardif, F. J., Preston, C., Holtum, J. A. M. & Powles, S. B. Resistance to acetyl-coenzyme a carboxylase-inhibiting herbicides endowed by a single major gene encoding a resistant target site in a biotype of *Lolium rigidum*. *Funct. Plant Biol.* **23**, 15–23 (1996).
32. Oliveira, M. C., Gaines, T. A., Jhala, A. J. & Knezevic, S. Z. Inheritance of mesotrione resistance in an *Amaranthus tuberculatus* (var. *rudis*) population from Nebraska, USA. *Front. Plant Sci.* **9**, 60 (2018).
33. Figueiredo, M. R. *et al.* Metabolism of 2,4-dichlorophenoxyacetic acid contributes to resistance in a common waterhemp (*Amaranthus tuberculatus*) population. *Pest Manag. Sci.* **74**, 2356–2362 (2018).
34. Shergill, L. S., Bish, M. D., Jugulam, M. & Bradley, K. W. Molecular and physiological characterization of six-way resistance in an *Amaranthus tuberculatus* var. *rudis* biotype from Missouri. *Pest Manag. Sci.* **74**, 2688–2698 (2018).
35. Giacomini, D. A. *et al.* Coexpression clusters and allele-specific expression in metabolism-based herbicide resistance. *Genome Biol. Evol.* **12**, 2267–2278 (2020).
36. Aarthy, T., Shyam, C. & Jugulam, M. Rapid metabolism and increased expression of CYP81E8 gene confer high level of resistance to tembotrione in a multiple-resistant Palmer amaranth (*Amaranthus palmeri* S. Watson). *Front. Agron.* **4**, 4 (2022).
37. Nakka, S. *et al.* Physiological and molecular characterization of hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitor resistance in Palmer amaranth (*Amaranthus palmeri* S. Wats). *Front. Plant Sci.* **8**, 555 (2017).
38. Nakka, S., Thompson, C. R., Peterson, D. E. & Jugulam, M. Target site-based and non-target site based resistance to ALS inhibitors in Palmer amaranth (*Amaranthus palmeri*). *Weed Sci.* **65**, 681–689 (2017).
39. Knezevic, S. Z., Streibig, J. C. & Ritz, C. Utilizing R software package for dose-response studies: The concept and data analysis. *Weed Technol.* **21**, 840–848 (2007).
40. Ritz, C., Baty, F., Streibig, J. C. & Gerhard, D. Dose-response analysis using R. *PLoS ONE* **10**, e0146021 (2015).
41. Wickham, H. *Ggplot2: Elegant Graphics for Data Analysis* (Springer, 2016).
42. Montgomery, J. S. *et al.* Draft genomes of *Amaranthus tuberculatus*, *Amaranthus hybridus*, and *Amaranthus palmeri*. *Genome Biol. Evol.* **12**, 1988–1993 (2020).
43. Han, H. *et al.* Cytochrome P450 *CYP81A10v7* in *Lolium rigidum* confers metabolic resistance to herbicides across at least five modes of action. *Plant J. Cell Mol. Biol.* **105**, 79–92 (2021).
44. Shyam, C., Chahal, P. S., Jhala, A. J. & Jugulam, M. Management of glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*) in 2,4-D-, glufosinate-, and glyphosate-resistant soybean. *Weed Technol.* **35**, 136–143 (2021).

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Author contributions
D.P. identified KCTR Palmer amaranth population in the conservation tillage field. M.J. conceived the research hypothesis, methodology, led, and supervised the research. C.S. conducted the research, collected, and analyzed data. A.J. critically reviewed the manuscript and provided valuable comments.

Competing interests
The authors declare no competing interests.

Additional information

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