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Molecular and Physiological Aspects of Glufosinate Resistance in Amaranthus palmeri

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Molecular and Physiological Aspects of Glufosinate Resistance in *Amaranthus palmeri*

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by

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Abstract

Palmer amaranth is one of the most troublesome weeds in worldwide agriculture. Among the traits that confers extreme weediness to this species, the ability to adapt to herbicide selection pressure stands out. The latest herbicide to which Palmer amaranth developed resistance is glufosinate-ammonium (GFA), an inhibitor of the enzyme glutamine synthetase (GS). The main objectives of this work were: 1) evaluate the resistance level of a Palmer amaranth population from Missouri, USA, 2) determine the mechanisms conferring GFA resistance in Palmer amaranth, 3) elucidate the behavior of amplified GS copies in GFA-resistant Palmer amaranth in terms of inheritance, stability and physical localization, and 4) evaluate the efficacy of herbicides from different modes of action. Our results indicates that Palmer amaranth achieves overproduction of the chloroplastic GS isoform (GS2) via gene amplification and overexpression. This theory was confirmed through biochemical (ammonia accumulation in Palmer amaranth resistant plants) and physiological (photosynthesis inhibition in transformed *Nicotiana benthamiana* plants) approaches. GS2 copy number varies within plants, as a result of somatic mosaicism observed at the cellular level. Segregation of GS2 copies does not follow Mendelian patters. GFA resistance in this population can be managed by most of the soil-applied commonly used herbicides, and foliar-applied glyphosate, acetolactate synthase- and protoporphyrinogen oxidase-inhibitors, are ineffective against this Palmer amaranth population. Knowledge generated in this research will serve as a basis for future, deeper investigations.

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General Introduction and Literature Review

Overview of herbicide technology

Although the recognition of Weed Science as a discipline is a contemporary event (Fryer, 1978), weeds have been part of human history since the inception of crop culture. It is not precisely known when humans started intentionally practicing weed control, but suggestive drawings of tools resembling modern hoes and mattocks were found in ancient Egypt and Mesopotamia, dated from 6000 B.C. (Timmons, 1970). As reviewed by Mesnage et al. (2021), the first known chemicals used as herbicides were the inorganic copper sulfate and sodium arsenate. Sodium chlorate is another salt used as herbicide, but like the others previously mentioned, is highly toxic and has terrible environmental profile even for the standards at that time (Frank, 1948). Likewise, a mixture of diesel and stove oil applied at 300 L ha^{-1} was also explored as a herbicide treatment (Benedict and Krofchek, 1946), something that seems preposterous considering today's standards. The discovery of 2,4-D as the first organic, selective, systemic and environmentally friendly herbicide widely used (Troyer, 2001) ushered the beginning of a new era, driven by industry innovation and frequent introductions of novel herbicides.

Evolution of weed resistance to herbicides

The evolution of herbicide-resistant (HR) weeds has been a concern to researchers since the early 1950s (Appleby, 2005). In the Second British Weed Control conference, held in 1952, crop and herbicide rotations were endorsed to manage resistance (Anonymous, 1954), even though at that time, the concept of HR was still abstract and far different from our current definitions. However, it was not until the rapid increase in reports of HR weeds between 1980 and 1995 (resulting from widespread use of the newly introduced acetolactate synthase (ALS)-

and photosystem II (PSII)-inhibitors), that agrichemical companies, universities and government joined forces to mitigate the evolution of resistance, leading to the creation of the Herbicide Resistance Action Committee (HRAC) and the International Survey of Herbicide Resistance (Shaner, 2014). A multi-approach to weed control was usually recommended, involving herbicide and crop rotations, tillage, grazing animals, use of cover crops, fallow and controlled burns (Heap, 1997).

In 1996, the introduction of RoundUp Ready (RR) crops was a game changer to weed control and agriculture in general. RR crops contained a transgene from an *Agrobacterium* sp. encoding a glyphosate-insensitive 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), the target site of glyphosate, which allowed the application of this herbicide on the resistant crops (Pollegioni et al., 2011). HR crops became widely adopted quickly in the United States: RR *Glycine max* (soybean) planted on 38% of the total acreage by 1998 (Carpenter and Gianessi, 1999), 85% by 2004 (Dill, 2005) and more than 90% in 2022 (USDA, 2023). Despite the several benefits associated with HR crops, their wide adoption also had detrimental effects, and the balance between pros and cons has been a subject of extensive study and public discourse as reviewed by Vencill et al. (2012) and Brunharo et al. (2022). In addition to industry consolidation and higher costs associated with herbicide registration, the economic success of RR crops led to a devaluation of the herbicide market, negatively affecting the introduction of novel active ingredients (Duke, 2012). In terms of weed management, it caused an even higher simplification of management strategies, with an overreliance on a single herbicide that was far more effective and practical than any other active ingredient at the time (Benbrook, 2016). According to Wilson et al. (2011), approximately two-thirds of growers in the US abandoned recommended diversified practices such as crop rotation and avoidance of annual applications of

the same herbicide, ultimately leading to a shift in weed populations and soil seedbanks (Owen, 2008, Wilson et al., 2011, Gibson et al., 2016).

Resistance to herbicides in Palmer amaranth

A steep increase in HR weeds was observed after several years of intense glyphosate use, not only to glyphosate itself, but to any other herbicide mistakenly taken as the next simple solution to manage the current resistance problem (Heap, 2023, Green, 2011). *Amaranthus palmeri* (Palmer amaranth) is a perfect example of this trend. Gossett et al. (1992) authored the first report of HR Palmer amaranth ever described: a dinitroaniline-resistant population collected from a farm with 24 consecutive years of trifluralin application, in South Carolina. The first ALS-resistant Palmer amaranth was detected in Kansas in 1993, just five years after registration of imazethapyr and thifensulfuron for Palmer amaranth control in the state (Horak and Peterson, 1995). Atrazine resistance in Palmer amaranth was first reported in 1993 in Texas (Heap, 2023), but no evidence other than personal communications and observations of control failures were found (Peterson, 1999, Neve et al., 2011, Ferrell and Leon, 2016). The occurrence of HR Palmer amaranth genotypes was limited to these three mechanisms of action until 2006, when a glyphosate-resistant biotype collected in 2004 from Georgia was reported by Culpepper et al. (2006). In the following years, GR populations were found in Arkansas (Norsworthy et al., 2008), North Carolina (Culpepper et al., 2008), Tennessee (Steckel et al., 2008), Mississippi (Nandula et al., 2012), Virginia (Ahmed, 2011) and New Mexico (Mohseni-Moghadam et al., 2013), among others. Multiple-resistant genotypes came soon after, as reported by Sosnoskie et al. (2011), Nandula et al. (2012) and Poirier et al. (2014), comprising usually a combination of resistance to glyphosate and ALS-inhibitors. Several other HR trait combinations were later found, such as to ALS + PPO-inhibiting herbicides (Salas et al., 2016), glyphosate + atrazine +

ALS-inhibiting herbicides (Kohrt et al., 2017, Faleco et al., 2022), atrazine + HPPD-inhibiting herbicides (Jhala et al., 2014) and glyphosate + ALS + PPO-inhibiting herbicides (Spaunhorst et al., 2019). Multiple-resistance in Palmer amaranth seems to be 'the new normal' as novel and more complex cases keep being reported, such as the 6-way resistant genotype described by Kumar et al. (2019).

The evolution of innumerous multiple-resistant Palmer amaranth genotypes is not only a result of inadequate management practices, but also of the extraordinary innate predisposition of this species to evolve herbicide resistance. Furthermore, its rapid seed germination (Steckel et al., 2004) and high growth rates (Horak and Loughin, 2000, Sellers et al., 2003); abundant seed production (Keeley et al., 1987) and efficient C4 metabolism (Ehleringer, 1983); obligatory cross-pollination, which enables the movement of adaptative traits over long distances (Sosnoskie et al., 2012); and ability to hybridize with other *Amaranthus* species (Franssen et al., 2001, Gaines et al., 2012, Wetzel et al., 1999, Molin et al., 2016) makes Palmer amaranth an extremely difficult weed to manage. Severe yield losses in *Gossypium hirsutum* (cotton) (MacRae et al., 2013), soybean (Basinger et al., 2019), *Arachis hypogaea* (peanut) (Burke et al., 2007), *Sorghum bicolor* (grain sorghum) (Moore et al., 2004), *Ipomoea batatas* (sweetpotato) (Meyers et al., 2010) and *Zea mays* (corn) (Massinga et al., 2001) have been reported.

Among all weeds, Palmer amaranth had the greatest shift in importance in the US Mid-south from 1994/1995 to 2008/2009 advancing from 25th to 7th (Webster and Nichols, 2012). By 2016, Palmer amaranth was already considered the most problematic and important weed in Mid-Southern US (Schwartz-Lazaro et al., 2018). Despite being native to the Sonoran Desert (Ehleringer, 1983), which encompasses southwestern United States and northwestern Mexico, Palmer amaranth has spread to most US states, and is present in all continents (Roberts and

Florentine, 2021). Introduced Palmer amaranth populations are oftentimes HR. For example, populations introduced to Brazil (Küpper et al., 2017), Argentina (Berger et al., 2016, Kaundun et al., 2019), and South Africa (Reinhardt et al., 2022) were resistant to glyphosate and ALS-inhibitors. Introduced populations in Spain were glyphosate-resistant (Manicardi et al., 2023), and those in Israel (Matzrafi et al., 2017) and Italy (Milani et al., 2021) were ALS-inhibitor-resistant. In several other cases, Palmer amaranth was found as a 'casual alien' or 'naturalized' in non-agricultural areas across Europe (EPPO, 2020). Climate change is likely to favor Palmer amaranth establishment in row-crop areas where infestations are absent or incipient (Kistner and Hatfield, 2018, Briscoe Runquist et al., 2019). Despite its success as an invasive species, Palmer amaranth introduction can be contained, and even reversed, if addressed properly and timely. Multiple strategies including intensive scouting, torching, prescribed burning and herbicide applications were implemented in Minnesota to eradicate this species from regions within the state a few years after its first detection (Yu et al., 2021).

Mechanisms of resistance to herbicides

As previously mentioned, Palmer amaranth adaptability to herbicide selection pressure is a key aspect to its success as a weed. The mechanisms through which Palmer amaranth (and other plants) can become resistant to herbicides can be classified into two categories: target-site resistance (TSR) or non-target-target site resistance (NTSR), as thoroughly reviewed by Gaines et al. (2020). Briefly, in this context, target-site (TS) refers to the specific enzyme or peptide to which the herbicide molecule binds, in order to disrupt an essential biochemical pathway and exert herbicidal activity. Mutations in the TS coding gene or TS overproduction are the two possible TSR mechanisms, the former being far more common than the latter.

TS mutations affect binding of the herbicide, making the enzyme insensitive – in various degrees – to inhibition. A comprehensive review was recently published summarizing known resistance-conferring TS mutation (Murphy and Tranel, 2019). Mutant TS can confer high or low resistance index to a plant, as seen in the *Myosoton aquaticum* carrying *ALS* P197E (Liu et al., 2015) and *Echinochloa phyllopogon* carrying *ALS* P197S (Liu et al., 2019), respectively. Mutations can be caused by polymorphisms in one [Palmer amaranth *PPO2* R128G, AGG to ATG (Giacomini et al., 2017)] or two [*Poa annua ALS* A205F, GCC to TTC (Brosnan et al., 2016)] nucleotides. Mutations may also happen as nucleotides deletions instead of polymorphisms, as seen in the G210 codon deletion in *PPO2* of *A. palmeri* and *A. tuberculatus* (Salas et al., 2016, Patzoldt et al., 2006), facilitated by the bi-GTG (or bi-TGG) repeat in positions 209 and 210 in those species. Genes generally carry a single mutation, but double- [*Eleusine indica EPSPS* T102I/P106S (Han et al., 2017)] or even triple-mutants [*A. hybridus EPSPS* T102I/A103V/P106S (García et al., 2019)] have been found. Single mutations may 'accumulate' at the population (Singh et al., 2019), plant or allele levels (Noguera et al., 2020), especially in outcrossing species such as Palmer amaranth.

TS overproduction is very rare in the context of herbicide resistance and may occur due to gene duplications and/or gene overexpression. Gene duplications may be a result of unequal crossover between sister chromatids leading to gene copies being in tandem arrays [*EPSPS* amplification in *Bassia scoparia* (Jugulam et al., 2014)]. Tandem arrays of repetitive DNA sequences may lead to DNA circularization and aneuploidy, as seen in *A. tuberculatus EPSPS* amplification (Koo et al., 2018a). Gene amplification can also be mediated by extrachromosomal circular DNAs (eccDNAs), as seen in *EPSPS* amplification in *A. palmeri* (Koo et al., 2018b). The mechanisms of gene overexpression are far less studied, and this information is missing in

most cases where increase in transcript levels was detected, such as *PsbA* overexpression in *Commelina communis* (Yang et al., 2022) and *ACCase*1 and 3 in *Echinochloa crus-galli* (González-Torralva and Norsworthy, 2023). Possible causes for TS gene overexpression include mutations in the promoter region or in the transcription factors, differential epigenetic regulation [as suggested by Margaritopoulou et al. (2018) in *EPSPS* overexpression in *Erigeron canadensis*] and gene duplications [*ACCase* overexpression in *Digitaria sanguinalis* (Laforest et al., 2017)].

NTSR involves a plethora of mechanisms that 1) reduces herbicide concentration at the target-site, either via enhanced herbicide metabolism, altered absorption/translocation or root exudation, 2) mitigates the detrimental downstream effects of TS inhibition, usually by activity of antioxidants and other cellular protectors (Gaines et al., 2020). NTSR has been reviewed by Jugulam and Shyam (2019) and Rigon et al. (2020), and is generally considered a complex subject as it is mainly polygenic in nature, and a single trait can confer resistance to multiple modes of action, potentially including herbicides not yet discovered (Délye, 2013). In fact, it was recently discovered that genes encoding enzymes related to herbicide metabolism have evolved even before the origin of land plants, as a result of chromosome duplications during the Palaeozoic period (Casey and Dolan, 2023).

As noted by Gaines et al. (2020), herbicide metabolism is usually divided in three phases: 1) activation, where a functional group is added to the herbicide by oxidation, reduction or hydrolysis; 2) conjugation, where the herbicide is complexed with another molecule such as glucose or glutathione; and 3) compartmentalization, where herbicide metabolites from previous phases are incorporated into cell walls or moved into the vacuole. Phase 1 reactions are usually catalyzed by cytochrome P450 monooxygenases, which are membrane-bound proteins coded by

a large gene superfamily (Dimaano and Iwakami, 2021). A single P450 may be specific to a certain herbicide (Zhao et al., 2022) or may metabolize multiple active ingredients from the same (Siminszky et al., 1999) or distinct modes of action (Han et al., 2021). Phase 2 reactions are performed by glutathione-S-transferases (GSTs) and glycosyltransferases (GTs), generally but not necessarily using metabolites from phase 1 reactions as substrates. GSTs have been widely recognized as the main mechanisms conferring resistance to atrazine in Palmer amaranth (Chahal et al., 2019). GTs seems to be a minor player in herbicide resistance; its overexpression has been identified in diclofop-resistant *Lolium rigidum* nevertheless (Gaines et al., 2014). Many times, the involvement of P450s and GSTs are indirectly assessed by application of herbicides in association with metabolic inhibitors such as malathion, phorate and NBD-Cl (Busi et al., 2017, Varanasi et al., 2019). The continuous advances in next generation sequencing and related technologies are likely to facilitate identification of novel metabolism-related genes in the near future (Patterson et al., 2019).

Reduced concentrations of active ingredient at the site of action may also be achieve by reduced translocation and absorption. This can happen through various mechanisms: 1) vacuolar sequestration, such as found in glyphosate-resistant *Erigeron canadensis* (Ge et al., 2010) and *Lolium* spp. (Ge et al., 2012); 2) inhibition of phloem loading, as in 2,4-D-resistant *Raphanus raphanistrum* (Goggin et al., 2016); 3) inhibition of intercellular transport, as in dicamba-resistance *Bassia scoparia* (Pettinga et al., 2018); or 4) rapid programmed cell death in response to herbicide perception, as in glyphosate-resistant *Ambrosia trifida* (Van Horn et al., 2018) and 2,4-D-resistant *Erigeron sumatrensis* (de Queiroz et al., 2020). Although reduced herbicide absorption has been observed in a few HR weeds, its contribution to overall resistance tend to be relatively small (Gaines et al., 2020).

Enhanced protection against herbicide downstream effects usually involves increased scavenging capacity of reactive oxygen species (ROS). ROS have important signaling functions in plants and its homeostasis in cells is tightly regulated to avoid lipid peroxidation and cell death (Czarnocka and Karpiński, 2018). Herbicides are known to disrupt this balance by causing an overproduction of ROS (Kaur, 2019). Although not many cases have been elucidated, few examples of enhanced ROS scavenging capacity as a main herbicide resistance mechanism have been described, such as those in paraquat-resistant *Erigeron canadensis* (Shaaltiel and Gressel, 1986) and PPO- and ALS-resistant *Euphorbia heterophylla* (Xavier et al., 2018). Oftentimes, protection against ROS acts as a complimentary mechanism, as seen in bensulfuron-resistant *Sagittaria trifolia* (Zou et al., 2022) and glyphosate-resistant *A. palmeri* (Maroli et al., 2015). Another mechanism, which is slightly different but would fall into the same category of increased protection, is increased detoxification of a metabolic product, which is lethal at high concentrations. One example is the expression and/or increased activity of β-cyanoalanine synthase, responsible for metabolizing cyanide that accumulates at toxic levels as a result of quinclorac activity in grasses (Grossmann and Kwiatkowski, 1995). Numerous *Echinochloa* species have evolved quinclorac resistance through this mechanism, including *E. oryzoides* (Haq et al., 2022), *E. crus-galli* var. *zelayensis* (Gao et al., 2017), *E. crus-galli* var. *mitis* (Haq et al., 2020) and *E. phyllopogon* (Yasuor et al., 2012).

The resistance mechanisms previously described exemplify the versatility weeds in adapting to herbicides. The complexity is increased if we consider that several mechanisms may be present in a single plant or population. The occurrence of both TSR and NTSR within the same population has been reported with respect to several herbicide modes of action, such as EPSPS-inhibitors (Alcántara-de la Cruz et al., 2016), ALS-inhibitors (Rey-Caballero et al.,

2017), PSII-inhibitors (Lu et al., 2019), Acetyl-coA Carboxylase (ACCase)-inhibitors (Chen et al., 2018) and mitosis inhibitors (Chen et al., 2020). In other cases, although not experimentally proven, the co-occurrence of TSR and NTSR has been suggested based on the contrasting response to herbicides of different populations with similar TSR profiles (Noguera et al., 2020). The presence of multiple mechanisms within a population hinders the inheritance prediction of resistance, as each allele may segregate independently and alleles providing strong phenotypic responses may mask minor-contributing alleles (Scarabel et al., 2015, Burns et al., 2018). In this case, more complex approaches such as quantitative trait loci analysis and genome-wide association studies are required, as reviewed by Leon et al. (2021). Inheritance patterns affects the dynamics of herbicide-resistance within and between weed populations, and its understanding may allow researchers to develop predictive models and strategies to mitigate resistance spread (Ghanizadeh et al., 2019).

A deep understanding of all of the abovementioned aspects of herbicide resistance is necessary to develop tools and approaches, to mitigate the spread of current HR weed populations and to slow down the selection of new ones. Because of its novelty, glufosinate resistance in Palmer amaranth presents a vast knowledge gap to be filled. In this work we shed light into the molecular mechanisms of glufosinate resistance in Palmer amaranth, which may serve as a basis for future, deeper explorations. The genetic factors driving segregation and stability of herbicide resistance traits and alternative chemical control options have also been studied.

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Involvement of GS2 amplification and overexpression in *Amaranthus palme*ri resistance to glufosinate

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Abstract

Amaranthus palmeri has recently evolved resistance to glufosinate herbicide. Several *A. palmeri* populations from Missouri and Mississippi, U.S.A. had survivors when sprayed with glufosinate-ammonium (GFA, 657 g ha^{-1}). One population, MO#2 (fourfold resistant) and its progeny (sixfold resistant), were used to study the resistance mechanism, focusing on the herbicide target glutamine synthetase (GS). We identified four GS genes in *A. palmeri*; three were transcribed: one coding for the plastidic protein (*GS2*) and two coding for cytoplasmic isoforms (*GS1.1* and *GS1.2*). These isoforms did not contain mutations associated with resistance. The 17 glufosinate survivors studied showed up to 21-fold increase in *GS2* copies. *GS2* was expressed up to 190-fold among glufosinate survivors. *GS1.1* was overexpressed > twofold in only 3 of 17, and *GS1.2* in 2 of 17 survivors. GS inhibition by GFA causes ammonia accumulation in susceptible plants. Ammonia level was analyzed in 12 F1 plants. *GS2* expression was negatively correlated with ammonia level ($r = -0.712$); therefore, plants with higher GS2 expression are less sensitive to GFA. The operating efficiency of photosystem II (ϕPSII) of *Nicotiana benthamiana* overexpressing *GS2* was four times less inhibited by GFA compared to control plants. Therefore, increased copy and overexpression of *GS2* confer resistance to GFA in *A. palmeri* (or other plants). We present novel understanding of the role of *GS2* in resistance evolution to glufosinate.

Introduction

Amaranthus palmeri S. Watson (Palmer amaranth) is a summer annual forb native of the Sonoran Desert (Ehleringer, 1983), which encompasses large regions of the southwestern United States and northwestern Mexico. Due to the globalization of agricultural markets and new habitat creation through agriculture expansion, this species has been introduced to several other countries and now can be found in all continents (Roberts and Florentine, 2021). Its biology, physiological characteristics and impressive adaptation potential has made this species a major threat to food security and the preservation of native ecosystems and wildlife (Roberts and Florentine, 2021, Ward et al., 2013). Climate change is likely to favor its establishment and expansion into key row-crop areas worldwide that are currently free of *A. palmeri*, or have incipient infestations, and enhance its competitive ability against crops (Kistner and Hatfield, 2018, Briscoe Runquist et al., 2019).

The adaptability of *A. palmeri* is demonstrated by its propensity to evolve resistance to herbicides. With resistance to nine sites of action (SoA) reported, *A. palmeri* is only behind *Lolium rigidum* globally, with the latter having resistance to 12 SoA (Heap, 2023). Resistance traits can accumulate in a plant. For instance, resistance to six SoA was reported in a genotype from Kansas, U.S.A. (Shyam et al., 2021). This characteristic reduces the already limited herbicide options for *A. palmeri* and hinders its management.

The latest addition to the list of herbicides to which *A. palmeri* has evolved resistance is glufosinate (Heap, 2023). This active ingredient is a glutamic acid analog, known by its fast, non-selective activity and reduced risk from the toxicological and environmental standpoints (Zhou et al., 2020, Duke et al., 2022). Glufosinate is mostly formulated as ammonium-salt

(hence, glufosinate-ammonium or GFA), and only the L-isomer has herbicidal activity (Hoerlein, 1994). The racemic mixture is commercially preferable due to lower production cost.

Glutamine synthetase (GS, EC 6.3.1.2), the target site of GFA, is an essential enzyme that catalyzes the ATP-dependent incorporation of ammonia to glutamate, yielding glutamine (Miflin and Habash, 2002). This reaction is the first step of N assimilation in plants, which also involves glutamate synthase (GOGAT, EC 1.4.1.13), to drive the GS/GOGAT cycle (Masclaux-Daubresse et al., 2010). The GS/GOGAT cycle also produces glutamate to serve as an N donor for the synthesis of glycine from glyoxylate, derived from photorespiration (Dellero et al., 2016). GS inhibition causes an accumulation of ammonia, glycolate and glyoxylate, inhibiting photosynthesis and leading to a state of extreme oxidative stress in the presence of light, which causes cell and plant death (Sauer et al., 1987, Takano et al., 2020, Coetzer and Al-Khatib, 2001, Oliver, 1980, Campbell and Ogren, 1990).

Resistance to GFA has evolved slower than to many herbicides with different SoAs. In 2009, *Eleusine indica* was the first species to be reported as GFA-resistant (Jalaludin et al., 2010). After that, resistance to GFA was documented in two species from the *Lolium* genus, and *A. palmeri* is the first dicot weed to evolve resistance to GFA (Heap, 2023). The only resistance mechanisms reported so far were the increased GFA metabolism in a *Lolium perenne* var. *multiflorum* (Brunharo et al., 2019) and the S59G mutation in the *GS1-1* gene from *E. indica* (Zhang et al., 2022). Resistance mechanism has yet to be determined in the remaining cases. Widespread weed resistance to the non-selective herbicide glyphosate has increased the use of this alternative non-selective herbicide glufosinate, increasing the selection pressure on weed species. The recent evolution of resistance to GFA in *A. palmeri* is a testament to that. The objectives of this research were to: (1) assess the level of GFA resistance in a selected

population; (2) determine if resistance is heritable; and (3) identify the mechanism(s) conferring resistance.

Materials and Methods

Plant materials, growth conditions and application parameters

The putative glufosinate-resistant Palmer amaranth population was collected from a soybean farm in Butler County, Missouri, at the end of 2020 growing season. The sensitive standard (SS) accession was collected in Crawford County, Arkansas, from a field with a history of minimal herbicide use. Sampling and collection were done according to standard protocols (Burgos, 2015). To generate the F1 population, ten plants from the MO#2 population that survived an application of 657 g ha^{-1} GFA were transplanted to 8L pots and grown together in a greenhouse until maturity. Female inflorescences were harvested, threshed and seeds were cleaned and stored in glass vials.

Plants were grown in a greenhouse maintained at 32/28 °C day/night temperature and a photoperiod of 14-h achieved with supplemental light. Irrigation was done via capillarity as needed and plants were fertilized once a week using a diluted water-soluble all-purpose plant food (Miracle-Gro, 15-30-15 NPK).

Herbicide applications were done using a benchtop sprayer, equipped with two Teejet Flat Fan 110 0067 nozzles, calibrated to deliver 187 L ha^{-1} of spray mix at 3.6 km h^{-1} and 275 KPa. Nozzle spacing was 50 cm and boom height was set to 45 cm above the plant canopy.

Response of MO#2 and its progeny to glufosinate

Seeds were sown in 50-cell trays filled with a commercial potting mix (Sun Gro Horticulture) and seedlings were thinned to 1 plant per cell a week after emergence. When plants were 5 to 8-cm tall, 7 rates of glufosinate (Liberty 280 SL, BASF SE) were sprayed. Putative

resistant populations (MO #2 and its progeny) were sprayed with 82, 164, 328, 657, 1314, 2628 and 5256 g ai ha^{-1} (corresponding to 0.125x, 0.25x, 0.5x, 1x, 2x, 4x and 8x of the labeled rate). The SS was sprayed with 5, 10, 20, 41, 82, 164 and 328 g ai ha^{-1} (covering 0.0078x to 0.5x the labeled rate). The 1x GFA rate is the herbicide label rate of 657 g ai ha^{-1}. The adjuvant ammonium-sulphate was added to all treatments at 10 g L^{-1} of spray mix. A nontreated check was included for all populations, two replications were used per treatment (1 rep = 25 plants), and the test was conducted twice. To avoid time-of-day effects on herbicide activity, both runs were sprayed from 1 to 2 PM. Applications of GFA during full sunlight tend to provide better weed control (Martinson et al., 2005). Fifteen days after treatment (DAT), live plants were counted and the data were converted to survival percentage. Survival data was fitted to a non-linear regression as described in the "Statistical analysis" section.

GS isoforms identification in *A. palmeri* genome

Gene annotation files of the *A. palmeri* genome (Montgomery et al. 2020) were parsed and four sequences were retrieved: g13234, g1417, g17049 and g17050. Upon comparison of their peptide sequences with 34 publicly available sequences representative of different plant families (retrieved from Phytozyme and Genbank databases, Supplementary Fig. S1 and File F1), it was determined that g13234 and g1417 (hereafter called *GS1.1* and *GS1.2*, respectively) encoded the cytosolic isoforms, whereas g17049 and g17050 (hereafter called *GS2.1* and *GS2*, respectively) encoded the plastidic proteins. A phylogenetic tree was built using the 34 sequences retrieved from online databases in addition to the sequences from *A. palmeri*. The tree was done using Geneious Prime software (Biomatters) and the neighbor-joining method, with no outgroups considered.

Because GS2 in plants is usually coded by a single nuclear gene, the two plastidic isoforms found in *A. palmeri* were further investigated by extracting a 40 kb surrounding genomic region and constructing a synteny dot plot using kmers (k = 10), where the region was compared to itself to identify genomic signatures of duplication and conservation.

Homology modeling

To identify the residues involved in GFA binding into GS1.1, the protein crystal structure of Zea mays GS1 (PDB 2D3A) was used as a template to build a homology model for *A. palmeri*. L-glufosinate was docked into the GS1.1 binding site. To guide the docking, we used the GFA binding mode from the protein crystal structure of Salmonella (1FPY). Molecular modeling was done using Molecular Operating Environment (MOE) 2020.09 software package (Chemical Computing Group ULC).

Sequencing of GS isozymes from the GFA survivors

RNA extraction and cDNA synthesis

Leaf sections (0.5 cm^2) were sampled, transferred into a collection microtube (Qiagen) and snap-frozen in liquid nitrogen. Samples were homogenized with steel beads in a shaker mill (TissueLyser II; Qiagen) and total RNA was extracted in a magnetic particle processor (Thermo Fisher Scientific) using the MagMAX™ Plant RNA Isolation Kit (Applied Biosystems) according to the manufacturer's instructions. An aliquot of 200 ng of total RNA was used for cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions.

Preparation of tailed cDNA for RACE PCR

The cDNA for RACE PCR was prepared using the SMARTer® RACE 5’/3’ Kit (Takara Bio Europe) according to the manufacturer's instructions. In brief, 10 µL of total RNA (200

ng/µL) was incubated with 1 µL of 5'-CDS Primer A for the 5' tailed cDNA or with 1 µL of 3'-CDS Primer A for the 3' tailed cDNA at 72°C. After 3 min, the temperature was decreased to 42 °C for 2 min. In addition, 1 µL of the SMARTer II A Oligonucleotide was added to the 5'-RACE preparation. The 3'-RACE preparation was used directly. To these solutions were added 4.0 µL of 5X First-Strand Buffer, 0.5 µL of DTT (100 mM), 1.0 µL of dNTPs (20 mM), 0.5 µL of RNase inhibitor (40 U/µL), and 2.0 µL of SMARTScribe Reverse Transcriptase (100 U). Reverse transcription was performed at 42 °C for 90 min. After heat inactivation for 10 min at 70 °C, the tailed cDNA was used for RACE PCR.

RACE PCR

For RACE PCR, cDNA was amplified in a 25-µL reaction containing 1 µL (10 pmol) of specific RACE primers, 2 µL of the Universal Primer A Mix, 12.5 µL SNP Pol 2X PCR Master Mix (Genaxxon bioscience GmbH) 4.5 µL PCR-Grade H2O and 5 µL of the tailed cDNA. The RACE PCR performed in a thermal cycler (T100, Bio-Rad Laboratories) under the following conditions: 3 min at 94 °C and 42 cycles of 10 s denaturation at 94 °C; 35 s annealing at 68 °C and 3 min elongation. Aliquots were taken and analysed on 1.5% agarose gels. Bands of the expected size were cut out and cleaned up (innuPREP DOUBLEpure Kit, IST Innuscreen GmbH).

The PCR products were verified with specific nested primers under the following conditions: 3 min at 94 °C and 35 cycles of 10 s denaturation at 94 °C; 35 s annealing at 65 °C and 90 s elongation at 72 °C; and a final elongation step at 72 °C for 5 min. Aliquots were taken and analysed on 1.5% agarose gels. Bands of the expected size were cut out, cleaned up, and subsequently cloned using StrataClone PCR Cloning Kit (Agilent). Positive white colonies were randomly picked and verified with colony PCR. For each clone, 10 positive PCR fragments were

randomly selected and verified via Sanger sequencing (SeqLab-Microsynth). Sequences were analysed using Geneious Prime software v. 9.1.8 (Biomatters).

End point PCR for entire coding sequences

Full-length amplification of GS coding sequences was performed in a final volume of 25 µL reaction, composed of 5 µL of cDNA, 1 µL (10 pmol) of F and R primers (Table S1), 12.5 of MyFi™ DNA Polymerase (Bioline GmbH) and 6.5 µL of H2O. Amplification was done in a thermal cycler (T100, Bio-Rad Laboratories) under the following conditions: 3 min at 95 °C and 35 cycles of 10 s denaturation at 95 °C; 35 s annealing at primer specific temperature (Table 1) and 2 min elongation at 72 °C, followed by a final elongation step at 72°C for 5 min. Aliquots were taken and submitted to gel electrophoresis to confirm the presence of a single amplicon. PCR products were Sanger-sequenced (SeqLab-Microsynth) and results were analysed using Geneious Prime software v. 9.1.8 (Biomatters).

GS copy number and expression analysis

Seventeen GFA survivors from the MO #20 population were sampled at 3 weeks after application for GS copy number and expression analysis. A 0.5 cm^2 of leaf tissue was transferred into a collection microtube (Qiagen) and homogenised in a shaker mill (Qiagen) with steel beads. DNA extraction was performed in magnetic particle processors (KingFisherTM, Thermo Fisher Scientific) using the Chemagic Plant 400 kit (Perkin Elmer) according to the manufacturer's instructions (modified by IDENTXX GmbH). RNA extraction and cDNA synthesis were done as described in section 2.5.1.

TaqMan™ assays were designed to allow a multiplex approach for the target and reference genes. GS1 isoforms plus Actin genes were run in a triplex reaction, while GS2 was

run in duplex with Actin, and each sample was run in triplicate. Gene expression and copy number were assayed using cDNA and gDNA as templates, respectively.

qPCR assays were performed in a 25 µL reaction composed of 5 µL of cDNA/gDNA, 1 µL (0.2 µM) of primers and 0.25 µL (0.2 µM) of probe, 0.25µL of SNP PolTaq DNA Polymerase and 2.5 µL 10X buffer (Genaxxon bioscience GmbH), 0.5 µL dNTP mix (10 mM) and 13 and 14.25 µL H2O for the triplex and duplex qPCR, respectively. Reactions were performed in a qPCR thermal cycler (Bio-Rad Laboratories) under the following conditions: 5 min at 95 °C, and 35 cycles of 95 °C for 10 s and 60 °C for 30 s. Real-time fluorescence data were captured during the amplification cycle.

Ammonia accumulation assay

Ammonia accumulation after GFA application has been used as an indicator of plant susceptibility to this herbicide, in both crops (Pornprom et al. 2003; Domínguez-Mendez et al. 2019) and weeds (Salas-Perez et al. 2018; Avila-Garcia et al. 2012). To verify if GS2 fold-change in expression correlates with ammonia levels, an in vitro assay was done using a modified methodology described by Dayan et al. (2015). In this assay, 12 survivors from the MO#2 F1 population were used and sampling occurred at 5 weeks after application. Briefly, three leaf discs (5 mm diameter) were cut from the youngest fully expanded leaf of each plant and placed in a microplate containing 150 uL of a 20 uM GFA (bathing) solution. Each well contained a single leaf-disc and represented a replication. The plate was sealed with two layers of micropore tape and kept in a growth chamber under continuous light at 28 °C for 24 h. The reaction was stopped by placing the plate at -80 °C. After two freeze-thaw cycles, a 50-uL aliquot of the bathing solution was transferred to a fresh plate for ammonia quantification as described by Molin and Khan (1995). Absorbance at 630 nm was read using a microplate reader

(SpectraMax iD3, Molecular Devices LLC) and converted to mM NH4+ g fresh biomass-1 using a standard curve produced with ammonium chloride.

GS isoforms quantification

To check if the higher number of GS copies and transcripts observed in resistant plants would result in higher protein levels, the three GS isoforms were quantified in the same 12 plants used in the previous study. Leaf samples were collected around 3 months after GFA application. For this reason, the assays for GS copy number and expression, which were done on these same plants 24 h after GFA application, were conducted again on these samples.

Protein extraction

Sampling was done by collecting and pooling the youngest fully expanded leaves from different branches into a 50-mL Falcon tube and immediately freezing it in liquid nitrogen. Samples were ground in liquid nitrogen using a mortar and pestle, and 400 mg of leaf powder was mixed with lysis buffer (5 % SDS; 50 mM TEAB; pH = 8,5) and incubated at 70 °C for 10 min. After centrifugation at 20,000 x g for 10 min, the remaining supernatant was filtered (0.45 µm filter). Total protein was quantified using the Pierce™ 660 nm kit (Thermo Fisher Scientific) and concentration adjusted to 150 µg per sample.

Protein digestion and peptide clean-up

Protein digestion and peptide clean-up was done using the S-trap™ micro spin columns kit (ProtiFi LLC) as per manufacturer instructions. In brief, reduction was conducted by adding dithiothreitol (DTT) to a final concentration of 20 mM and incubating at 60°C for 10 min. Alkylation was performed by addition of IAA to a final concentration of 60 mM and incubation in the dark at room temperature for 30 min. For protein digestion, 22 µL 12% H3PO4 plus 725 µL S-Trap™ binding buffer were added. The solution was loaded onto a S-Trap™ Micro column

and washed four times with the binding buffer. Digestion was carried out for 1h with 1.5 µg Lys-C and overnight with 3 µg trypsin diluted in 100 µL digestion buffer (50 mM TEAB). Elution of digested peptides was mediated by centrifuging for 1 min. Within two steps 40 µL of 0.2% FA and 40 µL of 0.2% FA in 50% ACN solution were loaded onto the column and centrifuged at same conditions. The flow through was vacuum-dried and dissolved in 100 µL of 1% FA. Desalting of the digested protein samples was performed by SDB Stage Tip purification. SDB Stage Tips were conditioned with 100 µL methanol and 100 µL SDB Stage Tip buffer B (80% ACN, 0.1% FA) and 2 x 100 µL SDB Stage Tip buffer A (0.1% FA). Samples were loaded and washed two times with 200 µL of SDB Stage Tip buffer A and 200 µL of SDB Stage Tip buffer. Elution was performed with 20 µL of elution buffer (5% NH4OH in 60% ACN, pH>9). The eluate was collected and vacuum dried. For mass spectrometry measurement, the dried sample was taken up in 100 µL of 0.1% FA and 2 % acetonitrile in water.

nanoLC-MS/MS analysis

Three technical replicates per sample were analyzed by a reversed-phase nano liquid chromatography system (EASY-Spray™ 1200, Thermo Scientific) connected to an Orbitrap Fusion™ mass spectrometer (Thermo Scientific). LC separations were performed on a 25 cm x 75 µm, C18 “Aurora” column (IonOpticks) packed with 1.7 µm particles at an eluent flow rate of 300 nL min−1 using a gradient of 2 to17% B in 72 min, 17 to 27% B in 28 min and 27 to 41% B in 20 min. Mobile phase A contained 0.1% FA and 2% acetonitrile in water, and mobile phase B consisted of 0.1% FA in 80% acetonitrile in water. Fourier transformed survey scans were acquired in a range from m z-1 375 to 1500 with a resolution of 240,000, at an automatic gain control target of 100% and a max injection time of 50 ms. In data-dependent mode monoisotopic precursor ions with charge states between 2 and 7 were selected for fragmentation. HCD MS/MS

spectra were acquired in the ion trap with a normalized collision energy of 35%, an automatic gain control target of 20% and a dynamic max injection time. Fragmented precursor ions were dynamically excluded from fragmentation for 20 s.

Raw data were search by MaxQuant 2.0.2.0 (Tyanova et al. 2016) against an inhouse database for *Amaranth palmeri* containing the different GS variants. Default MaxQuant parameters were used. Trypsin was chosen for digestion allowing up to two missed cleavages. N-terminal acetylation and methionine oxidation were considered as variable modifications and carbamidomethylation of Cys was specified as fixed modification. The false discovery rate was set to 1% for both peptide spectrum level and protein level. Label-free quantification (LFQ) including the match-between runs feature was enabled and LFQ min ratio count was set 2. At least two unique peptides were considered for quantification. A fold-change in protein levels was calculated by dividing the LFQ intensity of the sample by the average LFQ intensity of three plants from the SS population.

Nicotiana benthamiana **leaf infiltration with** *A. palmeri* ***GS2***

To provide further evidence that GS2 overexpression can lead to GFA resistance, transient expression of *A. palmeri* GS2 in *N. benthamiana* was done using the leaf infiltration technique (Sparkes et al., 2006), and leaf discs were incubated in a GFA solution. The operating efficiency of photosystem II (ϕPSII) was used as an indicator of photosynthetic activity in response to GFA (Murchie and Lawson 2013).

A plasmid containing *A. palmeri GS2* was inserted into an Agrobacterium strain and cultured. The culture was centrifuged for 20 min at 3000 rpm at 22 °C, and the pellet was washed with 50 mL H2O. After another centrifugation step, 20 mL of an infiltration medium (10 mM MgCl2, 10 mM MES pH 5.2, 10 µM acetosyringone) was used to re-suspend the pellet to

OD600 = 1. The solution was incubated for 2 h at room temperature. The abaxial surface *of N. benthamiana* leaves were infiltrated using a needleless 1 mL syringe, and incubated for 7 d at 23 °C. Control plants were infiltrated with the empty plasmid. Leaf discs (8 mm diameter) were sampled from control and transformed plants, and individually placed in the wells of a microtiter plate containing GFA solution (prepared with technical grade GFA and Milli-Q water). Nine rates were used ranging from 1 µM to 10 mM, and each rate was represented by three wells. Control treatments had water only. After 48 h of incubation in the herbicide solution, ϕPSII was measured using a DUAL-PAM-100 (Heinz Walz GmbH). Data was converted to percentage inhibition relative to control plants and fitted to a non-linear regression as shown in the "Statistical analysis" section.

Statistical analysis

Dead or alive counts from the dose-response experiment were transformed to survival percentage. A three-parameter log-logistic model was then fitted to the data (Ritz et al. 2016) using the package "drc" in R 4.0.3 (R Core Team 2019), as shown in Eq. 1. To assess fitness of the model, a Lack-of-fit test was done using the modelFit function from the drc package.

Equation 1. $$Y = \frac{d}{1+\mathrm{e}^{(b(\log x - \log ED_{50}))}}$$

In equation 1, Y is the percent survival, d is the upper asymptote, x is the GFA rate, and b is the slope around ED_{50}, which is the value of x giving a 50% response of Y. Differences in ED_{50} among populations were evaluated using the compParm function, and resistance index was calculated by dividing ED_{50} R/ED_{50} SS. Confidence intervals of the ED_{50} were estimated using the ED function. Similarly, the operating efficiency of photosystem II (ϕPSII) of *N. benthamiana*

samples was converted to percent inhibition relative to control plants and fitted with a 4-parameter Weibull II model (Eq. 2).

Equation 2. $$Y = c + (d - c)[1 - \exp(-(x/e)^b)]$$

In Eq. 2, Y is the percent inhibition, x is the herbicide concentration, c and d are the lower and upper asymptotes, respectively, and b is the slope around e, which is the inflection point of the dose-response curve. The I50 (dose of GFA required to cause a 50% reduction in Y) was estimated for the samples overexpressing *GS2* and the empty vector, and compared using the compParm function in drc.

Gene expression and copy number analysis was dose using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak 2008) using the software CFX Maestro 2.2 (Bio-Rad Laboratories). Dose-response graphs were done using the *drc* package in R, and all other graphs were generated using SigmaPlot 14.5 (Systat Software, Inc.).

Results

Resistance level of a GFA resistant *A. palmeri* from Missouri

None of the resistant populations were controlled 100% at the labeled herbicide rate (1x = 657 g ai ha^{-1}), whereas the SS was completely controlled at ¼x (Fig. 1). Early herbicide symptoms (leaves with water-soaked appearance) were observed as soon as 1 h after treatment (HAT), with severe necrosis developing from 24 HAT onwards. The estimated ED_{50} for MO#2 and MO#2 F1 were 256 and 381 g ai ha^{-1}, respectively, which were equivalent to 4.1- and 6.1-fold resistance index, respectively, compared to SS.

Identification of GS isoforms in *A. palmeri* and herbicide binding residues

The *A. palmeri* genome carries two cytosolic isoforms (*GS1.1* and *GS1.2*) and two chloroplastic isoforms (*GS2.1* and *GS2*). Phylogenetic analysis of GS isoforms from 11 species showed a close relation between *A. palmeri* isoforms and its homologs in other species from the *Amaranthaceae* family (Fig. 2). The *GS2.1* gene was located adjacent to *GS2* in the *A. palmeri* genome. At the protein and mRNA level, these two genes show a large degree of conservation as shown in the BLAST output, where 00779 (g17050) was used as the query. However, once the genomic level was assayed, the association fell apart and the second gene was not retrieved as a significant hit. Possible regions of synteny were assayed in the genomic surroundings of the *GS2* isoforms. Syntenic regions, which are indicative of duplication events (Tang et al., 2008), were not observed in the genomic regions flanking the *GS2* isoforms (Supplementary Fig. S2). Therefore *GS2.1* is unlikely a result of a duplication event of *GS2*.

By producing a homology model of *A. palmeri* GS1 and docking GFA into its binding site, we identified seven amino acids involved in GFA binding: E131, E192, G245, H249, R291, R311 and R332 in GS1 (Fig. 3). Their homologs in GS2 are E190, E251, G304, H308, R350, R370 and R391. Alignment of peptide sequences of *A. palmeri GS* isoforms with 34 other GSs (representing different plant families including *Fabaceae*, *Malvaceae*, *Brassicaceae*, *Poaceae*, *Asteraceae*) showed full conservation at these positions, suggesting that mutations at the substrate-binding residues are not tolerable (brown rectangles in Fig. S1). Mutations at the substrate-binding residues of *GS1* and *GS2* rendered inactive or severely impaired protein in a *E. coli*-based assay (A. Porri, unpublished data), corroborating with this hypothesis.

RACE primers were used for the amplification and sequencing of the untranslated regions (UTR). The lengths determined were: g13234: 5'UTR 83 bp, 3'UTR 268 bp; g1417:

5'UTR 77 bp, 3'UTR 67 bp; g17050: 5'UTR 132 bp, 3'UTR 163 bp. The *GS2.1* isoform was not detected in this experiment, indicating that it might be an unexpressed pseudogene (Chandrasekaran and Betrán, 2008).

Sequence analysis of *GS* isoforms in GFA survivors

Overall, all GS isoforms in 17 GFA survivors from MO#2 population showed a high level of conservation. Few mutations were detected in *GS1.1* and *GS1.2* (Supplementary Fig. S3 to S5). *GS2* from all 17 plants showed 100% sequence identity to the wild-type (WT), which illustrates the importance of this isoform in plant metabolism and the 'fixed' configuration of its catalytic site.

The most prevalent mutation was N41D, found in *GS1.1* of six plants. In this same isoform, four mutations were detected once (G27D, Y95N, V109D and E122K) and N109Y was detected twice. In the *GS1.2* isoform, only three mutations were detected: D173E was found in three plants, and F114I and I220L were found only once.

Copy number, transcript abundance and protein levels of GS isoforms in GFA-resistant plants

None of the samples showed increased copy of *GS1.1*, while only one sample showed increase in *GS1.2* copies (Fig. 4). On the other hand, 16 out of 17 samples showed a 4-fold or higher increase in *GS2* copies. The highest copy number was observed in sample #32, where a 21-fold increase was detected.

Fold change in *GS* expression followed a similar pattern: while both *GS1* isoforms had minimal or no increase in expression, *GS2* had a significant overexpression in all samples (Fig. 5). The lowest and highest fold change of *GS2* expression was 4- and 190-fold, respectively. There was no linear correlation between fold change in expression and in copy number of *GS2*,

while *GS1.1* was slightly over-expressed in some samples despite the absence of gene copy amplification (Fig 6).

In 12 survivors from the MO#2 F1 population, fold-changes in copy number, transcript abundance and protein levels were determined relative to three plants from the SS population. Although GS1.1 and GS1.2 were detected at similar amounts in R and S plants (data not shown), GS2 levels were higher in all samples, with a minimum and maximum of 2- and 16-fold change, respectively (Fig. 7). As seen previously in the dataset produced from 17 plants from the field population, the correlation between gene copies, transcript abundance and protein levels is weak, reinforcing the hypothesis that epigenetic or post-transcriptional mechanisms may play important roles in GS2 biosynthesis regulation in this resistant population.

Ammonia quantification using a leaf-disc assay

Ammonia accumulation is one of the physiological consequences of GS inhibition by GFA, and it has been used as a marker of plant susceptibility to this herbicide (Downs et al., 1994, Dayan et al., 2015). Therefore, plants with higher *GS* expression are expected to accumulate less ammonia. Twelve survivors from the MO#2 F1 population were submitted to an *in vitro* ammonia accumulation assay and had their *GS2* expression analyzed. As expected, there was a significant negative correlation between these two variables ($r = -0.712$, $P=0.00934$), as seen in Fig. 8.

Ectopic expression of *A. palmeri* GS2 in *N. benthamiana* leaf disc

Amaranthus palmeri GS2 was transiently overexpressed in *N. benthamiana* leaves, and an *in vitro* assay was performed by incubating leaf-discs in GFA solutions of increasing concentrations. The ϕPSII was determined and used as an indicator of the effect of GFA in photosynthetic activity.

Samples that received the empty vector had an estimated I_{50} of 40 µM GFA, while samples that overexpressed the *A. palmeri GS2* showed a 4-fold increase on that parameter (I_{50} = 160 µM) (Fig. 9). These results strongly suggest that overexpression of *GS2* is enough to increase plant tolerance to GFA.

Discussion

In the present work, we describe the discovery of the first GFA-resistant *A. palmeri* genotype from Missouri, USA. In the greenhouse around 20% of plants from the MO #2 field population survived the labeled rate of GFA (657 g ha^{-1}). The survival rate doubled in the progeny (MO #2 F1), equivalent to 6.1-fold resistance level relative to the susceptible population (Fig. 1). This level of resistance is already high, considering the frailty and uniformity of plant size under greenhouse conditions. The resistance problem is expected to be even higher in the field. GFA would be less effective, or could be inconsistent, under field conditions because of various mitigating factors including the large variability in plant growth stage and size; the 'hardened' condition of seedlings; sub-optimal environmental conditions around the time of herbicide application; and uneven spray coverage due to height differentials, plant crowding, and patchiness of plants. *Amaranthus palmeri* is a prolific seed producer and highly competitive (Ward et al., 2013). If these resistant plants are not controlled by other means, crop productivity will certainly be reduced (Massinga et al., 2001, Chandi et al., 2012) and the resistance problem to glufosinate will escalate. Hence, resistance to herbicides in general, and *A. palmeri* resistance to multiple herbicides (including GFA) in particular, is a threat to food security and economic sustainability.

Glutamine synthetase, the target of GFA, is a vital enzyme present in all living organisms. Enzymes in this family are classified as Type I, II or III based on its primary and

quaternary structures (dos Santos Moreira et al., 2019). In plants, GS can be further categorized according to their subcellular location: GS1 enzymes are cytosolic, and GS2 enzymes are plastidic (Bernard and Habash, 2009). While GS2 is encoded by a single, nuclear gene, GS1 is encoded by a multigene family generally composed of three to five isoforms (Swarbreck et al., 2010). In the present study, two cytosolic and one plastidic isoforms were discovered in the *A. palmeri* genome. The phylogenetic analysis of GS isoforms from 11 species (three monocots and eight dicots) showed a high similarity of *GS1.1*, *GS1.2* and *GS2* from *A. palmeri* with its respective homologs in *Spinacia oleracea* and *Chenopodium quinoa*, species that are also in the *Amaranthaceae* family (Fig. S1). *GS2* sequences from all species fall into a well-defined clade, as the divergence of cytosolic and plastidic *GS* genes pre-dates the divergence of monocots and dicots (Biesiadka and Legocki, 1997). The three monocots grouped together regardless of the isoform considered. With the clear distinction of GS between dicots and monocots, the involvement of GS in resistance to glufosinate in these two groups of species may differ. This question will remain until other cases of resistance to glufosinate evolve and the resistance mechanism identified.

With the homology model of *A. palmeri* GS1.1, produced using *Z. mays* GS1 (PDB 2D3A) as a template, we identified seven residues involved in GFA binding: E131, E192, G245, H249, R291, R311 and R332. The same amino acids were found to interact with methionine sulfoximine (another glutamate analog) in *Z. mays* GS1 (Unno et al., 2006), and are homologous to E190, E251, G304, H308, R350, R370 and R391 in *A. palmeri* GS2. Interestingly, no polymorphisms were observed in these loci in any of the 34 sequences included in the phylogenetic analysis (brown rectangles in Fig. S1), highlighting their importance to proper protein function (Capra and Singh, 2007, Unno et al., 2006). In accordance with that, mutations

introduced *in silico* at the above-mentioned positions from *GS1.1* and *GS2* produced either inactive or severely impaired enzymes, based on an *in vitro* assay (A. Porri, unpublished data).

Allosteric interactions are known to be one of the regulatory mechanisms of GS activity (Stadtman, 2001). Therefore, mutations outside the binding pocket could allosterically interfere with GFA binding and lead to herbicide resistance. To exclude that possibility, the three GS isoforms of 17 GFA survivors from the MO#2 population were sequenced. A total of six and three mutations were found in *GS1.1* and *GS1.2* isoforms, respectively, while no polymorphisms in *GS2* were detected in any of the 17 samples analyzed. The most prevalent mutation among GFA survivors was a N41D substitution in *GS1.1* (found in 6 out of 17 plants), but polymorphisms at that position are common (indicated by the pink rectangle in Fig. S1). The S59G substitution recently reported to confer GFA resistance in *E. indica* (Zhang et al., 2022) was not found in this experiment (blue rectangle in Supplementary Fig. S3 – S5). As no mutations were ubiquitous to all survivors analyzed, we conclude that GFA resistance in this *A. palmeri* genotype is not conferred by target-site mutations.

The *GS* copy number and expression level were also determined using the same 17 plants analyzed for *GS* polymorphisms. All isoforms (*GS 1.1*, *GS 1.2* and *GS2*) were assayed. *GS2* amplification was detected in all samples, but *GS1.1* and *GS1.2* copies were not augmented. Fold-change in transcript levels followed a similar pattern, with *GS1* isoforms showing little to no increase in expression and *GS2* being overexpressed to a great extent. It is intriguing that the correlation between *GS2* fold change in copy number and expression was weak (Fig. 6), suggesting that transcriptional regulation mechanisms might be involved in *GS2* overexpression. Similarly, the increase in GS2 protein levels were not always correlated with the fold-change increase in transcript abundance (Fig. 7), which points to a complex regulatory system of this

biochemical pathway. Changes in the methylation status of DNA and histones, as well as post-transcriptional mechanisms such as increase in mRNA stability are being investigated. It is possible that *GS2* expression is induced upon exposure to GFA. Corroborating this hypothesis, MO#2 plants that survived a 1x GFA application were not killed by a second application of 4x GFA applied 2 wk later (S. Bowe, unpublished data).

The negative correlation between *GS2* expression and ammonia content in *A. palmeri* leaf-discs, and the increased GFA tolerance observed in *N. benthamiana* overexpressing GS2 present strong evidence that this naturally evolved mechanism confers resistance to GFA in *A. palmeri*. GS overproduction in transgenic rice (Cai et al., 2009, James et al., 2018), tobacco (Eckes et al., 1989), wheat (Huang et al., 2005) and poplar (Pascual et al., 2008), all resulted in GFA tolerance at the plant level. The same was observed at the cell level in tobacco (Ishida et al., 1989) and alfalfa (Donn et al., 1984). The irreversible nature of GS inhibition by GFA matches very well with resistance through target-site overproduction. The enzyme abundance not only allows the biochemical pathways to be maintained, but also reduces the pool of available herbicide molecules with time. Furthermore, gene amplifications can facilitate evolution by reducing the selective constraints in one or more copies (Panchy et al., 2016, Flagel and Wendel, 2009). In other words, resistance-conferring mutations that would not be tolerated due to a strong fitness cost might be able to evolve as remaining copies are still functional. The close proximity of *GS2* and *GS2.1* provokes questions related to the evolution of this genomic region. Here we suggest that it is unlikely they originated from a duplication event. Expression of *GS2.1* was not detected in plants in normal physiological conditions, but assessing the effect of abiotic stresses on *GS2.1* expression might be an interesting follow-up study.

Among the naturally evolved mechanisms of herbicide resistance, target-site amplification is rare (Gaines et al., 2020). The first and most notable example is the glyphosate-resistant *A. palmeri* carrying increased *EPSPS* copies (Gaines et al., 2010). Interestingly, glyphosate tolerance in carrot cell lines was attributed to an increase in EPSPS activity at least 25 years prior the discovery of this mechanism in *A. palmeri* (Nafziger et al., 1984). This adaptation mechanism can now be found in at least eight weed species as a result of convergent evolution (Patterson et al., 2017). Resistance by target site amplification can also be introgressed into other genomically compatible species via pollen flow such as what occurred between *A. palmeri* and *A. spinosus* (Nandula et al., 2014).

EPSPS copies are distributed throughout the genome of *A. palmeri* due to self-replication of the *EPSPS cassette*, a ~300 kbp circular extra-chromosomal DNA structure that carries 58 genes plus the *EPSPS* gene itself (Molin et al., 2020, Gaines et al., 2010, Molin et al., 2017). Due to the large size and high copy number of the *EPSPS cassete*, genome size was shown to be up to 13% larger in R plants compared to S (Molin et al., 2017). Genome size analysis of plants from the MO#2 population did not detect any changes compared to plants from a SS population (M.M. Noguera, unpublished data). In *Bassia scoparia*, *EPSPS* copies are in tandem arrangement in a single chromosomal locus, likely originated from repeated unequal crossover (Jugulam et al., 2014). A greater understanding of the origin of these duplication events may facilitate the development of risk-prediction models, allowing proactive identification of 'high risk' species-by-chemistry combinations. Lastly, target-site amplification was also found in a *Digitaria sanguinalis* biotype cross-resistant to ACCase inhibitors (Laforest et al., 2017), but detailed information about its origin and distribution are not available.

In conclusion, our data strongly support the hypothesis that *GS* amplification and overexpression (particularly the plastidic isoform, *GS2*) is the main factor conferring resistance to GFA in this *A. palmeri* genotype. The co-occurrence of increased copy and increased expression of a herbicide target gene in the same plant is a novel adaptation mechanism that has not been detected previously. We hypothesize that epigenetic and post transcriptional mechanisms are likely to contribute to the overproduction of GS2 at the protein level, as these mechanisms are known to promote quick changes in transcript synthesis and translation (Zhang et al., 2018, Van Ruyskensvelde et al., 2018, Floris et al., 2009). Follow-up studies include determination of the distribution of *GS2* copies throughout the genome, the elucidation of the mechanism of *GS2* amplification, possible transcriptional and post-transcriptional regulation mechanisms involved in overexpression and protein synthesis, and contribution of additional traits towards resistance (such as ability to metabolize GFA, reduced absorption/translocation, or increased protection against oxidative damage). The multiple layers of regulation of protein biosynthesis in plants poses a challenge in elucidating herbicide resistance mechanisms related to target site overproduction, and the MO#2 population is a clear example of that. The history of *A. palmeri* adaptation to herbicide selection pressure shows that its management must not rely solely on the chemical approach. The use of a diversified strategy should be practiced, such as crop rotation, tillage, the use of preemergence herbicides, precise application time at young plant stage and herbicide mixtures of complementary mechanisms of action. The spread of GFA-resistant genotypes should be treated as a serious concern from the economical and humanitarian standpoints.

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Figures and Tables

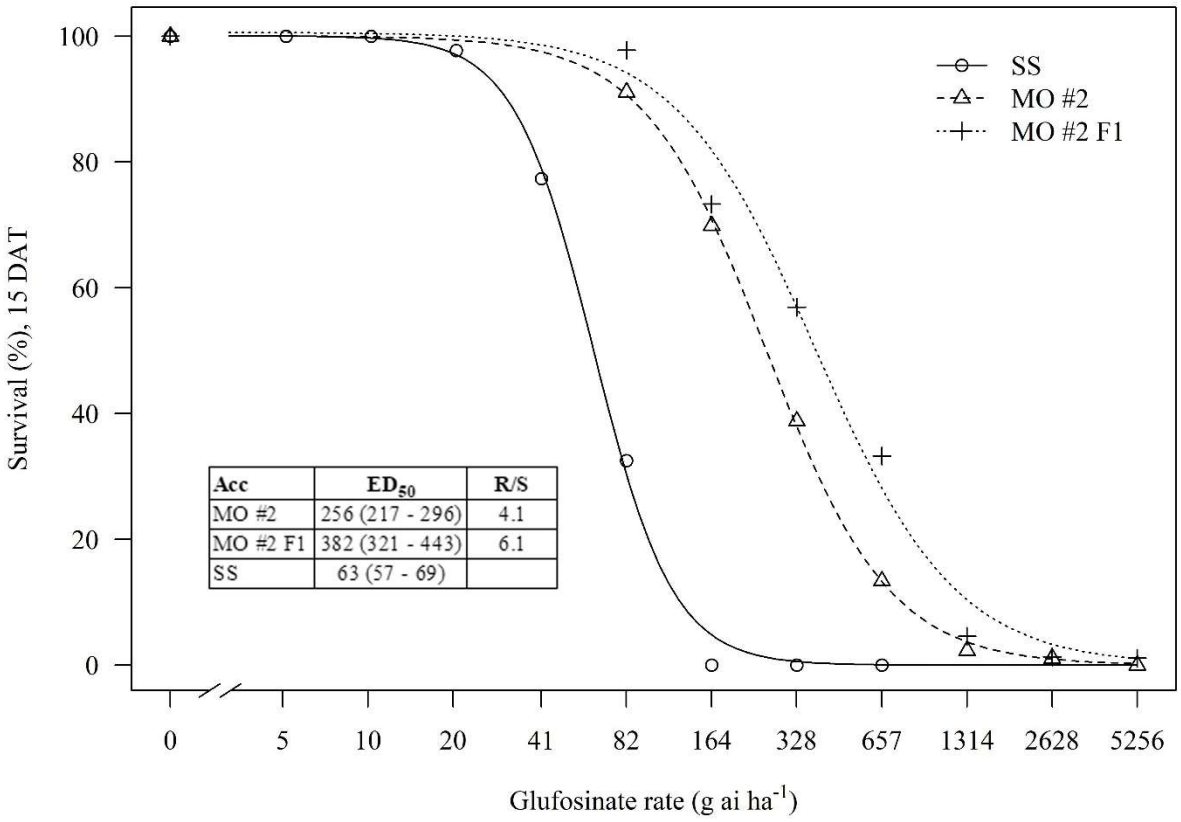

Figure 1. Response of MO #2, MO #2 F1 and SS to increasing rates of GFA. Labeled GFA rate is 657 g ha^{-1}. Percent survival data was fitted to a three-parameter log-logistic model and ED_{50} (GFA rate that controls 50% of plants) was estimated for each population; confidence intervals of this parameters are shown between brackets. Data points are means of two runs with four replications per treatment each (total *n*=8)

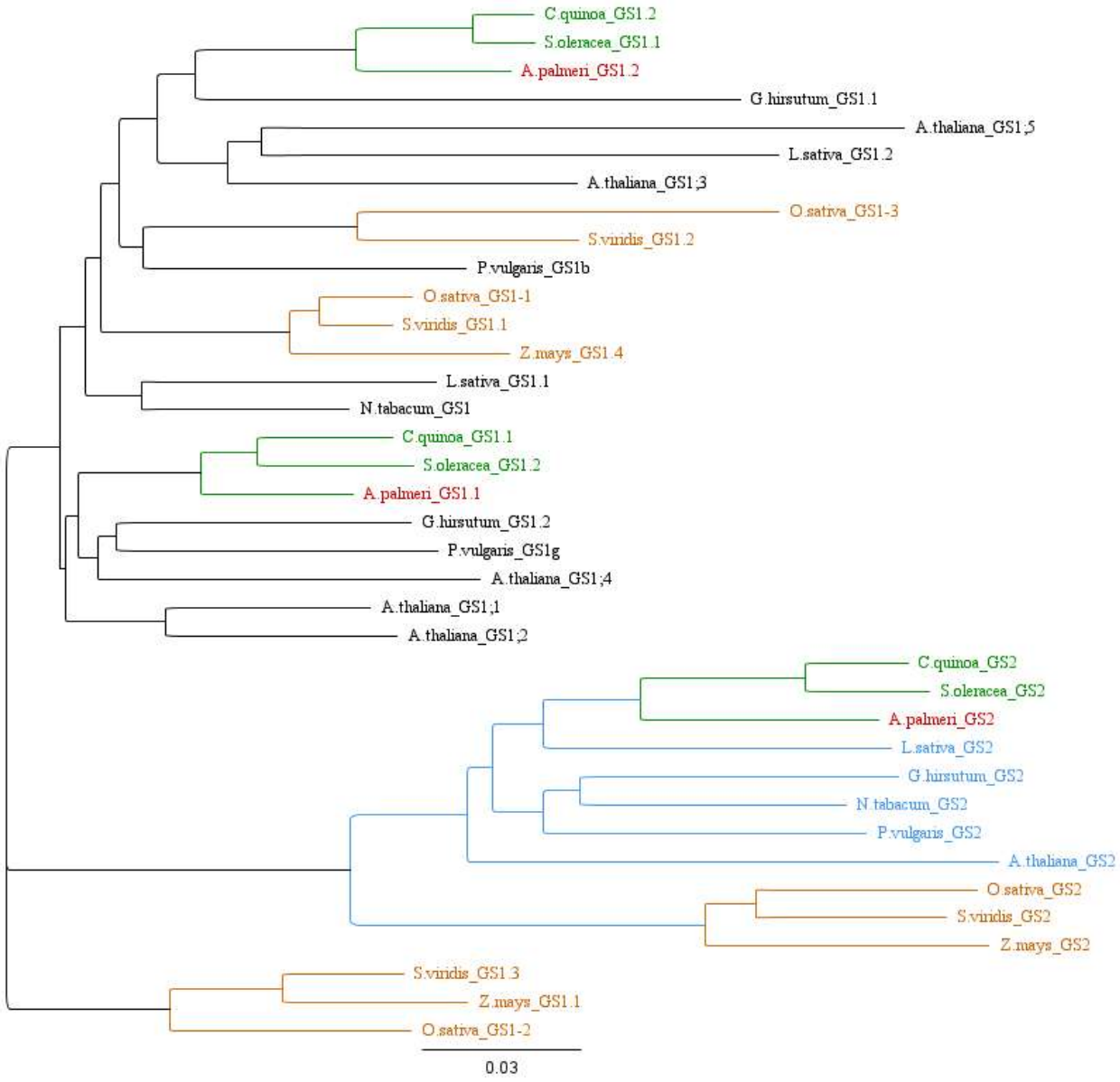

Figure 2. Phylogenetic tree composed of 37 *GS* isoforms from 12 plant species. Multiple alignment and tree construction was performed using Geneious Prime. *A. palmeri* isoforms are highlighted in red and clustered with other species from the *Amaranthaceae* family (green clades). GS isoforms in grasses were closely related (dark yellow clades). All GS2 sequences clustered in a well-defined clade (in blue). Sequences were obtained from Phytozeme and Genbank databases and entries are shown in the Supplementary file F1.

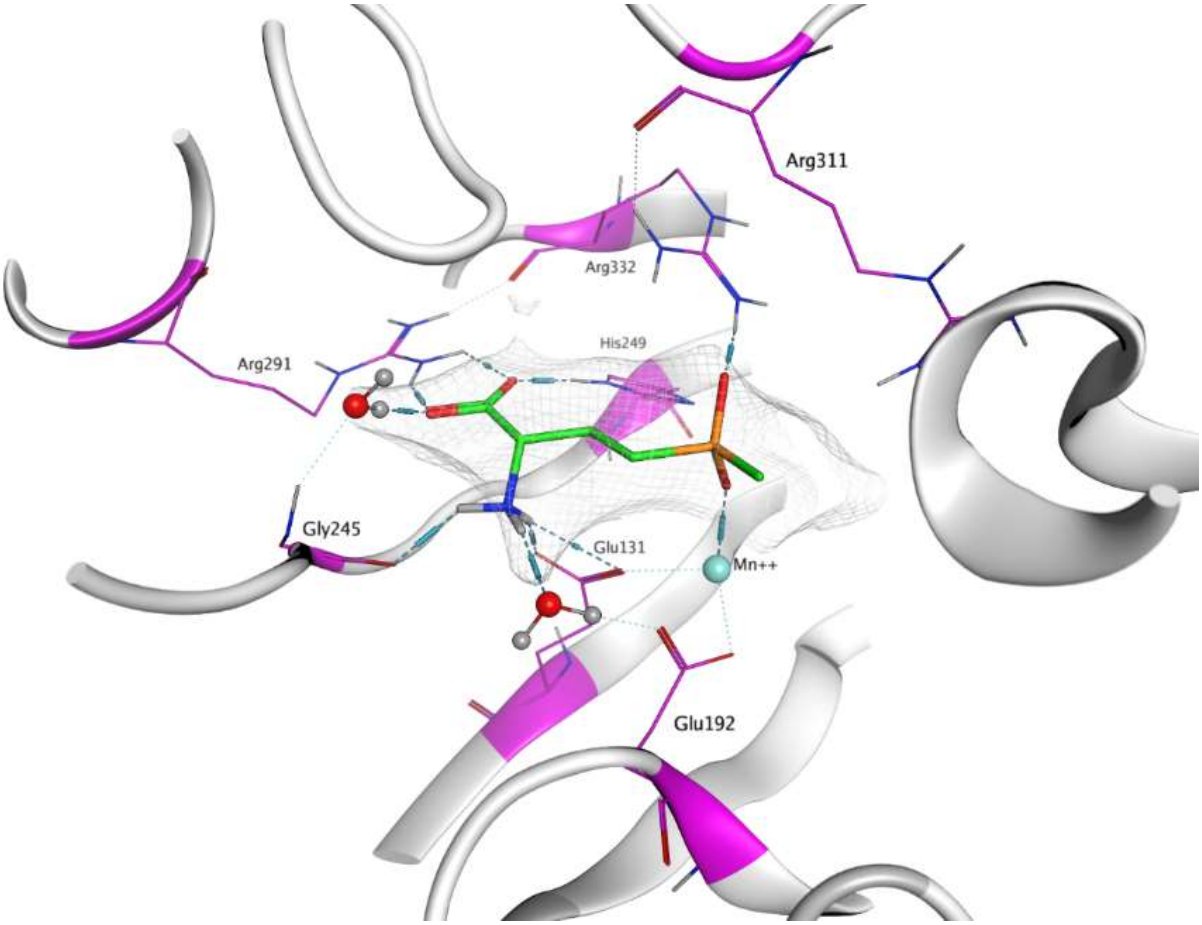

Figure 3. Homology model of *A. palmeri* GS1 with a GFA molecule bound into its catalytic site. The seven amino acids directly involved in GFA-binding are highlighted in fuchsia. Sticks in GFA molecule are color-coded: green = C, orange = P, red = O, blue = N and grey = H.

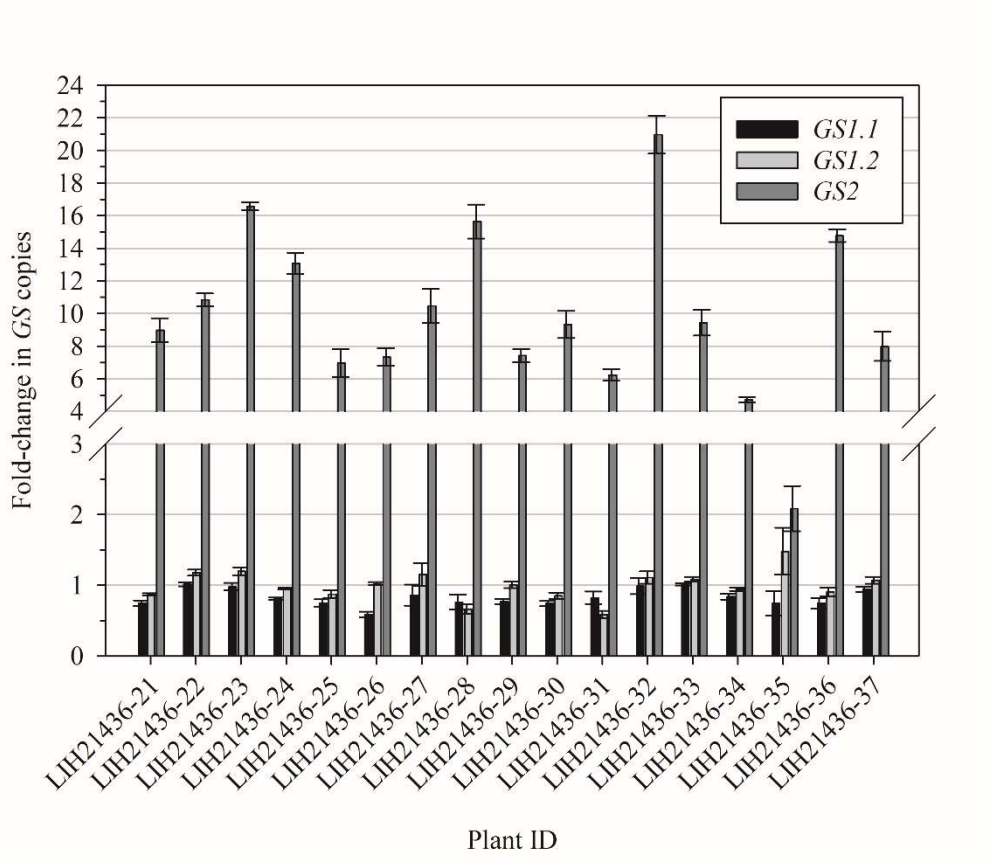

Figure 4. Fold-change in *GS* copies of 17 GFA survivors from the MO #2 population in relation to nontreated plants from a sensitive population. Fold-change was calculated using the $2^{-\Delta\Delta Ct}$ method as described in Schmittgen and Livak (2008). Bars represent means and lines represent the standard error of the mean.

SCHMITTGEN, T. D. & LIVAK, K. J. 2008. Analyzing real-time PCR data by the comparative CT method. *Nature protocols,* 3**,** 1101-1108.

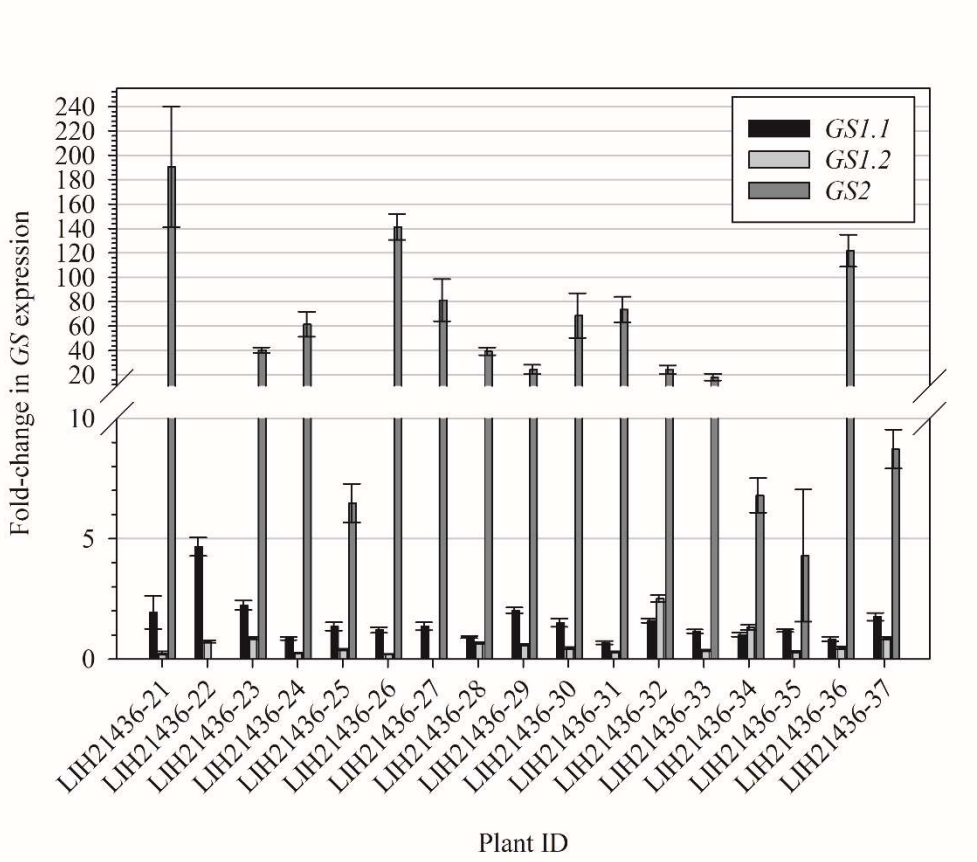

Figure 5. Fold-change in *GS* expression of 17 GFA survivors from the MO #2 population in relation to nontreated plants from a sensitive population. Fold-change was calculated using the $2^{-\Delta\Delta Ct}$ method as described in Schmittgen and Livak (2008). Bars represent means and lines represent the standard error of the mean.

SCHMITTGEN, T. D. & LIVAK, K. J. 2008. Analyzing real-time PCR data by the comparative CT method. *Nature protocols,* 3**,** 1101-1108.

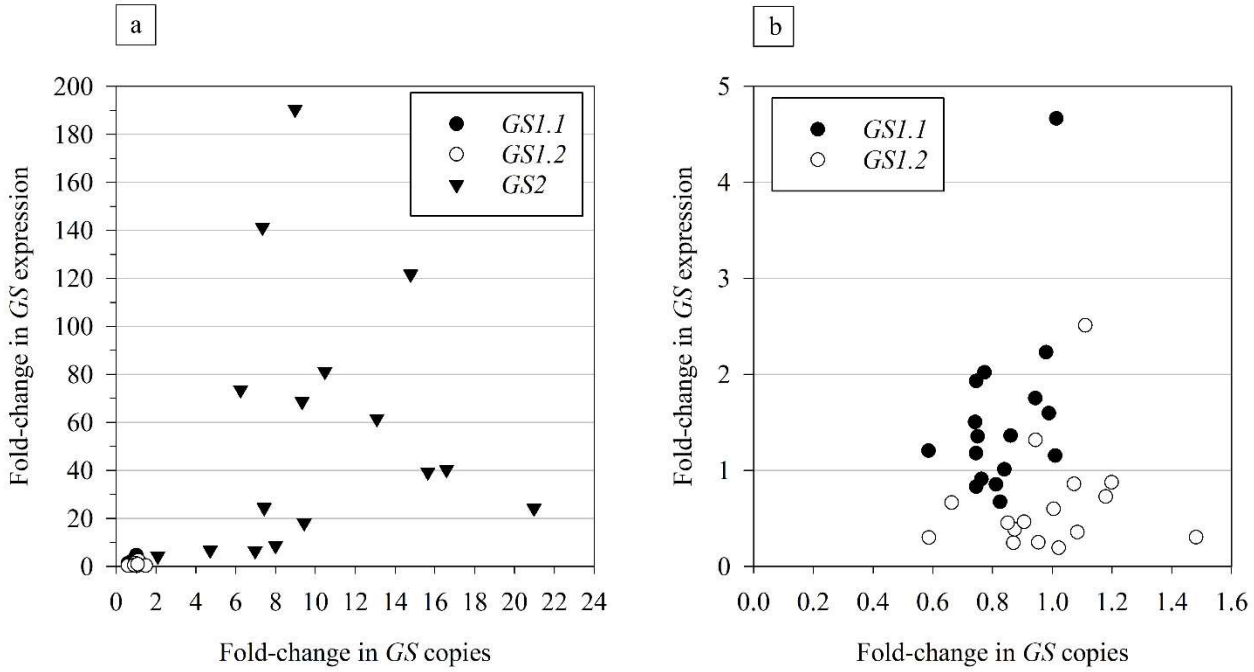

Figure 6. Correlation between fold-change in *GS* copies and expression, in 17 GFA survivors from the MO #2 population. Data points from *GS2* isoform were excluded from (b) for better visualization of data distribution.

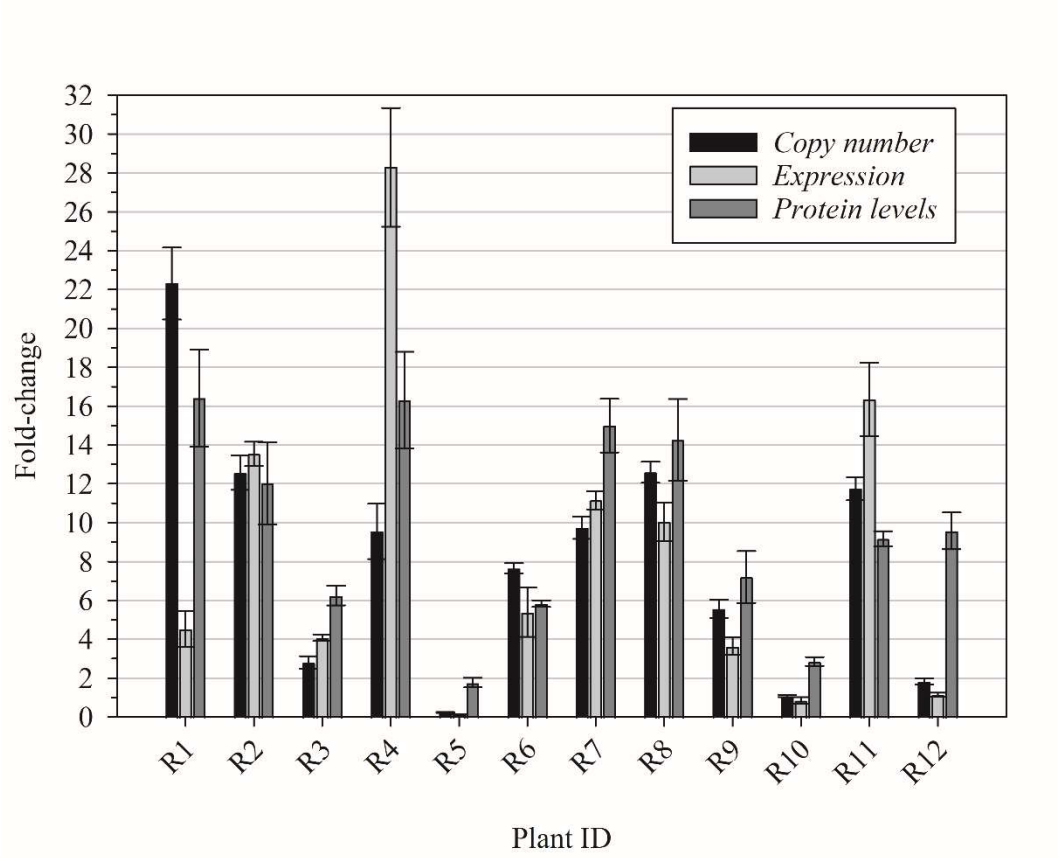

Figure 7. Fold-change in *GS2* copies, expression, and protein levels on 12 plants from the MO #2 F1 population compared to three plants from an SS population. Fold-change in *GS2* copies and expression was calculated using the $2^{-\Delta\Delta Ct}$ method as described in Schmittgen and Livak (2008). Fold-change in GS2 protein levels was calculated by dividing the LFQ intensity of the sample by the average of three SS plants. Bars represent means of three technical replicates and lines represent the standard error of the mean.

SCHMITTGEN, T. D. & LIVAK, K. J. 2008. Analyzing real-time PCR data by the comparative CT method. *Nature protocols,* 3**,** 1101-1108.

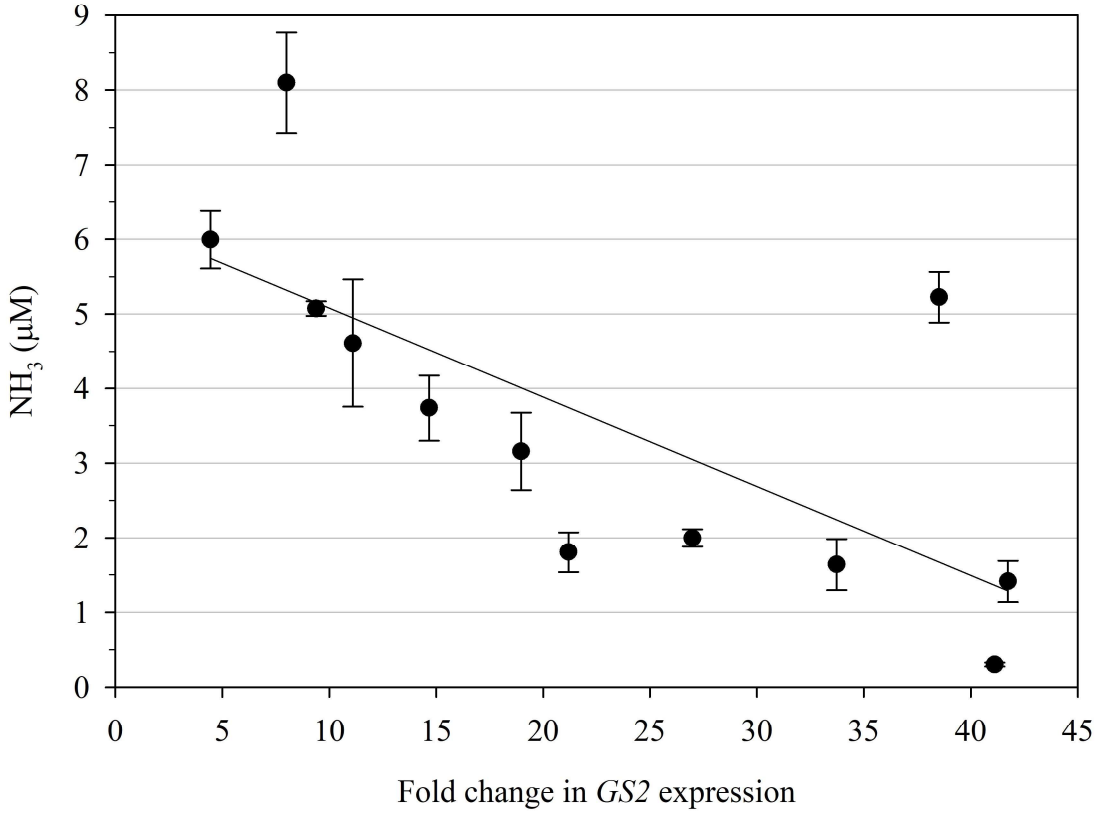

Figure 8. Correlation between ammonia accumulation and *GS2* expression fold-change relative to susceptible plants. Ammonia accumulation was determined spectrophotometrically at a wavelength of 630 nm. Absorbance was measured from leaf discs of 12 GFA-resistant plants incubated in 20mM GFA solution. Bars represent the standard error of the mean.

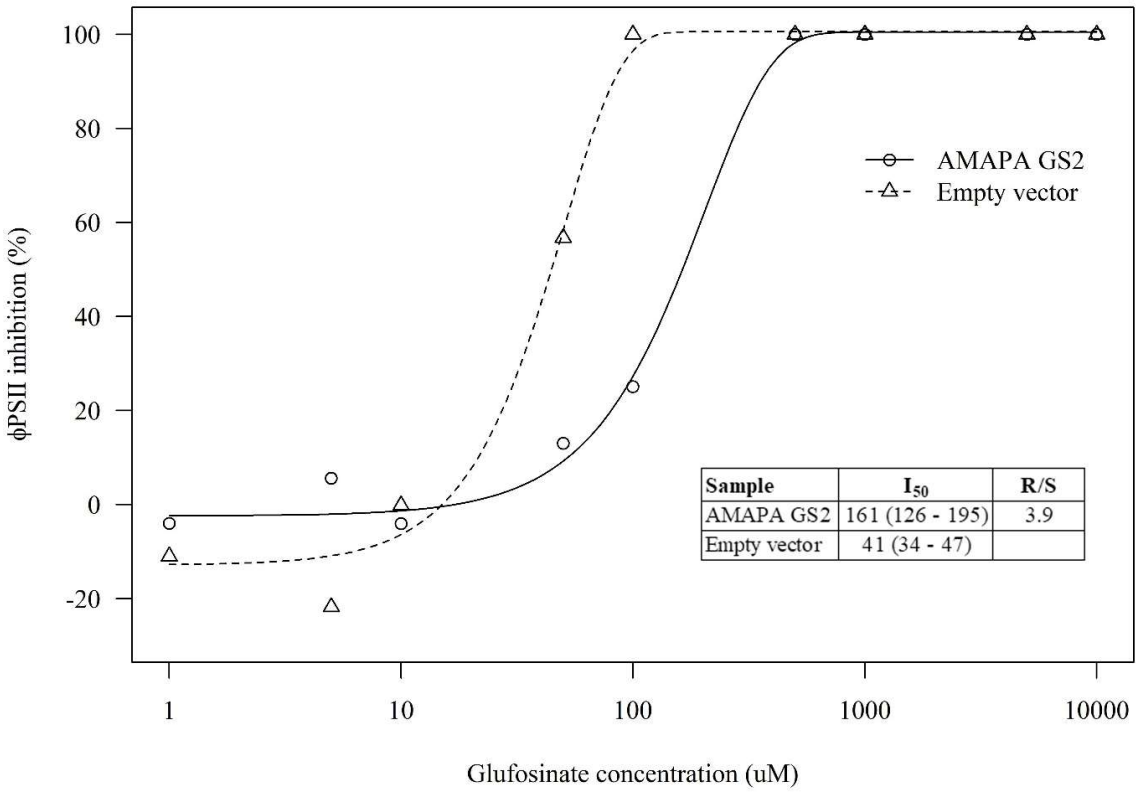

Figure 9. Response of *N. benthamiana* leaf-discs transiently overexpressing *A. palmeri GS2* or an empty vector to incubation in increasing concentrations of GFA. Photosystem II operating efficiency (ϕPSII) was obtained through chlorophyll fluorescence analysis and normalized to percent inhibition in relation to controls incubated in water. A Weibull II model was used to estimate I_{50} values; confidence intervals of this parameters are shown between brackets.

Appendix

Supplementary file F1. Genbank/Phytozyme entries used for construction of phylogenetic tree.

*A.thaliana*_GS1;1 (NP_198576.1);
*A.thaliana*_GS1;2 (NP_176794.1);
*A.thaliana*_GS1;3 (NP_188409.1);
*A.thaliana*_GS1;4 (AED92312.1);
*A.thaliana*_GS1;5 (NP_175280.1);
*A.thaliana*_GS2 (NP_001031969.1);
*C.quinoa*_GS1.1 (XP_021757390.1);
*C.quinoa*_GS1.2 (XP_021724688.1);
*C.quinoa*_GS2 (XP_021727743.1);
*G.hirsutum*_GS1.1 (XP_016737511.1);
*G.hirsutum*_GS1.2 (XP_016696747.1);
*G.hirsutum*_GS2 (XP_016670596.1);
*L.sativa*_GS1.1 (XP_023770002.1);
*L.sativa*_GS1.2 (XP_023754618.1);
*L.sativa*_GS2 (XP_023733962.1);
*N.tabacum*_GS1 (XP_016466322.1);
*N.tabacum*_GS2 (XP_016440217.1);
*O.sativa*_GS1-1 (XP_015626102.1);
*O.sativa*_GS1-2 (XP_015631679.1);
*O.sativa*_GS1-3 (XP_015628694.1);
*O.sativa*_GS2 (XP_015635322.1);
*P.vulgaris*_GS1b (XP_007152660.1);
*P.vulgaris*_GS1g (XP_007141923.1);
*P.vulgaris*_GS2 (XP_007147796.1);
*S.oleracea*_GS1.1 (Spov3_chr5.00751);
*S.oleracea*_GS1.2 (Spov3_chr4.02946);
*S.oleracea*_GS2 (Spov3_chr3.03310);
*S.viridis*_GS1.1 (Sevir.1G317300.1.p);
*S.viridis*_GS1.2 (Sevir.9G117000.1.p);
*S.viridis*_GS1.3 (Sevir.9G489700.1.p);
*S.viridis*_GS2 (Sevir.3G024800.1.p);
*Z.mays*_GS1.1 (BAA03432.1);
*Z.mays*_GS1.4 (AFP20991.1);
*Z.mays*_GS2 (NP_001352144.1).

Table S1. Primers used for sequencing and copy number/expression analysis of *GS* isoforms from *A. palmeri*. Fluorescent dyes for qPCR probes are shown in the footnote.

Objective	Name	Sequence (5' > 3')	Ta (°C)
Sequencing	GS1.1-F	GAAGAACATACTCATCTTCCACTTCTC	63
	GS1.1-R	TGCACAATAATGGCAGAGAAGATC	63
	GS1.2-F	TCTTCGTATTCTCTTTCATCTATGTCC	53
	GS1.2-R	CCAAGAAATTCCAAATTCACATTAACA	53
	GS2-F	CGACCACCCTTTTCCGATCA	60
	GS2-R	TGGGCACGTGAAASAGTTCC	60
qPCR	GS1.1-F	TGTGTGATGCCTATACTCCACA	60
	GS1.1-R	TACCATGGTTCCTCGGCAAC	60
	GS1.1-probe[1]	AGGAGAGCCAATCCCAACCAACA	60
	GS1.2-F	TGTGTGATGCATACACCCCG	60
	GS1.2-R	GACGTCGGGATGGCTAAAGA	60
	GS1.2-probe[2]	GCTGGAGAACCAATTCCAACAAACAAG	60
	GS2-F	TGGCACAAATACTTGCACCTT	60
	GS2-R	GCTGCTCCACCCTGTTTACT	60
	GS2-probe[1]	AGGCTCCACAAGTTCAATGACATCAA	60
	Actin-F	GCGGAAAGCTAAGCGTGAAC	60
	Actin-R	TCAGACCTGCTCTGGAGTCA	60
	Actin-probe[3]	GGAGGAAAAGGCGGATGCTGCA	60

[1]Fam/BMN-Q535
[2]Cy5/BMN-Q650
[3]Hex/BMN-Q535

Figure S1. Multiple alignment of *A. palmeri GS* isoforms with 34 other *GS* sequences representing 11 plant species. Pink rectangle under the consensus sequence indicates the 41st position *of A. palmeri GS1.1*, where polymorphisms were detected in 6 of 17 survivors from MO #2 population. Brown rectangles indicate the amino acids involved in substrate-binding, which are highly conserved across species.

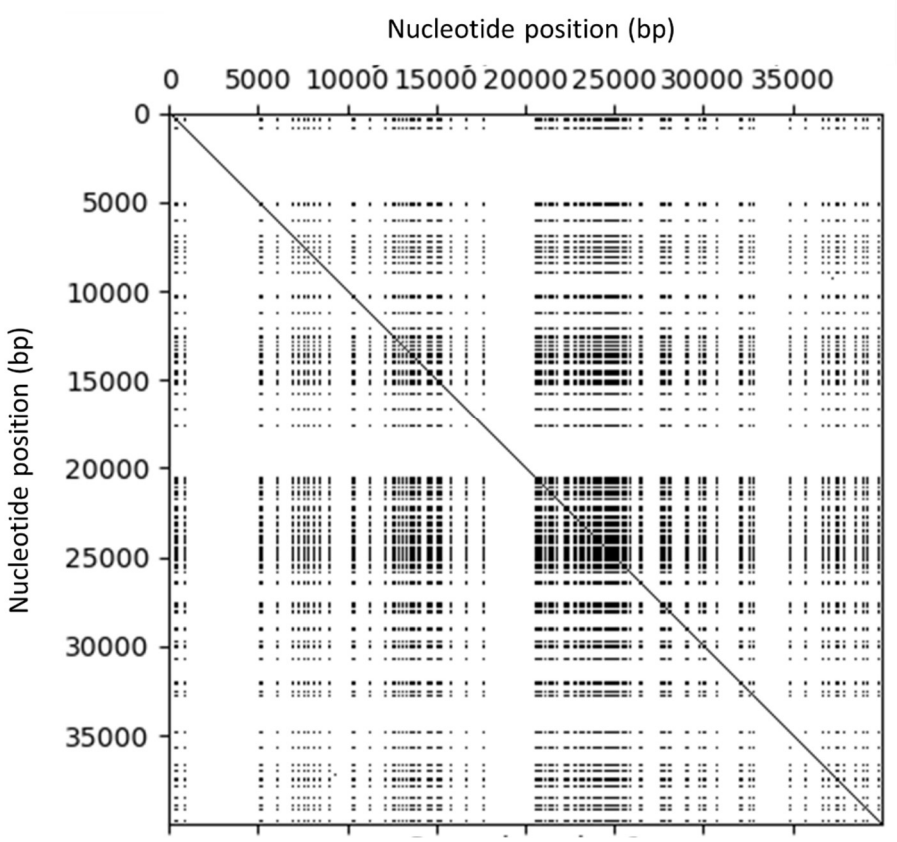

Figure S2. Nucleotide-to-nucleotide synteny plot of the genomic region containing *GS2.1* and *GS2*. Diagonal lines are indicative of synteny, while vertical/horizontal lines are indicative of repeats. A window size of 40 kb and kmer size of 10 bp was used.

Figure S3. Multiple alignment of *GS1.1* sequences of 17 GFA survivors. Brown rectangles indicate substrate-binding residues, and the blue rectangle locates S59, where a mutation was reported to confer resistance to GFA in *Eleusine indica* (Zhang et al., 2022).

ZHANG, C., YU, Q., HAN, H., YU, C., NYPORKO, A., TIAN, X., BECKIE, H. & POWLES, S. 2022. A naturally evolved mutation (Ser59Gly) in glutamine synthetase confers glufosinate resistance in plants. *Journal of Experimental Botany*.

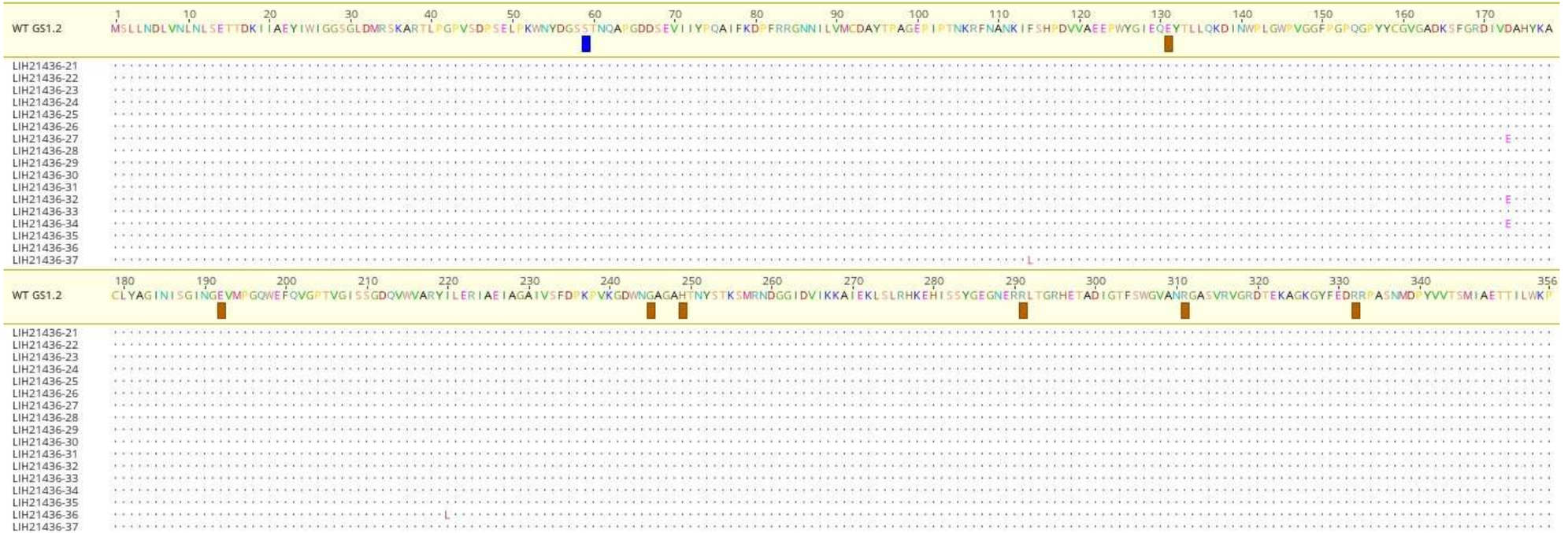

Figure S4. Multiple alignment of *GS1.2* sequences of 17 GFA survivors. Brown rectangles indicate substrate-binding residues, and the blue rectangle locates S59, where a mutation was reported to confer resistance to GFA in *Eleusine indica* (Zhang et al., 2022).

ZHANG, C., YU, Q., HAN, H., YU, C., NYPORKO, A., TIAN, X., BECKIE, H. & POWLES, S. 2022. A naturally evolved mutation (Ser59Gly) in glutamine synthetase confers glufosinate resistance in plants. *Journal of Experimental Botany*.

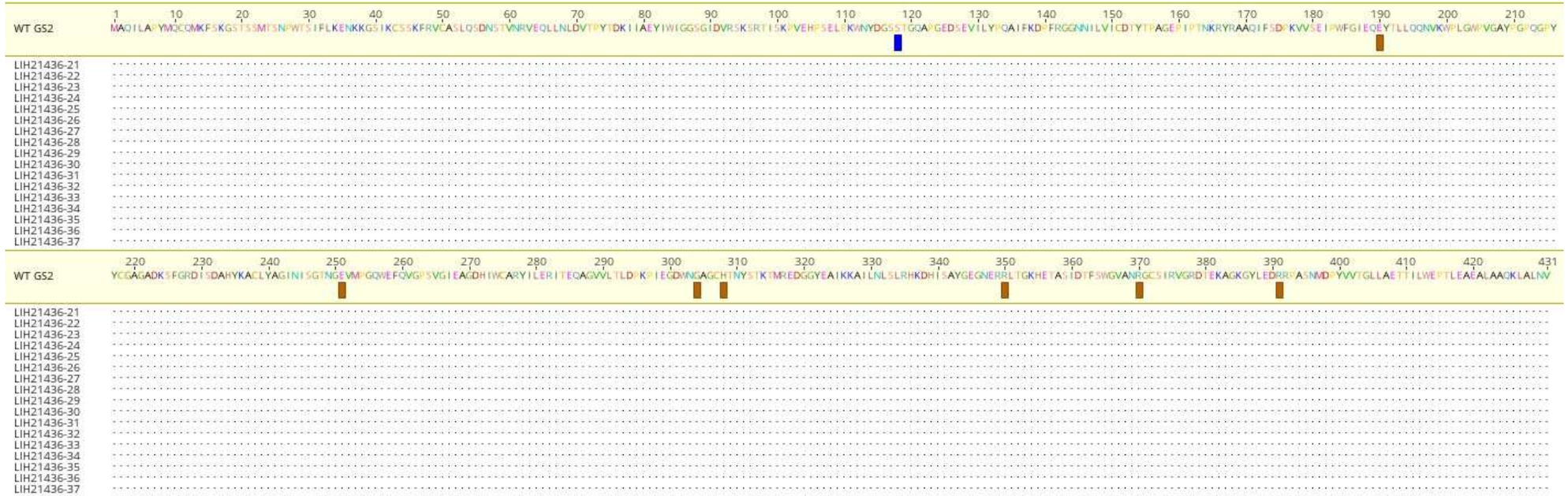

Figure S5. Multiple alignment of *GS2* sequences of 17 GFA survivors. Brown rectangles indicate substrate-binding residues, and the blue rectangle locates the residue homologous to S59, where a mutation was reported to confer resistance to GFA in *Eleusine indica* (Zhang et al., 2022).

ZHANG, C., YU, Q., HAN, H., YU, C., NYPORKO, A., TIAN, X., BECKIE, H. & POWLES, S. 2022. A naturally evolved mutation (Ser59Gly) in glutamine synthetase confers glufosinate resistance in plants. *Journal of Experimental Botany*.

Physical mapping, stability and inheritance of amplified *GS2* copies in glufosinate-resistant *Amaranthus palmeri* (Palmer amaranth)

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*Corresponding author

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Abstract

Glufosinate resistance in Palmer amaranth is achieved by an overproduction of the chloroplastic glutamine synthetase (GS2) protein, achieved by amplification and overexpression of the coding gene. In this research, the variability in *GS2* copy number among different plant tissues was investigated. Inheritance patterns were also analyzed and correlated to the physical location of *GS2* copies in Palmer amaranth genome. Our results indicate that *GS2* copy number varies randomly within a plant and also over time. Somatic mosaicism regarding GS2 copy number affects its segregation patterns, as inheritance did not follow classic Mendelian patterns. Segregation of resistance at the plant level and *GS2* amplification do not correlate well. Fluorescence in situ hybridization revealed that not all cells from a high GS2 copy individual contains *GS2* amplification, and rare cells from a low GS2 copy number plant may contain cells with several GS2 signals. Unpredictable inheritance, somatic mosaicism and presence of *GS2* copies in multiple chromosomes suggests the involvement of extrachromosomal circular DNAs in GS2 amplification. Further studies are needed to validate this hypothesis.

Introduction

Amaranthus palmeri is a troublesome weed with worldwide distribution and can significantly reduce yield of row crops if not properly managed (Roberts and Florentine, 2021). Among the traits that confer exceptional weediness to this species, its high genetic diversity stands out as it facilitates the evolution of herbicide resistance. Resistance to at least nine herbicide modes of action has been reported, which ranks Palmer amaranth second highest in resistance problems, behind only *Lolium rigidum* and *Poa annua*, with resistance to 12 modes of action (Heap, 2023).

Palmer amaranth resistance to glufosinate-ammonium (GFA), the only herbicide inhibitor of glutamine synthetase (GS) commercially developed, is the most recent case. Currently, populations from Missouri, North Carolina and Arkansas were found to be resistant to GFA (Noguera et al., 2022, Jones, 2022, Priess et al., 2022). In the population from Missouri, resistance was attributed to the overproduction of GS2, the chloroplast-located isoform, by means of gene amplification and overexpression acting concomitantly, but not independently (Noguera et al., 2022). In a population from Arkansas, *GS2* amplification was detected in four survivors of GFA applications, and *GS2* overexpression was detected in three nontreated plants from the same population. Whether GS2 amplification and overexpression happens in the same plant was not determined, nor if *GS2* overexpression leads to GS2 overproduction (Carvalho-Moore et al., 2022). The mechanism of resistance in GFA-resistant populations from North Carolina remains to be elucidated (Jones, 2022).

Herbicide resistance by target-site gene amplification is rare. The first report dates to 2010, when 5-Enolpyruvylshikimate-3-Phosphate synthase (*EPSPS*) amplification in glyphosate-resistant Palmer amaranth was described by Gaines et al. (2010). The same mechanism was

documented later in *L. perenne* ssp. *multiflorum* (Salas et al., 2012), *Bassia scoparia* (Jugulam et al., 2014), *A. spinosus* (Nandula et al., 2014), *Eleusine indica* (Chen et al., 2015), *Bromus diandrus* (Malone et al., 2016), *A. tuberculatus* (Dillon et al., 2017), *Chloris truncata* (Ngo et al., 2018), *P. annua* (Brunharo et al., 2019), *Hordeum glaucum* (Adu-Yeboah et al., 2021), and *Salsoga tragus* (Yanniccari et al., 2023). A relatively less-known case was amplification of Acetyl-CoA carboxylase (*ACCase*) in *Digitaria sanguinalis* cross-resistant to ACCase inhibitors (Laforest et al., 2017).

The physical location of additional copies in the plant genome can give hints about its heritability and mechanism of amplification (Jugulam and Gill, 2018). For example, *EPSPS* copies in *B. scoparia* are arranged in tandem at the telomeric regions of homologous chromosomes, which lead Jugulam et al. (2014) to suggest that amplification was due to unequal crossover. In addition, the close location of *EPSPS* copies resulted in single-locus inheritance (Jugulam et al., 2014). In *E. indica*, *EPSPS* copies are present in two pairs of homologous chromosomes, indicating a possible role of transposable elements in *EPSPS* amplification (Chen et al., 2020). In Palmer amaranth, *EPSPS* copies were found in several chromosomes (Gaines et al., 2010), and amplification was later determined to be eccDNA-mediated (Koo et al., 2018b). The apparent random distribution of *EPSPS* copies in Palmer amaranth genome have not allowed scientists to fully understand and predict its inheritance (Mohseni-Moghadam et al., 2013, Chandi et al., 2012, Giacomini et al., 2019).

This research focuses on understanding the distribution of amplified *GS2* copies and the inheritance pattern of GFA resistance in Palmer amaranth. The objective of this study was to determine the physical distribution of amplified *GS2* copies in Palmer amaranth genome, and how that relates to copy number stability and inheritance.

Materials and methods

Plant materials and *GS2* copy number (CN) analysis

All experiments herein described were conducted with two *A. palmeri* populations, namely MO20 #2 F1 and SS, which were previously characterized regarding their response to GFA (Noguera et al., 2022). Hereafter, these populations will be called GFA-R and GFA-S, respectively.

Unless otherwise stated, seeds were sown 50-cell trays filled with a commercial potting mix (ProMix LP15; Premier Tech Horticulture, Quakertown, PA, USA) and thinned to one plant per cell a week after planting. Plants were kept in a greenhouse maintained at 32/28 C day/night temperatures, with a 14-h photoperiod achieved by supplemental light.

GS2 CN was determined by TaqMan assays, adapted from Noguera et al. (2022). Actin was used as internal reference gene in duplex reactions and samples were ran in duplicate. Each qPCR reaction was composed of 12.5 µL of 2x GoTaq qPCR Probe Master Mix (Promega Corporation, Madison, WI, USA), 2.5 µL of each primer (0.5 µM), 0.5 µL of each probe (0.2 µM), 100 ng of DNA and water to a final volume of 25 µL. Assays were conducted in a CFX96 Real-Time System (Bio-Rad Laboratories, Hercules, CA, USA) under the following thermoprofile: 95 C for 2 min followed by 40 cycles of 95 C for 15 s, and 60 C for 1 min. Fluorescence measurements were taken at the end of each amplification step, and the $2^{-\Delta Ct}$ was used to calculate relative *GS2* CN (Schmittgen and Livak, 2008).

Stability of GS2 copies within plants

To determine the effect of GFA exposure and tissue localization in the number of *GS2* copies in Palmer amaranth, eight seedlings from the GFA-R accession were selected and individually transplanted to 500 mL pots. The average *GS2* CN in these plants was 10,

determined as previously described. When plants reached the 15-leaf stage, leaf samples were collected from all plants. After 24 h, half of the plants were sprayed with 82 g ai ha^{-1} of GFA, and a second set of samples was collected 24 h from application. Concomitantly, samples were also taken from the four nontreated plants. The sampling protocol is illustrated in Figure 1. Briefly, four leaves from each plant were selected, and from each leaf, two 5 mm leaf discs were collected. Each leaf disc was considered a sample. Therefore, each plant was represented by 16 samples: 4 leaves x 2 locations per leaf x 2 sampling times. DNA was extracted using a modified CTAB protocol (Doyle and Doyle, 1987), quantified spectrophotometrically (Nanodrop 2000; ThermoFisher Scientific, Waltham, MA, USA), and diluted to 20 ng μL^{-1}. *GS2* CN was determined in all samples as previously described.

Data from each plant was analyzed separately using JMP Pro 16.0.0 (SAS Institute Inc, Cary, NC, US), considering sample location, leaf and timing as factors. ANOVAs were not significant for any of the plants tested. Therefore, t-tests were used to check for statistical differences between two sampling timings for a same plant, and box-whiskers plots were prepared on SigmaPlot 14.5 (Systat Software, San Jose, CA) to visualize data distribution.

To assess the GS2 CN variation in tissues from fully-grown plants, nine Palmer amaranth seedlings with contrasting GS2 CN were selected and transplanted to 10-L pots. When plants reached the reproductive stage (>1.5 m tall), branches from the top, middle and bottom portions were selected (to have canopy location representation within each plant), and a single leaf was collected. All 70 samples were submitted to *GS2* CN assessment.

Inheritance of amplified GS2 copies and GFA resistance

To study the inheritance pattern of amplified *GS2* copies, 35 nontreated plants from the GFA-R population were used. A single sample was collected from each plant's youngest, fully

expanded leaf. DNA was extracted and *GS2* CN was assessed. Selected plants were transplanted to 5 L pots and grouped according to desired crosses, as shown in Table 1. After maturity, female inflorescences were harvested, air dried and threshed, and seeds were cleaned with an air blower and stored in glass vials.

Two experiments were conducted with the seeds from designed crosses. In the first experiment, 50 plants (7- to 10-cm tall) from each cross were sprayed with a 1x rate of GFA (657 g ai ha^{-1}, Liberty 280 SL, BASF Corporation, Research Triangle Park, NC, USA). Alive plants were counted at 15 days after application (DAA), and data were converted to survival percentage. The experiment was repeated, with 150 plants per cross. The second experiment aimed to quantify *GS2* CN in the offspring from each cross. A total of 250 unsprayed plants were studied, as shown in Table 2. Tissue collection, DNA extraction and *GS2* CN quantification was done as previously described.

Fluorescence *in situ* hybridization (FISH)

Seeds from cross RR-1 were germinated in a plastic tray containing potting mix, and 15 seedlings were transplanted to a hydroponics system at the 1-leaf stage. Plants were grown in a full-strength Hoagland basal salt solution, prepared by diluting a commercial salt mixture (MP Biomedicals LLC, Solon, OH, USA). Solution was constantly aerated by an aquarium air pump and replenished as needed. Three plants were selected for FISH assay, hereby designated as #1, #2 and #5, based on their GS2 CN of 20, 10 and 1, respectively.

To prepare the *GS2* FISH probe, a 2.8 kb region of the genomic sequence of *GS2* was PCR-amplified using primers F: 5'-TGGCACAAATACTTGCACCTT-3' and R: 5'-ACACTTGGGCCAACTTGGAA-3'. Genomic DNA from plant #1 was used as a template for PCR amplification, with the following conditions: 98 C for 30 sec, followed by 30 cycles of 98 C

for 10 sec, 60 C for 30 sec and 72 C for 3 min. A final extension step was added at the end of the run, being at 72 C for 5 min. The reaction consisted of 25 µL of EmeraldAmp Max HS Buffer, 1 µL of each primer (10 µM), 1 µL of DNA (50 ng μL^{-1}) and 22 µL of nuclease-free water. Gel electrophoresis was performed to confirm amplicon size, and a commercial kit (PureLink Quick Gel Extraction Kit, ThermoFisher Scientific) was used to purify DNA fragments. Four identical PCR reactions were done, and purified DNA was bulked and concentrated to achieve a concentration of 200 ng μL^{-1}. GS2 nick-translated probe was prepared using Texas Red-5-dCTP (Kato et al., 2006).

Somatic chromosome spreads were prepared based on published protocols (Kato et al., 2004) with minor modifications. Roots were individually digested for 15 to 20 min at 37C after 1.5 h of N_2O treatment at 160 psi. Chromosomes were counterstained with DAPI in Vectashield antifade solution (Vector Laboratories, Burlingame, CA, USA) and visualized with a BX61 fluorescence microscope (Olympus America Inc., Center Valley, PA, USA). Pictures were acquired with a mounted camera and GenASIs software (Applied Spectral Imaging, Carlsbad, CA). Pictures were later processed using Adobe Photoshop 24.3.0 (Adobe Inc, San Jose, CA).

Results

Stability of *GS2* copies across plant tissues

Results of the assessment of *GS2* copies across various location in the plant are shown in Figure 2. Neither the interaction nor the main effects of GFA application, collection timing and sample location were significant, so t-tests were used to compare plant average *GS2* CN between two sampling times.

Statistically significant differences between sampling times were observed in only two out of eight plants (t-test, $p<0.05$). In the remaining plants, even though the mean *GS2* CN were

not differentiated statistically with time for the same plant, the wide range and distribution of values that compose the average were apparent in several cases, such as in plants B, D, F and H.

GS2 CN was determined in 10 samples collected from different branches of nine fully-grown Palmer amaranth plants. *GS2* amplification was detected at the seedling stage in seven of these plants (red squares, plants P1 to P7, Figure 3). All plants showed high variability in *GS2* CN at the adult stage. Only plants P5 and P7 showed *GS2* amplification in all samples analyzed. Therefore, the majority of plants did not show *GS2* amplification in all leaf tissues. A single *GS2* copy was detected in plant P8 at the seedling stage; however, *GS2* amplification (4 and 5 copies) was observed in two tissues collected from the adult plant without herbicide treatment. This indicates that *GS2* copies can be generated even in the absence of herbicide application or other abiotic stresses.

GS2 inheritance

In terms of range and average of *GS2* CN, RR-1 and RR-2 (described in Table 1) behaved similarly. The highest *GS2* CN observed in siblings from these crosses was 31 and 37, respectively (Table 2). The average *GS2* CN was slightly higher in RR-1 siblings (8.2) than in RR-2 (7.2). The most remarkable difference between these two populations was the percentage of plants having *GS2* amplification: RR-1 had 23 percent points difference compared to RR-2 (77.5 and 54.5%, respectively). Crosses between individuals without *GS2* amplification are represented by SS-1 and SS-2. None of the 15 siblings analyzed from SS-2 showed more than 3 copies (Table 2). Conversely, 13% of the 37 siblings analyzed from SS-1 showed *GS2* amplification.

To determine possible gender-related effects in *GS2* segregation, populations SR and RS were created by crossing parents with and without *GS2* amplification. The RS cross had a high-

copy female parent while the SR cross had a high-copy male parent. Interestingly, the RS progeny originated from a mother plant with the highest *GS2* CN (17.4) and showed the highest average for this variable (3.1 copies). The percentage of RS siblings with *GS2* amplification was more than three times higher than that of the SR siblings, indicating possible maternal effect on *GS2* inheritance.

In terms of GFA susceptibility, RR-1 and both SS crosses showed the highest and lowest survival percentages, respectively. The remaining crosses generated offspring that behaved similarly in response to GFA. The survival percentage among SS-2 progeny was lower than the percentage of individuals with *GS2* amplification in all cases but SR.

Physical mapping of amplified GS2 copies

In plants #1 and #2, which contained 20 and 10 *GS2* copies, respectively, *GS2* signals were observed in several mitotic chromosomes and dispersed throughout the interphase nuclei (Fig S1 and S2). Interestingly, the number and intensity of GS2 signals varied from cell to cell, and from root to root. For example, Figures S3 and S4 were taken from the same root spread, and *GS2* signals were observed in 2 and 11 chromosomes, respectively. Figure S5 captured two cells at distinct stages and with contrasting *GS2* copies: the interphase nuclei contain multiple dispersed signals, while only three of the metaphase chromosomes carry *GS2* genes. Similarly in plant #5, cells from the same root differed in number and intensity of *GS2* signals. Intriguingly, five out of 27 interphase nuclei imaged showed three clear *GS2* signals (Fig S6), and four signals were observed in three cells. In every interphase nucleus where more than two signals were observed, *GS2* copies appear to be arranged in pairs (white arrows, Fig S7 and S8). FISH on extended chromatin fibers (Fiber-FISH) can answer whether GS2 duplications in tandem have occurred on those cells.

Discussion

GS2 copies are not stable in the plant. It varies by location and time but does not seem affected by GFA application, at least within the short timeframe considered. In addition, *GS2* copies can be spontaneously generated during the plant's cycle, and these extra copies can be transmitted to the next generation. Despite being extremely rare, this behavior of amplified copies is not novel in herbicide-resistant Palmer amaranth. Giacomini et al. (2019) observed drastic differences in *EPSPS* CN among six branches of the same plant, and an even higher variation across clonal generations originated from a single plant. The authors also found *EPSPS* amplification in plants from a cross between two low CN parents, similar to what occurred among progeny of the SS-1 cross from our study.

The uncertainty associated with *GS2* CN determination in a given plant hinders the elucidation of *GS2* inheritance patterns, as the quantified copy number pertains to the specific group of cells from which DNA was extracted and may not be representative of the whole plant CN. This applies to the parental lines and offspring. In addition, CN is generally assessed in vegetative tissues and may differ from CN in the gametes. In the most comprehensive examination of *EPSPS* copies inheritance performed to date, positive and negative transgressive segregation was observed (Giacomini et al., 2019), suggesting that identifying a precise model for inheritance of *EPSPS* amplification in Palmer amaranth is not possible. Our data indicates *GS2* copies behave similarly but with an additional layer of complexity, as the proportion of offspring with GS2 amplification and the percent survival to GFA may differ. For example, GS2 amplification was detected in 54% of seedlings from cross RR-2, but survival to GFA was only 23%, not statistically different from crosses involving one low CN parent (SR and RS). This difference may be attributed to the requirement of a CN higher than 3 (which was the threshold

for classification as amplified *GS2*) to endow resistance at the plant level; the lack of correlation between *GS2* CN, expression and protein levels, or the presence of additional herbicide resistance traits segregating independently. Lastly, facultative apomixis may influence segregation ratios of amplified copies as reported by Ribeiro et al. (2014) pertaining to *EPSPS* inheritance. Whether apomixis is responsible for the apparent maternal effect in GS2 inheritance remains to be tested.

The variation in *GS2* CN at the tissue level can be explained by the large variability at the cellular level, as shown in our FISH assay. In the high CN plants, signals of different intensities were detected in up to 16 chromosomes, but several cells with only two *GS2* signals were also found. In this sense, the mechanism of *GS2* amplification in Palmer amaranth seems to contrast with *EPSPS* amplification in *B. scoparia* (Jugulam et al., 2014) and *A. tuberculatus* (Koo et al., 2018a, Dillon et al., 2017). Tandem amplification of *EPSPS* was found in these two species, with the latter also presenting aneuploidy in the form of an additional circular chromosome with multiple *EPSPS* copies. No evidence of aneuploidy was found in Palmer amaranth in the present study, as no cells deviated from the normal chromosome number of 34 (Grant, 1959). Chen et al. (2020) suggested transposable elements could be involved in *EPSPS* amplification in *E. indica*, as copies were found in two pairs of homologous chromosomes.

The involvement of eccDNA in *GS2* amplification can be hypothesized based on 1) the somatic mosaicism observed in relation to presence/absence and localization of multiple GS2 signals; 2) the unpredictable inheritance patterns of amplified copies; and 3) the spatiotemporal variation of *GS2* CN in plants. The same behavior had been previously described by Koo et al. (2018b) and Giacomini et al. (2019) in eccDNA-mediated *EPSPS* amplification in Palmer amaranth. Conversely, in most cells, *GS2* signals appear to be paired in two sister chromatids,

indicating possible integration of some of the *GS2* copies into the chromosome. Although the factors driving integration of eccDNAs to linear chromosomes in plants are not well understood (Krasileva, 2019), such phenomenon was observed, for example, in the amplification of *vasa* genes in *Oreochromis niloticus* (Fujimura et al., 2011), *XylA* genes in *Saccharomyces cerevisiae* (Demeke et al., 2015), and in several human oncogenes (Yang et al., 2022). This hypothesis could explain the lack of correlation between *GS2* copies and *GS2* expression described in our first work (Noguera et al., 2022), as reintegration may occur near strong promoter motifs or genomic regions under epigenetic regulation. Thus, the level of *GS2* expression could vary widely depending on where the copies are integrated.

It should be noted that plants used in the FISH assay were grown under ideal conditions and were not exposed to GFA, which can affect the ratio of cells presenting or not *GS2* amplification, and the extent of amplification in those cells (Arrey et al., 2022). Due to the dynamic character of eccDNAs, Jugulam (2021) hypothesized that removing glyphosate selection pressure from resistant plants could lead to a reduction in *EPSPS* CN over time, restoring susceptibility. To our knowledge this hypothesis remains to be tested. In the case of *GS2* amplification, if our hypothesis that some eccDNAs are incorporated into linear chromosomes is true, removing glufosinate selection pressure would have limited effect in restoring population susceptibility.

The fact that Palmer amaranth was the first weed to evolve GFA resistance through a complex mechanism such as *GS2* amplification and overexpression is a testament to the superior adaptability of this species, and reinforces the necessity of adopting alternative control methods, reducing the overreliance on herbicide use. Time will tell if the same mechanism will evolve in other species, similar to what happened with *EPSPS* amplification (Patterson et al., 2017).

Follow up studies such as mobilome sequencing (Lanciano et al., 2017) and Fiber-FISH are scheduled and should provide a clearer understanding of the mechanism of *GS2* amplification.

Conclusions

GS2 copies are unstable in the GFA-resistant Palmer amaranth plants. At the tissue level, CN randomly varies across time and space, and is not influenced by GFA application. Seedlings without *GS2* amplification may contain tissues with high CN at the adult stage. *GS2* inheritance does not follow a Mendelian pattern. The prevalence of *GS2* amplification and survival to GFA are weakly correlated. *GS2* copy number variability in plant tissues is due to the somatic mosaicism regarding the presence and extent of *GS2* amplification in cells from the same plant. *GS2* copies are spread across multiple chromosomes, and on many occasions, seem to be integrated to them. The unpredictable inheritance pattern of *GS2* copies along with its variability at the cell and tissue level suggests the involvement of eccDNAs. Further studies are needed to test this hypothesis.

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Tables and Figures

Figure 1. Sampling protocol used in the GS2 stability study; leaves 1 and 2 were selected from the bottom ⅓, while leaves 3 and 4 were selected from the top ⅓.

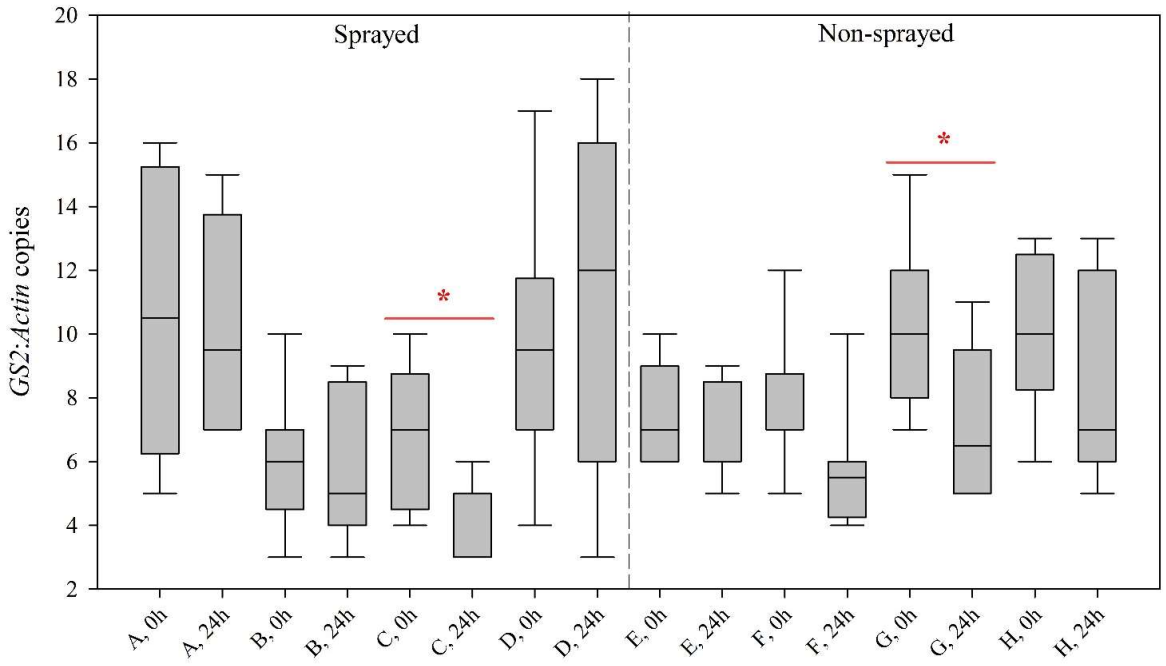

Figure 2. Distribution of *GS2* CN values in eight Palmer amaranth plants, sprayed (left side) or not (right side) with 82 g ai ha^{-1} of GFA. Each box-whiskers represents eight samples from a single plant, at a certain sampling time. Red asterisk indicates statistical difference between averages of two sampling times for a same plant.

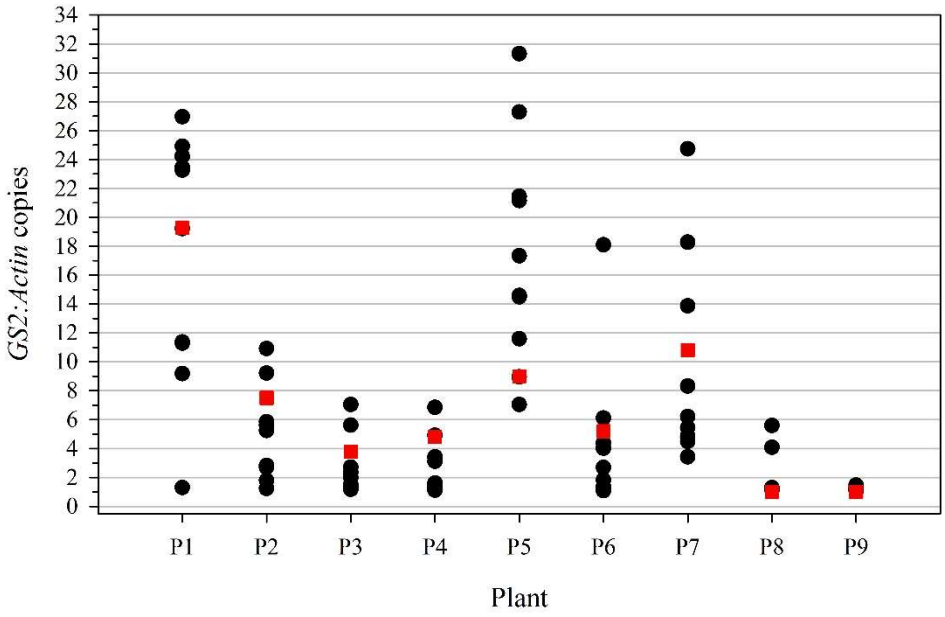

Figure 3. *GS2* CN in 10 different tissues collected from nine plants at maturity. Each black dot represents a sample. Red squares represent the *GS2* CN determined at the 1-leaf stage.

Table 1. Designed crosses used in the GS2 copies inheritance. CN was determined via qPCR using a single tissue per plant, collected at the 10-leaf stage.

Cross	*GS2:Actin* copy number	
	♀	♂
RR-1	12	13
RR-2	23	14
SR	3	13
RS	8	2
SS-1	2	2
SS-2	2	3

Table 2. *GS2* CN in parent plants and offspring from six designed crosses.

Cross	♀ CN x ♂ CN	Plants assessed	*GS2:Actin* copy number		% amplified	Survival (%)
			Range	Average		
RR-1	12x13	32	1.3 - 31.0	8.2	77.5	60 A
RR-2	23x14	55	0.9 - 37.0	7.2	54.5	23 B
SR	3x13	37	1.4 - 7.6	2.2	8.1	18 BC
RS	8x2	53	1.1 - 17.4	3.1	30.2	18 BC
SS-1	2x2	37	1.3 - 4.6	2.3	13.5	7 CD
SS-2	2x3	15	1.2 - 2.5	1.9	0	0 D

Appendix

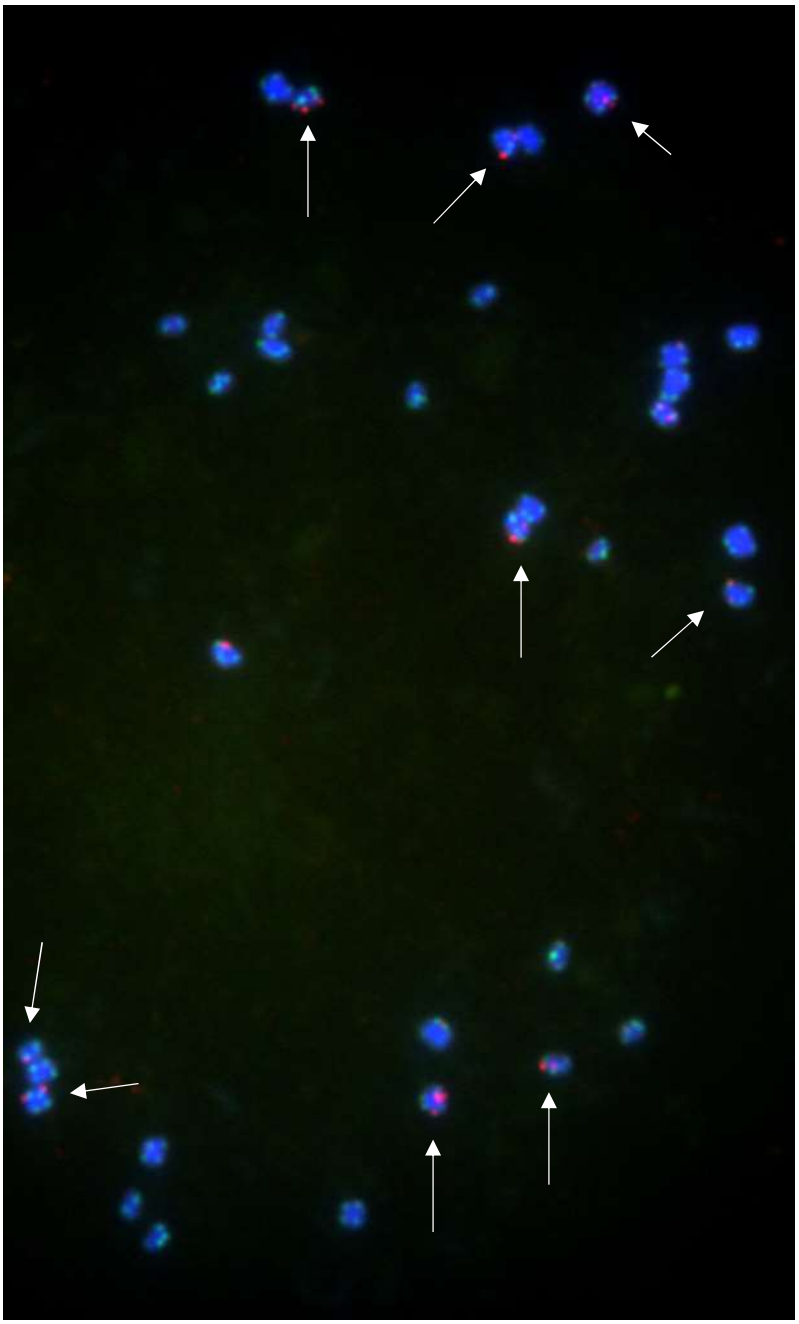

Figure S1: *GS2* FISH in a root spread from plant #1, estimated to have GS2 CN = 20 copies). White arrows indicate chromosomes where signal is visible.

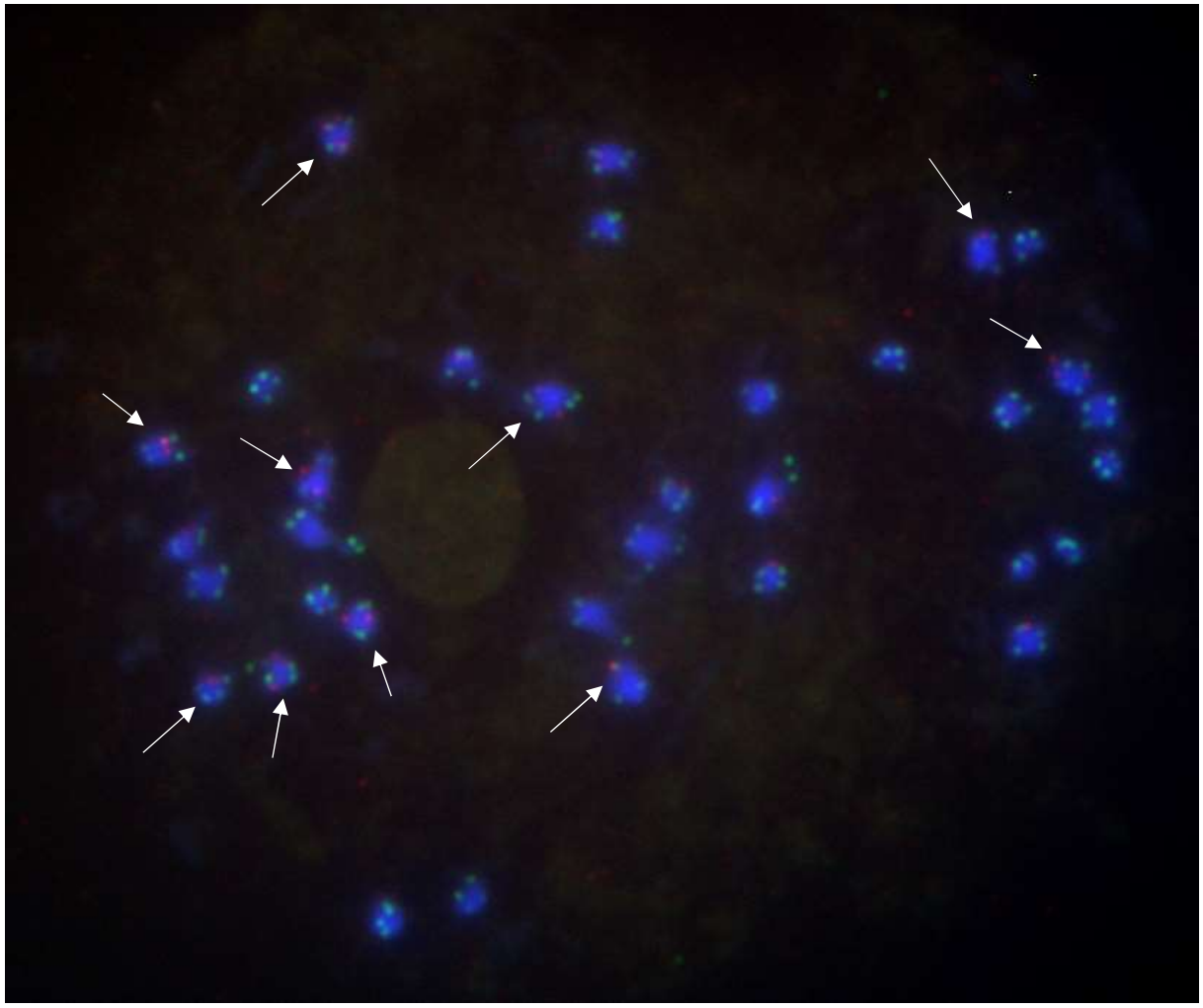

Figure S2: *GS2* FISH in a root spread from plant #2, estimated to have GS2 CN = 10 copies). White arrows indicate chromosomes where signal is visible

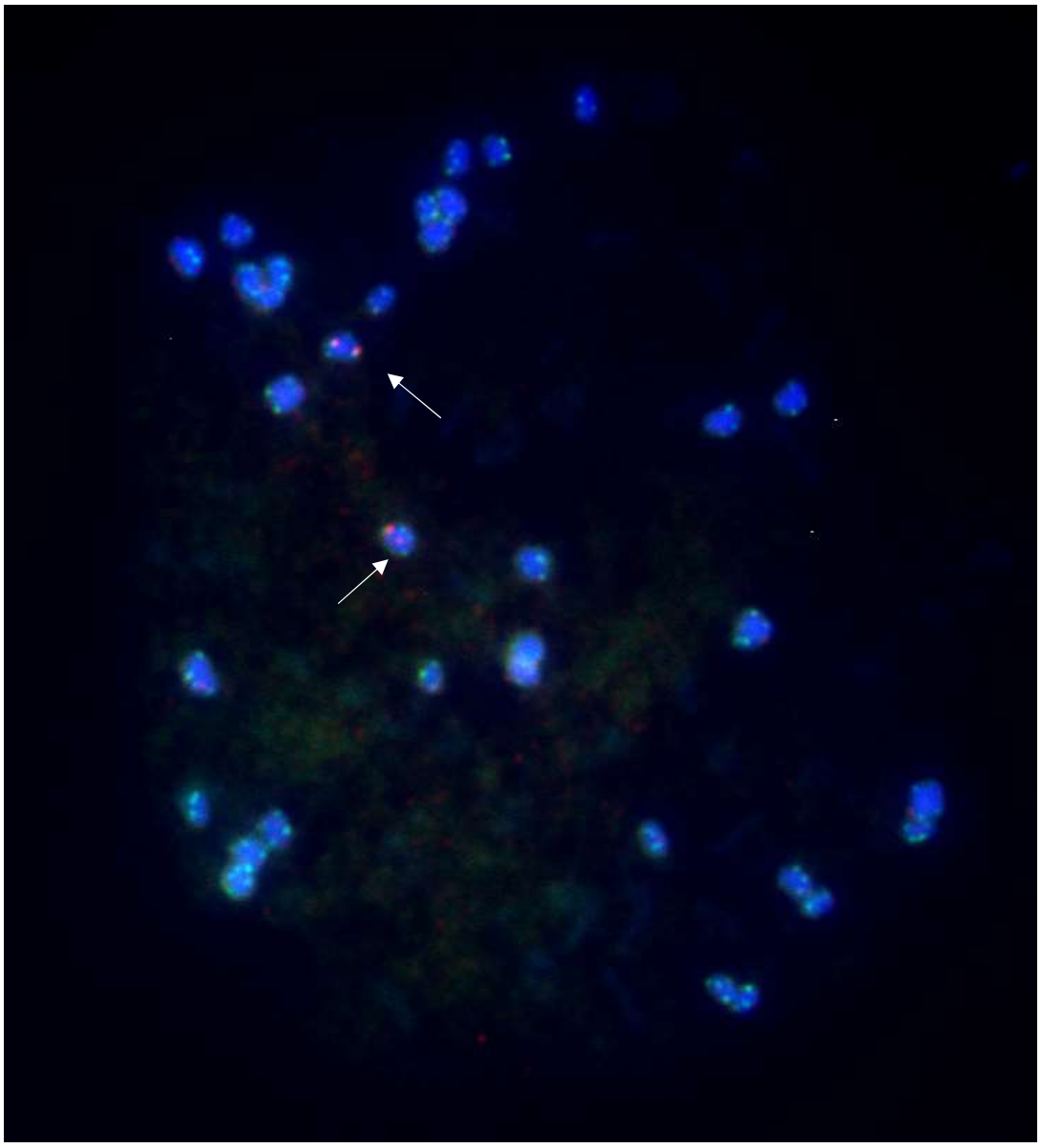

Figure S3: *GS2* FISH in a root spread from plant #1, estimated to have GS2 CN = 20 copies. White arrows indicate chromosomes where signal is visible.

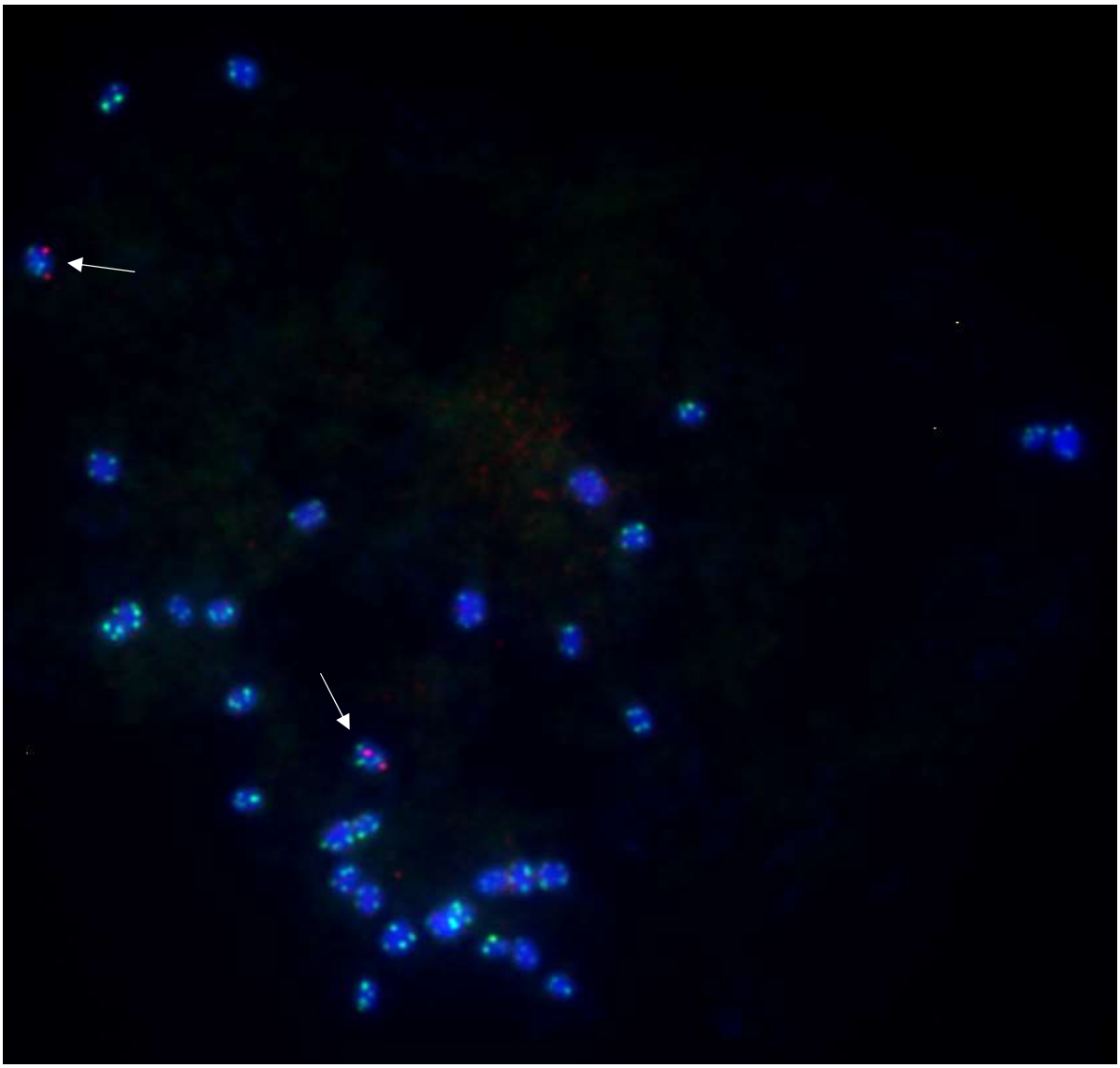

Figure S4: *GS2* FISH in a root spread from plant #1, estimated to have GS2 CN = 20 copies. White arrows indicate chromosomes where signal is visible.

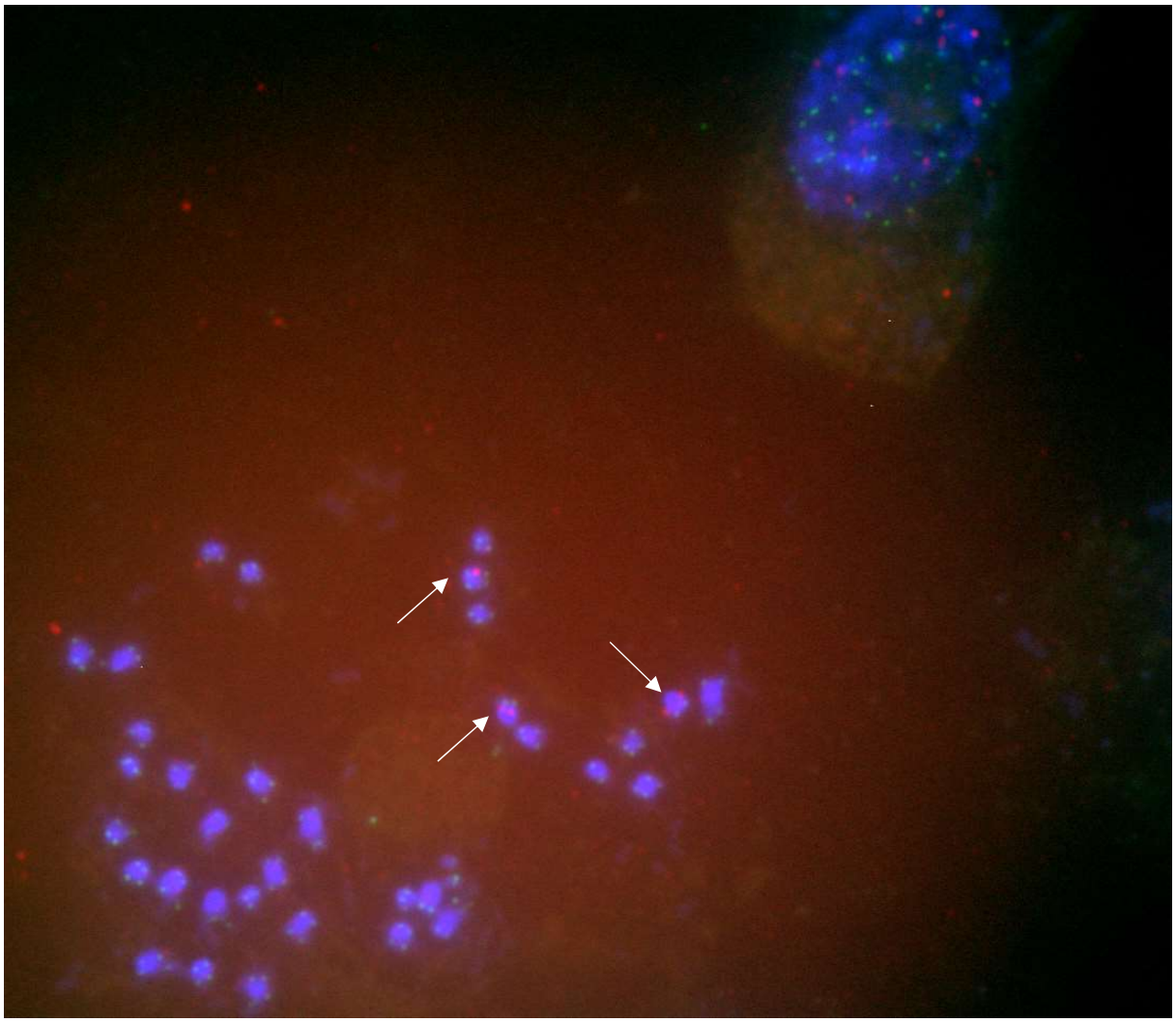

Figure S5: *GS2* FISH in a root spread from plant #2, estimated to have *GS2* CN = 10 copies). White arrows indicate chromosomes where signal is visible. Several *GS2* signals are seen in an interphase nucleus from the same root spread.

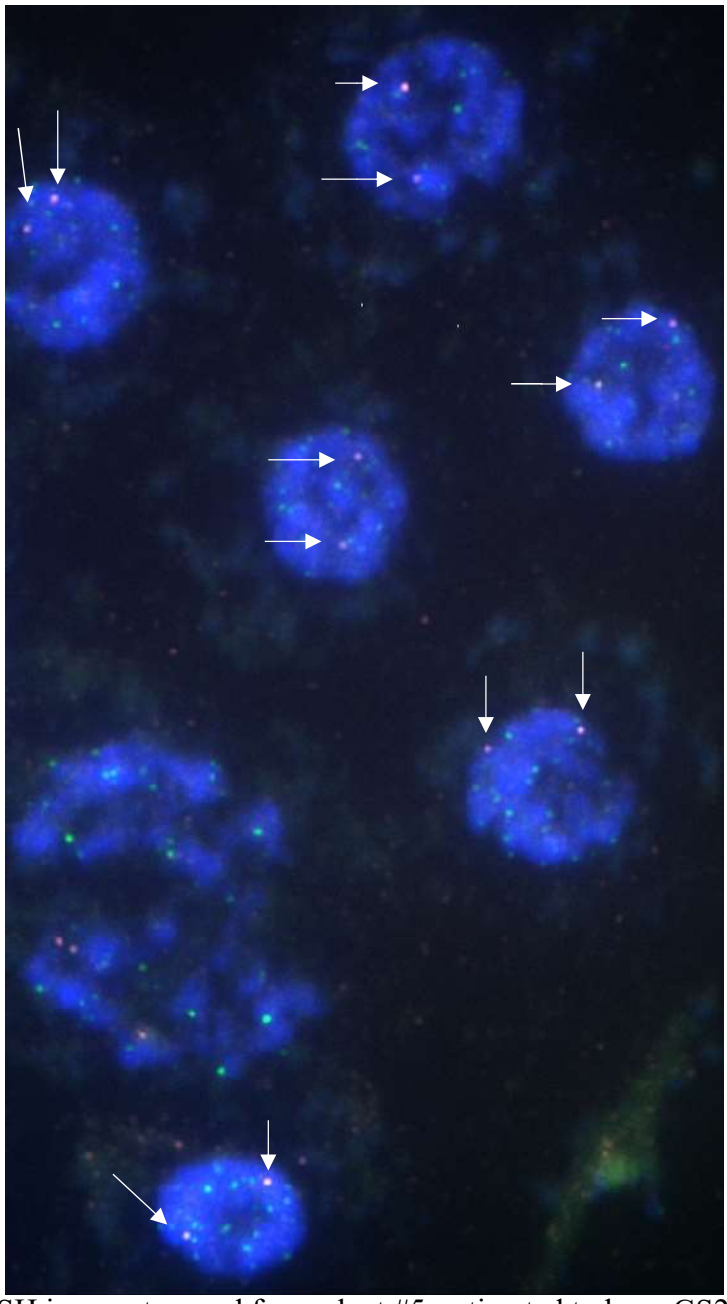

Figure S6: *GS2* FISH in a root spread from plant #5, estimated to have GS2 CN = 1 copy. White arrows indicate *GS2* signals.

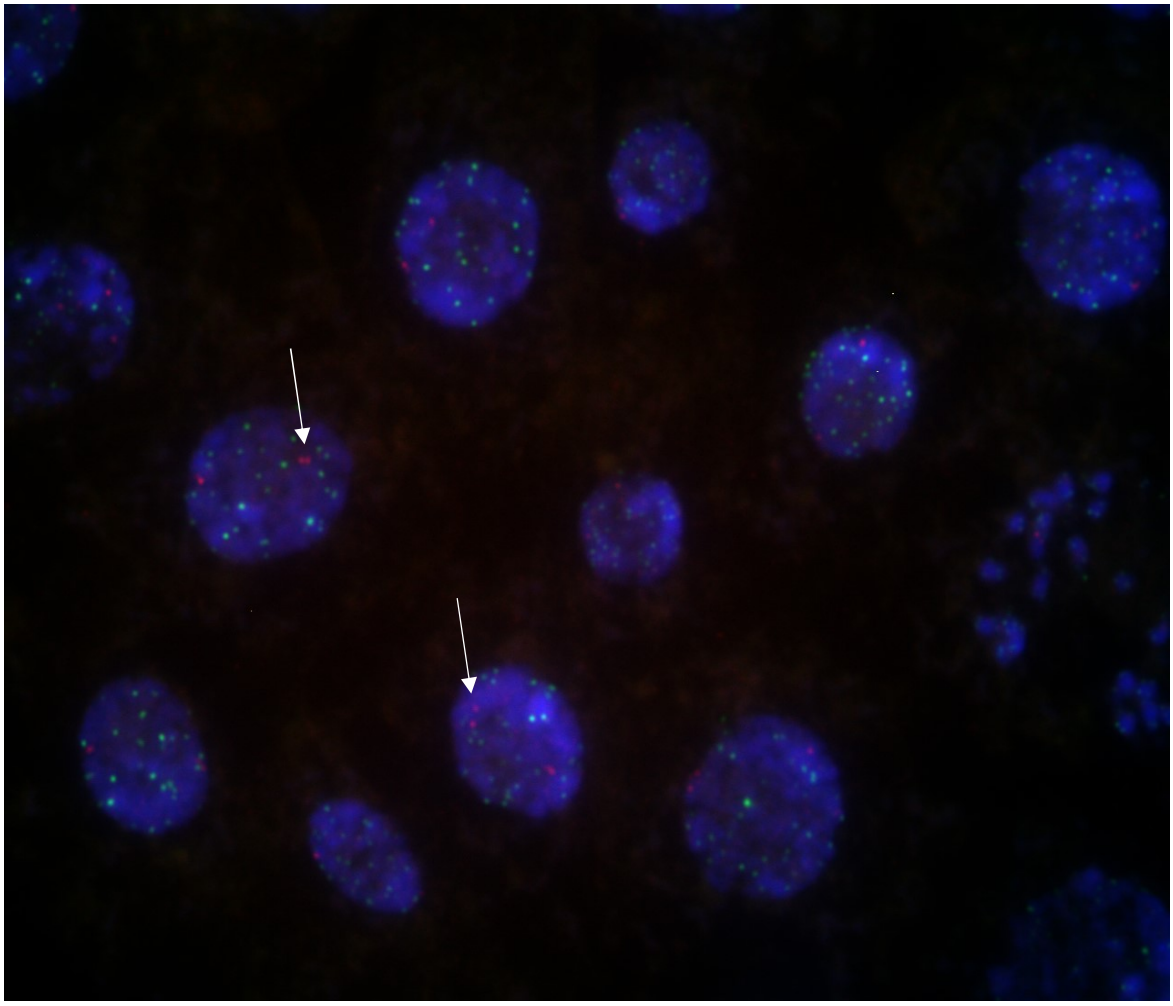

Figure S7: *GS2* FISH in a root spread from plant #5, estimated to have GS2 CN = 1 copy. White arrows indicate two *GS2* signals in tandem.

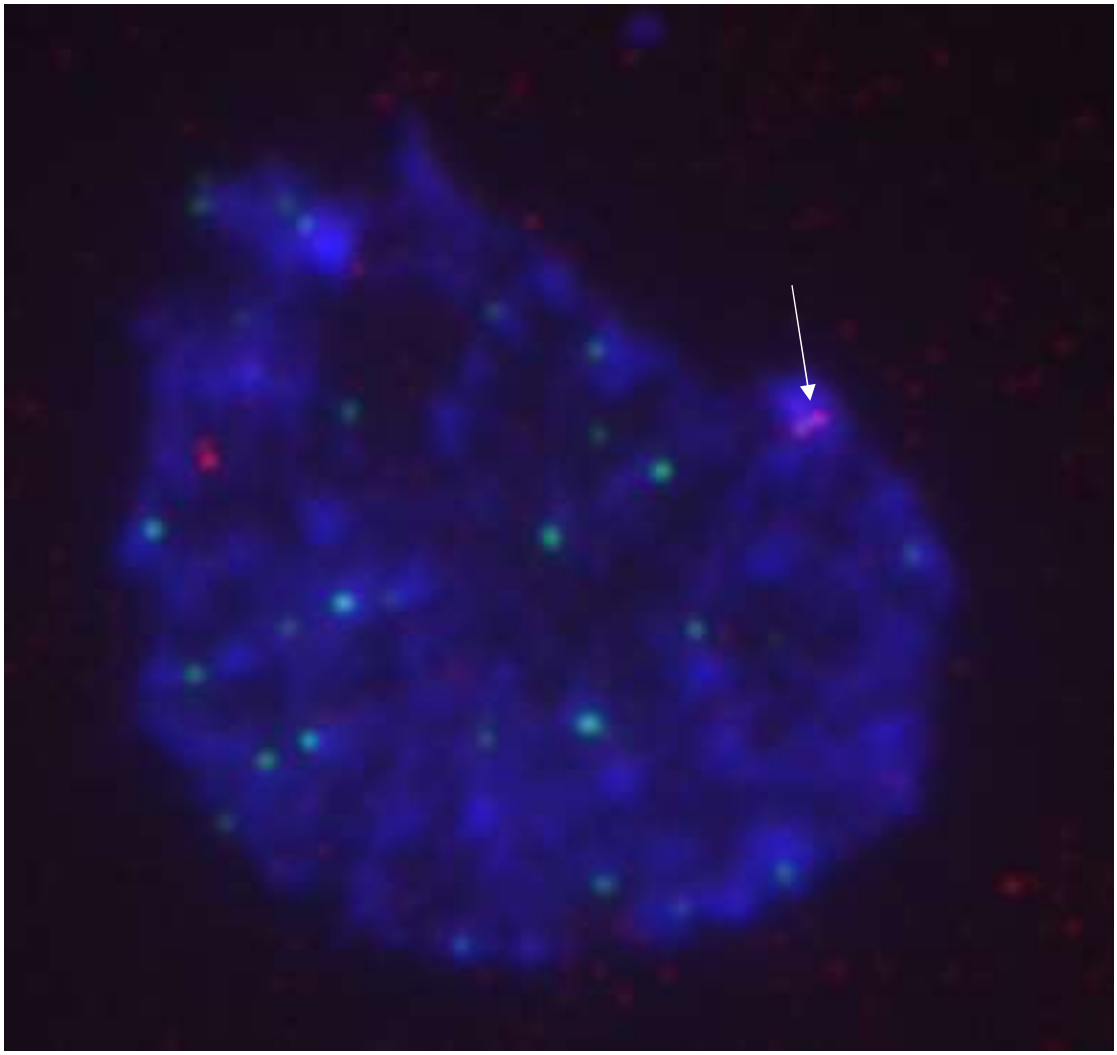

Figure S8: *GS2* FISH in a root spread from plant #5, estimated to have *GS2* CN = 1 copy. White arrow indicate two *GS2* signals in tandem arrangement.

Alternative management options for a glufosinate-resistant Palmer amaranth biotype from Missouri, USA

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Abstract

Now having evolved resistance to nine different herbicidal modes of action, Palmer amaranth stands out as one of the most troublesome weeds to manage in row crops. The latest herbicide to which this species evolved resistance is glufosinate (GFA), an inhibitor of glutamine synthetase. Two greenhouse tests were done to evaluate the performance of commonly used herbicides in soybean, cotton and corn to manage glufosinate-resistance in a Palmer amaranth population from Missouri. Fourteen herbicides from seven modes of action were tested as preemergent, and 15 herbicides from seven modes of action were tested in foliar applications. Soil-applied herbicides provided good control in general, with few exceptions such as imazethapyr and mitosis inhibitors. Foliar applied herbicides showed a great range of activity: glyphosate did not cause any significant effects on GFA-resistant Palmer amaranth, whereas synthetic auxins and HPPD inhibitors showed the greatest activities. Despite several candidate herbicides found in this research, Palmer amaranth control must not solely rely on herbicide applications but a holistic approach is encouraged.

Introduction

The occurrence of herbicide resistant (HR) weeds results from an evolutionary process driven by the intensive selection pressure exerted by recurrent applications of herbicides from a single site of action (SoA) (Peterson et al., 2018). More than 500 unique cases of resistance have been reported in the International Survey of HR weeds (Heap, 2023), but that database does not contain all HR reports known to date (Torra et al., 2022).

Weeds tend to accumulate HR traits, leading to cross- and multiple-resistant biotypes. According to Heap (2014), cross-resistance occurs when a single resistance mechanism confers resistance to more than one herbicide, while multiple-resistance is defined by more than one mechanism occurring within an individual plant. Both categories are prejudicial to weed management as they reduce the already limited number of efficient herbicides available.

Species such as *Amaranthus palmeri* (Palmer amaranth) and *Lolium rigidum* (rigid ryegrass) have demonstrated remarkable propensity to evolve HR: these species have evolved resistance to 9 and 12 different SoA, respectively (Heap, 2023). In addition, extreme cases of multiple-resistance have been reported, such as the 9-way resistant rigid ryegrass described by Burnet et al. (1994), or the 6-way resistant Palmer amaranth describe by Shyam et al. (2021). Multiple-resistance has become the new normal: in Australia, 60% of the 1441 rigid ryegrass populations analyzed were resistant to two or more SoA (Broster et al., 2022). Several cases of multiple-resistance in Palmer amaranth have been reported (Kumar et al., 2019a, Faleco et al., 2022, Aulakh et al., 2021), but few attempts have been made to characterize the distribution of such populations (Bagavathiannan and Norsworthy, 2016, Garetson et al., 2019).

The recent discovery of a glufosinate-resistance Palmer amaranth biotype (Noguera et al., 2022) adds another layer of complexity to the management of this species under field conditions.

Information regarding possible cross- and multiple-resistance is needed for the adoption of control strategies. Therefore, the objectives of this study were: 1) to evaluate the efficacy of soil-applied herbicides in controlling a glufosinate-resistant Palmer amaranth genotype; 2) to evaluate the efficacy of foliar-applied herbicides in controlling a glufosinate-resistant Palmer amaranth.

Materials and methods

Plant materials and herbicide applications

All experiments herein described were conducted with two *A. palmeri* populations, namely MO20 #2 F1 and SS, which were previously characterized regarding their response to glufosinate (Noguera et al., 2022). These populations will be hereafter called GFA-R and GFA-S, respectively.

Herbicide applications were performed using a laboratory sprayer fitted with two flat-fan 110 0067 nozzles (Teejet, Wheaton, IL), calibrated to deliver 187 L ha^{-1} of spray solution at 275 kPa. The spray boom was set at 45 cm above the plant canopy or soil surface.

Efficacy of soil-applied herbicides

The efficacy of soil-applied herbicides against GFA-R and GFA-S was assessed in a greenhouse experiment. The bi-factorial test was conducted in a randomized complete block design, with 15 herbicides applied at a single rate (factor A) and two Palmer amaranth populations as previously mentioned (factor B). Herbicides and rates are shown in Table 1.

Thirty seeds were placed on the soil surface of 400-mL pots filled with a 4:1 mixture of field soil:commercial potting mix, with the following characteristics: pH = 6.6, organic matter = 2.6%, silt = 68%, clay = 8%, sand = 24 %. Field soil was collected from an area with minimal history of herbicide use, in the Vegetable Research Station of the University of Arkansas, Kibler, AR. Seeds were covered with a thin layer of the same mix and immediately sprayed. Pots were

taken to the greenhouse and the soil surface was misted to incorporate and activate the herbicides. The greenhouse was maintained at 32/28 C day/night temperatures, with a 14-h photoperiod achieved by supplemental light. Soil moisture was maintained at pot capacity with watering by capillarity as needed. Each pot was considered an experimental unit.

Weed control ratings in relation to the untreated check were performed at 21 days after treatment (DAT), using a 0-100% scale, where 0% means absence of visible injury and 100% represents plant death/no emergence. Data was submitted to ANOVA using the *ExpDes* package in R (R Core Team, 2023), and means separation was done using the Tukey's test at 5% significance.

Efficacy of foliar-applied herbicides

The test with foliar-applied herbicide was conducted with the same experimental design from the soil-applied test. Herbicides and rates used are shown in Table 2.

Seeds were sown in 50-cell trays filled with commercial potting mix (ProMix LP15; Premier Tech Horticulture, Quakertown, PA, USA) and thinned to one plant per cell a week after planting. Herbicide applications were done when plants were 7- to 10-cm tall. Each treatment was sprayed on two half-trays (1 replication = 25 plants), on a total of 50 seedlings.

Weed control ratings and statistical analysis was done as previously described.

Results and discussion

Efficacy of soil-applied herbicides

The interaction between Herbicide and Population was not significant at α=5%, neither was Population significant as a main factor (Table 3). Therefore, herbicide means were compared across both populations

With few exceptions, all soil-applied herbicides provided excellent control of Palmer amaranth (Figure 1). Four modes of action were represented by more than one herbicide: inhibitors of PPO, PSII, ALS and mitosis. Of those, only PPO- and PSII-inhibitors performed consistently well. Both herbicides associated with carotenoid biosynthesis inhibition, mesotrione and fluridone, performed equally well.

The mitosis inhibitors pendimethalin and trifluralin, along with the ALS-inhibitor imazethapyr were the only herbicides showing lower Palmer amaranth control than the remaining 11 herbicides. Among these three active ingredients, trifluralin performed the best, and pendimethalin, the worst.

Interestingly, imazethapyr and flumetsulam inhibit the same enzyme in plants, but showed differential activity. A possible explanation for such behavior is the much higher water solubility of flumetsulam (5.6 g L^{-1}) compared to imazethapyr (1.4 g L^{-1}) (2007), which may have allowed the former to percolate to a higher degree, reaching the seed zone in higher amounts than imazethapyr.

Physicochemical properties may also partially explain the differences between pendimethalin and trifluralin: their log octanol-water partition coefficient is 5.18 and 5.07 respectively, which means pendimethalin is more tightly bound to soil non-polar portion and thus, less available for plant absorption (Gavrilescu, 2005). Volatilization and photolysis are known to be major dissipation routes for dinitroanilines (Curran, 2016), which justifies the recommendation to incorporate these herbicides into soil mechanically or by irrigation (Prostko et al., 2001). Insufficient incorporation by irrigation might have also contributed to the lower activity of these herbicides.

It must be noted that the lower efficacy of these herbicides does not completely preclude its use in an Integrated Weed Management (IWM), considering that: 1) weeds escaping pre-emergence herbicide applications have reduced competitive ability and are more easily controlled by post-emergence herbicides (Liphadzi and Dille, 2006, de Sanctis et al., 2021), and 2) mitosis inhibitors are among the herbicide groups less prone to evolve herbicide resistance, which is supported by the low number of cases reported (Heap, 2023). In other words, pendimethalin may not be the best option to manage GFA-R, but its use in a tank-mix may be justified by the good control of grasses it generally provides, for example.

Efficacy of foliar-applied herbicides

Analysis of variance for weed control data showed a significant interaction between Herbicides and Populations, as shown in Table 4.

For GFA-R, glyphosate was the least effective herbicide, and five other active ingredients showed intermediate efficacy: fomesafen, saflufenacil, fluometuron, imazethapyr and flumetsulam. For GFA-S, only three herbicides showed reduced activity: fluometuron, imazethapyr and flumetsulam. Palmer amaranth populations did not vary statistically in response to 9 out of 15 herbicides. Synthetic auxins and HPPD-inhibitors were the only MOA groups where all representative active ingredients behaved similarly.

Regarding the ALS-inhibitors, two interesting aspects should be noticed: the low control provided by imazethapyr and flumetsulam in the GFA-S population, and the superior performance of trifloxysulfuron on both populations. Firstly, the GFA-S population was collected from an organic farm in Arkansas in 2013, when resistance to ALS herbicides was already widespread in the state (Singh et al., 2019, Norsworthy et al., 2008). In a similar way, Priess et al. (2022) reported a 0% control of two supposedly sensitive Palmer amaranth

populations (collected in 2001) in response to a labeled rate of imazethapyr. It is possible that alleles from surrounding ALS-resistant populations have been transferred to the GFA-S population, causing the observed efficacy reduction. Secondly, it is known that ALS resistance can be manifested in several cross-resistance patterns according to the mutation endowing it (Singh et al., 2019, Palmieri et al., 2022). For example, mutations at positions P197 and A122 confer resistance exclusively to sulfonylureas and imidazolinones, respectively. However, T574 render the PPO enzyme insensitive to both chemical groups (Yu and Powles, 2014). Therefore, A122 could be, in theory, responsible for the inadequate Palmer amaranth control by imazethapyr in this study.

PPO-inhibitors is another interesting case where active ingredients from the same mode of action behave differently. In GFA-R, trifludimoxazin provided higher control than saflufenacil and fomesafen. Resistance to PPO herbicides in Palmer amaranth was first reported by Salas et al. (2016), and its spread was further documented by Noguera et al. (2020). Although non-target site resistance to PPO inhibitors has been documented (Varanasi et al., 2018), mutations in the *PPO2* gene are known to be the main mechanism associated with resistance (Gaines et al., 2020), and mutated residues differ in their importance towards resistance. Trifludimoxazin is a novel active ingredient with superior affinity to its target site compared to fomesafen and saflufenacil (Porri et al., 2022), resulting in efficient inhibition of mutant PPO2s and control of PPO-resistant populations (Steppig, 2022, Witschel et al., 2021).

Glyphosate showed the lowest control of GFA-R among all herbicides. This is particularly interesting considering that *EPSPS* amplification is the main resistance mechanism to glyphosate in Palmer amaranth (Patterson et al., 2017). Amplification and overexpression of GS2, the target site of glufosinate, was recently established as the source of resistance in GFA-R

(Noguera et al., 2022). The existence and extent of commonalities between these two target-site gene amplifications deserves follow-up studies.

All auxin mimics (2,4-D, dicamba and florpyrauxifen-benzyl) provided excellent control of GFA-R and would allow a successful use of the Enlist™ and XtendFlex™ technologies. These weed management systems have been studied in depth for several years and substantial information about possible benefits is available (Kumar et al., 2019b, Johnson et al., 2010, Hedges et al., 2018, Peterson et al., 2017, Miller and Norsworthy, 2016, Manuchehri et al., 2017). Likewise, florpyrauxifen-benzyl was proven to be efficient in managing Palmer amaranth in furrow-irrigated rice (Beesinger et al., 2022, Wright et al., 2021). On the other hand, the emerging resistance to auxin mimics in Palmer amaranth (Foster and Steckel, 2022, Kumar et al., 2019a) deserves attention and reminds us that no technology is foolproof.

The overreliance on herbicides for weed management and lack of crop rotations are two of the main factors contributing to the evolution of herbicide resistant biotypes (Storkey et al., 2019). Therefore, herbicides that performed satisfactorily in this study must be integrated to other management practices. For example, Palhano et al. (2018) observed a 83% reduction in Palmer amaranth emergence when cereal rye was used as a cover crop, compared to bare-soil treatments. Price et al. (2016) observed that Palmer amaranth escapes declined exponentially as a function of cover crop biomass. Singh et al. (2022) identified highly allelopathic sweet potato cultivars, able to cause up to 98% reduction in Palmer amaranth biomass in a pot experiment. In a follow-up study, Werle et al. (2022) observed up to 50% reduction in weed biomass under field conditions, and also pointed out the importance of morphological characteristics of sweet potato plants to increase crops competitive ability against weeds. Seed destruction either by burning (Spoth et al., 2022) or use of specialized equipment (Schwartz-Lazaro et al., 2017) can reduce

inputs into soil seed seedbank, and decreasing soybean row widths consistently reduced pigweeds biomass (Hay et al., 2019). These are some of the several examples of potential methods to reduce herbicide reliance and thus, to improve weed control sustainability.

Conclusions

Glyphosate, PPO- and ALS inhibitors tend to perform poorly on GFA-R, but HPPD inhibitors and synthetic auxins are good options for post-emergence control. Several herbicides are still viable for pre-emergence applications. The understanding of GFA-R herbicide resistance mechanisms can aid its management and mitigate the evolution of additional resistance traits. Resistance levels and mechanisms are being currently investigated. Extensive practice of non-chemical weed control methods such as use of cover crops, crop scouting, crop rotations, and use of allelopathic crops is necessary for weed management sustainability.

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Tables and figures

Table 1. Herbicides, modes of action, and rates applied at pre-emergence

Herbicide		Mode of Action	Rate (g ae ha^{-1} or g ai ha^{-1})
Active Ingredient	**Brand name**		
Flumetsulam	Python	ALS	50
Imazethapyr	Pursuit	ALS	70
Mesotrione	Callisto	HPPD	224
Fluridone	Brake	PDS	337
Trifludimoxazin	Tirexor	PPO	24.9
Flumioxazin	Valor SC	PPO	89
Saflufenacil	Sharpen	PPO	25
Atrazine	AAtrex	PSII	1800
Prometryn	Caparol	PSII	2700
Fluometuron	Cotoran	PSII	1680
Metribuzin	Tricor	PSII	260
Pendimethalin	Prowl H2O	Mitosis	1070
Trifluralin	Treflan	Mitosis	1120
Pyroxasulfone	Zidua	VLCFA	146

Table 2. Herbicides, modes of action and rates, applied at pre-emergence

Herbicide		Mode of Action	Rate (g ae ha^{-1} or g ai ha^{-1})
Active Ingredient	**Brand name**		
Glyphosate	RoundUp PM II	EPSPS	860
Fomesafen	Flexstar	PPO	280
Saflufenacil	Sharpen	PPO	25
Trifludimoxazin	Tirexor	PPO	24.9
Dicamba	XtendiMax	Synthetic auxin	560
2,4-D	Enlist One	Synthetic auxin	1070
Florpyrauxifen-benzyl	Loyant	Synthetic auxin	30
Paraquat	Gramoxone	PSI	840
Atrazine	AAtrex	PSII	2240
Fluometuron	Cotoran	PSII	1680
Mesotrione	Callisto	HPPD	105
Topramezone	Armezon	HPPD	24.6
Trifloxisulfuron	Envoke	ALS	13.1
Imazethapyr	Pursuit	ALS	52.6
Flumetsulam	Python	ALS	56

Table 3. ANOVA for weed control data; soil-applied herbicides.

Sourve of variation	Degrees of freedom	Sum of Squares	Mean Squares	F-Value	P-value
Herbicide	13	25598.4	1969.11	124.248	0
Population	1	50.2	50.22	3.169	0.07866
Herbicide*Population	13	209.2	16.09	1.015	0.44478
Residuals	84	1331.3	15.85		
Total	111	27189.1			

CV = 4.29%

Table 4. ANOVA for weed control data; foliar-applied herbicides.

Sourve of variation	Degrees of freedom	Sum of Squares	Mean Squares	F-Value	P-value
Herbicide	14	32208	2300.6	54.936	$0.00E^{+00}$
Population	1	3763	3762.6	89.847	$1.57E^{-10}$
Herbicide*Population	14	12950	925	22.088	$5.11E^{-12}$
Residuals	30	1256	41.9		
Total	59	50177			

CV = 8.31%

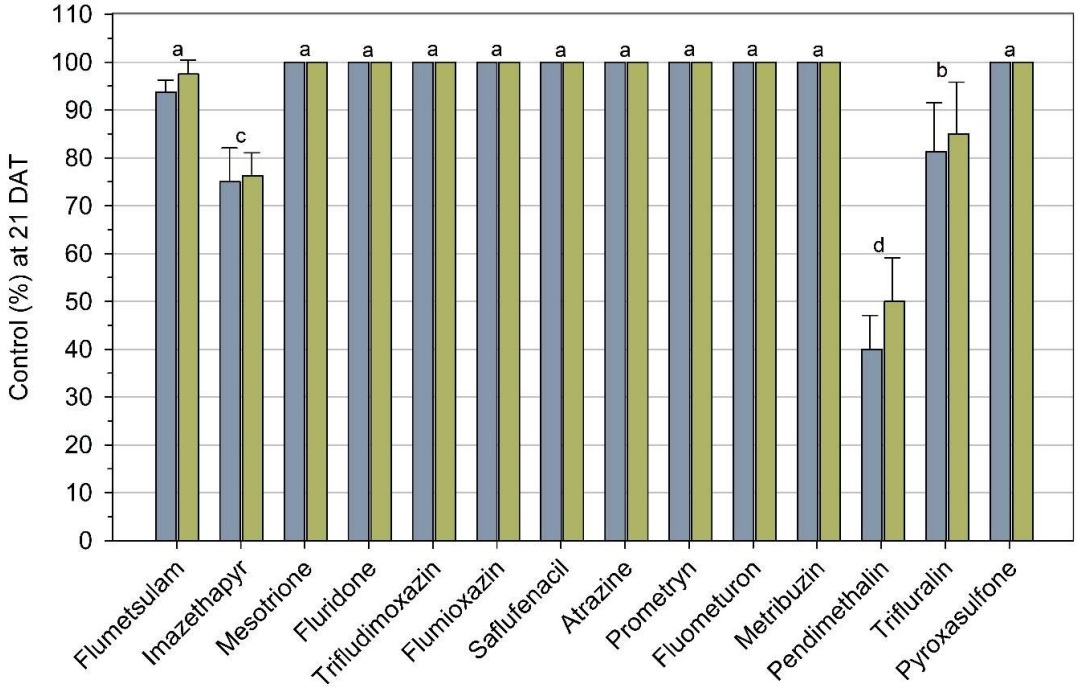

Figure 1. Efficacy of soil-applied herbicides on GFA-R (gray bars) and GFA-S (yellow bars) populations. Populations did not differ from each other in any of the herbicides studied. Letters compare herbicide across the average of both populations. Error bars represents the standard error of the means.

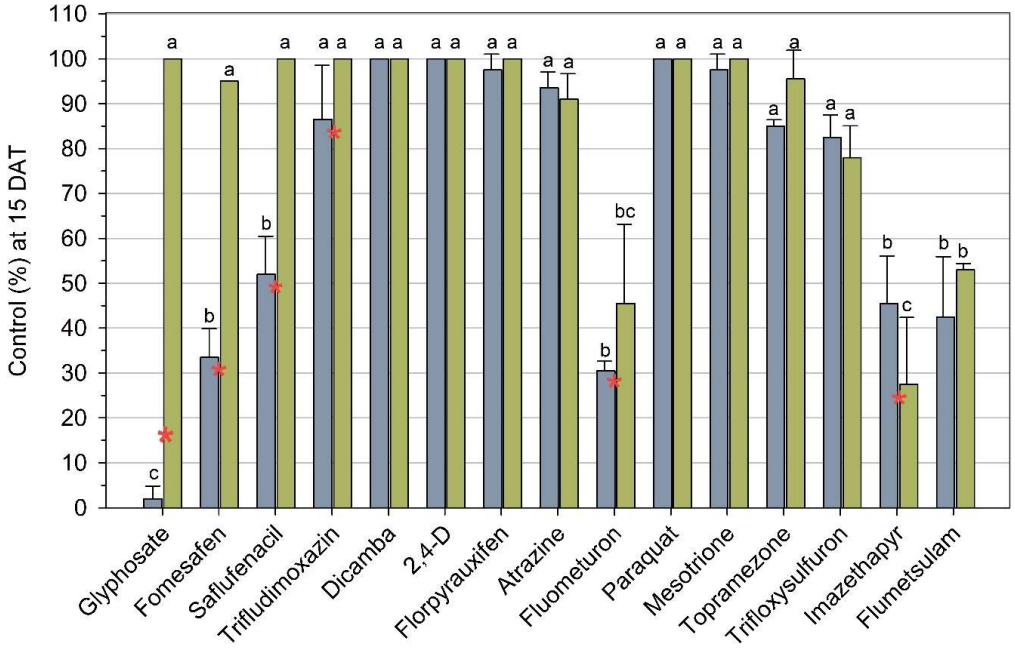

Figure 2. Efficacy of foliar-applied herbicides on GFA-R (gray bars) and GFA-S (yellow bars) populations. Letters compare herbicides within populations, and red asterisks indicates statistical differences of populations within herbicides. Error bars represents the standard error of the means.

Conclusion

Palmer amaranth resistance to glufosinate has recently been detected in Mid-Southern US. Resistance is due to amplification and overexpression of chloroplastic glutamine synthetase (GS2), acting concomitantly but not independently. No correlation between fold change in GS2 copies and transcripts was observed. Ammonia accumulation, a biochemical marker of GS2 inhibition, was inversely correlated with GS2 expression. Inheritance, stability and genomic location of GS2 copies were investigated. GS2 copy number shows great variability both within the plant and over time, but no effects of glufosinate application were observed. Segregation of GS2 copies does not follow a classic Mendelian model. Somatic mosaicism was observed in cells from a same root. The variability in number and strength of GS2 signals suggests the involvement of extrachromosomal circular DNAs in GS2 amplification, with possible reintegrations to the linear genome. Soil-applied herbicides represent the best opportunity to manage this Palmer amaranth population, as all but mitosis inhibitors and imazethapyr provided high levels of control. Foliar herbicide application had variable efficacy, being glyphosate the least effective and synthetic auxins and HPPD inhibitors, the most effectives. Chemical control of weeds will remain to be essential in the future, but additional strategies must be incorporated in order to preserve herbicide efficacy.