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GENETICS AND BREEDING OF HERBICIDE TOLERANCE IN SUNFLOWER*

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SUMMARY

Although there are some examples of gene discovery for non-target site mechanisms of tolerance in sunflower, the main focus of research and development during the last decade was directed to the discovery of altered acetohydroxyacid synthase (AHAS) genes and enzymes. In this way, several natural or induced mutant alleles of the sunflower *Ahasl1* locus were reported and characterized. Four of these alleles were utilized to develop different non-GMO traits and technologies of weed control: Clearfield®, Clearfield Plus®, Sures, and ExpressSun®. Each one of these technologies has their own characteristics, cross-tolerance pattern, benefits and drawbacks, which are briefly reviewed. Some methods to speed up the introgression of these traits into the breeding program are described, as well as the dominance relationships between some members of the multiallelic *Ahasl1* locus. Proper utilization of these technologies allowed, and will continue to allow, an excellent weed control for the sunflower crop. However, some of these genes and their allelic interactions remain to be tested and developed in the years to come in order to create novel technologies. Additionally, it is clear that only one mode of action - the inhibition of the AHAS enzyme - is being exploited so far in sunflower. This will prompt the rapid selection of tolerant weeds that may jeopardize the sustainability of all these technologies. Selection over cultivated germplasm, wild *Helianthus* species and mutagenized libraries will allow the discovery of new sources of HT, especially to other modes of action apart from the inhibition of AHAS, in order to complement the current technologies.

Key words: **herbicide resistance, AHAS inhibitor herbicides, mutation breeding**

INTRODUCTION

Weeds compete with sunflower for moisture, nutrients, and depending on species for light and space. Weed competition can cause substantial yield losses in sun-

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flower, with reports ranging from 20 to 70% (Blamey *et al.*, 1997). Herbicides are the most desirable method for weed control, especially under no-tilling conditions. However, the availability of selective herbicides for the sunflower crop is quite limited and, due to the high cost of herbicide registration, new molecules of herbicides are unlikely to be specifically developed for weed control in this crop. Growers have traditionally relied on preemergence herbicides for weed control in sunflower. However, soil-active preemergence herbicides are expensive and require timely rainfall or irrigation for activation. Also, some are marginally effective because of the narrow spectrum of weeds controlled (Miller and Alford, 2000). For this reason, gene discovery and trait development for herbicide tolerance (HT) in sunflower, particularly imidazolinones (IMI) and sulfonylureas (SU), was an active area of research during the past decade to provide non-GMO strategies of weed control in this crop (Sala *et al.*, 2012b).

TYPES OF HERBICIDE TOLERANCE

There are two primary mechanisms of HT in sunflower: (i) tolerance caused by mutations in target sites of the herbicide (*target-site tolerance*), and (ii) tolerance caused by mutations in non-target sites (*non-target site tolerance*). Target-site tolerance involves a reduced sensitivity of target specific enzymes or proteins and, thus, this type of tolerance is mostly monogenic (Tranel and Wright, 2002). Non-target tolerance, on the other hand, involves several mechanisms, such as reduced uptake or translocation of the herbicide, increased rate of herbicide detoxification, decreased rate of herbicide activation, or sequestration of the herbicide away from the target site into the vacuole or the apoplast (Yuan *et al.*, 2006). Both types of HT are present in sunflower, and, in fact the tolerance of one of the current technologies of weed control, Imisun sunflowers, is the result of the additive interaction between target and non-target site mechanisms (Breccia *et al.*, 2012; Sala *et al.*, 2012c).

Non-target-site herbicide tolerance

Herbicides can cause several injury problems to the sunflower crop (Blamey *et al.*, 1997). As a matter of fact, sunflower genotypes varied widely in their response to soil-applied and to post-emergence herbicides. For several molecules, genotypic response can range from susceptibility to tolerance and the tolerance level also varied according to growth stage of the plants, herbicide rate and environmental conditions, like temperature and relative humidity (Gillespie and Miller, 1983). Natural variation for tolerance was recently investigated by inhibiting the activity of P450 monooxygenases (P450) and one line was selected showing significantly higher tolerance to imazamox, prosulfuron, and atrazine than susceptible lines (Kaspar *et al.*, 2011). In fact, the P450 gene family in plants encodes the most versatile class of enzymes involved in the metabolic detoxification of xenobiotics and in non-target-site herbicide tolerance in plants (Yuan *et al.*, 2006). One of the first P450 genes identified for HT, which metabolizes with high efficiency a wide range of xenobiotics

and several herbicides, was cloned from *Helianthus tuberosus* (Battard *et al.*, 1988; Cabello Hurtado *et al.*, 1988; Robineau *et al.*, 1988). A mutant P450 gene showing increased levels of HT was also reported in sunflower (León *et al.*, 2008). These observations indicate that there exists enough natural and induced variation for P450s genes in sunflower to be used in developing new HT traits.

Target-site-herbicide tolerance

IMI and SU herbicides have been demonstrated to have a broad spectrum of weed control activity, flexibility in timing of application, low usage rates, and low mammalian toxicity (Brown, 1990; Tan *et al.*, 2005). These herbicides inhibit the enzymatic activity of acetohydroxyacid synthase (AHAS, EC 4.1.3.18, also known as acetolactate synthase, ALS; Shaner *et al.*, 1984; Ray, 1984), the first enzyme in the pathway for the synthesis of the branched chain amino acids valine, leucine, and isoleucine (Singh, 1999). This same enzyme has been shown to be the site of action for the triazolopyrimidines (TZ, Subramanian and Gerwick, 1989), pyrimidyloxybenzoates (POB, Subramanian *et al.*, 1990), and sulfonylaminocarbonyl-triazolinones (Santel *et al.*, 1999).

Given their high effectiveness and low-toxicity, IMI and SU herbicides are favored for agricultural use. However, the ability to use both types of herbicides in a sunflower production system depends upon the availability of IMI- and SU-tolerant hybrid cultivars. To produce such tolerant cultivars, it is imperative to develop IMI- or SU-tolerant plants with altered AHAS genes and enzymes. These plants have been discovered in sunflower, which permitted the development and commercialization of several herbicide-tolerant traits. Tolerance in these traits is due to a form of the AHAS large subunit enzyme (AHASL) that is less sensitive to herbicide inhibition and is conferred by a single, partially dominant nuclear gene.

The reduction in herbicide binding is caused by mutations at key sites in the genes coding for the catalytic subunit of AHAS. Several authors have reviewed known mutations of the AHAS genes that confer tolerance to AHAS-inhibiting herbicides in weeds and crops (Preston and Mallory-Smith, 2001; Tranel and Wright, 2002; Tan *et al.*, 2005; Tan *et al.*, 2006). Based on molecular studies, Kolkman *et al.* (2004) identified and characterized three genes coding for the AHAS catalytic subunits in sunflower (*Ahasl1*, *Ahasl2* and *Ahasl3*). *Ahasl1* is a multilallelic locus and the only member of this small gene family where all the induced and natural mutations for herbicide resistance were described thus far in sunflower (Figure 1). *Ahasl1-1* (also known as *Imr1* or Ar_{pur}, Bruniard and Miller, 2001; Kolkman *et al.*, 2004; respectively) harbors a C-to-T mutation in codon 205 (*Arabidopsis thaliana* nomenclature), which confers a moderate tolerance to IMI. *Ahasl1-2* (also known as Ar_{kan}) shows a C-to-T mutation in codon 197 conferring high levels of SU- tolerance (Kolkman *et al.*, 2004). *Ahasl1-3* presents a G-to-A mutation in codon 122, which confers high levels of IMI-tolerance (Sala *et al.*, 2008c), and *Ahasl1-4* harbors a G-to-T mutation in codon 574, which endows broad range tolerance to four families of

herbicides targeting AHAS (Sala and Bulos 2012a). Other resistant alleles at the *Ahasl1* locus do not have a formal designation yet. One of them is an allele that shows a mutation in codon 203 conferring slight tolerance to IMI and susceptibility to SU herbicides (León *et al.*, 2007). Other alleles obtained by EMS mutagenesis are present in line M7 (Gabard and Huby, 2001) and MUT28 (León *et al.*, 2006). Both of them have a mutation at the same codon as *Ahasl1-2* conferring tolerance to SU (Hawley, 2005; León *et al.*, 2006).

HERBICIDE TOLERANCE TRAITS

Many of the above-mentioned alleles are being used for the production of sunflower hybrids tolerant to herbicides or they are being developed to produce new tolerance traits. In the following sections the current technologies for weed control in sunflower using AIH are briefly summarized.

Clearfield® technology based on Imisun sunflowers

The first commercial HT trait in sunflowers is known as 'Imisun' and its development started in 1996, when IMI-tolerant wild sunflowers were discovered in a field in Kansas, USA. Subsequent crossing of these plants with cultivated sunflowers lines gave rise to IMI-tolerant populations and lines (Al-Khatib *et al.*, 1998), which were released as donor materials for developing hybrid varieties (Jocic *et al.*, 2004; Tan *et al.*, 2005). The inheritance of Imisun is additively controlled by two genes, where one of them is the partially dominant allele *Ahasl1-1* and the other a modifier or enhancer factor (Miller and Al-Khatib, 2002; Bruniard and Miller, 2001). Variability for tolerance to IMI in homozygous Imisun inbred lines, together with the synergistic effects of IMI and malathion over tolerance in certain Imisun genotypes indicate that several physiological mechanisms are involved in the non-target site component of tolerance of this trait (Sala *et al.*, 2012c). In fact, to produce Imisun sunflower hybrids with commercial levels of tolerance to IMI, both target and non-target components of tolerance need to be expressed in the final variety. Another important aspect of this technology is that the linkage drag from the wild parent around the resistant gene determined a decrease in oil percent in the seed (Trucillo Silva *et al.*, 2010).

Clearfield Plus® technology based on CLPlus sunflowers

The second IMI tolerance trait in sunflowers, known as CLPlus, is controlled by the expression of the partially dominant nuclear allele *Ahasl1-3*, which was developed by seed mutagenesis and selection with imazapyr (Sala *et al.*, 2008b). Based on a vast array of environmental conditions and in biochemical studies, it was determined that the CLPlus trait provides superior herbicide tolerance to IMI to the Imisun trait (Sala *et al.*, 2008a & c, 2012d; Weston *et al.*, 2012b). In fact, the CLPlus trait displays the lowest level of inhibition of the AHAS enzyme extracts by IMI, which results in a higher level of accumulation of biomass after IMI application

at the above-ground (Sala *et al.*, 2012a) and root levels (Sala *et al.*, 2012e). Moreover, this superior level of tolerance also provides a better stability of the tolerance to cope with the unpredictable portion of the environmental variation. In turn, this greater stability determines a better reliability of the Clearfield Plus® technology than the Imisun trait when both of them are challenged with different IMI molecules and doses (Sala and Weston, 2010; Sala *et al.*, 2012d). Due to the high levels of tolerance, only one homozygous component, namely *Ahasl1-3*, or the combination of both *Ahasl1-1* and *Ahasl1-3* alleles in the final hybrid variety, are required to achieve commercial tolerance levels (Sala *et al.*, 2008a, 2012a; Sala and Weston, 2010). High level of tolerance of this trait over Imisun sunflowers also permits to develop new herbicide formulations providing more flexible and reliable weed control (Pfenning *et al.*, 2012). The absence of genes from a wild source around *Ahasl1-3* determines that the oil contents in the hybrids carrying the CLPlus trait show the same oil yield per hectare as those of their conventional counterparts (Weston *et al.*, 2012b).

Sures sunflowers

SU-tolerant sunflowers were developed from wild sunflower populations discovered in USA (Al-Khatib *et al.*, 1999). The tolerance allele *Ahasl1-2* was introgressed into cultivated sunflower by forward crossing and selection with the herbicide tribenuron and gave rise to the trait known as Sures (Miller and Al-Khatib, 2004). Even though the inheritance of this trait has not been reported yet, it is well established that the target-site-tolerance is the result of the mutation P197L at the *Ahasl1* locus (Kolkman *et al.*, 2004) and that differences in crop injury among Sures-tolerant breeding lines (*Ahasl-2/Ahasl1-2*) are the result of the presence of modifier genes (Miller and Zollinger, 2004). This trait was used to develop SU-tolerant hybrid cultivar in many countries, increasing the range of available herbicides in sunflower (Jocić *et al.*, 2011).

ExpressSun® technology

The same type of tolerance as Sures sunflowers was obtained by EMS mutagenesis over the line HA89 (Gabard and Huby, 2001) and was developed and commercialized under the name ExpressSun® (Streit, 2012).

CROSS-TOLERANCE TO DIFFERENT AHAS INHIBITORS HERBICIDES

Cross tolerance is defined as the expression of a genetically-endowed mechanism conferring the ability to withstand herbicides from different chemical classes (Hall *et al.*, 1994). Different HT traits in sunflower showed striking differences in their cross-tolerance level to AHAS inhibitor herbicides (Table 1). *Ahasl1-1*, for example, confers tolerance to imazethapyr, imazamox, slight tolerance to thifensulfuron and chlorimuron, but no tolerance to cloransulam-methyl, pyrithiobac or

high doses of imidazolinones (Al-Khatib *et al.*, 1998; Bruniard and Miller 2001; White *et al.*, 2002; Sala *et al.*, 2008b). Plants carrying the Sures trait show tolerance to tribenuron (Miller and Al-Khatib, 2004), metsulfuron, and chlorsulfuron, but complete susceptibility to imazapyr, imazapic, and imazamox (Sala *et al.*, 2008b). The mutant which gave rise to the ExpressSun® trait shows also good tolerance to tribenuron and ethametsulfuron-methyl but it is somewhat sensitive to other SU (Gabard and Huby, 2001). By the contrary, the Clearfield Plus® trait confers high levels of tolerance to IMI but complete susceptibility to SU (Sala *et al.*, 2008b). *Ahasl1-4* presents a completely new pattern of cross-tolerance for sunflower, since it shows a broad range level of tolerance to different AHAS-inhibiting herbicides (IMI, SU, TZ and POB). Furthermore, this allele also presents a higher level of tolerance to IMI and SU than lines carrying the Imisun and the Sures traits, respectively (Sala and Bulos, 2012a).

Table 1: Cross-tolerance of several herbicide-tolerance traits of sunflower

Trait	Type of herbicide tolerance			
	SU	IMI	TZ	POB
Conventional (wild type)	S	S	S	S
Imisun	S/MS	T	S	S
Sures	T	S	S	S
CLPlus	S	HT	S	S
AIR	HT	HT	T	T

References: SU, sulfonylureas; IMI, imidazolinones; TZ, triazolopyrimidines; POB, pyrimidyloxybenzoates; S, susceptible; T, tolerant; HT, highly tolerant; MS, medium susceptible.

It is known that sunflower lines developed to tolerate some AHAS-inhibiting herbicides are susceptible to foliar applications and, in many cases, to soil residues of other AHAS-inhibiting herbicides (Howatt and Endress, 2006). In these cases, the cross-tolerance of *Ahasl1-4* could allow sunflower hybrids carrying this allele to cope with the soil residues of other types of AHAS inhibiting herbicides from the fallow or the previous crop.

DOMINANCE RELATIONSHIPS AT THE *Ahasl1* LOCUS

Literature about herbicide tolerance indicates that almost all tolerances are inherited as partially to totally dominant traits. However, most studies reporting the degree of dominance of a herbicide-tolerance trait were designed to assess the inheritance of the tolerance mutation. As a consequence, a single threshold herbicide is generally used and this single dose approach may not be appropriate to correctly assess dominance since the applied dose may affect apparent dominance and recessivity (Roux *et al.*, 2005).

Using the Imisun and CLPlus alleles it was found that the degree of dominance in the presence of IMI can vary from dominance to recessivity depending on the resistant allele, the applied dose of herbicide and the variable considered (*i.e.*: bio-

mass accumulation or enzymatic activity, Sala *et al.*, 2012a). Both resistant alleles are recessive with respect to the wildtype allele at the enzymatic level but they showed from dominance to recessivity at the phenotypic level. This discrepancy was explained by the margin of error of the enzyme and by differences in AHAS functionality of each of the tolerance-conferring AHAS gene mutations. Interestingly, *Ahasl1-3* showed dominance over *Ahasl1-1* both at the phenotypic and enzymatic levels and at all the tested doses, an observation that can be interpreted taking into account the protein structure of the AHAS catalytic subunit (Sala *et al.*, 2012a). On the other hand, it was also observed that *Ahasl1-2* shows dominance over *Ahasl1-1* when challenged with SU-herbicides (Miller and Zollinger, 2004). Besides its inherent importance to understand the evolution of the dominance, this type of research has several practical implications ranging from plant breeding to weed management. As a matter of fact, multiallelism at the *Ahasl1* locus permits the exploitation of several other allelic interactions in designing new technologies for weed control.

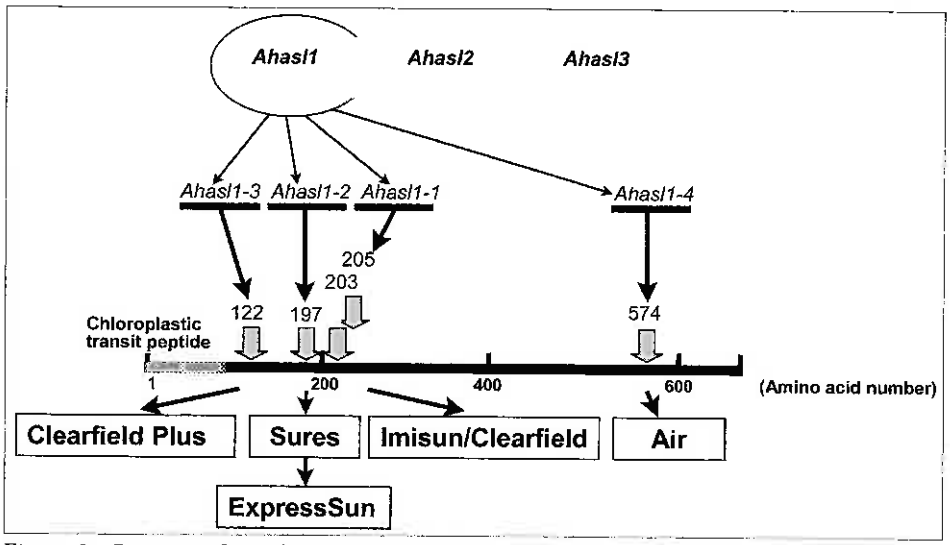

Figure 1: Genetics of AHAS-inhibitor herbicide tolerance in sunflower. There exist three genes encoding for catalytic subunits of the AHAS enzyme: Ahasl1, Ahasl2, and Ahasl3. Known mutations for herbicide tolerance so far described were located in Ahasl1. Formally described alleles of this gene, the site of the aminoacidic substitution controlling tolerance in each case (following Arabidopsis thaliana nomenclature), and the herbicide tolerance trait developed from each allele/mutant are provided.

BREEDING FOR HT AND HT-ASSISTED BREEDING IN SUNFLOWER

Phenotypic identification of HT involves the spraying of herbicide onto plants grown in the field or greenhouse at early stages of development, usually V2-V4 and selection of tolerant genotypes. Screening a large number of genotypes for HT in the

field is time consuming and requires a large amount of resources and space. Under these circumstances, the development of efficient and reliable diagnostic bioassays or molecular markers for early screening of HT is needed. In this sense, immature-embryo (Breccia *et al.*, 2009) and seed germination bioassays were developed for screening IMI-tolerance (Vega *et al.*, 2008; Breccia *et al.*, 2011; Gil *et al.*, 2012) as well as SU-tolerance (Dimitrijević *et al.*, 2012). Moreover, introgression of genes for herbicide resistance into high yielding sunflower germplasm is being facilitated by marker assisted selection with diagnostic markers for each one of the resistance alleles at the *Ahasl1* locus (Kolkman *et al.*, 2004; Bulos *et al.*, 2010).

It is well known, on the other hand, that the final value of a sunflower inbred line can only be determined by testing its general and specific combining ability in hybrid combinations. In this sense, HT traits can also be used as useful tools to assist the breeding process. In fact, a male-sterilization system was developed recently based on the adequate manipulation of the type of mutant at the *Ahasl1* locus, doses and timing of herbicide application, plant zygosity and stage of development. This system not only permits the sterilization of testers in the breeding program, but also allows increasing the frequency of HT plants in the progeny due to gametophytic selection (Sala and Bulos, 2012b).

QUALITY ASSESSMENT OF HT COMMERCIAL HYBRIDS

Quality assessment should provide accurate and on time information to take the best decisions in order to reduce production cost, meet commercialization standards and customer expectations. To reach this goal, quality assurance should start in the production field and should continue until the seed is dispatched to the farmer. Hence, the integration of quality assurance with the production practices led to a complete traceability of the seed. This, in turn, gives the opportunity to introduce timely corrective actions and to improve the whole process continuously (de Estrada *et al.*, 2012). Considering the negative impact of genetic contaminations (*i.e.* susceptible seed in an Imisun hybrid, for example) over yield performance of HT commercial hybrids, seed purity testing in HT sunflower is an area of concern, continuous research and new developments. Currently, determination of off-types in HT sunflowers is carried out in off season nurseries and is complemented by the assessment of trait purity by bioassays and molecular markers (see, for example, Katz *et al.*, 2012). Likewise, by using Real Time PCR technology it is possible to detect up to 0.2% of contaminant seeds in bulks of 1000 to 3000 seeds of a HT hybrid (Sensolini *et al.*, 2012).

WEED CONTROL

IMI and SU herbicides provide excellent broad-spectrum weed control in sunflower, including some of the most problematic weeds for the sunflower crop. In addition, both families of herbicides allow the possibility to control broomrapes (*Orobanche cernua* and *O. cumana*), obligate root holoparasitic weeds that are

insufficiently controlled by other herbicides traditionally used in sunflower. Rapid changes in broomrape race composition have forced sunflower breeders and geneticists to search continuously for new sources of resistance to the new races of *Orobanche*. The development of sunflower hybrids resistant to the IMI and SU herbicides has made it possible to successfully control broomrape regardless of the race composition of the populations of these weeds (Škorić and Pacureanu, 2010)

Different HT traits, herbicides and methods of application - such as seed-coating, single or sequential post-emergent herbicide applications, timing of application, or their combinations - have been devised, tested and/or validated in order to control these parasitic weeds (Alonso *et al.*, 1998; Aly *et al.*, 2001; Gabard and Huby, 2001; Sala *et al.*, 2008d, among others). However, HT traits should be integrated with resistant genes to different races of *Orobanche* in order to avoid the rapid evolution of HT broomrapes. This combined strategy will provide the most sustainable and durable method of broomrape control in the following years.

ENVIRONMENTAL SAFETY AND CONTROL OF VOLUNTEER HT SUNFLOWERS

No competitive advantage other than that conferred by tolerance to SU or IMI herbicides is conferred to HT sunflowers. In fact, no significant differences could be detected when HT sunflowers and their conventional isohybrids were evaluated and compared in several environmental situations by different agronomic traits covering a broad range of characteristics that encompass the entire life cycle of the sunflower plant. It is therefore not expected that HT sunflowers would present traits that would render them invasive of natural habitats since none of the reproductive or growth characteristics were modified. HT in itself will not cause Clearfield®, Clearfield Plus® or ExpressSun® sunflowers to become more weedy or invasive in managed habitats than conventional *H. annuus*. Gene flow from HT sunflowers to wild sunflower populations is very likely to occur in many parts of the world. However, some of the genes controlling the HT traits are already present at various levels in wild sunflower populations (Sala and Bulos, 2012a and references therein). In addition, gene flow from HT sunflowers to wild sunflowers populations would not be expected to result in increased invasiveness of the offspring, as the HT traits are not associated with enhanced weediness. The occurrence of IMI- or SU-tolerant wild sunflowers will not cause weed management issues as HT wild sunflowers will still be easily controlled by herbicides with other modes of action or by cultivation (Canadian Food Inspection Agency 2005, 2008, 2010).

It is likely that SU- or IMI-tolerant sunflower volunteers will not be controlled in subsequent crops if an AHAS inhibitor herbicide is used as the sole weed control tool. However, control of HT sunflowers as volunteer weeds in other crops or in fallow ground can readily be achieved by the use of herbicides with other modes of action or by mechanical means (Canadian Food Inspection Agency 2005, 2008, 2010).

CONCLUSION

Several herbicide resistant genes were discovered, and many HT traits and technologies for weed control were developed for the sunflower crop in the last decade. Proper utilization of these technologies allowed, and will continue to allow, an excellent weed control for the sunflower crop. However, some genes and allelic interactions remain to be tested and developed in the following years in order to create novel technologies. Additionally, it is clear that only one mode of action - the inhibition of the AHAS enzyme - is being exploited until the present. This will prompt the rapid selection of tolerant weeds that may jeopardize the sustainability of all these technologies. Selection over cultivated germplasm, wild *Helianthus* species and mutagenized libraries will allow the discovery of new sources of herbicide tolerance, especially to other modes of action apart from the inhibition of AHAS, in order to complement the current technologies. In addition, new interactions between target and non-target site tolerance mechanisms should be explored as potential novel HT traits for the sunflower crop.

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