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Decoding Non-Target-Site Herbicide Resistance in Sunflower: The Beginning of the Story

https://doi.org/10.1515/helia-2019-0002
Received January 16, 2019; accepted May 07, 2019

Abstract: In the last years, many efforts have been made to develop sunflower cultivars showing important agronomical characteristics such as herbicide resistance. These approaches have been focused mainly on resistance to herbicides with the same mode of action, that is acetohydroxyacid synthase (AHAS) inhibitors. To date, four induced and natural AHAS mutations have been found that confer resistance to these herbicides and many of these alleles are being used for the production of sunflower hybrids resistant to herbicides and to develop different non-transgenic technologies for weed control. However, little is known about the bases of non-target-site-based resistance (NTSR) developing cross-resistance to herbicides with different modes of action in sunflower. These mechanisms diminish the number of active herbicide molecules that reach the target and are generally polygenic. Elucidating the nature of NTSR would allow evaluating maximal efficiency conditions for the herbicide and would enable to establish weed management strategies in sunflower crop. Nowadays, mining of NTSR genes can be more easily accomplished taking advantage of up-to-date omics-based approaches: high-throughput techniques involving genomics, transcriptomics, proteomics and metabolomics. Considering the difficulties in the discovery of new compounds with a broad spectrum of weed control, it results essential to broaden the use of former herbicides which are highly efficient and ecologically desirable. Full understanding of NTSR mechanisms in sunflower would allow detecting specific genes potentially useful as biotechnological tools for the phytoremediation of herbicides and modern plant breeding.

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Keywords: gene expression, genome sequencing, *Helianthus annuus* L., herbicide resistance, plant breeding

Sunflower breeding: Important agronomical traits

According to the Food and Agriculture Organization of the United Nations (FAO) by the year 2050 food production must increase by 70 % in order to ease potential increase in world's population and changes in diets (http://www.fao.org). This percentage becomes highly influenced by continuous climate changes, weed competition and yield losses in crops. To deal with these challenges, crops must be developed that combine several traits including improved weed control, insect and disease resistance, enhanced resistance to abiotic stresses and product quality (Cantamutto and Poverene, 2007). Genetic improvement of crop performances under disadvantageous conditions is necessary as a sustainable alternative (Roche and Hewezi, 2009) and cultivated sunflower is a main candidate to achieve this goal.

Sunflower (*Helianthus annuus* L.) is an economically important major crop belonging to the Compositae or Asteraceae family. In 2016, the global planting area of this crop was greater than 24,970,000 hm^2, reaching position 12 in global harvested area (http://www.fao.org) and achieving an average global annual production of 47 million tonnes (National Sunflower Association 2016/2017). Besides its importance in food and oil production, sunflower has also become a model crop for ecological and evolutionary studies. One of the most interesting features of sunflower relies on its promise for adaptation to extreme environmental conditions (Kane and Rieseberg, 2007) and its potential to hybridize freely with their wild relatives (Massinga *et al*., 2003), allowing the detection of new alleles endowing interesting agronomical traits that can be used in breeding programs.

In the last years, many efforts have been made to develop sunflower cultivars showing important agronomical characteristics such as drought and salinity stress tolerance (Blum, 1987; Liu and Baird, 2003; Roche and Hewezi, 2009; Sala *et al*., 2012a) and herbicide resistance (Sala *et al*., 2012b). The latter has been one of the most critical areas of research, aiming to obtain improved sunflower germplasms with resistance to herbicides in order to control weeds and reduce economic losses all over the world. These approaches have been focused mainly on physiological and molecular insights on the target site mechanisms of resistance to herbicides with the same mode of action, that is acetohydroxyacid synthase (AHAS) inhibitors or group B herbicides.

Herbicide NTSR mechanisms: Pool of opportunities

According to the Weed Science Society of America, herbicide resistance is defined as the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type. Resistance may occur naturally or be induced by techniques such as genetic engineering or mutagenesis (WSSA, 1998).

Sometimes a particular plant biotype express a single (cross-resistance) or more than one resistance mechanism (multiple resistance) that endows the ability to withstand herbicides from different chemical classes (Hall *et al.*, 1994). Apart from the serious economic damage on cropping, these phenomena are interesting from a mechanistic viewpoint because different biochemical processes could account for them: a reduced sensitivity of one or more herbicide target sites and a reduction in the effective concentration of herbicides at their respective target sites (Matthews *et al.*, 1990). The latter could be caused by several factors including a reduction in herbicide absorption and translocation, a reduced conversion of herbicides to their active forms, changes in the inter- or intracellular sequestration of herbicides or an increased capacity to detoxify the herbicides (De Prado and Franco, 2004; Matthews *et al.*, 1990; Powles and Yu, 2010). Whatever the mechanisms, they must be general enough to generate resistance to structurally distinct herbicides yet specific enough to be responsible for the herbicide specificity that is still observed (Matthews *et al.*, 1990).

Among the mechanisms that diminish the number of active herbicide molecules that reach the target, herbicide detoxification has been one of the most intriguing. Throughout the last decade, different approaches have revealed the presence of a multitude of enzymes that metabolize herbicides and other xenobiotics to non-phytotoxic products in plants (Kreuz *et al.*, 1996). These enzymes confer non-target-site resistance (NTSR): mechanisms not specifically related to the xenobiotic site of action, which are considered the principal type of resistance to the most important herbicide groups currently commercialized (Délye *et al.*, 2013).

Herbicide metabolism conferring NTSR follows a four-step schema: first, activation of herbicide molecules by an oxidation typically carried out by P450 monooxygenases and other oxidases. Second, conjugation of a hydrophilic molecule by glutathione S-transferases (GSTs), glycosyltransferases and UDP-glucoronosyl/UDP-glucosyltransferases, followed by an active transportation of herbicide conjugates and their metabolites by ABC transporters. Finally, herbicide conjugates are sequestrated in the vacuole for further degradation (Bartholomew *et al.*, 2002; Klein *et al.*, 2006) and protection mechanisms against reactive oxygen species are triggered (Yuan *et al.*, 2007) (Figure 1).

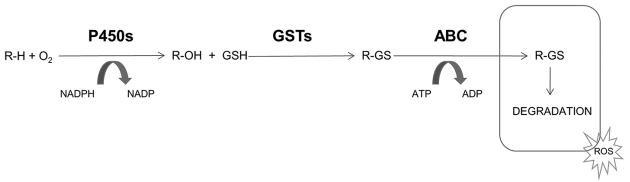

Figure 1: Four-phase schema of xenobiotic metabolism in higher plants. Cytochrome P450 (P450) monooxygenase activity, glutathione S-transferase (GST) conjugation and ATP-dependent transport via ABC transporters is followed by subsequent detoxification in vacuoles and depletion of collateral effects such as the generation of reactive oxygen species.
Source: Yuan *et al.* (2007).

Target-site resistance (TSR) is mostly monogenic, involves a point mutation that decrease herbicide binding to the target enzyme or an increased activity (increased expression or increased intrinsic activity) of the target protein (Délye *et al.*, 2015; Yuan *et al.*, 2007) and therefore its molecular mechanisms are relatively easy to study. In contrast, NTSR mechanisms are generally polygenic and may include several biochemical modifications to the herbicide molecule through metabolism. NTSR is a dynamic stress response that involves two major groups of proteins: effectors that function directly in the protection of plant cells against stresses such as heat stress proteins or chaperones, osmoprotectants and detoxification enzymes; the second group includes those regulators that control gene expression and signal transduction (Délye, 2012; Roche and Hewezi, 2009).

Why to focus on NTSR in sunflower?

Herbicides are crucial for weed control strategies in current production systems. However, the availability of selective herbicides in sunflower crop is limited and because of high costs of research, test and registration, new molecules are unlikely to be specifically developed (Sala and Bulos, 2012). Given the difficulty of discovering new herbicides, expanding the utility of existing herbicides that have a broad weed-control spectrum and good environmental profile through genetically enhanced resistance in crops is a useful strategy (Tan *et al.*, 2005).

During the last decade, gene discovery followed by development of herbicide resistant cultivars is one of the most important issues concerning productivity gains and competitive ability in sunflower crop. The focus of research was directed to the discovery of new acetohydroxyacid synthase (AHAS) genes leading to resistance to AHAS-inhibitor herbicides. Kolkman *et al.* (2004) identified

and characterized three genes coding for the AHAS catalytic subunits in sunflower (Ahas1, Ahas2 and Ahas3). Moreover, Ahas1 is a multiallelic locus where all induced and natural mutations for herbicide resistance have been found (Sala *et al*., 2008a). Many of these alleles are being used for the production of sunflower hybrids resistant to herbicides and to develop different non-transgenic technologies for weed control (Table 1). Breeding for resistance to AHAS

Table 1: Herbicide-resistance traits in sunflower. Ahas1 is a multilallelic locus where all the induced and natural mutations for herbicide resistance were found in sunflower. Imidazolinone (IMI), sulfonylurea (SU), triazolo- pyrimidines (TZ), pyrimidinyloxybenzoates (POB).

Allele	Mutation	Trait	Type of mutation	Information
Ahasl1-1	Ala 205 Val	Imisun	Natural	First commercial herbicide resistance trait in sunflower. Clearfiled® technology. IMI resistance genes from a sunflower wild population collected in Kansas, USA in 1996 were introgressed to inbred lines (Al-Khatib *et al*., 1998; Miller and Al-Khatib, 2002). Confers resistance to IMI herbicides.
Ahasl1-2	Pro 197 Leu	Sures	Natural	Resistance allele introgressed into cultivated sunflower by forward crossing and selection with the herbicide tribenuron (Miller and Al-Khatib, 2004). Provides resistance to SU herbicides.
		ExpressSun	Induced	Obtained by seed mutagenesis over the susceptible line HA89 (Gabard and Huby, 2001). Provides resistance to SU herbicides. ExpressSun® technology.
Ahasl1-3	Ala 122 Thr	CL Plus	Induced	Developed by seed mutagenesis and selection with imazapyr (Sala *et al*., 2008b). Provides resistance to IMI. Clearfield Plus® technology.
Ahasl1-4	Trp 574 Leu	AIR	Natural	Discovered in a wild sunflower population from Jovita, Argentina (Sala and Bulos, 2012). Shows a broad range level of resistance to different AHAS-inhibiting herbicides (IMI, SU, TZ and POB).

inhibitors represented a major advance in weed control technology in sunflower. However, the use of herbicide families with the same mode of action generated soon after a rapid appearance and subsequent spread of resistant weeds, herbicide residue problems and gene flow to wild species. A potential alternative would be to strengthen efforts in the characterization of mechanisms that delay this resistance emergence, such as those that confer cross-resistance.

Two mechanisms of AHAS-inhibitor herbicide resistance coexist in sunflower: an altered target site and enhanced herbicide metabolism (Sala *et al.*, 2012b). The presence of NTSR mechanisms in sunflower resistant lines that are related specifically to herbicide metabolism has been suggested in several works (Balabanova *et al.*, 2018; Breccia *et al.*, 2017; Gil *et al.*, 2018a; Kolkman *et al.*, 2004) although very few genes endowing NTSR have been identified to date.

Herbicide metabolism depends on the xenobiotic, the organism and environmental conditions and generally confers cross-resistance to different classes of herbicide (Van Eerd *et al.*, 2003). In this way, researching the genetic bases of NTSR to herbicides in sunflower represents a major challenge for weed and crop science.

Understanding the molecular basis of NTSR mechanisms in sunflower is crucial since these resistance genes would confer cross-resistance to herbicides with different modes of action including those that have not been commercialized yet. Considering the difficulties in the discovery of new biodegradable and safe active compounds with a broad spectrum of weed control, it results essential to broaden the use of those molecules that possess ecologically desirable properties and high efficacy. Elucidating the nature of NTSR would allow evaluating maximal efficiency conditions for the herbicide and would enable to establish weed management strategies in sunflower crop.

Full understanding of herbicide-resistance mechanisms would allow detecting a diversity of detoxification-gene families that might provide an important source for engineering herbicide tolerance, biosafening, bioremediation and green chemistry (Werck-Reichhart *et al.*, 2000). The identification and recovery of individual genes and the development of transgenic plants has revealed their potential as useful biotechnological tools for the phytoremediation system involved in the degradation of herbicide pollutants in agricultural fields (Karavangeli *et al.*, 2005).

Plant breeding has been very successful in developing improved varieties using conventional methodologies. Currently, genome-wide expression studies are providing breeders with new tools that allow a step forward in the genetic dissection and breeding particularly for complex traits such as NTSR. An understanding of the molecular basis of complex traits together with these genomic tools and resources facilitate studying the genetic diversity, which is a

requirement for a precision breeding approach involving germplasm management, enhancement and use (Pérez-de-Castro *et al.*, 2012).

High-throughput approaches in sunflower

The patterns of resistance due to NTSR mechanisms are unpredictable (Petit *et al.*, 2010). NTSR is a quantitative trait, meaning that it is under the control of many genes that individually contribute only a small proportion of genetic variation. Its characterization becomes a challenge since the majority of modern tools in quantitative and population genetics, including genome wide association studies and selection mapping protocols, are designed to identify individual genes with large effects. NTSR genes may be identified by quantitative trait loci (QTL) mapping or via genetic transformation (forward or reverse genetics) but both approaches are complex, time consuming and not easily applied to non-model organisms (Délye, 2012).

In sunflower, phenotypic and RT-qPCR molecular approaches have been performed to stablish the participation of particular gene families such as P450s and GSTs (Balabanova *et al.*, 2018; Breccia *et al.*, 2017) in NTSR (Table 2). However, the large number of those and others NTSR-related gene families present in vegetal genomes makes their individual characterization a very complex challenge. Global gene expression analysis have been carried out by microarrays to determine complex traits such as leaf senescence and seed dormancy in sunflower (Bazin *et al.*, 2011; Fernandez *et al.*, 2012), although its utility becomes restricted because they have a limited sensitivity and present trouble in distinguishing transcripts belonging to homologous genes. These constraints are overcame by alternative methods based on sequencing or PCR amplification (Breyne *et al.*, 2003). A transcript profiling characterization of NTSR by cDNA-AFLP methodology was carried out in Imisun sunflower confirming the contribution of these mechanisms in imazethapyr resistance (Gil *et al.*, 2018a) (Table 2). An important number of genes related to metabolism of xenobiotics and stress was found: P450 monooxygenases, UDP-glucuronosyl/UDP-glucosyltransferases, glycosyltransferases and ATP-binding cassette transporters, among others, suggesting that herbicide detoxification processes are involved in imidazolinone resistance in sunflower.

Nowadays, mining of NTSR genes can be more easily accomplished taking advantage of omics-based approaches: high-throughput techniques involving genomics, transcriptomics, proteomics and metabolomics, destined to identify

Table 2: Different approaches to characterize non-target-site resistance (NTSR) in the *Helianthus* genera. ATP-binding cassette transporter (ABC), 1-aminobenzotriazole (ABT), glutathione S-transferase (GST), glycosyltransferase (GT), piperonyl butoxide (PBO), cytochrome P450 monooxygenase (P450).

Evidence of NTSR in *Helianthus*		
Identification of particular isoforms	Detoxifying enzymes inhibition	A cultivated sunflower line (*Helianthus annuus* L.) with multiple herbicide resistance was selected with the herbicide imazamox and resistance levels were reverted by the inhibitor of P450s malathion (Kaspar *et al*., 2011). The increased susceptibility to imazapyr after P450s-inhibitors treatment (ABT, PBO and malathion) indicated that herbicide metabolism by different P450s isozymes is a mechanism involved in *Helianthus annuus* L. resistance (Breccia *et al*., 2017). Drawbacks: the use of P450s inhibitors particularly selective for specific isoforms fails to elucidate the involvement of multiple P450s in the detoxification process.
	RT-qPCR	Gene expression analysis after application of imazamox using real time qPCR technique suggested that GSTs are involved in IMI detoxification in *Helianthus annuus* L. (Balabanova *et al*., 2018). Drawbacks: false positives due to post-transcriptional control. Not possible to analyse complete response pathways.
	Mutagenesis	Herbicide-resistant sunflower plants were obtained by mutagenesis and selection program. Imazamox resistance was found to be controlled by induced P450 detoxification process (León *et al*., 2012). Drawbacks: time consuming, necessity of large growing spaces and human sources.
	Transgenesis	A P450 gene was isolated from *Helianthus tuberosus* L. and it was found to increase phenylurea herbicide metabolism and resistance when expressed in tobacco and Arabidopsis (Robineau *et al*., 1998; Didierjean *et al*., 2002). Drawbacks: characterization of individual isoforms. Complicated procedures in polyploid crops.

(*continued*)

Table 2: (*continued*)

Evidence of NTSR in *Helianthus*		
Global gene expression analysis	Transcript profiling	Characterization of the gene expression of resistant and susceptible sunflower lines in response to imazethapyr herbicide by cDNA-AFLP allowed to detect candidate detoxification related genes: P450s, UDP-glucuronosyl/UDP-glucosyltransferases, GTs and ABCs, among others (Gil *et al*., 2018a). Drawbacks: false positives due to post-transcriptional control.
	High throughput sequencing	Genome-wide expression analysis by RNA-seq suggested that constitutive NTSR mechanisms might account for imazethapyr resistance in sunflower. P450s, ABCs, GTs, UDPglucuronosyl/glucosyltransferases and GSTs involved in NTSR were identified (Gil *et al*., 2018b). Drawbacks: false positives due to post-transcriptional control.

traits that have been under selection and are controlled by large numbers of loci. In this manner, systems biology approaches integrating transcriptomic and metabolomic analyses were carried out in sunflower using microarrays, physiological measurements and chromatography assays to understand natural leaf senescence and drought stress response (Moschen *et al*., 2016a, 2017).

The analysis of the raw data coming from high-throughput technologies requires deep knowledge of bioinformatics, statistics, and data mining methods and allows the reconstruction of genetic circuits and the deciphering of complex regulatory networks associated with biological processes. In this way, the complementary use of network and molecular signature software applications provided a useful tool for identifying candidate genes and metabolites to characterize leaf senescence process in sunflower based on transcriptomic and metabolomic data (Moschen *et al*., 2016b).

One of the possibilities to address the complex genetic control of herbicide NTSR in sunflower involves global sequencing of the transcriptome by RNA-seq (Giacomini *et al*., 2017). RNA-seq is the most powerful sequence-based tool available for identifying differentially expressed genes without the necessity of previous genomics or transcriptomic sequences and may be used for detecting cellular pathway alterations during herbicide treatment. RNA-seq is considered a highly promising way of unravelling the genetic control of complex traits in

plants (Duhoux *et al.*, 2015) since it may yield information about the affiliations between genes and their products and allow isolation of genes for traits whose biochemistry is difficult (Malik, 2016). It offers several advantages over other transcriptome techniques such as high sensitivity and specificity in the detection of lower abundance transcripts and the possibility of discriminating highly similar sequences.

Recently, gene expression profiling by RNA-seq has been carried out to study the response to drought stress (Liang *et al.*, 2017) and *Verticillium dahliae* disease (Guo *et al.*, 2017) in sunflower. Moreover, a characterization of NTSR in response to imazethapyr was performed using RNA-seq in Imisun sunflower aiming to determine the nature of this resistance (Gil *et al.*, 2018b) (Table 2). Numerous genes related to xenobiotic metabolism were found: cytochromes P450s, ABC transporters, glycosyltransferases, UDPglucuronosyl/glucosyltransferases and glutathione S-transferases. None of these genes showed differential expression between control and imazethapyr-treated plants suggesting that NTSR mechanisms were constitutive.

RNA-seq technique involves total or poly-A RNA conversion into a cDNA library followed by amplification and next generation sequencing. Short reads are obtained (30 to 400 bp depending on the sequencer) and two strategies can be applied to construct transcripts using these short reads. The first strategy is an 'align-then- assemble' approach: based on this method, the transcript can be reconstructed by aligning the short reads to the genome and then accounting for possible splice events (Guttman *et al.*, 2010; Trapnell *et al.*, 2010). The second strategy is called 'assemble-then-align': de novo assembly is applied to construct the transcripts, and then the assembled transcripts build a reference transcriptome for these no-model organisms without genome sequences. However, in order to perform *de novo* assembly and to build a reference transcriptome, very abundant short reads, samples in different conditions and 3 or more replica per sample are required (Fan *et al.*, 2013). That means that to build a good reference numerous efforts have to be made.

Sunflower genome: A turning point

Despite the large interest in sunflower crop, until 2016 no reference genome of cultivated sunflower had been completely sequenced, which made difficult the application of molecular approaches for crop breeding and evolutionary studies. Cultivated sunflower is not only one of the most important annual crops grown for edible oil worldwide (Putt, 1997) but its 3.6 gigabase genome turns it into a

model system for molecular and biochemical studies. It is an annual crop that belongs to the Compositae family, one of the biggest among flowering plants. It has a diploid genome (2n = 34) slightly longer than the human genome, a reason for a particularly slow genomic characterization in relation to other crops (Kane *et al.*, 2011).

Finally, in June 2016 a high-quality reference for the sunflower genome was presented to the scientific society as an achievement of the SUNRISE project (http://www.sunrise-project.fr) in collaboration with the International Sunflower Genome Consortium (University of British Columbia, Canada and Institut National de la Recherche Agronomique, France) and it was published online in May 2017 (Badouin *et al.*, 2017). Assembling the sunflower genome has been extremely difficult as it mainly consists of long and highly similar repeats (~78 %), the majority of which are transposons (Badouin *et al.*, 2017; Cavallini *et al.*, 2010; Gill *et al.*, 2014). This complexity has demanded de development of innovative assembly protocols for almost 10 years (Kane *et al.*, 2011).

Genome and sequence analysis tools are now available at the Sunflower Genome Portal (https://www.heliagene.org/HanXRQ-SUNRISE/). Genome assembly, structural annotations and protein alignments are some of the valuable data accessible to researchers for designing experiments aiming to contribute to specific knowledge about sunflower crop and its weedy relatives. The reference genome sets the bases for future research programs aiming to generate new varieties with improved biotic and abiotic stress resistance necessary for current production strategies.

New genome, new chances: Future perspectives

In sunflower, molecular markers for simple traits such as fertility restoration and high oleic acid content have been successfully used in marker-assisted breeding programs for years. However, agronomically important complex quantitative traits are a challenge and require genome-wide approaches which have been simplified by the availability of the sunflower genome sequences. Genotype-by-sequencing, and whole genome sequencing based on next generation sequencing technologies facilitated the production of large amounts of SNP markers for high density maps as well as SNP arrays and allowed genome-wide association studies and genomic selection in sunflower. Genome wide or candidate gene based association studies have been performed for traits like branching, flowering time, resistance to Sclerotinia head and stalk rot (Dimitrijević and Horn, 2018).

The sunflower reference genome generated by Badouin *et al.* (2017) is a landmark in sunflower molecular research, providing new opportunities for gene discovery and multigenic traits characterization. It broadens the potential of high-throughput techniques such as RNA-seq, enabling the characterization of gene expression patterns of complex traits such as NTSR and improving phenotyping selection techniques involved in breeding programs in sunflower crop.

NTSR mechanisms constitute a potential source of variability available for breeding programs in sunflower species and proteins involved in these mechanisms have been suggested as novel metabolic sources for herbicide resistance (Thyssen *et al.*, 2014).

Proteomic studies should follow RNA sequencing to continue with the identification of NTSR-related genes. Particularly, a high-throughput technique with high potential for evaluating the differentially expressed genes between two or more conditions and for detecting genes associated with quantitative traits in sunflower is liquid chromatography combined with electrospray tandem mass spectrometry (LC-MS). This technique generates big amount of data that can be aligned against the reference genome and proteome sequences in order to achieve technique full potentiality.

It has been extremely difficult to predict cross-resistance due to increased herbicide detoxification in crops, so elucidating the genetic basis of these mechanisms in sunflower will also provide new predictive tools useful to breeders. Allocating efforts and resources to these studies will allow generating resistance to new active ingredients, with different modes of action. Moreover, new technologies can be developed that improve herbicide product formulations and tend to more efficient and sustainable weed management strategies. It has been estimated that millions of tonnes of herbicide are applied annually but less than 5 % of these molecules reach their target sites, while the rest deposes in the field and moves towards atmosphere and nearby water sources (Van Eerd *et al.*, 2003). Since proteins involved in NTSR are versatile and develop cross-resistance to chemically different xenobiotics, they could be implemented as potentially useful tools for bioremediation of contaminated soil and water (Siminszky, 2006; Werck-Reichhart *et al.*, 2000).

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