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Characterization of Non-Target-Site Mechanisms in Imidazolinone-Resistant Sunflower by RNA-seq

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Abstract: Imisun sunflowers (*Helianthus annuus* L.) are imidazolinone-resistant cultivars in which the two mechanisms of herbicide resistance coexist: (i) mutation in herbicide target-site (target-site resistance) and (ii) non-target-site resistance (NTSR). In Imisun technology, NTSR could be related to herbicide metabolism and might occur as a result of a constitutive up-regulation of resistance genes, or it can appear only after herbicide treatment. The objective of this study was to characterize NTSR in Imisun sunflower in response to

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imazethapyr using RNA-Seq and to determine whether these mechanisms are constitutive or herbicide-induced. Cypsels were germinated in plastic pots, watered by capillarity and growth in chamber under controlled conditions. Seven-day-old plants were treated with 0 (control) and 1 µM imazethapyr for 12 h. After leaf RNA purification, stranded, paired-end cDNA libraries were constructed. Sequencing was performed with Illumina HiSeq2000. Local mapping, with and without multihits, was carried out over the reference transcriptome HaT13l and differential expression was analysed. Sixty one and 47 contigs (according to mapping strategy) related to xenobiotic metabolism were found: cytochromes P450s, ABC transporters, glycosyltransferases, UDPglucuronosyl/glucosyltransferases and glutathione S-transferases. None of these contigs showed differential expression between control and imazethapyr-treated plants. Seventeen interesting contigs were verified by qRT-PCR. These results suggest that constitutive NTSR mechanisms may account for imidazolinone resistance in Imisun sunflower.

Keywords: herbicide resistance, gene expression, imazethapyr, high-throughput sequencing

Introduction

Imisun is the first commercial imidazolinone resistance trait in sunflower species and therefore it was widely used in subsequent breeding programs (Miller and Al-Khatib, 2002; Sala *et al*., 2012; Tan *et al*., 2005). Two mechanisms of herbicide resistance occur simultaneously in Imisun sunflowers (*Helianthus annuus* L.): (i) mutation in herbicide target-site (target-site resistance, TSR) and (ii) non-target-site resistance (NTSR) (Sala *et al*., 2012). TSR is conferred by a partially dominant allele at the *Ahasl1* locus (Bruniard and Miller, 2001) coding for a less sensitive acetohydroxyacid synthase (AHAS) catalytic subunit (Kolkman *et al*., 2004), which is the target of imidazolinone herbicides. The nature of the resistance endowed by NTSR mechanisms in Imisun sunflower has not been determined yet, but it could be related to xenobiotic metabolism (Al-Khatib *et al*., 1998; Breccia *et al*., 2017).

TSR involves a reduced sensitivity of specific enzymes and is generally a monogenic trait (Délye *et al*., 2013). In contrast, NTSR mechanisms consist of a reduction in herbicide penetration and translocation, an enhanced herbicide degradation (metabolic resistance) and/or a protection against the collateral damage of herbicide action (Délye, 2012), being mostly polygenic (Busi *et al*.,

2010; Petit *et al.*, 2010). Particularly, metabolic resistance can involve several processes: oxidation of herbicide molecules by cytochrome P450 monooxygenases (P450s), sugar-conjugation involving glycosyltransferases and glutathione S-transferases (GSTs) and transport of conjugated molecules into the vacuole or extracellular space by ABC transporters for subsequent degradation (Yuan *et al.*, 2007).

NTSR can occur either as a result of a constitutive up-regulation of resistance genes before and after herbicide treatment, or of herbicide-induced up-regulation (Duhoux and Délye, 2013; Zhang *et al.*, 2007). In addition, these dynamic mechanisms involve both proteins directly interfering with herbicide action and regulators controlling the expression of such proteins (Délye, 2012; Délye *et al.*, 2013). In this way, a high-throughput sequencing approach such as RNA-seq becomes a promising tool for studying such a complex trait (Duhoux *et al.*, 2015; Malik, 2016).

Only a few studies have described the role of xenobiotic metabolism in herbicide resistance in *Helianthus* genera and they were particularly focused on P450s (Breccia *et al.*, 2017; Didierjean *et al.*, 2002; Kaspar *et al.*, 2011) and GSTs (Balabanova *et al.*, 2018). Recent transcriptomic characterization by cDNA-AFLP allowed detecting sequences related to imazethapyr metabolism in sunflower that corresponded to four gene families: ABC transporters, glycosyltransferases, P450s and UDP-glucuronosyl/UDP-glucosyltransferases (Gil *et al.*, 2018). The objective of this study was to characterize NTSR in Imisun sunflower in response to imazethapyr using RNA-seq and to determine whether these mechanisms are constitutive or herbicide-induced.

Materials and methods

Two assays were carried out to determine: (i) optimal herbicide treatment concentration and (ii) duration of herbicide treatment (Gil *et al.*, 2018).

RNA-seq analysis was performed on Imisun HA 425 sunflower line. This resistant line is a BC2 F6 maintainer line resulting from the cross HA 89 * 3 and PUR *H. annuus* (Miller and Al-Khatib, 2002). Cypsels were germinated in plastic pots (70 cm^3) filled with perlite and watered by capillarity with 1.1 $g.L^{-1}$ of Murashige and Skoog's salts (Murashige and Skoog, 1962) during incubation at 25 ± 2 °C with a 16/8 h light/dark photoperiod (100 $\mu mol.m^{-2}.s^{-1}$). The herbicide imazethapyr (active ingredient: 5-ethyl-2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-il nicotinic acid) belonging to the imidazolinone familiy was used as AHAS inhibitor.

Seven-day-old plants were treated with 0 (control) and 1 µM imazethapyr for 12 h. RNA from 8-day-old leaves was extracted using *PureLink RNA Mini Kit* (Life Technologies) according to the manufacturer´s instruction. RNA`s integrity and concentration were estimated by Agilent Bioanalyzer RNA 6000 Nano (Agilent Technologies) and RiboGreen Fluostar Optima BMG (BMG Labtech), respectively. The experiment consisted of two biological replicates per treatment.

Stranded, pair-ended cDNA libraries were constructed with TruSeq Stranded mRNA protocol (Illumina). Briefly, 1 µg RNA was purified by RNA purification beads coupled with poly(dT) oligo-primers (AMPure XP Beads). After cleanup procedures in magnetic stand (Life Technologies), mRNA was fragmented and primed for cDNA synthesis with Fragment, Prime and Finish mix. First strand cDNA synthesis was performed with SuperScript II + First Strand Synthesis ActD mix in thermal cycler and followed by second strand synthesis (Second Strand Marking Master mix). At this step, RNA template was removed and a replacement strand was synthesized, incorporating dUTP in place of dTTP to generate double stranded blunt-ended cDNA. After adenylation of 3´ends (A-Tailing mix), multiple indexing adapters were ligated to the ends of the ds cDNA allowing subsequent hybridization onto the flow cell (Ligation and RNA Adapter Index mix). Finally, PCR was performed to selectively enrich DNA fragments having adapter molecules on both ends and to amplify DNA amount in the library. Quality control analyses of final libraries were carried out with Agilent Bioanalyzer DNA 1000 (Agilent Technologies) and accurate quantitation of DNA was performed by RiboGreen Fluostar Optima BMG (BMG Labtech) and real time qPCR.

cBOT^{TM} (Illumina) was used to made bridge amplification over the flow cell in order to clone each single-molecule DNA template simultaneously. All the samples were sequenced with Hiseq2000 sequencing system (Illumina) in one lane, using different bar-coded adapters. On average, 50 million paired-end reads (100 pb) per sample were generated after stranded, paired-end sequencing. SortMeRNA (v2.0) software was used to filter rRNA fragments from transcriptomic data and quality assessment was performed by FastQC (v0.10.1). The RNA-Seq data were deposited in the NCBI Sequence Read Archive (SRA) database under the accession SRP133609.

Two strategies of local mapping: including and filtering multihits, were carried out using Bowtie2 tool (v2.2.2) over the reference sunflower transcriptome HaT13l available at www.heliagene.org\HaT13l and generated by Badouin *et al.* (2017). This reference transcriptome includes 91,860 contigs with a N_{50} of 557 pb.

Reads quantification was performed by Sam2Count package (v2.2.2). After TMM normalization (Li *et al.*, 2015), differential expression analysis was performed by EdgeR package (v2.4.6) of R software (R Development Core Team, 2010) with a FDR cut-off of 0.05.

RNA-seq data was examined to detect herbicide-detoxifying contigs potentially involved in NTSR. This search was limited to contigs with change in expression $|\log_2 FC| \geq 0.9$.

RNA-seq data were validated by measuring the expression of 17 contigs related to herbicide detoxification using qRT-PCR. Two contigs were chosen from RNA-seq data and used as reference genes. These genes presented the highest expression stability among all samples and their expression levels were similar to those of the 17 validating contigs. The stability of the reference genes was determined by calculating two quality measures: the coefficient of variation ($\overline{CV}$) and the M-value (Hellemans *et al.*, 2007). Specific primers were designed with Primer3 tool (web v4.0.0, http://bioinfo.ut.ee/primer3-4.0.0/primer3/) (Rozen and Skaletsky, 2000). Primer specificity was evaluated *in silico* over the reference transcriptome using Blast tool in HeliaGene database (http://heliagene.org) and in agarose gels. PCR efficiency for contigs and reference genes was evaluated in four dilutions of pooled cDNA samples. Primers used in RT-qPCR analysis are shown in Supplementary Table 1. Quantification cycle (Cq) values for each amplicon were obtained by the slope of the regression line. Analysis were carried out using the software CFX ManagerTM Software Security Edition (BIORAD). Normalised expression value for each contig was calculated based on E and Cq in comparison to the reference genes. Data were tested for statistical significance using t-tests on R statistical software (R Development Core Team, 2010).

Tissue expression patterns were studied using the Genomics/Clusters tool (Badouin *et al.*, 2017) available at HeliaGene database (www.heliagene.org \HaT13l) for the 17 contigs verified by qRT-PCR. This interface allows data visualization of tissue-specific expression patterns of RNAs belonging to the reference transcriptome HaT13l.

Results and discussion

Optimal imazethapyr concentration was found to be 1 µM and the duration of herbicide treatment was 12 h (Gil *et al.*, 2018). Based on these data, RNA-seq analysis was performed on RNA extracted from leaf tissue of 8-day-old seedlings treated with 0 (control) or 1 µM imazethapyr for 12 h.

High quality stranded cDNA libraries were obtained, achieving an average fragment size of 260 pb. The GC content in the libraries was approximately 45 % and 97.42 % had a quality score higher than Q30 (0.1 % chance of error).

Sunflower genomic redundancy was analyzed using two mapping strategies over the reference transcriptome: filtering and including multihits. Percentage of paired-reads mapped to a contig for each strategy was 72 and 91 %, respectively, indicating compatibility with the reference. Differential expression analysis results obtained with EdgeR package are shown in Table 1. Differentially expressed contigs (FDR < 0.05) which were overexpressed in 0 µM imazethapyr (control) treatment corresponded to the functional groups involved in: aminoacid biosynthesis, DNA binding proteins, allergens involved in defense response, ions and oligopeptide transporters, among others. On the other hand, contigs overexpressed in 1 µM imazethapyr treatment were related to: ATPase activity, transcription factors, lipid transfer, thaumatins related to pathogenesis, among others.

Table 1: EdgeR differential expression analysis after two mapping strategies: including (yes) and filtering (no) multihits. Expression levels were adjusted by optimized FDR approach (Storey and Tibshirani, 2003).

EdgeR analysis		
Expressed contigs	**Mapping**	
	Multihits (Yes)	**Multihits (No)**
Total number[a]	37,849	32,251
Mean length (pb)	1310	1335
Total length (Mb)	49.59	43.05
Contigs ≥ 1000 pb	16,505 (43.6 %)	18,569 (57.6 %)
$\log_2FC > 0$[b]	19,249	16,427
$\log_2FC < 0$	18,600	15,824
ED[c]	52	46
EDI[d]	8	8
EDC[e]	44	38
SED[f]	37,797	32,205

[a]Total number of expressed contigs analysed after *EdgeR* filtration of no-expressed genes. [b]$\log_2FC = \log_2$ (IMI/C), IMI: contig expression level in 1 µM imazethapyr treatment, C: contig expression level in 0 µM imazethapyr (control) treatment.
[c]Differentially expressed contigs (FDR < 0.05).
[d]Differentially expressed contigs which were overexpressed in 1 µM imazethapyr treatment. [e]Differentially expressed contigs which were overexpressed in 0 µM imazethapyr (control) treatment.
[f]Contigs with no differential expression between treatments.

After RNA-seq data examination of contigs with $|\log_2 FC| \geq 0.9$, 61 and 47 contigs (previous mapping including and filtering multihits, respectively) related to xenobiotic metabolism were found. The corresponding gene families and subfamilies are shown in Table 2. None of these contigs showed differential expression between control and 1 µM imazethapyr-treated plants (FDR > 0.05).

Table 2: Protein function classification of xenobiotic metabolism-related contigs from RNA-seq data and their corresponding families and sub-families.

Protein function classification	Family†	Sub family†
Cytochrome P450 monooxygenase	71	A
		B
	72	A
	74	A
	75	B
	76	C
	77	B
	82	G
	83	B
	85	A
	93	D
	97	C
	704	A
	706	A
	707	A
ABC transporter	C8	na*
	G	na
	T1	na
	T2	na
Glycosyltransferase	8	na
UDPglucuronosil/UDP-glucosyltransferase	na	na
Glutathion S-transferase	na	na

†Data available from HAT13l sunflower transcriptome (www.heliagene.org \HaT13l).

*na: classification description not available at www.heliagene.org\HaT13l.

The primers chosen for RT-qPCR validation showed an amplification efficiency (E) of 100 % ± 10 % and adjusted values (R^2) of ~ 0.98. Reference genes showed an average $\overline{CV}$ value of 8.14 % and a M-value of 0.24, indicating high expression stability among samples. T-tests showed no differential expression of all

validating contigs between control and 1 µM treatment ($p > 0.05$). Tissue expression patterns showed maximum expression levels in leaves for 10 of the contigs verified by qRT-PCR. Three contigs showed maximum expression levels in the ligule, while the others were expressed in roots, seeds and bracts.

NTSR mechanisms involve constitutive or herbicide-induced up-regulation of metabolism-related gene expression. This up-regulation can result from an overproduction of enzymes (gene amplification or upregulation of expression) or from modified enzymes, leading to more efficient herbicide detoxification (R4P Network, 2016). In the present study, the herbicide-detoxifying genes analyzed showed no differential expression between control and 1 µM imazethapyr treated plants. Previous cDNA-AFLP characterization of Imisun technology has demonstrated that imazethapyr resistance is based on several metabolism routes that are present in both, resistant and susceptible lines. Moreover, no particular trend was found in relation to the duration of herbicide treatment (Gil *et al.*, 2018). The present study broadens available information about gene expression and imidazolinone resistance mechanisms in sunflower and supports the hypothesis that herbicide metabolism in Imisun sunflower is constitutive. In most cases, NTSR in plants appears to result from a constitutively increased metabolism such as reported for *Lolium sp*. in which the constitutive expression of cytochrome P450s was found to be higher in resistant than susceptible populations (Duhoux and Délye, 2013). NTSR mechanisms would be more efficient in avoiding permanent physiological damage when constitutive (Duhoux *et al.*, 2015).

RNA-seq analysis allowed detecting constitutively expressed detoxification genes potentially related to imidazolinone resistance and broadened general knowledge available on sunflower species transcriptome. This approach is suitable to characterize overall expression patterns of interesting genes and is remarkably useful to improve phenotypic selection techniques and to identify putative NTSR genes (Duhoux *et al.*, 2015).

Gene families potentially involved in herbicide response and NTSR in Imisun sunflower are an important source of variability for crop breeding. Since NTSR mechanisms generate cross resistance to herbicides with different modes of action, further research on genes detected in the present study will allow more effective weed management strategies. Furthermore, they become potential target genes to transform alternative crops and to be included in soil and waste-water bioremediation approaches.

Conclusion

The present study allowed identifying detoxification genes potentially related to imazethapyr resistance in sunflower: cytochrome P450 monooxygenases, ABC transporters, UDPglucuronosil/UDP-glucosyltransferases, glycosyltransferases and glutathion S-transferases. These herbicide-detoxifying genes showed no differential expression between control and imazethapyr treated plants supporting the hypothesis that herbicide metabolism in Imisun sunflower is constitutive.

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Resumen

Caracterización de mecanismos de resistencia no relacionados al sitio de acción en girasol mediante RNA-seq

Los girasoles Imisun son cultivares resistentes a imidazolinonas en los cuales coexisten dos mecanismos de resistencia: (i) una mutación puntual en el sitio blanco del herbicida (resistencia relacionada al sitio de acción) y (ii) resistencia no relacionada al sitio de acción (NTSR). En la tecnología Imisun, NTSR podría estar relacionada con el metabolismo del herbicida y podría resultar de una sobreexpresión constitutiva de los genes detoxificadores, o inducida luego del tratamiento con herbicida. El objetivo de este trabajo fue caracterizar NTSR en girasoles Imisun en respuesta al tratamiento de imazetapir mediante RNA-seq, y determinar si estos mecanismos son constitutivo o inducidos por el herbicida. Las cipselas fueron germinadas en multimacetas, regadas por capilaridad y se mantuvieron en una cámara bajo condiciones controladas. Plantas de 7 días fueron tratadas con imazetapir 0 (control) y 1 µM por 12 h. Luego de la purificación del RNA de hoja, se construyeron bibliotecas de cDNA stranded y paired-end. La secuenciación se llevó a cabo en Illumina HiSeq2000. Se realizaron dos tipos de estrategias de mapeo local contra el transcriptoma de referencia HaT13l, incluyendo y filtrando multihits, respectivamente y se realizó el análisis de la expresión diferencial. Se identificaron 61 y 47 contigs (de acuerdo

a la estrategia de mapeo) relacionados a metabolismo de xenobióticos: citocromos P450, transportadores ABC, glicosiltransferasas, UDPglucuronosil/glucosiltransferasas y glutatión S-transferasas. No se encontró expresión diferencial entre las plantas tratadas y sin tratar con imazetapir para ninguno de los contigs. La expresión de 17 contigs de interés fue validada mediante qRT-PCR. Estos resultados sugieren que mecanismos NTSR constitutivos estarían involucrados con la resistencia a imidazolinonas en girasol.

Résumé

Caractérisation de la réponse NTSR à l'herbicide imazethapyr chez le tournesol Imisun en utilisant la technologie RNA-seq

Les tournesols (génotype Imisun) sont des cultivars imidazolidone-résistants au sein desquels cohabitent 2 mécanismes distints de résistance à cet herbicide: (1) mutation de type « gène cible » et (2) une résistance non géniquement ciblé (NTSR: Non-Target-Site-Resistance). Dans le génotype Imisun, la résistance de type NTSR pourraient être soit reliée au métabolisme de l'herbicide en lui-même, conduisant ainsi à une régulation positive et constitutive des gènes de résistance, soit apparaitrait seulement après chaque traitement herbicide. L'objectif de ce projet était de caractériser la réponse NTSR à l'herbicide « imazethapyr » chez le tournesol Imisun en utilisant la technologie RNA-seq et déterminer ainsi si ce mécanisme était constitif ou induit par l'herbicide seulement. Pour ce faire, des graines ont été mis à germer dans des pôts en plastiques arrosés par capilarité et mis en culture dans des chambres climatiques en conditions environnementales controlées. Des plants de 7 jours ont été traités avec 0 µM d'imazethapyr (condition « controle ») et 1 µM d'imazethapyr (condition « traitement ») pendant 12 h. Après extraction des ARN totaux des feuilles, des banques cDNA orientées et pairées ont été construites. Le séquençage a été réalisé sur des machines Illumina HiSeq2000. Un mapping « local », avec ou sans prise en compte des hits multiples, a été réalisé contre le transcriptome de référence HaT13l. L'analyse différentielle de l'expression génique entre les 2 conditions (« contrôle » vs « traitement ») a été réalisée. Respectivement 61 et 47 contigs ont été trouvés relatif au métabolisme des xenobiotiques selon à méthode de mapping: cytochromes P450, ABC transporters, glycotransferases, UDPglucuronosyl/glucosyltransferases et des glutathione-S-transferases. Aucun de ces contigs n'a été trouvés comme différentiellement exprimés entre la condition controle et le traitement à l'imazethapyr. Dix-sept contigs d'intérets ont été vérifiés par qRT-PCR. Ces résultats suggèrent qu'une réponse constitutive des mécanismes NTSR pourrait expliquer la résistance à l'imidazolinone chez le tournesol (génotype Imisun).