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MECANISMOS ENVOLVIDOS NA REGULAÇÃO EPIGENÉTICA RELACIONADA À TOLERÂNCIA A HERBICIDAS EM PLANTAS

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À minha família.

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não existe para ele.
Por isso o universo de cada um,
se resume ao tamanho de seu saber.”

Albert Einstein

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MECANISMOS ENVOLVIDOS NA REGULAÇÃO EPIGENÉTICA RELACIONADA À TOLERÂNCIA A HERBICIDAS EM PLANTAS [1]

Autor: Catarine Markus
Orientador: Aldo Merotto Jr.

RESUMO

A regulação mediada por mecanismos epigenéticos tem sido sugerida recentemente como um dos fatores relacionados à variação de efeito e à resistência a herbicidas. Os objetivos deste estudo foram identificar como a exposição a herbicidas pode desencadear alterações epigenéticas em *Arabidopsis thaliana* e se essas alterações podem estar relacionadas com mecanismos de resistência a herbicidas. Os experimentos foram realizados com *A. thaliana* Columbia-0 (tipo silvestre-WT), 11 mutantes epigenéticos, e a linhagem L5 de *A. thaliana*. Os herbicidas utilizados foram glyphosate, imazethapyr e 2,4-D em doses sub-letais de 72, 10,6 e 40,3 g ha^{-1}, respectivamente. Nas plantas L5, a expressão relativa analisada por qRT-PCR mostrou que β- glucuronidase (GUS) foi de 7 a 12 vezes mais expresso nas plantas tratadas com esses herbicidas. Isso indica que os herbicidas ocasionaram modificações globais na metilação do DNA, que afetam no silenciamento gênico transcricional (SGT). A suscetibilidade aos herbicidas foi afetada em seis dos 11 mutantes epigenéticos testados. O mutante *ros1* teve aumento de 20 a 30% na suscetibilidade para glyphosate, imazethapyr e 2,4-D. ROS1 (REPRESSOR OF SILENCING 1) é uma 5-metil-citosina glicosilase, que atua como repressor de SGT. O efeito do imazethapyr sobre a metilação global do DNA (5mdC) foi analisado por cromatografia líquida de alto desempenho (HPLC). Plantas WT tratadas com imazethapyr apresentaram níveis inferiores de 5mdC (5,65%) em comparação ao *ros1* tratado e não tratado. A expressão diferencial de genes avaliada por sequenciamento de RNA (RNA-Seq) revelou que 2464 genes foram induzidos no WT e 3323 no mutante *ros1*. Imazethapyr induziu a expressão de genes relacionados a processos epigenéticos. Ainda, foram identificados 31 genes candidatos envolvidos com a tolerância a imazethapyr, sendo que cinco genes (*TT7, HMTDSP*, *SCAMP*, *MFSP* e *XTH10*) mostraram a região promotora metilada na análise *in silico* e revelaram variação nos níveis de metilação nos sítios CG, CHG e CHH em decorrência da aplicação de imazethapyr. O mutante *tt4* mostrou que o acúmulo de flavonóides pode ser importante para a tolerância ao imazethapyr em *A. thaliana* e que genes dessa via biossintética são regulados epigeneticamente por ROS1. Os resultados deste estudo sugerem que ROS1 atua na demetilação do DNA induzido pelos herbicidas. Os herbicidas avaliados podem alterar vias epigenéticas específicas e alguns genes putativos envolvidos na resistência a herbicidas estão sob regulação epigenética. Esses resultados podem contribuir para a compreensão do efeito do herbicida na regulação epigenética associado à evolução da resistência aos herbicidas.

[1] Tese de Doutorado em Fitotecnia, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil. (160f.) Março, 2017.

MECHANISMS INVOLVED IN THE EPIGENETIC REGULATION RELATED TO THE TOLERANCE OF HERBICIDES IN PLANTS[2]

Author: Catarine Markus
Advisor: Aldo Merotto Jr.

ABSTRACT

The regulation mediated by epigenetic mechanisms has been recently suggested to be related to variation of herbicide effect and herbicide resistance in weeds. The objectives of this study were to identify how exposure to herbicides can trigger epigenetic changes *in Arabidopsis thaliana* and whether these changes may be related to the known mechanisms of herbicide resistance. The experiments were performed with *A. thaliana* Columbia-0 (wild-type WT), 11 epigenetic mutants, and the *A. thaliana* line L5. The herbicides used were glyphosate, imazethapyr and 2,4-D at sublethal doses of 72, 10.6 and 40.3 g ha^{-1}, respectively. In L5 plants, the relative expression analyzed by qRT-PCR showed that β-glucuronidase (GUS) was 7 to 12 times more expressed in plants treated with these herbicides. These results indicate the occurrence of global modifications in DNA methylation affecting transcriptional gene silencing (TGS). Susceptibility to herbicides was affected in six out of 11 epigenetic mutants tested. The *ros1* mutant showed 20 to 30% increase in susceptibility for the three herbicides. ROS1 (REPRESSOR OF SILENCING 1) is a 5-methylcytosine glycosylases, which act as a repressor of TGS. The effect of imazethapyr on global DNA methylation (5mdC) was evaluated by high-pressure liquid chromatography (HPLC). WT plants treated with imazethapyr presented lower levels of 5mdC (5.65%) in comparison to treated and not-treated plants of *ros1*. Differential expression of genes assessed by RNA sequencing (RNA-Seq) revealed 2464 genes induced in WT and 3323 in *ros1* mutant. Imazethapyr induced the expression of genes related to epigenetic processes. Additionally, 31 candidate genes putatively involved in imazethapyr tolerance were identified. An *in silico* analysis indicated that five of them (*TT7*, *HMTDSP*, *SCAMP*, *MFSP* and *XTH10*) have a methylated promoter region and presented varying levels of methylation at GC, CHG and CHH sites. The *tt4* mutant showed that the accumulation of flavonoids may be important for tolerance to imazethapyr in *A. thaliana* and that genes of this biosynthetic pathway are epigenetically regulated by ROS1. The results of this study suggest that ROS1 presents importance to the demethylation process induced by the herbicides, the evaluated herbicides can change specific epigenetic pathways and some putative genes involved in herbicide resistance are under epigenetic regulation. These results can contribute for understanding the herbicide effect associated with the evolution of herbicide resistance.

[2] Doctoral thesis in Plant Science, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. (160p.) March, 2016.

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1 INTRODUCTION

Weeds are considered the most limiting biotic factor for agricultural production and are responsible for 34%, on average, of crop yield losses worldwide (Oerke, 2006). Weed control has become increasingly problematic in recent decades due to the evolution of herbicide-resistant weed biotypes. Herbicides are the most used tool for weed control due to high efficiency, low price, and practical use in comparison with other methods. The intense use of this technology favors the evolution of resistance since the herbicides are strong selection agents (Yuan *et al*., 2007). Herbicide resistance can be classified into two primary mechanisms; target-site resistance (TSR) and non-target-site resistance (NTSR) (Yuan *et al*., 2007; Powles & Yu, 2010; Délye, 2013).

TSR is caused mainly by mutation that change an amino acid causing alteration in the enzyme conformation, preventing the binding of the herbicide in the site of action (Sammons & Gaines, 2014; Varanasi *et al*., 2016). Herbicide resistance caused by mutation on target enzyme is the most frequent process and has occurred to almost all herbicide mechanisms of action. Additionally, TSR can also be caused by overexpression of a target enzyme through gene amplification or changes in a gene promoter (Gaines *et al*., 2010; Powles & Yu, 2010) or codon deletion (Patzoldt *et al*., 2006).

NTSR is a more complex process that we know much less about in comparison with TSR. The mechanisms associated with NTSR include decreased herbicide absorption and translocation, enhanced detoxification, or rapid oxidative stress response (Yuan *et al*., 2007; Yang *et al*., 2017). NTSR caused by enhanced detoxification is considered of particular importance because it is often associated with resistance to herbicides through of multiple mechanisms of action, including compounds never previously exposed to the herbicide resistant weed (Yu & Powles, 2014). The enhanced herbicide detoxification is caused by the common xenobiotic detoxification process mediated primarily by cytochrome P450 monooxygenases (CytP450) (Iwakami *et al*., 2014a; Iwakami *et al*., 2014b), glutathione S-transferase (GST) (Cummins *et al*., 2013) and ATP-dependent (ATP-binding cassette, ABC) transporters (Lane *et al*., 2016). However, gene expression and regulation associated with NTSR is still poorly understood. Recently it was suggested that in addition to DNA mutations or *indels* in TSR or NTSR related genes, herbicide resistance can also be related to epigenetic processes (Gressel, 2009; Powles & Yu, 2010; Délye, 2013). However, the elucidation of the relationship of herbicide resistance and epigenetic control is in its infancy.

In humans, several studies have indicated that the evolution of drug resistance is associated with epigenetic regulation (Ingelman-Sundberg & Cascorbi, 2016). Together with genetic variation, epigenetics is one of the factors that contribute in the inter-individual variability in drug response (Neul *et al*., 2016). In addition, the variability of expression and function of ABC transporters that mediate drug efflux in cancer cells has been recently explained by epigenetic processes (Kozyra *et al*., 2017). Most of the detoxification and trans-membrane movement of herbicides in plants and drugs in animals are similar (Lane *et al*., 2016; Neul *et al*., 2016), indicating similar processes could occur in both systems.

Epigenetic modulation consists of chemical modification of DNA or histone proteins that result in alleles with the same DNA sequence but different patterns of biochemical modifications (called 'epialleles') (Cortijo *et al*., 2014). Epigenetics have been shown to have an important role in the evolutionarily development of plant response to stress (Hauser *et al*., 2011; Chinnusamy & Zhu, 2009). This regulation occurs with modification in the 'open-ness' of chromatin that represses or activates gene transcription, transposition of transposable elements (TEs), nucleosome occupancy and recombination (Tricker, 2015). Some of the modifications only occur during stress exposure and normally revert soon after its occurrence (Pecinka & Scheid, 2012), classified as a transient process. In some cases, this response is maintained over generations, being inherited by the progeny of the exposed individuals it results in a transgenerational character, thus epigenetic changes are considered as inheritable (Tricker, 2015). However, the transgenerational inheritance of stress-induced epigenetic modifications remains poorly understood (Pecinka & Scheid, 2012).

The discovery of epigenetic-based stress tolerance and the possibility of transgenerational inheritance brought exciting possibilities to explain the heritability of environmentally induced traits (Boyko & Kovalchuk, 2011). In humans, some studies indicate that the pattern of food intake or the emotions we feel may affect gene regulation through epigenetics, generating hypotheses whether this pattern of regulation can be passed on to our descendants (Heard & Martienssen, 2014). Similar questions are being raised for pesticide resistance, particularly whether sub-lethal herbicide doses lead to epigenetic alterations that are 'remembered' in subsequent generations, leading to enhanced survival and eventual resistance (Gressel, 2015). In addition, this phenomenon could explain the variability of unknown responses of herbicide resistance in some weed species.

The plasticity under stress conditions is provide by the dynamics of epigenetic changes that plays an important role in immediate and long-term response (Mirouze & Paszkowski, 2011). DNA methylation of the gene promoter for instance correlates with transcriptional gene silencing (TGS), which is an efficient gene regulation process (Morel *et al*., 2000; Law & Jacobsen, 2010). In *Arabidopsis thaliana*, ROS1 (REPRESSOR OF SILENCING) is one of the enzymes responsible for the DNA demethylation (Zhu *et al*., 2007). During abiotic stress, ROS1 contributes to epigenetic plasticity and avoids hypermethylation in thousands of specific genomic loci (Qian *et al*., 2012). Several pathways and enzymes are involved to regulate chromatin structure and gene expression that are able to control a set of specific genes.

Herbicides cause intense alteration in the basal defense pathways of plants, similar to abiotic stresses (Radwan, 2012). Based on that, the main hypothesis of this study is that herbicides may cause alterations in epigenetic modulations, that can regulate genes important to herbicide resistance. However, epigenetic studies in weeds are still initial and there is information only for two herbicides, glyphosate (Nardermir *et al*., 2015) and atrazine (Lu *et al*., 2016), which induce alteration of DNA methylation. Due to the lack of inforFmation on the genome even of the most important weeds, the preset study used the model plant *Arabidopsis thaliana*. Subsequently, these understandings can be used for weeds with agricultural importance.

Main objectives:

The present study aims to analyze the possible involvement of the epigenetic mechanisms in the regulation of herbicide tolerance in *Arabidopsis thaliana* and to understand how epigenetics can contribute to the regulation of genes involved in herbicide resistance.

Specific objectives:

- To analyze if herbicides can cause alteration in the gene expression through transcriptionally gene silencing (TGS) in *A. thaliana*.

- To evaluate the importance of specific epigenetic pathways for herbicide detoxification.

- To analyze the effect of the herbicide imazethapyr on the global pattern of DNA methylation in *A. thaliana* and in the epigenetic mutant *ros1*.

- To verify the effect of the herbicide imazethapyr on the plant transcriptome of *A. thaliana* and to contrast its effect on the epigenetic mutant *ros1*.

- To identify candidate genes involved with herbicide detoxification that are under epigenetic regulation.

2 LITERATURE REVIEW

This literature review is part of the review submitted to the Journal *Pest Management Science*, entitled “Epigenetic regulation: Contribution to herbicide resistance in weeds?”.

2.1 What is epigenetics?

Epigenetics refers to alterations of chromatin states that change gene expression patterns without modification in DNA sequence (Cortijo *et al*., 2014). These mechanisms involve a wide range of biochemical processes, such as DNA methylation, histones variants, histone post-translational modifications and small or long non-coding RNAs (Chinnusamy & Zhu, 2009). Among the eukaryotic organisms, plants have the most complex epigenetic regulation (Pikaard & Scheid, 2014). In plants, most information about epigenetics is presented in the model species *Arabidopsis thaliana* (Zemach *et al*., 2013), although the epigenetic regulatory mechanisms of the crop plants rice (*Oryza sativa*) (Li *et al*., 2011), maize (*Zea mays*) (Forestan *et al*., 2016), soybean (*Glycine max*) (Chen *et al*., 2010) and sugar beet (*Beta vulgaris*) (Hébrard *et al*., 2016) have also been studied (Pikaard & Scheid, 2014). However, our knowledge of epigenetic processes in weeds is poorly understood, with some studies in ryegrass (*Lolium perenne*) (De Block & Van Lijsebettens, 2011), wild radish (*Raphanus raphanistrum*) (Agrawal *et al*., 2002) and

common dandelion (*Taraxacum officinale*) (Verhoeven & Van Gurp, 2012). Moreover, recent studies have indicated that some allelopathic compounds inhibit histone deacetylases (HDACs) (Hofmann, 2015). Epigenetic mechanisms have been associated with numerous cellular processes such as developmental programming, gene expression, embryonic development, transposon inactivation, genome stability and plant stress response (Chinnusamy & Zhu, 2009). Much remains unknown about the initiation and consequences of the various epigenetic mechanisms and their effects on plant biology and ecology.

2.1.1 DNA methylation in plant genomes

DNA methylation involves the addition of a methyl group to the fifth carbon of cytosine (5mC) and is highly correlated with chromatin remodeling and repression of gene transcription (Law & Jacobsen, 2010). In plants, DNA methylation is presented in three sequence contexts, CG, CHG, and CHH (where H = A, C, or T). The level of methylation can vary with each sequence context and species. In *Arabidopsis,* the percent of all 5mC cytosines are 24% for CG, 7% for CHG and 2% for CHH (Cokus *et al*., 2008). In comparison, the percent of 5mC in rice are 59% for CG, 21% for CHG, and 2.2% for CHH (Feng *et al*., 2010). Whereas for unfertilized ears of maize the level of 5mC are 86% for CG, 74% for CHG and 5% for CHH (Gent *et al*., 2013). This information is not available for any weed species. Methylation levels are governed by processes that reflect the balance between enzymes involved in activities of establishment, maintenance, or removal of methylation (Pikaard & Scheid, 2014).

DNA methylation is established *de novo* mainly by the RNA-directed DNA methylation (RdDM) pathway guided by small interfering RNAs (siRNAs), which is unique to plants (Law & Jacobsen, 2010). Studies have demonstrated that RdDM is

responsible for methylation in all sequence contexts, but CHH methylation is a specific hallmark of RdDM (Pikaard & Scheid, 2014). After the first occurrence, DNA methylation can be perpetuated by a process called DNA methylation maintenance, through mitoses and sometimes also meiosis (Quadrana & Colot, 2016). Three different maintenance pathways exist in *Arabidopsis*, depending on the RdDM, DNA methyltransferase 1 (MET1) and chromomethylases 2 and 3 (CMT2–CMT3). MET1 appears to maintain CG methylation over both genes and repeat sequences, while CMT2 methylates both CHG and CHH contexts *de novo* and CMT3 maintains CHG methylation (Zemach *et al*., 2013).

The localization of methylation in the genome is important to understand the dynamic of gene expression patterns. The three sequence contexts occur in transposable elements (TEs) and in the CG context of active genes (Law & Jacobsen, 2010). In plants, CG methylation is generally found within gene bodies, and in *Arabidopsis* around 33% of genes have CG methylation in the coding region (Cokus *et al*., 2008). TE methylation results in transcriptional silencing, while non-TE methylation is not correlated with repression of transcription (Law & Jacobsen, 2010). DNA methylation in promoters and heterochromatin is also associated with gene repression.

2.1.2 Histone modifications and variants in plants

The nucleosome is an octamer of two copies of histones H2A, H2B, H3, and H4, wrapped by 147 bp of DNA, where 14 contact points between histones and DNA are found (Luger *et al*., 1997). Histone modification can occur in the N-terminal region called the histone tail. This region is enriched with the amino acid residues lysine and arginine, which can result in various post-translational modifications such as methylation, acetylation, phosphorylation, and ubiquitination (Zhang *et al*., 2007;

Arya & Schlick, 2009). The main histone modifiers were identified in plants and are named histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), and histone demethylases (HDMs) (Kim *et al*., 2015). Histone modifications are able to change the activity of genes that are located around the nucleosome increasing transcription or repressing gene expression. Generally, the acetylation of lysine residues of histone 3 and histone 4 (H3 and H4) neutralizes the positive charge of the histone tails, resulting in gene activation, because of the decreased affinity for DNA due to its negative charge (Luger *et al*., 1997; Arya & Schlick, 2009). In addition, tri-methylation of the fourth lysine of H3 (H3K4me3) activates transcription, while tri-methylation in the twenty-seventh lysine of H3 (H3K27me3) represses transcription (Zhang *et al*., 2007). The magnitude of gene expression depends on the residue where the modification occurs, the type of modification and its spatial distribution across the gene region (Kim *et al*., 2015).

Histone variants can also change the chromatin structure and result in different transcriptional level by changing the properties of nucleosomes. This process results from the replacement of the canonical histones with non-allelic histone variants. The differences between both can be related to the key amino acid residues in the histone tails or in the histone domains (Li *et al*., 2007). Canonical and variant histones mainly differ because histone variants are expressed outside of S phase and are assimilated into chromatin in a DNA replication-independent mode. The exchange with the canonical histones can occur during development and differentiation, described as replacement histones. The histone variants H2A.Z and CENH3 act in precise regulation of gene activity and genome structure (Coleman-Derr & Zilberman, 2012). In *Arabidopsis,* nucleosomes that present the alternative histone H2A.Z are important to temperature sensitivity (Kumar & Wigge, 2010).

Although it is accepted that modifications and histone variants are important

response under environmental stresses, it is still unknown which is the first step, if the transcription patterns or chromatin changes, and why some modifications present rapid response, while other present gradual and prolonged responses (Kim *et al*., 2015).

2.2 How do epigenetic mechanisms regulate gene expression and mitigate stresses in plants?

Epigenetic pathways contribute to survival in unfavorable environmental conditions by triggering defense responses through a network of specific genes (Hauser *et al*., 2011). Recently, experimental evidence shows that plant metabolism and cell redox status play an important role in epigenetic control (Shen *et al*., 2016). This study indicates that reactive oxygen species (ROS) produced in plant cells during basal processes (respiration, photosynthesis) and stress conditions, affect not only metabolic flux, but also control of chromatin modifications and epigenetic reprogramming of gene expression (Shen *et al*., 2016). Plants under stress often experience an energy imbalance that contributes to increased epigenetic changes. This may result in changes in metabolite control of stress-induced chromatin, and usually involves the consumption of redox-active molecules such as adenosine triphosphate (ATP), methyl donor sadenosylmethionine (SAM), nicotinamide adenine dinucleotide (NAD) and the acetyl donor acetyl coenzyme A (acetyl-CoA) (Vriet & Laloi, 2015).

SAM is synthesized from the amino acid methionine, is the universal methyl donor in methylation reactions, and acts as substrate for histone and DNA methyltransferases (HMT and DMT, respectively) (Shen *et al*., 2016; Vriet & Laloi, 2015). The importance of SAM was demonstrated in a study where genes encoding SAM synthetases were knocked out that resulted in suppressing DNA and

H3K4me3 transmethylations, which resulted in late flowering in rice (Li *et al*., 2011). In this species, the utilization of 5-azacytidine, a DNA methylation inhibitor, caused expression of oxidative stress-related genes, favoring tolerance to salinity (Zhong *et al*., 2010). In *Nicotiana tabacum*, oxidative stress induced demethylation and transcriptional activation of *NtGPDL* (glycerophosphodiesteraselike protein) (Choi & Sano, 2007).

Numerous TEs are also involved in environmental stress adaptation in plants. The modification of methylation in TEs targeted by siRNA-mediated DNA methylation is correlated with expression of nearby genes (Wang *et al*., 2013). Several genes involved with plant response to stresses are present in the flanking regions enriched in TEs. Gene expression is negatively correlated with the number of siRNA-targeted that are located next to methylated TEs (Hollister & Gaut, 2009). Stresses can destabilize transcriptional gene silencing (TGS), enabling the activation of specific genes under adverse conditions. Heat stress in *Arabidopsis* seedlings occasioned transcriptional activation of *ONSEN* (*copia*-type retrotransposon) that conferred heat responsiveness through nearby genes by the siRNA pathway (Ito *et al*., 2011). Although the mechanisms and enzymes that silence transposons can occur through different forms (RNA interference, histone modification, and DNA methylation), there is considerable interaction between these epigenetic processes (Vriet & Laloi, 2015).

In plants, histone modification has been reported in the response to a wide range of abiotic stresses, including salt (Arya & Schlick *et al*., 2009), heat (Weng *et al*., 2014) and cold (Kwon *et al*., 2009). The enzymes that perform histone acetylation and deacetylation, HATs and HDACs respectively, present antagonistic activities and are influenced by the pool of acetyl-CoA, because HATs use acetyl-CoA as substrates to acetylate histone lysine residues (Shen *et al*., 2016).

Additionally, HATs interact with transcription factors and are involved in activating stress-response genes (Chinnusamy & Zhu, 2009). HDACs have their activity limited under conditions of oxidative stress, indicating that H_2O_2 administration induced tyrosine nitration of HDAC2, which correlates with a significant increase in acetylated H4 at the IL-8 promoter (Ito *et al*. 2004). In addition, HDACs, such as HDA6 and HDA19 are able to perform histone deacetylation in response of biotic and abiotic stresses in *Arabidopsis* (Zhou *et al*., 2005). In rice, the expression of different HDACs is also differentially regulated by osmotic stress by the hormones abscisic, gibberellic, and salicylic acid (Zhou *et al*., 2005; Kou et al., 2011). These effects suggest that a 'metabolic decision' may epigenetically regulate responses to stresses.

2.3 Transgenerational epigenetic inheritance

The heritability and transgenerational 'memory of stress' through epigenetic alterations induced by stresses remains ambiguous (Asensi-Fabado *et al*., 2017). Global erasure of epigenetic changes occurs naturally in germline cells, referred to as epigenetic reprogramming. In this situation, it is necessary to reset the epigenetic signatures of imprinted genes (Heard & Martienssen, 2014). With the purpose to transmit epigenetic states from one generation to the next, it is necessary to bypass this very effective reprogramming process (Calarco *et al*., 2012). Thus, certain genomic regions can escape erasure strategies that allows for the persistence of epigenetic states to be passed to progeny resulting in the transgenerational epigenetic inheritance (Lange & Schneider, 2010). This knowledge is important to formulate questions about the permanence and heritability of epigenetic information.

DNA methylation has been the major focus to understand how these processes occur over generations. Plants appear to show less DNA methylation

reprogramming during their life cycle compared to mammals (Quadrana & Colot, 2016). In mammals, epigenetic reprogramming occurs in at least two rounds in sexual reproduction, during gametogenesis and early embryonic development. Studies with mice showed that their genome suffers several rounds of DNA methylation and demethylation, starting after fertilization and being more important during the reprogramming process of the germline, where the paternal and maternal somatic programs are erased together with imprints (Heard & Martienssen, 2014). This explains the fact that mammals had rare epigenetic inheritance and normally related with nonessential genes (Asensi-Fabado *et al*., 2017).

In plants, for some period it was considered that the patterns of DNA methylation are stably maintained through sexual reproduction. Currently it has been revealed that DNA methylation activity fluctuates during sexual reproduction (Jullien *et al*., 2012). In *Arabidopsis,* a study that analyzed three haploid cell types (sperm cell, the vegetative cell, and the postmeiotic microspore) found that in pollen the symmetric CG and CHG methylations are largely retained in the germline, while the asymmetric CHH methylation of transposons is reduced in the microspore and sperm cells. CHH methylation is restored in the embryo after fertilization by *de novo* DNA methyltransferase and is suggested to reflect an ancient mechanism for transposon recognition (Calarco *et al*., 2012).

Transgenerational epigenetic inheritance has been reported in *A. thaliana*, demonstrating that plants exposed to abiotic stress induced epigenetic states that are inherited by non-stressed progeny. Epigenetic responses to abiotic stresses such as heat, UV-B, heavy metal contamination and oxidative stresses have been reported to be passed transgenerationally (Rahavi *et al*., 2011; Müller-Xing *et al*., 2014). Two main hypotheses are discussed regarding transgenerationally inherited changes (Vriet & Laloi, 2015). The first is related with the possibility of stress

tolerance through direct result from specific stress-defense genes present in progeny of stressed plants. The second indicated that the increased genetic or epigenetic variability in the progeny of stressed plants could be just a consequence of undirected effects, being a stochastic event in the DNA methylation induced by stress (Pecinka & Scheid, 2012). In the case of herbicide resistance, transgenerationally inherited changes may be important to explain cases of resistance that clearly are not inheritable in a Mendelian manner.

2.4 How epigenetic process can be involved with herbicide resistance?

Herbicide resistance is increasing rapidly worldwide, and it is an important example of rapid evolution in plants. Currently, there are 480 unique cases (species X site of action) of herbicide resistance reported globally distributed in 251 species and in almost all herbicides mechanism of action (Heap, 2017). The high natural variation present in weed populations and the high number of individuals exposed greatly contributes to the selection of herbicide resistant biotypes. High herbicide doses are suggested to select individuals with high resistance levels, selecting for rare resistance alleles (Délye, 2013) mainly associated with mutations on the gene that codes for the herbicide target enzyme. However, lower sub-lethal herbicide doses can also select for resistance, and it is quite important for the recurrent enrichment of several minor additive genes (Neve & Powles, 2005a), mainly related with non-target-site resistance (NTSR). Sub-lethal doses are commonly experienced by weeds in crop fields due to drift, incomplete coverage, over-topping crop canopies, or intentionally applied low rates. Over time, low doses can increase the frequency of plants that have accumulated different alleles causing a reduction in herbicide sensitivity at the population level (Délye, 2013). This process may occur

faster in cross-pollinated species that can rapidly accumulate resistance genes (Powles & Yu, 2010).

Plants are especially proficient at transgenerational epigenetics because their germlines arise from somatic cells that have developed under specific environmental conditions during which epigenetic changes may have occurred in response to stress. Floral meristems arising from such tissues would contain epigenetic changes that could be transmitted to future generations, thereby transmitting ‘epigenetic memories’ of stressful environments. In other words, progeny whose mothers passed ‘epigenetic memory', and thus tolerance, to a specific stress will outperform individuals without that prior generational experience. Several researchers have speculated that epigenetic gene regulation may play a role in herbicide resistance (Gressel, 2009; Délye *et al*. 2013), but no specific theories or data have been produced.

Repeated cycles of selection by sub-lethal herbicide doses has been shown to lead to eventual development of herbicide resistant biotypes. Sub-lethal rates of ACCase herbicides over generations of *Lolium rigidum* increased the mean population survival after three cycles of selection (Neve & Powles, 2005b). In *L. multiflorum,* the application of a series of ACCase herbicides increased their level of resistance, though it was not transmitted to their offspring, denoting the nature of acclimation response (Vila-Aiub & Ghersa, 2005).

The evolution of herbicide resistance is still not fully understood, especially whether the stress caused by the herbicide could trigger epigenetic changes, resulting in alterations of the expression patterns of specific genes without changes in the DNA sequence that can be transgenerational or transient. This knowledge could modify the way that resistance is defined based on an exclusively ‘inherited’ trait (Gressel, 2015), because the epigenetic regulation could contribute to plants

survive to an herbicide stress application regulating gene regulation only with a transient manner. Epigenetic mechanisms could help explain rapid adaptation of weeds to herbicide selection pressure through alterations in gene expression or changes in activity of transposons that can also affect gene expression or even lead to gene duplication.

In fact, a recent study with atrazine in rice indicated that most of DNA methyltransferases, histone methyltransferases and DNA demethylase were differentially regulated in response to the herbicide (Lu *et al*., 2016). In this study, epigenetic alterations were suggested to be involved with activation of specific genes responsible for atrazine detoxification. In a study carried out with glyphosate in *Triticum aestivum*, different herbicide concentrations changed the levels of DNA methylation from 28.3 to 73.9%, primarily caused by DNA hypermethylation (Nardemir *et al*., 2015). Kim *et al*. (unpublished data) recently showed that sub-lethal doses of glyphosate induce dose-dependent differentially methylated regions across the *A. thaliana* genome. Interestingly, >90% of the affected genes are not shared by responses to other abiotic stresses, suggesting that epigenetic changes may be stress-specific.

These epigenetic mechanisms could be related to the ‘flipped on’ or ‘flip off’ control of genes such as CytP450, GSTs and ABC transporters or transcription factors important for herbicide detoxification. In addition, herbicides can cause oxidative stress similar to some abiotic stresses in plants (Radwan, 2012). Thus, most of the enzymes involved in metabolizing herbicides are enzymes involved in the basal stress response pathways in plants, as detoxification of ROS resulting from stress conditions (Tausz, 2001). This contributes to the hypothesis that herbicide resistance may result from epigenetic mechanisms involved in strategies

to survive under stress caused by the herbicide, similar to that observed in other abiotic stresses.

The baseline genetic mutation rate of 10^{-7}–10^{-8} per nucleotide may not provide sufficient phenotypic variation for establishment of new traits at the short timescales (Boyko & Kovalchuk, 2011) of herbicide resistance evolutions. Therefore, epigenetic processes can act alone or interact with DNA changes resulting in the evolution of herbicide resistance. Mistakes in the maintenance of methylation states cause increasing in single methylation polymorphisms over evolutionary timescales, similar as observed for spontaneous DNA mutation (Boyko and Kovalchuk, 2011; Schmitz *et al*., 2011). The analysis of spontaneous variation in DNA methylation in *A. thaliana* plants originated from single-seed descendent for 30 generations identified 114,287 CG single methylation polymorphisms and 2485 CG differentially methylated regions (DMRs) that differed from the ancestral state, contributing to phenotypic diversity (Schmitz *et al*., 2011). Nevertheless, DNA methylation has lower stability compared to mutations in the primary DNA sequence and can be lost at a high frequency in segregating of F2 (Vaughn *et al*., 2007).

A model related to the role of epigenetic changes on the evolution of herbicide resistance in weeds is suggested (Figure 1). This model was based on the system proposed to explain the epigenetic regulation caused by abiotic stresses (Chinnusamy & Zhu, 2009) and the overall model of NTSR that indicates how plant cells receive herbicide stress signals via sensors (Délye, 2013). After the application of sub-lethal herbicide doses, it is suggested that signaling pathways that trigger general and specific responses are activated. Primary and secondary signals involved with metabolite alteration could induce changes in specific pathways. This response may be involved with the evolution of plant stress response since the herbicide response may be comparable to other abiotic stresses (Ramel *et al*.,

2012). The signal is transduced to regulators and triggers regulation cascade(s). Processes of transcriptional and post-transcriptional control can act in expression regulation of important genes to herbicide resistance (P450, GST, ABC transporters). The herbicide signal (regulation cascade) could also trigger metabolite alteration that induces changes in expression and/or activity of RdDM pathways, histone variants and histone modification enzymes that induce epigenetic changes. The alteration in epigenetic enzymes and pathways can induce changes in genes involved indirectly or directly with herbicide resistance, which can be heritable. Non-heritable modifications are reverted when the plant overcomes the stress, showing transient changes that are involved with acclimation. While other alterations can be heritable mitotically and/or meiotically providing a ‘stress memory’, where if the mitotic heritability is present the ‘stress memory’ will occur within generation, and if both mitotically and meiotically heritability exist it will result in a transgenerational inheritance (Figure 1).

Thus, herbicide resistance development, particularly through NTSR, may involve epigenetic mechanisms. Some of these responses may be general stress responses, but others may be herbicide specific. Much remains to be studied regarding epigenetics and NTSR, especially considering the large number of herbicide mechanism of action. Indeed, there is likely to be species-herbicide specific changes that lead to resistance development. Additionally, the intra-generational stability and transgenerational potential of these epigenetic changes must be studied to identify their potential to confer herbicide resistance. Rapid local adaptation to herbicide stress may be partly mediated by epigenetic control over gene expression, which can act much faster than traditional Darwinian evolutionary processes.

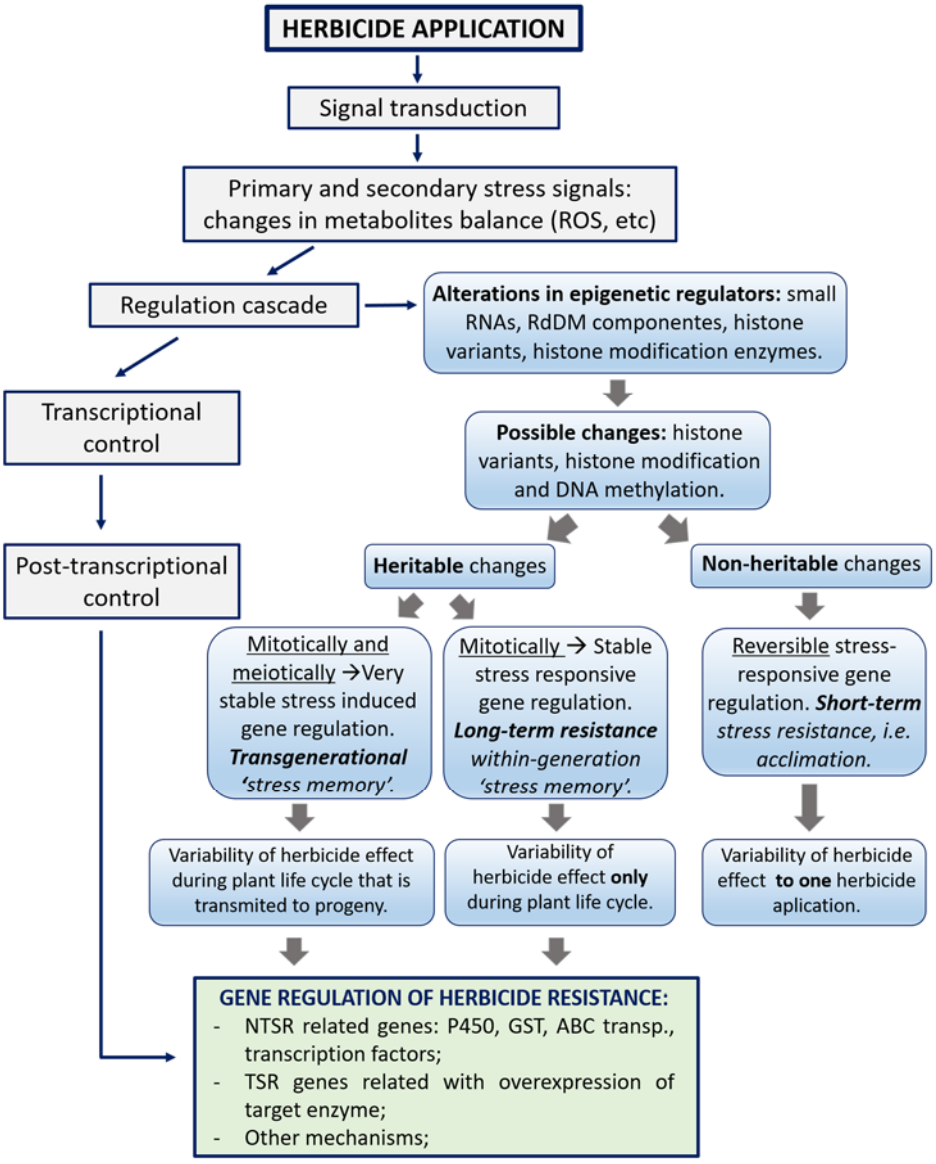


FIGURE 1. Model of epigenetic regulation triggered by herbicide stress that can induce heritable epigenetic modifications or transient changes associated with herbicide resistance. Gray arrows propose the involvement of epigenetics with the herbicide resistance regulation. Adapted of Chinnusamy & Zhu (2009) and Délye (2013). UFRGS, Porto Alegre, RS, Brazil. 2017.

2.5 How can epigenetic processes be studied?

Molecular tools have been invaluable for the understanding of the genetic regulation of herbicide resistance mechanisms. However, there remains much to be learned about NTSR, because of the restricted genomic information available for weedy species (Yuan *et al*., 2007; Délye *et al*., 2015). Some of these difficulties are related to the high genome size of some weed species and ploidy level, while most

model plant species normally have small genomes. The model plant, *A. thaliana,* has a genome size of ~135 Mbp, while the genome sizes of *Lolium* ssp. is ~4,067 Mbp (Evans *et al*., 1972). Some *Amaranthus* spp. have moderate genome sizes, such as *A. palmeri* of ~900 Mbp and A. *tuberculatus* of ~1,400 Mbp (Rayburn *et al*., 2005).

Many of the major weeds are polyploids (Gressel, 2009), which increases the complexity of the genome and hampers genomics analyses because of the high number of repeats and gene redundancy. Genomic research of weeds lags far behind that of crops and model species, but recent advances have been made regarding the effect of herbicides in weeds mainly via transcriptomic analyses and *de novo* genome assembly (Gaines *et al*., 2014; Velmurugan *et al*., 2016). Epigenetic analyses and other molecular studies are further hampered in weeds due to limited reference genomes for most weeds. However, with the recent advent of 'omics' tools these studies can be performed, with some restriction, even in non-model species such as weeds (Délye, 2013).

For the identification of epigenetic process in weeds first we need to decide if the study will focus on the detection of modifications - DNA methylation, histone modifications, histone variation - some of which focus on single gene (locus-specific) and some on the genome-wide scale (Chen *et al*., 2010). Each technique provides various levels of information and inherent potential for bias, the selection of which requires further research - a summary of each epigenetic methods is presented in the Table 1.

TABLE 1. Comparison of methods for DNA methylation, histone modification and histone variants analyses. UFRGS, Porto Alegre, RS, Brazil. 2017.

Methods	Accuracy*	Specialized equipment/ reagents*	Coverage*	Cost*
DNA methylation				
Bisulfite conversion	High	Yes	100% or specific genes/regions of interest	High
Methylation-sensitive enzymes	Intermediate	Specific enzymes	Gene-specific	Low
HPLC	High	Yes	Whole genome assessment	Intermediate
ELISA	Intermediate	Availability as kit	Whole genome assessment	Low
Cytosine-extension assay	High	Yes	Whole genome assessment	Low
Histone modification				
ChIP	High	Specific antibody	Whole genome Assessment or specific genes/regions of interest	Intermediate
MS	High	Yes	Whole genome Assessment or specific genes/regions of interest	Intermediate
HPLC	Intermediate	Yes	Whole genome assessment	Intermediate
ELISA	Intermediate	Availability as kit	Whole genome assessment	Low
Histone variants				
ChIP	High	Specific antibody	Whole genome Assessment or specific genes/regions of interest	Intermediate

* Compiled from Fraga & Esteller, 2002; Bonaldi *et al*., 2004; Saleh *et al*., 2008; Schones *et al*., 2008; Li & Tollefsbol, 2011; Soldi *et al*., 2014; Kurdyukov & Bullock, 2016; Bilichak & Kovalchuk, 2017;

2.5.1 DNA methylation

If the candidate genes to be studied are known, methods such as bisulfite conversion and/or methylation-sensitive enzymes (or digestion-based assays) can be used for analyzing DNA methylation of specific genes/regions of interest (Kurdyukov & Bullock, 2016). However, when a broader methylation profile is sought, analyses such as whole genome bisulfite sequencing (WGBS), high-performance liquid chromatography (HPLC) and ELISA are the primary methods of choice. Other methods can be utilized for whole genome methylation profiling, but they will not be the focus of this review, such as: LUMA (luminometric methylation

assay) technique, traditional polymerase chain reaction (PCR) based in amplification fragment length polymorphism (AFLP) (Aung *et al*., 2010), restriction fragment length polymorphism (RFLP) (Jaligot *et al*., 2002), and PCR amplification of LINE-1 followed by pyrosequencing that involves the bisulfite conversion of DNA (Kurdyukov & Bullock, 2016).

2.5.1.1 Bisulfite sequencing

Bisulfite sequencing involves treatment of DNA with sodium bisulfite, which converts cytosine into uracil while methylated cytosine remains intact in the DNA. For detection of DNA methylation in a particular gene, methylation specific primers are utilized for the PCR amplification and subsequent sequencing (Li & Tollefsbol, 2011). Unmethylated cytosine converts into thymine, whereas the presence of cytosine peak in the sequencing result indicates the presence of methylated cytosine (Clark *et al*., 1994). DNA methylation status of the gene is interpreted by comparing the sequencing results and the original DNA sequence (Chen *et al*., 2010). Bisulfite conversion is a commonly used approach for gene-specific DNA methylation analyses. The main limitations of this method are primer design, PCR amplification, and the difficulty to amplify long DNA fragments from bisulfite-treated samples, the current limit is 100–300 bp (Kurdyukov & Bullock, 2016). However, it can be used for whole-epigenome profiling technology, as WGBS, with single-base-resolution using bisulfite genomic sequencing technology (Lu *et al*., 2016; Kurdyukov & Bullock, 2016). This technology provides a qualitative, quantitative, and efficient approach with single base-pair resolution (Li & Tollefsbol, 2011). Single cell methylome sequencing will have additional advantage in understanding of gene regulation and phenotype development of an organisms (Clark *et al*., 2016). Others bisulfite conversion techniques such as pyrosequencing, COLD-PCR for the

detection of unmethylated island, methylation-specific PCR, PCR with high resolution melting have been frequently used, but can be applied when candidate genes are known (Herman *et al*., 1996; Wojdacz *et al*., 2008).

2.5.1.2 Methylation-sensitive enzymes

This method is based on the advantage of the differential digestion of DNA by particular restriction endonucleases, named as methylation-sensitive restriction enzymes (MREs). For example, if the internal CpG in the 5'-CCGG-3' tetranucleotide sequence is methylated, cleavage with *Hpa*II is blocked, but cleavage with *Msp*I is not affected. *Hpa*II and *Msp*I MREs are isoschizomers that recognizes the same DNA sequence, presenting the same point of cleavage but exhibit different sensitivities to the DNA methylation state for fragment verification of the DNA methylation status of the genetic region under study, which is robust and simple (Chen *et al*., 2010) (Table 1). However, other methods such as bisulfite-based DNA methylation are more accurate, sensitive, and efficient (Li & Tollefsbol, 2011). Additionally, MRE digestion can be combined with sequencing of the resulting DNA fragments. The obtained results provide the locations of the unmethylated and methylated sites in the DNA.

2.5.1.3 Cytosine extension assay

CpG methylation status in the genome of plants can be studied by utilizing the differential cleavage abilities of MREs and incorporation of radiolabeled [^{3}H]dCTP corresponding to the guanine overhangs (Bilichak & Kovalchuk, 2017). In this method, a high quality genomic DNA of a sample under study is treated with methylation sensitive, methylation insensitive isochizomers in separate reactions, while non-enzyme treated DNA of the same sample acts as a background control.

Incubation of digested DNA with [^{3}H] dCTP and AmpliTaq DNA polymerase, incorporates [^{3}H] dCTP into the digested DNA due to single nucleotide extension at the overhangs. Radioactivity incorporation is measured by liquid scintillation counter. Readings obtained from non-enzyme treated DNA is used for correction of background incorporation. Ratio of background corrected readings of methylation sensitive and methylation insensitive enzymatic digestion show the percentage of unmethylated restriction sites. Here, amount of incorporated radionucleotides is inversely proportional to the methylation level of the genome. A non-radioactivity method can be performed by utilizing biotinylated dCTP (Fujiwara & Ito, 2002).

2.5.1.4 High-performance liquid chromatography (HPLC)

The content of methylcytosine can be measured by high-performance liquid chromatography (HPLC), that is highly quantitative and reproducible (Alonso *et al*., 2015). This method requires high-quality genomic DNA. Genomic DNA is hydrolyzed by chemical or enzymatic treatments and resulting deoxyribonucleosides are separated by HPLC for the quantification of methylcytosine levels by comparing the relative absorbance of cytosine and methylcytosine at 254 nm in the sample (Fraga & Esteller, 2002). This analyzes present a global methylation information in the genome of an organism without providing any evidence about the location of methylation (Kurdyukov & Bullock, 2016).

2.5.1.5 Enzyme-linked immunosorbent assay (ELISA)-Based Methods

The enzyme-linked immunosorbent assay (ELISA) permits the quantification of global DNA methylation by using several commercially available kits containing primary antibodies raised against 5-methylcytosine (5-mC). This provides great

agility in the rough estimation of cytosine methylation in the genome compared to other methods (Table 1). This analysis is appropriate to recognize large changes in global DNA methylation (~1.5–2 times) (Kurdyukov & Bullock, 2016). In this method, DNA is denatured and immobilized on ELISA plate, then incubated with a 5-mC monoclonal primary antibody. Quantification of methylated cytosine is carried out by colorimetric/fluorometric detection of staining intensity produced by fluorescein-conjugated secondary antibodies bound to the 5-mC primary antibodies.

2.5.2 Histone modification and variants

Although histone modifications are involved in PTMs, histone variants are encoded by separate genes. Among the various strategies available for detection of histone modifications and variants, mass spectrometry (MS) based proteomics (Soldi *et al*., 2014) and chromatin immunoprecipitation (ChIP) (Saleh *et al*., 2008) are best approaches. Additionally, histone modifications are also analyzed by ELISA test. Methods such as reversed phase HPLC, can also be used to determine the state of histone modifications in chromatin. However, these methods together with ELISA can present restrictions to identify specific modifications, showing the number of modifications but not the site of histone modifications (Bonaldi *et al*., 2004).

2.5.2.1 Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) is a method that uses specifically-raised antibodies against histone variants to analyze histone modifications (Saleh *et al*., 2008). The main advantages of this technique is the sensitivity, reliability, and quickness (Table 1). However, it is necessary to have *a prior* knowledge about the antibody-based assays for the studies of the histone modifications of interest (Bonaldi *et al*., 2004), including H3K4me2, H3K4me3, H3K9me2, H3K27me2 and

H3K27me3 among a large number of tissue-specific histones (Zhang *et al*., 2007, Saleh *et al*., 2008). Histone modifications can rigorously disturb antibody binding to its epitope (region that is recognized by the antibody) and then mimic the loss of a particular modification (Bonaldi *et al*., 2004). Additionally, this method permits the use of a combination of other techniques. For example, ChIP followed by mass spectrometry (ChIP-MS) facilitates the parallel analysis of histone marks and their binding proteins at functionally distinct chromatin regions (Soldi *et al*., 2014). Additionally, ChIP can be combined with serial analyses of gene expression (SAGE), genome-wide mapping technique (GMAT), ChIP combined with paired-end ditag sequencing (ChIP–PET) and next-generation sequencing (ChIP–seq) (Schones *et al*., 2008).

2.5.2.2 Mass spectrometry (MS) and MS-based proteomics

The recent technical advances to study proteins and peptides by mass spectroscopy (MS) permit the study of histone modifications particularly in its quantitative format, and is a powerful tool to analyze the histone code (Soldi *et al*., 2014). One of the advantages of this technique is that it is not necessary to generate antibodies against specific modifications as is required in case of ChIP (Bonaldi *et al*., 2004). The modifications are identified in MS by calculating the “deltamass” (Δm), through the difference between the theoretical and experimentally-measured masses of a peptide. This method presents accurate measurement based on the Δm value, showing high precision regarding the position, number and combinations of marks present on a polypeptide. Additionally, this method is useful to discover novel marks associated with epigenetic control (Soldi *et al*., 2014).

2.6 Perspectives for epigenetic regulation in herbicide resistance

The rapid evolution of herbicide resistance is a challenge for weed management and herbicide use, especially with the increasing problem of resistance to herbicides of multiple sites of action. Epigenetic mechanisms have been well described as important regulators of plant-environment interactions, mainly associated with stress adaptation. The effect of sub-lethal doses of herbicides may act similar to other abiotic environmental stresses, triggering epigenetic responses that lead to stress tolerance. Epigenetic mechanisms likely play an important, yet currently under-studied, contribution to the development of herbicide resistance, particularly NTSR. Future exploration of epigenetics may provide a new phase on the study of environmental stresses on crop performance and herbicide resistance in weeds, such as what is occurring about stress responses and drug resistance in humans. The possibility of transgenerational epigenetic effects presents a tantalizing ‘memory’ of expression patterns of the parental plants that survived herbicide stress, accelerating the evolution of resistance. Advances in ‘omics’ technologies, along with the development of basic molecular knowledge in weeds will allow important epigenetic knowledge to open new avenues for weed research, which is likely to happen in the next few decades.

REFERENCES

AGRAWAL, A.A. Herbivory and maternal effects: Mechanisms and consequences of transgenerational induced plant resistance. **Ecology**, Washington, v.83, n.12, p.3408-3415, 2002.

ALONSO, C. et al. Global DNA cytosine methylation as an evolving trait: phylogenetic signal and correlated evolution with genome size in angiosperms. **Frontiers in Genetics**, Lausanne, v.6, n.1, p.1-9, 2015.

ARYA, G.; SCHLICK, T. A Tale of Tails: How Histone Tails Mediate Chromatin Compaction in Different Salt and Linker Histone Environments. **Journal of Physical Chemistry A**, Washington, v.113, n.16, p.4045-4059, 2009.

ASENSI-FABADO M.A.; AMTMANN A.; PERRELLA G. Plant responses to abiotic stress: The chromatin context of transcriptional regulation. **Biochimica et Biophysica Acta**, Amsterdam, v.1860, n.1, p.106-122, 2017.

AUNG, H.T. et al. Stringent Programming of DNA Methylation in Humans. **Twin Research and Human Genetics**, Bowen Hills, v.13, n.5, p.405-411, 2010.

BILICHAK A.; KOVALCHUK I. Analysis of Global Genome Methylation Using the Cytosine-Extension Assay. **Methods in Molecular Biology**, Clifton, v.1456, n.1, p.73-79, 2017.

BONALDI, T.; REGULA, J.T.; IMHOF, A. The use of mass spectrometry for the analysis of histone modifications. **Chromatin and Chromatin Remodeling Enzymes,** San Diego, v.377, p.111-130, 2004.

BOYKO, A.; KOVALCHUK, I. Genome instability and epigenetic modification - heritable responses to environmental stress? **Current Opinion in Plant Biology**, London, v.14, n.3, p.260-266, 2011.

CALARCO, J.P. et al. Reprogramming of DNA Methylation in Pollen Guides Epigenetic Inheritance via Small RNA. **Cell**, Cambridge, v.151, n.1, p.194-205, 2012.

CHEN, M.; LV, S.L.; MENG, Y.J. Epigenetic performers in plants. **Development Growth & Differentiation**, Malden, v.52, n.6, p.555-566, 2010.

CHINNUSAMY, V.; ZHU, J.K. Epigenetic regulation of stress responses in plants. **Current Opinion in Plant Biology**, London, v.12, n.2, p.133-139, 2009.

CHOI, C.S.; SANO, H. Abiotic-stress induces demethylation and transcriptional activation of a gene encoding a glycerophosphodiesterase-like protein in tobacco plants. **Molecular Genetics and Genomics**, New York, v.277, n.5, p.589-600, 2007.

CLARK, S.J. et al. High-sensitivity mapping of methylated cytosines. **Nucleic Acids Research**, Oxford, v.22, n.15, p.2990-2997, 1994.

CLARK, S.J. et al. Single-cell epigenomics: powerful new methods for understanding gene regulation and cell identity. **Genome Biology**, London, v.17, art.72, 2016.

COKUS, S.J. et al. Shotgun bisulphite sequencing of the Arabidopsis genome reveals DNA methylation patterning. **Nature**, London, v.452, n.7184, p.215-219, 2008.

COLEMAN-DERR, D.; ZILBERMAN, D. Deposition of Histone Variant H2A.Z within Gene Bodies Regulates Responsive Genes. **Plos Genetics**, San Francisco, v.8, n.10, 2012.

CORTIJO, S. et al. Mapping the Epigenetic Basis of Complex Traits. **Science**, Washington**,** v.343, n.6175, p.1145-1148, 2014.

CUMMINS, I. et al. Key role for a glutathione transferase in multiple-herbicide resistance in grass weeds. **Proceedings of the National Academy of Sciences of the United States of America**, Collins, v.110, n.15, p.5812-5817, 2013.

DE BLOCK, M.; VAN LIJSEBETTENS, M. Energy efficiency and energy homeostasis as genetic and epigenetic components of plant performance and crop productivity. **Current Opinion in Plant Biology**, London, v.14, n.3, p.275-282, 2011.

DÉLYE, C. et al. Molecular Mechanisms of Herbicide Resistance. **Weed Science**, Champaign**,** v.63, p.91-115, 2015.

DÉLYE, C.; JASIENIUK, M.; LE CORRE, V. Deciphering the evolution of herbicide resistance in weeds. **Trends in Genetics**, London, v.29, n.11, p.649-658, 2013.

DÉLYE, C. Unravelling the genetic bases of non-target-site-based resistance (NTSR) to herbicides: a major challenge for weed science in the forthcoming decade. **Pest Management Science**, Malden, v.69, n.2, p.176-187, 2013.

EVANS G.M. et al. The relation between nuclear DNA amount and the duration of the mitotic cycle. **Chromosomes Today**, Basel, n.3, p.24–31, 1972.

FENG, S.H. et al. Conservation and divergence of methylation patterning in plants and animals. **Proceedings of the National Academy of Sciences of the United States of America**, Collins, v.107, n.19, p.8689-8694, 2010.

FORESTAN, C. et al. Stress-induced and epigenetic-mediated maize transcriptome regulation study by means of transcriptome reannotation and differential expression analysis. **Scientific Reports**, Legnaro, v.6, n.1, p.1-20, 2016.

FRAGA, M.E.; ESTELLER, M. DNA methylation: A profile of methods and applications. **Biotechniques**, Natick, v.33, n.3, p.632-649, 2002.

FUJIWARA, H.; ITO, M. Nonisotopic cytosine extension assay: A highly sensitive method to evaluate CpG island methylation in the whole genome. **Analytical Biochemistry**, San Diego, v.307, n.2, p.386-389, 2002.

GAINES, T.A. et al. Gene amplification confers glyphosate resistance in Amaranthus palmeri. **Proceedings of the National Academy of Sciences of the United States of America**, Collins, v.107, n.3, p.1029-1034, 2010.

GAINES, T.A. et al. RNA-Seq transcriptome analysis to identify genes involved in metabolism-based diclofop resistance in Lolium rigidum. **Plant Journal**, Oxford, v.78, n.5, p.865-876, 2014.

GENT, J.I. et al. CHH islands: de novo DNA methylation in near-gene chromatin regulation in maize. **Genome Research**, Irvine, v.23, n.4, p.628-637, 2013.

GRESSEL J. Perspective: consider removing 'inherited' from definitions of pesticide resistance. **Outlooks on Pest Management**, Malden, v.26, n.5, p.220-222, 2015.

GRESSEL, J. Evolving understanding of the evolution of herbicide resistance. **Pest Management Science**, Malden, v.65, n.11, p.1164-1173, 2009.

HAUSER, M.T. et al. Transgenerational epigenetic inheritance in plants. **Biochimica Et Biophysica Acta-Gene Regulatory Mechanisms**, Amsterdam, v.1809, n.8, p.459-468, 2011.

HEAP, I. **The International survey of herbicide resistant weeds.** 2017. Available in:<www.weedscience.com>. Accessed in: 3 jan. 2017.

HEARD, E.; MARTIENSSEN, R.A. Transgenerational Epigenetic Inheritance: Myths and Mechanisms. **Cell**, Cambridge, v.157, n.1, p.95-109, 2014.

HÉBRARD, C. et al. Epigenomics and bolting tolerance in sugar beet genotypes. **Journal of Experimental Botany**, Oxford, v.67, n.1, p.207-225, 2016.

HERMAN, J.G. et al. Methylation-specific PCR: A novel PCR assay for methylation status of CpG islands. **Proceedings of the National Academy of Sciences of the United States of America**, Collins, v.93, n.18, p.9821-9826, 1996.

HOFMANN, N.R. Epigenetic Battles Underfoot: Allelopathy among Plants Can Target Chromatin Modification. **Plant Cell**, Rockville, v.27, n.11, p.3021-3021, 2015.

HOLLISTER, J.D.; GAUT, B.S. Epigenetic silencing of transposable elements: A trade-off between reduced transposition and deleterious effects on neighboring gene expression. **Genome Research**, Irvine, v.19, n.8, p.1419-1428, 2009.

INGELMAN-SUNDBERG, M.; CASCORBI, I. Pharmacogenomic or -Epigenomic Biomarkers in Drug Treatment: Two Sides of the Same Medal? **Clinical Pharmacology & Therapeutics**, Hoboken, v.99, n.5, p.478-480, 2016.

ITO, H. et al. An siRNA pathway prevents transgenerational retrotransposition in plants subjected to stress. **Nature**, London, v.472, n.7341, p.115-U151, 2011.

ITO, K. et al. Oxidative stress reduces histone deacetylase 2 activity and enhances IL-8 gene expression: role of tyrosine nitration. **Biochemical and Biophysical Research Communications**, London, v.315, n.1, p.240-245, 2004.

IWAKAMI S. et al. Cytochrome P450 genes induced by bispyribac-sodium treatment in a multiple-herbicide-resistant biotype of Echinochloa phyllopogon. **Pest Management Science**, Malden, n.70, p.549-558, 2014b.

IWAKAMI, S. et al. Cytochrome P450 CYP81A12 and CYP81A21 Are Associated with Resistance to Two Acetolactate Synthase Inhibitors in Echinochloa phyllopogon. **Plant Physiology**, Rockville, v.165, n.2, p.618-629, 2014a.

JALIGOT, E.; BEULE, T.; RIVAL, A. Methylation-sensitive RFLPs: characterisation of two oil palm markers showing somaclonal variation-associated polymorphism. **Theoretical and Applied Genetics**, New York, v.104, n.8, p.1263-1269, 2002.

JULLIEN, P.E. et al. DNA Methylation Dynamics during Sexual Reproduction in Arabidopsis thaliana. **Current Biology**, Cambridge, v.22, n.19, p.1825-1830, 2012.

KIM, J.M. et al. Chromatin changes in response to drought, salinity, heat, and cold stresses in plants. **Frontiers in Plant Science**, Lausanne, v.6, art.114, 2015.

KOZYRA M.; INGELMAN-SUNDBERG M.; LAUSCHKE V.M. Rare genetic variants in cellular transporters, metabolic enzymes, and nuclear receptors can be important determinants of interindividual differences in drug response. **Genetics in Medicine**, New York, v.19, n.1, p.20-29, 2017.

KUMAR, S.V.; WIGGE, P.A. H2A.Z-Containing Nucleosomes Mediate the Thermosensory Response in Arabidopsis. **Cell**, Cambridge, v.140, n.1, p.136-147, 2010.

KURDYUKOV S.; BULLOCK M. DNA Methylation Analysis: Choosing the Right Method. **Biology,** Basel, v.5, n.3, p1-21, 2016.

KWON, C.S. et al. Histone occupancy-dependent and -independent removal of H3K27 trimethylation at cold-responsive genes in Arabidopsis. **Plant Journal**, Oxford, v.60, n.1, p.112-121, 2009.

LANE, T. S. et al. Diversity of ABC transporter genes across the plant kingdom and their potential utility in biotechnology. **Bmc Biotechnology**, London, v.16, n.1, p.1-10, 2016.

LANGE, U.C.; SCHNEIDER, R. What an epigenome remembers. **Bioessays**, Hoboken, v.32, n.8, p.659-668, 2010.

LAW, J.A.; JACOBSEN, S.E. Establishing, maintaining and modifying DNA methylation patterns in plants and animals. **Nature Reviews Genetics**, London, v.11, n.3, p.204-220, 2010.

LI Y.; TOLLEFSBOL T.O. DNA methylation detection: Bisulfite genomic sequencing analysis. **Methods in Molecular Biology**, Clifton, v.791, n.1, p.11-21, 2011.

LI, B.; CAREY, M.; WORKMAN, J.L. The role of chromatin during transcription. **Cell**, Cambridge, v.128, n.4, p.707-719, 2007.

LI, W.X. et al. Knockdown of SAMS genes encoding S-adenosyl-L-methionine synthetases causes methylation alterations of DNAs and histones and leads to late flowering in rice. **Journal of Plant Physiology**, Jena, v.168, n.15, p.1837-1843, 2011.

LU, Y.C. et al. Genome-wide identification of DNA methylation provides insights into the association of gene expression in rice exposed to pesticide atrazine. **Nature Scientific Reports**, London, v.6, n.1, p.1-15, 2016.

LUGER, K. et al. Crystal structure of the nucleosome core particle at 2.8 angstrom resolution. **Nature**, London, v.389, n.6648, p.251-260, 1997.

MULLER-XING, R.; XING, Q.; GOODRICH, J. Footprints of the sun: memory of UV and light stress in plants. **Frontiers in Plant Science**, Lausanne, v.5, art.474, 2014.

NARDEMIR, G. et al. Determination of genetic and epigenetic effects of glyphosate on Triticum aestivum with RAPD and CRED-RA techniques. **Theoretical and Experimental Plant Physiology**, Campinas, v.27, n.2, p.131-139, 2015.

NEUL, C. et al. Impact of Membrane Drug Transporters on Resistance to Small-Molecule Tyrosine Kinase Inhibitors. **Trends in Pharmacological Sciences**, London, v.37, n.11, p.904-932, 2016.

NEVE, P.; POWLES, S. High survival frequencies at low herbicide use rates in populations of Lolium rigidum result in rapid evolution of herbicide resistance. **Heredity**, London, v.95, n.6, p.485-492, 2005a.

NEVE, P.; POWLES, S. Recurrent selection with reduced herbicide rates results in the rapid evolution of herbicide resistance in Lolium rigidum. **Theoretical and Applied Genetics**, New York, v.110, n.6, p.1154-1166, 2005b.

OERKE, E.C. Crop losses to pests. **Journal of Agricultural Science**, New York, v.144, n.1, p.31-43, 2006.

PATZOLDT, W.L. et al. A codon deletion confers resistance to herbicides inhibiting protoporphyrinogen oxidase. **Proceedings of the National Academy of Sciences of the United States of America**, Collins, v.103, n.33, p.12329-12334, 2006.

PECINKA, A.; SCHEID, O.M. Stress-Induced Chromatin Changes: A Critical View on Their Heritability. **Plant and Cell Physiology**, Oxford, v.53, n.5, p.801-808, 2012.

PIKAARD, C.S.; SCHEID, O.M. Epigenetic Regulation in Plants. **Cold Spring Harbor Perspectives in Biology**, Bungtown, v.6, n.12, 2014.

POWLES, S.B.; YU, Q. Evolution in Action: Plants Resistant to Herbicides. **Annual Review of Genetics**, Palo Alto, v.61, p.317-347, 2010.

QUADRANA, L.; COLOT, V. Plant Transgenerational Epigenetics. **Annual Review of Genetics**, Palo Alto, v.50, p.467-491, 2016.

RADWAN, D.E.M. Salicylic acid induced alleviation of oxidative stress caused by clethodim in maize (Zea mays L.) leaves. **Pesticide Biochemistry and Physiology**, San Diego, v.102, n.2, p.182-188, 2012.

RAHAVI, M.R. et al. Transgenerational adaptation to heavy metal salts in Arabidopsis. **Frontiers in Plant Science**, Lausanne, v.2, art.91, 2011.

RAMEL, F. et al. Xenobiotic sensing and signalling in higher plants. **Journal of Experimental Botany**, Oxford, v.63, n.11, p.3999-4014, 2012.

RAYBURN, A.L. et al. Genome size analysis of weedy Amaranthus species. **Crop Science**, Madison, v.45, n.6, p.2557-2562, 2005.

SALEH, A.; ALVAREZ-VENEGAS, R.; AVRAMOVA, Z. An efficient chromatin immunoprecipitation (ChIP) protocol for studying histone modifications in Arabidopsis plants. **Nature Protocols**, London, v.3, n.6, p.1018-1025, 2008.

SAMMONS, R.D.; GAINES, T.A. Glyphosate resistance: state of knowledge. **Pest Management Science**, Malden, v.70, n.9, p.1367-1377, 2014.

SCHMITZ, R.J. et al. Transgenerational Epigenetic Instability Is a Source of Novel Methylation Variants. **Science**, Washington**,** v.334, n.6054, p.369-373, 2011.

SCHONES, D.E.; ZHAO, K. Genome-wide approaches to studying chromatin modifications. **Nature Reviews Genetics**, London, v.9, n.3, p.179-191, 2008.

SHEN, Y.; ISSAKIDIS-BOURGUET, E.; ZHOU, D.X. Perspectives on the interactions between metabolism, redox, and epigenetics in plants. **Journal of Experimental Botany**, Oxford, v.67, n.18, p.5291-5300, 2016.

SOLDI, M.; BREMANG, M.; BONALDI, T. Biochemical systems approaches for the analysis of histone modification readout. **Biochimica Et Biophysica Acta-Gene Regulatory Mechanisms**, Amsterdam, v.1839, n.8, p.657-668, 2014.

TAUSZ, M. The role of glutathione in plant response and adaptation to natural stress. In: GRILL, D.; TAUSZ M.; KOK, L.J. (Ed.) **Significance of Glutathione in Plant Adaptation to the Environment.** Netherlands: Kluwer Academic Publishers, 2001. p.101-122

TRICKER, P.J. Transgenerational inheritance or resetting of stress-induced epigenetic modifications: two sides of the same coin. **Frontiers in Plant Science**, Lausanne, v.6, art.699, 2015.

VARANASI, V.K. et al. A Target-Site Point Mutation in Henbit (Lamium amplexicaule) Confers High-Level Resistance to ALS-Inhibitors. **Weed Science**, Champaign, v.64, n.2, p.231-239, 2016.

VAUGHN, M.W. et al. Epigenetic natural variation in Arabidopsis thaliana. **Plos Biology**, San Francisco, v.5, n.7, p.1617-1629, 2007.

VELMURUGAN, J. et al. An ultra-high density genetic linkage map of perennial ryegrass (Lolium perenne) using genotyping by sequencing (GBS) based on a reference shotgun genome assembly. **Annals of Botany**, Oxford v.118, n.1, p.71-87, 2016.

VERHOEVEN, K.J.F.; VAN GURP, T.P. Transgenerational Effects of Stress Exposure on Offspring Phenotypes in Apomictic Dandelion. **Plos One**, San Francisco**,** v.7, n.6, e38605, 2012.

VILA-AIUB, M.M.; GHERSA, C.M. Building up resistance by recurrently exposing target plants to sublethal doses of herbicide. **European Journal of Agronomy**, Amsterdam, v.22, n.2, p.195-207, 2005.

VRIET, C.; HENNIG, L.; LALOI, C. Stress-induced chromatin changes in plants: of memories, metabolites and crop improvement. **Cellular and Molecular Life Sciences**, Basel, v.72, n.7, p.1261-1273, 2015.

WANG, X.; WEIGEL, D.; SMITH, L.M. Transposon Variants and Their Effects on Gene Expression in Arabidopsis. **Plos Genetics**, San Francisco, v.9, n.2, e1003255, 2013.

WENG, M.J. et al. Histone chaperone ASF1 is involved in gene transcription activation in response to heat stress in Arabidopsis thaliana. **Plant Cell and Environment**, Hoboken, v.37, n.9, p.2128-2138, 2014.

WOJDACZ, T.K.; DOBROVIC, A.; HANSEN, L.L. Methylation-sensitive high-resolution melting. **Nature Protocols**, London, v.3, n.12, p.1903-1908, 2008.

YANG, X. et al. Quantitative proteomics reveals ecological fitness cost of multi-herbicide resistant barnyardgrass (Echinochloa crus-galli L.). **Journal of Proteomics**, Amsterdam, v.150, p.160-169, 2017.

YU, Q.; POWLES, S. Metabolism-Based Herbicide Resistance and Cross-Resistance in Crop Weeds: A Threat to Herbicide Sustainability and Global Crop Production. **Plant Physiology**, Rockville, v.166, n.3, p.1106-1118, 2014.

YUAN, J.S.; TRANEL, P.J.; STEWART, C.N. Non-target-site herbicide resistance: a family business. **Trends in Plant Science**, London, v.12, n.1, p.6-13, 2007.

ZEMACH, A. et al. The Arabidopsis Nucleosome Remodeler DDM1 Allows DNA Methyltransferases to Access H1-Containing Heterochromatin. **Cell**, Cambridge, v.153, n.1, p.193-205, 2013.

ZHANG, K.L. et al. Distinctive Core Histone Post-Translational Modification Patterns in Arabidopsis thaliana. **Plos One**, San Francisco**,** v.2, n.11, e1210, 2007.

ZHONG, L.; XU, Y.H.; WANG, J.B. The effect of 5-azacytidine on wheat seedlings responses to NaCl stress. **Biologia Plantarum**, Dordrecht, v.54, n.4, p.753-756, 2010.

ZHOU, C.H. et al. HISTONE DEACETYLASE19 is involved in jasmonic acid and ethylene signaling of pathogen response in Arabidopsis. **Plant Cell**, Rockville**,** v.17, n.4, p.1196-1204, 2005.

3 CHAPTER 1

Specific epigenetic pathways are requied to tolerance of herbicides glyphosate, imazethapyr and 2,4-D in *A. thaliana*

1 INTRODUCTION

Herbicides are designed as the most effective method of controlling plants, they have chemical properties capable of killing or suppressing plant growth (Foster *et al*., 1993). However, the intense use of these molecules has selected weed populations resistant to herbicides, reducing the efficiency of these compounds (Heap, 2017). Herbicide resistance is related to the occurrence of two primary mechanisms: target-site resistance (TSR) and non-target-site resistance (NTSR) (Powles & Yu, 2010).

Gene expression is highly regulated in plants (Nakashima *et al*., 2009). In addition to genetic regulation, the epigenetic mechanisms are also suggested to be involved with processes of herbicide resistance (Gressel, 2009; Powles & Yu, 2010; Délye, 2013). Although very few studies address the epigenetic relationship with herbicide resistance (Nardemir *et al*., 2015; Lu *et al*., 2016), it is possible that epigenetic acts regulating specific genes important to herbicide detoxification.

Epigenetics refers to the alteration of gene expression patterns without change in DNA sequence (Grossniklaus *et al*., 2013). The epigenetic control involves chief mechanisms such as DNA methylation, non-coding RNA (related to RNA-directed DNA methylation – RdDM) and histone modifications (Allis & Jenuwein, 2016). Several pathways and enzymes are associated with epigenetic marks that regulate gene expression and suppress transposon activity (Du *et al*., 2015).

Among the epigenetic mechanisms, DNA methylation is the most understood process in plants. It consists in a biochemical process attaching a methyl group in to the 5-position of cytosine (5mC), which can be found in CG, CHG and CHH contexts (with H standing for C, A, T) (Cokus *et al*., 2008). Stress conditions can induce changes in gene expression through hypomethylation or hypermethylation

of DNA, where a decline in the level of methylation leads to an increase in gene expression (Finnegan *et al*., 1998). In *Arabidopsis*, heat stress for instance can involve active demethylation of transposon elements (TEs) that leads to transcriptional activation of specifics genes (Pecinka *et al*., 2010). The demethylation of 5mC is performed by enzymes such as ROS1 (REPRESSOR OF SILENCING 1), DME (DEMETER), DML2 (DEMETER-LIKE) and DML3 (Penterman *et al*., 2007).

At least three classes of enzymes perform the addition of methyl groups into cytosine, called DNA methyltransferase: DNA METHYLTRANSFERASE 1 (MET1), CHROMOMETHYLTRANSFERASES 3 (CMT3) and DOMAINS REARRANGED METHYLTRANSFERASE (DRM) (Huang *et al*., 2010). MET1 and CMT3 appear to maintain CG and CHG methylation, respectively, during DNA replication (Matzke & Birchler, 2005). The DRM is the major DNA methyltransferase that catalyzes *de novo* methylation by RdDM pathway (Cao *et al*., 2003).

The RdDM is unique for plants because it depends on the specialized transcriptional machinery plant-specific RNA polymerases IV and V (Pol IV and V) (Matzke & Mosher, 2014). The RdDM mediate the transcriptional gene silencing (TGS or RdTGS) (Mirouze & Paszkowski, 2011). In this pathway, small interfering RNAs (siRNAs) through the recognition of homologous regions of the genome are able to directing repressive epigenetic modifications (Matzke & Mosher, 2014). TGS controls the expression of transposable elements (TEs) and of endogenous genes and it is associated with increased of DNA methylation (Elmayan *et al*., 2005). These mechanisms play an important role in plant development and in the tolerance to environmental stresses (Zheng *et al*., 2010).

Histone modification and variants can be also correlated with gene expression in response to abiotic stresses, such as water deficit, high-salinity, and

temperature shifts (Kim *et al*., 2008; Luo *et al*., 2012). A study showed extensive links and crosstalk between histone modification and DNA methylation (Du *et al*., 2015). The exclusion of active marks such as trimethylation of histone 3 at lysine 4 (H3K4me3) and histone acetylation may function as indirect regulators of DNA methylation (Greenberg *et al*., 2013). Although the epigenetic pathways present some connection it is suggested that epigenetic modifications are definite for the stress condition and regulate a specific set of genes (Luo *et al*., 2012; Du *et al*., 2015).

A recent study with rice (*Oryza sativa*) showed that atrazine-induced changes in DNA methylation marks, which were associated with activation of specific genes responsible for atrazine degradation and detoxification (Lu *et al*., 2016). However, the understanding of the role of epigenetics on herbicide resistance is still unknown and currently this area of research is in its beginning. Furthermore, it may be considered that the epigenetic effect on herbicide resistance could be associated directly with the herbicide target enzyme or indirectly by affecting herbicide detoxification or movement in the plant.

The herbicides glyphosate, imazethapyr and 2,4-D belong to different mechanisms of action and are widely used for controlling weeds to improve crop productivity. Moreover, the number of cases of resistance is continuously rising (Heap, 2017). Base on that, these herbicides were chosen to analyze the effect on specific epigenetic pathways. The present study is based on *Arabidopsis thaliana*, which has a wide range of well-characterized mutants and epigenetic regulators (Pikaard & Scheid, 2014). Once understood if herbicides can cause epigenetic alterations in this specie, this knowledge can be applied to other plants and weeds, where herbicide resistance is causing high economic impact. The aims of this study were to analyze the effect of herbicides of different mechanisms of action on the

TGS in *A. thaliana* and to evaluate the importance of specific epigenetic pathways for herbicide detoxification.

2 MATERIALS AND METHODS

Seeds were sown on moist soil in plastic pots (7 x 7 cm) and stratified for 48 hours at 4°C in a dark room in order to obtain uniform seed germination and plant emergence. Afterward, pots were transferred to a growth chamber (Percival AR-95L3; Percival Scientific/USA) where plants were cultivated under a long day regime (16h 21°C light/ 8h 19°C dark), relative humidity of 70 – 75% and a 150 µmol m^{-2} s^{-1} light intensity. After one week, plants were singled to one plant per pot to avoid stress by plant competition and to obtain independent biological replicates.

2.1 Determination of sub-lethal dose of the herbicides

The experiment was carried out with *Arabidopsis thaliana* accession Columbia-0 (Col-0), using the methodology of dose–response curve, organized in completely randomized design, with four replicates. Three herbicides of different mode of action were used: glyphosate (EPSPS - 5 enolpyruvylshikimate-3-phosphate synthase inhibitor), imazethapyr (ALS - acetolactate synthase inhibitor) and 2,4-D (auxinic herbicide, 2,4-dichlorophenoxyacetic acid). This experiment was performed to select the sub-lethal dose for each herbicide that was used for all subsequent experiments. The sub-lethal dose was considered the maximum dose that caused plant injury, but did not cause plant death at 20 days after treatment (DAT). Each herbicide was evaluated at 0, 2.5, 5, 10 and 20% of the label dose. The doses used were 0, 18, 36, 72, 144 g ha^{-1} for glyphosate (Roundup Original, 480 g/L CS, Monsanto S/A), 0, 2.6, 5.3, 10.6, 21.28 g ha^{-1} in addition of 0.5% v/v Dash for imazethapyr (Imazethapyr plus Nortox, 106 g/L CS, Nortox S/A) and 0, 20.1,

40.3, 80.6, 161.2 g ha^{-1} for 2,4-D (DMA 806 BR, 806 g/L CS, Dow AgroSciences S/A). The treatments were applied in 21 days old plants using an automatic spray chamber (Greenhouse Spray Chamber; Generation III), with the TJ8002E spray nozzle, constant pressure of 42 lb pol^{-2} and velocity of 1.16 m s^{-1}, providing a spray volume of 200 L ha^{-1}.

Plant injury and shoot dry weight were evaluated at 20 DAT. The plant injury was evaluated visually on a scale of 0% (no injury) to 100% (plant dead). Shoot dry weight was obtained by harvesting the plants and drying in an oven forced air at 60°C until constant weight. Data were tested for normality using PROC UNIVARIATE and subjected to analysis of variance (ANOVA) ($p \leq 0.05$) in SAS (SAS Institute, 2004). To satisfy the ANOVA premise for normality, plant injury and dry weight data were transformed with $X=10+\text{arcsen}\sqrt{X100}$ and $X=1/X$, respectively. After that, complementary regression analysis was performed, fitted to a non-linear logistic model with three parameters [$y= a/1+(x/x_0)^b$], proposed by Streibig, (1988). Where, y= is the percentage of plant injury or dry weight (grams per plant); x = dose of the herbicide; a= is the difference between the maximum and minimum points of the curve; b= is the slope of the curve, and x0= is the dose which provides 50% of the response of the variable, when minimum and maximum point in the curve are 0 and 100, respectively. The determination of 50% plant injury and 50% growth reduction (GR50) was obtained by replacing “y” in the equation with 50, as proposed by Carvalho *et al*. (2005).

2.2 Effect of herbicides in transcriptional gene silencing (TGS)

Plant material consisted of the *A. thaliana* line L5, and seeds obtained by crossing L5 and the epigenetic *Arabidopsis ddm1* mutant (L5X*ddm1*), used as positive control. *A. thaliana* line L5 is in the Columbia-0 (Col-0) background carrying

an insert consisting of the 35S promoter of *Cauliflower mosaic* virus and the ß-glucuronidase (GUS) marker gene (*P35S:GUS*) (Morel *et al*., 2000; Probst *et al*., 2004). The insert of a multicopy *P35S:GUS* gene is suppressed by transcriptional gene silencing (TGS) (Pecinka *et al*., 2010). In this experiment, plants L5 were used to analyze the effect of herbicides on epigenetically regulated transcription. The sub-lethal doses of herbicides glyphosate, imazethapyr and 2,4-D were applied in 21 days old plants. The experiment was conducted as a completely randomized design with four replications. The treated and non-treaded plants were collected 48 hours after herbicide application. The measurement of transcriptional activation GUS was detected by histochemical staining and by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).

2.2.1 Histochemical staining

Shoots were excised, stored in plastic tubes, and immediately placed on ice. Afterward, plants were exposed to 40 min vacuum infiltration and overnight incubation in GUS staining solution in dark condition at 37 °C. The GUS staining solution was performed using 0.1 M sodium phosphate buffer, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 100 mg/mL chloramphenicol, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, and 0.5 mg/mL X-glucuronide. Subsequent washes with 70% ethanol at 37°C were performed to remove chlorophyll and enhance contrast. Images were acquired with the Leica Application Suite and processed with Adobe Photoshop (Adobe).

2.2.2 Quantitative reverse transcriptase PCR

The entire plant rosette was collected and immediately frozen in liquid nitrogen. RNA extraction was performed using the RNeasy Plant Mini Kit (Qiagen,

Hilden/Germany) according to the manufacture's protocol with an additional on-column DNase I digestion (Roche, Basel/Switzerland). RNA concentrations were quantified with the NanoDrop ND-1000 spectral photometer (peqLab, Erlangen/Germany).

An amount of 1 µg of total RNA was reverse transcribed into complementary DNA (cDNA) with the First Strand cDNA Synthesis Kit using oligo(dT) primers (Thermo Scientific, St. LeonRot/Germany) according to the manufacture's protocol and analyzed by qRT-PCR with the SensiMix SYBR & Flourescein Kit (Bioline, Berlin/Germany). Each reaction was set up in a 12 µl total volume, which contained 6 µl of 2x SensiMix SYBR & Fluorescein, 1 µl of primers (in final concentration of 250nM for each primer), 1 µl of water and 4 µl of the cDNA template. Primer sets used are shown in Table 1. The cDNA samples were diluted at a cDNA: distilled water ratio of 1:100. The reactions were carried out using the following cycling parameters: 95°C for 5 min, followed by 40 cycles of 94°C for 15 s, 60°C for 10 s, 72°C for 15 s, and 60°C for 35 s.

TABLE 1. Primers used in the analysis of transcription levels of GUS. UFRGS, Porto Alegre, RS, Brazil. 2017.

Target	Primer Name	Sequence (5' → 3')	Product Size (bp)
GUS	GUS_F	TTAACTATGCCGGAATCCATCGC	
GUS	GUS_R	CACCACCTGCCAGTCAACAGACGC	
GAPC-2	GAPC-2_F	ATCGGTCGTTTGGTTGCTAGAGT	251
GAPC-2	GAPC-2_R	ACAAAGTCAGCTCCAGCCTCA	
UBC28	UBC28qF	TCCAGAAGGATCCTCCAACTTCCTGCAGT	124
UBC28	UBC28qR	ATGGTTACGAGAAAGACACCGCCTGAATA	

PCR was calibrated using logarithmic serial dilutions from 10^{-1} to 10^{-3} of cDNA preparations. The threshold cycle (Ct value) for reactions was determined in technical triplicates. Samples with mean Ct values ± 0.25 cycles were included in calculations. The evaluation of qPCR data was performed according to the MIQE

(Minimum Information for publication of Quantitative real-time PCR experiments) (Bustin *et al*., 2009). The values for the correlation coefficient of the PCR between 80 and 110% were accepted. After running the PCR, the melting curve of the PCR products was analyzed to control the homogeneity of the amplification product, where a sharp, narrow peak was required. Transcription levels of GUS were estimated by the standard curve method (Larionov *et al*., 2005) and normalized to the herbicide stable reference genes GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE C-2 (GAPC-2; AT1G13440) and UBIQUITIN-CONJUGATING ENZYME 28 (UBC28; AT1G64230). Data sets were submitted to the *t*-test ($p \leq 0.05$).

2.3 Evaluation of herbicide effect on epigenetic Arabidopsis mutants

The experiment was performed with 11 different mutants of *A. thaliana* associated with specific epigenetic pathways (Table 2) in comparison with wild type (WT) plants. The analyzed mutants are involved with RdDM pathway, chromatin formation or remodeling, histone modification and DNA methylation and are represented in bold letters in Figures 1, 2 and 3. The epigenetic mechanism of the proteins UPF1-5 and RPA2 are not completely elucidated. All plants were cultivated as described above and the herbicides were applied in 20 days old plants. Non-treated checks of each mutant and WT plants were included. The herbicides glyphosate, imazethapyr and 2,4-D were applied in sub-lethal doses according to the results of the first experiment. The experiment was organized in completely randomized design, with four repetitions and conducted twice.

TABLE 2. Description of epigenetic mutants associated with specific pathways of epigenetic modifications, according to Pikaard & Scheid (2014). UFRGS, Porto Alegre, RS, Brazil. 2017.

Gene/ mutant	Gene or mutant name	Confirmed or putative function of protein
Indirectly involved with epigenetics control		
UPF1-5 (not shown)	Up-Frameshift	Nonsense-mediated mRNA decay (NMD).
small interfering RNAs (siRNAs)		
AGO6 (Figure 1)	Argonaute 6	PAZ-PIWI domain protein, siRNA-binding, RNA-directed DNA methylation (RdDM).
NRPE1 (Figure 1)	Nuclear RNA Pol V, defective in RNA-directed DNA methylation	RNA Pol V largest subunit. RNA-directed DNA methylation (RdDM).
NRPE2a (Figure 1)	Nuclear RNA polymerases IV and V; defective in RNA-directed DNA methylation 2	2nd subunit of Pol IV and PolV. RNA-directed DNA methylation (RdDM).
RDR6 (Figure 3)	RNA-dependent RNA polymerase	RNA-dependent RNA polymerase. Post-Transcriptional Gene Silencing (PTGS).
Chromatin formation or chromatin remodeling		
DDM1 (Figure 2)	Decreased-DNA methylation 1	SWI2/SNF2 chromatin remodeling ATPase. Transcriptional Gene Silencing (TGS).
FAS1 (Figure 2)	Fasciata 1	Chromatin assembly factor subunit H3/H4. Transcriptional Gene Silencing (TGS).
RPA2 (not shown)	Replication protein A	Single-stranded DNA-binding protein. Transcriptional Gene Silencing (TGS).
Histone modification		
IBM1 (Figure 2)	Increase in bonsai methylation 1	Histone demethylase.
RTS1 or HDA6 (Figure 2)	Histone deacetylase, RNA-mediated transcriptional silencing	Histone deacetylase.
DNA methylation		
ROS1 (Figure 3)	Repressor of silencing 1	DNA glycosylase-domain protein, cytosine demethylation. Anti-Transcriptional Gene Silencing (antiTGS).

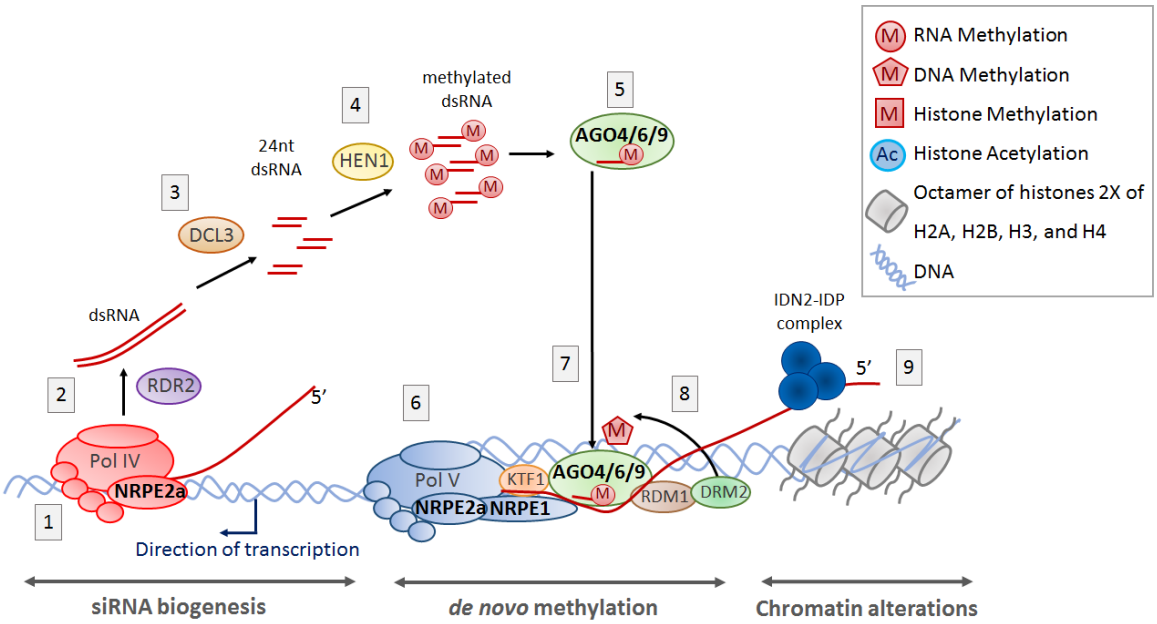


FIGURE 1. Canonical RNA-directed DNA methylation (RdDM) pathway. *siRNA biogenesis*: Polymerase IV (Pol IV) present the subunit NRPE2a and initiates the RdDM pathway [1], forming transcripts that are copied into double-stranded RNA (dsRNA) by RNA-DEPENDENT POLYMERASE 2 (RDR2) [2]. DICER-LIKE 3 (DCL3) cleaves the dsRNA into 24-nucleotide small interfering RNA (siRNA) duplexes [3] that are methylated by HUA ENHANCER 1 (HEN1) [4] and incorporate to ARGONAUTE (AGO4/5 or 6) [5]. *de novo methylation*: Independent of siRNA biogenesis, Pol V-mediate *de novo* methylation [6]. AGO4 binds Pol V transcripts via base-pairing with the siRNA and interact with the NRPE1 carboxyl-terminal domain (CTD) and KTF1 [7]. The RNA-DIRECTED DNA METHYLATION 1 (RDM1) links AGO4 and DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2), which catalyzes *de novo* methylation of DNA [8]. *Chromatin alterations*: Pol V transcripts interact with the IDN2 (INVOLVED IN DE NOVO 2) – IDP (IDN2 PARALOGUE) and result in histone modifications from the RdDM pathway [9] (more details in Figure 2) (Adapted from Matzke & Mosher, 2014). UFRGS, Porto Alegre, RS, Brazil. 2017.

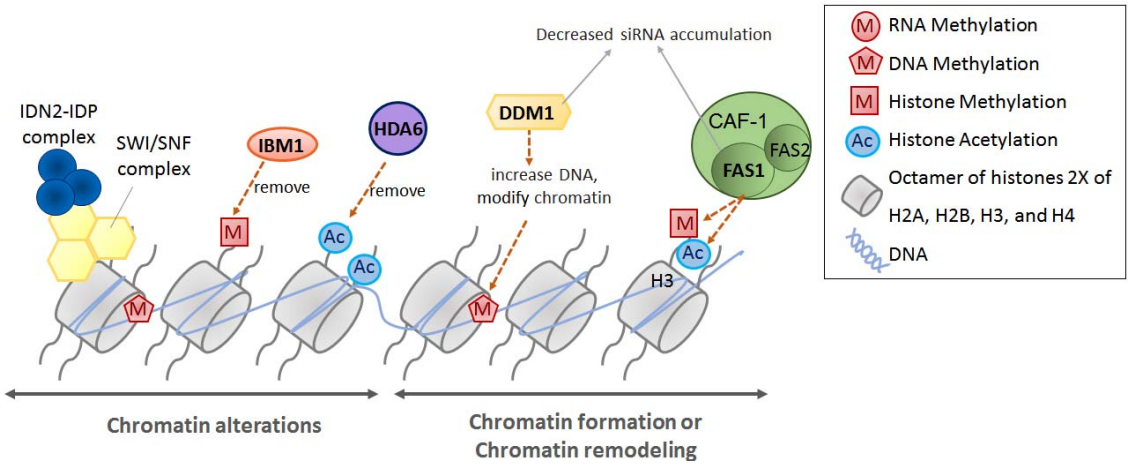


FIGURE 2. Nucleosome positioning is adjusted by the SWI/SNF complex, which interacts with the IDN2–IDP complex that binds Pol V transcripts scaffold. Histone modifications include HISTONE DEACETYLASE 6 (HDA6) that acts to maintain CG methylation and to promote H3K9me by deacetylating histones. INCREASE IN BONSAI METHYLATION 1 (IBM1) is a histone demethylase and removes H3K9me2 from gene bodies. FAS1, belongs to the Chromatin Assembly Factor 1 (CAF-1) complex and cause changes in histone H3 acetylation and methylation. DECREASED-DNA METHYLATION 1 (DDM1) is a SWI2/SNF2 chromatin remodeling that increase DNA methylation (Adapted from Zemach *et al*., 2013; Matzke & Mosher, 2014). UFRGS, Porto Alegre, RS, Brazil. 2017.

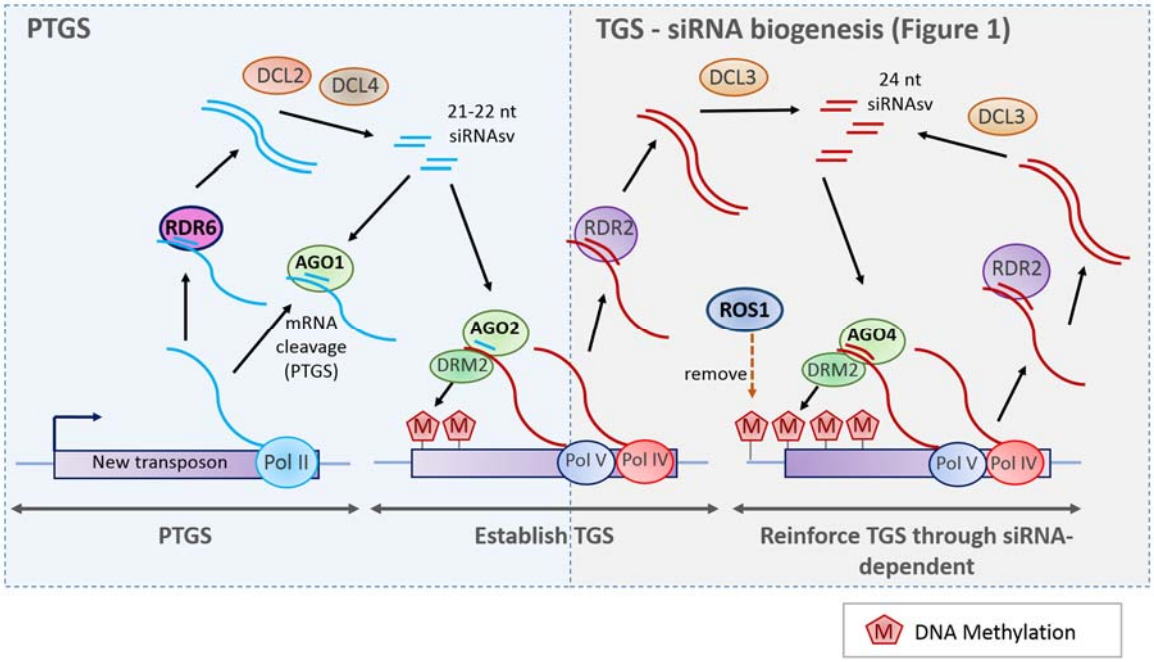


FIGURE 3. Non-canonical Pol II–RDR6-dependent RdDM pathway. A new transposon is initially a target of post-transcriptional gene silencing (PTGS). Some of the transcripts are copied by RNA-DEPENDENT RNA POLYMERASE 6 (RDR6) to produce double-stranded RNAs (dsRNAs) which are processed by DICER-LIKE 2 (DCL2) and DCL4 into 21–22-nucleotide (nt) small interfering RNAs (siRNAs). The ARGONAUTE 1 (AGO1) guide cleavage of transposon transcripts in a classic PTGS pathway. This pathway can provide resources to establish the transcriptional gene silencing (TGS) via RdDM (as showed in Figure 1) and reinforce TGS. The reverse process can be performed by four enzymes, one of them is REPRESSOR OF SILENCING 1 (ROS1) (Adapted from Matzke & Mosher, 2014). UFRGS, Porto Alegre, RS, Brazil. 2017.

The evaluations of leaf length and shoot dry weight were performed ten days after treatment (DAT). The leaf length measurement was based on pictures of the plant shoot followed by analysis with the ImageJ software. For each plant the length of the fifth to the ninth leaves were analyzed according to Farmer *et al*., (2013). The measurement of dry weight was performed as described in the first experiment. Significant differences between the effect occasioned by the herbicide in the mutant and WT was statistically evaluated by ANOVA ($p \leq 0.05$). Data were tested for normality using PROC UNIVARIATE in SAS (SAS Institute, 2004). To satisfy the ANOVA premise for normality, leaf length data were transformed with $X=\sqrt{X}+0.5$. If

statistical significance was found, the means were compared by the Tukey-test (p≤ 0.05).

3 RESULTS AND DISCUSSION

3.1 Herbicides sub-lethal dose

The analysis of variance showed significance for the visual plant injury and dry weight for the herbicides glyphosate, imazethapyr and 2,4-D (Appendix 1 and 2). Therefore, data of *Arabidopsis thaliana* plant injury and dry weight were fitted to the sigmoidal logistic regression model with three parameters (Table 3, Figure 4, and Figure 5). The values of 50% plant injury for glyphosate, imazethapyr and 2,4-D were 39.11, 4.43 and 24.74 g ha^{-1}, respectively (Table 3). Herbicide effects on the shoot dry weight are shown on Figure 5. The GR_{50} value for *Arabidopsis* was 31.31 g ha^{-1} of glyphosate, 4.07 g ha^{-1} of imazethapyr and 27.05 g ha^{-1} of 2,4-D (Table 3).

TABLE 3. Parameters of logistic equation of plant injury and shoot dry weight of *Arabidopsis thaliana* treated with glyphosate, imazethapyr and 2,4-D. UFRGS, Porto Alegre, RS, Brazil. 2017.

Treatment	[1]a	[2]b	[3]X_0	[4]R^2	[5]PI_{50} (g ha^{-1})
			Plant injury		
Glyphosate	77.76**	-1.72*	27.78**	0.99	39.11
Imazethapyr	97.04**	-1.67*	4.27*	0.98	4.43
2,4-D	87.95**	-3.54*	22.89**	0.99	24.74
Treatment	[1]a	[2]b	[3]X_0	[4]R^2	[6]GR_{50} (g ha^{-1})
			Shoot dry weight		
Glyphosate	1.07**	1.49*	31.47*	0.99	31.31
Imazethapyr	1.02**	1.28*	4.07**	0.99	4.07
2,4-D	0.79**	1.45*	27.05*	0.98	27.05

[1]a: the difference between the maximum and minimum points of the curve;
[2]b: the slope of the curve;
[3]X_0: the dose which provides 50% of the response of the variable;
[4]R^2: coefficient of determination;
[5]PI_{50}: dose required to provide 50% plant injury, obtained by replacing “y” of equation with 50;
[6]GR_{50}: dose required to reduce shoot weight by 50%; obtained by replacing “y” of equation with 50% of the shoot dry weight;
** p<0.01; *p<0.05; NS non-significant;

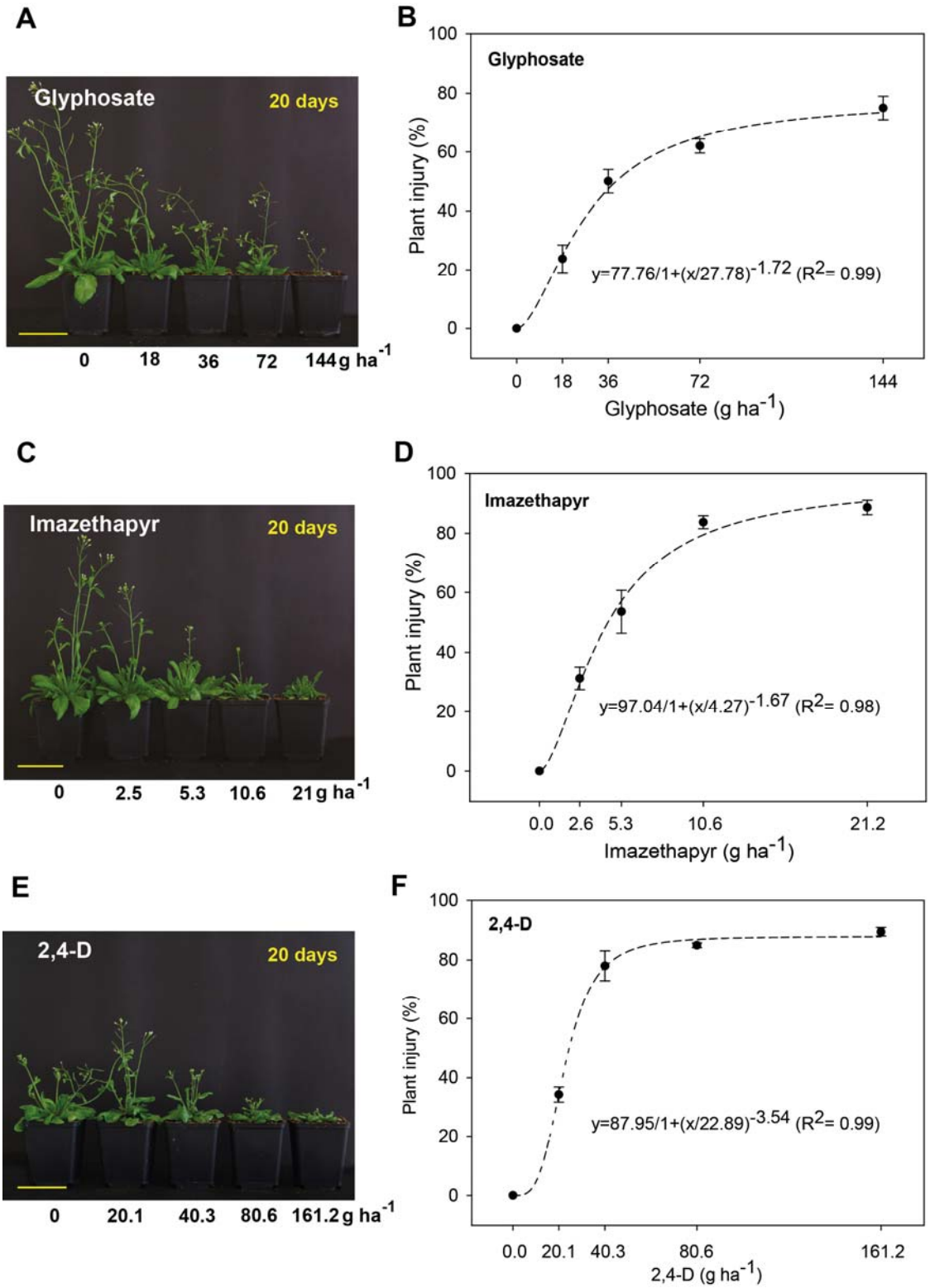


FIGURE 4. (A, C, and E) Visual effect of *Arabidopsis thaliana* 20 days after treatment (DAT) of glyphosate, imazethapyr and 2,4-D, respectively. Yellow bars correspond to 7 cm. (B, D, and F) Plant injury (%) of *A. thaliana* treated with glyphosate, imazethapyr and 2,4-D, respectively, at 20 DAT. The graphs were plotted with the average and the vertical bars indicate the confidence interval. UFRGS, Porto Alegre, RS, Brazil. 2017.

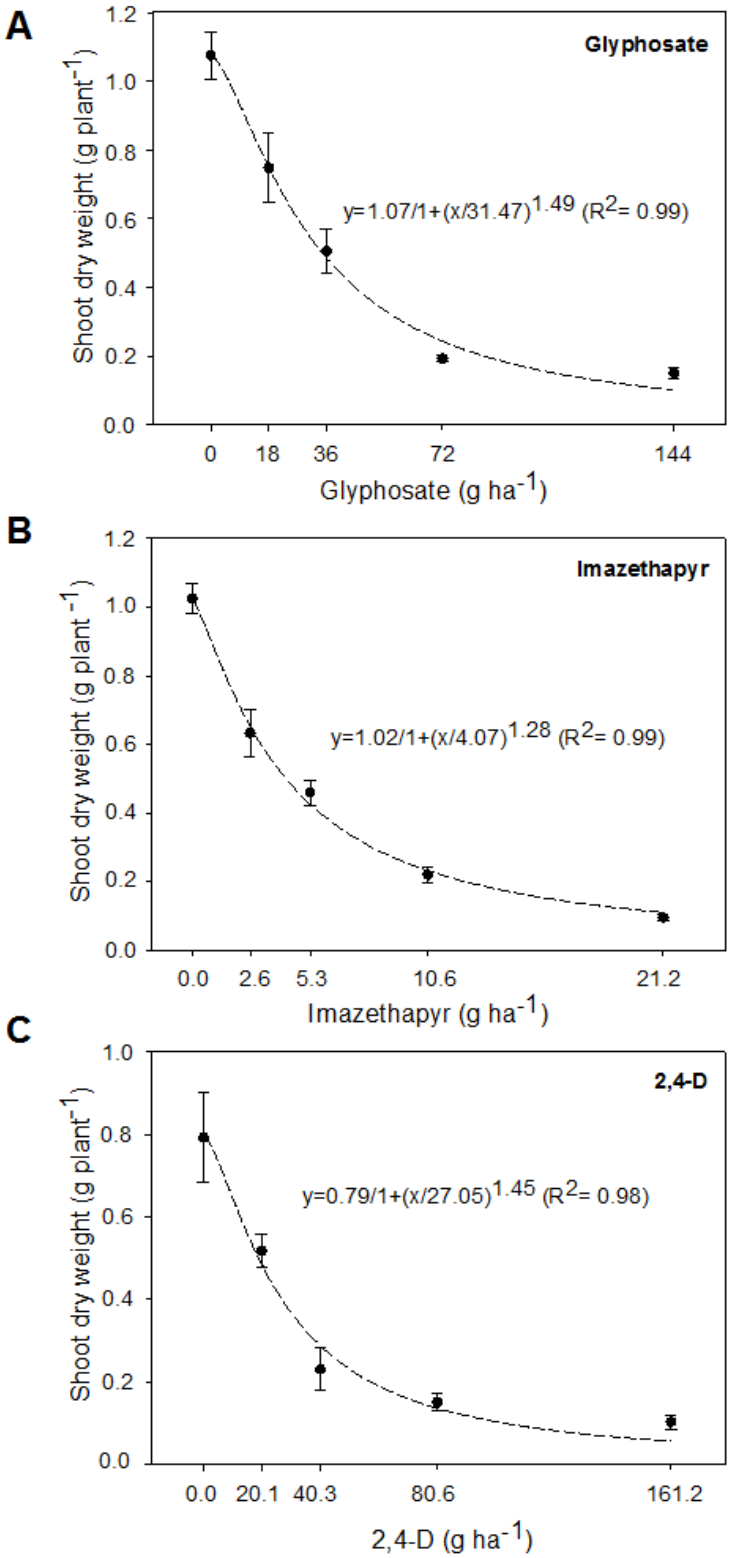


FIGURE 5. Shoot dry weight (g plant-1) of *Arabidopsis thaliana* 20 days after treatment (DAT) of glyphosate (A), imazethapyr (B) and 2,4-D (C). The graphs were plotted with the average and the vertical bars indicate the confidence interval. UFRGS, Porto Alegre, RS, Brazil. 2017.

The sub-lethal dose for each herbicide was selected based on the dose treatment that provided drastic plant injury (Figure 4B, D and F) and biomass reduction (between 65-85%) (Figure 5), but that still resulted in seed production (Figure 4A, C and E). Based on that, the selected doses for glyphosate, imazethapyr and 2,4-D were 72, 10.6 and 40.3 g ha^{-1}, respectively, that correspond to 10%, 10% and 5%, respectively, of the field application rate. Other study also identified the sub-lethal dose for glyphosate as 10% of the field rate for *Brassica napus*, *Brassica rapa*, *Brassica nigra* and *Brassica juncea*, all plants belonging to the same family as *A. thaliana* (Londo *et al*., 2014). Although the values found in the present study and by Londo *et al*. (2014) are close, it is desired to obtain the herbicide sub-lethal dose considering the used genotypes, herbicide application and the experimental plant growth conditions.

The effect of herbicide sub-lethal dose simulates field situations where plants may receive reduced rates of herbicide caused by failures in herbicide application, plant canopy protection or herbicide drift. These plants will suffer the herbicide effect but will not die and as consequence may alter the composition of weedy or native plant communities (Pfleeger *et al*., 2012; Londo *et al*., 2014). Furthermore, herbicide sub-dose is described to be involved in evolution of non-target-site resistance (NTSR) mainly by accumulation of important alleles for herbicide detoxification in plants (Délye *et al*., 2011). Diclofop-methyl applied in 10% of the recommended field application rate increased the level of resistance in *L. rigidum* progeny after three cycles of selection with sub-lethal dose (Neve & Powles, 2005). However, the study does not describe the mechanism related with the increasing level of resistance. In the present study, the sub-lethal doses of herbicides are used to evaluate possible effect on epigenetic processes in *A. thaliana*.

3.2 Effect of herbicides in transcriptional gene silencing (TGS)

Gene silencing in plants can occur in transcriptional (Wolffe & Matzke, 1999) and post-transcriptional context (Fagard & Vaucheret, 2000). Methylation of the gene (or transgene) promoter correlates with transcriptional gene silencing (TGS) while methylation of the coding sequence is associated with post-transcriptional gene silencing (PTGS) (Morel *et al*., 2000). The transgenic line L5 is homozygous for an insert carrying multiple and methylated copies of a transgene consisting of *P35S:GUS* which makes possible the analysis of the effect of specifics stresses on TGS (Morel *et al*., 2000; Probst *et al*., 2004). According to the histochemical staining, the sub-lethal doses of the herbicides glyphosate, imazethapyr and 2,4-D leads to global alterations of DNA methylation at 48 hours after treatment (Figure 6A). Quantitative RT-PCR revealed GUS expression of 12.2, 6.7 and 8.7 times higher in plants treated with herbicides glyphosate, imazethapyr and 2,4-D, respectively, in comparison with the control treatment ($p < 0.05$) (Figure 6B).

Although the tested herbicides significantly changed the amount of GUS transcript, it is not as high as the positive control L5 *ddm1* (Figure 6B). Quantitative RT-PCR revealed that GUS expression increased significantly by 1,878.8 times compared with control plants L5 ($p<0.05$) (Figure 6B). According to Pecinka *et al*. (2010) *Arabidopsis* present numerous repeats in the genome that are especially proper for studying epigenetic regulatory mechanisms. Generally, expression of repeats is suppressed by TGS, and they represent suitable indicators to score the epigenetic regulation under stress conditions, through high levels of DNA methylation, inactive chromatin marks, and chromatin compaction (Soppe *et al*., 2000).

Plants originated from crossing the line L5 with the epigenetic *A. thaliana ddm1* mutant were used as positive control because the lack of DDM1 (DECREASE

IN DNA METHYLATION 1) can release TGS and methylation of various transcriptionally silenced loci (Morel *et al*., 2000), causing an approximately 70% reduction in genomic 5-methylcytosine content in *Arabidopsis* mutant (Vongs *et al*., 1993). In *ddm1*, DNA methylation is reduced at all of CG, CHG and CHH sites (Yan *et al*., 2016) and it can explain the high GUS expression presented by L5 *ddm1* plants in this experiment (Figure 6A and B).

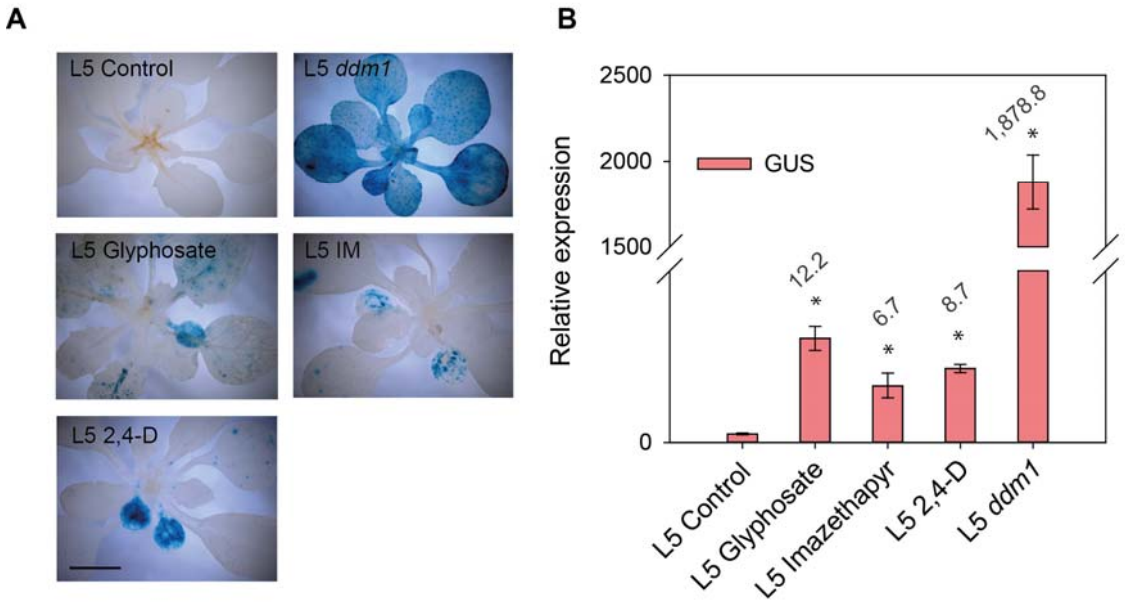


FIGURE 6. (A) Representative GUS-stained shoot of the L5 plants 48 hours after treatment with glyphosate, imazethapyr (IM) and 2,4-D, non-treated plant (L5 control) and L5x*ddm1* mutant, used as positive control. (B) Relative expression by qRT-PCR analysis of TGS targets (GUS) after 48 hours of treatment. Error bars indicate standard deviation of four replications measurement. Statistically significant differences between treatments contrasted with L5 control are indicated by asterisks (*t* test, $p < 0.05$). UFRGS, Porto Alegre, RS, Brazil. 2017.

Arabidopsis plants exposed to the stresses of salinity, drought, and UV radiation destabilized TGS at some specific loci (Pecinka *et al*., 2010; Mirouze & Paszkowski, 2011). However, the TGS destabilization can be transient, re-establishing the silencing within 2 days after the stress ceased (Pecinka *et al*., 2010). A study related to heat stress in *A. thaliana* L5 showed strong GUS

expression achieved with heat stress for 30 hours at 37°C, where qRT-PCR revealed more than 10,003 times induction of GUS transcript compared with plants in control conditions (Pecinka *et al*., 2010). However, when plants were exposed for a short period in heat stress (three hours at 37°C) no visible effect in GUS expression was observed (Pecinka *et al*., 2010). This indicates that *A. thaliana* presents repetitive elements under epigenetic regulation by TGS at normal conditions and it depends on the exposure of the stress condition to become activated. The results of the present study indicated that the herbicides glyphosate, imazethapyr and 2,4-D treatment leads to some change in TGS. However, it is not intense as in *ddm1* mutant, indicating that herbicides can change specific epigenetics pathways and possibly these results may also vary according to the dose and herbicide mode of action.

3.3 Evaluation of specific epigenetic pathways

The ANOVA of leaf length and shoot dry weight showed significant interaction between the studies and the different genotypes analyzed (*A. thaliana* epigenetic mutants and WT) (Appendix 3 and 4). The leaf length and shoot dry weight of the mutants *upf1-5, ago6*, *nrpe1*, *nrpe2a* and *rpa2* did not differed statistically from the WT, or did not presented consistent results among the two studies for herbicides glyphosate, imazethapyr and 2,4-D (Figure 7 and 8). In *A. thaliana*, Argonaute (AGO) AGO4, AGO6 and AGO9 act in canonical RdDM and/or TGS, AGO4 interacts with the Pol V subunit Nuclear RNA Polymerase E1 (NRPE1) (Figure 1) that is required for RdDM (El-Shami *et al*., 2007). Additionally, the NRPE2 is the shared second largest subunit of Pol IV and Pol V (Figure 1). The similar sensibility for the herbicides glyphosate, imazethapyr and 2,4-D observed for the mutants *ago6*, *nrpe1* and *nrpe2a* compared to the WT (Figure 7 and 8) shows that the involvement of

RdDM components is not required for the detoxification of herbicides glyphosate, imazethapyr and 2,4-D.

The leaf length and shoot dry weight analysis for the herbicides glyphosate and 2,4-D showed that *rdr6* mutant did not differ significantly from the WT, or did not show consistent phenotype during the analysis (Figure 7 and 8). However, this mutant presented increased susceptibility in comparison with WT for the herbicide imazethapyr (Figure 7A, C and 8B). The RDR6 (RNA-DEPENDENT RNA POLYMERASE 6) is involved in non-canonical Pol II–RDR6-dependent RdDM pathway (Figure 3). This pathway normally is related to a newly inserted transposon that is originally a target of post-transcriptional gene silencing (PTGS). The transposons are active and transcribed by RNA polymerase II (Pol II). Some of those transcripts are copied by RDR6 providing small interfering RNAs (siRNAs) that favors the establishment of RdDM and can also guarantee stability of TGS (Matzke & Mosher, 2014). The different effect of the herbicides on the *rdr6* mutant can be related with the fact that herbicides glyphosate, imazethapyr and 2,4-D have different modes of action and probably require different detoxification genes, plant transport or other metabolizing processes.

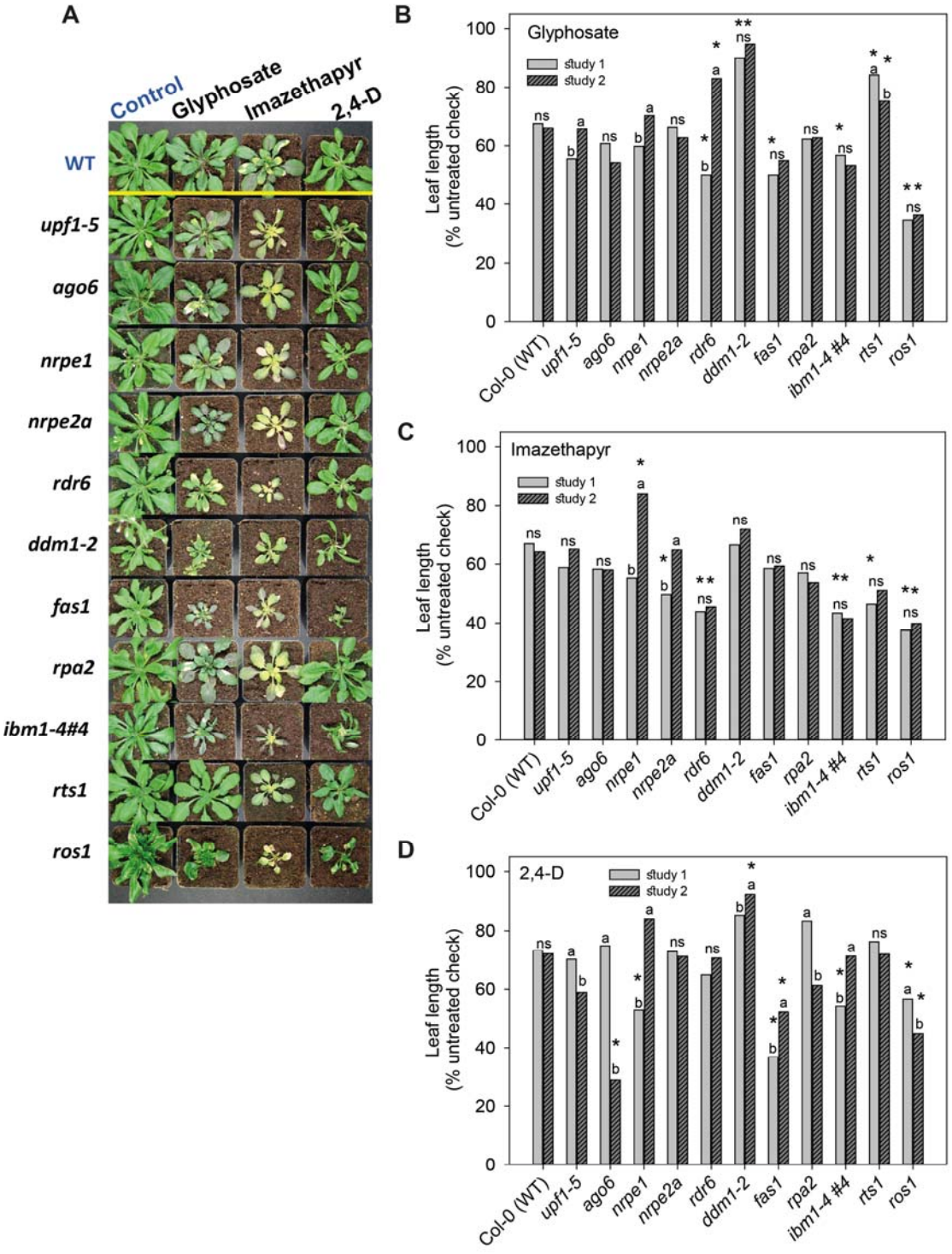


FIGURE 7. (A) Visual effect of *Arabidopsis thaliana* wild type (WT) and epigenetic mutants, at 10 days after treatment (DAT) of glyphosate, imazethapyr, 2,4-D, and non-treated plants. (B, C and D) Leaf length (% untreated check). Means of studies (1 and 2) followed by different letter differ significantly after Tukey ($p<0.05$); ns=non-significant. Mutant followed by asterisk differ significantly and presented different letter from WT according to Tukey ($p<0.05$). UFRGS, Porto Alegre, RS, Brazil. 2017.

The herbicide glyphosate reduced approximately 15% of the leaf length on the mutant *ddm1* in comparison with the WT plants that reduced approximately 35% (Figure 7B). This result agrees with the effect observed for shoot dry weight (Figure 8A). For the herbicide imazethapyr *ddm1* did not differ from WT for leaf length and shoot dry weight (Figure 7C and 8B). For the herbicide 2,4-D, *ddm1* showed lower reduction of leaf length after herbicide treatment in comparison to WT in the second study (Figure 7D). Additionally, the shoot dry weight analysis *ddm1* differed statistically from WT, showing increase in biomass for herbicide 2,4-D (Figure 8C). Together with *ddm1* and *fas1*, the *rpa2* is also classified as chromatin formation or chromatin remodeling and acts in TGS. The RPA2 is a REPLICATION PROTEIN A2 and mutation in *RPA2* declining the siRNA accumulation and/or chromatin modification (Elmayan *et al*., 2005). It is important to emphasize that even if these mutants are involved in TGS, they can affect a different subset of targets (Zilberman *et al*., 2003).

Although RdDM contributes to the transcriptional repression of TEs (Elmayan *et al*., 2005; Mirouze & Paszkowski, 2011), it has a smaller role in comparison with DDM1 pathway. In *A. thaliana*, *ddm1* mutant presented a 58%, 57% and 32% overall reduction of CG, CHG and CHH methylation, respectively (Zemach *et al*., 2013). The DDM1 (DECREASED DNA METHYLATION 1) encodes a nucleosome remodeling ATPase (Brzeski & Jerzmanowski, 2003) and is involved with chromatin formation or chromatin remodeling (Figure 2). The *ddm1* mutation induces hypomethylation of repeated sequences (Kakutani *et al*., 1996). Beyond of genome-wide reduction of DNA methylation the lack of DDM1 causes reduction in H3K9me2, transcriptional activation of repetitive elements and changes the expression of numerous genes (Pikaard & Scheid, 2014). Thus, it is suggested that genes that

were demethylated in the absence of DDM1 are important to cause a slight increase in the tolerance of the *ddm1* mutant to the herbicides glyphosate and 2,4-D.

The mutant *fas1* differed statistically from the WT only for the herbicide 2,4-D (Figure 7A, D and 8C). FAS1, together with FAS2, belongs to the two large subunits of Chromatin Assembly Factor 1 (CAF-1) complex (Figure 2) (Ramirez-Parra & Gutierrez, 2007). Loss of FAS1 is accompanied by up-regulation of the expression of a subset of genes. In all these evaluations, the obtained result is the consequence of selective epigenetic changes in histone H3 acetylation and methylation in their promoters and not of global changes in chromatin remodeling (Ramirez-Parra & Gutierrez, 2007).

The mutants related to histone modification *rts1* and *ibm1#4* were also affected by the herbicide treatments. The mutant *rts1* was statistically different from the WT for the herbicide glyphosate, showing increase of approximately 5 to 20% in leaf length and biomass compared to WT (Figure 7A, B and 8A). RTS1 is a RNA-mediated transcriptional silencing (or HDA6 - HISTONE DEACETYLASE 6) that interact directly and act together with MET1 to maintain CG methylation and to promote H3K9me by histone deacetylation (Figure 2) (Liu *et al*., 2012). A study with *A. thaliana* revealed that TEs were transcriptionally reactivated in *hda6* mutant, associated with elevated histone H3 and H4 acetylation as well as increased levels of H3K4Me3 and H3K4Me2 (Liu *et al*., 2012). It is suggested that HDA6 silences the TEs by regulating histone acetylation and methylation as well as the DNA methylation status of the TEs, indicating that HDA6 is required for the maintenance of TGS (Probst *et al*., 2004). This information corroborates with results obtained in the present study and can indicate that important genes for detoxification of glyphosate can be silenced by HDA6 pathway.

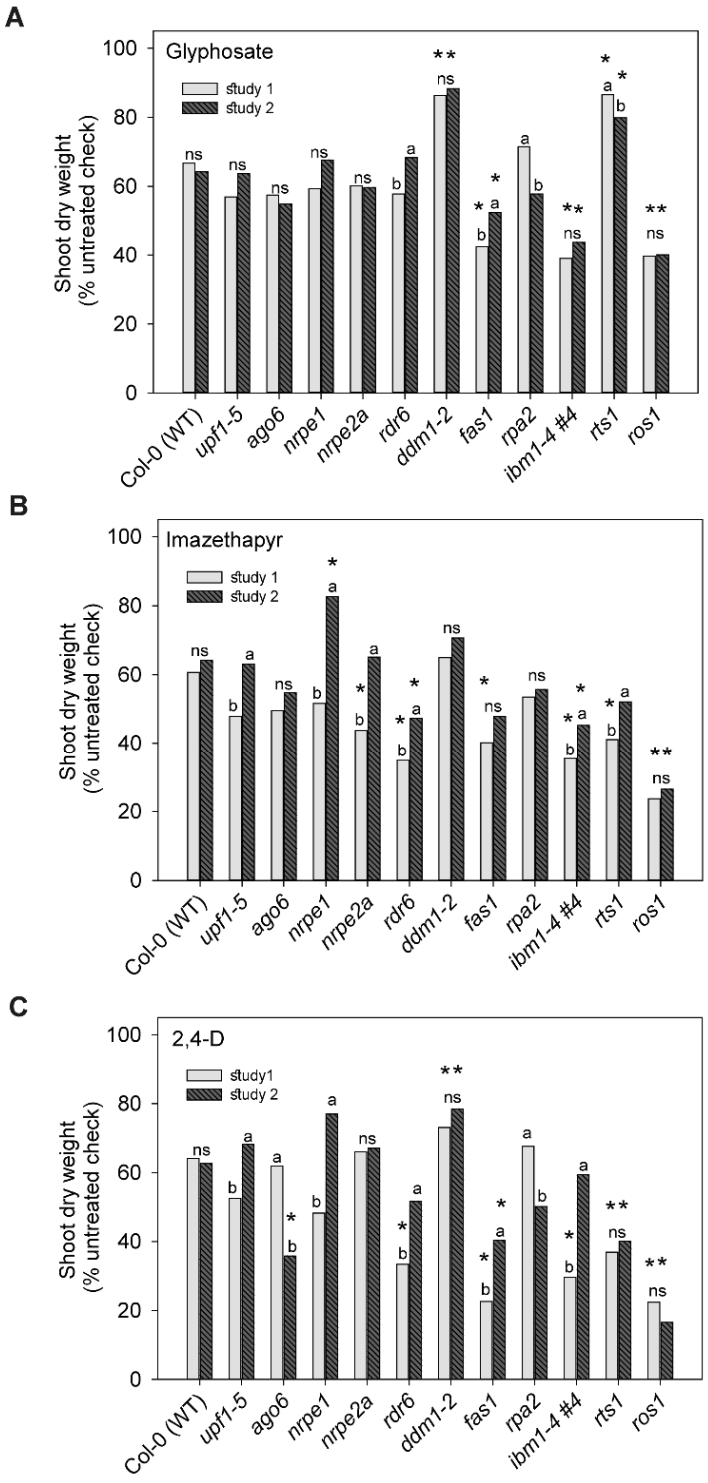


FIGURE 8. Shoot dry weight (% untreated check) of *Arabidopsis thaliana* wild type (WT) and epigenetic mutants, at 10 days after treatment (DAT) of glyphosate (A), imazethapyr (B) and 2,4-D (C). Means of studies one and two followed by different letter differ significantly according with Tukey ($p<0.05$); ns=non-significant. Mutant followed by asterisk differ significantly from WT according to Tukey ($p<0.05$). UFRGS, Porto Alegre, RS, Brazil. 2017.

The *ibm1#4* mutant differed statistically from WT only for the herbicide imazethapyr, according to the results for leaf length and shoot dry weight (Figure 7A, C and 8B). The JmjC protein, increase in BONSAI METHYLATION 1 (IBM1), counteracts H3K9 methylation and CHG DNA methylation (Figure 2). A study with *A. thaliana* revealed that IBM1 mutation induced extensive hypermethylation in thousands of genes (Miura *et al*., 2009). The hypermethylation leads to drastically decrease in genes transcription. Probably, according to results obtained in the present study, the IBM1 can be involved with expression regulation of genes that are important for imazethapyr detoxification.

The *ros1* mutant reduced the leaf length and shoot dry weight for all tested herbicides comparted to WT (Figure 7 and 8). The visual injury analysis also confirms the increase in herbicides susceptibility in *ros1* (Figure 7A). The results discussed above indicate that glyphosate, imazethapyr and 2,4-D affected some mutants related to with DNA and specific histone methylation modifications (Figure 7 and 8). However, the consistent results for these herbicides were presented by *ros1*. The *ros1* presented approximately 20%, 30% and 20% reduction in leaf length for herbicides glyphosate, imazethapyr, and 2,4-D, respectively, compared to WT (Figure 7).

In order to confirm the results obtained for *ros1* the experiment was repeated independently three times more. These evaluations were performed only with glyphosate and imazethapyr because there is more information about the mode of action and mechanisms of herbicide-resistant for these compounds. The ANOVA of leaf length and shoot dry weight indicated the occurrence of significance of the studies and genotypes (Appendix 5 and 6). The obtained results (Figure 9) were similar to previous outcomes and confirmed the high susceptibility presented by *ros1* mutant. It suggests that ROS1 is important for detoxification of sub-lethal doses of

glyphosate and imazethapyr, for this reason *ros1* was selected for subsequent molecular studies (Chapter 2).

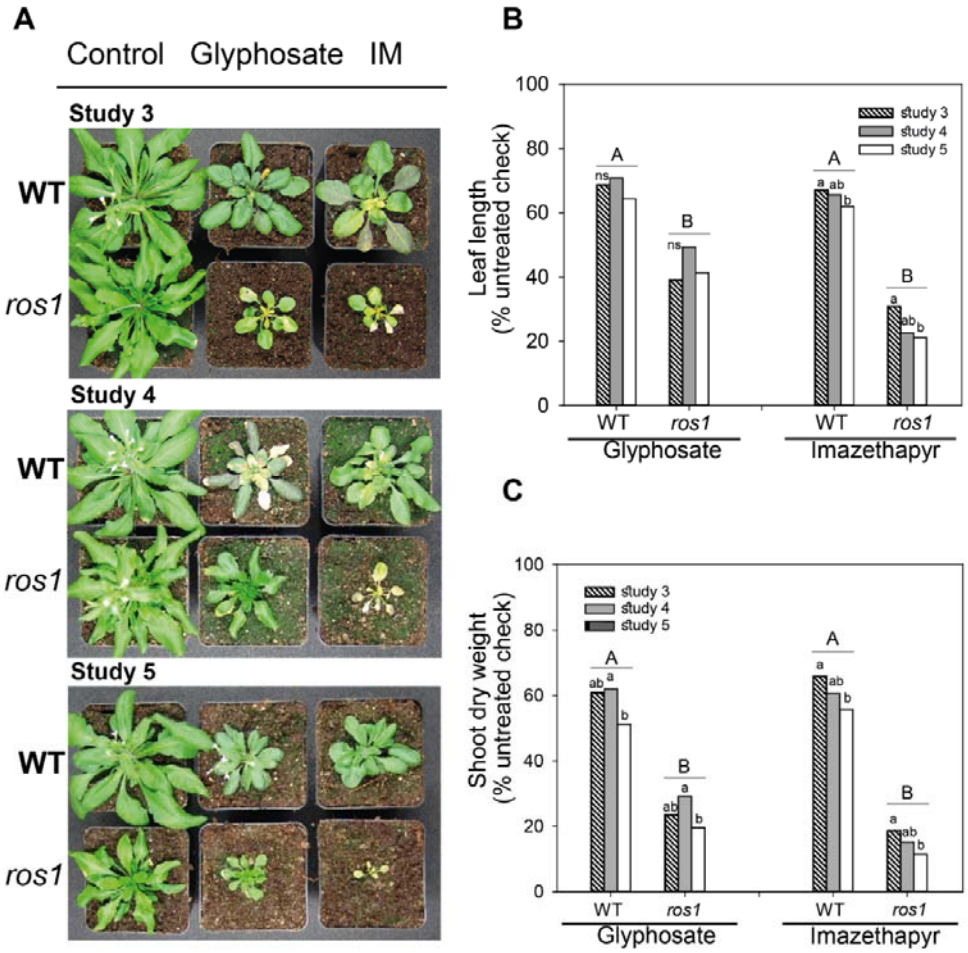


FIGURE 9. (A) Visual effect (B) leaf length and (C) shoot dry weight of *Arabidopsis thaliana* wild type (WT) and *ros1* mutant, in study 3, 4 and 5, 10 days after application of herbicide of glyphosate and imazethapyr (IM). Means followed by different letter (lower case for studies; capital for genotypes) differ significantly according to Tukey ($p<0.05$); ns=non-significant. UFRGS, Porto Alegre, RS, Brazil. 2017.

The ROS1 is a 5-methylcytosine glycosylases, a repressor of TGS (Figure 3). Studies show that ROS1 plays an important role in the dynamic processes of DNA demethylation, responding to developmental and environmental cues that allows plant epigenome plasticity (Zhu *et al*., 2007). In transgenic tobacco, *AtROS1* overexpression showed importance of ROS1 during salt stress (Bharti *et al*., 2015). Loss-of-function mutations in *ROS1* result in hypermethylation of the RD29A

promoter and silencing of the RD29A-promoter-driven luciferase transgene (Gong *et al*., 2002). In *Arabidopsis*, ROS1 is important for avoiding DNA hypermethylation at thousands of specific genomic loci. However, how ROS1 and other demethylation enzymes target to specific genomic regions is poorly understood (Qian *et al*., 2012).

A hypothetical proposition of the effect of ROS1 on the activation of genes important to herbicide detoxification is presented in the Figure 10. ROS1 acts in DNA demethylation, which permits the expression of specific genes, induced by stimulus perception to adaptive plant behavior (Figure 10A). The stimulus could be related with abiotic stresses such as high temperature, drought, and salt or by a stress occasioned by the herbicide application, as proposed in this study. In *ros1* methylation patterns are altered and in this model, important genes to herbicide detoxification, such as P450, GSTs, ABC transporters are not demethylated after herbicide application (Figure 10B). It is emphasized that methylation on the promoter region normally represses the gene transcription.

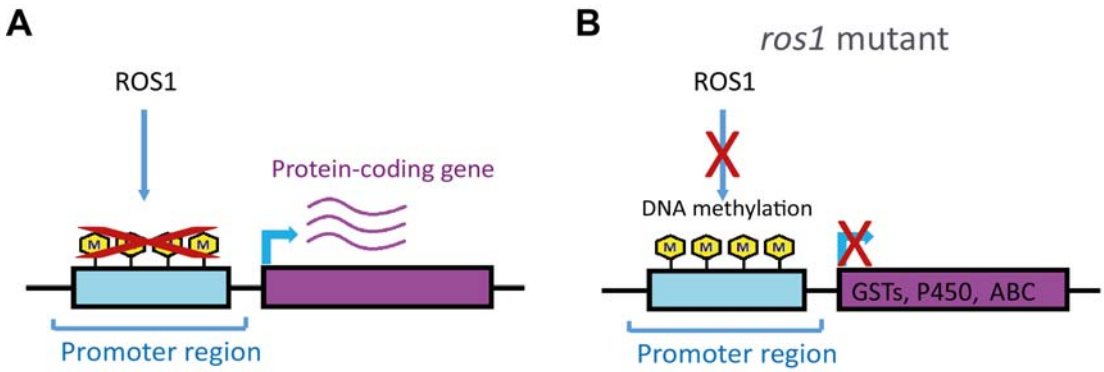


FIGURE 10. (A) Schematic representation of ROS1 (REPRESSOR OF SILENCING 1) acting as cytosine demethylation. (B) ROS1 can be important for activation of genes important to herbicide detoxification. UFRGS, Porto Alegre, RS, Brazil. 2017.

Epigenetic control of gene expression involves dynamic regulation of DNA methylation and histone modification marks, mainly under stress conditions. The present study revealed that some epigenetic pathways could be important for

herbicide detoxification, although many of them are likely very specific for a given herbicide. The differences find out for the effect of different epigenetic pathway and herbicide, as mentioned above, is probably because the herbicides glyphosate, imazethapyr and 2,4-D belong to different herbicides mode of action.

The herbicide glyphosate is a specific inhibitor of the chloroplast enzyme EPSPS. The inhibition of EPSPS activity disrupts the shikimate pathway and inhibits aromatic amino acid production, ultimately causing plant death (Dill, 2005). While imazethapyr is a potent inhibitor of ALS, thereby stopping synthesis of the branched-chain amino acids valine, leucine, and isoleucine, with subsequent plant death (Sales *et al*., 2008). Additionally, the 2,4-D is an auxinic herbicide and the mode of action, and subsequently, the resistance mechanisms in weeds are not fully understood (Mithila *et al*., 2011).

The differences in mode of action can contribute to the herbicide resistance evolution. For herbicides that inhibit EPSPS besides the cases of point mutation of target enzyme, the resistance was related to some weed species as the result of an increased number of copies of the target gene (TSR) (Gaines *et al*., 2010; Vila-Aiub *et al*., 2014). Among the mechanisms of NTSR for EPSPS, reduced absorption or reduced translocation in the plant (Vila-Aiub *et al*., 2012) and sequestration into vacuoles were described (Ge *et al*., 2010). The mechanism of herbicide resistance of ALS-inhibitors is normally involved with point mutation on the target enzyme (TSR), but detoxification processes mediated by cytochrome P450 and GST also occur (NTSR) (Cummins *et al*., 2013; Iwakami *et al*., 2014; Saika *et al*., 2014). While for auxinic herbicides new findings including nuclear auxin receptors F-box proteins, influx and efflux carriers and plasma membrane bound receptors have provided basic clues as to the molecular mechanisms of resistance of these herbicides (Krecek *et al*., 2009; Song, 2014). New recent findings indicate large diversity and

complexity of herbicide resistance mechanisms. These results suggest involvement of epigenetic regulation on the herbicide effect and potentially on the evolution herbicide resistance on weeds.

4 CONCLUSIONS

The obtained results indicate that application of the herbicides glyphosate, imazethapyr and 2,4-D leads to global alterations of DNA methylation presenting action in transcriptional gene silencing (TGS). The alteration in TGS is not intense as verified in *ddm1* mutant, suggesting that these herbicides change the TGS using specific epigenetic pathways in *A. thaliana*.

A. thaliana susceptibility to the herbicides glyphosate, imazethapyr and 2,4-D is affect by the lack of specific enzymes that belong to epigenetic pathways involved to DNA methylation, non-coding RNA and/or histone modifications. However, the effect on each mutant was specific for each herbicide. The lack of proteins DDM1, RST1 and ROS1 respectively involved to chromatin remodeling, histone modification and DNA methylation mechanisms interfere with the effect of the herbicide glyphosate. While for imazethapyr, the lack of expression of the proteins RDR6, IBM1 and ROS1 results in high susceptibility to imazethapyr. Additionally, the effect of 2,4-D in *A. thaliana* change when proteins involved with chromatin remodeling and DNA methylation mechanisms are not expressed. The deficiency of FAS1 and ROS1 promote increase in A. *thaliana* susceptibly to 2,4-D. The mutant *ros1* was the unique evaluated mutant that alters the effect for all tested herbicides. The lack of ROS1 increases the susceptibility to the herbicides.

The results indicate that the evaluated herbicides can change specific epigenetic pathways according to the herbicide and suggest regulation of specific

genes. Additionally, demethylation process may be important for *A. thaliana* tolerance to the herbicides glyphosate, 2,4-D and, especially to imazethapyr.

5 REFERENCES

ALLIS, C.D. JENUWEIN, T. The molecular hallmarks of epigenetic control. **Nature Reviews Genetics**, London, v.17, n.8, p.487-500, 2016.

BHARTI, P. et al. AtROS1 overexpression provides evidence for epigenetic regulation of genes encoding enzymes of flavonoid biosynthesis and antioxidant pathways during salt stress in transgenic tobacco. **Journal of Experimental Botany**, Oxford, v.66, n.19, p.5959-5969, 2015.

BRZESKI, J.; JERZMANOWSKI, A. Deficient in DNA methylation 1 (DDM1) defines a novel family of chromatin-remodeling factors. **Journal of Biological Chemistry**, Bethesda, v.278, n.2, p.823-828, 2003.

BUSTIN, S.A. et al. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. **Clinical Chemistry**, Washington, v.55, n.4, p.611-622, 2009.

CAO, X.F. et al. Role of the DRM and CMT3 Methyltransferases in RNA-directed DNA methylation. **Current Biology**, Cambridge, v.13, n.24, p.2212-2217, 2003.

CARVALHO, S.J. P. et al. Curvas de dose-resposta para avaliação do controle de fluxos de emergência de plantas daninhas pelo herbicida imazapic. **Planta Daninha**, Viçosa, v.23, n.3, p.535-542, 2005.

COKUS, S.J. et al. Shotgun bisulphite sequencing of the Arabidopsis genome reveals DNA methylation patterning. **Nature**, London, v.452, n.7184, p.215-219, 2008.

CUMMINS, I. et al. Key role for a glutathione transferase in multiple-herbicide resistance in grass weeds. **Proceedings of the National Academy of Sciences of the United States of America**, v.110, n.15, p.5812-5817, 2013.

DÉLYE, C. et al. Non-target-site-based resistance should be the centre of attention for herbicide resistance research: Alopecurus myosuroides as an illustration. **Weed Research**, Malden, v.51, n.5, p.433-437, 2011.

DÉLYE, C.; JASIENIUK, M.; LE CORRE, V. Deciphering the evolution of herbicide resistance in weeds. **Trends in Genetics**, London, v.29, n.11, p.649-658, 2013.

DÉLYE, C. Unravelling the genetic bases of non-target-site-based resistance (NTSR) to herbicides: a major challenge for weed science in the forthcoming decade. **Pest Management Science**, Malden, v.69, n.2, p.176-187, 2013.

DILL, G.M. Glyphosate-resistant crops: history, status and future. **Pest Management Science**, Malden, v.61, n.3, p.219-224, 2005.

DU, J. et al., DNA methylation pathways and their crosstalk with histone methylation. **Nature Reviews**, London, v.16, n.1, p.519-532, 2015.

ELMAYAN, T.; PROUX, F.; VAUCHERET, H. Arabidopsis RPA2: A genetic link among transcriptional gene silencing, DNA repair, and DNA replication. **Current Biology**, Cambridge, v.15, n.21, p.1919-1925, 2005.

EL-SHAMI, M., et al. Reiterated WG/GW motifs form functionally and evolutionarily conserved ARGONAUTE-binding platforms in RNAi-related components. **Genes & Development**, Woodbury, v.21, n.20, p.2539-2544, 2007.

FAGARD, M.; VAUCHERET, H. (Trans)gene silencing in plants: How many mechanisms? **Annual Review of Plant Physiology and Plant Molecular Biology**, Palo Alto, v.51, n.1, p.167-194, 2000.

FARMER, E.; MOUSAVI S.; LENGLET A. Leaf numbering for experiments on long distance signalling in Arabidopsis. **Protocol exchange Nature**, [On-line], 2013.

FINNEGAN, E. J. et al. DNA methylation and the promotion of flowering by vernalization. **Proceedings of the National Academy of Sciences of the United States of America**, Collins, v.95, n.10, p.5824-5829, 1998.

GAINES, T.A. et al. Gene amplification confers glyphosate resistance in Amaranthus palmeri. **Proceedings of the National Academy of Sciences of the United States of America**, Collins, v.107, n.3, p.1029-1034, 2010.

GE, X. et al. Glyphosate-resistant horseweed made sensitive to glyphosate: low-temperature suppression of glyphosate vacuolar sequestration revealed by 31P NMR. **Pest Management Science**, Malden, v.67, n.10, p.1215-1221, 2010.

GONG, Z.H. et al. ROS1, a repressor of transcriptional gene silencing in Arabidopsis, encodes a DNA glycosylase/lyase. **Cell**, Cambridge, v.111, n.6, p.803-814, 2002.

GREENBERG, M.V. et al. Interplay between active chromatin marks and RNA-directed DNA methylation in Arabidopsis thaliana. **Plos Genetics**, San Francisco, v.9, n.11, p. 1-11, 2013.

GRESSEL, J. Evolving understanding of the evolution of herbicide resistance. **Pest Management Science**, Malden, v.65, n.11, p.1164-1173, 2009.

GROSSNIKLAUS, U. et al. Transgenerational epigenetic inheritance: how important is it? **Nature Reviews Genetics**, London, v.14, n.11, p.228-235, 2013.

HEAP, I. **The International survey of herbicide resistant weeds.** 2017. Available in:<www.weedscience.com>. Accessed in: 3 jan. 2017.

HUANG, J.J. et al. Roles of DNA methyltransferases in Arabidopsis development. **African Journal of Biotechnology**, Victoria Island, v.9, n.50, p.8506-8514, 2010.

IWAKAMI, S. et al. Cytochrome P450 CYP81A12 and CYP81A21 Are Associated with Resistance to Two Acetolactate Synthase Inhibitors in Echinochloa phyllopogon. **Plant Physiology**, Rockville, v.165, n.2, p.618-629, 2014.

KAKUTANI, T. et al. Meiotically and mitotically stable inheritance of DNA hypomethylation induced by ddm1 mutation of Arabidopsis thaliana. **Genetics**, Baltimore, v.151, n.2, p.831-838, 1999.

KIM, J.M. et al. Alterations of lysine modifications on the histone H3 N-tail under drought stress conditions in Arabidopsis thaliana. **Plant and Cell Physiology**, Oxford, v.49, n.10, p.1580-1588, 2008.

KRECEK, P., et al. The PIN-FORMED (PIN) protein family of auxin transporters. **Genome Biology**, London, v.10, n.12, p.1-10, 2009.

LARIONOV, A.; KRAUSE, A.; MILLER, W. A standard curve based method for relative real time PCR data processing. **Bmc Bioinformatics**, Oxford, v.6, n.1 p.1-16, 2005.

LIU, X. C. et al. HDA6 Directly Interacts with DNA Methyltransferase MET1 and Maintains Transposable Element Silencing in Arabidopsis. **Plant Physiology**, Rockville, v.158, n.1, p.119-129, 2012.

LONDO, J.P. et al. Sub-lethal glyphosate exposure alters flowering phenology and causes transient male-sterility in Brassica spp. **Bmc Plant Biology**, London, v.14, n.70, p.1-10, 2014.

LU, Y.C. et al. Genome-wide identification of DNA methylation provides insights into the association of gene expression in rice exposed to pesticide atrazine. **Nature Scientific Reports**, London, v.6, n.1, p.1-15, 2016.

LUO, M., et al. Chromatin modifications and remodeling in plant abiotic stress responses. **Biochimica Et Biophysica Acta-Gene Regulatory Mechanisms**, Amsterdam, v.1819, n.2, p.129-136, 2012.

MATZKE, M.A.; BIRCHLER, J.A. RNAi-mediated pathways in the nucleus. **Nature Reviews Genetics**, London, v.6, n.1, p.24-35, 2005.

MATZKE, M.A.; MOSHER, R. A. RNA-directed DNA methylation: an epigenetic pathway of increasing complexity. **Nature Reviews Genetics**, London, v.15, n.6, p.394-408, 2014.

MIROUZE, M.; PASZKOWSKI, J. Epigenetic contribution to stress adaptation in plants. **Current Opinion in Plant Biology**, London, v.14, n.3, p.267-274, 2011.

MITHILA, J. et al. Evolution of Resistance to Auxinic Herbicides: Historical Perspectives, Mechanisms of Resistance, and Implications for Broadleaf Weed Management in Agronomic Crops. **Weed Science**, Champaign, v.59, n.4, p.445-457, 2011.

MIURA, A. et al. An Arabidopsis jmjC domain protein protects transcribed genes from DNA methylation at CHG sites. **Embo Journal**, New York, v.28, n.8, p.1078-1086, 2009.

MOREL, J.B. et al. DNA methylation and chromatin structure affect transcriptional and post-transcriptional transgene silencing in Arabidopsis. **Current Biology**, Cambridge, v.10, n.24, p.1591-1594, 2000.

NAKASHIMA, K.; ITO, Y. AND YAMAGUCHI-SHINOZAKI, K. Transcriptional regulatory networks in response to abiotic stresses in Arabidopsis and grasses. **Plant Physiology**, Rockville, v.149, n.1, p.88–95, 2009.

NARDEMIR, G. et al. Determination of genetic and epigenetic effects of glyphosate on Triticum aestivum with RAPD and CRED-RA techniques. **Theoretical and Experimental Plant Physiology**, Campinas, v.27, n.2, p.131-139, 2015.

NEVE, P.; POWLES, S. Recurrent selection with reduced herbicide rates results in the rapid evolution of herbicide resistance in Lolium rigidum. **Theoretical and Applied Genetics**, New York, v.110, n.6, p.1154-1166, 2005.

PECINKA, A., et al. Epigenetic Regulation of Repetitive Elements Is Attenuated by Prolonged Heat Stress in Arabidopsis. **Plant Cell**, Rockville, v.22, n.9, p.3118-3129, 2010.

PENTERMAN, J.; UZAWA, R.; FISCHER, R.L. Genetic interactions between DNA demethylation and methylation in Arabidopsis. **Plant Physiology**, Rockville, v.145, n.4, p.1549-1557, 2007.

PFLEEGER, T. et al. The effects of glyphosate and aminopyralid on a multi-species plant field trial. **Ecotoxicology**, Dordrecht, v.21, n.7, p.1771-1787, 2012.

PIKAARD, C.S.; SCHEID, O.M. Epigenetic Regulation in Plants. **Cold Spring Harbor Perspectives in Biology**, Bungtown, v.6, n.12, 2014.

POWLES, S.B.; YU, Q. Evolution in Action: Plants Resistant to Herbicides. **Annual Review of Genetics**, Palo Alto, v.61, p.317-347, 2010.

PROBST, A.V. et al. Arabidopsis histone deacetylase HDA6 is required for maintenance of transcriptional gene silencing and determines nuclear organization of rDNA repeats. **Plant Cell**, Rockville, v.16, n.4, p.1021-1034, 2004.

QIAN, W.Q. et al. A Histone Acetyltransferase Regulates Active DNA Demethylation in Arabidopsis. **Science**, Washington**,** v.336, n.6087, p.1445-1448, 2012.

RAMIREZ-PARRA, E.; GUTIERREZ, C. E2F regulates FASCIATA1, a chromatin assembly gene whose loss switches on the endocycle and activates gene expression by changing the epigenetic status. **Plant Physiology**, Rockville, v.144, n.1, p.105-120, 2007.

SAIKA, H. et al. A novel rice cytochrome P450 gene, CYP72A31, confers tolerance to acetolactate synthase-inhibiting herbicides in rice and Arabidopsis. **Plant Physiology**, Rockville, v.166, n.3, p.1232-1240, 2014.

SALES M.A., et al. Amino acid substitutions in the acetolactate synthase gene of red rice (Oryza sativa) confer resistance to imazethapyr. **Weed Science**, Champaing, v.56, n.4, p.485-489, 2008.

SAS INSTITUTE. **Base SAS_9.1:** procedures guide. Cary: SAS Institute, 2004.

SONG, Y.L. Insight into the mode of action of 2,4-dichlorophenoxyacetic acid (2,4-D) as an herbicide. **Journal of Integrative Plant Biology**, Hoboken, v.56, n.2, p.106-113, 2014.

SOPPE, W.J.J. et al. The late flowering phenotype of fwa mutants is caused by gain-of-function epigenetic alleles of a homeodomain gene. **Molecular Cell**, Cambridge**,** v.6, n.4, p.791-802, 2000.

STREIBIG, J.C. Herbicide bioassay. **Weed Research**, Malden, v.28, n.6, p.479-484, 1988.

VILA-AIUB, M.M. et al. Glyphosate resistance in perennial Sorghum halepense (Johnsongrass), endowed by reduced glyphosate translocation and leaf uptake. **Pest Management Science**, Malden, v.68, n.3, p.430-436, 2012.

VILA-AIUB, M.M. et al. No fitness cost of glyphosate resistance endowed by massive EPSPS gene amplification in Amaranthus palmeri. **Planta**, New York, v.239, n.4, p.793-801, 2014.

VONGS, A. et al. Arabidopsis thaliana DNA methylation mutants. **Science**, Washington**,** v.260, n.5116, p.1926-1928, 1993.

WOLFFE A.; MATZKE M. Epigenetic regulation through repression. **Science**, Washington**,** v.286, n.1 p.481-486, 1999.

YAN, X.J. et al. DNA methylation signature of intergenic region involves in nucleosome remodeler DDM1-mediated repression of aberrant gene transcriptional read-through. **Journal of Genetics and Genomics**, Beijing, v.43, n.8, p.512-522, 2016.

YUAN, J.S.; TRANEL, P.J.; STEWART, C.N. Non-target-site herbicide resistance: a family business. **Trends in Plant Science**, London, v.12, n.1, p.6-13, 2007.

ZEMACH, A. et al. The Arabidopsis Nucleosome Remodeler DDM1 Allows DNA Methyltransferases to Access H1-Containing Heterochromatin. **Cell**, Cambridge, v.153, n.1, p.193-205, 2013.

ZHENG, Q. et al. Genome-Wide Double-Stranded RNA Sequencing Reveals the Functional Significance of Base-Paired RNAs in Arabidopsis. **Plos Genetics**, San Francisco, v.6, n.9, p.1-14, 2010.

ZHU, J.H. et al. The DNA glycosylase/lyase ROS1 functions in pruning DNA methylation patterns in Arabidopsis. **Current Biology**, Cambridge, v.17, n.1, p.54-59, 2007.

ZILBERMAN, D.; CAO, X.F.; JACOBSEN, S.E. ARGONAUTE4 control of locus-specific siRNA accumulation and DNA and histone methylation. **Science**, Washington**,** v.299, n.5607, p.716-719, 2003.

4 CHAPTER 2

Genome-wide expression analysis provides insights into the effect of imazethapyr in gene expression in the epigenetic *Arabidopsis thaliana* mutant *ros1*

1 INTRODUCTION

Imazethapyr is a common imidazolinone herbicide that provides selectively control of several grasses and broadleaf weeds (Soltani *et al*., 2008). Imazethapyr is a systemic herbicide, absorbed by both roots and shoots with rapid translocation in the xylem and phloem to the meristematic regions. The mechanism of action of imazethapyr is the inhibition of the plant enzyme acetolactate synthase (ALS) (E.C.4.1.3.18), which catalyzes the first reaction in the biosynthetic pathway of branched chain amino acids (BCAAs; valine, leucine, and isoleucine) (York *et al*., 1995). Characteristics such as low use rates, low mammalian toxicity, effective and prolonged control of a broad spectrum of weeds, and minimum crop injury, have favored the intense use of ALS-inhibiting herbicides including imazethapyr (Tan *el al*., 2005).

The occurrence of resistance to ALS-inhibiting herbicides is a large problem in many crops around the world, and had increased in recent years. Currently there are 159 species with resistance to ALS-inhibiting herbicides (Heap, 2017). The mechanism of resistance in most species is associated with alteration of the target ALS enzyme (target site resistance- TSR) (Devine & Eberlein, 1997). There are 22 resistance substitutions at seven sites across ALS gene, but Pro-197-Ser substitution is the most often observed (Tranel & Wright, 2002; Yu *et al*., 2010). In addition, non- target site resistance (NTSR) is also reported to resistance to ALS-inhibiting herbicides (Powles & Yu, 2010). NTSR normally present involvement of genes associated with xenobiotic detoxification. For example, imazethapyr resistance in *Digitaria sanguinalis* is related with enhanced degradation mediated by cytochrome P450 monooxigenases (cytP450) (Powles & Yu, 2010), and in *Echinochloa crus-galli* the genes *CYP81A6* and *GSTF1* had larger expression in plants resistant to imazethapyr (Dalazen *et al*., 2015).

The main processes associated with xenobiotics detoxification are mediated by cytP450, glutathione S-transferases (GSTs) and ABC transporters (Yu & Powles, 2014; Lane *et al*., 2016). Herbicide resistance caused by enhanced detoxification is considered important because frequently results in resistance to herbicides of different mechanism of action, similarly to what occur with antibiotics (Gressel, 2009). Only recently the genes associated with herbicide detoxification had been identified in some species of weeds such as *Lolium rigidum* (Yu *et al*., 2009; Busi *et al*., 2011), *Alopercurus myosuroides* (Délye *et al*., 2011), *Echinochloa phyllopogon* (Yasour *et al*., 2009; Iwakami *et al*., 2014). However, the molecular and genetic regulatory mechanism of enhanced detoxification related with herbicide resistance is unknown.

Exposure of plants to ALS-inhibiting herbicides can modify the expression of several genes. A microarray analysis performed in *Arabidospsis thaliana* identified 478 genes significantly and coordinately regulated by four ALS-inhibiting herbicides, including one imidazolinone (imazapyr), one triazolopyrimidine (cloransulam-methyl), and two sulfonylureas (primisulfuron-methyl and sulfometuron methyl) (Das *et al*., 2010). In the same specie, a proteomic analysis identified 1,322 and 987 proteins differentially expressed in response to root and shoot- imazethapyr treatments, respectively (Qian *et al*., 2015).

Recently, epigenetic mechanisms, such as DNA methylation, started to be related to the regulation of genes involved to pesticides detoxification, as described to atrazine herbicide (Lu *et al*., 2016). DNA methylation is a biochemical process in which the methyl group is attached to the 5-position of cytosine (5mC), found in CG, CHG and CHH sequence contexts (H represents A, C, or T). A group of enzymes known as DNA methyltransferases (DMT) catalyzes the DNA methylation (Huang *et al*., 2010). In addition, four proteins, ROS1 (REPRESSOR OF SILENCING), DME

(DEMETER), DML2 (DEMETER-LIKE) and DML3 are responsible for the active demethylation of 5mC in *A. thaliana* (Penterman, *et al*., 2007). The dynamic of DNA methylation, mainly in gene promoters, is important to control plant development and gene expression in environmental-stressed plants (Law & Jacobsen, 2010). DNA methylation may also be an important mechanism involved in the regulation of plant response to pesticide compounds.

In previous study with *A. thaliana* (Chapter 1), the lack of ROS1 increased the susceptibility of imazethapyr herbicide, indicating that DNA methylation may be important for *A. thaliana* tolerance to this herbicide. Understanding the complex genetic control of herbicide response and NTSR can be reached using whole-transcriptome sequencing (RNA-Seq) that is currently the most powerful tool to identify genes differentially regulated (Duhoux *et al*., 2015). The aims of the present study were firstly to identify whether imazethapyr leads to global alteration in DNA methylation patterns in *A. thaliana* and if this pattern change in lack of ROS1. Secondly, to identify differential expression genes between *A. thaliana* and *ros1* mutant in exposure to imazethapyr, and to analyze if genes putatively involved with herbicide resistance are epigenetically regulated.

2 MATERIALS AND METHODS

The *A. thaliana* accessions Columbia-0 (Col-0) and the epigenetic mutant *ros1* were used in all experiments, which were performed at Max Planck Institute for Plant Breeding Research, Cologne/Germany. Seeds were sown on moist soil in 7 x 7 cm plastic pots and stratified for 48 hours at 4°C in a dark room. Subsequently, pots were transferred to a growth chamber (Percival AR-95L3; Percival Scientific/USA) and plants were cultivated under a long day regime (16h 21°C light/ 8h 19°C dark), relative humidity of 70 – 75% and 150 µmol m^{-2} s^{-1} light intensity.

After one week, plants were singled to one plant per pot. The herbicide imazethapyr was applied in sub-lethal dose of 10% of the label dose (10.6 g ha^{-1} in addition of 0.5% v/v Dash), according previous study (Chapter 1) in 14 days old plants for all experiments.

2.1 Global DNA methylation analysis by isocratic cation-exchange high-pressure liquid chromatography

Plants of *A. thaliana* Col-0 wild type (WT) and *ros1* mutant were cultivated as described above. Treated (sub-lethal dose of imazethapyr) and non-treated plants were collected 48 hours after herbicide application. The *ddm1* epigenetic mutant line, lambda phage DNA (N3011S; New England Biolabs, Frankfurt/Germany) and herring DNA (Sigma-Aldrich, Hamburg/Germany) were used as experiment controls (Vongs *et al*., 1993). The experiment was organized in completely randomized design, with three repetitions. DNA isolation was performed by using Nucleon PhytoPure gDNA Kit (GE Healthcare, Munich/Germany) according to the manufacturer's instructions. DNA concentrations were quantified with the NanoDrop ND-1000 spectral photometer (peqLab, Erlangen/Germany). Treatment with RNase A (Thermo Fischer, Langenselbold/Germany) for 20 min at 37°C was performed for all samples. Visualization of DNA quality on samples was confirmed by electrophoresis on 1.5 % agarose gel.

An amount of 1 µg genomic DNA of each sample was sent to Fachgebiet Biotechnologie Gartenbaulicher Kulturen, Technical University of Munich, Munich/Germany for HPLC analysis. The quantification of global cytosine methylation was based on enzymatic hydrolysis of DNA, dephosphorylation, and subsequent HPLC measurement, as described by Rozhon *et al*. (2008). Nucleosides were separated under isocratic conditions on a benzenesulfonic acid-

modified silica phase and detected by UV absorption (Rozhon *et al*., 2008). For digestion, DNA was treated with Nuclease P1 and subsequently with alkaline phosphatase to obtain the free dNs. The HPLC system included a Dionex P680 pump, an ASI-100 autosampler, and a PDA-100 photodiode array detector. The system was equipped with a Macherey–Nagel 125 x 4 mm Nucleosil 100-10 SA column preceded by a Valco 2 µm inline filter. The mobile phase consisted of 60 mM acetic acid dissolved in 15% acetonitrile and was set to pH 4.8 with NaOH. A constant flow rate was maintained at 1.5 ml/min, and UV detection was performed at 277 nm with a bandwidth of 10 nm. The obtained chromatograms were analyzed with Chromeleon 7 (Dionex, Sunnyvale/USA).

The 5-mdC values were expressed as percent of total cytosine. Data were tested for normality using PROC UNIVARIATE in SAS (SAS Institute, 2004) and statistically evaluated by ANOVA ($p \leq 0.05$). If statistical significance was found, means were compared by the Tukey-test ($p \leq 0.05$).

2.2 High-throughput mRNA sequencing (RNA-seq)

Plant material consisted of Col-0 wild type (WT) and *ros1* mutant plants, both analyzed in control condition (non-treated plants) and treated plants with sub-lethal dose of imazethapyr. The treated and non-treated plants were collected 48 hours after herbicide application (Figure 1). Each sample consisted of the entire rosette of two plants that were collected and immediately frozen in liquid nitrogen. Two replicates for each treatment were used.

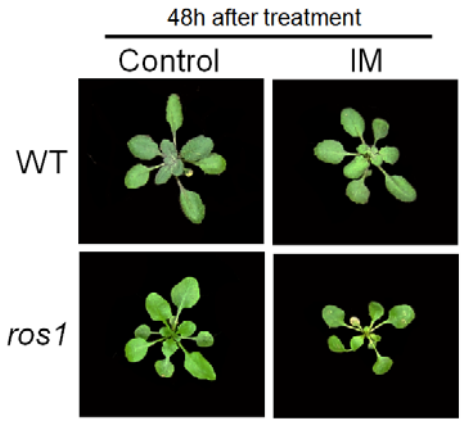


FIGURE 1. *Arabidopsis thaliana* wild type (WT) and *ros1* mutant plants at time of collection, 48 hours after imazethapyr (IM) treatment. UFRGS, Porto Alegre, RS, Brazil. 2017.

Total RNA was isolated by using the RNeasy Plant Mini Kit (Qiagen, Hilden/Germany) according to the manufacture's protocol and with an additional on-column DNase I digestion (Roche, Basel/Switzerland). RNA concentrations were quantified by spectrophotometry using the Qubit RNA HS Assay kit and the Qubit Fluorometer (Life Technologies, Karlsruhe/Germany). Afterwards, the RNA integrity was evaluated in an agarose-formaldehyde gel electrophoresis of RNA. This method improves the separation of long RNAs on gel and permits to distinguish the RNAs bands. The present method uses the combination of 3-(N-morpholino) propanesulfonic acid (MOPS) and sodium acetate as the conductive medium (Mansour *et al*., 2013). For preparation of 200ml of gel with 1.5% agarose, 3 g agarose were melt in 162 ml of autoclaved water, followed by addition of 20 ml 10X MOPS (0.2 M MOPS pH 7.0, 10 mM EDTA, 50 mM NaOAc) in the running buffer to 1X final concentration, and 11.6 ml of 37% formaldehyde to 0.7 M. For sample preparation, 5 µg RNA was added in to RNAse-free tube, and the volume was adjusted to 6 µl with DEPC-treated water, followed by 10 µl formamide, 4 µl formaldehyde, 2 µl 10 X MOPS buffer, 1 µl bromophenol blue dye mix (6x Loading dye) in a total volume of 23 µl. The gel was photographed after running for 30 min

in a 1X MOPS buffer with 15 µl Ethidium Bromide and washed in 1X MOPS for 10 min to remove unbound Ethidium Bromide.

RNA Integrity Numbers (RIN) were determined on a Bioanalyzer assay using the Agilent RNA 6000 Nano Kit (Böblingen/Germany). Samples with a RIN between 8 and 10 were used for library construction. RNA Libraries were made using Illumina TruSeq RNA Sample Prep Kit (San Diego/USA) following manufacturer's instructions. Subsequently, library concentrations were measured with the Qubit dsDNA HS Assay Kit on the Qubit Fluorometer and its insert size and integrity analyzed on a Bioanalyzer using the Agilent DNA 1000 Kit (Böblingen/Germany). High throughput sequencing was performed on an Illumina HiSeq2500 sequencer with a requested sequencing depth of 18.7 million 100bp single end reads per library at the Max Planck Genome Center (Cologne/Germany).

Obtained RNA-seq raw reads were quality controlled using FASTQC (Version 0.10.1) and low quality bases were trimmed with the FASTX-toolkit (García-Alcalde *et al*., 2012) using standard parameters. The protocol used for differential gene and transcript expression analysis of RNA-seq was described by Trapnell *et al*., (2012) and is represented in Figure 2. The libraries with sufficient quality were mapped to the corresponding reference genome *A. thaliana* Col-0 TAIR10 (Lamesch *et al*., 2012) using bowtie2 and TopHat2 with default parameters (Trapnell *et al*., 2012; Kim *et al*., 2013). Reads for each biological replicate were mapped independently. After mapping, the resulting alignment files were submitted to Cufflinks, which produces one file of assembled transfrags for each replicate to generate the transcriptome assembly. These assemblies were then merged together using the Cuffmerge utility of the Cufflinks package. The combined assembly was fed to Cuffdiff, which offers a uniform basis for calculating expression levels of genes and tests the statistical significance of observed changes in each

condition (Trapnell *et al*., 2012). The obtained files were indexed and visualized with CummeRbund (performed in the statistical software 'R') to facilitate exploration of genes identified by Cuffdiff as differentially expressed genes.

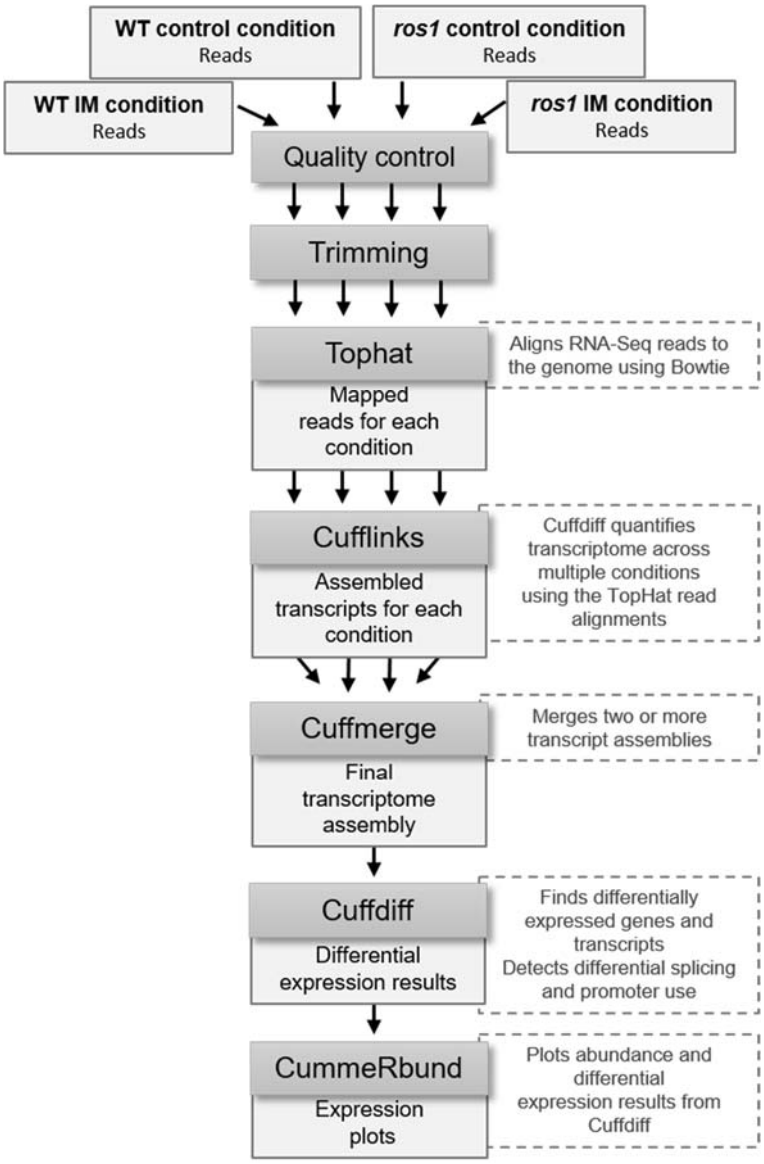


FIGURE 2. Overview of protocol steps described by Trapnell *et al*. (2012) to perform the differential gene and transcript expression analysis RNA-seq. UFRGS, Porto Alegre, RS, Brazil. 2017.

2.3 DNA methylation analysis by methylation-sensitive restriction cleavage

Plants of Col-0 (WT) and *ros1* mutant were grown as described above. The treated (sub-lethal dose of imazethapyr) and non-treated plants were collected 48 hours after herbicide application. The experiment was conducted as a completely

randomized design with three replications. DNA isolation was performed by using Nucleon PhytoPure gDNA Kit (GE Healthcare, Munich/Germany) according to the manufacturer's instructions. DNA concentrations were quantified with the NanoDrop ND-1000 spectral photometer (peqLab, Erlangen/Germany).

For methylation analysis, approximately 50 ng of extracted DNA were dissolved in 400 µl of distilled water, 50 µl of 10x CutSmart buffer and 50 µl of bi-distilled water were added to a final volume of 500 µl. Aliquots of 100 µl were incubated with 10 U of restriction enzymes *Alu*I, *Hpa*I, *Hpa*II, *HpyCh4*IV, *Msp*I, *Rsa*I (New England Biolabs, Ipswich/USA) and without restriction enzyme, and incubated at 37°C for 16 h. Each enzyme was used according to the restriction cleavage site for each gene (Table 1). Subsequently, the restriction enzymes were heat-inactivated by incubation for five minutes at 85°C. Finally, 399 µl of bi-distilled water was added to a final volume of 500 µl.

Approximately 500 bp of promoter region of candidate genes involved with herbicide resistance were analyzed in at least two contexts of CG, CHG and/or CHH. Six different methylation sensitive enzymes (*Alu*I, *Hpa*I, *Hpa*II, *HpyCh4*IV, *Msp*I and *Rsa*I) were used in order to assess the methylation level in specific sites, according to Table 1. Methylation of cytosine blocks cleavage according to REBASE (<*http://rebase.neb.com*>).

TABLE 1. Restriction enzymes used for qPCR assay and number of cleavage sites for which gene sequence analyzed. UFRGS, Porto Alegre, RS, Brazil. 2017.

Target	Sequence context	Restriction enzymes	Cleavage site
AT3G18780	CG	HpyCh4IV	
(Actin2)	CHG	HpaI/MspI	Negative control
	CHH	AluI	
AT5G07990	CG	HpyCh4IV	3
(TT7)	CHG	HpaI	1
	CHH	AluI	1
AT1G51090	CG	HpyCh4IV	3
(HMTDSP)	CHG		
	CHH	AluI	1
AT1G03550	CG	HpaII	1
(SCAMP)	CHG	MspI	1
	CHH	AluI	2
AT1G04570	CG	HpyCh4IV	4
(MFSP)	CHG	HpaI	1
	CHH		
AT4G10050	CG	HpyCh4IV	2
(Esterase)	CHG	RsaI	1
	CHH	AluI	4
AT2G14620	CG	HpyCh4IV	2
(XTH10)	CHG	HpaI	1
	CHH	AluI	1

Quantitative polymerase chain reaction (qPCR) was performed using the 7300 Real-Time PCR System® (Applied Biosystems). Each reaction contained 12.5 µl of SYBR Green® (Invitrogen), 1.25 µl of primers forward and reverse with final concentration of 0.25 µM each (primer sets used are showed in Table 2), 10 µl of cleaved DNA or control DNA templates, to reach a final volume of 20 µl for each sample. The reactions were carried out using the following cycling parameters: 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 62°C for 30 s, 72°C for 30 s, and 72°C for 5 min.

PCR was calibrated using logarithmic serial dilutions from 10^{-1} to 10^{-3} of genomic DNA preparations. The threshold cycle (Ct value) for reactions with serial dilution samples was determined in technical triplicates. Ct values of reactions with cleaved and control DNA samples were determined in triplicates. Samples with mean Ct values ± 0.25 cycles were included in calculations. The evaluation of qPCR

data was performed according to the MIQE (Minimum Information for publication of Quantitative real-time PCR experiments) (Bustin *et al*., 2009). The melting curve of the PCR products was analyzed to control the homogeneity of the amplification products, where a sharp and narrow peak was required. The values for the correlation coefficient of the PCR between 80 and 110% were accepted.

TABLE 2. Primers used for methylation analysis by methylation sensitive restriction cleavage. UFRGS, Porto Alegre, RS, Brazil. 2017.

Target	Name of primer	Primer Sequence (5'→ 3')	Product Size (bp)
AT3G18780	At-Actin2_F	cactgtcaatgttagattgaatctgaacactg	
	At-Actin2_R	atcatctcctgcaaatccagccttcaccat	258
AT5G07990	At-3-TT7_F	accaaacggtgaaaatccagc	
	At-3-TT7_R	cacccgattcgcaatgcttc	489
AT1G51090	At-8- HMTDSP_F	tctgaggacggatctcacga	
	At-8- HMTDSP_R	tgaatttatgaaacacggtgtatcaca	413
AT1G03550	At-15-SCAMP_F	tcgagaattaaagtggcggct	
	At-15-SCAMP_R	cggaggcaccacaatcac	386
AT1G04570	At-17-MFSP_F	cgtaaccgaactttggggct	
	At-17-MFSP_R	agctaggctcctacattggga	475
AT4G10050	At-26-esterase_F	tgcgttgattaaatgacgacact	
	At-26-esterase_F	tgctggacactggaaatttttgt	471
AT2G14620	At-30-XTH10_F	agagtgagtgtttcatgaagca	
	At-30-XTH10_R	tgtggagaagcaaagagaataca	552

The calculation of the relative amount of amplified target was performed according to Livak & Schmiitgen (2001) with $2^{-(\Delta\Delta CT)}$ formula. Results were presented as percent of the mean signal obtained for the control samples without restriction enzyme (set to 100%). Additionally, Actin2 region was used as known not methylated control. Data were transformed with $X=10+\text{arcsen}\sqrt{X100}$ to satisfy the ANOVA premise for normality. If statistical significance was found, the means were compared by the Tukey-test ($p \leq 0.05$).

2.4 Quantitative reverse transcriptase PCR (qRT-PCR) validation of genes by RNA-seq

For validation of RNA-seq the experiment was carried out as described in item 2.2 for plant grown, treatments, material collection and RNA extraction. The experiment was conducted as a completely randomized design with three replications. After RNA extraction, RNA concentrations were quantified with the NanoDrop ND-1000 spectral photometer (peqLab, Erlangen/Germany). An amount of 1 µg of total RNA was reverse transcribed into complementary DNA (cDNA) with the RevertAid First Strand cDNA Synthesis Kit using oligo(dT) primers (Thermo Scientific, St.LeonRot/Germany) according to the manufacture's protocol. Each reaction was set up in a 12 µl total volume, which contained 6 µl of SensiMix SYBR & Flourescein Kit (Bioline, Berlin/Germany), 1.25 µl of primers forward and reverse (with final concentration of 0.25 µM each) and 10 ng of the cDNA sample (1:100). The reactions were carried out using the following cycling parameters: 95°C for 5 min, followed by 40 cycles of 94°C for 15 s, 60°C for 10 s, 72°C for 15 s, and 60°C for 35 s. All reactions were run in triplicate by monitoring the dissociation curve to detect and exclude the possible nonspecific amplifications.

Six candidate genes to be involved with herbicide tolerance to imazethapyr were selected for validation using qRT-PCR. Primers were designed using the program Primer3Plus (<*http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi*>), based on the nucleotide sequences available on NCBI (<*http://www.ncbi.nlm.nih.gov/genbank/*>). Primer sets used are showed in Table 3. Transcription levels of genes were estimated by $2^{-(\Delta\Delta CT)}$ formula (Livak & Schmiitgen, 2001) and normalized to the imazethapyr stable reference genes GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE C-2 (GAPC-2; AT1G13440) and UBIQUITIN-CONJUGATING ENZYME 28 (UBC28; AT1G64230).

The qPCR was calibrated using logarithmic serial dilutions from 10^{-1} to 10^{-3} of cDNA preparations. The evaluation of qPCR data was performed according to the MIQE (Minimum Information for publication of Quantitative real-time PCR experiments) (Bustin *et al*., 2009) as previously described in item 2.3. Data sets were submitted to the *t*-test (p≤0.05).

TABLE 3. Primers used for quantitative RT-PCR validation of genes by RNA-Seq. UFRGS, Porto Alegre, RS, Brazil. 2017.

Target	Name of primer	Primer Sequence (5'→ 3')	Product Size (bp)
GAPC-2	GAPC-2_F GAPC-2_R	atcggtcgtttggttgctagagt acaaagtcagctccagcctca	251
UBC28	UBC28qF UBC28qR	tccagaaggatcctccaacttcctgcagt atggttacgagaaagacaccgcctgaata	124
TT7	TT7q_F TT7q_R	ctcgccggagtattcaacat ccgttcatttcgtgctcttt	143
HMTDSP	HMTDSPq_F HMTDSPq_R	agcctcaaccacctcagaag agcggaagacgaaacaagaa	110
SCAMP	SCAMPq_F SCAMPq_R	ccaccagtcatcttccaagg cagaagaacccagcaccaat	113
MFSP	MFSPq_F MFSPq_R	ccccaaccaagttcttgaaa aatgtggtcgctcttcttgg	124
ESTERASE	esterase_F esterase_F	caaatttgaatcctcttcgtga gcgaatacttctccgacgaat	126
XTH10	XTH10q_F XTH10q_R	agaaaccatggggagaaagg atggacccttttgaccaatca	130

3 RESULTS AND DISCUSSION

3.1 Global DNA methylation by isocratic cation-exchange high-pressure liquid chromatography (HPLC)

The genomic DNA quality of samples used for HPLC analysis were satisfactory, according to 1.5% agarose gel (Appendix 1). Global 5-mdC levels of samples were analyzed as percentage of 5-mdC in relation to total deoxycytidine (dC) levels using cation exchange HPLC (Rozhon *et al*., 2008). This analysis determined the overall DNA methylation regardless of any sequence context. The ANOVA of global DNA methylation analysis showed significance of F test (Appendix

2). The *ddm1* mutant line was used as control as it has been shown that causes dramatic change in global 5-mdC level (Vongs *et al*., 1993), lambda phage DNA (for very low 5-mdC level) and herring DNA (for high 5-mdC level) as additional controls. The results obtained for the controls corroborates with previous studies (Vongs *et al*., 1993), since *ddm1* showed drastic decrease in 5-mdC level of 3.18% (±0.02), lambda DNA showed 0.21% (±0.03) and herring DNA had 7.65% (±0.32), as expected (Figure 3). In general plants have levels of 5mC ranging from approximately 6 to 25 % of total cytosines, depending on the specie (Steward *et al*., 2002). In *A. thaliana*, close to 7% of the cytosines in cellular DNA are methylated (Rozhon *et al*., 2008).

5mdC levels of 5.84% (±0.10) was found for WT control whereas after imazethapyr treatment presented 5mdC of 5.65% (±0.01), however they were not statistically different (Figure 3). Plants of the *ros1* treated and non-treated showed 5mdC levels of 6.30% (±0.20) and 6.22% (±0.24), respectively. Nevertheless, it was possible to identify the statistical difference presented between WT treated with imazethapyr and *ros1* mutant (treated and not-treated plants) (Figure 3). It can be part of explanation of the higher susceptibility of imazethapyr presented by *ros1* mutant compared to WT (Chapter 1).

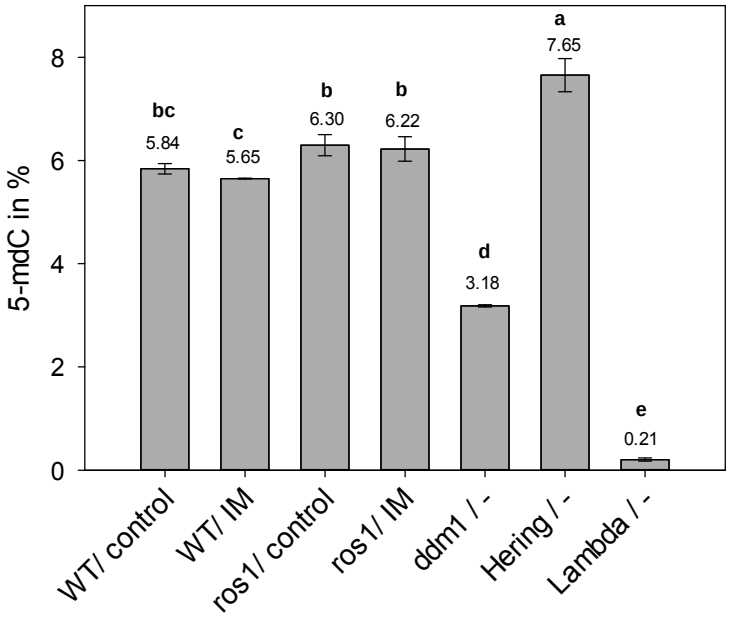


FIGURE 3. HPLC measurement of global 5-mdC (%) in *A. thaliana* WT and *ros1*, in control condition and treated with imazethapyr (IM), 48 hours after treatment. *ddm1*, lambda phage DNA and herring DNA were used as controls. Error bars denote standard deviations from three replicates. Means followed by different letter differ significantly after Tukey ($p \leq 0.05$). UFRGS, Porto Alegre, RS, Brazil. 2017.

According to previous study (Chapter 1), *ros1* plants presented 30% reduction in leaf length when treated with imazethapyr, compared to WT. As discussed before, ROS1 works with a demethylation role. A study showed that even a ‘‘silent’’ transposon presents dynamic control by both methylation and demethylation. This dynamic control is important for keeping the plant epigenome plastic, which allows the plant to respond to environment changes (Zhu *et al*., 2007). As suggested in Chapter 1, the *ros1* plants could have genes important for herbicide detoxification under methylation control that are not able to be demethylate by ROS1. Although HPLC analysis allowed to verify the different overall level of DNA methylation between WT- imazethapyr and *ros1* (treated and non-treated), this analysis does not permit to verify any DNA sequence sites, as well as genome location where this occurs. In this context, the next step for understanding the

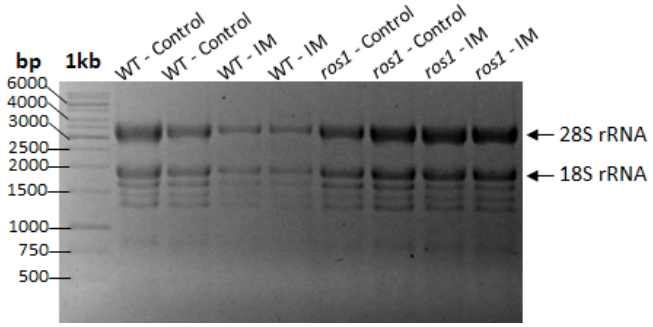
epigenetic mechanisms involved with regulation of gene important for herbicide detoxification was to verify which genes are differently expressed in WT and *ros1* after imazethapyr application.

3.2 High-throughput mRNA sequencing (RNA-seq)

3.2.1 Transcriptome sequencing and mapping of the reads

The quality of RNA samples was checked by agarose-formaldehyde gel electrophoresis and showed good quality of total RNA presenting sharp, clear 28S and 18S rRNA bands, without smeared appearance that is present in degraded RNA (Figure 4A). Additionally, the RNA Integrity Numbers (RIN) of each sample showed satisfactory numbers for library construction, between 8 and 10 (Figure 4B). The obtained RNA-seq reads were quality controlled using FastQC. This procedure permitted to analyze per base sequence quality, per base sequence content, per base GC content, per sequence GC content, per base N content, sequence length distribution, duplicate sequences, overrepresented sequences and adapter content. According to per base sequence the trimming was necessary only for low quality bases position in reads of 40 bp, the filtering was running with the FASTX-toolkit (García-Alcalde *et al*., 2012). After trimming per base sequence showed a satisfactory overview of the range of quality values across all bases at each position in the FastQ file (Appendix 3). The basic statistics provided by FastQC is presented in (Appendix 4).

A



B

Sample	RIN
WT – Control – R1	8.9
WT – Control – R2	8.9
WT – IM – R1	8.5
WT – IM – R1	8.5
ros1 – Control – R1	8.9
ros1 – Control – R2	8.9
ros1 – IM – R1	9.2
ros1 – IM – R2	9.3

FIGURE 4. (A) Agarose-formaldehyde gel electrophoresis and (B) RNA Integrity Numbers (RIN) of each sample used for RNA-seq. UFRGS, Porto Alegre, RS, Brazil. 2017.

After quality control of reads, a total of 163,214,163 short reads (Table 4) were mapped to corresponding reference genome *A. thaliana* Col-0 TAIR10. The *A. thaliana* Col-0 TAIR10 genome annotation present about 125 Mbp in size with 27,416 protein-coding genes (Lamesch *et al*., 2012). Approximately 150,7 million reads were perfectly aligned to the reference genome, for WT approximately 97% of total reads aligned to the reference genome, while for *ros1* this number was close to 88% (Table 4). The mapping data generated by TopHat was processed by Cufflinks and the abundance of gene transcripts was expressed as FPKM (fragments per kilobase of transcript per million fragments mapped).

TABLE 4. Summary of reads obtained by Tophat analysis based on the RNA-seq data. UFRGS, Porto Alegre, RS, Brazil. 2017.

Sample	Total Reads	Mapped Reads	Uniquely Mapped Reads	Non-uniquely Mapped Reads	Unmapped Reads
WT Control – R1	20355943	19608630 **96.33%**	18790352 **92.31%**	747313 **3.67%**	1565591 **7.69%**
WT Control – R2	22575110	22049429 **97.67%**	20941997 **92.77%**	525681 **2.33%**	1633113 **7.23%**
WT IM – R1	20502313	19819046 **96.67%**	19027463 **92.81%**	683267 **3.33%**	1474850 **7.19%**
WT IM – R1	20286614	19552987 **96.38%**	18758219 **92.47%**	733627 **3.62%**	1528395 **7.53%**
ros1 Control – R1	20342194	18001244 **88.49%**	17262400 **84.86%**	2340950 **11.51%**	3079794 **15.14%**
ros1 Control – R2	17373574	14891533 **85.71%**	14282008 **82.21%**	2482041 **14.29%**	3091566 **17.79%**
ros1 IM – R1	20012495	17719864 **88.54%**	16998840 **84.94%**	2292631 **11.46%**	3013655 **15.06%**
ros1 IM – R2	21765920	19080090 **87.66%**	18326741 **84.20%**	2685830 **12.34%**	3439179 **15.80%**
TOTAL:	**163214163**				

The initial analysis and visualization of the gene expression data produced by Cuffdiff was performed by using CummeRbund. As shown in the Figure 5A, the expression level distribution for all genes is presented for WT and *ros1* in conditions of control and imazethapyr treatment. The density plot reveled similar distribution of FPKM in $\log_{10}$ across individual conditions (Figure 5A). The volcano plot Illustrates the relationship between the p-values ($-\log_{10}$) and the magnitude of the difference in expression values represented by 'Fold Change' ($\log_2$) between the pairs of comparisons. According to Figure 5B a large number of genes were significant (p-values<0.05) differentially expressed between pairs of conditions that are indicated with red color.

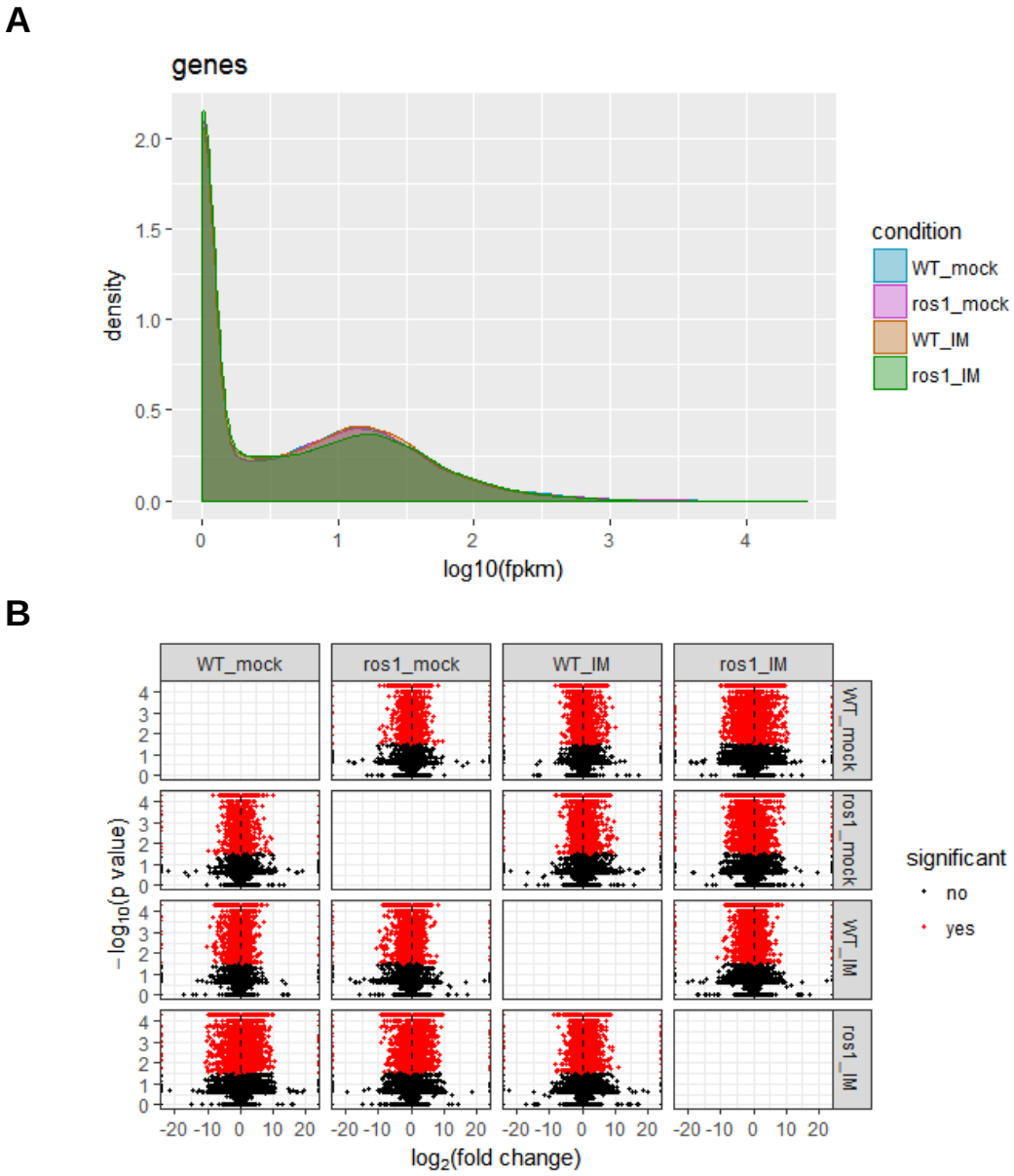


FIGURE 5. Bioinformatic analysis of RNA-seq data by using CummeRbund plots. (A) Expression level distribution for all genes in WT and *ros1*, in control (mock) condition and imazethapyr treatment (IM); FPKM, fragments per kilobase of transcript per million fragments mapped reads. (B) Volcano plots showing significant (adjusted p-value <0.05) differentially expressed genes, in red color. UFRGS, Porto Alegre, RS, Brazil. 2017.

3.2.2 Global analysis of gene expression

Differential expression of genes (DEGs) in response to the herbicide imazethapyr compared to control plants revealed 1172 significantly (adjusted p-value<0.05 in Cuffdiff) down-regulated genes in WT and 4471 in *ros1* mutant (Figure 6). The up-regulated genes for same conditions reveled 2464 significantly (adjusted

p-value<0.05 in Cuffdiff) in WT and 3323 in *ros1* mutant (Figure 6). This shows that the effect of the imazethapyr in the WT was more intense in induction than repression of gene expression. While for *ros1* the imazethapyr effect was intense in down-regulation genes (4471) compared to up-regulation genes (3323) (Figure 6). Other comparisons showing up and down-regulated genes are also presented in Figure 6.

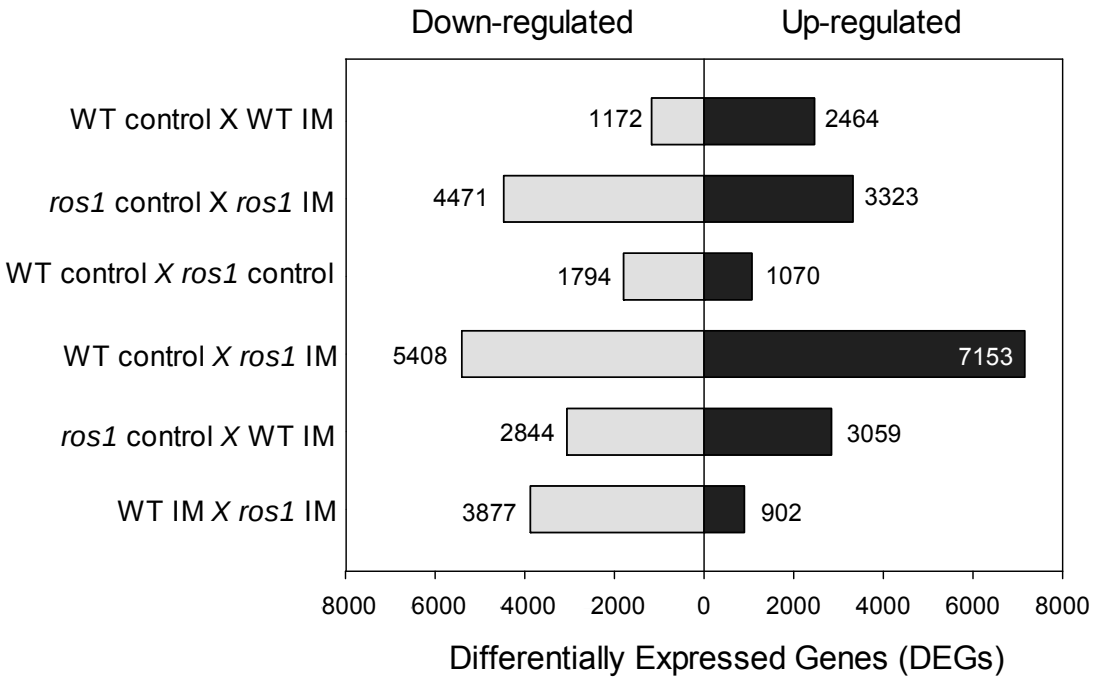


FIGURE 6. Differential expression of genes (DEGs) in response to herbicide imazethapyr (IM) and in control condition in *A. thaliana* wild type (WT) and *ros1* mutant. Number of up and down-regulated genes are represented in black and gray bars, respectively. The differences in gene expression were obtained based on the Log_2 Fold Change ≥2 and adjusted p-value < 0.05 in Cuffdiff. UFRGS, Porto Alegre, RS, Brazil. 2017.

Venn diagrams showed overlap of DEGs in response to IM treatment in WT and *ros1*, for up and down-regulated genes (Figure 7). The Venn diagrams represent the overlaps of genes between each pairwise comparison (Khraiwesh *et al*., 2015). For the comparisons of pairs tested, a large number of DEGs were specific for WT or *ros1* plants. For instance, 937 (22%) genes were induced for

imazethapyr treatment in WT. The overlap of up-regulated genes between WT and *ros1* corresponded to 35.8% of total up-regulated genes and 1796 (42.2%) genes were induced for imazethapyr treatment only in *ros1* plants. While for down-regulated genes the overlap genes corresponded to 18.5% of total. Additionally, 291 (6.1%) were repressed in WT with imazethapyr treatment, while 75.4% of down-regulated genes were presented when ROS1 was knocked out (Figure 7).

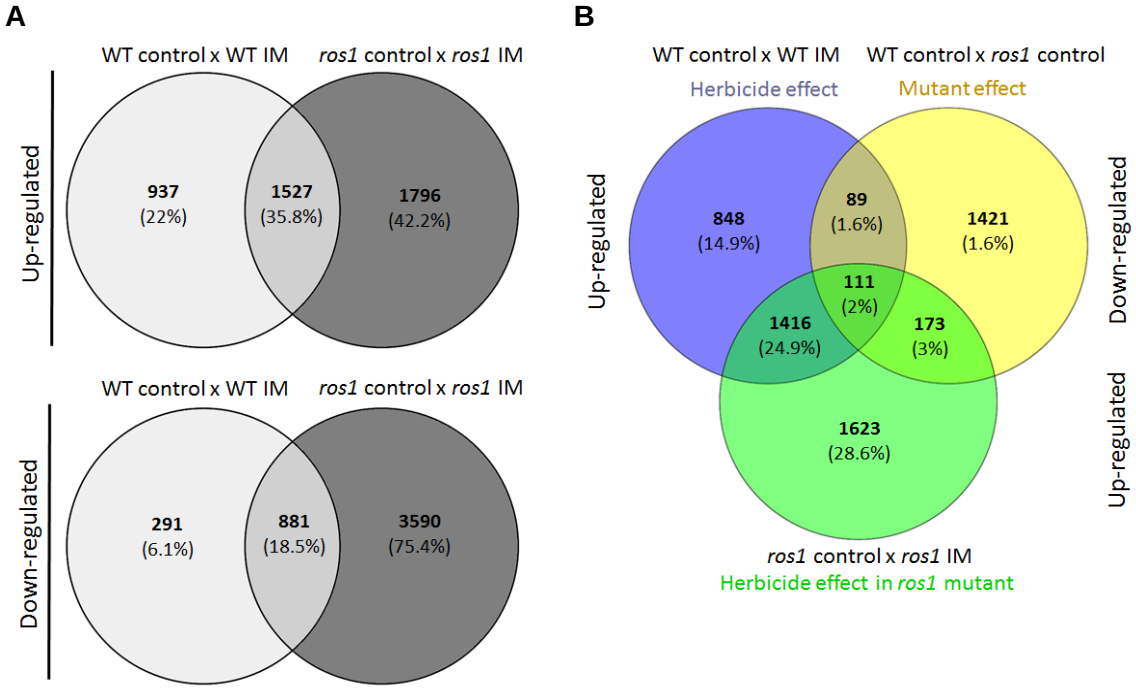


FIGURE 7. Venn diagrams. (A) Overlap of expressed genes in response to herbicide imazethapyr (IM) in *A. thaliana* wild type (WT) and *ros1*, for up and down-regulated genes. (B) Overlap of genes comparing genes inducted by IM in WT (herbicide effect – blue circle), with repressed genes in *ros1* (mutant effect – yellow circle) and effect of IM in *ros1* (herbicide effect on mutant – green circle). UFRGS, Porto Alegre, RS, Brazil. 2017.

The main analysis of interest genes was performed in Figure 7B. The comparison of the genes inducted by imazethapyr in WT (herbicide effect – blue circle) with all repressed genes in *ros1* in control condition (mutant effect – yellow circle) indicates an overlap of 200 genes. The complete list of the 200 genes is present in Appendix 5. In addition, the effect of imazethapyr in *ros1* was included in

this analysis to discount the genes mutually induced by imazethapyr, in WT and *ros1* (111 genes) (herbicide effect on *ros1* mutant – green circle). Thus, 89 genes show to be genes of interest that are induced by imazethapyr only in WT and present down-regulation in *ros1* mutants (Figure 7B). The differential expression of these genes is probable responsible for the tolerance difference to imazethapyr in WT and *ros1*. The ontology of the 89 genes were analyzed and some of them are putative involved with herbicide detoxification (Appendix 5).

3.2.3 Gene ontology (GO) for differential expression of genes (DEGs)

To provide a better analysis of gene expression, DEGs were assigned to different functional categories using PANTHER Classification System. The annotations were verified manually and integrated using gene ontology (GO) classification in three categories: biological process, molecular function, and cellular component. The detail information of biological process was performed using singular enrichment analysis (SEA) in agriGO. GO enrichment analysis was performed to reveal the biological processes overrepresented under imazethapyr treatment in WT, and also focused on the alteration of the expression of genes with epigenetic involvement. Additionally, GO classification and enrichment were accomplished in the genes of interest (89 genes identified with venn diagrams).

3.2.3.1 Herbicide effect in WT – gene ontology (GO)

GO enrichment analysis was performed to reveal genes categories of processes overrepresented under imazethapyr treatment in WT (Figure 8). The up-regulated genes involved in cellular component were classified in six main categories, biological processes were related to ten processes, while the involvement with molecular function was divided in eight main categories (Figure 8).

Singular enrichment analysis (SEA) indicate that multiple biological processes (32 pathways) are responsive to imazethapyr application in *A. thaliana* (Figure 9). However, it also indicated the predominance of genes linked to response to stimulus (including chemical stimulus) and stress (Figure 9). Secondary metabolism was also observed in Figure 9 as an effect of imazethapyr application. The secondary metabolism processes are known to be affected by herbicide application (Das *et al*., 2010). In the present study, the significant enrichment of included subcategories was more instructive. For the secondary metabolism showed an influence of imazethapyr on genes potentially related to herbicide metabolism and detoxification, e.g. *GSTF5* - Glutathione S-Transferase (Class PHI) 5 (AT1G02940), *GSTU25* - Glutathione S-transferase TAU 25 (AT1G17180) and *GSTF12* - Glutathione S-Transferase PHI 12 (AT5G17220). Additionally, genes involved with flavonoid biosynthesis were identified, such as chalcone-flavanone isomerase family protein (AT5G05270), *F3H* - Flavanone 3-Hydroxylase (AT3G29590), *TT4*, *TT5* and *TT7* (TRANSPARENT TESTA 4, 5 and 7) (AT5G13930, AT3G55120 and AT5G07990, respectively).

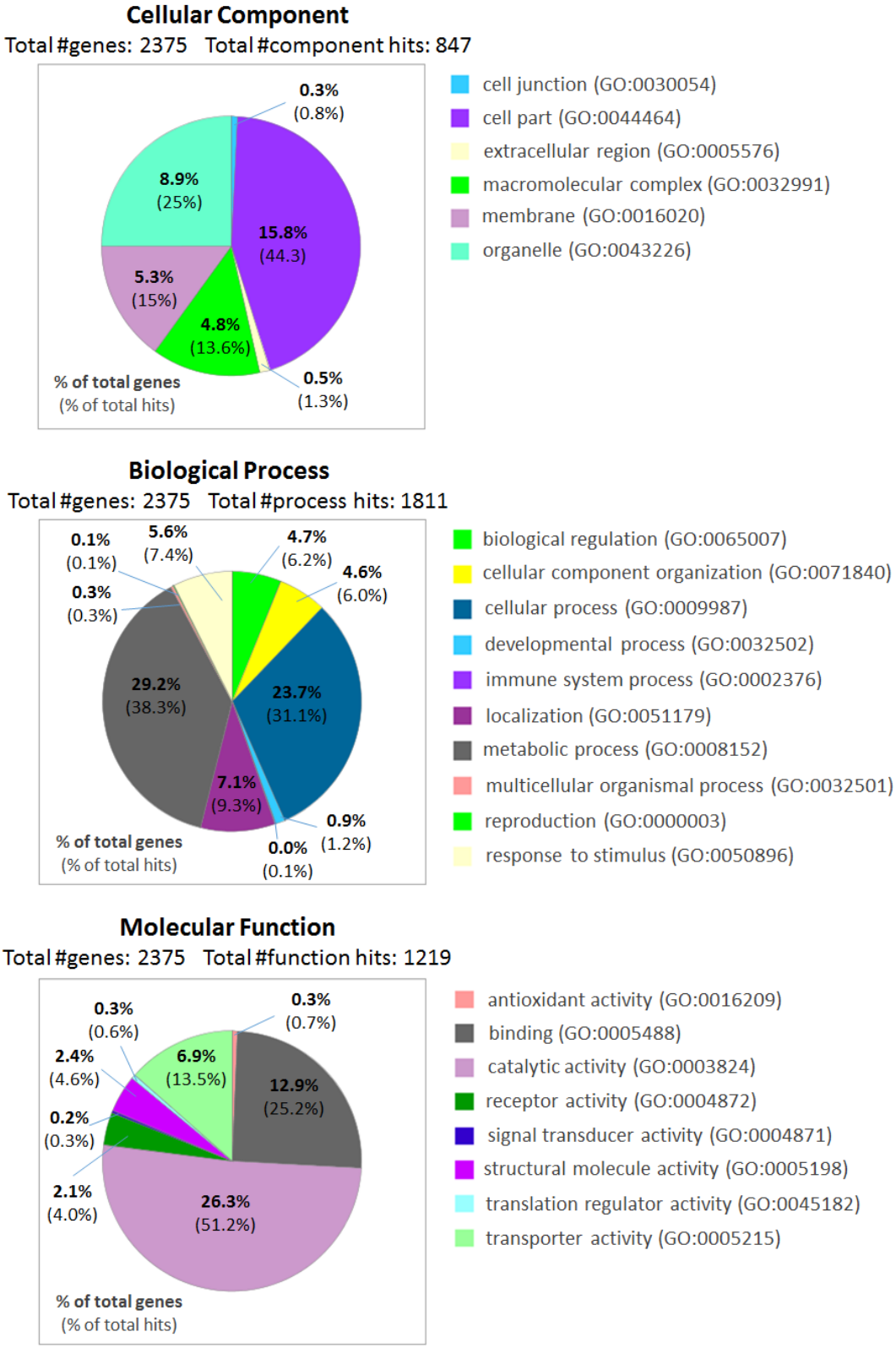


FIGURE 8. Pie chart representing Gene Ontology (GO) of up-regulated genes in wild type (WT) 48 hours after imazethapyr (IM) treatment. 2375 differential expression of genes (DEGs) were annotated in at least one of the three GO categories: cellular component, biological process and molecular function. UFRGS, Porto Alegre, RS, Brazil. 2017.

A transcriptome study based on Affymetrix ATH1 arrays in *Arabidopsis thaliana* reveled that an herbicide of a different mode of action, glyphosate, induced genes linked to secondary metabolism and defense of which seven were also induced by ALS-inhibitors (Das *et al*., 2010). All of them were potentially related to herbicide metabolism and detoxification, e.g., *CYP71A13*, *UGT74E2*, ABC transporter gene ATPDR12 and two MATE transporter genes (Das *et al*., 2010).

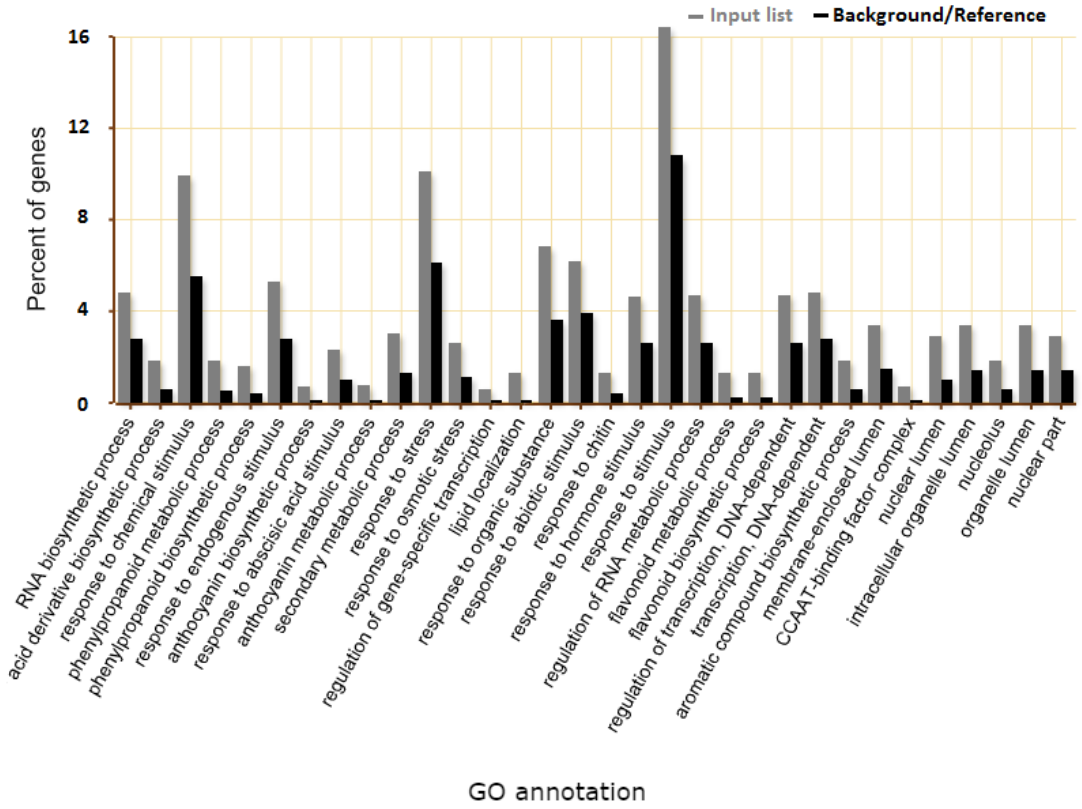


FIGURE 9. Detail information of biological process representing percent of genes involved in biological process pathways of up-regulated in wild type (WT) 48 hours after imazethapyr (IM) treatment, performed by using singular enrichment analysis (SEA). Gray and black bars indicate the percent of genes related to input list and the percent of genes compared to genome reference, respectively. UFRGS, Porto Alegre, RS, Brazil. 2017.

Down-regulated genes also presented similar division of classification in categories of cellular component, biological processes and molecular function compered to up-regulated genes (Figure 10). However, the analysis revealed that

subcategories of repressed genes were different affected, where the main involved processes were the regulation of cell cycle, response to endogenous stimulus, response to gibberellin, anatomical structure, and morphogenesis (Figure 11). Changes in the expression of genes involved in central energy pathways were also observed. Several genes encoding components of chloroplast, light stimulus and photosystems indicated reduction in photosynthesis compounds due to the application of imazethapyr (Figure 11). Results from previous study showed that 20 µg/L imazethapyr markedly affected the growth of the three ecotypes of *A. thaliana* (Sun *et al*., 2016). The results of the biochemical assays indicated more anthocyanin and reactive oxygen species (ROS) were produced and photosynthetic activity was substantially decreased (Sun *et al*., 2016).

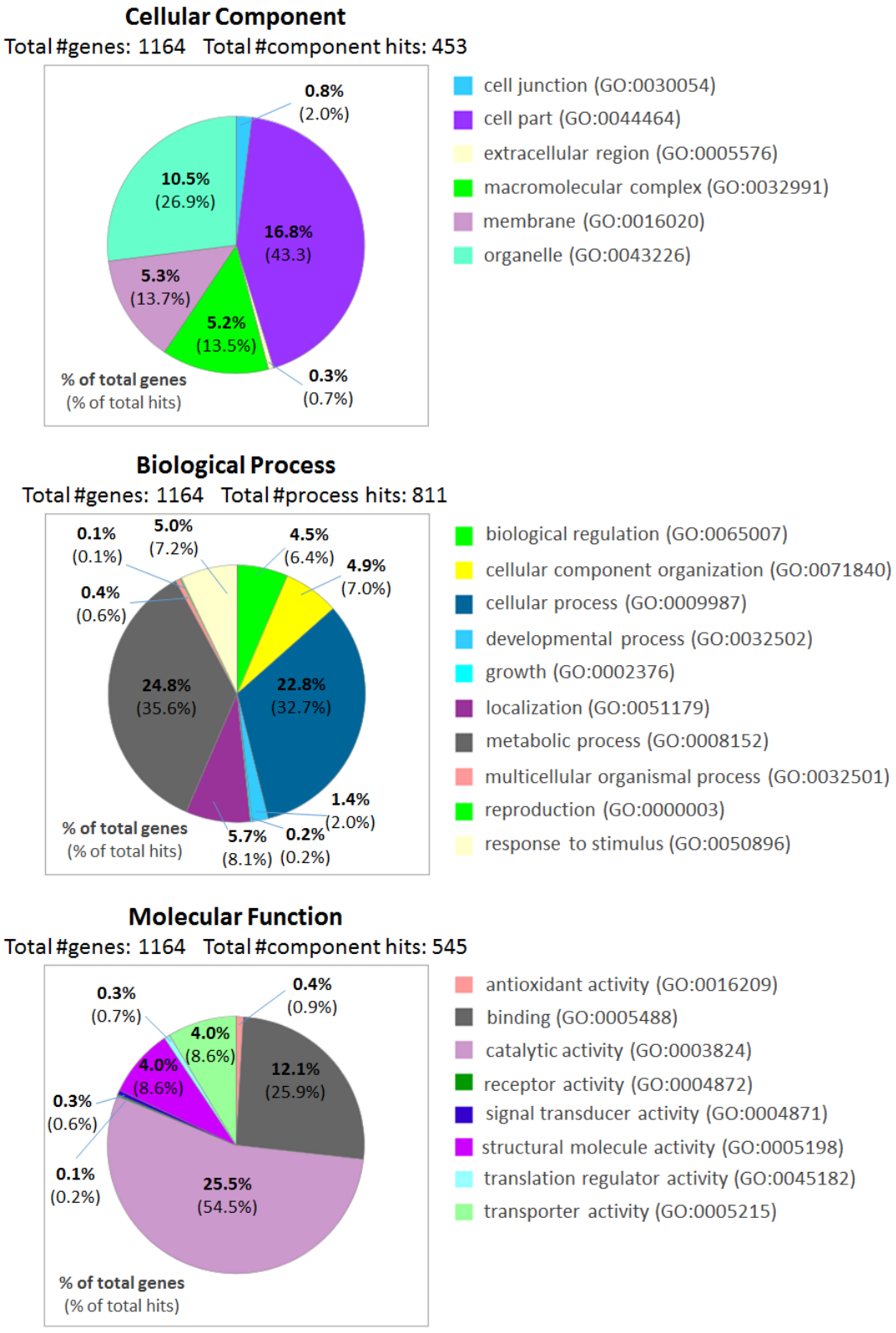


FIGURE 10. Pie chart representing Gene Ontology (GO) of down-regulated genes in wild type (WT) 48 hours after imazethapyr (IM) treatment. 1164 differential expression of genes (DEGs) were annotated in at least one of the three GO categories: cellular component, biological process, and molecular function. UFRGS, Porto Alegre, RS, Brazil. 2017.

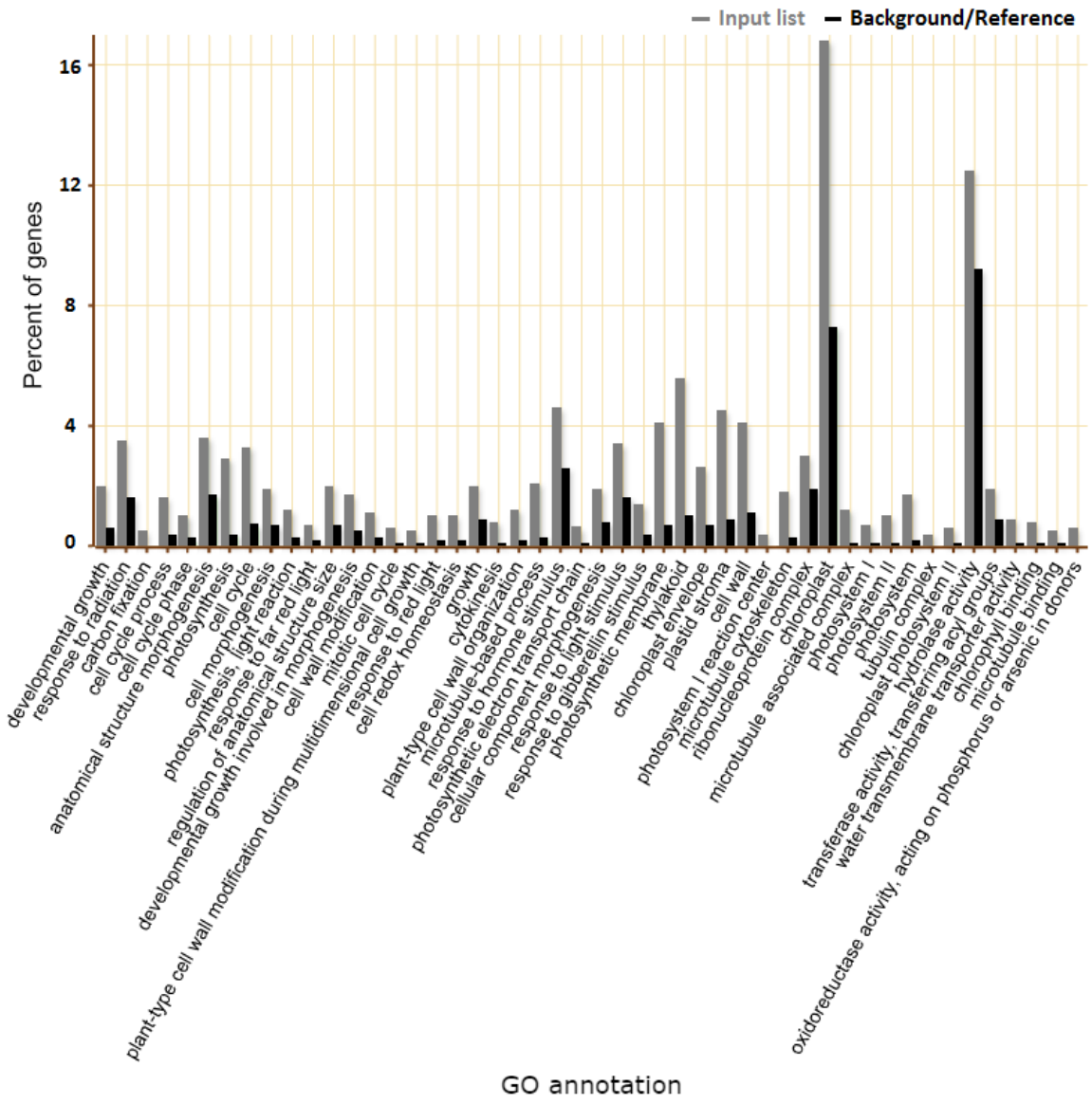


FIGURE 11. Detail information of biological process representing percent of genes involved in biological process pathways of down-regulated in wild type (WT) 48 hours after imazethapyr (IM) treatment, performed by using singular enrichment analysis (SEA). Gray and black bars indicate the percent of genes related to input list and the percent of genes compared to genome reference, respectively. UFRGS, Porto Alegre, RS, Brazil. 2017.

Additionally, in order to understand the effects of imazethapyr in the epigenetic regulation, genes involved with this process were analyzed comparing WT control with WT treated plants. Imazethapyr treatment caused significant (adjusted p-value<0.05) up-regulation of 40 genes and down-regulation of 33 genes involved with epigenetic mechanisms, according to The Arabidopsis Information

Resource 10 (TAIR 10) (Table 5). This indicated that imazethapyr leads to alteration of genes involved with DNA methylation, non-coding RNA, histone modification, chromatin modification and chromatin assembly or disassembly (Table 5). Some of these genes are involved with more than one process, such as *IBM1* (INCREASE IN BONSAI METHYLATION 1) that is related to DNA methylation, histone, and chromatin modification.

Some of the found genes involved with epigenetic mechanisms presented in Table 5 are well described in literature. According to TAIR eight genes presented in the list are designated to be related with DNA methylation: *AGO4*, *CMT2*, *CMT3*, *DML1*, *DRM2*, *IBM1*, *RDR1* and *SUVH4*. Eleven of them, *AGO4*, *CMT3*, *DRM2*, *HDA05*, *HDA08*, *HDA14*, *HDA15*, *HDA18*, *IBM1*, *SUVH4* and *SWIB* complex are associated with histone modification. Additionally, the genes *AGO4*, *CHR4*, *CMT3*, *DRM2*, *FAS1*, *FAS2*, *HDA04*, *HDA05*, *HDA08*, *HDA14*, *HDA15*, *HDA18*, *HIS1-3*, *histone H3*, *histone H3.2*, *histone H4*, *HMGB1*, *IBM1*, *NRP2*, *putative histone H2B*, *putative histone H3*, *SUVH4*, *SWIB complex* are involved with chromatin modification and/or chromatin assembly (Table 5).

TABLE 5. Genes involved with epigenetic mechanisms (TAIR10) significantly up and down-regulated 48 hours after imazethapyr (IM) treatment, according to RNA-seq data. UFRGS, Porto Alegre, RS, Brazil. 2017.

Gene ID	Gene Annotation	Control	IM	Log2 Fold Change	Adjusted P Value[2]
		FPKM [1]			
Up-regulated genes					
AT2G21450	*chromatin remodeling 34 (CHR34);*	0.08	5.95	6.15	0.00215
AT2G39030	Acyl-CoA N-acyltransferases (NAT) superfamily protein;	5.19	148.67	4.84	5.00E-05
AT2G18050	*histone H1-3 (HIS1-3);*	8.72	202.63	4.54	5.00E-05
AT2G32020	Acyl-CoA N-acyltransferases (NAT) superfamily protein;	0.53	7.70	3.87	0.00045
AT1G31290	*ARGONAUTE 3 (AGO3);*	0.20	2.20	3.49	5.00E-05
AT2G32030	Acyl-CoA N-acyltransferases (NAT) superfamily protein;	0.81	5.06	2.64	5.00E-05

continuation TABLE 5. Genes involved with epigenetic mechanisms (TAIR10) significantly up and down-regulated 48 hours after imazethapyr (IM) treatment, according to RNA-seq data. UFRGS, Porto Alegre, RS, Brazil. 2017.

Gene ID	Gene Annotation	Control	IM	Log2 Fold Change	Adjusted P Value[2]
		FPKM [1]			
Up-regulated genes					
AT1G64490	DEK, chromatin associated protein;	13.20	47.99	1.86	5.00E-05
AT1G13370	Histone superfamily protein;	1.12	3.46	1.63	0.005
AT5G42060	DEK, chromatin associated protein;	21.09	63.74	1.60	6.45371
AT1G05490	*chromatin remodeling 31 (chr31);*	0.40	1.09	1.46	5.00E-05
AT1G02740	*MRG family protein; chromatin assembly or disassembly;*	4.41	11.33	1.36	5.00E-05
AT5G20420	*chromatin remodeling 42 (CHR42);*	0.55	1.36	1.30	5.00E-05
AT3G44750	*histone deacetylase 3 (HDA3);*	55.69	135.64	1.28	5.00E-05
AT1G08460	*histone deacetylase 8 (HDA08);*	14.65	33.87	1.21	5.00E-05
AT1G03750	*switch 2 (SWI2);*	2.47	5.61	1.18	3.48208
AT1G77540	Acyl-CoA N-acyltransferases (NAT) superfamily protein;	26.68	56.79	1.09	4.38593
AT3G26850	*histone-lysine N-methyltransferases;*	6.32	13.02	1.04	0.0013
AT5G26040	*histone deacetylase 2 (HDA2);*	6.15	12.60	1.04	0.00965
AT1G18800	NAP1-related protein 2 (NRP2);	26.31	51.29	0.96	5.00E-05
AT5G02560	*histone H2A 12 (HTA12);*	17.17	33.40	0.96	5.00E-05
AT1G31280	*argonaute 2 (AGO2);*	7.50	14.33	0.93	5.00E-05
AT2G39000	Acyl-CoA N-acyltransferases (NAT) superfamily protein;	20.53	38.64	0.91	5.00E-05
AT1G62310	transcription factor jumonji (jmjC) domain-containing protein;	8.48	15.14	0.84	5.00E-05
AT2G44980	SNF2 domain-containing protein/ helicase domain-containing protein;	3.64	6.49	0.84	5.00E-05
AT2G30280	*RNA-directed DNA methylation 4 (RDM4);*	8.18	14.53	0.83	5.00E-05
AT4G17080	Histone H3 K4-specific methyltransferase SET7/9 family protein;	8.66	15.30	0.82	5.00E-05
AT1G03650	Acyl-CoA N-acyltransferases (NAT) superfamily protein;	4.67	8.19	0.81	0.00195
AT3G51880	*high mobility group B1 (HMGB1);*	89.36	155.70	0.80	5.00E-05
AT3G22680	*RNA-DIRECTED DNA METHYLATION 1 (RDM1);*	16.21	27.36	0.76	0.0001
AT2G27840	*histone deacetylase activity HDT4;*	32.81	54.97	0.74	5.00E-05

continuation TABLE 5. Genes involved with epigenetic mechanisms (TAIR10) significantly up and down-regulated 48 hours after imazethapyr (IM) treatment, according to RNA-seq data. UFRGS, Porto Alegre, RS, Brazil. 2017.

Gene ID	Gene Annotation	Control	IM	Log2 Fold Change	Adjusted P Value[2]
		FPKM [1]			
Up-regulated genes					
AT3G07610	*increase in bonsai methylation 1 (IBM1);*	10.69	17.79	0.73	5.00E-05
AT2G06990	*hua enhancer 2 (HEN2);*	14.40	23.17	0.69	5.00E-05
AT5G14620	*domains rearranged methyltransferase 2 (DRM2);*	6.11	9.66	0.66	0.00015
AT2G38950	*Transcription factor jumonji (jmj) family protein;*	17.57	27.18	0.63	5.00E-05
AT2G39020	Acyl-CoA N-acyltransferases (NAT) superfamily protein;	56.00	77.09	0.46	0.00035
AT1G21920	Histone H3 K4-specific methyltransferase SET7/9 family protein;	8.86	11.94	0.43	0.00935
AT5G61060	*histone deacetylase 5 (HDA05);*	16.08	21.06	0.39	0.0029
AT2G39030	*RNA polymerase II large subunit (NRPB1);*	27.83	36.38	0.39	0.0012
AT3G18520	*histone deacetylase 15 (HDA15);*	17.94	22.28	0.31	0.0178
AT1G14790	*RNA-dependent RNA polymerase 1 (RDR1);*	8.59	10.65	0.31	0.02225
Down-regulated genes					
AT5G44800	*chromatin remodeling 4 (CHR4);*	15.47	12.40	-0.32	0.0071
AT2G44150	*histone-lysine N-methyltransferase ASHH3 (ASHH3);*	11.68	9.05	-0.37	0.0264
AT5G56740	*histone acetyltransferase of the GNAT family 2 (HAG2);*	17.65	13.55	-0.38	0.0137
AT5G08430	*SWIB/MDM2 domain;*	13.58	9.96	-0.45	0.00395
AT2G28720	Histone superfamily protein;	96.07	69.51	-0.47	0.0002
AT5G27670	*histone H2A 7 (HTA7);*	120.74	87.28	-0.47	0.0001
AT4G29730	*nucleosome/chromatin assembly factor group C5 (NFC5);*	7.22	5.17	-0.48	0.00955
AT4G40030	Histone superfamily protein;	415.57	292.48	-0.51	5.00E-05
AT5G18620	*chromatin remodeling factor17 (CHR17);*	18.97	13.29	-0.51	5.00E-05
AT4G13460	*SU(VAR)3-9 homolog 9 (SUVH9);*	23.67	16.09	-0.56	5.00E-05
AT2G36490	*demeter-like 1 (DML1);*	25.00	16.68	-0.58	5.00E-05
AT5G13960	*SU(VAR)3-9 homolog 4 (SUVH4);*	7.64	5.06	-0.60	0.00105
AT2G27040	*ARGONAUTE 4 (AGO4);*	24.06	15.48	-0.64	5.00E-05

continuation TABLE 5. Genes involved with epigenetic mechanisms (TAIR10) significantly up and down-regulated 48 hours after imazethapyr (IM) treatment, according to RNA-seq data. UFRGS, Porto Alegre, RS, Brazil. 2017.

Gene ID	Gene Annotation	Control	IM	Log2 Fold Change	Adjusted P Value[2]
		FPKM [1]			
Down-regulated genes					
AT4G40030	Histone superfamily protein;	415.57	292.48	-0.51	5.00E-05
AT5G18620	*chromatin remodeling factor17 (CHR17);*	18.97	13.29	-0.51	5.00E-05
AT4G13460	*SU(VAR)3-9 homolog 9 (SUVH9);*	23.67	16.09	-0.56	5.00E-05
AT2G36490	*demeter-like 1 (DML1);*	25.00	16.68	-0.58	5.00E-05
AT5G13960	*SU(VAR)3-9 homolog 4 (SUVH4);*	7.64	5.06	-0.60	0.00105
AT2G27040	*ARGONAUTE 4 (AGO4);*	24.06	15.48	-0.64	5.00E-05
AT5G43990	*zinc ion binding, histone-lysine N-methyltransferase activity SUVR2;*	6.64	4.04	-0.72	0.00015
AT5G63950	*chromatin remodeling 24 (CHR24);*	7.89	4.30	-0.88	5.00E-05
AT5G61070	*histone deacetylase of the RPD3/HDA1 superfamily 18 (HDA18);*	1.16	0.63	-0.88	0.0135
AT3G27180	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein;	27.91	14.62	-0.93	5.00E-05
AT5G64150	RNA methyltransferase family protein;	13.56	6.41	-1.08	5.00E-05
AT4G33470	*histone deacetylase 14 (hda14);*	48.01	20.00	-1.26	5,00E-05
AT4G27230	*histone H2A 2 (HTA2);*	84.29	35.01	-1.27	5.00E-05
AT5G64630	*FASCIATA 2 (FAS2);*	6.91	2.74	-1.33	5.00E-05
AT1G65470	*FASCIATA 1 (FAS1);*	9.50	3.71	-1.36	5.00E-05
AT5G66750	*chromatin remodeling 1 (CHR1);*	8.73	3.06	-1.51	5.00E-05
AT3G28460	*methyltransferases;*	40.87	13.87	-1.56	5.00E-05
AT4G19020	*chromomethylase 2 (CMT2);*	10.60	3.31	-1.68	5.00E-05
AT1G51060	*histone H2A 10 (HTA10);*	263.45	70.29	-1.91	5.00E-05
AT2G28740	*histone H4 (HIS4);*	303.29	80.88	-1.91	5.00E-05
AT1G69770	*chromomethylase 3 (CMT3);*	19.41	4.95	-1.97	5.00E-05
AT3G20670	*histone H2A 13 (HTA13);*	120.90	30.31	-2.00	5.00E-05
AT3G54560	*histone H2A 11 (HTA11);*	76.58	16.47	-2.22	5.00E-05
AT3G46320	histone superfamily protein;	165.57	34.32	-2.27	5.00E-05
AT5G59870	*histone H2A 6 (HTA6);*	267.30	51.66	-2.37	5.00E-05
AT5G65350	*histone 3 11 (HTR11);*	9.38	1.55	-2.60	5.00E-05

[1] Fragments per kilobase of transcript per million fragments mapped (FPKM) are an average of two biological replicates 48 hours after imazethapyr (IM) treatment;
[2]Adjusted P values were calculated using Cuffdiff statistics.

Limited information is available for the effect of herbicide on epigenetic mechanisms. A recent study with atrazine in rice showed that DNA methyltransferases, histone methyltransferases and DNA demethylase were differentially regulated by this compound (Lu *et al*., 2016). The results obtained in the present study not only provide a comprehensive analysis of imazethapyr effects in *A. thaliana* pathways such as the changes in genes linked to response to chemical stimulus, secondary metabolism and stress, but also provide evidence that imazethapyr-induced changes in epigenetic regulation genes.

3.2.3.2 Genes of interest – gene ontology (GO)

GO analysis of genes of interest classified 75 from 89 genes in categories of biological process, molecular function and cellular component. The genes involved with cellular component (15 genes) were divided in four main categories including cell part, macromolecular complex, membrane, and organelle that corresponded to 10.7%, 4.0% 2.7% and 2.7% of total genes, respectively (Figure 12). For biological processes, 53 genes were identified and divided in classes as biological regulation (8.0%), cellular process (17.3%), developmental process (1.3%), localization (6.7%), metabolic process (32.0%), multicellular organismal process (1.3%) and response to stimulus (4.0%). The 30 molecular function genes were classified in three categories, binding (8.0%), catalytic activity (26.7%) and transporter activity (5.3%) (Figure 12).

Detail information of biological process showed that the profile of these genes is involved in a total of 22 pathways overrepresented, according to SEA (Figure 13). The candidate genes putatively involved with NTSR (cytochrome P450, GST, transporters, oxidases, glycosyl-transferase and esterases/hydrolase) were selected on the basis of high variation in expression through imazethapyr application

in WT and low expression in *ros1* mutant. Thirty-one candidate genes were identified, including two cytP450, three GSTs, thirteen transporters, one oxidase, five glycosyl-transferase and six esterases/hydrolase (Appendix 5).

CytP450, GST, glycosyltransferase and transporter genes can be involved in herbicide NTSR via enhanced expression (Délye, 2013). Among these mechanisms, the oxidization of herbicides by endogenous cytP450 is thought to be a major pathway in plants (Siminszky, 2006). In the present study, the promoter region of these candidate genes was also analyzed to verified if they can be epigenetically regulated via DNA methylation as showed in items 3.3 and 3.4.

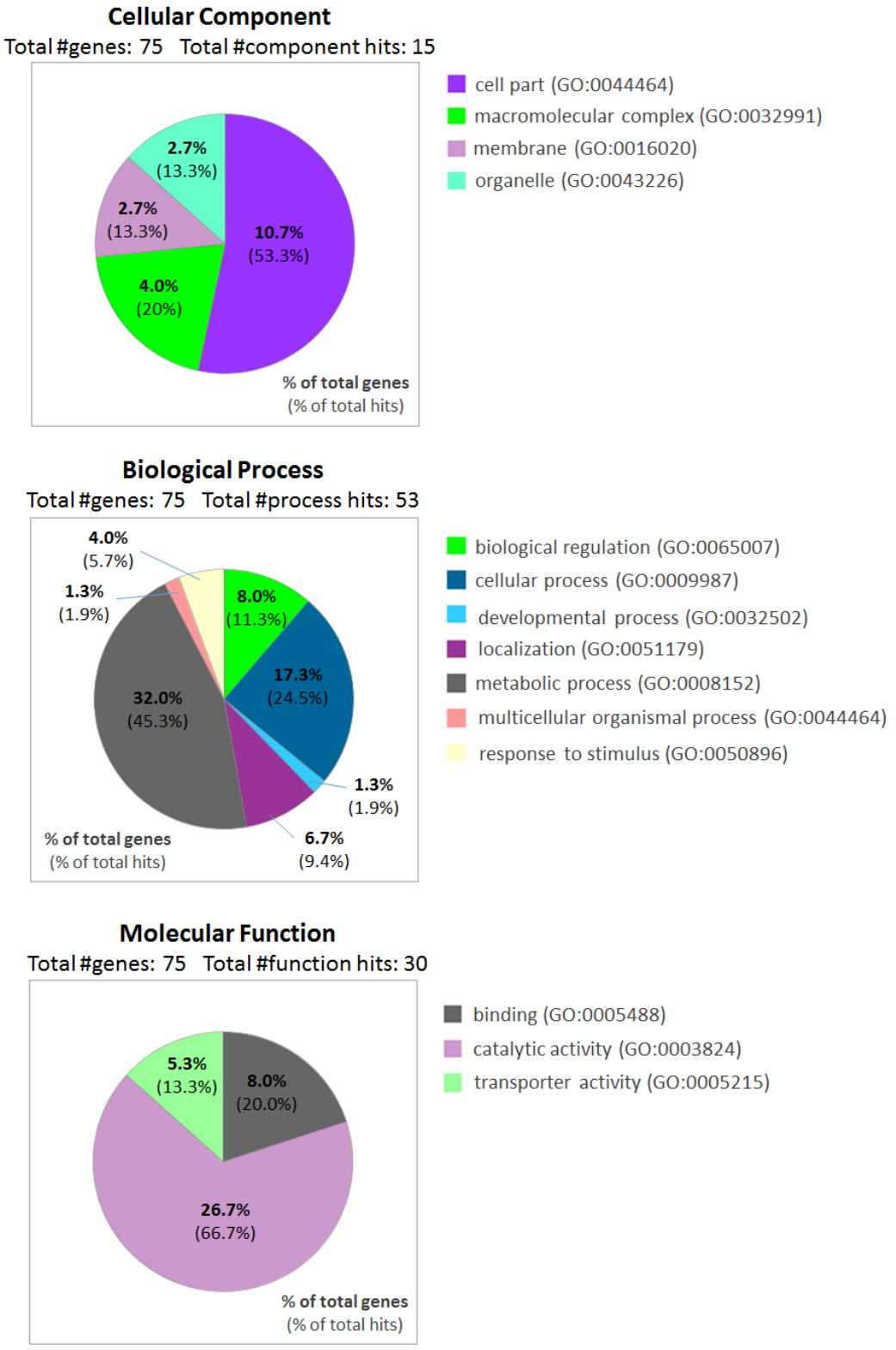


FIGURE 12. Pie chart representing Gene Ontology (GO) of 89 genes of interest induced by imazethapyr (IM) in wild type (WT) and down-regulated in *ros1* mutant. 75 differential expression of genes (DEGs) were annotated in at least one of the three GO categories: cellular component biological process and molecular function. UFRGS, Porto Alegre, RS, Brazil. 2017.

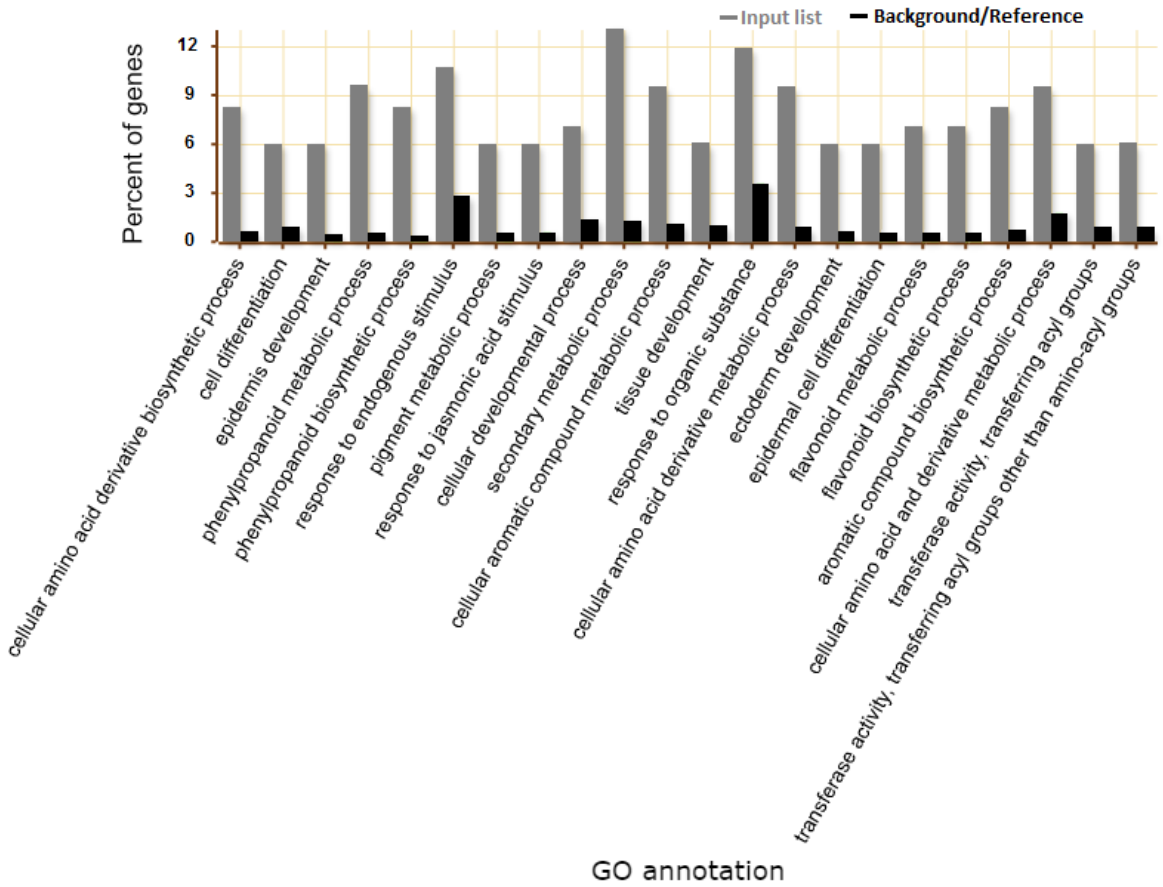


FIGURE 13. Detail information of biological process representing percent of genes involved in biological process pathways of 89 genes of interest induced by imazethapyr (IM) in wild type (WT) and down-regulated in *ros1* mutant, performed by using singular enrichment analysis (SEA). Gray and black bars indicate the percent of genes related to input list and the percent of genes compared to genome reference, respectively. UFRGS, Porto Alegre, RS, Brazil. 2017.

In order to analyze the pathway connections of genes of interest an overview showing the predominance of genes involved with metabolic processes, cellular processes and response to stimulus is represented in Figures 14 and 15. These results are consistent with GO presented in Figure 13. In order to improve the visualization of pathways the figure was divided in parts I and II that correspond to Figure 14 and 15, respectively. The first part (Figure 14) shows that the metabolic processes most significantly enriched pathways were cellular amino acid derivative biosynthetic process, aromatic compound biosynthetic process, secondary metabolic process that are connected to the flavonoid biosynthetic process and

flavonoid metabolic process. The second part (Figure 15) represent the pathways involved with cellular processes and response to stimulus. For cellular processes, the enriched pathways were mainly involved with cell differentiation, tissue development related to epidermal cell differentiation.

The accumulation of flavonoids in plants is induced under the influence of abiotic stresses such as nitrogen, light, temperature, UV, and drought (Dixon & Paiva, 1995). In grasses, studies have revealed that multiple herbicide resistance is connected to changes in endogenous antioxidant and secondary metabolism, particularly an accumulation of cytoprotectants such as glutathione, flavonoids and anthocyanins (Cummins *et al*., 2009). A proteome study in *A. thaliana* showed that imazethapyr does not affect the gene transcription or translation of the ALS enzyme. In contrast, imazethapyr induced the biosynthesis of anthocyanins, which include antioxidants that protect plants against biotic and abiotic stressors and decrease reactive oxygen species (ROS) accumulation and toxicity (Qian *et al*., 2015). Transformed *Arabidopsis* show that *Alopecurus myosuroides* (Am) *AmGSTF1* conferred tolerance multiple herbicides and exerted a direct regulatory control on metabolism that led to an accumulation of protective flavonoids (Cummins *et al*., 2013). To better understand the involvement of accumulation of flavonoids in the tolerance to imazethapyr in *A. thaliana* a flavonoid-deficient mutant, *transparent testa4* (*tt4*), was tested and contrasted with WT results for imazethapyr treatment (Figure 16).

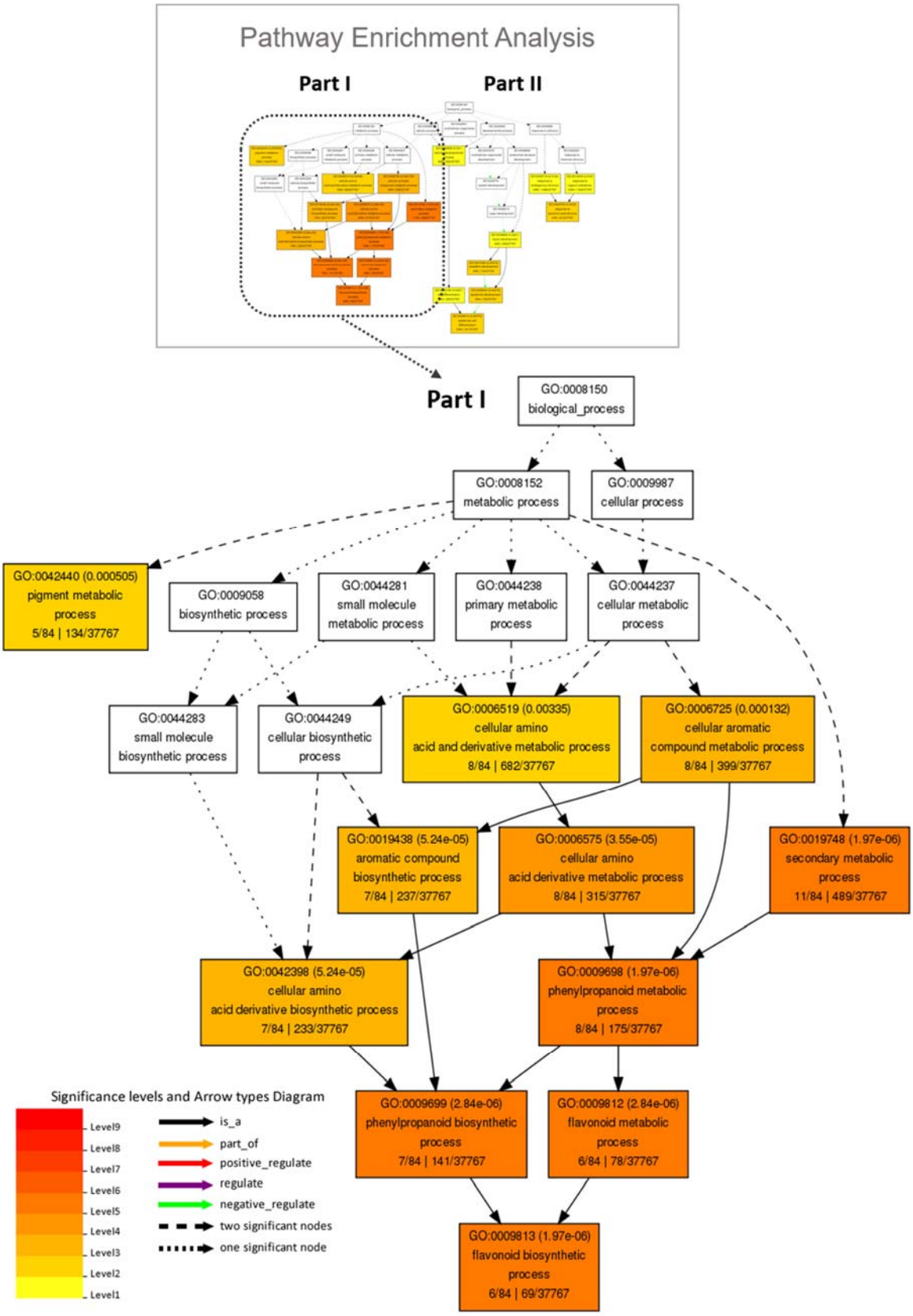


FIGURE 14. Part I of overview of pathways overrepresented, according to singular enrichment analysis (SEA). The color scale indicates significance levels of enrichment analysis. The arrows represent the relationship between parent–child terms. UFRGS, Porto Alegre, RS, Brazil. 2017.

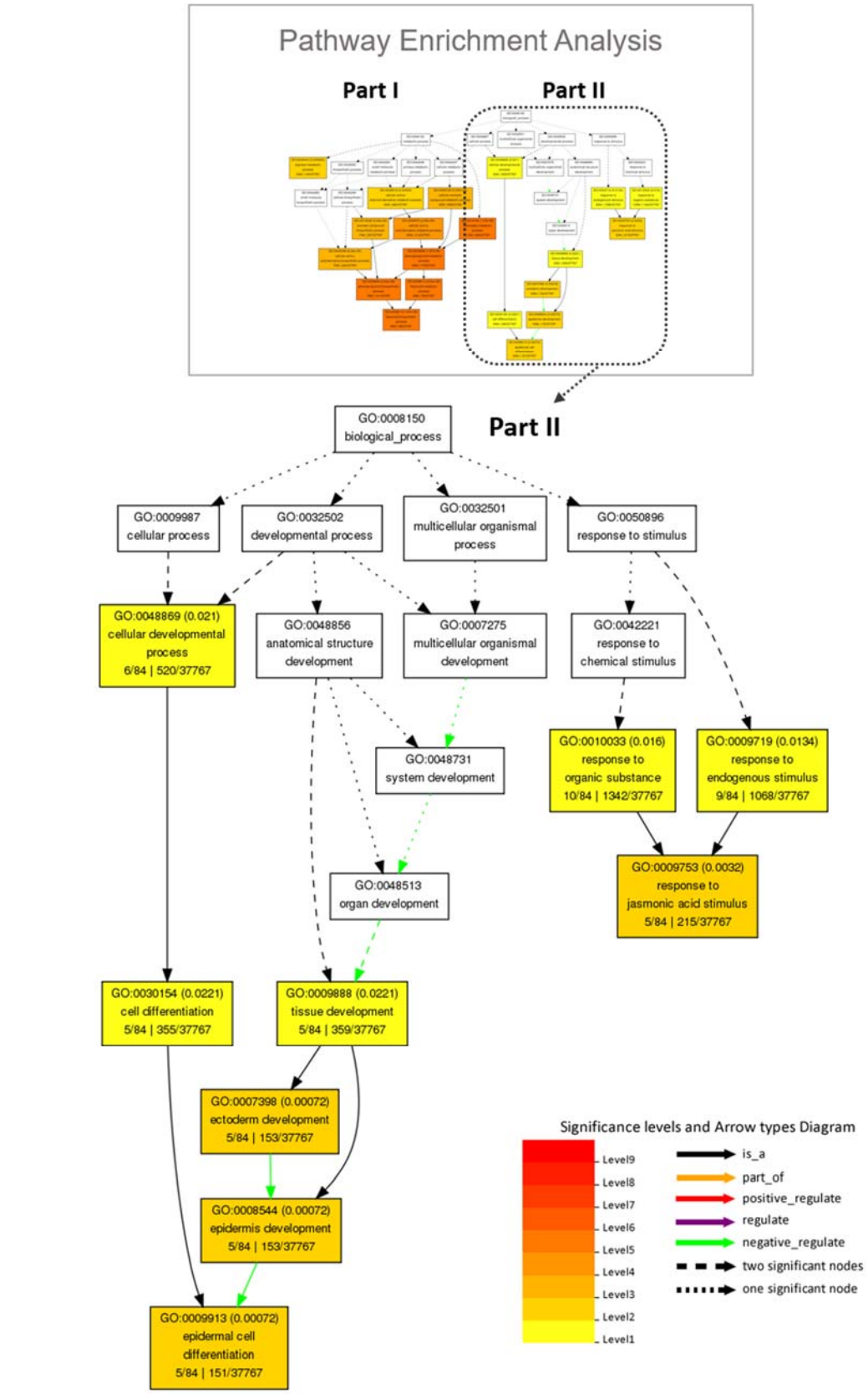


FIGURE 15. Part II of overview of pathways overrepresented, according to singular enrichment analysis (SEA). The color scale indicates significance levels of enrichment analysis. The arrows represent the relationship between parent–child terms. UFRGS, Porto Alegre, RS, Brazil. 2017.

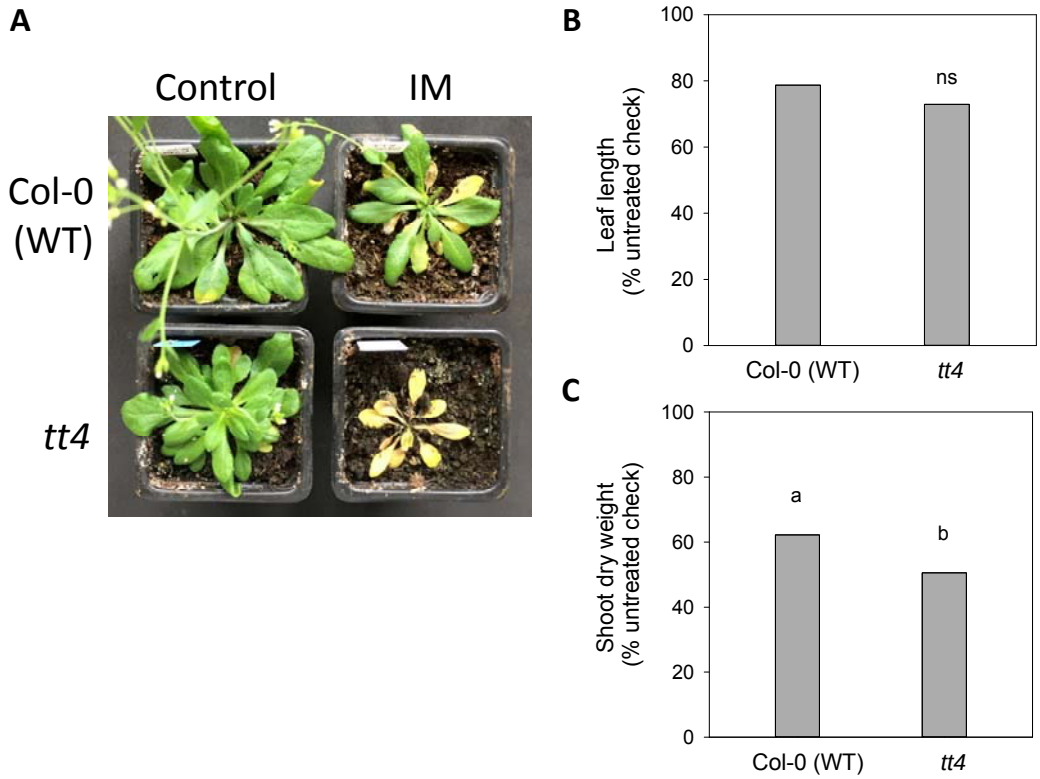


FIGURE 16. (A) Visual effect (B) Leaf length and (C) Shoot dry weight of *Arabidopsis thaliana* wild type (WT) and *tt4* mutant, 14 days after application of herbicide imazethapyr (IM). Means followed by different letter differ significantly after Tukey ($p<0.05$); UFRGS, Porto Alegre, RS, Brazil. 2017.

The F test showed significance for the shoot dry weight and absence of significance for leaf length (Appendix 6 and 7). The *tt4* mutant revealed decrease in approximately 10% of shoot dry weight 14 days after imazethapyr treatment compared to WT (Figure 16C). The visual effect of imazethapyr in *tt4* corroborates with shoot dry weight results, showing an increase in imazethapyr susceptibility (Figure 16A). Interestingly, similar result was found to saline stress in tobacco (Bharti *et al*., 2015). Thirty-five genes are involved to encoding biosynthetic enzymes or transcription factors that are responsible for aglycone formation and subsequent tailoring modifications to produce flavonols and anthocyanins (Yonekura-Sakakibara *et al*., 2008). Transgenic tobacco overexpressing AtROS1 increased the demethylation level at promoter gene involved with the flavonoid biosynthetic pathway, glutathione S-transferase, ascorbate peroxidase, glutathione

peroxidase, and glutathione reductase (Bharti *et al*., 2015). The demethylation was increased during salt-stress conditions, and showed the importance of AtROS1 in the epigenetic regulation of flavonoid biosynthetic and antioxidant pathways during salt-stress exposure of plants (Bharti *et al*., 2015). These data are similar with results found in the present study and suggest that flavonoid accumulation have also effect on imazethapyr tolerance in *A. thaliana* and that some genes of this pathway are epigenetically regulated since they present low expression in *ros1* mutant.

3.3 *In silico* analysis of promoter region of candidate genes

In order to verify if candidate genes described in Appendix 5 are epigenetically regulated via DNA methylation, the promoter region of them was analyzed *in silico.* Thirty-one candidate genes potentially involved in NTSR were evaluated and are described in Table 6. The *in silico* analysis was performed according to Epigenomics Data provided by Jacobsen Labs (*http://genomes.mcdb.ucla.edu/AthBSseq/*). Each gene was observed in upstream region conferring to the presence of transposable elements (TE) and DNA methylation, according to figure presented in Appendix 8. *Arabidopsis* encodes four DNA demethylases, DME, ROS1, DML2 and DML3. The mutant *rdd* is a triple DNA demethylase mutant (ros1 dml2 dml3). Additionally, to the *in silico* examination, the upstream region of *rdd* mutant was verified for each gene (Appendix 8; Table 6). Genes that presented at least two of these items in upstream region (presence of TE, DNA methylation and/or change in DNA methylation in *rdd* mutant) were selected for the study of the promoter region determined by quantitative PCR after cleavage with methylation sensitive restriction enzymes. Six out of 31 genes were selected and are present as outlined in Table 6.

TABLE 6. *In silico* analysis of upstream region of putative herbicide resistance genes. UFRGS, Porto Alegre, RS, Brazil. 2017.

		Putative herbicide resistance genes	Upstream region/presence of[1]: TE[2]	5mC[3]	*rdd* change[4]
		Cytochromes P450			
1	AT4G19230	cytochrome P450, family 707, subfamily A, polypeptide 1 (CYP707A1);	yes	no	no
2	AT1G19630	cytochrome P450, family 722, subfamily A, polypeptide 1 (CYP722A1);	no	no	no
3	AT5G07990	TRANSPARENT TESTA 7 (TT7); or CYTOCHROME P450 75B1	no	yes	yes
		GST			
4	AT2G29490	glutathione S-transferase TAU 1 (GSTU1);	no	no	no
5	AT5G17220	glutathione S-transferase phi 12 (GSTF12);	no	no	no
6	AT1G17170	glutathione S-transferase TAU 24 (GSTU24);	no	yes	no
		Transporters			
7	AT2G04070	MATE efflux family protein;	no	no	no
8	AT1G51090	Heavy metal transport/detoxification superfamily protein ;	no	yes	yes
9	AT1G43890	RAB GTPASE HOMOLOG B18 (RAB18);	no	yes	yes
10	AT4G21910	MATE efflux family protein;	yes	no	no
11	AT4G35060	Heavy metal transport/detoxification superfamily protein;	no	no	no
12	AT1G70300	K+ uptake permease 6 (KUP6);	yes	no	no
13	AT5G47560	tonoplast dicarboxylate transporter (TDT);	no	no	no
14	AT1G09180	secretion-associated RAS super family 1 (SARA1A);	no	no	no
15	AT1G03550	Secretory carrier membrane protein (SCAMP) family protein;	yes	yes	yes
16	AT1G31820	Amino acid permease family protein;	no	no	no
17	AT1G04570	Major facilitator superfamily protein;	yes	yes	yes
18	AT2G41190	Transmembrane amino acid transporter family protein;	no	no	no
19	AT3G46450	SEC14 cytosolic factor family protein/ phosphoglyceride transfer family protein;	no	no	no
		Oxidases			
20	AT4G20860	FAD-binding Berberine family protein;	no	no	no
		Glycosyl-transferase			
21	AT2G43820	UDP-glucosyltransferase 74F2 (UGT74F2);	no	no	no
22	AT5G54060	UDP-glucose:flavonoid 3-o-glucosyltransferase (UF3GT);	no	no	no
23	AT1G24070	cellulose synthase-like A10 (CSLA10);	no	no	no
24	AT1G56600	galactinol synthase 2 (GolS2);	no	no	no
25	AT1G05675	UDP-Glycosyltransferase superfamily protein;	no	no	no
		Esterases/hydrolase			
26	AT4G10050	esterase/lipase/thioesterase family protein;	STR	yes	yes
27	AT1G54020	GDSL-like Lipase/Acylhydrolase superfamily protein;	no	no	no
28	AT1G47510	inositol polyphosphate 5-phosphatase 11 (5PTASE11);	yes	no	no

continuation TABLE 6. *In silico* analysis of upstream region of putative herbicide resistance genes. UFRGS, Porto Alegre, RS, Brazil. 2017.

		Putative herbicide resistance genes	Upstream region/presence of[1]: TE[2]	5mC[3]	*rdd* change[4]
		Esterases/hydrolase			
29	AT3G43580	Beta-galactosidase related protein;	STR	yes	yes
30	AT2G14620	xyloglucan endotransglucosylase/ hydrolase 10 (XTH10);	yes	no	yes
31	AT5G50400	purple acid phosphatase 27 (PAP27);	yes	yes	yes

[1]Analyzed data in Epigenomics Data Jacobsen labs, Dept. of MCDB, UCLA;
[2]Presence of Transposable Element (TE);
[3]Presence of DNA methylation in 5-position of cytosine (5mC);
[4]DNA methylation change in rdd triple DNA demethylase mutant, *rdd* (*ros1 dml2 dml3*).

3.4 DNA methylation analysis by methylation-sensitive restriction cleavage and quantitative RT-PCR validation of genes by RNA-Seq

The global DNA methylation was reduced in WT after imazethapyr treatment compared to *ros1* as described previously in item 3.1 (Figure 3). In order to check and compare the effect of imazethapyr in DNA methylation of specific sequence, the analysis of cytosine methylation determined by quantitative PCR after cleavage with methylation sensitive restriction enzymes was performed. Six genes outlined in the Table 6 were chosen to proceed the analysis by methylation-sensitive restriction cleavage. According to RNA-seq, these genes increased this expression as a consequence of imazethapyr application in WT and showed reduced expression compared to *ros1* (Figure 17A), which can be better observed through heat map (Figure 17B). First of all, in order to check these results and to confirm RNA-Seq data, these six genes were selected for qRT-PCR analyses. It is shown that all genes analyzed by qRT-PCR (Figure 18) had an expression pattern similar to RNA-Seq (Figure 17), providing RNA-seq validation.

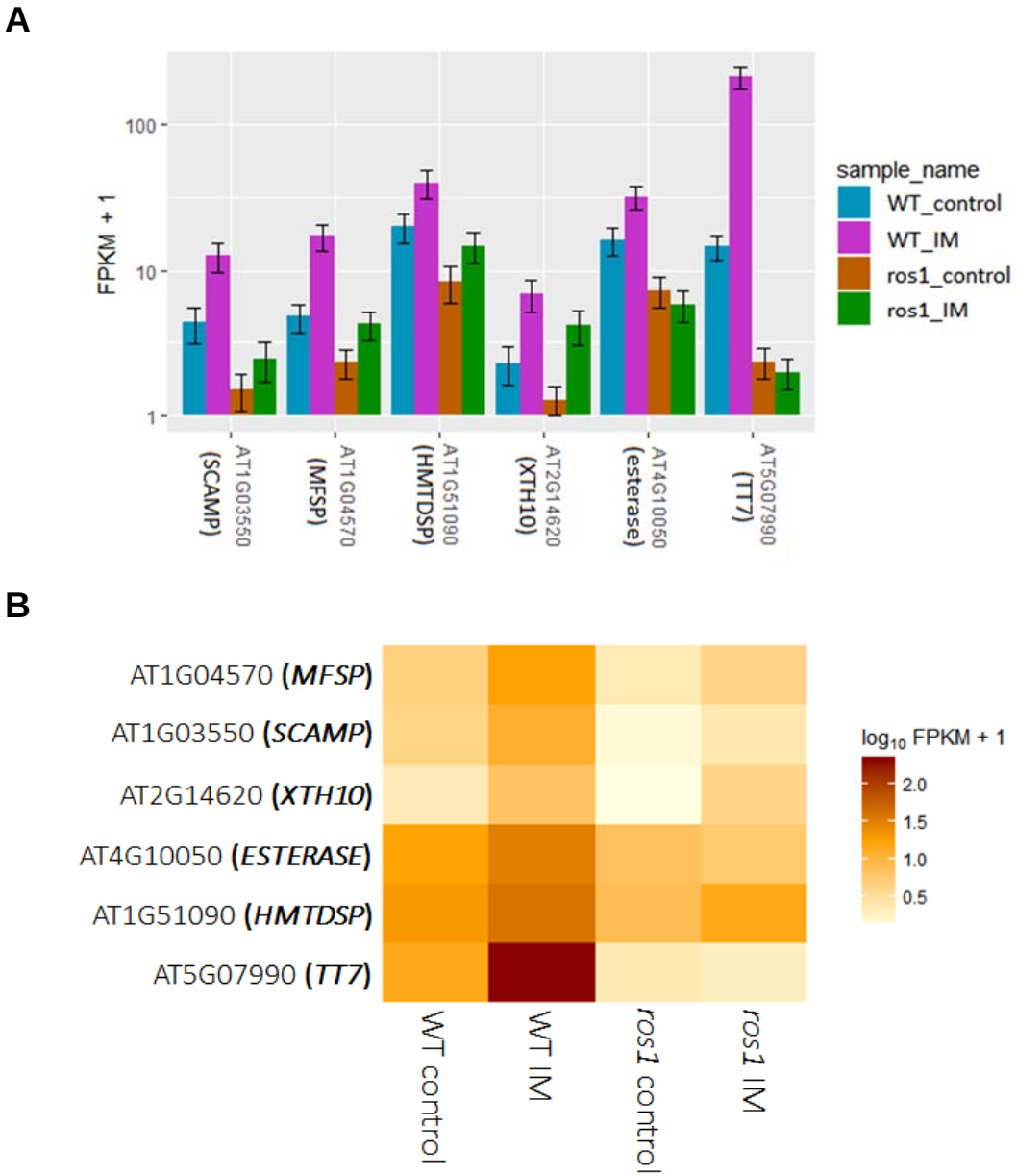


FIGURE 17. Differentially expressed genes (DEGs) performed by using CummeRbund, comparing gene in response to herbicide imazethapyr (IM) in *A. thaliana* wild type (WT) and *ros1,* relative expression values computed from the Fragments Per Kilobase of exon per Million fragments mapped (FPKM) counts. (A) Bar graph shows each gene and transcript expression value annotated with error bars that capture both cross-replicate variability and measurement uncertainty as estimated by Cuffdiff's statistical model of RNA-seq (adjusted p-value < 0.05) (B) The gene-normalized signal intensities is show in the heat map. UFRGS, Porto Alegre, RS, Brazil. 2017.

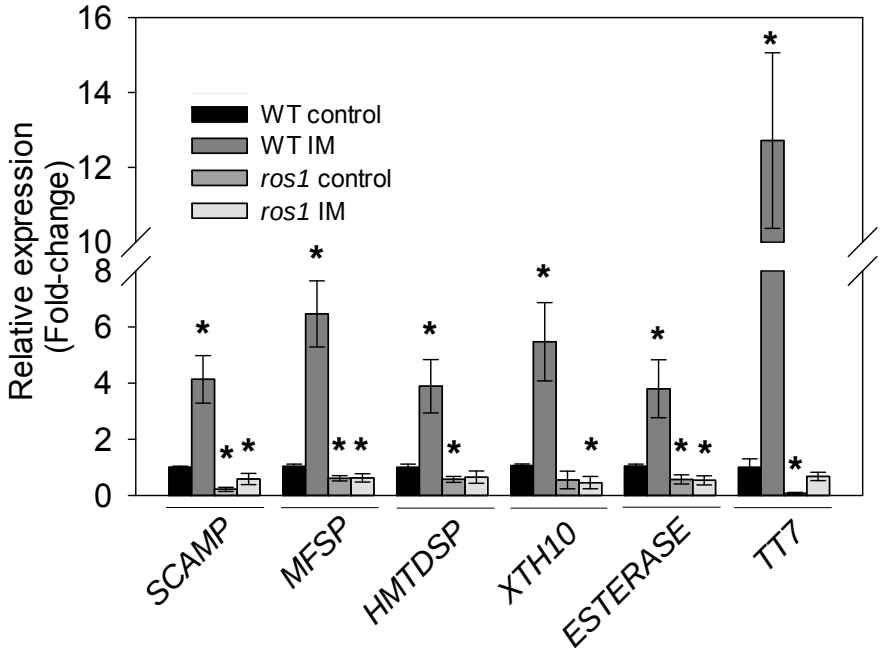


FIGURE 18. Quantitative PCR validation (Fold-change) of genes from the differentially expressed genes (DGEs) profiling. Error bars indicate standard deviation of three biological replicates and asterisks indicate significant differences between the treatments and wild type (WT) control according to t-test ($p<0.05$). UFRGS, Porto Alegre, RS, Brazil. 2017.

Secondly, part of promoter region (approximately 500 bp) of each gene was analyzed in at least two contexts of CG, CHG and/or CHH. Different methylation sensitive enzymes were used in order to assess the methylation level in each context. The percentage of un-cleaved DNA was normalized to the DNA that was not cut by any enzyme. The ANOVA of DNA methylation showed significant interaction between the genotypes and sites of DNA methylation (CG, CHG and CHH) (Appendix 9).

The level of DNA methylation in *TT7* reduced after imazethapyr treatment in WT and *ros1* for the sites CG and CHH (Figure 19A). The genes *HMTDSP*, *SCAMP* and *MFSP* showed similar behavior for WT, reducing respectively 50% in CHH, 45% in CHH and 20% in CG of DNA methylation after imazethapyr application (Figure 19B, C and D). For these genes, it is suggested that imazethapyr cause effect on

DNA demethylation. However, this pattern was not observed for all genes. For *esterase* gene (Figure 19E), the level did not significant change after imazethapyr treatment and for *XTH10* (Figure 19F) the imazethapyr including increased the DNA methylation level in CG and CHH contexts. In *XTH10,* high methylation level was found for CHG site in *ros1* for treated and non-treated plants, which was proximally 75%. Additionally, imazethapyr reduced the level of DNA methylation in *ros1* only in CHH site for the gene *TT7*. The analyzed sequence region showed the dynamic activity of DNA methylation, especially for WT, and suggests the effect of the herbicide imazethapyr to enhance these changes and lead to regulation of gene expression. The confirmation of these results requires a bisulfite sequencing analysis.

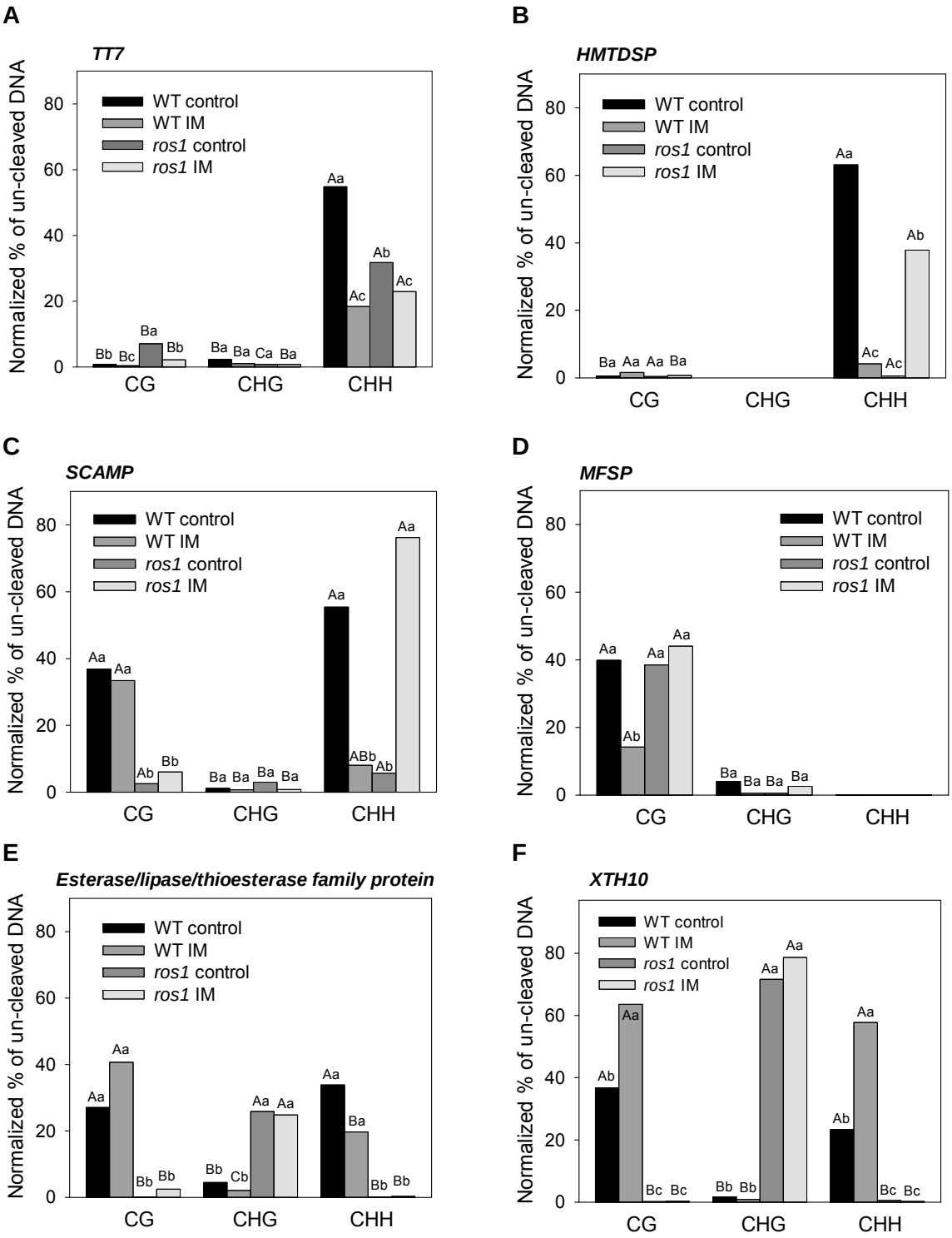


FIGURE 19. Normalized % of un-cleaved DNA that represent cytosine methylation at CG, CHG and CHH contexts, for genes: (A) *TT7*; (B) *HMTDSP*; (C) *SCAMP*; (D) *MFSP*; (E) *Esterase/lipase thioesterase family protein* and (F) *XTH10*. Means followed by different letters differ significantly by Tukey ($p<0.05$). Lowercase letters indicate comparison among treated and non-treated genotypes at each context (CG, CHG and CHH). Capital letters indicate comparison of the same genotype among the CG, CHG and CHH contexts. UFRGS, Porto Alegre, RS, Brazil. 2017.

4 CONCLUSIONS

The application of sub-lethal dose of imazethapyr leads to global alteration in DNA demethylation in *A. thaliana.* Plants lacking ROS1 show no alteration in DNA methylation level after imazethapyr treatment. These results indicate that ROS1 may have an important role in DNA demethylation induced by imazethapyr.

In *A. thaliana*, imazethapyr treatment induces the expression of genes linked to response to chemical stimulus, stress, and secondary metabolism. In contrast, this herbicide reduces the expression of genes involved to cell cycle, response to hormones, anatomical structure, and morphogenesis, and involved in central energy (photosynthesis compounds) pathways. Plants lacking ROS1 do not show the expression of several genes involved with flavonoid biosynthesis. A flavonoid mutant *tt4* presented increase in imazethapyr susceptibility. Thence, flavonoid accumulation appears to be important on imazethapyr tolerance in *A. thaliana* and some genes of this pathway are epigenetically regulated since they present low expression in *ros1* mutant.

Additionally, some putatively genes involved with imazethapyr tolerance in *A. thaliana* show the presence of TEs and DNA methylation near to/or in promoter region, which favors the epigenetic regulation mainly via DNA methylation. The analysis of CG, CHG and CHH contexts of genes *TT7, HMTDSP*, *SCAMP*, *MFSP* and *XTH10* show that imazethapyr is able to chance the DNA methylation levels. These results together suggest that imazethapyr-induced changes in DNA methylation marks are possibly involved in an epigenetic mechanism associated with activation of specific genes responsible for imazethapyr degradation and detoxification.

5 REFERENCES

BHARTI, P. et al. AtROS1 overexpression provides evidence for epigenetic regulation of genes encoding enzymes of flavonoid biosynthesis and antioxidant pathways during salt stress in transgenic tobacco. **Journal of Experimental Botany**, Oxford, v.66, n.19, p.5959-5969, 2015.

BUSI, R.; VILA-AIUB, M.M.; POWLES, S.B. Genetic control of a cytochrome P450 metabolism-based herbicide resistance mechanism in Lolium rigidum. **Heredity**, London, v.106, n.5, p.817-824, 2011.

BUSTIN, S.A. et al. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. **Clinical Chemistry**, Washington, v.55, n.4, p.611-622, 2009.

CHINNUSAMY, V.; ZHU, J.K. Epigenetic regulation of stress responses in plants. **Current Opinion in Plant Biology**, London, v.12, n.2, p.133-139, 2009.

CUMMINS, I. et al. Key role for a glutathione transferase in multiple-herbicide resistance in grass weeds. **Proceedings of the National Academy of Sciences of the United States of America**, Collins, v.110, n.15, p.5812-5817, 2013.

CUMMINS, I.; BRYANT, D.N.; EDWARDS, R. Safener responsiveness and multiple herbicide resistance in the weed black-grass (Alopecurus myosuroides). **Plant Biotechnology Journal**, Malden, v.7, n.8, p.807-820, 2009.

DALAZEN, G. et al. Genes relacionados à detoxificação de imazethapyr em capim-arroz (Echinochloa crus-galli). In: CONGRESSO BRASILEIRO DE ARROZ IRRIGADO, n.9, 2015, Pelotas. **Anais...** Pelotas: SOSBAI, 2015. p.840-843.

DAS, M. et al. A composite transcriptional signature differentiates responses towards closely related herbicides in Arabidopsis thaliana and Brassica napus. **Plant Molecular Biology**, Dordrecht, v.72, n.4-5, p.545-556, 2010.

DÉLYE, C. Unravelling the genetic bases of non-target-site-based resistance (NTSR) to herbicides: a major challenge for weed science in the forthcoming decade. **Pest Management Science**, Malden, v.69, n.2, p.176-187, 2013.

DÉLYE, C. et al. Non-target-site-based resistance should be the centre of attention for herbicide resistance research: Alopecurus myosuroides as an illustration. **Weed Research**, Malden, v.51, n.5, p.433-437, 2011.

DEVINE, M.D.; EBERLEIN. C.V. Physiological, biochemical and molecular aspects of herbicide resistance based on altered target sites. pp. 159-185. In: HERBICIDE activity: toxicology, biochemistry and molecular biology. Amsterdam: IOS Press, 1997.

DIXON R.A.; PAIVA N.L. Stress-induced phenylpropanoid metabolism. **Plant Cell**, Rockville, n.7, p.1085-1097, 1995.

DUHOUX, A. et al. RNA-Seq analysis of rye-grass transcriptomic response to an herbicide inhibiting acetolactate-synthase identifies transcripts linked to non-target-

site-based resistance. **Plant Molecular Biology**, Dordrecht, v.87, n.4-5, p.473-487, 2015.

GARCÍA-ALCALDE F. et al. Qualimap: evaluating next-generation sequencing alignment data. **Bioinformatics**, Oxford, v.28, p.2678–2679, 2012.

GRESSEL, J. Evolving understanding of the evolution of herbicide resistance. **Pest Management Science**, Malden, v.65, n.11, p.1164-1173, 2009.

HEAP, I. **The International survey of herbicide resistant weeds.** 2017. Available in:<www.weedscience.com>. Accessed in: 26 jan. 2017.

HUANG, J.J. et al. Roles of DNA methyltransferases in Arabidopsis development. **African Journal of Biotechnology**, Victoria Island, v.9, n.50, p.8506-8514, 2010.

IWAKAMI, S. et al. Cytochrome P450 CYP81A12 and CYP81A21 Are Associated with Resistance to Two Acetolactate Synthase Inhibitors in Echinochloa phyllopogon. **Plant Physiology**, Rockville, v.165, n.2, p.618-629, 2014.

KHRAIWESH, B. et al. Genome-wide expression analysis offers new insights into the origin and evolution of Physcomitrella patens stress response. **Nature Scientific Reports**, London, v.5, n.1, p.1-15, 2015.

KIM, D. et al. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. **Genome Biology**, London, v.14, n.4, p. 1-13, 2013.

LAMESCH, P. et al. The Arabidopsis Information Resource (TAIR): improved gene annotation and new tools. **Nucleic Acids Research**, Oxford, v.40, n.1, p.1202-1210, 2012.

LANE, T. S. et al. Diversity of ABC transporter genes across the plant kingdom and their potential utility in biotechnology. **Bmc Biotechnology**, London, v.16, n.1, p.1-10, 2016.

LAW, J.A.; JACOBSEN, S.E. Establishing, maintaining and modifying DNA methylation patterns in plants and animals. **Nature Reviews Genetics**, London, v.11, n.3, p.204-220, 2010.

LIVAK, K.J.; SCHMITTGEN, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T) (-Delta Delta C) method. **Methods**, San Diego, v.25, n.4, p.402-408, 2001.

LU, Y.C. et al. Genome-wide identification of DNA methylation provides insights into the association of gene expression in rice exposed to pesticide atrazine. **Nature Scientific Reports**, London, v.6, n.1, p.1-15, 2016.

MANSOUR, F.H.; PESTOV, D.G. Separation of long RNA by agarose-formaldehyde gel electrophoresis. **Analytical Biochemistry**, v.441, n.1, p.18-20, 2013.

PENTERMAN, J.; UZAWA, R.; FISCHER, R.L. Genetic interactions between DNA demethylation and methylation in Arabidopsis. **Plant Physiology**, Rockville, v.145, n.4, p.1549-1557, 2007.

POWLES, S.B.; YU, Q. Evolution in Action: Plants Resistant to Herbicides. **Annual Review of Genetics**, Palo Alto, v.61, p.317-347, 2010.

QIAN, H.F. et al. Analyzing Arabidopsis thaliana root proteome provides insights into the molecular bases of enantioselective imazethapyr toxicity. **Scientific Reports**, Legnaro, v.5, n.1, p.1-13, 2015.

QIAN, H.F. et al. Enantioselective phytotoxicity of the herbicide imazethapyr and its effect on rice physiology and gene transcription. **Environmental Science & Technology**, Washington, v.45, n.16, p.7036-7043, 2011.

SAS INSTITUTE. **Base SAS_9.1:** procedures guide. Cary: SAS Institute, 2004.

SIMINSZKY, B. Plant cytochrome p450-mediated herbicide metabolism. **Phytochemistry** Reviews, Dordrecht, v.5, n.1, p.445–458, 2006.

SOLTANI, N. et al. Efficacy of imazethapyr application timing on the control of long-spined sandbur Cenchrus longispinus (Hack. in Kneuck.) Fernald in soybean. **Canadian Journal of Plant Science**, Ottawa, v.89, n.1, p.205-207, 2009.

STEWARD, N. et al. Periodic DNA methylation in maize nucleosomes and demethylation by environmental stress. **Journal of Biological Chemistry**, Bethesda, v.277, n.40, p.37741-37746, 2002.

SUN, C.C. et al. Effects of the Herbicide Imazethapyr on Photosynthesis in PGR5- and NDH-Deficient Arabidopsis thaliana at the Biochemical, Transcriptomic, and Proteomic Levels**. Journal of Agricultural and Food Chemistry**, Washington, v.64, n.22, p.4497-4504, 2016.

TAN, S.Y. et al. Imidazolinone-tolerant crops: history, current status and future. **Pest Management Science**, Malden, v.61, n.3, p.246-257, 2005.

TRANEL, P.J.; WRIGHT, T.R. Resistance of weeds to ALS-inhibiting herbicides: what have we learned? **Weed Science**, Champaign, v.50, n.6, p.700-712, 2002.

TRAPNELL, C. et al. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. **Nature Protocols**, London, v.7, n.3, p.562-578, 2012.

VONGS, A. et al. Arabidopsis thaliana DNA methylation mutants. **Science**, Washington**,** v.260, n.5116, p.1926-1928, 1993.

YONEKURA-SAKAKIBARA, K. et al. Comprehensive flavonol profiling and transcriptome coexpression analysis leading to decoding gene-metabolite correlations in Arabidopsis. **Plant Cell**, Rockville, v.20, n.8, p.2160-2176, 2008.

YORK, A.C. et al. Efficacy of imazethapyr in peanut (Arachis-hypogaea) as affected by time of application. **Weed Science**, Champaign, v.43, n.1, p.107-116, 1995.

YU, Q. et al. AHAS herbicide resistance endowing mutations: effect on AHAS functionality and plant growth. **Journal of Experimental Botany**, Oxford, v.61, n.14, p.3925-3934, 2010.

YU, Q. et al. Distinct non-target site mechanisms endow resistance to glyphosate, ACCase and ALS-inhibiting herbicides in multiple herbicide-resistant Lolium rigidum. **Planta**, New York, v.230, n.4, p.713-723, 2009.

YU, Q.; POWLES, S. Metabolism-Based Herbicide Resistance and Cross-Resistance in Crop Weeds: A Threat to Herbicide Sustainability and Global Crop Production. **Plant Physiology**, Rockville, v.166, n.3, p.1106-1118, 2014.

ZHU, J.H. et al. The DNA glycosylase/lyase ROS1 functions in pruning DNA methylation patterns in Arabidopsis. **Current Biology**, Cambridge, v.17, n.1, p.54-59, 2007.

5 FINAL CONSIDERATIONS

Weeds are constantly exposed to non-lethal herbicide doses, and this process could trigger epigenetic changes. The present study identified that the application of sub-lethal dose of the herbicides glyphosate, imazethapyr and 2,4-D leads to global alterations of DNA methylation in *A. thaliana.* Additionaly, showed that specific epigenetic pathways related to DNA methylation, non-coding RNA, and histone modifications affect in plants susceptibility to the herbicide. These findings suggest that field situations where plants may receive reduced rates of herbicide can cause epigenetic alteration that are important to the plant to tolerate the herbicide. This is the first study which revealed that sub-lethal doses of the herbicides glyphosate, imazethapyr and 2,4-D can cause epigenetic alterations in *A. thaliana*.

The specific analyses for the herbicide imazethapyr show that this compound induce the expression of genes linked to response to chemical stimulus, stress, and secondary metabolism. In contrast, this herbice reduces the expression of genes mainly involved to cell division and central energy pathways. Additionally, imazethapyr also alters genes related to DNA methylation, non-coding RNA, histone modification and chromatin modifications, showing effect on epigenetic regulation enzymes. This suggests that specific epigenetic alteration (mainly involved with DNA methylation/demethylation) during imazethapyr exposure are directly or

indirectly associated with the activation of functional genes responsible for imazethapyr degradation or detoxification.

The discoveries found in this study also revealed that flavonoid accumulation appears to be important for imazethapyr tolerance in *A. thaliana* and that some genes of flavonoid biosynthesis pathway are epigenetically regulated by ROS1. Additionally, from some genes putative involved with imazethapyr tolerance show the presence of DNA methylation in promoter region, which favors the epigenetic regulation mainly via DNA methylation. The DNA methylation levels of CG, CHG and CHH sites vary in response to imazethapyr treatment and the behavior for DNA methylation in these sites is different for the mutant *ros1.* This result suggests that imazethapyr cause effect on DNA methylation not only in global DNA methylation but also in specific sites and that ROS1 is important to regulation of the genes *TT7, HMTDSP*, *SCAMP*, *MFSP* and *XTH10* through the dynamic process of DNA demethylation.

These results together accept the hypothesis that herbicides glyphosate, imazethapyr and 2,4-D cause epigenetic change specially for the herbicide imazethapyr and that this changes can be important to herbicide tolerance. In an evolutionary scenario, it can add a new perspective to our knowledge of the evolution of herbicide resistance in plants.

6 APPENDIX

CHAPTER 1

APPENDIX 1. ANOVA of plant injury of *A. thaliana* plants 20 days after treatment (DAT), treated with herbicides (A) glyphosate, (B) imazethapyr, (C) 2,4D.

A	**Glyphosate at 20 DAT** (CV% = 6.98)				
Sources of variation	Degrees of freedom	Sum of squares	Mean square	F	Significance
Doses	4	9524.51	2381.13	166.99	<0.01
Residue	15	213.88	14.26		
Total	19	9738.39			
B	**Imazethapyr at 20 DAT** (CV% = 5.44)				
Sources of variation	Degrees of freedom	Sum of squares	Mean square	F	Significance
Doses	4	11133.46	2783.37	282.78	<0.01
Residue	15	147.64	9.84		
Total	19	11281.10			
C	**2,4-D at 20 DAT** (CV% = 7.52)				
Sources of variation	Degrees of freedom	Sum of squares	Mean square	F	Significance
Doses	4	11950.85	2987.71	147.36	<0.01
Residue	15	304.11	20.27		
Total	19	12254.96			

APPENDIX 2. ANOVA of shoot dry weight of *A. thaliana* plants 20 days after treatment (DAT), treated with herbicides (A) glyphosate, (B) imazethapyr, (C) 2,4D.

A	**Glyphosate at 20 DAT** (CV% = 7.39)				
Sources of variation	Degrees of freedom	Sum of squares	Mean square	F	Significance
Doses	4	78.18	19.54	233.58	<0.01
Residue	15	1.25	0.08		
Total	19	79.43			
B	**Imazethapyr at 20 DAT** (CV% = 17.49)				
Sources of variation	Degrees of freedom	Sum of squares	Mean square	F	Significance
Doses	4	218.41	54.60	65.59	<0.01
Residue	15	12.49	0.83		
Total	19	230.90			
C	**2,4-D at 20 DAT** (CV% = 14.42)				
Sources of variation	Degrees of freedom	Sum of squares	Mean square	F	Significance
Doses	4	220.74	55.18	80.60	<0.01
Residue	15	10.27	0.68		
Total	19	231.01			

APPENDIX 3. ANOVA of leaf length of *A. thaliana* plants (wild type and mutants) 10 days after treatment (DAT), treated with herbicides (A) glyphosate, (B) imazethapyr, (C) 2,4D.

A Glyphosate at 10 DAT (CV% = 6.69)

Sources of variation	Degrees of freedom	Sum of squares	Mean square	F	Significance
Study (S)	1	237.64	237.64	18.40	<0.01
Genotype (G)	11	7837.63	712.51	55.18	<0.01
Int. SxG	11	2077.35	188.85	14.62	<0.01
Treatment	23	10152.62	441.42	34.18	<0.01
Residue	72	929.75	12.91		
Total	95	11082.37			

B Imazethapyr at 10 DAT (CV% = 7.19)

Sources of variation	Degrees of freedom	Sum of squares	Mean square	F	Significance
Study (S)	1	667.84	667.84	52.05	<0.01
Genotype (G)	11	2941.04	267.37	20.84	<0.01
Int. SxG	11	1518.18	138.02	10.76	<0.01
Treatment	23	5127.06	222.91	17.37	<0.01
Residue	72	923.78	12.83		
Total	95	6050.84			

C 2,4-D at 10 DAT (CV% = 8.13)

Sources of variation	Degrees of freedom	Sum of squares	Mean square	F	Significance
Study (S)	1	9.43	9.43	0.46	ns
Genotype (G)	11	5793.02	526.64	25.78	<0.01
Int. SxG	11	3573.50	324.86	15.90	<0.01
Treatment	23	9375.95	407.65	19.96	<0.01
Residue	72	1470.74	20.43		
Total	95	10846.69			

APPENDIX 4. ANOVA of shoot dry weight of *A. thaliana* plants (wild type and mutants) 10 days after treatment (DAT), treated with herbicides (A) glyphosate, (B) imazethapyr, (C) 2,4D.

A Glyphosate at 10 DAT (CV% = 10.35)

Sources of variation	Degrees of freedom	Sum of squares	Mean square	F	Significance
Study (S)	1	717.83	717.83	19.29	<0.01
Genotype (G)	11	23145.05	2104.09	56.55	<0.01
Int. SxG	11	5255.49	477.77	12.84	<0.01
Treatment	23	29118.37	1266.02	34.02	<0.01
Residue	72	2679.13	37.21		
Total	95	31797.50			

B Imazethapyr at 10 DAT (CV% = 12.91)

Sources of variation	Degrees of freedom	Sum of squares	Mean square	F	Significance
Study (S)	1	4251.35	4251.35	92.51	<0.01
Genotype (G)	11	11985.65	1089.60	23.72	<0.01
Int. SxG	11	2645.43	240.49	5.23	<0.01
Treatment	23	18882.43	820.97	17.86	<0.01
Residue	72	3308.68	45.95		
Total	95	22191.11			

C 2,4-D at 10 DAT (CV% = 16.94)

Sources of variation	Degrees of freedom	Sum of squares	Mean square	F	Significance
Study (S)	1	1030.71	1030.71	12.89	<0.01
Genotype (G)	11	28600.47	2600.04	32.52	<0.01
Int. SxG	11	7453.89	677.63	8.47	<0.01
Treatment	23	37085.07	1612.39	20.16	<0.01
Residue	72	5757.14	79.96		
Total	95	42842.21			

APPENDIX 5. ANOVA of leaf length of *A. thaliana* plants (wild type and *ros1*) 10 days after treatment (DAT), treated with herbicides (A) glyphosate, (B) imazethapyr, (C) 2,4D.

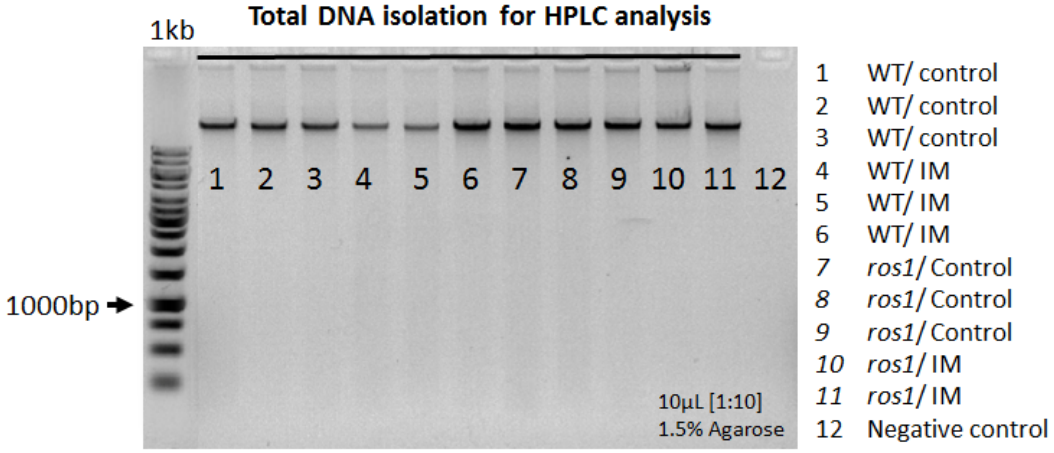
A	**Glyphosate at 10 DAT** (CV% =10.35)				
Sources of variation	Degrees of freedom	Sum of squares	Mean square	F	Significance
Study (S)	2	247.71	123.85	3.74	<0.05
Genotype (G)	1	3654.86	3654.86	110.35	<0.01
Int. SxG	2	77.24	38.62	1.17	ns
Treatment	5	3979.81	795.96	24.03	<0.01
Residue	18	596.15	33.12		
Total	23	4575.96			
B	**Imazethapyr at 10 DAT** (CV% = 8.59)				
Sources of variation	Degrees of freedom	Sum of squares	Mean square	F	Significance
Study (S)	2	225.60	112.80	7.59	<0.01
Genotype (G)	1	9608.00	9608.00	646.88	<0.01
Int. SxG	2	48.42	24.21	1.63	ns
Treatment	5	9882.02	1976.40	133.06	<0.01
Residue	18	267.35	14.85		
Total	23	10149.37			

APPENDIX 6. ANOVA of shoot dry weight *A. thaliana* plants (wild type and *ros1*) 10 days after treatment (DAT), treated with herbicides (A) glyphosate, (B) imazethapyr, (C) 2,4D.

A	**Glyphosate at 10 DAT** (CV% = 16.77)				
Sources of variation	Degrees of freedom	Sum of squares	Mean square	F	Significance
Study (S)	2	431.82	215.91	4.58	<0.05
Genotype (G)	1	6915.27	6915.27	146.29	<0.01
Int. SxG	2	36.06	18.03	0.38	ns
Treatment	5	7383.15	1476.63	31.24	<0.01
Residue	18	850.90	47.27		
Total	23	8234.05			
B	**Imazethapyr at 10 DAT** (CV% = 10.88)				
Sources of variation	Degrees of freedom	Sum of squares	Mean square	F	Significance
Study (S)	2	299.03	149.52	8.80	<0.01
Genotype (G)	1	12499.43	12499.43	735.34	<0.01
Int. SxG	2	8.72	4.36	0.26	ns
Treatment	5	12807.18	2561.44	150.69	<0.01
Residue	18	305.97	16.99		
Total	23	13113.15			

CHAPTER 2

APPENDIX 1. Quality of total DNA used for global DNA methylation by isocratic cation-exchange high-pressure liquid chromatography (HPLC) analysis, checked in 1.5% agarose gel.

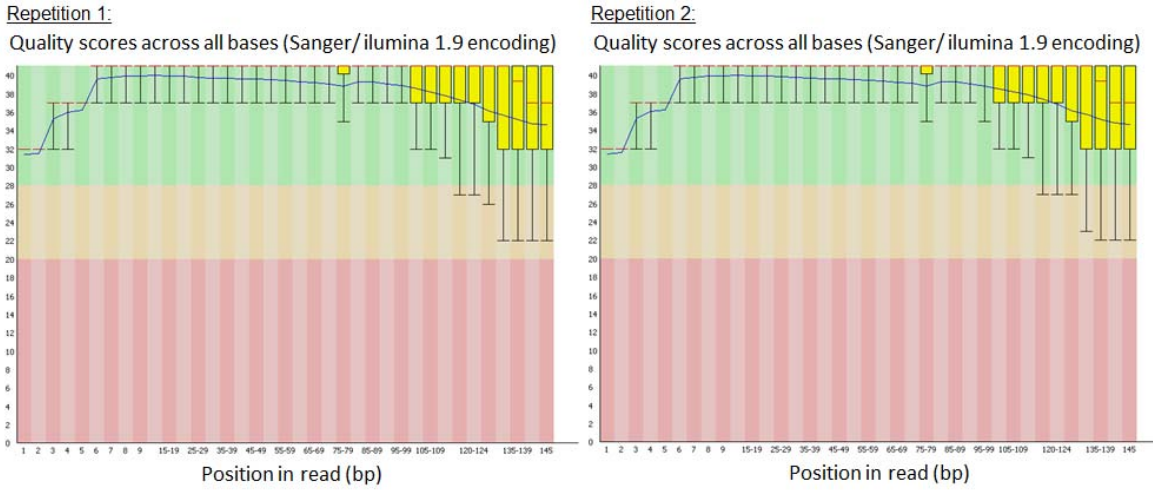


APPENDIX 2. ANOVA of global DNA methylation obtained by isocratic cation-exchange high-pressure liquid chromatography (HPLC) analysis.

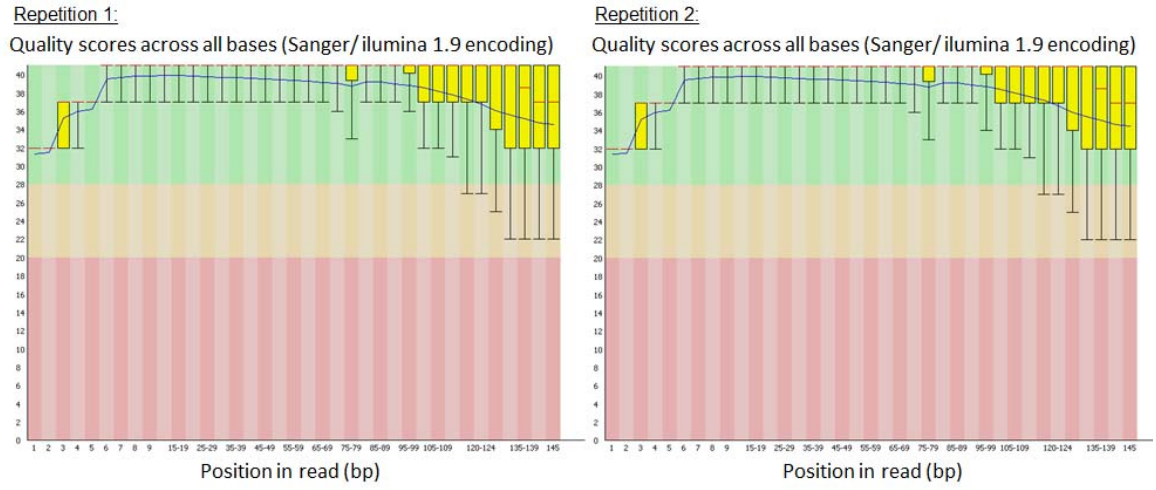
Sources of variation	Degrees of freedom	Sum of squares	Mean square	F	Significance
Treatments	6	112.92	18.82	618.82	<0.01
Residue	14	0.43	0.03		
Total	20	113.35			
					(CV% = 3.48)

APPENDIX 3. Per base sequence quality of high-throughput mRNA sequencing (RNA-seq) data for wild type (WT) control (A), WT imazethapyr (IM) (B), *ros1* control (D) and *ros1* imazethapyr (IM) (D). The graphics show an overview of the range of quality values across all bases at each position in the FastQ file. For each position a BoxWhisker type plot is drawn as follows: The central red line is the median value. The yellow box represents the inter-quartile range (25-75%). The upper and lower whiskers represent the 10% and 90% points. The blue line represents the mean quality.

A. WT control

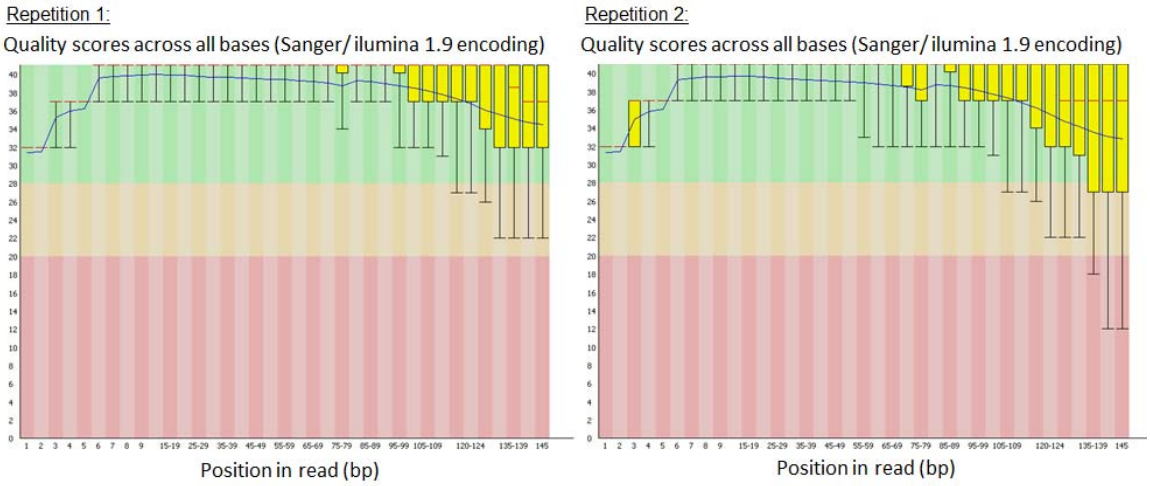


B. WT IM

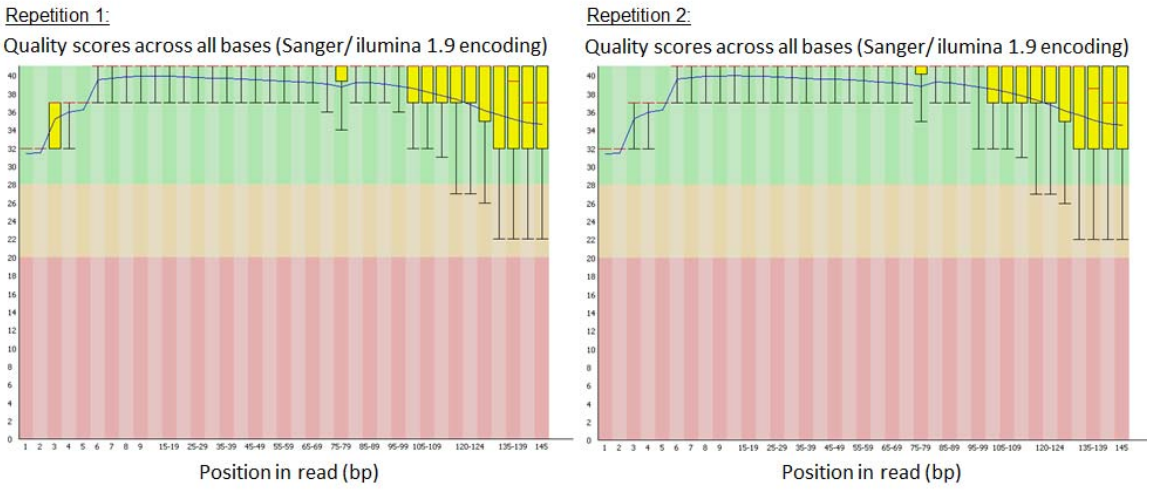


continuation APPENDIX 3. Per base sequence quality of high-throughput mRNA sequencing (RNA-seq) data for wild type (WT) control (A), WT imazethapyr (IM) (B), *ros1* control (D) and *ros1* imazethapyr (IM) (D). The graphics show an overview of the range of quality values across all bases at each position in the FastQ file. For each position a BoxWhisker type plot is drawn as follows: The central red line is the median value. The yellow box represents the inter-quartile range (25-75%). The upper and lower whiskers represent the 10% and 90% points. The blue line represents the mean quality.

C. *ros1* control



D. *ros1* IM



APPENDIX 4. Number of reads, sequence length, sequences flagged as poor quality and % GC obtained for each sample submitted to RNA-sequencing.

	Sample	Number of reads	Sequence length	Sequences flagged as poor quality	%GC
1	WT – Control – R1	20,355,943	15-145	0	45
2	WT – Control – R2	22,575,110	15-145	0	45
3	WT – IM – R1	20,502,313	15-145	0	45
4	WT – IM – R1	20,286,614	15-145	0	45
5	*ros1* – Control – R1	20,342,194	15-145	0	45
6	*ros1* – Control – R2	17,373,574	15-145	0	45
7	*ros1* – IM – R1	20,012,495	15-145	0	45
8	*ros1* – IM – R2	21,765,920	15-145	0	44
	TOTAL:	**163,214,16**			

APPENDIX 5. Table of gene identification (ID) and gene annotation according to TAIR10 of 200 genes that were significantly ($p<0.05$) induced by imazethapyr (IM) in wild type (WT) and present down-regulation in *ros1* mutants, according with RNA-sequencing data.

	Gene ID	Gene Annotation
1	AT4G22820	A20/AN1-like zinc finger family protein;
2	AT1G69260	ABI five binding protein (AFP1);
3	AT3G29575	ABI five binding protein 3 (AFP3);
4	AT2G15310	ADP-ribosylation factor B1A (ARFB1A);
5	AT1G77120	alcohol dehydrogenase 1 (ADH1);
6	AT1G31820	Amino acid permease family protein;
7	AT1G20490	AMP-dependent synthetase and ligase family protein;
8	AT5G61160	anthocyanin 5-aromatic acyltransferase 1 (AACT1);
9	AT4G39210	APL3;
10	AT3G29590	AT5MAT;
11	AT2G04160	AUXIN-INDUCED IN ROOT CULTURES 3 (AIR3);
12	AT4G00870	basic helix-loop-helix (bHLH) DNA-binding superfamily protein;
13	AT5G24800	basic leucine zipper 9 (BZIP9);
14	AT1G73870	B-box type zinc finger protein with CCT domain;
15	AT2G46410	CAPRICE (CPC);
16	AT1G24070	cellulose synthase-like A10 (CSLA10);
17	AT5G05270	Chalcone-flavanone isomerase family protein;
18	AT5G37440	Chaperone DnaJ-domain superfamily protein;
19	AT3G58020	Chaperone DnaJ-domain superfamily protein;
20	AT2G43590	Chitinase family protein;
21	AT5G43860	chlorophyllase 2 (CLH2);
22	AT2G42540	cold-regulated 15a (COR15A);
23	AT4G19230	cytochrome P450, family 707, subfamily A, polypeptide 1 (CYP707A1);
24	AT1G19630	cytochrome P450, family 722, subfamily A, polypeptide 1 (CYP722A1);
25	AT3G49620	DARK INDUCIBLE 11 (DIN11);
26	AT5G42800	dihydroflavonol 4-reductase (DFR);
27	AT4G14370	Disease resistance protein (TIR-NBS-LRR class) family;
28	AT2G33830	Dormancy/auxin associated family protein;

continuation APPENDIX 5. Table of gene identification (ID) and gene annotation according to TAIR10 of 200 genes that were significantly ($p<0.05$) induced by imazethapyr (IM) in wild type (WT) and present down-regulation in *ros1* mutants, according with RNA-sequencing data.

	Gene ID	Gene Annotation
29	AT5G05410	DRE-binding protein 2A (DREB2A);
30	AT4G15910	drought-induced 21 (DI21);
31	AT3G63060	EID1-like 3 (EDL3);
32	AT4G14590	embryo defective 2739 (emb2739);
33	AT4G10050	esterase/lipase/thioesterase family protein;
34	AT1G17870	ETHYLENE-DEPENDENT GRAVITROPISM-DEFICIENT AND YELLOW-GREEN-LIKE 3 (EGY3);
35	AT5G64260	EXORDIUM like 2 (EXL2);
36	AT4G20860	FAD-binding Berberine family protein;
37	AT2G36090	F-box family protein;
38	AT1G62540	flavin-monooxygenase glucosinolate S-oxygenase 2 (FMO GS-OX2);
39	AT1G62570	flavin-monooxygenase glucosinolate S-oxygenase 4 (FMO GS-OX4);
40	AT5G14780	formate dehydrogenase (FDH);
41	AT1G56600	galactinol synthase 2 (GolS2);
42	AT1G54020	GDSL-like Lipase/Acylhydrolase superfamily protein;
43	AT5G17220	glutathione S-transferase phi 12 (GSTF12);
44	AT2G29490	glutathione S-transferase TAU 1 (GSTU1);
45	AT1G17170	glutathione S-transferase TAU 24 (GSTU24);
46	AT2G05380	glycine-rich protein 3 short isoform (GRP3S);
47	AT4G30460	glycine-rich protein;
48	AT1G51090	Heavy metal transport/detoxification superfamily protein ;
49	AT4G35060	Heavy metal transport/detoxification superfamily protein ;
50	AT1G07430	highly ABA-induced PP2C gene 2 (HAI2);
51	AT5G54080	homogentisate 1,2-dioxygenase (HGO);
52	AT3G50480	homolog of RPW8 4 (HR4);
53	AT4G37580	HOOKLESS 1 (HLS1);
54	AT5G39090	HXXXD-type acyl-transferase family protein;
55	AT1G03495	HXXXD-type acyl-transferase family protein;
56	AT1G23040	hydroxyproline-rich glycoprotein family protein;
57	AT4G14560	indole-3-acetic acid inducible (IAA1);
58	AT1G15580	indole-3-acetic acid inducible 5 (IAA5);
59	AT5G42810	inositol-pentakisphosphate 2-kinase 1 (IPK1);
60	AT1G64180	intracellular protein transport protein USO1-related;
61	AT1G18870	isochorismate synthase 2 (ICS2);
62	AT1G70300	K+ uptake permease 6 (KUP6);
63	AT5G15960	KIN1;
64	AT5G06760	Late Embryogenesis Abundant 4-5 (LEA4-5);
65	AT1G02050	LESS ADHESIVE POLLEN 6 (LAP6);
66	AT1G66830	Leucine-rich repeat protein kinase family protein;
67	AT3G50970	LOW TEMPERATURE-INDUCED 30 (LTI30);
68	AT2G18170	MAP kinase 7 (MPK7);

continuation APPENDIX 5. Table of gene identification (ID) and gene annotation according to TAIR10 of 200 genes that were significantly ($p<0.05$) induced by imazethapyr (IM) in wild type (WT) and present down-regulation in *ros1* mutants, according with RNA-sequencing data.

	Gene ID	Gene Annotation
69	AT4G21910	MATE efflux family protein;
70	AT2G04070	MATE efflux family protein;
71	AT1G64660	methionine gamma-lyase (MGL);
72	AT3G13540	myb domain protein 5 (MYB5);
73	AT4G05100	myb domain protein 74 (MYB74);
74	AT1G66390	myb domain protein 90 (MYB90);
75	AT1G14520	myo-inositol oxygenase 1 (MIOX1);
76	AT1G52040	myrosinase-binding protein 1 (MBP1);
77	AT1G32870	NAC domain protein 13 (NAC13);
78	AT2G23910	NAD(P)-binding Rossmann-fold superfamily protein;
79	AT2G37770	NAD(P)-linked oxidoreductase superfamily protein;
80	AT5G22300	nitrilase 4 (NIT4);
80	AT5G22300	nitrilase 4 (NIT4);
81	AT3G28007	Nodulin MtN3 family protein;
82	AT1G72830	nuclear factor Y, subunit A3 (NF-YA3);
83	AT1G30500	nuclear factor Y, subunit A7 (NF-YA7);
84	AT1G57590	Pectinacetylesterase family protein;
85	AT1G09680	Pentatricopeptide repeat (PPR) superfamily protein;
86	AT3G57680	Peptidase S41 family protein;
87	AT4G33905	Peroxisomal membrane 22 kDa (Mpv17/PMP22) family protein;
88	AT5G48880	PEROXISOMAL-3-KETO-ACYL-COA THIOLASE 1 (PKT1);
89	AT3G53260	phenylalanine ammonia-lyase 2 (PAL2);
90	AT2G17280	Phosphoglycerate mutase family protein;
91	AT2G25590	Plant Tudor-like protein;
92	AT2G02850	plantacyanin (ARPN);
93	AT5G43980	plasmodesmata-located protein 1 (PDLP1);
94	AT1G66480	plastid movement impaired 2 (PMI2);
95	AT4G05320	polyubiquitin 10 (UBQ10);
96	AT1G56650	production of anthocyanin pigment 1 (PAP1);
97	AT1G30160	Protein of unknown function (DUF295);
98	AT3G19520	Protein of unknown function (DUF626);
99	AT4G02360	Protein of unknown function, DUF538;
100	AT5G43180	Protein of unknown function, DUF599;
101	AT3G11410	protein phosphatase 2CA (PP2CA);
102	AT5G50400	purple acid phosphatase 27 (PAP27);
103	AT3G62460	Putative endonuclease or glycosyl hydrolase;
104	AT1G43890	RAB GTPASE HOMOLOG B18 (RAB18);
105	AT3G24982	receptor like protein 40 (RLP40);
106	AT3G25010	receptor like protein 41 (RLP41);
107	AT1G46768	related to AP2 1 (RAP2.1);
108	AT3G58350	RESTRICTED TEV MOVEMENT 3 (RTM3);
109	AT2G39100	RING/U-box superfamily protein;

continuation APPENDIX 5. Table of gene identification (ID) and gene annotation according to TAIR10 of 200 genes that were significantly ($p<0.05$) induced by imazethapyr (IM) in wild type (WT) and present down-regulation in *ros1* mutants, according with RNA-sequencing data.

	Gene ID	Gene Annotation
110	AT5G38895	RING/U-box superfamily protein;
111	AT3G46450	SEC14 cytosolic factor family protein / phosphoglyceride transfer family protein;
112	AT1G09180	secretion-associated RAS super family 1 (SARA1A);
113	AT1G03550	Secretory carrier membrane protein (SCAMP) family protein;
114	AT1G55740	seed imbibition 1 (SIP1);
115	AT5G22860	Serine carboxypeptidase S28 family protein;
116	AT2G23000	serine carboxypeptidase-like 10 (scpl10);
117	AT1G53160	squamosa promoter binding protein-like 4 (SPL4);
118	AT5G66170	sulfurtransferase 18 (STR18);
119	AT3G60980	Tetratricopeptide repeat (TPR)-like superfamily protein;
120	AT1G21400	Thiamin diphosphate-binding fold (THDP-binding) superfamily protein;
110	AT5G38895	RING/U-box superfamily protein;
111	AT3G46450	SEC14 cytosolic factor family protein / phosphoglyceride transfer family protein;
112	AT1G09180	secretion-associated RAS super family 1 (SARA1A);
113	AT1G03550	Secretory carrier membrane protein (SCAMP) family protein;
114	AT1G55740	seed imbibition 1 (SIP1);
115	AT5G22860	Serine carboxypeptidase S28 family protein;
116	AT2G23000	serine carboxypeptidase-like 10 (scpl10);
117	AT1G53160	squamosa promoter binding protein-like 4 (SPL4);
118	AT5G66170	sulfurtransferase 18 (STR18);
119	AT3G60980	Tetratricopeptide repeat (TPR)-like superfamily protein;
120	AT1G21400	Thiamin diphosphate-binding fold (THDP-binding) superfamily protein;
121	AT5G42850	Thioredoxin superfamily protein;
122	AT5G47560	tonoplast dicarboxylate transporter (TDT);
123	AT1G49450	Transducin/WD40 repeat-like superfamily protein;
124	AT2G45290	Transketolase;
125	AT4G03320	translocon at the inner envelope membrane of chloroplasts 20-IV (tic20-IV);
126	AT2G41190	Transmembrane amino acid transporter family protein;
127	AT5G13930	TRANSPARENT TESTA 4 (TT4);
128	AT5G07990	TRANSPARENT TESTA 7 (TT7); or CYTOCHROME P450 75B1;
129	AT4G09820	TRANSPARENT TESTA 8 (TT8);
130	AT2G37260	TRANSPARENT TESTA GLABRA 2 (TTG2);
131	AT2G47770	TSPO(outer membrane tryptophan-rich sensory protein)-related (TSPO);
132	AT5G53970	Tyrosine transaminase family protein;
133	AT5G08600	U3 ribonucleoprotein (Utp) family protein;
134	AT5G42300	ubiquitin-like protein 5 (UBL5);
135	AT5G54060	UDP-glucose:flavonoid 3-o-glucosyltransferase (UF3GT);
136	AT2G43820	UDP-glucosyltransferase 74F2 (UGT74F2);
137	AT3G24780	Uncharacterised conserved protein UCP015417, vWA;

continuation APPENDIX 5. Table of gene identification (ID) and gene annotation according to TAIR10 of 200 genes that were significantly ($p<0.05$) induced by imazethapyr (IM) in wild type (WT) and present down-regulation in *ros1* mutants, according with RNA-sequencing data.

	Gene ID	Gene Annotation
138	AT2G17570	Undecaprenyl pyrophosphate synthetase family protein;
139	AT2G14620	xyloglucan endotransglucosylase/hydrolase 10 (XTH10);
140	AT2G40110	Yippee family putative zinc-binding protein;
141	AT5G60250	zinc finger (C3HC4-type RING finger) family protein;
127	AT5G13930	TRANSPARENT TESTA 4 (TT4);
128	AT5G07990	TRANSPARENT TESTA 7 (TT7);
129	AT4G09820	TRANSPARENT TESTA 8 (TT8);
130	AT2G37260	TRANSPARENT TESTA GLABRA 2 (TTG2);
131	AT2G47770	TSPO(outer membrane tryptophan-rich sensory protein)-related (TSPO);
132	AT5G53970	Tyrosine transaminase family protein;
133	AT5G08600	U3 ribonucleoprotein (Utp) family protein;
134	AT5G42300	ubiquitin-like protein 5 (UBL5);
135	AT5G54060	UDP-glucose:flavonoid 3-o-glucosyltransferase (UF3GT);
136	AT2G43820	UDP-glucosyltransferase 74F2 (UGT74F2);
137	AT3G24780	Uncharacterised conserved protein UCP015417, vWA;
138	AT2G17570	Undecaprenyl pyrophosphate synthetase family protein;
139	AT2G14620	xyloglucan endotransglucosylase/hydrolase 10 (XTH10);
140	AT2G40110	Yippee family putative zinc-binding protein;
141	AT5G60250	zinc finger (C3HC4-type RING finger) family protein;
138	AT2G17570	Undecaprenyl pyrophosphate synthetase family protein;
139	AT2G14620	xyloglucan endotransglucosylase/hydrolase 10 (XTH10);
140	AT2G40110	Yippee family putative zinc-binding protein;
141	AT5G60250	zinc finger (C3HC4-type RING finger) family protein;

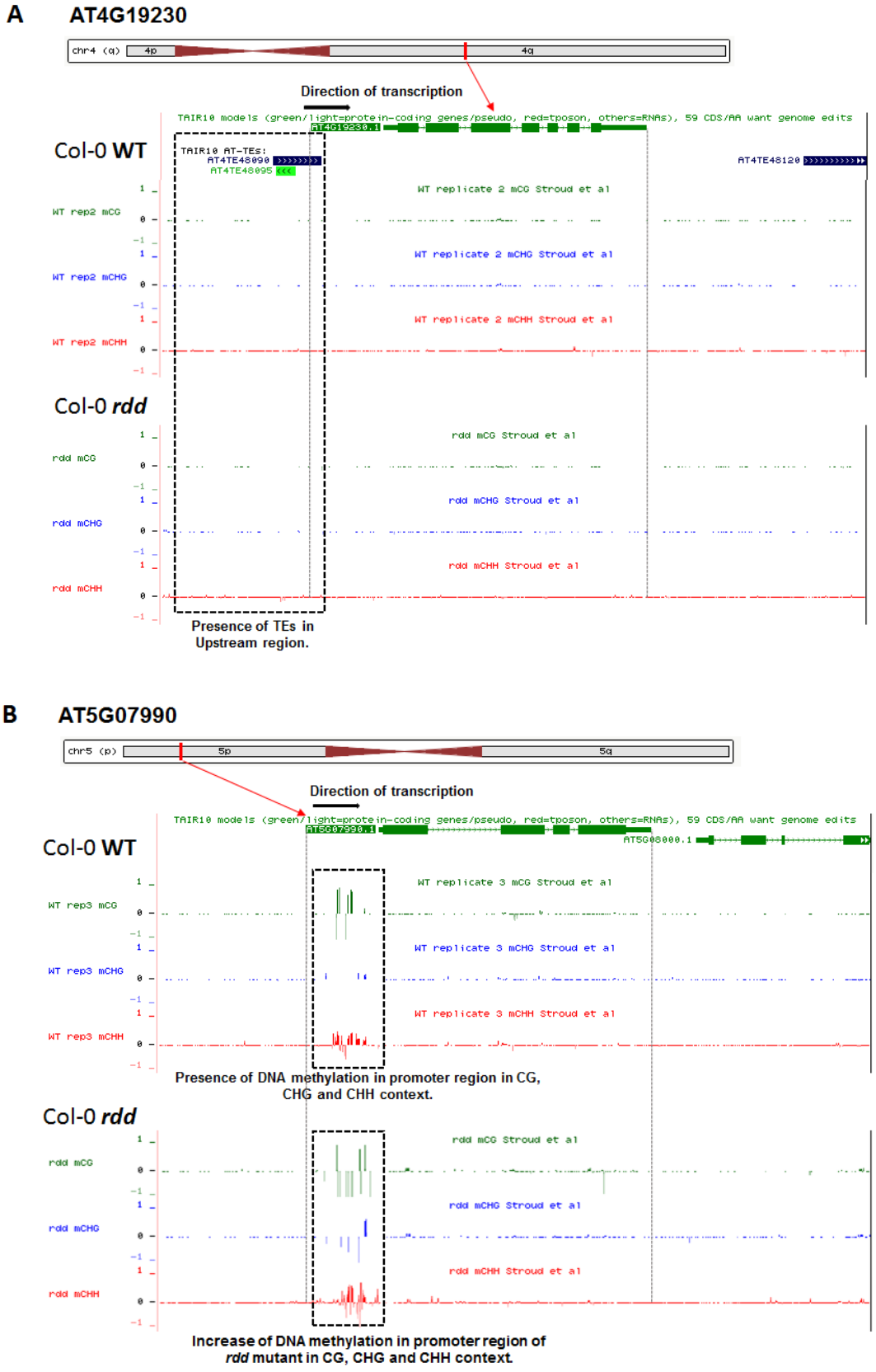
APPENDIX 6. ANOVA of leaf length of *A. thaliana* plants, wild type and *tt4* mutant.

Imazethapyr at 14 DAT (CV% = 5.82)					
Sources of variation	Degrees of freedom	Sum of squares	Mean square	F	Significance
Treatment	1	67.33	67.33	3.46	ns
Residue	6	116.68	19.45		
Total	7	184.01			

APPENDIX 7. ANOVA of shoot dry weight of *A. thaliana* plants, wild type and *tt4* mutant.

Imazethapyr at 14 DAT (CV% = 7.55)					
Sources of variation	Degrees of freedom	Sum of squares	Mean square	F	Significance
Treatment	1	273.15	273.15	15.06	<0.01
Residue	6	108.82	18.14		
Total	7	381.97			

APPENDIX 8. *In silico* DNA methylation analysis of upstream region. (A) Analysis of presence of transposable element (TE) near to promoter region of gene of interest. (B) Analysis of DNA methylation in CG (green), CHG (blue) and CHH (red) context near to promoter region of gene of *A. thaliana* Col-0 wild type (WT) and triple epigenetic mutant *rdd*.



APPENDIX 9. ANOVA of DNA methylation analysis of genes *TT7* (A), Heavy metal transport/detoxification superfamily protein (B), *SCAMP* (C), *Major facilitator superfamily protein* (D); Esterase/lipase/thioesterase family protein (E), *XTH10* (F).

A. *TT7* (CV% =11.49)

Sources of variation	Degrees of freedom	Sum of squares	Mean square	F	Significance
Genotype (G)	3	432.92	144.30	42.37	<0.01
Sequence contexts (S)	2	5805.10	2902.55	852.34	<0.01
Int. GxS	6	716.67	119.44	35.07	<0.01
Treatments	11	6954.69	632.24	185.66	<0.01
Residue	24	81.73	3.40		
Total	35	7036.42			

B. *Heavy metal transport/detoxification superfamily protein* (CV% = 31.15)

Sources of variation	Degrees of freedom	Sum of squares	Mean square	F	Significance
Genotype (G)	3	2329.43	776.48	31.89	<0.01
Sequence contexts (S)	1	2886.79	2886.79	118.56	<0.01
Int. GxS	3	2419.33	806.44	33.12	<0.01
Treatments	7	7635.55	1090.79	44.80	<0.01
Residue	16	389.58	24.34		
Total	23	8025.14			

C. *Secretory carrier membrane protein (SCAMP) family protein* (CV% = 41.22)

Sources of variation	Degrees of freedom	Sum of squares	Mean square	F	Significance
Genotype (G)	3	2051.49	683.83	8.74	<0.01
Sequence contexts (S)	2	4940.02	2470.00	31.57	<0.01
Int. GxS	6	4852.57	808.76	10.34	<0.01
Treatments	11	11844.07	1076.73	13.76	<0.01
Residue	24	1877.80	78.24		
Total	35	13721.88			

D. *Major facilitator superfamily protein* (CV% = 17.84)

Sources of variation	Degrees of freedom	Sum of squares	Mean square	F	Significance
Genotype (G)	3	588.54	196.18	13.65	<0.01
Sequence contexts (S)	1	4690.04	4690.04	326.45	<0.01
Int. GxS	3	237.41	79.14	5.51	<0.01
Treatments	7	5516.00	788.00	54.85	<0.01
Residue	16	229.87	14.37		
Total	23	5745.87			

continuation APPENDIX 9. ANOVA of DNA methylation analysis of genes *TT7* (A), Heavy metal transport/detoxification superfamily protein (B), *SCAMP* (C), *Major facilitator superfamily protein* (D); Esterase/lipase/thioesterase family protein (E), *XTH10* (F).

E. *Esterase/lipase/thioesterase family protein;* (CV% = 24.51)

Sources of variation	Degrees of freedom	Sum of squares	Mean square	F	Significance
Genotype (G)	3	1700.79	566.93	26.97	<0.01
Sequence contexts (S)	2	96.45	48.23	2.29	ns
Int. GxS	6	5244.66	874.11	41.58	<0.01
Treatments	11	7041.91	640.17	30.45	<0.01
Residue	24	504.56	21.02		
Total	35	7546.47			

F. *Xyloglucan endotransglucosylase/hydrolase 10 (XTH10);* (CV% = 20.09)

Sources of variation	Degrees of freedom	Sum of squares	Mean square	F	Significance
Genotype (G)	3	1195.68	398.56	14.52	<0.01
Sequence contexts (S)	2	997.84	498.92	18.18	<0.01
Int. GxS	6	17780.48	2963.41	108.00	<0.01
Treatments	11	19974.01	1815.82	66.18	<0.01
Residue	24	658.54	27.43		
Total	35	20632.55			

7 VITA

Catarine Markus is daughter of Vilmar Markus and Bernadeth Markus, was born in Taquari/RS on February 21th, 1988. In 2004, completed the Technical Course in Business Administration at the Instituto Cenecista General Canabarro (IECEG) in Teutônia/RS. In 2005, completed High School in the same school. In 2006, joined the Agronomy School at the Universidade Federal de Pelotas (UFPEL) in Pelotas/RS and graduated as Agronomist in 2010. From 2007 to 2010, took a Scholarship for Scientific Initiation (IC) in Herbology. In 2010, completed the curricular traineeship at Texas A & M University - AgriLive Research & Extension, College Station/USA, in behavior and dynamics of herbicides in the soil. In 2011, joined the Master's degree program in Agronomy, at the Universidade Federal do Rio Grande do Sul (UFRGS) with a CNPq scholarship. Holds a Master Degree in Herbology since February 2013. In the same year, joined the PhD in Plant Science with emphasis on Herbology, at UFRGS, in Porto Alegre/RS, and was a CNPq scholarship student during the period. In the year of 2015, joined the Max Planck Institute as a Guest Student with a CAPES scholarship. After 12 months, returned to Brazil for finishing the PhD.