

Trace concentrations of imazethapyr (IM) affect floral organs development and reproduction in *Arabidopsis thaliana*: IM-induced inhibition of key genes regulating anther and pollen biosynthesis

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Abstract Understanding how herbicides affect plant reproduction and growth is critical to develop herbicide toxicity model and refine herbicide risk assessment. Although our knowledge of herbicides toxicity mechanisms at the physiological and molecular level in plant vegetative phase has increased substantially in the last decades, few studies have addressed the herbicide toxicity problematic on plant reproduction. Here, we determined the long-term (4–8 weeks) effect of a chiral herbicide, imazethapyr (IM), which has been increasingly used in plant crops, on floral organ development and reproduction in the model plant *Arabidopsis thaliana*. More specifically, we followed the effect of two IM enantiomers (*R*- and *S*-IM) on floral organ structure, seed production, pollen viability and the transcription of key genes involved in anther and pollen development. The results showed that IM strongly inhibited the transcripts of genes regulating *A. thaliana* tapetum development (*DYT1*: DYSFUNCTIONAL TAPETUM 1), tapetal differentiation and function (*TDF1*: TAPETAL DEVELOPMENT AND FUNCTION1), and pollen wall formation and developments (*AMS*: ABORTED

MICROSPORES, *MYB103*: MYB DOMAIN PROTEIN 103, *MS1*: MALE STERILITY 1, *MS2*: MALE STERILITY 2). Since *DYT1* positively regulates 33 genes involved in cell-wall modification (such as, *TDF1*, *AMS*, *MYB103*, *MS1*, *MS2*) that can catalyze the breakdown of polysaccharides to facilitate anther dehiscence, the consistent decrease in the transcription of these genes after IM exposure should hamper anther opening as observed under scanning electron microscopy. The toxicity of IM on anther opening further lead to a decrease in pollen production and pollen viability. Furthermore, long-term IM exposure increased the number of apurinic/apyrimidinic sites (AP sites) in the DNA of *A. thaliana* and also altered the DNA of *A. thaliana* offspring grown in IM-free soils. Toxicity of IM on floral organs development and reproduction was generally higher in the presence of the *R*-IM enantiomer than of the *S*-IM enantiomer. This study unraveled several IM toxicity targets and mechanisms at the molecular and structural level linked to the toxicity of IM trace concentrations on *A. thaliana* reproduction.

Keywords *Arabidopsis thaliana* · Imazethapyr · Floral organs · Enantiomer · Reproduction

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Introduction

Environmental deterioration due to herbicide application in crops around the world is a serious concern since low herbicide residual concentrations in soils (on the order of a few $\mu\text{g a.i. L}^{-1}$, where a.i. means active ingredient) and water may lead to deleterious effects on non-target plants and animals and ultimately humans (Jettner et al. 1999; Lambrev et al. 2003; Gavrilesco 2005; Boutin et al. 2014). The vast majority of applied herbicide does not reach the target organisms (Pimentel 1995) and enter the soil

environment by three pathways: (1) direct interception of spray by the soil surface during the applications, (2) runoff of the herbicide from plants, and (3) leaching from dead plant material (Zabaloy et al. 2011). The residual herbicide concentration in soil near agricultural areas may vary from a few μg to mg per kg of soil (Dalton and Boutin 2010). The dispersion of residuals herbicide in soils and adjacent ecosystems has been linked to marked population decline of many plants species living near agricultural areas (Olofsdotter et al. 1998; Boatman et al. 2007; Boutin et al. 2014). Herbicide application is even believed to be one of the most important factor affecting the biodiversity of wild plants in agricultural areas (Strandberg et al. 2012).

Imazethapyr (IM) is a common imidazolinone herbicide widely applied in rice, soybeans, groundnuts and other crops around the world to control dicot weeds (York et al. 1995). Imazethapyr is absorbed by the roots and foliage, and then transported into the meristematic region of weeds. The mechanism of action of IM is to inhibit the plant enzyme acetohydroxyacid synthase (E.C.4.1.3.18), also known as acetolactate synthase (ALS). This enzyme catalyzes the first common reaction in the biosynthetic pathway of branched-chain amino acids (BCAA; valine, leucine and isoleucine) in plants, but is absent in animals (Singh 1999). However, since IM has a half-life of 53–122 days in aerobic soil (Mills and Witt 1989; Curran et al. 1992). IM residues in soils adjacent to crops will persist affecting not only the ALS enzyme of non-target wild plants, but also several other metabolic pathways and plant functions. For instance, exposure of *Arabidopsis thaliana* plantlets for 2–4 weeks to trace IM concentrations ($2\text{--}2.5 \mu\text{g L}^{-1}$) has been shown to strongly affect chlorophyll synthesis, several genes related to photosynthesis, increased ROS production while inhibiting the activity of several ROS-detoxifying enzymes (Peroxidase and catalase) (Qian et al. 2011a, b; 2013). Short exposure of rice seedlings for 6 days to IM ($50\text{--}500 \mu\text{g L}^{-1}$) induced damages to lipid membranes and negatively affects the transcription of a number of genes involved in many metabolic pathways, including amino acid metabolism, photosynthesis, starch and sugar metabolism and the tricarboxylic acid cycle (Qian et al. 2009, 2011a, b). Previous reports have mainly focused on the cellular and molecular effects of IM (as well as other herbicide) on plants. However, little is known regarding the toxicity mechanisms of IM on plant reproduction (Carpenter and Boutin 2010) even though there is increasing evidence that plant reproduction constitutes generally a more sensitive endpoint than classical short-term physiological measurements of herbicide toxicity (Boutin et al. 2014). Only very recently, it was shown that trace imazethapyr concentrations promote early flowering of *A. thaliana* (Qian et al. 2014), but the toxicity mechanisms at the cellular and molecular level leading to the strong effect of imazethapyr on *A. thaliana* reproduction are still unknown.

In the present study, we examined the effect of a long-term (4–8 weeks) exposure to *R*- and *S*-IM enantiomers on floral organ structure, seed production, pollen viability and transcription of key genes involved in anther and pollen development in the model dicotyledon plant *A. thaliana*. Our findings shade new lights on the mechanisms by which residual IM concentrations in soils may affect the development of floral organs in *A. thaliana*.

Materials and methods

Plant material and growth conditions

Seeds of *A. thaliana* (Col-0) were germinated on agar plates containing MS (Murashige and Skoog) medium. One-week-old seedlings were transferred to soil (Custom growing mix, Conrad Fafard Inc, Agawam, MA) and irrigated with water with or without IM enantiomers. The concentrations of the IM enantiomers (*R*- and *S*-IM) were 10 and 30 $\mu\text{g L}^{-1}$ and treatments were designated as R30 (30 $\mu\text{g L}^{-1}$ *R*-IM), R10 (10 $\mu\text{g L}^{-1}$ *R*-IM), S30 (30 $\mu\text{g L}^{-1}$ *S*-IM) and S10 (10 $\mu\text{g L}^{-1}$ *S*-IM) as in our previous study (Qian et al. 2014). The culture medium with or without IM enantiomers was replenished every fourth day. The chamber temperature was maintained at $25 \pm 0.5 \text{ }^\circ\text{C}$ and the seedlings were grown under a 12 h light/12 h dark cycle at a light intensity of $300 \mu\text{mol m}^{-2} \text{ s}^{-1}$ under cool-white fluorescent lights for 3–8 weeks depending on the experiments.

Pollen viability and silique observation

Pollen viability was assessed by staining control and IM enantiomers-treated pollen with the dye, tetrazolium red (TTR). Note that this dye allows discriminating between dead and alive pollens; living pollen is capable of reducing the colorless chemical TTR into a red-colored compound, which leads to red staining of pollen tissues (Wang et al. 2009). Pollen stained into red in the presence of TTR typically has high germination rates, but light orange to yellow pollen has low germination rates (Lansac et al. 1994).

To measure pollen viability, the pollen from 6-week-old seedlings (stage 14 of anther development) was kept on glass slides in the presence of a 400 μL TTR solution (2 % TTR in distilled water with 60 % sucrose, which prevents the pollen from bursting) at $35 \text{ }^\circ\text{C}$ for 15 min. Then, the slides were kept at $25 \text{ }^\circ\text{C}$ and the degree of pollen staining was visualized using a light microscope at $200\times$ magnification 6 h after the TTR treatment.

To determine the seed number per silique, fully developed siliques from the main stem of 8-week-old seedlings (stage of fruit formation) were transferred to 70 % ethanol

to desaturate silique chlorophyll. Seeds per cleared silique were then counted under the dissecting microscope. Five replicates were used in each treatment and each replicate consisted of 12 randomly selected seedlings.

Scanning electron microscopy visualization of anther and pollen

When floral buds were visible at the center of the rosette, whole inflorescences were collected from 6-week-old control and IM-treated plants and fixed with 2.5 % glutaraldehyde in 0.025 M sodium phosphate buffer (pH 6.8) at 4 °C overnight. The samples were then rinsed with phosphate buffer (0.1 M, pH 7.0) three times, and then fixed in 1 % OsO₄ in 0.05 M sodium cacodylate buffer (pH 7.0) for 2 h. Subsequently, samples were dehydrated through a graded ethanol series before treatment with a mixture of ethanol and isoamyl acetate. The samples were observed under a TM-1000 tabletop scanning electron microscope (SEM) from Hitachi High-Technologies.

qRT-PCR analysis of gene expression

SYBR Green based real-time qRT-PCR was used for measuring relative expression of several genes related to floral organ development (Table 1). We followed the gene DYT1 (DYSFUNCTIONAL TAPETUM 1) involved in *A. thaliana* tapetum development as well as several downstream genes regulated by DYT1, i.e. the gene TDF1 (*TAPETAL DEVELOPMENT AND FUNCTION1*) regulating tapetal differentiation and function, and several other key genes involved in formation of pollen exine and pollen cytosolic components as well as tapetum development (*AMS: ABORTED MICROSPORES, MYB103: MYB DOMAIN PROTEIN 103, MS1: MALE STERILITY 1, MS2: MALE STERILITY 2*) (Ó'Maoiléidigh et al. 2013) (Table 1). Total RNA was extracted from 4-week-old control and herbicide-treated seedlings using RNAiso (TaKaRa Company, Dalian, China) following the manufacturer's instructions. RNA quality was verified by visualizing the samples on ethidium bromide-stained agarose gels. A 1-µg sample of total RNA was used as a template for cDNA synthesis using M-MLV reverse transcriptase (Toyobo, Tokyo, Japan) with oligod (T). Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad) in an Eppendorf MasterCycler[®] ep RealPlex⁴ (Wesseling-Berzdorf, Germany). The specific primer pairs used for each gene are listed in Table 1. Expression of the gene *Actin 2* was used as a housekeeping gene to normalize the expression profiles. About 50 ng of cDNA were added into 10 µL reaction mixture. The following two-step PCR protocol was used: one denaturation step at 95 °C for

1 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Each treatment had 4 replicates and 5 seedlings were used to extract RNA in each replicate. The relative quantification of gene expression among the treatment groups was analyzed according to the method of Livak and Schmittgen (2001).

Quantification of damaged DNA

Plant leaves from control as well as R30- and S30-treated groups for 3 weeks were collected and the DNA of each group was extracted by phenol–chloroform extraction. Seeds were harvested from R30 and S30-treated groups and cultivated, again for 3 weeks, but in soil and culture medium not contaminated with IM (both treatments hereafter designated OR30 and OS30). After 3 weeks of growth, plant leaves of OR30 and OS30-treated groups were also collected and their DNA were extracted. DNA damage was quantified with a DNA Damage Quantification Kit (Dojindo, Kumamoto, Japan) based on the detection of abasic sites in genomic DNA with aldehyde reactive probe reagent, which reacts specifically with the open ring form of the abasic sites. This assay was performed according to the manufacturer's instructions. Each treatment had 6 replicates and 5 seedlings were pooled for each replicate.

Data analysis

Differences among the means of different treatments were tested for statistical significance by one-way ANOVAs followed by Dunnett's post hoc test using the StatView 5.0 software. When the probability (*p*) was less than 0.05 (*p* < 0.05), the means were considered to be significantly different. Errors are given as ±one standard error of the mean (SEM).

Results

The effect of IM on floral organ structure and pollen viability

Plants treated for 6 weeks days to 10 or 30 µg L⁻¹ of both IM enantiomers developed complete flower organ structure consisting of four organ types: sepals and petals, as well as stamens and a pistil in the center of the flower, but all of these floral organs were deformed for the R30 and R10-treated groups (Fig. 1). SEM analyses also revealed that all the floral organs (sepal, petal, stamen, and carpel) in IM-treated groups were developed (Fig. 2a–e), but anthers in the R10 and R30 groups were deformed and were frequently collapsed (Fig. 2d, e). In the *A. thaliana* Col-0 wild-type, the anther dehisced easily and released many

Table 1 Genes and their IDs as well as the sequences of primer pairs used in real-time PCR

Gene	Orientation	Sequence (5'–3')	Gene ID
<i>DYT1</i>	Forward	CATGTCCCCATTGTCACCAAC	827883
	Reverse	CTTCATCAATCTCAGGAGGAGC	
<i>TDF1</i>	Forward	GCTGATGAAAAATGGGGATAGAC	822477
	Reverse	GCTGAGTTGGATTGTGTGAGTA	
<i>AMS</i>	Forward	TGCAAGGGCTTGGGATTATTG	816194
	Reverse	CTTCTGTTCCATTCTCCGCTATGA	
<i>MYB103</i>	Forward	CCCTAACCCAAACAACCTTTTAC	835710
	Reverse	ATCCCAAACGAAGACGAAGAG	
<i>MS1</i>	Forward	CACGGCTCTTTCACTCCAACG	832286
	Reverse	GCGTCATTCAACCCTATTTTCCTG	
<i>MS2</i>	Forward	GCCTAGCGTCATCGAAAGCA	821147
	Reverse	GCCCTTCCCGTAACATAAAAC	
<i>Action2</i>	Forward	ACCTTGCTGGACGTGACCTTACTGAT	821141
	Reverse	GTTGTCTCGTGGATTCCAGCAGCTT	

DYT1 DYSFUNCTIONAL TAPETUM 1, *TDF1* TAPETAL DEVELOPMENT AND FUNCTION1, *AMS* ABORTED MICROSPORES, *MS1* MALE STERILITY, *MS2* MALE STERILITY 2, *MYB103* MYB DOMAIN PROTEIN103

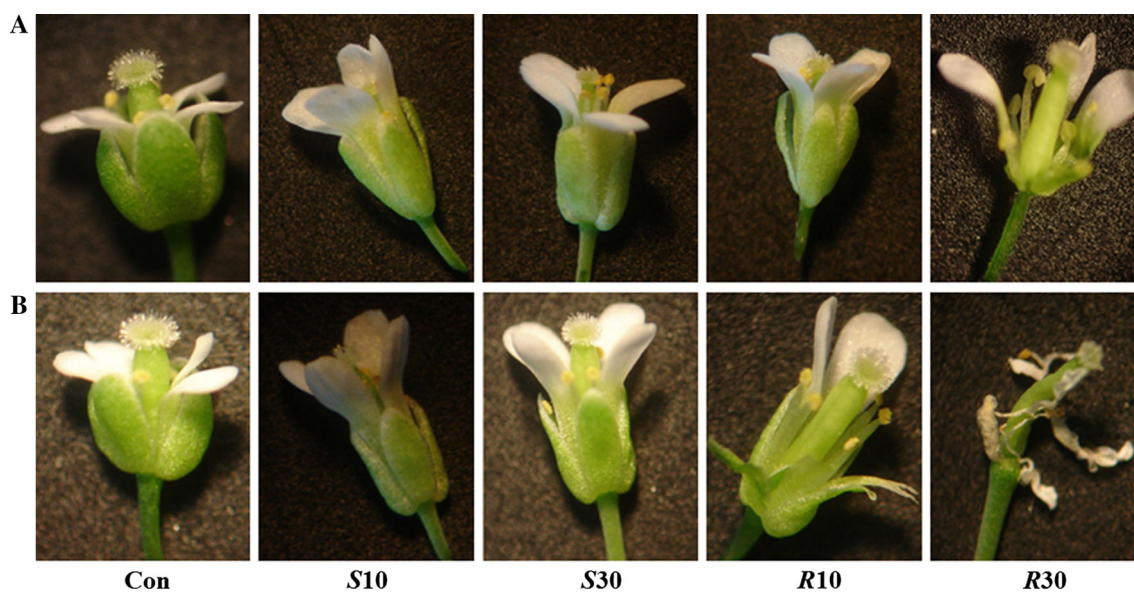


Fig. 1 The effect of a 6-week exposure of *A. thaliana* to 10 and 30 $\mu\text{g L}^{-1}$ IM enantiomers (*R*- and *S*-IM) on floral organ macroscopic structure (Control: no IM added; *S10*: 10 $\mu\text{g L}^{-1}$ *S*-IM; *S30*:

30 $\mu\text{g L}^{-1}$ *S*-IM; *R10*: 10 $\mu\text{g L}^{-1}$ *R*-IM; *R30*: 30 $\mu\text{g L}^{-1}$ *R*-IM). Line (a) and (b) represent two independent replicates on different seedlings

round-shaped pollen grains (Fig. 2e, k). Anthers in the *S10* group were also easily dehisced, with a pollen yield per anther similar to that of wild-type as judged by visual observation (Fig. 2g, l). However, the anthers in the *S30*, *R10* and *R30* groups were not easily dehisced and the number of pollen per plant decreased significantly compared to that of the control and *S10* groups (Fig. 2h–j, m–o), the pollen grains of both the control and the *S10* group being partially collapsed. Pollen was particularly difficult to find in the *R30* group (Fig. 2j, o). In the control group,

the pollen grains appeared pink and were round in shape, implying that the pollen was still alive. In the *R10* and *S10* groups, pollen grains still appeared round in shape, but only stained light pink or dark red. By contrast, pollen grains were small and squashed in the *R30* and *S30* groups. Furthermore, TTR-stained pollen grains in the *S30* group appeared reddish brown and, in the *R30* group, the grains were yellow or almost transparent showing that they are not viable (Fig. 3). Therefore, the results demonstrated that IM trace concentrations potentially inhibited pollen viability

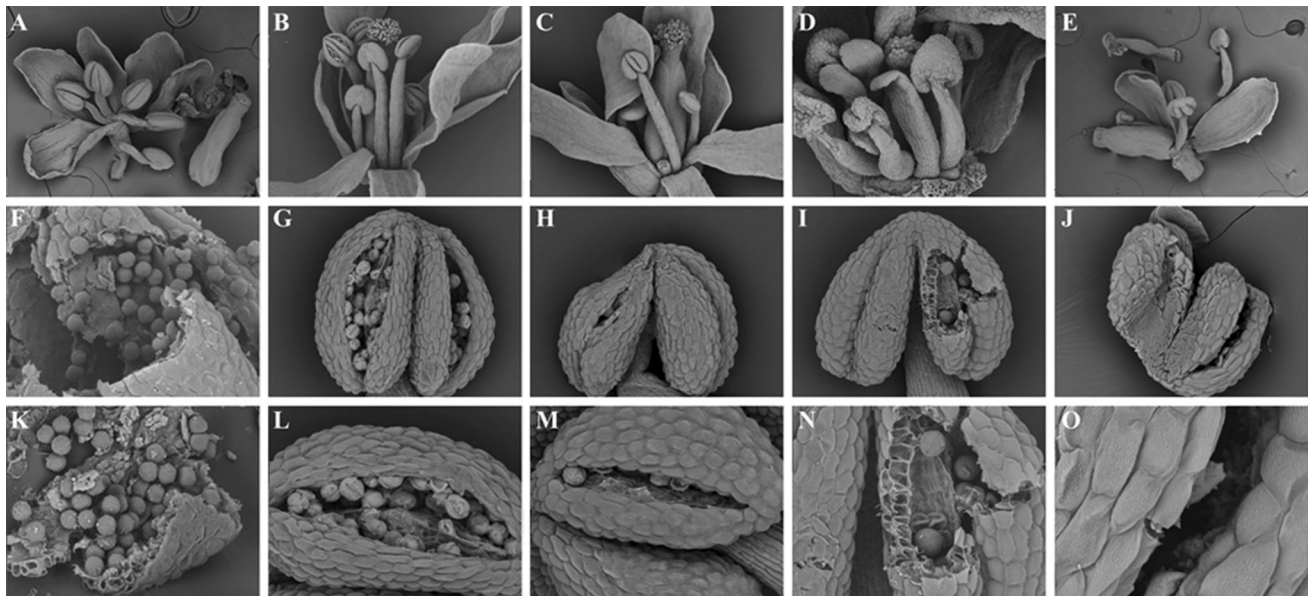


Fig. 2 The effect of a 6-week exposure of *A. thaliana* to 10 and 30 $\mu\text{g L}^{-1}$ IM enantiomers (*S*-IM and *R*-IM) on floral organ substructure observed with scanning electron microscopy. Symbols of each treatment are the same than those in Fig. 1. a–e show the

floral organs of *A. thaliana* in the control, S10, S30, R10 and R30 groups, respectively. f–j show the mature anther in the control, S10, S30, R10 and R30 groups, respectively. k–o show the pollen in anther of the control, S10, S30, R10 and R30 groups, respectively

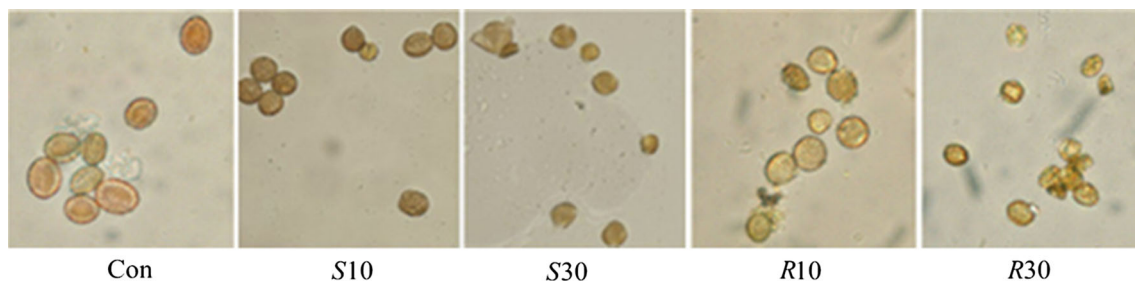


Fig. 3 The effect of a 6-week exposure of *A. thaliana* to 10 or 30 $\mu\text{g L}^{-1}$ IM enantiomers (*S*-IM or *R*-IM) on pollen viability. The stained pollens were observed under a light microscope at 200 \times magnification. Symbols of each treatment are the same than those in Fig. 1

and that the *R*-IM enantiomer affects more strongly pollen activity than the *S*-IM enantiomer.

The effect of IM on the transcription of pollen development-related genes and on DNA defects

The development of the tapetum layer in anthers is highly coupled with meiosis and pollen development (Zhu et al. 2008), which is controlled by a complex transcriptional regulatory network (Thorstensen et al. 2008). *DYT1* is a crucial component in the transcriptional regulatory network that controls anther development and function (Feng et al. 2012) by regulating the transcription of down-stream genes (*TDF1*, *AMS*, *MYB103*, *MS1* and *MS2*) to precisely control pollen development (Zhu et al. 2011). In this study, the transcripts of several pollen development-related genes were measured, such as *DYT1*, *TDF1*, *AMS*, *MYB103*, *MS1* and *MS2*, at stage 6–8 of anther development. The Fig. 4

showed that expression of all the genes tested did not decrease significantly in the S10 group compared with the control. However, the relative abundance of all genes transcripts decreased significantly by 2- to 3-fold in the S30 and R10 groups relative to the transcription level of the control, but the highest decrease in gene transcription occurred for the R30 group; The transcripts of *DYT1*, *TDF1*, *AMS*, *MYB103*, *MS1* and *MS2* being reduced by 4.6-, 6.0-, 4.1-, 5.8-, 13.6- and 2.6-fold, respectively, relative to the control transcription level.

Under herbicide-free conditions, the level of DNA damage was very low and only 47 apurinic/aprimidinic (AP) sites out of 10^4 nucleotides were detected. In the S30 and R30 groups, the number of AP sites increased significantly by 2.7- and 2.4-fold relative to the control value respectively (Fig. 5). However, there were no significant differences in the number of AP sites between the S30 and R30 treatments. The presence of DNA defects (AP sites)

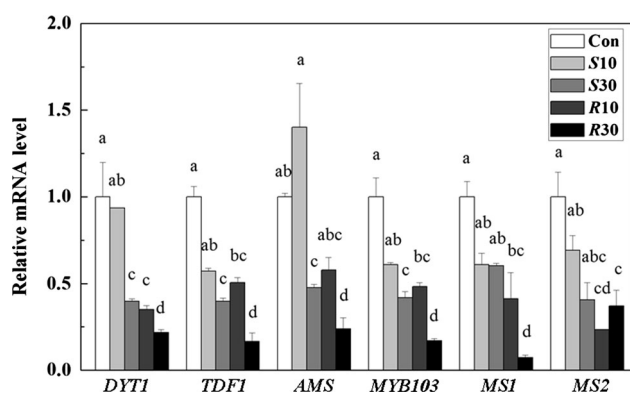


Fig. 4 The effect of a 4-week exposure of *A. thaliana* to 10 or 30 $\mu\text{g L}^{-1}$ IM enantiomers (*S*-IM or *R*-IM) on the transcripts of several genes related to pollen development. Transcription levels were normalized against *Actin 2*, and represent the mean relative mRNA expression value \pm SEM of three replicate cultures. Different letters represent statistically significant differences at $p < 0.05$ obtained from a one way ANOVA. Symbols of each treatment are the same than those in Fig. 1

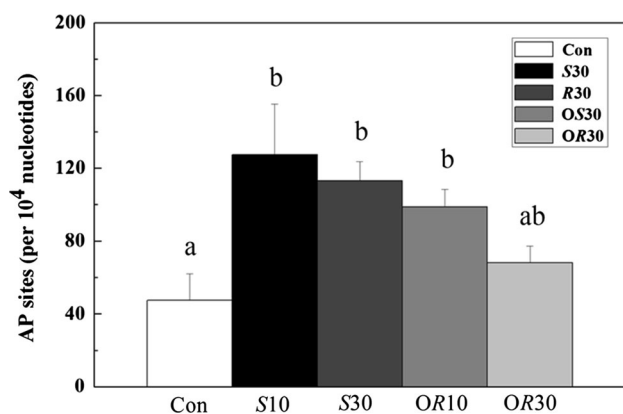


Fig. 5 The effect of a 3-week exposure of *A. thaliana* to 30 $\mu\text{g L}^{-1}$ IM enantiomers (*R*-IM and *S*-IM; denote *R*30 and *S*30 as explained in Fig. 1) on the number of DNA defects per 10^4 nucleotides. The *OS*30 and *OR*30 treatments represent the offspring of *A. thaliana* plants that have been pre-exposed to 30 $\mu\text{g L}^{-1}$ *R*-IM and *S*-IM for 3 weeks, but that were grown for 3 weeks in IM-free soil before the measurements of the number of AP sites. Different letters represent statistically significant differences at $p < 0.05$ obtained from a one way ANOVA

was also observed in the offspring of the IM-treated groups (*OS*30 and *OR*30) grown in IM-free medium; the mean number of AP sites in *OS*30 and *OR*30 groups decreasing slightly compared with those of the *S*30 or *R*30 groups but still remaining higher than that of the control.

The effect of IM on the number of seeds per silique

In the later stages of floral organ development, the fertilized pistil will give rise to the silique. The silique number per plant and the number of seeds per silique after IM

treatment were measured. As shown in Fig. 6a, the mean silique number per plant was approximately 11 in the control group, and significantly decrease to only approximately 8, 4, 4 and 2 per plant in the *S*10, *S*30, *R*10 and *R*30 group, respectively. Siliques in the control and *S*10 groups were plump, but they were relatively thin and short in *R*10 and *S*30 (Fig. 6b). However, the siliques became slender and contained almost no seeds in the *R*30 group. To carefully observe the capsule and seed number, we decolorized the mature capsule with ethanol and found that the length of the capsule in *S*10 plants was similar to that of the control, but with fewer seeds. The siliques in the *S*30 and *R*10 groups were very short and had several seeds, while there were only a few seeds in the siliques of the *R*30 group. The weight of seeds per plant in the control, *S*10, *S*30 and *R*10 groups were 1.8, 0.25, 0.005, and 0.0038 mg/plant, respectively and no seeds were found in the *R*30 group. The above results show that IM, especially the *R*-IM enantiomer, decreased seed production in *A. thaliana*.

Discussion

Imazethapyr toxicity on anther and pollen formation

Floral organ development is considered as one of the most important processes in plant reproduction, as abnormal floral organs can dramatically decrease the number of offspring produced by a plant. Anther structure was deformed by the treatment with 10 or 30 $\mu\text{g L}^{-1}$ *R*-IM or 30 $\mu\text{g L}^{-1}$ *S*-IM and the *R*-IM enantiomer was more toxic on floral organ development than the *S*-IM enantiomer. Higher toxicity of the *R*-IM than the *S*-IM enantiomer on several physiological functions in rice seedlings and *A. thaliana* plantlets was also demonstrated in our previous studies (Qian et al. 2009; 2011a, b). The potent effect of trace IM concentrations on anther development is important since anther is the part of the stamen that is responsible for the production of pollen and thus plays a key part in plant reproduction. Pollen production was indeed severely compromised in the presence of IM. TTR pollen coloration indicates that the pollen collected in the *S*-30 and *R*-30 groups were not as viable as that of the control suggesting that the fertility of *A. thaliana* exposed to IM was strongly compromised. Furthermore, pollen production and size dramatically decreased in the presence of low trace concentrations of IM. Thus, the results clearly showed that chronic exposure of *A. thaliana* to trace IM concentrations adversely affected anther development and nearly abolished pollen production (for the *R*30 group) as severe long-term drought stresses do (Su et al. 2013). Plant reproduction (seed production and germination) is routinely more sensitive than other conventional endpoints

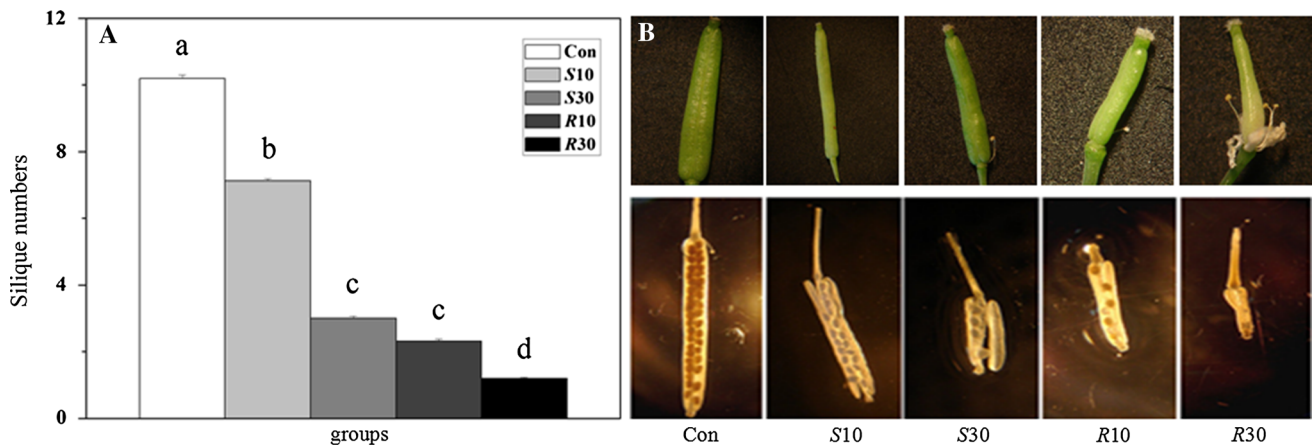


Fig. 6 The effect of a 8-week exposure of *A. thaliana* to 10 or 30 $\mu\text{g L}^{-1}$ IM on the number of seeds per silique (a) or the overall appearance of siliques (b). Different letters represent statistically

significant differences at $p < 0.05$ obtained from one way ANOVA. Symbols of each treatment are the same than those in Fig. 1

(e.g. plant biomass) to the presence of herbicides (Boutin et al. 2014). A range of species exposed to different herbicides consistently exhibit a much higher sensitivity to herbicides when exposed in the flowering period than during the vegetative phase (Boutin et al. 2014). Our study performed with *A. thaliana* add to the current paradigm of a high pesticide toxicity to non-target plants in the reproductive phase.

Similarly to our findings, low herbicide doses have been shown to dramatically decrease seed germination and reproduction in a range of plant species (Fletcher et al. 1993, 1996; Gealy et al. 1995; Boutin et al. 2014). Several studies have sought to demonstrate mechanisms by which herbicides may affect reproductive structures. Ratsch et al. (1986) found that for the herbicides DGME, dalapon and TCA, flower deformations at even the lowest doses tested precluded pollination and seed formation. Other studies have uncovered herbicide effects on male structures, especially with glyphosate tested on crops (Baucom et al. 2008; Pline et al. 2002; Thomas et al. 2004; Yasuor et al. 2006). However, the mechanisms of herbicide toxicity on plants are still lacking.

Toxicity mechanisms at the level of gene transcription

Only very recently, genome-wide approaches have been applied to obtaining a global view of the gene regulatory networks underlying flower formation (Ó'Maoiléidigh et al. 2013). This powerful tool opens up new possibilities to better understand the toxic effect of herbicide toxicity in plants. Transcriptional regulation is a major mechanism controlling anther development in *A. thaliana*. Several genes encoding transcription factors were shown to be essential for male fertility in plants. *DYT1* encodes a putative basic helix-loop-helix transcription factor, which lies upstream of at least 22 genes encoding transcription

factors and regulates the expression of a large number of genes, such as *TDF1*, *AMS*, *MYB103*, *MS1* and *MS2*. Therefore, *DYT1* is a crucial component in the control of the development and function of anthers (Zhang et al. 2006; Feng et al. 2012). In this study, we show that IM enantioselectively inhibited *DYT1* transcription and also down-regulated several down-stream gene transcripts (Fig. 4). This strong effect of IM on the transcription of key genes involved in floral organ development could be one of the main mechanisms explaining the strong IM effect on pollen development that we observed in *A. thaliana*. Indeed, *DYT1* positively regulates genes related to lipid metabolism that are important for pollen development. As observed in this study as well as in Feng et al. (2012), the herbicide-induced down-regulation of *DYT1* also caused a decrease in the transcription of *MS2*, which encodes a fatty acid reductase that is required for pollen wall formation (Aarts et al. 1997). Similarly, since *DYT1* positively regulates the expression of 33 genes involved in cell wall modification that can catalyze the breakdown of polysaccharides to facilitate anther dehiscence (Feng et al. 2012), the decrease in *DYT1* transcription measured in *A. thaliana* exposed to IM should lead to the production of anthers that could not easily dehisce to allow pollen dissemination, as observed under SEM observation. Several experiments demonstrated that *TDF1*, *AMS*, *MYB103*, *MS1* and *MS2* suppression inhibited pollen grain production or caused male sterility (Zhu et al. 2008; Feng et al. 2012). Thus, the inhibition of these key genes in *A. thaliana* exposed to IM may well explained the measured decrease in pollen production and pollen viability, which ultimately led to a decrease in the number of seeds per silique (Fig. 6). To the best of our knowledge, this is the first study unraveling the mechanisms of toxicity of imazethapyr on the transcription level of several key genes involved in plant reproduction.

Multi-generational effects of imazethapyr: IM-induced irreversible DNA defects

Information about the genotoxic potential of imidazolinone herbicide such as IM on plant cells is surprisingly scarce in the literature. It is known that low concentrations of IM can induce ROS formation in *A. thaliana* (Qian et al. 2011a) and that ROS can damaged proteins, lipids, and also DNA (Apel and Hirt 2004). We speculate that the above mechanism could explain the DNA damages observed in *A. thaliana* exposed to trace IM concentrations for 3 weeks (Fig. 5). Even more interestingly, our results show that the adverse genotoxic effect of IM is inherited by the offspring grown in IM-free medium (on a time frame of one generation) (Fig. 5). If ROS have damaged nucleotides in generative cells of *A. thaliana*, then the DNA defects is expected to be passed on to the offspring. To our knowledge, this study is the first to document severe genotoxic effect of IM in plants.

Environmental implications: herbicide toxicity on *A. thaliana* life cycles

Flowering time is an important trait in crops that also affects the life cycles of pollinator species. The transition to flowering is under the control of a complex genetic network that integrates information from various endogenous and environmental cues such as temperature, photoperiod and nutrient availability (Amasino 2010). Our previous report demonstrated that the herbicide IM can affect the transition from the vegetative phase to the reproductive phase (Qian et al. 2014). The correct timing of the transition to flowering is of the utmost importance to all plants (Huijser and Schmid 2011): premature flowering usually results in reduced biomass and fruit setting. On the contrary, prolonged vegetative growth might lead to an increase in biomass and often results in a reduce production of seeds and seed filling (Demura and Ye 2010). Furthermore, in the case of non-self-fertile species, flowering also needs to be synchronized within a population and with the occurrence of potential pollinators. The crucial effect of imazethapyr on *A. thaliana* reproduction revealed in the present study could affect the timing of floral transition altering global plant phenology and distribution. This fundamental and still understudied effect of herbicide on plant reproduction pose a new threat to plant biodiversity besides the ongoing global climate change perturbation, which promotes early flowering and perturbs plant life cycles (Kumar et al. 2012; Satake et al. 2013). The complex and important toxicity effects of herbicide on the reproduction of non-target plants in agricultural areas clearly deserve more attention in future study in order to improve current herbicide toxicity assessment.

Conclusions

Understanding how herbicides affect the life cycle of terrestrial plants is critical to evaluating ecosystem vulnerability to environmental pollutants. In the present study, trace IM concentrations inhibits the transcription of several key genes related to the anther and pollen development in *A. thaliana*, which lead to deformed anthers and decreased pollen number and viability. Prolonged exposure to IM also increased the number of DNA defects in *A. thaliana* as well as in their offspring, which were cultivated in IM-free medium, demonstrating the strong multi-generational toxic effect of IM on DNA sequences of *A. thaliana*. Our findings document crucial molecular targets of IM by which this imidazolinone herbicide may exert its toxic effect on the reproduction of *A. thaliana*. The strong toxic effect of IM on *A. thaliana* reproduction revealed in this study underscores the need to consider reproduction endpoints in herbicide ecological risks assessments.

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Conflict of interest The authors declare that they have no conflict of interest.

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