Induction of the PDH bypass and upregulation of the *ALDH7B4* in plants treated with herbicides inhibiting amino acid biosynthesis

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HIGHLIGHTS

- Imazamox and glyphosate provoke induction of the pyruvate dehydrogenase bypass and upregulation of ALDH7B4 expression

- Analysis of *pdc1-pdc2* mutant seedlings confirmed the role of ALDH7B4 in the PDH bypass.

- ALDH7B4 induction is not related with detoxification of the aldehydes or changes in lipid content or composition

- ALDH7B4 upregulation partially alleviates the toxicity of herbicides on root growth, carbohydrate and organic acid content

ABSTRACT

Imazamox and glyphosate represent two classes of herbicides that inhibit the activity of acetohydroxyacid synthase in the branched-chain amino acid biosynthesis pathway and the activity of 5-enolpyruvylshikimate-3-phosphate synthase in the aromatic amino acid biosynthesis pathway, respectively. However, it is still unclear how imazamox and glyphosate lead to plant death. Both herbicides inhibit amino-acid biosynthesis and were found to induce ethanol fermentation in plants, but an *Arabidopsis* mutant deficient in *alcohol dehydrogenase 1* was neither more susceptible nor more resistant than the wild-type to the herbicides. In this study, we investigated the effects of the amino acid biosynthesis inhibitors, imazamox and glyphosate, on the pyruvate dehydrogenase bypass reaction and fatty acid metabolism in *A. thaliana*. We found that the pyruvate dehydrogenase bypass was upregulated following the treatment by the two herbicides. Our results suggest that the *Arabidopsis aldehyde dehydrogenase 7B4* gene might be participating in the pyruvate dehydrogenase bypass reaction. We evaluated the potential role of the aldehyde dehydrogenase 7B4 upon herbicide treatment in the plant defence mechanism. Plants that overexpressed the *ALDH7B4* gene accumulated less soluble sugars, starch, and fatty acids and grew better than the wild-type after herbicide treatment. We discuss how the upregulation of the *aldehyde dehydrogenase 7B4* alleviates the effects of the herbicides, potentially through the detoxification of the metabolites produced in the pyruvate dehydrogenase bypass.

KEYWORDS

Acetohydroxyacid synthase, 5-enolpyruvylshikimate-3-phosphate synthase, ethanol fermentation, fatty acids, glyphosate, imazamox.

ABBREVIATIONS

ABIH, amino acid biosynthesis-inhibiting herbicide; ACS, acetyl-CoA synthetase; AHAS, acetohydroxyacid synthase; ALDH, aldehyde dehydrogenase; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; PDC, pyruvate decarboxylase; PDH, pyruvate dehydrogenase.

1 INTRODUCTION

Herbicide application contributes to the large-scale agronomic crop production by efficient weed removal. There are three groups of commercialized herbicides that inhibit the biosynthesis of amino acids, these are the most common herbicides used worldwide. Imazamox represents a herbicide that inhibits the activity of the enzyme acetohydroxyacid synthase (AHAS, EC 2.2.1.6) in the branched-chain amino acid biosynthesis pathway. Glyphosate represents another group that inhibits the activity of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (EC 2.5.1.19) in the aromatic amino acid biosynthesis pathway [1]. Although the enzyme targets of the herbicides in the branched-chain amino acid and aromatic amino acid biosynthesis branches have been known since the early 1980s [2–4], it is still unclear how the inactivation of AHAS or EPSPS results in plant death. Previous findings showed that both AHAS and EPSPS inhibitors cause growth arrest followed by a slow plant death of the herbicide-treated plants although they act upon different pathways [5,6]. Both types of herbicides provoke an accumulation of free amino acids [3,7–11]. This effect

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is accompanied by a decrease in the soluble protein content [9,11,12] and may be the key to the common response that has been associated with a plant proteolysis response [9]. Besides the amino acid metabolism, induction of ethanol fermentation has been described in plants treated with amino acid biosynthesis-inhibiting herbicides (ABIHs) including AHAS and EPSPS inhibitors [12–16]. However, the pyruvate dehydrogenase (PDH) bypass, a metabolic route that is intimately connected with the ethanol fermentation, has so far been overlooked in the response of plants to ABIHs.

The PDH bypass involves the action of three enzymes: pyruvate decarboxylase (PDC, EC 4.1.1.1), aldehyde dehydrogenase (ALDH, EC 1.2.1.3) and acetyl-CoA synthetase (ACS, EC 6.2.1.1) [17]. In the first reaction of the PDH bypass, the PDC catalyses the conversion of pyruvate to acetaldehyde (it is the first step of the ethanol fermentation pathway). Then acetaldehyde is metabolized to acetate by ALDH, and in the last step of the PDH bypass, acetyl-CoA is produced from acetate in a reaction catalysed by ACS. Compared to the reaction catalysed by the PDH complex, a small amount of acetyl-CoA is generated by the PDH bypass. The PDH bypass has been hypothesized to play a specialized role in certain cells and tissues [18]. Different studies reported the presence of the PDH bypass during pollen development [19,20]. In parallel with an increase in the ethanol fermentation during tobacco pollen development, which is primarily controlled by sugar supply rather than by oxygen availability [21,22], the existence of the PDH bypass has been suggested to prevent the accumulation of fermentation products including acetaldehyde and ethanol to toxic levels. In agreement with this, the inactivation of *ADH1* in pollen did not affect normal pollen development, suggesting the existence of an alternative pathway to metabolize the acetaldehyde produced by PDC [23]. The existence of the PDH bypass in vegetative tissues has also been described [24,25]. In vegetative tissues, the PDH bypass has been proposed to contribute to the detoxification of the metabolites

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produced during fermentation. On the one hand, one member of the ALDH family 2 has been suggested to detoxify the acetaldehyde produced during reaeration (by the oxidation of the ethanol produced during anoxia) in rice [26,27]. On the other hand, plants lacking the *ACS1* gene presented higher susceptibility to exogenously applied ethanol, acetaldehyde, and/or acetate than wild-type plants, indicating a role for the PDH bypass in the detoxification of these chemicals [24].

The induction of the ethanol fermentation in plants treated with ABIHs suggests that the PDH bypass might also be affected by the ABIHs, given that *A. thaliana* plants lacking the *ADH1* gene did not present higher susceptibility to ABIH application [15]. To gain new insights into the physiological effects triggered by ABIHs, the current study examined their effect on the PDH bypass, particularly on the ALDHs. We found that the PDH bypass and the *ALDH7B4* were upregulated following the treatment with the ABIHs. We discuss how the ALDH7B4 might be implicated in the PDH bypass and how the induction of ALDH7B4 alleviates the effects of the herbicides.

2   MATERIALS AND METHODS

2.1   Plant material, growth conditions and herbicide treatments

Arabidopsis thaliana Col-0 wild-type plants were used (wt). The *A. thaliana* Col-0 T-DNA insertion mutants defective in the *ALDH7B4* gene (SALK line 143309) (*aldh7b4*) [28,29], the transgenic line expressing the *ALDH7B4* under the control of the CaMV 35S promoter (*35S::ALDH7B4*) [29], and the transgenic line expressing the *ALDH7B4-promoter::GUS gene* cassette were described previously [30].

Plants were grown as described by Zulet et al. [15]. Briefly, seeds were surface sterilized before sowing them on Seedholders (Araponics SA, Belgium) filled with 0.65% (w/v) plant agar. Seedholders were placed in tanks and plants

were grown in a growth chamber under 150 μmol m^{-2}s^{-1} PPF, 65% RH and 25/20ºC day/night. The plants were maintained in a 12 h/12 h day/night photoperiod for the first 4 weeks and grown in 8/16 h day/night photoperiod afterward to prevent flowering. The nutrient solution was slightly modified from [31]: 1 mM NH_4NO_3, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 250 mM $CaCl_2$, 0.1 mM Na-Fe-EDTA, 50 mM KCl, 50 mM H_3BO_3, 5 mM $MnSO_4$, 1 mM $ZnSO_4$, 1 mM $CuSO_4$, and 0.1 mM $(NH_4)_6Mo_7O_{24}$. Aeration was set in the tanks when the plants were six-week-old and maintained from then onwards.

When plants were approximately eight weeks old (rosette stage), herbicides were applied to the nutrient solution. The two herbicides were applied as commercial formulations at a final concentration of 1.5 mg active ingredient L^{-1} (4.9 μM) of imazamox (Pulsar$^®$40, BASF Española SA, Barcelona, Spain) or 20 mg active ingredient L^{-1} (87.65 μM) of glyphosate (Glyfos$^®$, Bayer CropScience, S.L, Paterna, Valencia, Spain). The experiment was performed in triplicate.

Samples were taken after three days of herbicide application, before obvious visual plant death was observed. This time point was chosen in order to allow the evaluation of physiological and biochemical plant responses induced by the herbicides but not directly resulting from cell death. Intact leaf and root samples were immediately frozen in liquid nitrogen and stored at -80°C for further analyses. Later, frozen samples were ground under liquid nitrogen using a Retsch mixer mill (MM200, Retsch$^®$, Haan, Germany), the required amount of tissue for each analysis was separated and stored at -80°C. Fresh material was used for the histochemical detection and measurement of GUS activity.

2.2 Plants grown under axenic conditions

Wild-type *A. thaliana* Col-0 (wt) and a double mutant of *A. thaliana* Col-0 with a T-DNA insertion line for *PDC1* and *PDC2* (*pdc1-pdc2*) (kindly provided by Francesco Licausi PlantLab, Scuola Superiore Sant'Anna, Pisa, Italy) were used.

To ensure axenic conditions all growth containers and medium were sterilized before use. Plants were grown in sterile six-well plates in liquid half-strength MS medium (pH 5.7) (Sigma-Aldrich Co., St. Louis, MO, USA) enriched with 1% (w/v) sucrose under continuous shaking. Ten seeds were placed in each well and plates were incubated for 3 days at 4°C in darkness for stratification. Plates were then placed in a growth chamber and seedlings were grown under 80 µmol m^{-2} s^{-1} light, at 23°C/18°C day/night temperature and at a 12/12 h day/night photoperiod.

When plants were six days old, the old growth medium was removed and replaced with fresh sucrose-free medium, and treatments were started. Seedlings were treated with imazamox, glyphosate for 5 days. The two herbicides were applied as commercial formulations at a final concentration of 1.5 mg active ingredient L^{-1} (4.9 µM) of imazamox (Pulsar®40, BASF Española SA, Barcelona, Spain) or 20 mg active ingredient L^{-1} (87.65 µM) of glyphosate (Glyfos®, Bayer CropScience, S.L, Paterna, Valencia, Spain). Seedlings from one individual well were collected as a biological sample and different wells were harvested as replicates. The plant material was immediately frozen.

2.3 Determination of root growth on agar plates

Wild-type *Arabidopsis thaliana* Col-0 plants (wt), *A. thaliana* Col-0 T-DNA insertion mutants defective in the *ALDH7B4* gene (SALK line 143309) (*aldh7b4*) [28,29], and three independent transgenic lines expressing the *ALDH7B4* under the

control of the CaMV 35S promoter (*35S::ALDH7B4, 35S::ALDH7B4*_2 and *35S::ALDH7B4*_3) [29] were used.

Seeds were sterilized (section 2.1) and were then transferred to petri dishes containing 0.1% (w/v) plant agar. Plants were incubated for 3 days at 4°C in darkness before they were transferred to the growth chamber. Plants were grown under 120-150 µmol m^{-2} s^{-1} light, 65% relative humidity at 23°C/18°C day/night temperature and 12/12h day/night cycle. After 4 days in the growing chamber, when the root length was about 1 cm, seedlings were transferred to 12 x 12 cm plates containing half-strength Murashige and Skoog (MS) medium (pH 5.7), 1% sucrose (w/v) and 0.9% (w/v) plant agar (8 seeds per plate) and, where corresponding, the selected herbicide dose. The two herbicides were sterilized using 0.20 µm filters and were added to the medium before it solidified.

Since the applied herbicide concentration in the hydroponically grown *A. thaliana* plants was too high for agar plates (plants died within two days), preliminary studies were conducted to find a herbicide concentration that was not too aggressive and killed the plant in a few days, but that was sufficiently aggressive to have an effect on the plant growth. Thus, 0.005 mg active ingredient L^{-1} of imazamox (0.016 µM) and 0.4 mg active ingredient L^{-1} of glyphosate (1.75 µM) were chosen as herbicide concentrations. Plants not treated with herbicide were used as control plants. Root elongation was measured 15 days after the transfer to 12 x 12-plates. The experiment was done in duplicate.

2.4 Semi-quantitative Reverse-Transcription-Polymerase Chain Reactions (RT-PCRs)

Total RNA was extracted from about 0.1 g of previously ground frozen leaf or root samples as described by Missihoun et al. [32].

Extracted RNA was subsequently quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). OD 260 and 280 nm were read for every sample. The RNA quality was also checked in a 1% agarose gel. For the PCR analysis 2 µg of total RNA was treated with 10 U RNase-free DNase I (Thermo Fisher Scientific Inc., Waltham, MA, USA) in a 10 µL reaction containing 1 x DNase I buffer (10 mM Tris/HCl (pH 7.5), 0.5 mM CaCl$_2$ and 2.5 mM MgCl$_2$) at 37°C for 10 min. Then, 1 µL of 25 mM EDTA was added and the reaction was heated at 65°C for 10 min to deactivate the DNase I.

First-strand cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions.

After reverse transcription, RT-PCR was performed on an Eppendorf Mastercycler ep Gradient S (Eppendorf, AG, Hamburg, Germany). Each reaction was performed for 1 µL of cDNA in a total volume of 20 µL containing: 1 x ammonium buffer (Tris-HCl (pH 8.5), (NH$_4$)$_2$S0$_4$, 15 mM MgCl$_2$, 1% Tween 20®), 0.2 mM of each dNTPs, 0.4 µM specific forward primer, 0.4 µM specific reverse primer and 1 U Taq polymerase (Ampliqon A/S, Odense, Denmark).

The specific primers used for the RT-PCR analysis are detailed in appendix B, Suppl. Table S1. The parameters of the PCR programme were as follows: 5 min 94°C; 25-32 cycles (30 s 94°C, 45 s 62°C and 2 min (3 min for *ALDH3F1*) 72°C); 5 min 72°C and pause at 4°C. The specific number of cycles used for each gene is presented in appendix B, Suppl. Table S2.

Two-fold diluted PCR amplified products were loaded on a 1% agarose gel and run at 135 mA for 35 min. A 1Kb Gene Ruler was used as size marker. The

gels were visualized in a Bio-Rad Gel Documentation Gel Doc™ 2000 System (Bio-Rad Laboratories Inc., Hercules, CA, USA). To quantify the intensity of the bands given by the PCR amplified products the Quantity One® version 4.6.9 Software (Bio-Rad Laboratories Inc., Hercules, CA, USA) was used. The signal intensity value for each sample and for a specific gene was divided by that of the *ACTIN2* (At3g18780) gene for the same sample.

2.5 Quantitative Real-Time-Polymerase Chain Reactions (qPCR)

Total RNA was extracted from ground frozen seedlings (about 0.1 g FW) as described in Kosmacz et al. [33]. Total RNA was subjected to a DNase treatment using the RQ1-DNase kit (Promega Biotech Ibérica, SL., Alcobendas, Spain). Five hundred ng RNA were reverse transcribed into cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) following the manufacturer's instructions.

The qPCR amplification was carried out with the ABI Prism 7300 sequence detection system (Applied Biosystems, Life Technologies, Darmstadt, Germany) using the iQ™ SYBR® Green Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA). The parameters of the PCR program were as follows: 10 s 50°C, 3 min 95°C, 40 cycles (15 s 95°C, 30 s 60°C), a dissociation curve (15 s 95°C, 30 s 60°C and 15 s 95°C). *ACTIN2* was used as reference. The primer pairs used in the qPCRs are presented in appendix B, Suppl. Table S3. Relative quantification of the expression of each individual gene was performed using the $2^{-\Delta\Delta C_T}$ method [34].

2.6 Histochemical detection and measurement of the GUS activity

GUS staining was performed as described by Jefferson et al. [35]. Fluorometric GUS activity of crude plant samples was determined according to Jefferson et al. [35].

2.7 Lipid peroxidation assay

The level of lipid peroxidation products was measured in the plant tissues by the thiobarbituric acid-reactive-substances assay. Modifications were introduced to correct interference generated by nonspecific turbidity, thiobarbituric acid-sugar complexes and other no-thiobarbituric acid reactive-substances absorbing at 532 nm [36].

2.8 PDC and ADH activities

The *in vitro* activities of PDC were assayed in ground tissue samples as described in [12].

2.9 Free amino acid extraction and determination

Total free amino acids were measured from ground tissue samples. Amino acids were extracted with 1M HCl. After protein precipitation, amino acid concentrations were analysed in the supernatant. After derivatization with FITC, the amino acid content was measured using capillary electrophoresis equipped with a laser-induced fluorescence detector [37]. The cysteine contents were determined from the same acid extracts derivatized with 5-iodoacetamide fluorescein and reduced with tributylphosphine [38].

2.10 Carbohydrate extraction and determination

Soluble carbohydrate (glucose, fructose and sucrose) contents were determined in ethanol-soluble extracts. The ethanol-insoluble residue was extracted for starch analysis [39]. The determination of starch and soluble sugar concentrations was performed using capillary electrophoresis [39].

2.11 Organic acid extraction and determination

Long-chain organic acids were measured in *A. thaliana* leaves and roots, the same extracts were used for the determination of ethanol-soluble sugars (as described above for carbohydrates extraction, section 2.9).

Short-chain organic acids were extracted from ground *A. thaliana* leaf and root samples. About 0.1 g of plant samples were homogenized in 0.5 mL 1M HCl. Tubes were centrifuged at 18,000 *g* for 25 min at 4°C and 250 µL of supernatant was transferred to a new tube and diluted to 1:10 in deionized water. Extracts were filtered with Ag filters (to eliminate Cl^-) and H^+ filters (to eliminate cations).

The organic acid content was determined by ion chromatography in a DX-500 IC System (Dionex Corporation, Sunnyvale, CA, USA) that included a GP40 Gradient Pump and an ED40 Electrochemical Detector. Ion-Pak AG11 and AS11 columns were used for the separation. The gradient was used from 0.2 mM NaOH to 45 mM NaOH and from 10% methanol to 20% methanol, in 27 min, at a flux of 1 mL min^{-1} for long-chain organic acid determination, and from 0.2 mM NaOH to 15 mM NaOH in 25 min, at a flux of 1 mL min^{-1} for short-chain organic acid determination.

2.12 Total fatty acid extraction and determination

For total fatty acid extraction glass tubes with screw caps containing Teflon septa were used. Freshly collected ground plant samples were transmethylated after submergence in 1 M HCl in methanol, and extracted after addition of 0.9% NaCl: hexane (1:1).

Total fatty acid determination was performed by gas chromatography with flame ionization detector on an Agilent 7890A Gas Chromatograph (Agilent

Technologies Inc., Santa Clara, CA, USA) using pentadecanoic acid (15:0) as internal standard [40].

2.13 Statistical analyses

The data obtained from this study were analysed by the IBM SPSS Statistics (v.22) software package. Data are presented as mean ± SE and was calculated using samples from different individual plants as replicates.

First, for each studied parameter, the untreated plants of each genotype were compared to the untreated wild-type plants employing Student's t-test to obtain the significance of the difference between the means of two independent samples (significance level of 5%, $p < 0.05$).

Second, the data of the herbicide-treated and non-treated plants of each genotype were compared using the one-way ANOVA test, after log transformations of the data if needed. To confirm homoscedasticity of variances, the Levene's test was used. The HSD Tukey and Dunnett T3 *post hoc* statistical tests were applied to the homogeneity and non-homogeneity of variances cases, respectively. When the results were expressed in percentages, the data were first transformed according to the following formula: $arcsin\sqrt{x/100}$. In all cases, statistical analyses were conducted at a significance level of 5% ($p < 0.05$).

For the root elongation measurements, herbicide effects were expressed in percentages with respect to the untreated plants for each genotype. Percentages of transgenic genotypes (after transformation to $arcsin\sqrt{x/100}$ were compared to the percentage in wild-type plants employing Student's t-test to obtain the significance of the difference between the means of two independent samples (significance level of 5%, $p < 0.05$).

3　RESULTS AND DISCUSSION

3.1　Induction of *ACS* gene expression in *Arabidopsis* plants treated with imazamox or glyphosate

The induction of ethanol fermentation is a well-known physiological effect provoked by ABIHs in plants, although oxygen is not limited under these conditions. To examine the effects of ABIHs on the PDH bypass, the expression (mRNA transcript levels) of genes involved in the PDH bypass in *A. thaliana* plants was measured following the application of imazamox or glyphosate, which inhibit AHAS and EPSPS, respectively. The *A. thaliana ALDH2B4* gene was found to be the primary contributor to the PDH bypass pathway in both vegetative and floral tissues [25], one step before of the unique ACS-coding gene in *A. thaliana*. Thus, the expression pattern of *ALDH2B4* and *ACS* were measured in the leaves and the roots of *A. thaliana* plants treated with imazamox or glyphosate (Fig. 1). The results showed an increase in the expression of the *ACS* gene in the leaves and roots after ABIH treatment (Fig. 1). In the leaves, glyphosate provoked a higher increase in the *ACS* transcript levels, while in the roots, the effect of the two herbicides was similar. The induction of the *ACS* gene expression by ABIHs has not been previously described. This result indicates that the effect of these herbicides on primary plant metabolism has broader physiological consequences than a lack of certain amino acids alone, which suggests that that both AHAS and EPSPS inhibitors provoke similar physiological effects on treated plants, even though they act upon different pathways.

In contrast, the expression of *ALDH2B4* was not induced as a consequence of ABIH application (Fig. 1). This observation led us to analyse the expression of other ALDHs in response to herbicide treatments.

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3.2 Induction of the *ALDH7B4* gene expression is a new effect triggered in plants after ABIH application

Sixteen genes code for members of ten ALDH protein families in *A. thaliana* [41,42]. We focused on the family 2, family 3 and family 7 because members of these three families have been described to play a role in the response of plants to abiotic stresses (such as flooding, salinity and dehydration) [29,43–46]. The transcript levels of the ALDHs belonging to family 2 *(ALDH2B7* and *ALDH2C4*), family 3 *(ALDH3F1, ALDH3H1, ALDH3I1)* and family 7 *(ALDH7B4)* were analysed by RT-PCRs in leaves and roots of imazamox- or glyphosate-treated wild-type *A. thaliana* Col-0 plants (Fig. 2). Except for *ALDH7B4* in roots and *ALDH7B4* and *ALDH2C4* in leaves, no changes in expression of the monitored ALDHs were found following imazamox or glyphosate application. The expression of the *ALDH2C4* increased in the leaves of imazamox-treated plants. This gene encodes a protein known to play a role in the biosynthesis of ferulic acid and sinapic acid [47,48]. The increase in the *ALDH2C4* expression suggests an increase of ferulic acid and sinapic acid, which have been reported to accumulate in imidazolinone-treated pea plants [49]. Interestingly, the upregulation of *ALDH7B4* was the only common effect triggered by both herbicide treatments in leaves and roots, with glyphosate inducing a stronger response than imazamox.

To confirm the induction of *ALDH7B4* after herbicide treatment, *ALDH7B4-promoter::GUS* transgenic plants were grown and treated with imazamox or glyphosate and the promoter activity was monitored by histochemical GUS staining and by GUS enzyme activity measurements (Fig. 3). The activity of the promoter of *ALDH7B4* is strongly induced in both leaves and roots in response to imazamox or glyphosate (Fig. 3.A and 3.B). The activity of the *ALDH7B4*

promoter dramatically increased after glyphosate treatment while the increase detected after imazamox application was lower. The histochemical GUS staining indicated a higher GUS activity in leaves of *ALDH7B4::GUS* transgenic plants treated with imazamox or glyphosate. These increases were not observed for roots, probably due to the high GUS activity already present in the roots of untreated plants [30]. RT-PCRs were also conducted for the *ALDH7B4* and *GUS* genes in the leaves and the roots of *ALDH7B4::GUS* transgenic plants (Fig. 3.C). The *GUS* and *ALDH7B4* transcript levels of both leaves and roots increased as a consequence of the two herbicide treatments. Glyphosate caused the highest increase in the expression of the two genes. The results observed in the analysis of the *ALDH7B4* promoter (Fig. 3) correlated with the ones obtained for the *ALDH7B4* gene expression analysis carried out in wild-type *A. thaliana* Col-0 plants (Fig. 2), and confirmed the induction of *ALDH7B4* at the transcriptional level. The prominent increase in the expression of the *ALDH7B4* was comparable for the two herbicides and indicates that this enzyme plays an important role in response to ABIH-treatment.

It is important to elucidate the physiological implications of the ALDH7B4 induction in the toxicity of ABIHs. Two, non-contradictory explanations, can be considered. Firstly, the induction of these two pathways could be a plant defence mechanism that promotes better tolerance of the herbicide, and/or secondly, it could be a consequence of the herbicide activity thus contributing to the chemical's toxicity.

3.3 Analysis of *pdc1-pdc2* defective mutant seedlings shows a role of ALDH7B4 in the PDH bypass

The only *ALDH* upregulated by the two herbicides in leaves and roots was the cytosolic *ALDH7B4*. This makes ALDH7B4 a good candidate for the cytosolic conversion of acetaldehyde into acetate in the PDH bypass following the herbicide treatment. Indeed, in the PDH bypass, acetaldehyde is produced in the cytosol from the decarboxylation of pyruvate in a reaction catalysed by the PDC, and the ACS enzyme converts acetate into acetyl-CoA in the plastids. It was proposed that either the conversion of acetaldehyde into acetate takes place in the cytosol and then acetate is imported to the plastids from the cytosol, or the conversion of acetaldehyde into acetate takes place in the plastids. However, it is very unlikely that acetaldehyde would freely cross the membrane due to its high reactivity and toxicity. Moreover, the expression of *ALDH3I1*, the only known plastid localized ALDH in *A. thaliana,* was not affected by the herbicides (Fig. 2). Consistent with our hypothesis, acetaldehyde has been shown to be metabolized by members of the family 7 ALDHs in rice [50]. To confirm that the conversion of the acetaldehyde derived from the induced PDC into acetate was catalysed by the ALDH7B4, an experiment was performed with a double *pdc1-pdc2* mutant. The use of this mutant offered the opportunity of assessing the physiological role of ALDH7B4 with an expected lower availability of acetaldehyde, as this mutant lacks the two predominant pyruvate-consuming *PDC* enzymes. Seedlings of *A. thaliana* (wild-type and *pdc1-pdc2* mutants) were grown under sterile conditions and were treated with imazamox or glyphosate for 5 days. The transcript levels of the *ALDH7B4* and *ACS* genes were measured by qPCR (Fig. 4). In wild-type seedlings, the expression of *ALDH7B4* was induced after the two ABIH treatments (Fig. 4), as it was

detected in the plants grown in the non-sterile hydroponic system (Fig. 2). Additionally, both herbicides provoked an increase in the expression of the *ACS*. The concomitant increases in the expression of *ALDH7B4* and *ACS* after ABIH treatment support the induction of the PDH bypass by the herbicides. However, the increase of the *ALDH7B4* expression upon imazamox treatment was absent in the *pdc1-pdc2* seedlings whereas the induction of *ACS* observed in wild-type plants after both ABIH applications was abolished in the *pdc1-pdc2* double mutants (Fig. 4). The simultaneous decrease in the expression of the ALDH7B4 and ACS enzymes in the *pdc1-pdc2* mutants supports the hypothesis that the PDH bypass is induced in ABIH-treated plants, as an alternative pathway for pyruvate consumption. The results evidence that ALDH7B4 is involved in the PDH bypass, at least in the case of imazamox, because no induction of the expression of this gene was observed in the *pdc1-pdc2* mutants after the application of this herbicide.

3.4   ALDH7B4 is not involved in the detoxification of aldehydes derived from lipid peroxidation

Lipid peroxidation can be initiated by ROS produced under impaired photosynthesis conditions, and a decrease in the net photosynthesis rate has been described in several plant species (e.g. pea, sugar beet and barley) after AHAS- or EPSPS-inhibiting herbicide application [8,39,51–53]. The ALDH7B4 is the only member belonging to the ALDH family 7 present in *A. thaliana*. This protein has been shown to play a role during different stress conditions such as, dehydration, salinity, heavy metals and abscisic acid treatment [29,43]. ALDH7B4 contributes to stress tolerance since *A. thaliana* mutants overexpressing the *ALDH7B4* gene showed improved stress tolerance, while *ALDH7B4* mutant plants presented higher stress sensitivity [29]. Ectopic expression of a soybean ALDH7 gene in transgenic

A. thaliana and tobacco plants increased abiotic stress tolerance, and the plants contained lower levels of malondialdehyde, confirming the protective role of ALDH7 [45]. Additionally, rice plants lacking *OsALDH7B6*, a gene necessary for seed maturation and maintenance of seed viability, showed higher stress sensitivity, and this increased sensitivity has been related to the accumulation of malondialdehyde and of the yellow pigment oryzamutaic acid A [50]. Recently, *A. thaliana* plants ectopically overexpressing the wheat stress-inducible *TraeALDH7B1-5A* gene showed improved tolerance to water deficit [54]. Based on these reports and on the conclusion that the *ALDH7B4* enzyme increases stress tolerance by detoxifying aldehydes [29,45], malondialdehyde content was measured as an indicator of lipid peroxidation, using the thiobarbituric acid-reactive-substances assay. *A. thaliana* T-DNA mutants defective for *ALDH7B4* (*aldh7b4*) and a transgenic line expressing *ALDH7B4* under the control of the CaMV 35S promoter (*35S::ALDH7B4*) were assayed alongside the wild-type. The validation of the mutant lines is presented in the Appendix C, Suppl. Figs. S1, S2. No phenotypical differences with respect to the wild-type plants were found in the *aldh7b4* and *35S::ALDH7B4* mutants in the experimental conditions used. Our results showed that the malondialdehyde content only increased in the leaves of glyphosate-treated wild-type plants, and no increase in the malondialdehyde content was found in the roots of imazamox- or glyphosate-treated plants (Fig. 5). The transgenic lines showed the same pattern as in the wild-type plants, indicating that the malondialdehyde content is not influenced by the lack or the overexpression of the *ALDH7B4* gene in ABIH-treated plants. Similar effects of ABIHs have been reported before: imazethapyr (another AHAS inhibitor) did not provoke oxidative stress [55] whereas glyphosate application was related with an

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oxidative stress in plants [56,57]. Therefore, *ALDH7B4* seems not to be related to the detoxification of aldehydes derived from lipid peroxidation because lipid peroxidation was not a common effect of the two types of herbicides and no differences were detected in the mutant lines.

3.5 Effect of ABIHs on fatty acid content and *de novo* fatty acid biosynthesis

Acetyl-CoA can follow different routes in the cellular compartments. In the plastids, acetyl-CoA is the substrate for *de novo* fatty acid biosynthesis. Although the acetyl-CoA pool generated by ACS from acetate seems to be redundant for fatty acid biosynthesis, ACS is hypothesized to play a specialized role in certain cells and tissues in which the PDH bypass is activated [18]. It was proposed that activation of the PDH bypass is needed to enable a high rate of lipid biosynthesis under nitrogen deprivation in the green alga *Chlorella desiccata* [58]. Different studies confirmed the incorporation of acetyl-CoA produced in the PDH bypass into fatty acids. Radiolabelled ethanol was shown to be incorporated into CO_2, and amino acids derived from intermediates of the TCA cycle and lipids in tobacco pollen [19]. In vegetative tissues, incorporation of ^{14}C-ethanol into fatty acids following the PDH bypass was described for *A. thaliana* [25].

To analyse whether the acetyl-CoA produced in the PDH bypass in plants exposed to herbicides is redirected to the biosynthesis of *de novo* fatty acids, the total fatty acid content and the expression pattern of different genes involved in the *de novo* fatty acid biosynthesis (*ACC2, KASIII, KASI, KASII*) (Fig. 6) were measured in leaves and roots of imazamox- or glyphosate-treated *A. thaliana* plants.

In general, the total fatty acid content was not significantly affected by the ABIH treatment, although some changes were observed. In leaves, a non-significant decrease in the total fatty acid content was detected after application of the two herbicides (Fig. 6.A), even though the expression of the genes that participate in *de novo* fatty acid biosynthesis (*ACC2*, *KASI*, *KASIII* and *KASII*) was not affected by the herbicides. The only changes detected were an increase in the expression of *KASIII* following imazamox application and a decrease in the expression of *KASI* after treatment with glyphosate (Fig. 6.B). No changes in the total fatty acid content was detected in the roots after ABIH application (Fig. 6.A), even though the expression of the *ACC2*, *KASIII, KASI* and *KASII* genes decreased as a consequence of ABIH application. The expression of these genes was more affected through glyphosate than imazamox (Fig. 6.B).

Although an increase in the acetyl-CoA pool would be expected by the concomitant activation of the PDH bypass upon ABIH treatment, it was not clear whether the extra acetyl-CoA is employed for *de novo* fatty acid biosynthesis. Nevertheless, our results suggest that acetyl-CoA levels are limiting for fatty acid synthesis probably due to an affected PDH complex, which is the main pathway of acetyl-CoA biosynthesis. Although the specific effects of ABIHs on the PDH complex have not been studied yet, a negative effect of the ABIHs can be hypothesized on this complex, because carbon metabolism is often affected by ABIHs [14,39]. A decrease in some TCA intermediates has been described in pea roots treated with AHAS inhibitors [16], suggesting a blockage at the level of the synthesis of acetyl-CoA, the main substrate for the TCA cycle.

Although the total fatty acid content was not modified by ABIHs, changes cannot be excluded in the relative amounts of individual fatty acids. The effect of

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herbicides on the percentage of the individual fatty acid content in leaves and the roots of *A. thaliana* wild-type plants was measured (Fig. 7). The results showed that the effect of imazamox in the leaves was minor. Imazamox provoked a slight increase in the percentage of 16:2, and a small decrease in the percentage of 18:0, 16:3 and 18:1 fatty acids. In contrast, the effect of glyphosate was stronger and it provoked an accumulation of 16:0, 16:1, 16:2, 18:1 and 18:2 fatty acids and a decrease in the 16:3 and 18:3 fatty acids (Fig. 7). In roots, the percentage of 14:0, 16:1, 16:3 and 18:1 fatty acids increased as a consequence of imazamox application and, in contrast, the percentage of 16:0 fatty acids species decreased. The percentage of 14:0, 16:0 and 18:1 species increased, the 18:3 content decreased in response to glyphosate treatment.

The fact that the percentage of 16:0 fatty acids increases and of 18:3 decreases as a consequence of glyphosate application suggests that the synthesis of 18:3 fatty acids from the precursor 16:0 is affected. Indeed, a decrease in the transcript levels of *KASII* in roots (a gene encoding the condensing enzyme involved in the elongation of 16:0-ACP to 18:0-ACP, i.e. the precursor for 18:3 acyl groups) was detected as a consequence of glyphosate application, while imazamox did not affect the expression of this gene (Fig. 6). Thus, it seems that, in the case of glyphosate-treated roots, the decrease in the 18:3 content is due to a reduced synthesis rate. Nevertheless, a specific degradation of 18:3 species could also contribute to the decrease in the 18:3 content. 18:3 is the substrate for jasmonic acid and other oxylipins. A connection between the 18:3 content and the expression of *ALDH7B4* has been proposed, since it has been observed that in the triple mutant *fad3-2fad7-2fad8*, which does not accumulate 18:3, the expression of the *ALDH7B4* was impaired [59,60]. A regulatory pathway for the expression of the gene *ALDH7B4*

upon wounding has been proposed recently whereby the oxylipins derived from linolenic acid (18:3) may activate the transcription factors for the activation of the expression of the *ALDH7B4* [30]. This kind of parallelism between wounding and glyphosate has been proposed before, as both stresses have been reported to induce phenylalanine ammonia lyase activity [61].

To further elucidate a potential role of the induction of ALDH7B4 in fatty acid accumulation, the total fatty acid content was analysed in the *aldh7b4* and the *35S::ALDH7B4* mutants (Appendix C, Suppl. Fig. S3-4). Interestingly, the effects of ABIHs on the fatty acid content in the leaves and the roots of *aldh7b4* and *35S::ALDH7B4* mutants were very similar compared to the effects found in the wild-type plants, with few exceptions. A significant decrease in the total fatty acid content was detected in the leaves of *ALDH7B4* overexpressing mutants after imazamox or glyphosate application and it was related to a decrease in the expression of the genes involved in the *de novo* fatty acid biosynthesis, especially the *ACC2* and *KASIII* genes.

The fatty acid compositions (Appendix C, Suppl. Fig. S5) were similar between the wild-type plants, the *aldh7b4* and *35S::ALDH7B4* mutants, so it seems that the ALDH7B4 does not affect the overall fatty acid composition.

3.6 ALDH7B4 alleviates the physiological effects of ABIHs on root carbon metabolism

We examined the role of the upregulation of ALDH7B4 in response to ABIHs, as the induction of this enzyme could be a plant defence mechanism promoting improved tolerance to the herbicide or it could be a consequence of the herbicide activity, thus contributing to the chemical toxicity. To this end, we evaluated ethanol fermentation, free amino acid content, soluble sugars and starch contents,

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as well as the pyruvate content in leaves and roots of the wild-type compared to *aldh7b4* and *35S::ALDH7B4* mutant *Arabidopsis* lines. A summary of the results (Fig. 8) shows where differences in the response to the herbicides have been detected between the wild-type and transgenic line. The PDC activity did not increase in the roots of the *35S::ALDH7B4* line after imazamox and glyphosate treatment compared to the wild-type plants. In leaves, the ADH activity significantly increased in the *35S::ALDH7B4* plants compared to the wild-type and the *aldh7b4* mutant (Suppl. Fig. S6). No difference was seen for the total free amino acid content between the wild-type and the mutants (Suppl Fig. S7). In contrast, the increase in the total soluble sugars and in starch content after imazamox treatment was very minor in the leaves of *35S::ALDH7B4* mutants compared to the increased found in the leaves of wild-type and *aldh7b4* plants. Similarly, the sucrose, glucose, and total soluble sugars contents increased less in a*ldh7b4* and *35S::ALDH7B4* leaves than in the wild-type after glyphosate treatment (Suppl Fig. S8). The untreated wild-type and two mutant plants showed similar values for almost all evaluated parameters, except for glucose and starch contents in the leaves (Suppl. Fig. S8), total soluble sugars (Suppl Fig. S9), and pyruvate and malate contents (Suppl Fig. S11) in the roots. As for the organic acids, notably pyruvate, the content of this metabolite did not increased after herbicide treatments in the leaves or roots of the two studied mutants compared to the wild-type plants (Suppl Figs. S10, S11). Overall, the results showed that the free amino acid profiles of the three genotypes were similar in contrast to the carbohydrate content. In leaves, only starch and pyruvate accumulation elicited by glyphosate were attenuated in the two transgenic lines. The effect of glyphosate was alleviated in the roots of the treated *aldh7b4* plants as no total soluble sugars, starch or pyruvate

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accumulation or PDC induction were detected (Fig. 8b, 8d, 8f, 8h). The accumulation of carbohydrate and pyruvate, and the induction of PDC detected in treated roots of wild-type plants were abolished in plants overexpressing the *ALDH7B4* gene, indicating an attenuation of the typical effects of the two herbicides on carbohydrate accumulation and PDC induction. It seems that the increased expression of ALDH7B4 has increased the capacity of the plant to metabolize the acetaldehyde and thus helped the plant to survive the stress provoked by herbicide application. The PDH bypass may therefore serve to metabolize the accumulated pyruvate after the inhibition of AHAS or EPSPS, and to detoxify the acetaldehyde produced during ethanol fermentation. Indeed, the *A. thaliana ALDH7B4* gene has been found to contribute to the survival of the plants to different stress conditions (such as, drought and salinity) [29]. Members of the ALDH7 family have also been observed to contribute to stress tolerance in soybean [45] and wheat [54]. Although the physiological disturbances on carbon metabolism of ABIHs on roots were slightly alleviated in *ALDH7B4* overexpressing plants, almost no changes were detected in *aldh7b4* mutants. Possibly other ALDH enzymes compensate for the loss of ALDH7B4 in the *aldh7b4* null mutant.

3.7 Overexpression of *ALDH7B4* alleviates the inhibition of root elongation provoked by ABIHs

Besides using physiological markers to evaluate the role of ALDH7B4 in plants treated with ABIHs, root elongation was used as an overall marker to compare the sensitivity among the genotypes (wild-type, *aldh7b4*, *35S::ALDH7B4*). The objective of this experiment was to elucidate the possible physiological implications of the ALDH7B4 induction in the toxicity of ABIHs, whether as a plant stress response

phenomenon (a detoxification mechanism after the application of herbicides, which the plants use to protect their viability) or a contribution to the chemical toxicity.

To monitor herbicide effects, *A. thaliana* seedlings were grown on vertical agar plates and root growth was measured. This parameter was used to check sensitivities to ABIHs in different genotypes. Root growth assays have been frequently used to check sensitivities to other abiotic stresses such as exposition to heavy metals [62–65], osmotic stress [66] or application of the herbicide 2,4-D [67].

Figure 9 shows the results of the experiment. All genotypes were severely affected by both herbicides, as shown in the representative photograph of each treatment (Fig. 9a) and root growth arrest was significant in all cases (Fig. 9b). Interestingly, the arrest of root elongation provoked by ABIHs was significantly alleviated in *35S::ALDH7B4* genotype compared to the wild-type plants: imazamox and glyphosate effects were alleviated from 37% to 55% of root growth inhibition and from 8% to 14.6% of root growth inhibition (Fig. 9c). The relative effect detected in the *aldh7b4* genotype was similar to the effect detected in the wild-type plants.

To confirm that the alleviation of the effect of herbicides on root growth observed in the *35S::ALDH7B4* plants was due to the overexpression of the *ALDH7B4* gene, an additional experiment was performed including using three independent *A. thaliana 35S::ALDH7B4* lines (*35S::ALDH7B4, 35S::ALDH7B4_2* and *35S::ALDH7B4_*3) and the wild-type (Suppl. Fig. S12). Although the absolute growth of the plants (Suppl. Fig. S12a,S12b) was higher in this experiment compared to the results shown in Figure 9, the relative effect of the herbicides on root elongation on wild-type was maintained (Suppl. Figs. S12c). Interestingly,

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alleviation of the effect of ABIHs in the three *35S:ALDH7B4* lines was significant the alleviation was from 38.3% to 46.3-68.1% in the case of imazamox and from 7.9% to 10.4-12.9 % in the case of glyphosate (Suppl. Fig. S12c). These results showed that the root growth inhibition provoked by imazamox or glyphosate was alleviated in *35S::ALDH7B4* evaluated lines comparing to the wild-type plants. Thus, this experiment confirmed that the slight increase in the tolerance to the herbicides observed in the *35S::ALDH7B4* lines is due to *ALDH7B4* overexpression.

These results evidence that the overexpression of *ALDH7B4* decreases the sensitivity to ABIHs, supporting that the induction of ALDH7B4 is a plant stress response helping in the tolerance to the herbicide. Further research would be necessary to confirm that the alleviation due to ALDH7B4 induction is directly related to its metabolic activity in the PDH bypass by the detoxification of toxic compounds. In this context, the comparison of the herbicide effects on mutant lines lacking other enzymes of the PDH bypass would be very helpful.

4   CONCLUSIONS

Even though modern agriculture depends on herbicides for a large-scale production, herbicide efficacy is now compromised by the rapid evolution of resistant weeds. Investigation into the processes that lead to lethality of current herbicides can help to elucidate why plants die as a consequence of herbicide treatment and would help in the discovery of new herbicides with new sites of action that will reduce the selection pressure for resistant weeds. Moreover, physiological studies would help in the discovery of possible biochemical markers for differential characterization of resistant and susceptible populations. Here new insights were provided in the processes following application of two widely used

herbicides with different targets. Our study has shown two effects that were not described before but are commonly provoked by ABIHs in treated plants: the induction of the PDH bypass and the upregulation of the *ALDH7B4*. We focused on the physiological role of ALDH7B4 in plants upon herbicide treatment and found out that the function of ALDH7B4, however, seemed not to be related to the detoxification of aldehydes derived from lipid peroxidation or to a higher rate of the *de novo* fatty acid synthesis, but rather to the carbon metabolism. No increase in the *ALDH7B4* gene expression was detected in imazamox-treated plants lacking the *PDC1* and *PDC2* genes, suggesting a role of ALDH7B4 in the PDH bypass. Plants overexpressing *ALDH7B4* were less sensitive to ABIHs supporting that ALDH7B4 induction is a plant defence mechanism that promotes better tolerance of the herbicide and plant viability. The ALDH7B4 would function in the PDH bypass to detoxify the compounds produced during the aerobic fermentation. Nevertheless, further experiments to unravel the whole role of the PDH bypass in plants upon herbicide treatment would be needed.

5 ACKNOWLEDGMENTS

APPENDIX A. SUPPLEMENTARY METHODS

APPENDIX B. SUPPLEMENTARY TABLES

APPENDIX C. SUPPLEMENTARY FIGURES

REFERENCES

[1] S.O. Duke, Overview of herbicide mechanisms of action, Environ. Health Perspect. 87 (1990) 263–271.

[2] T.B. Ray, Site of action of chlorsulfuron: inhibition of valine and isoleucine biosynthesis in plants, Plant Physiol. 75 (1984) 827–831.

[3] D.L. Shaner, M.L. Reider, Physiological responses of corn (*Zea mays*) to AC 243,997 in combination with valine, leucine, and isoleucine, Pestic. Biochem. Physiol. 25 (1986) 248–257.

[4] H.C. Steinrücken, N. Amrhein, The herbicide glyphosate is a potent inhibitor of 5-enolpyruvylshikimic acid-3-phosphate synthase, Biochem. Biophys. Res. Commun. 94 (1980) 1207–1212.

[5] V. Wittenbach, L. Abell, Inhibition of valine, leucine and isoleucine biosynthesis, in: B.K. Singh (Ed.), Plant Amino Acids Biochem. Biotechnol., Marcel Dekker, New York, 1999: pp. 385–416.

[6] K. Gruys, J. Sikorski, Inhibitors of tryptophan, phenylalanine, and tyrosine biosynthesis as herbicides, in: B.K. Singh (Ed.), Plant Amino Acids Biochem. Biotechnol., Marcel Dekker, New York, 1999: pp. 357–384.

[7] C.-Y. Wang, Effect of glyphosate on aromatic amino acid metabolism in purple nutsedge (*Cyperus rotundus*), Weed Technol. 15 (2001) 628–635.

[8] L. Orcaray, M. Igal, D. Marino, A. Zabalza, M. Royuela, The possible role of quinate in the mode of action of glyphosate and acetolactate synthase inhibitors, Pest Manag. Sci. 66 (2010) 262–269.

[9] A. Zulet, M. Gil-Monreal, J.G. Villamor, A. Zabalza, R.A.L. van der Hoorn, M. Royuela, Proteolytic pathways induced by herbicides that inhibit amino acid biosynthesis, PLoS One 8 (2013) e73847.

[10] A.S. Maroli, V.K. Nandula, F.E. Dayan, S.O. Duke, P. Gerard, N. Tharayil, Metabolic profiling and enzyme analyses indicate a potential role of antioxidant systems in complementing glyphosate resistance in an *Amaranthus palmeri* biotype, J. Agric. Food Chem. 63 (2015) 9199–9209.

[11] A.S. Maroli, V.K. Nandula, S.O. Duke, N. Tharayil, stable isotope resolved metabolomics reveals the role of anabolic and catabolic processes in glyphosate-induced amino acid accumulation in *Amaranthus palmeri* biotypes, J. Agric. Food Chem. 64 (2016) 7040–7048.

[12] S. Gaston, A. Zabalza, E.M. González, C. Arrese-Igor, P.M. Aparicio-Tejo, M. Royuela, Imazethapyr, an inhibitor of the branched-chain amino acid biosynthesis, induces aerobic fermentation in pea plants, Physiol. Plant. 114 (2002) 524–532.

[13] A. Zabalza, E.M. González, C. Arrese-Igor, M. Royuela, Fermentative metabolism is induced by inhibiting different enzymes of the branched-chain amino acid biosynthesis pathway in pea plants, J. Agric. Food Chem. 53 (2005) 7486–7493.

[14] L. Orcaray, A. Zulet, A. Zabalza, M. Royuela, Impairment of carbon

metabolism induced by the herbicide glyphosate, J. Plant Physiol. 169 (2012) 27–33.

[15] A. Zulet, M. Gil-Monreal, A. Zabalza, J.T. van Dongen, M. Royuela, Fermentation and alternative oxidase contribute to the action of amino acid biosynthesis-inhibiting herbicides, J. Plant Physiol. 175 (2015) 102–112.

[16] A. Zabalza, L. Orcaray, M. Igal, N. Schauer, A.R. Fernie, P. Geigenberger, J.T. van Dongen, M. Royuela, Unraveling the role of fermentation in the mode of action of acetolactate synthase inhibitors by metabolic profiling, J. Plant Physiol. 168 (2011) 1568–1575.

[17] J.T. Pronk, T.J. Wenzel, M.A.H. Luttik, C.C.M. Klaassen, W.A. Scheffers, H.Y. Steensma, J.P. van Dijken, Energetic aspects of glucose metabolism in a pyruvate-dehydrogenase-negative mutant of *Saccharomyces cerevisiae*, Microbiology 140 (1994) 601–610.

[18] J. Ke, R.H. Behal, S.L. Back, B.J. Nikolau, E.S. Wurtele, D.J. Oliver, The role of pyruvate dehydrogenase and acetyl-Coenzyme A synthetase in fatty acid synthesis in developing Arabidopsis seeds, Plant Physiol. 123 (2000) 497–508.

[19] S. Mellema, W. Eichenberger, A. Rawyler, M. Suter, M. Tadege, C. Kuhlemeier, The ethanolic fermentation pathway supports respiration and lipid biosynthesis in tobacco pollen, Plant J. 30 (2002) 329–336.

[20] N. Gass, T. Glagotskaia, S. Mellema, J. Stuurman, M. Barone, T. Mandel, C. Kuhlemeier, U. Roessner-Tunali, Pyruvate decarboxylase provides growing pollen tubes with a competitive advantage in Petunia, Plant Cell. 17 (2005) 2355–2368.

[21] M. Bucher, K.A. Brander, S. Sbicego, T. Mandel, C. Kuhlemeier, Aerobic fermentation in tobacco pollen, Plant Mol. Biol. 28 (1995) 739–750.

[22] M. Tadege, C. Kuhlemeier, Aerobic fermentation during tobacco pollen development, Plant Mol. Biol. 35 (1997) 343–354.

[23] M. Freeling, D.C. Bennett, Maize *Adh1*, Annu. Rev. Genet. 19 (1985) 297–323.

[24] M. Lin, D.J. Oliver, The Role of acetyl-coenzyme A synthetase in Arabidopsis, Plant Physiol. 147 (2008) 1822–1829.

[25] Y. Wei, M. Lin, D.J. Oliver, P.S. Schnable, The roles of aldehyde dehydrogenases (ALDHs) in the PDH bypass of Arabidopsis, BMC Biochem. 10 (2009) 7.

[26] H. Tsuji, N. Meguro, Y. Suzuki, N. Tsutsumi, A. Hirai, M. Nakazono, Induction of mitochondrial aldehyde dehydrogenase by submergence facilitates oxidation of acetaldehyde during re-aeration in rice, FEBS Lett. 546 (2003) 369–373.

[27] I. Sadiq, F. Fanucchi, E. Paparelli, E. Alpi, A. Bachi, A. Alpi, P. Perata, Proteomic identification of differentially expressed proteins in the anoxic rice coleoptile, J. Plant Physiol. 168 (2011) 2234–2243.

[28] J.M. Alonso, A.N. Stepanova, T.J. Leisse, C.J. Kim, H. Chen, P. Shinn, D.K. Stevenson, J. Zimmerman, P. Barajas, R. Cheuk, C. Gadrinab, C. Heller, A. Jeske, E. Koesema, C.C. Meyers, H. Parker, L. Prednis, Y. Ansari, N. Choy, H. Deen, M. Geralt, N. Hazari, E. Hom, M. Karnes, C. Mulholland, R. Ndubaku, I. Schmidt, P. Guzman, L. Aguilar-Henonin, M. Schmid, D. Weigel, D.E. Carter, T. Marchand, E. Risseeuw, D. Brogden, A. Zeko, W.L. Crosby, C.C. Berry, J.R. Ecker, Genome-wide insertional mutagenesis of *Arabidopsis thaliana*, Science 301 (2003) 653–657.

[29] S.O. Kotchoni, C. Kuhns, A. Ditzer, H.-H. Kirch, D. Bartels, Over-expression of different *aldehyde dehydrogenase* genes in *Arabidopsis thaliana* confers tolerance to abiotic stress and protects plants against lipid peroxidation and oxidative stress, Plant, Cell Environ. 29 (2006) 1033–1048.

[30] T.D. Missihoun, Q. Hou, D. Mertens, D. Bartels, Sequence and functional analyses of the *aldehyde dehydrogenase 7B4* gene promoter in *Arabidopsis thaliana* and selected Brassicaceae: regulation patterns in response to wounding and osmotic stress, Planta 239 (2014) 1281–1298.

[31] D. Loque, Gene expression of the NO3- transporter NRT1.1 and the nitrate reductase NIA1 is repressed in Arabidopsis roots by NO2-, the product of NO3- reduction, Plant Physiol. 132 (2003) 958–967.

[32] T.D. Missihoun, J. Schmitz, R. Klug, H.-H. Kirch, D. Bartels, Betaine aldehyde dehydrogenase genes from Arabidopsis with different sub-cellular localization affect stress responses, Planta 233 (2011) 369–382.

[33] M. Kosmacz, S. Parlanti, M. Schwarzländer, F. Kragler, F. Licausi, J.T. van Dongen, The stability and nuclear localization of the transcription factor RAP2.12 are dynamically regulated by oxygen concentration, Plant Cell Environ. 38 (2015) 1094–1103.

[34] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method, Methods 25 (2001) 402–408.

[35] R.A. Jefferson, T.A. Kavanagh, M.W. Bevan, GUS fusions: B-glucuronidase as a sensitive and versatile gene fusion marker, EMBO J. 6 (1987) 3901–3907.

[36] D.M. Hodges, J.M. DeLong, C.F. Forney, R.K. Prange, Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds, Planta 207 (1999) 604–611.

[37] A. Zulet, A. Zabalza, M. Royuela, Phytotoxic and metabolic effects of exogenous quinate on *Pisum sativum* L., J. Plant Growth Regul. 32 (2013) 779–788.

[38] A. Zinellu, S. Sotgia, A.M. Posadino, V. Pasciu, M.G. Perino, B. Tadolini, L. Deiana, C. Carru, Highly sensitive simultaneous detection of cultured cellular thiols by laser induced fluorescence-capillary electrophoresis, Electrophoresis 26 (2005) 1063–1070.

[39] A. Zabalza, L. Orcaray, S. Gaston, M. Royuela, Carbohydrate accumulation

in leaves of plants treated with the herbicide chlorsulfuron or imazethapyr is due to a decrease in sink strength, J. Agric. Food Chem. 52 (2004) 7601–7606.

[40] J. Browse, P.J. McCourt, C.R. Somerville, Fatty acid composition of leaf lipids determined after combined digestion and fatty acid methyl ester formation from fresh tissue, Anal. Biochem. 152 (1986) 141–145.

[41] C. Brocker, M. Vasiliou, S. Carpenter, C. Carpenter, Y. Zhang, X. Wang, S.O. Kotchoni, A.J. Wood, H.-H. Kirch, D. Kopečný, D.W. Nebert, V. Vasiliou, Aldehyde dehydrogenase (ALDH) superfamily in plants: gene nomenclature and comparative genomics, Planta 237 (2013) 189–210.

[42] N. Stiti, T.D. Missihoun, S.O. Kotchoni, H.-H. Kirch, D. Bartels, Aldehyde dehydrogenases in *Arabidopsis thaliana*: biochemical requirements, metabolic pathways, and functional analysis, Front. Plant Sci. 2 (2011) 65.

[43] H.-H. Kirch, S. Schlingensiepen, S. Kotchoni, R. Sunkar, D. Bartels, Detailed expression analysis of selected genes of the aldehyde dehydrogenase (ALDH) gene superfamily in *Arabidopsis thaliana*, Plant Mol. Biol. 57 (2005) 315–332.

[44] M. Nakazono, H. Tsuji, Y. Li, D. Saisho, S. Arimura, N. Tsutsumi, A. Hirai, Expression of a gene encoding mitochondrial aldehyde dehydrogenase in rice increases under submerged conditions, Plant Physiol. 124 (2000) 587–598.

[45] S.M. Rodrigues, M.O. Andrade, A.P.S. Gomes, F.M. Damatta, M.C. Baracat-Pereira, E.P.B. Fontes, Arabidopsis and tobacco plants ectopically expressing the soybean antiquitin-like *ALDH7* gene display enhanced tolerance to drought, salinity, and oxidative stress, J. Exp. Bot. 57 (2006) 1909–1918.

[46] T.D. Missihoun, H.-H. Kirch, D. Bartels, T-DNA insertion mutants reveal complex expression patterns of the *aldehyde dehydrogenase 3H1* locus in *Arabidopsis thaliana*, J. Exp. Bot. 63 (2012) 3887–3898.

[47] R.B. Nair, K.L. Bastress, M.O. Ruegger, J.W. Denault, C. Chapple, The *Arabidopsis thaliana reduced epidermal fluorescence1* gene encodes an aldehyde dehydrogenase involved in ferulic acid and sinapic acid biosynthesis, Plant Cell 16 (2004) 544–554.

[48] T.D. Missihoun, S.O. Kotchoni, D. Bartels, Active sites of reduced epidermal fluorescence1 (ref1) isoforms contain amino acid substitutions that are different between monocots and dicots, PLoS One 11 (2016) e0165867.

[49] L. Orcaray, M. Igal, A. Zabalza, M. Royuela, Role of exogenously supplied ferulic and p-coumaric acids in mimicking the mode of action of acetolactate synthase inhibiting herbicides, J. Agric. Food Chem. 59 (2011) 10162–10168.

[50] J.-H. Shin, S.R. Kim, G. An, Rice aldehyde dehydrogenase7 is needed for seed maturation and viability, Plant Physiol. 149 (2009) 905–915.

[51] D.R. Geiger, S.W. Kapitan, M.A. Tucci, Glyphosate inhibits photosynthesis and allocation of carbon to starch in sugar beet leaves, Plant Physiol. 82 (1986) 468–472.

[52] M. Royuela, A. Gonzalez, E.M. Gonzalez, C. Arrese-Igor, P.M. Aparicio-Tejo, C. Gonzalez-Murua, Physiological consequences of continuous, sublethal imazethapyr supply to pea plants, J. Plant Physiol. 157 (2000) 345–354.

[53] C.F. Olesen, N. Cedergreen, Glyphosate uncouples gas exchange and chlorophyll fluorescence, Pest Manag. Sci. 66 (2010) 536–542.

[54] J. Chen, B. Wei, G. Li, R. Fan, Y. Zhong, X. Wang, X. Zhang, *TraeALDH7B1-5A*, encoding aldehyde dehydrogenase 7 in wheat, confers improved drought tolerance in Arabidopsis, Planta 242 (2015) 137–151.

[55] A. Zabalza, S. Gaston, L.M. Sandalio, L.A. del Río, M. Royuela, Oxidative stress is not related to the mode of action of herbicides that inhibit acetolactate synthase, Environ. Exp. Bot. 59 (2007) 150–159.

[56] L.P.E. Miteva, S. V. Ivanov, V.S. Alexieva, Alterations in glutathione pool and some related enzymes in leaves and roots of pea plants treated with the herbicide glyphosate, Russ. J. Plant Physiol. 57 (2010) 131–136.

[57] N. Ahsan, D.-G. Lee, K.-W. Lee, I. Alam, S.-H. Lee, J.D. Bahk, B.-H. Lee, Glyphosate-induced oxidative stress in rice leaves revealed by proteomic approach, Plant Physiol. Biochem. 46 (2008) 1062–1070.

[58] O. Avidan, U. Pick, Acetyl-CoA synthetase is activated as part of the PDH-bypass in the oleaginous green alga *Chlorella desiccata*, J. Exp. Bot. 66 (2015) 7287–7298.

[59] S. Berger, Jasmonate-related mutants of Arabidopsis as tools for studying stress signaling, Planta 214 (2002) 497–504.

[60] K. Matsui, Green leaf volatiles: hydroperoxide lyase pathway of oxylipin metabolism, Curr. Opin. Plant Biol. 9 (2006) 274–280.

[61] K. Hahlbrock, D. Scheel, Physiology and molecular biology of phenylpropanoid metabolism, Annu. Rev. Plant Physiol. Plant Mol. Biol. 40 (1989) 347–369.

[62] W.-Y. Song, J. Park, D.G. Mendoza-Cozatl, M. Suter-Grotemeyer, D. Shim, S. Hortensteiner, M. Geisler, B. Weder, P.A. Rea, D. Rentsch, J.I. Schroeder, Y. Lee, E. Martinoia, Arsenic tolerance in Arabidopsis is mediated by two ABCC-type phytochelatin transporters, Proc. Natl. Acad. Sci. 107 (2010) 21187–21192.

[63] W.-Y. Song, K.S. Choi, D.Y. Kim, M. Geisler, J. Park, V. Vincenzetti, M. Schellenberg, S.H. Kim, Y.P. Lim, E.W. Noh, Y. Lee, E. Martinoia, Arabidopsis PCR2 is a zinc exporter involved in both zinc extrusion and long-distance zinc transport, Plant Cell 22 (2010) 2237–2252.

[64] Y. Tang, Y. Cao, J. Qiu, Z. Gao, Z. Ou, Y. Wang, Y. Zheng, Expression of a vacuole-localized BURP-domain protein from soybean (SALI3-2) enhances tolerance to cadmium and copper stresses, PLoS One 9 (2014) e98830.

[65] J.L. Freeman, D.E. Salt, The metal tolerance profile of *Thlaspi goesingense* is mimicked in *Arabidopsis thaliana* heterologously expressing serine acetyl-transferase, BMC Plant Biol. 7 (2007) 63.

[66] H. Fujii, P.E. Verslues, J.-K. Zhu, Arabidopsis decuple mutant reveals the importance of SnRK2 kinases in osmotic stress responses *in vivo*, Proc. Natl. Acad. Sci. 108 (2011) 1717–1722.

[67] M.C. Teixeira, P. Duque, I. Sá-Correia, Environmental genomics: mechanistic insights into toxicity of and resistance to the herbicide 2,4-D, Trends Biotechnol. 25 (2007) 363–370.

Figure captions

Fig. 1. Relative expression levels (transcripts) of the genes *ALDH2B4* and *ACS* in the leaves and the roots of wild-type *Arabidopsis thaliana* Col-0 plants untreated (control, C) or treated with imazamox (IMX) or glyphosate (GLP) for 3 days. Gels shown are representative examples of the six RT-PCRs carried out. Graphs represent the relative band intensity (*GENE OF INTEREST / ACTIN2*) measured by the Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA, USA). Values represent mean ± SE (n=6, biological replicates). ◯ and ● indicate significant difference between control and imazamox- or glyphosate-treated plants, respectively (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

Fig. 2. Relative expression levels (transcripts) of the genes *ALDH2B7, ALDH2C4, ALDH3F1, ALDH3H1, ALDH3I1* and *ALDH7B4* in the leaves and the roots of wild-type *Arabidopsis thaliana* Col-0 plants untreated (control, C) or treated with imazamox (IMX) or glyphosate (GLP) for 3 days. Gels shown are representative examples of the six RT-PCR assays. Graphs represent the relative band intensity (*GENE OF INTEREST / ACTIN2*) measured by the Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA, USA). Values represent mean ± SE (n=6, biological replicates). ◯ and ● indicate significant difference between control and imazamox- or glyphosate-treated plants, respectively (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

Fig. 3. Activity of the *ALDH7B4* promoter upon herbicide treatments. **A.** *In situ* detection of the activity of *ALDH7B4* promoter in leaves and roots of wild-type *Arabidopsis thaliana* Col-0 plants untreated (C) or treated with imazamox (IMX) or glyphosate (GLP) for 3 days. **B.** Measurement of the *ALDH7B4* promoter-driven GUS activity in leaves and roots of *ALDH7B4::GUS Arabidopsis thaliana* plants untreated (control) or treated with imazamox or glyphosate for 3 days. **C.** Relative transcript levels of the *ALDH7B4* and *GUS* genes in leaves and roots of *ALDH7B4::GUS*

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Arabidopsis thaliana plants untreated (control, C) or treated with imazamox (IMX) or glyphosate (GLP) for 3 days. Gels shown are representative examples of the six RT-PCR assays. The relative band intensity (*GENE OF INTEREST / ACTIN2*) was measured using the Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA, USA). Values represent the mean ± SE (n = 6, biological replicates). Significant variations are marked with ⬤ for differences between control and imazamox-treated plants, and with ⬤ for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

Fig. 4. Relative expression levels (transcripts) of the *ALDH7B4* and *ACS* genes in untreated (control) or imazamox- or glyphosate-treated (for five days) wild-type (wt) *Arabidopsis thaliana* Col-0 and *pdc1-pdc2* mutant seedlings grown under sterile conditions. Values represent mean ± SE (n=5, biological replicates). ⬤ and ⬤ indicate significant difference between control and imazamox- or glyphosate-treated plants, respectively (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

Fig. 5. As a marker of lipid peroxidation, the malondialdehyde (MDA) content was measured in the leaves and the roots of wild-type (wt) *Arabidopsis thaliana* Col-0, *aldh7b4* and *35S::ALDH7B4* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n=5, biological replicates). Significant variations are marked with ⬤ for differences between control and imazamox-treated plants, and with ⬤ for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

Fig. 6. Effect of herbicides on the fatty acid content and biosynthesis. **A.** Total fatty acid content in the leaves and the roots of wild-type (wt) *Arabidopsis thaliana* Col-0 plants, untreated (control) or treated with imazamox or glyphosate for 3 days.

Values represent mean ± SE (n=5, biological replicates). **B.** Expression pattern of *ACC2, KASIII, KASI* and *KASII* in the leaves and the roots of wild-type *Arabidopsis thaliana* Col-0 plants untreated (control, C) or treated with imazamox (IMX) or glyphosate (GLP) for 3 days. Gels shown are representative examples of the six RT-PCRs carried out. Graphs represent the relative band intensity (*GENE OF INTEREST / ACTIN2*) measured by the Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA, USA). Values represent mean ± SE (n=6, biological replicates). ○ and ● indicate significant difference between untreated and imazamox- or glyphosate-treated plants, respectively (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

Fig. 7. Individual fatty acid content (expressed as the percentage of the total fatty acids) in the leaves and the roots of wild-type *Arabidopsis thaliana* Col-0 plants, untreated (control) or treated with imazamox or glyphosate for 3 days. In inset figure a portion of main figure data is plotted in close-up. Values represent mean ± SE (n=5). ○ and ● indicate significant difference between control and imazamox- or glyphosate-treated plants, respectively (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

Fig. 8. Differences in soluble sugar, starch, pyruvate contents and pyruvate decarboxylase (PDC) activity between herbicide-treated wild-type (wt) *Arabidopsis thaliana* and the *aldh7b4* or *35S::ALDH7B4* mutants. The total soluble sugars content (a and b), the starch content (c and d), the pyruvate content (e and f) and the activity of PDC (g and h) in the leaves and the roots are presented. Values represent the mean ± SE (n = 5, biological replicates). ▽ indicates differences between the untreated (control) plants of the corresponding genotype and the untreated wt plants (*t*-Test, $p < 0.05$). Significant variations are marked with ○ for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

Fig. 9. As a marker of sensitivity to the herbicide, root length of seedlings was monitored in wild-type (wt) *Arabidopsis thaliana* Col-0, *aldh7b4* and *35S::ALDH7B4* mutants, untreated (control) or treated with imazamox or glyphosate **A.** Photographs shown are representative examples of the treatments. **B**. Root length was measured in seedlings of wild-type (wt) *Arabidopsis thaliana* Col-0, *aldh7b4 and 35S::ALDH7B4* mutant plants, untreated (control) or treated with imazamox or glyphosate. Significant variations are marked with ○ for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$). **C.** Comparison of the inhibitory effect of each herbicide in each genotype. * indicates differences between the plants of the corresponding genotype and wild-type plants (*t*-Test, $p < 0.05$). Values represent the mean ± SE (n = 48, biological replicates).

LEAVES

ROOTS

LEAVES

□ Control ▨ Imazamox ■ Glyphosate

A.

C   IMX   GLP

B.

LEAVES   ROOTS

□ Control   ▨ Imazamox   ■ Glyphosate

C.

LEAVES

□ Control   ▨ Imazamox   ■ Glyphosate

C   IMX   GLP   C   IMX   GLP

GUS   *ALDH7B4*

ROOTS

□ Control   ▨ Imazamox   ■ Glyphosate

C   IMX   GLP   C   IMX   GLP

GUS   *ALDH7B4*

LEAVES

Control · Imazamox · Glyphosate

MDA equivalents (nmol g^{-1} FW)

wt · aldh7b4 · 35S::ALDH7B4

ROOTS

Control · Imazamox · Glyphosate

MDA equivalents (nmol g^{-1} FW)

wt · aldh7b4 · 35S::ALDH7B4

A.

LEAVES | ROOTS
Control | Imazamox | Glyphosate

B.

Control | Imazamox | Glyphosate

LEAVES
C IMX GLP
ACC2

ROOTS
C IMX GLP
ACC2

Acetate

Pyruvate → Acetyl-CoA → Malonyl-CoA

CoA CO₂
NAD⁺ NADH

ACS

CO₂ ADP+Pi
ATP

PDH complex   ACC2

MCMT

KAS III   Malonyl-ACP

CO₂   CO₂

FAS

KAS I

16:0-ACP → 18:0-ACP → 18:1-ACP

KAS II   SAD

FAT   FAT

16:0   18:1

Prokaryotic
glycerolipid
pathway

LEAVES
C IMX GLP
KASIII

ROOTS
C IMX GLP
KASIII

LEAVES
C IMX GLP
KASI

ROOTS
C IMX GLP
KASI

LEAVES
C IMX GLP
KASII

ROOTS
C IMX GLP
KASII

LEAVES

wild-type

ROOTS

wild-type

LEAVES

ROOTS

□ Control ■ Imazamox (grey) ■ Glyphosate (black)

a) Total Soluble Sugars (mg g⁻¹ FW)

b) Total Soluble Sugars (mg g⁻¹ FW)

c) Starch (mg glucose g⁻¹ FW)

d) Starch (mg glucose g⁻¹ FW)

e) Pyruvate (mg g⁻¹ FW)

f) Pyruvate (mg g⁻¹ FW)

g) PDC activity (nmol NADH min⁻¹ mg protein⁻¹)

h) PDC activity (nmol NADH min⁻¹ mg protein⁻¹)

wt   aldh7b4   35S::ALDH7B4

A.

Control   Imazamox   Glyphosate

wt

aldh7b4

35S::ALDH7B4

B.

Control   Imazamox   Glyphosate

Root length (cm)

wt   aldh7b4   35S::ALDH7B4

C.

wt   37.2%
aldh7b4   43.8%   Imazamox
35S::ALDH7B4   55.8% *

wt   8.0%
aldh7b4   10.1%   Glyphosate
35S::ALDH7B4   14.6% *

Root growth
(% with respect to the untreated plants of the corresponding genotype)

Appendix A. Supplementary Methods.

Methods S1. Polymerase Chain Reactions for the screening of the *aldh7b4* mutants.

Genomic DNA was extracted from about 0.1 g of previously frozen leaves. The plant material was homogenized in 375 μL of 2× lysis buffer (0.6 M NaCl, 0.1 M Tris-HCl (pH 8.0), 40 mM EDTA (pH 8.0), 4% sarcosyl, and 1% SDS) and 375 μL of 2 M urea. One volume (750 μL) of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the mixture and mixed briefly. The homogenates were centrifuged at 20,000 g for 10 min at room temperature. To precipitate the DNA, 0.7 volume (525 μL) of cold isopropanol was added to the supernatants, and the tubes were centrifuged at 20,000 g for 15 min at 4 °C. The DNA pellet was washed twice with 1 mL of 70% ethanol, air-dried, and resuspended in 25 μL of resuspension buffer (10 mM Tris-HCl (pH 8.0), containing 30 μg mL^{-1} RNase A). Samples were briefly incubated at 37 °C for 5 min to degrade contaminating RNAs.

Extracted DNA was subsequently quantified and analysed using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). OD 260 and 280 nm were read for every sample. The DNA quality was also checked in a 1% agarose gel. Ten-fold diluted DNA samples were loaded onto a 1% agarose gel and run at 75 mA for 35 min. The gels were visualized in a Gel DocTM 2000 system (Bio-Rad Laboratories Inc., Hercules, CA, USA).

A PCR was performed on an Eppendorf Mastercycler ep Gradient S (Eppendorf, AG, Hamburg, Germany). Each reaction was performed for 2 μL of genomic DNA in a total volume of 10 μL containing: 1x PCR Buffer (Takara Bio Inc., Shiga, Japan), 2.5 mM MgCl2, 0.25 mM of each dNTPs, 0.6 μM specific forward primer, 0.25 μM specific reverse primer and 0.25 U Takara Taq polymerase (Takara Bio Inc., Shiga, Japan).

The specific primers used are detailed in Appendix B: Supplementary Table S4. The parameters of the PCR carried out for the screening of the *aldh7b4* mutants were as follows: 5 min 95°C; 34 cycles (30 s 95°C and 8 min 30 s 68°C); 10 min 68°C and pause at 4°C. Five-fold diluted PCR amplified products were loaded in a 1% agarose gel and run at 135 mA for 35 min. A 1Kb Gene Ruler was used as control for the length of the bands. The gels were visualized in a Bio-Rad Gel Documentation Gel DocTM 2000 System (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Appendix B. Supplementary tables.

Table S1. Primers used in the RT-PCRs.

GENE	FORWARD	REVERSE
ALDH2B4 (At3g48000)	cctcttcttcaaccaggggca	cttcgtctcgttcgccctct
ALDH2B7 (At1g23800)	aggtgacaggcttggttcca	ccaggcagggttcttgaggg
ALDH2C4 (At3g24503)	gggtgaaatttgcgtggcga	tgcgcatttgatcccttcct
ALDH3F1 (At4g36250)	gaagccatggaagctatgaaggagac	gtctctgtctctcactttcccccctt
ALDH3H1 (At1g44170)	cgtttcgccggactatatcttgacg	tcaaccaactaagtccatgtttga
ALDH3I1 (At4g34240)	ctactggatgtgcctgaagcatc	catgagtctttagagaacccaaag
ALDH7B4 (At1g54100)	gaagcaatagccaaagacacacgc	gatatctcgattatcgtaggctcc
ACS (At5g36880)	gtcaaaggttcatggcccgg	tcgtctttggcaaccctggt
ACC2 (At5g16390)	cgtctctctgctaagcccaa	ctggagtaggtggggatggt
KASI (At5g46290)	tcctccaaacccacttcgct	aacccacggatctgaccacc
KASII (At1g74960)	cgtgatgggagagggagctg	tccgtatcgcctgcacagtt
KASIII (At1g62640)	agctccaatggctcggtgtt	atccccacgttaaaccggct
GUS	cgtcctgtagaaaccccaacc	gatagtctgccagttcagttcg
ACTIN2 (At3g18780)	ggaatccacgagacaacctataac	gaaacattttctgtgaacgattcct

Table S2. The specific number of cycles used in the PCR programme for each gene.

GENE	LEAVES	ROOTS
ALDH2B4 (At3g48000)	25 cycles	27 cycles
ALDH2B7 (At1g23800)	30 cycles	30 cycles
ALDH2C4 (At3g24503)	25 cycles	25 cycles
ALDH3F1 (At4g36250)	30 cycles	30 cycles
ALDH3H1 (At1g44170)	27 cycles	25 cycles
ALDH3I1 (At4g34240)	30 cycles	30 cycles
ALDH7B4 (At1g54100)	25 cycles	26 cycles
ACS (At5g36880)	27 cycles	26 cycles
ACC2 (At5g16390)	27 cycles	28 cycles
KASI (At5g46290)	27 cycles	28 cycles
KASII (At1g74960)	27 cycles	32 cycles
KASIII (At1g62640)	27 cycles	28 cycles

Table S3. Primers used in the qPCRs

GENE	FORWARD	REVERSE
ALDH7B4 (At1g54100)	gagccgacaactcaatggatcg	tgccaagaggattccacatctcc
ACS (At5g36880)	aagagatgtgtggtggcaggatg	ccattccacctcacacgatgttgg
ACTIN2 (At3g18780)	tcttccgctctttctttccaagc	accattgtcacacacgattggttg

Table S4. Primers used for the screening of the *aldh7b4* mutants.

	FORWARD	REVERSE
ALDH7B4 (At1g54100)	catacgaggatgatcgtggcaatgt	
T-DNA		cagtcatagccgaatagcctctcca

Appendix C. Supplementary Figures.
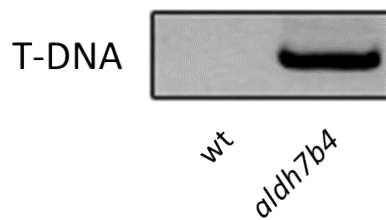
Fig. S1. Validation of the T-DNA mutant line defective for *ALDH7B4* (*aldh7b4*) used in this study. The presence of the T-DNA insertion in the *ALDH7B4* gene was checked (See Supplementary Methods S1). The wild-type (wt) line was used as a negative control.
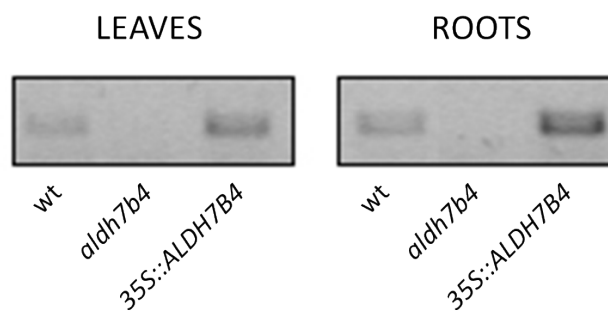

Fig. S2. Validation of the T-DNA mutant line defective for *ALDH7B4* (*aldh7b4*) and the transgenic line expressing *ALDH7B4* under the control of the CaMV 35S promoter (*35S::ALDH7B4*) used in this study. A comparative analysis of the accumulation of the *ALDH7B4* transcripts (mRNA levels) in the leaves and the roots of wild-type (wt) *Arabidopsis thaliana*, the T-DNA insertion mutants defective for *ALDH7B4* (*aldh7b4)* and the *ALDH7B4* overexpressing line (*35S::ALDH7B4*). See Materials and Methods section 2.3.
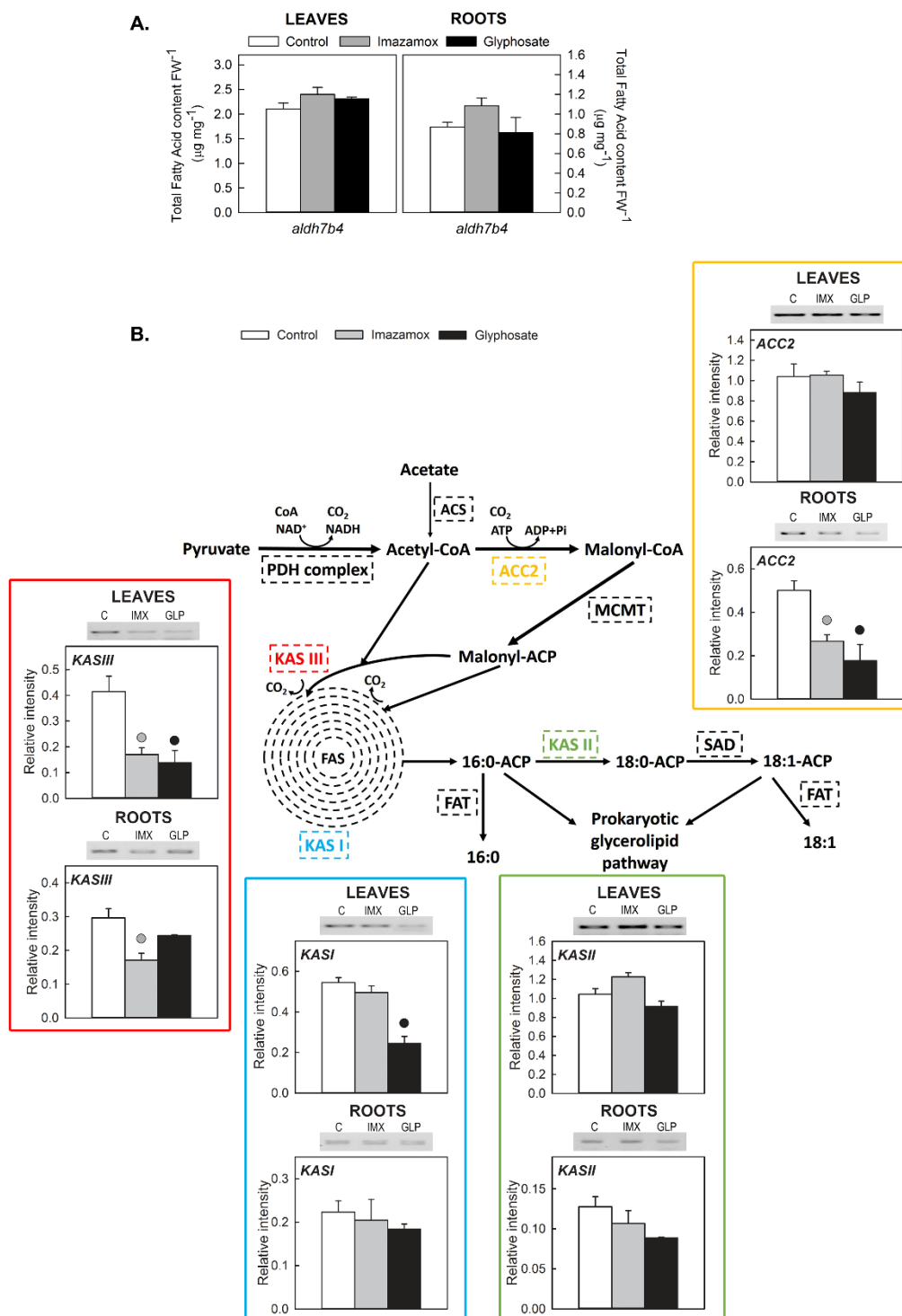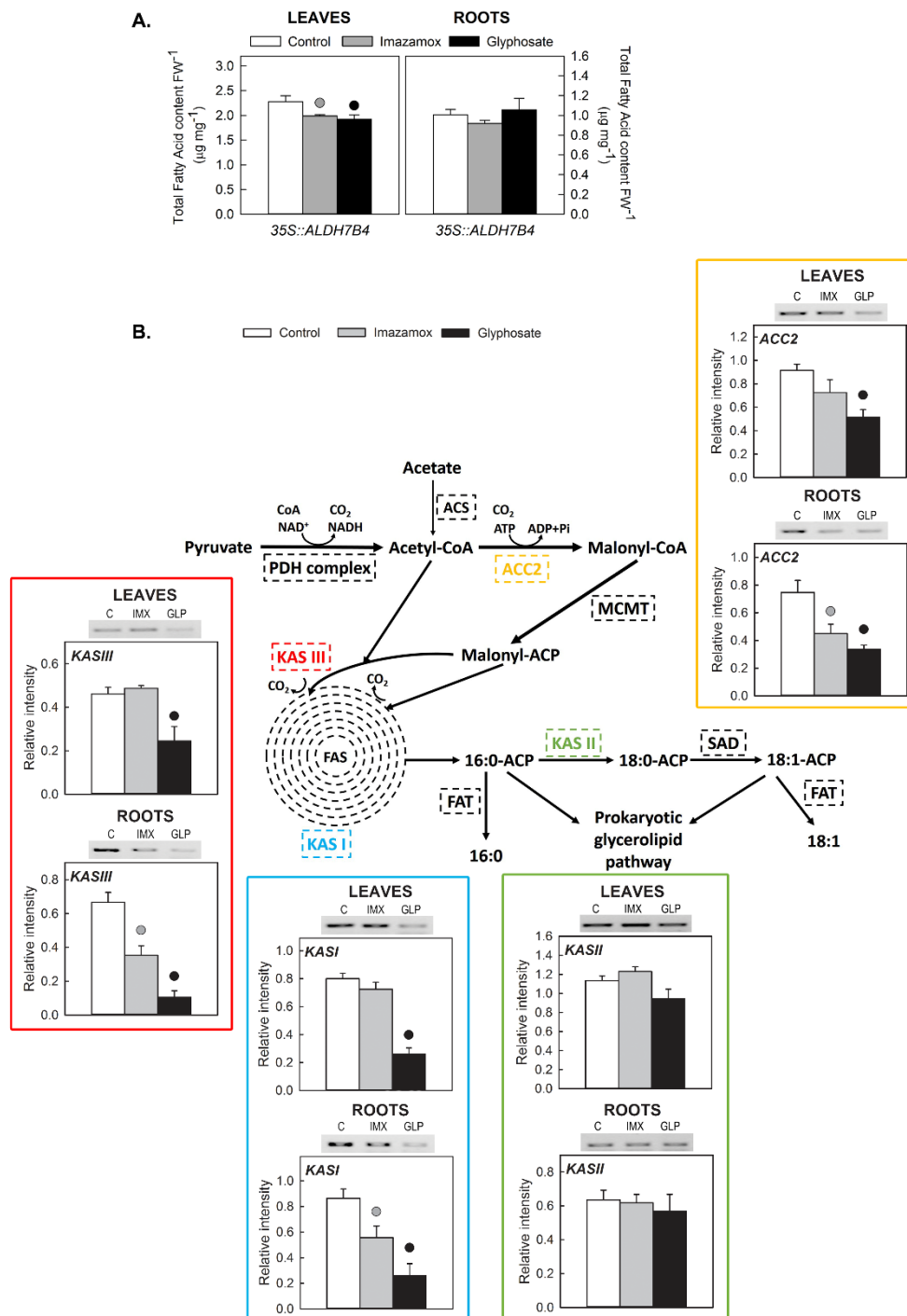
Fig. S3. Effect of herbicides on the fatty acid content and biosynthesis. **A.** Total fatty acid content in the leaves and the roots of mutant *Arabidopsis thaliana* plants defective for the *ALDH7B4* gene (*aldh7b4*), untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent mean ± SE (n=5, biological replicates). **B.** Expression pattern of *ACC2, KASIII, KASI* and *KASII* in the leaves and the roots of *aldh7b4* mutant plants untreated (C) or treated with imazamox (IMX) or glyphosate (GLP) for 3 days. Gels shown are representative examples of the six RT-PCRs carried out. Graphs represent the relative band intensity (*GENE OF INTEREST / ACTIN2*) measured by the Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA, USA). Values represent mean ± SE (n=6, biological replicates). ◎ and ● indicate significant difference between untreated and imazamox- or glyphosate-treated plants, respectively (ANOVA, HSD Tukey/T3 Dunnet; *p* < 0.05).

Fig. S4. Effect of herbicides on the fatty acid content and biosynthesis. **A.** Total fatty acid content in the leaves and the roots of transgenic *Arabidopsis thaliana* plants expressing *ALDH7B4* under the control of the CaMV 35S promoter (*35S::ALDH7B4*), untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent mean ± SE (n=5, biological replicates). **B.** Expression pattern of *ACC2, KASIII, KASI* and *KASII* in the leaves and the roots of *35S::ALDH7B4* mutant plants untreated (C) or treated with imazamox (IMX) or glyphosate (GLP) for 3 days. Gels shown are representative examples of the six RT-PCRs carried out. Graphs represent the relative band intensity (*GENE OF INTEREST / ACTIN2*) measured by the Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA, USA). Values represent mean ± SE (n=6, biological replicates). ⊙ and ● indicate significant difference between untreated and imazamox- or glyphosate-treated plants, respectively (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).
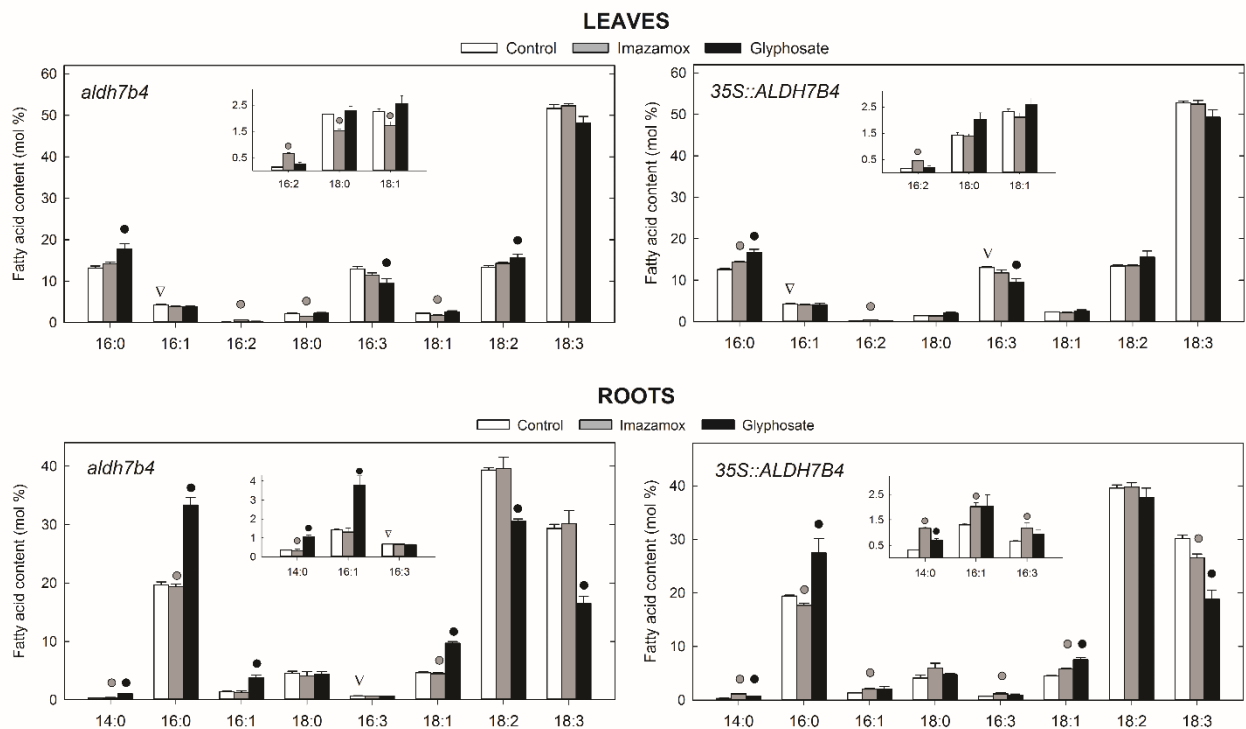
Fig. S5. Individual fatty acid content (expressed as the percentage of the total fatty acids) in the leaves and the roots of the T-DNA mutant line defective for *ALDH7B4* (*aldh7b4*) and the transgenic line expressing *ALDH7B4* under the control of the CaMV 35S promoter (*35S::ALDH7B4*), untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent mean ± SE (n=5, biological replicates). ∇ indicates differences between the untreated plants of the corresponding genotype and the untreated wild-type plants (*t*-Test, $p < 0.05$). Significant variations are marked with ⊙ for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).
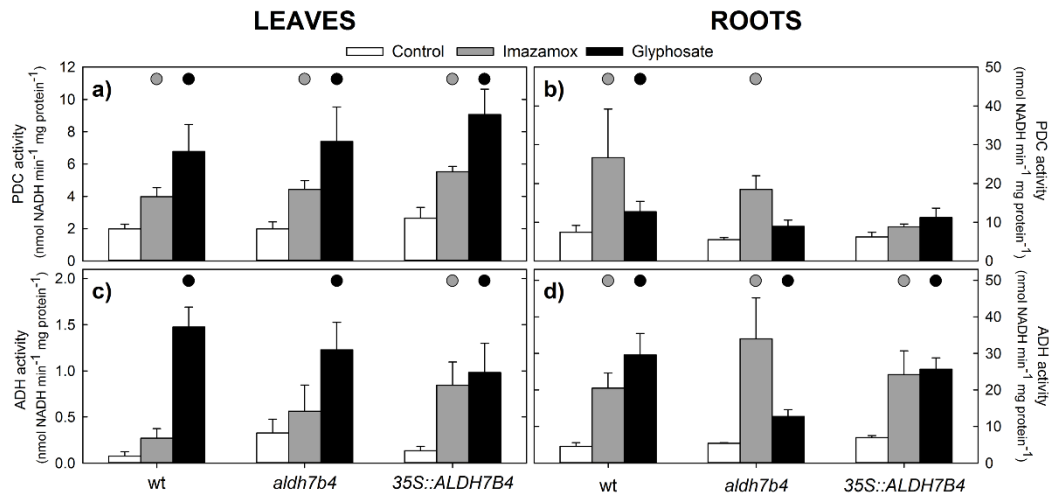
Fig. S6. The *in vitro* activities of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) in the leaves (a and c) and the roots (b and d) of wild-type (wt) *Arabidopsis thaliana* Col-0, *aldh7b4 and 35S::ALDH7B4* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5, biological replicates). Significant variations are marked with ⬤ for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).
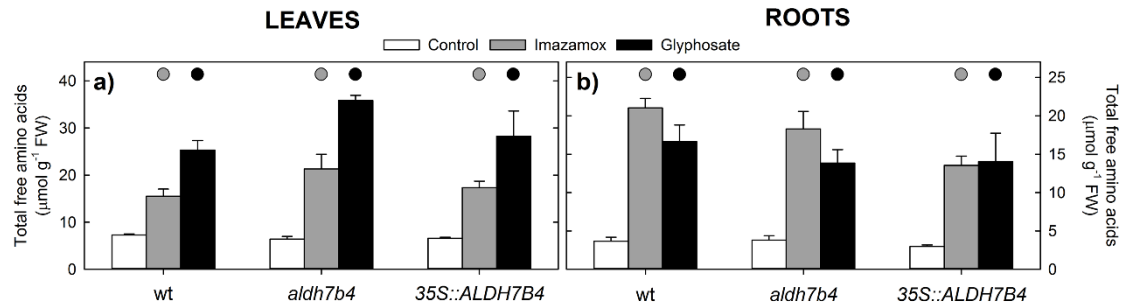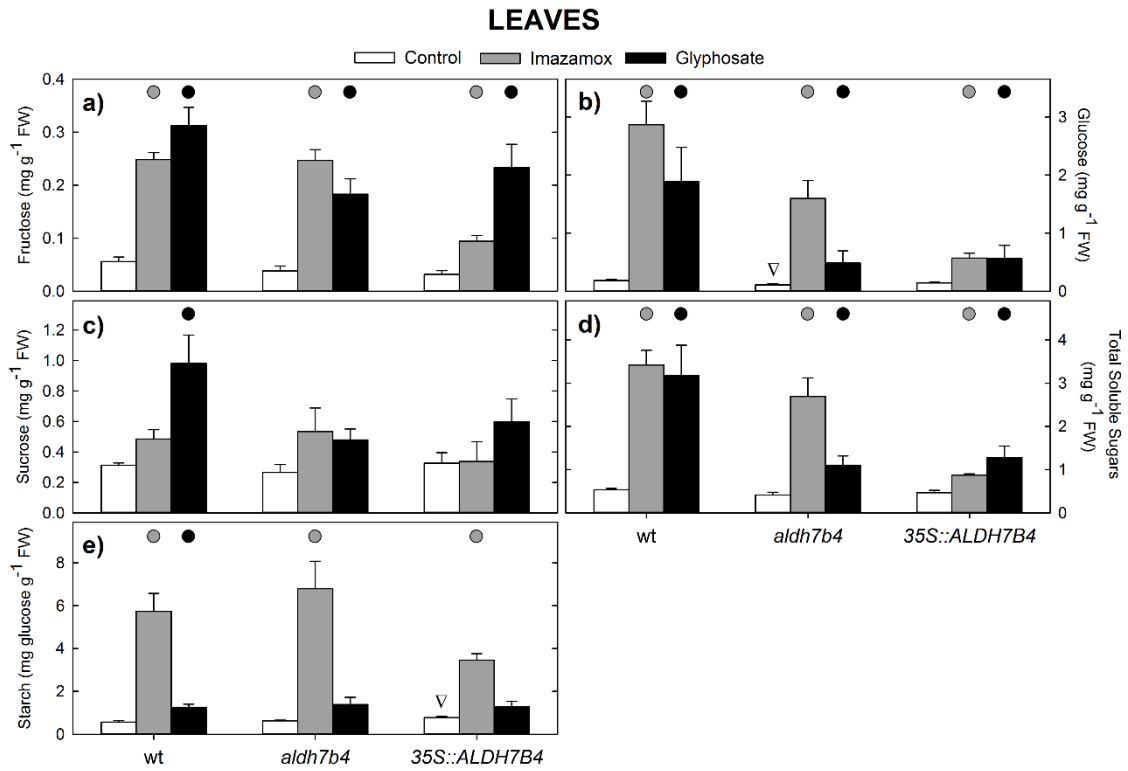
Fig. S7. Total free amino acid content in the leaves (a) and the roots (b) of wild-type (wt) *Arabidopsis thaliana* Col-0, *aldh7b4 and 35S::ALDH7B4* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5, biological replicates). Significant variations are marked with ⬤ for differences between control and imazamox-treated plants, and with ⬤ for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).
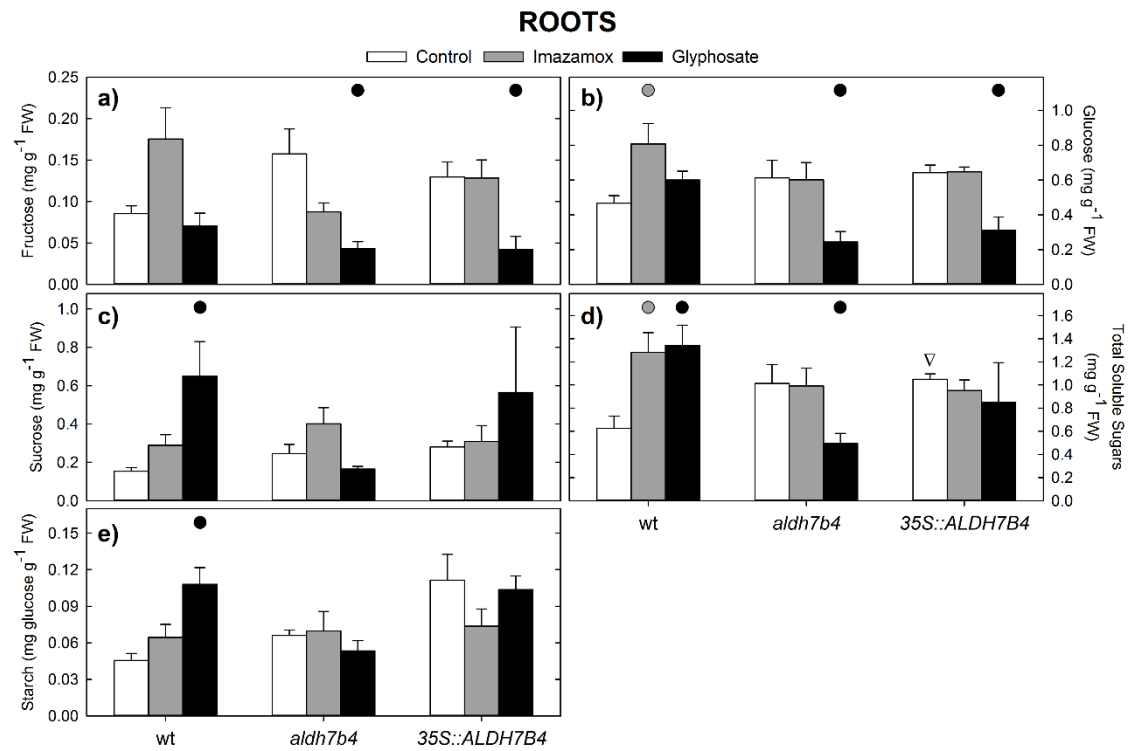
Fig. S8. The carbohydrate content in the leaves of wild-type (wt) *Arabidopsis thaliana* Col-0, *aldh7b4 and 35S::ALDH7B4* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5, biological replicates). ∇ indicates differences between the untreated plants of the corresponding genotype and the untreated wt plants (*t*-Test, $p < 0.05$). Significant variations are marked with ◐ for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

Fig. S9. The carbohydrate content in the roots of wild-type (wt) *Arabidopsis thaliana* Col-0, *aldh7b4 and 35S::ALDH7B4* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5, biological replicates). ∇ indicates differences between the untreated plants of the corresponding genotype and the untreated wt plants (*t*-Test, $p < 0.05$). Significant variations are marked with ⬤ for differences between control and imazamox-treated plants, and with ⬤ for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).
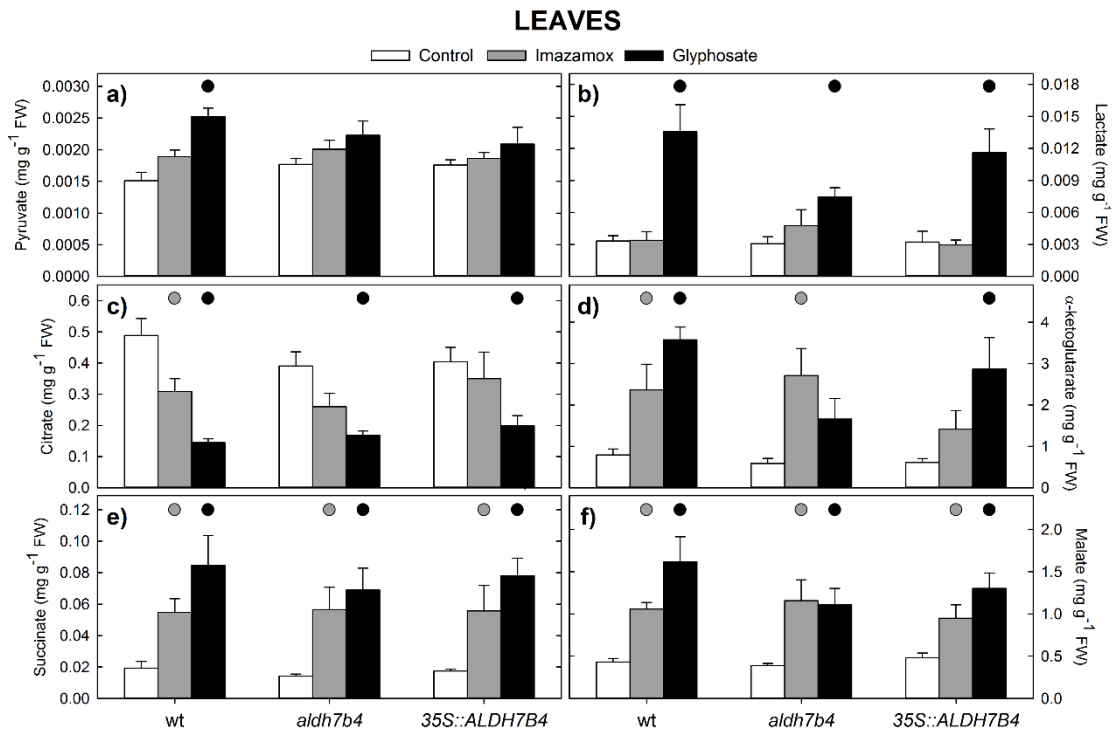
Fig. S10. The organic acid content in the leaves of wild-type (wt) *Arabidopsis thaliana* Col-0, *aldh7b4 and 35S::ALDH7B4* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5, biological replicates). Significant variations are marked with ⦾ for differences between control and imazamox-treated plants, and with ⬤ for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).
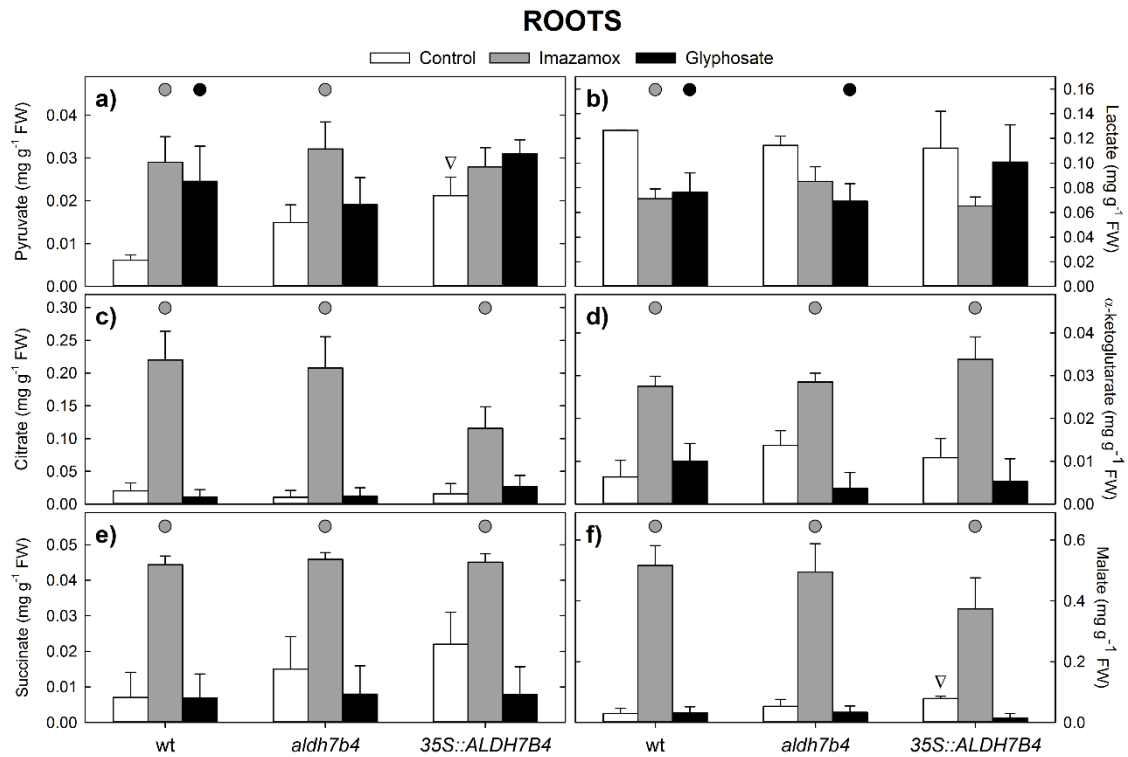
Fig. S11. The organic acid content in the roots of wild-type (wt) *Arabidopsis thaliana* Col-0, *aldh7b4 and 35S::ALDH7B4* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5, biological replicates). ∇ indicates differences between the untreated plants of the corresponding genotype and the untreated wt plants (*t*-Test, $p < 0.05$). Significant variations are marked with ◐ for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).
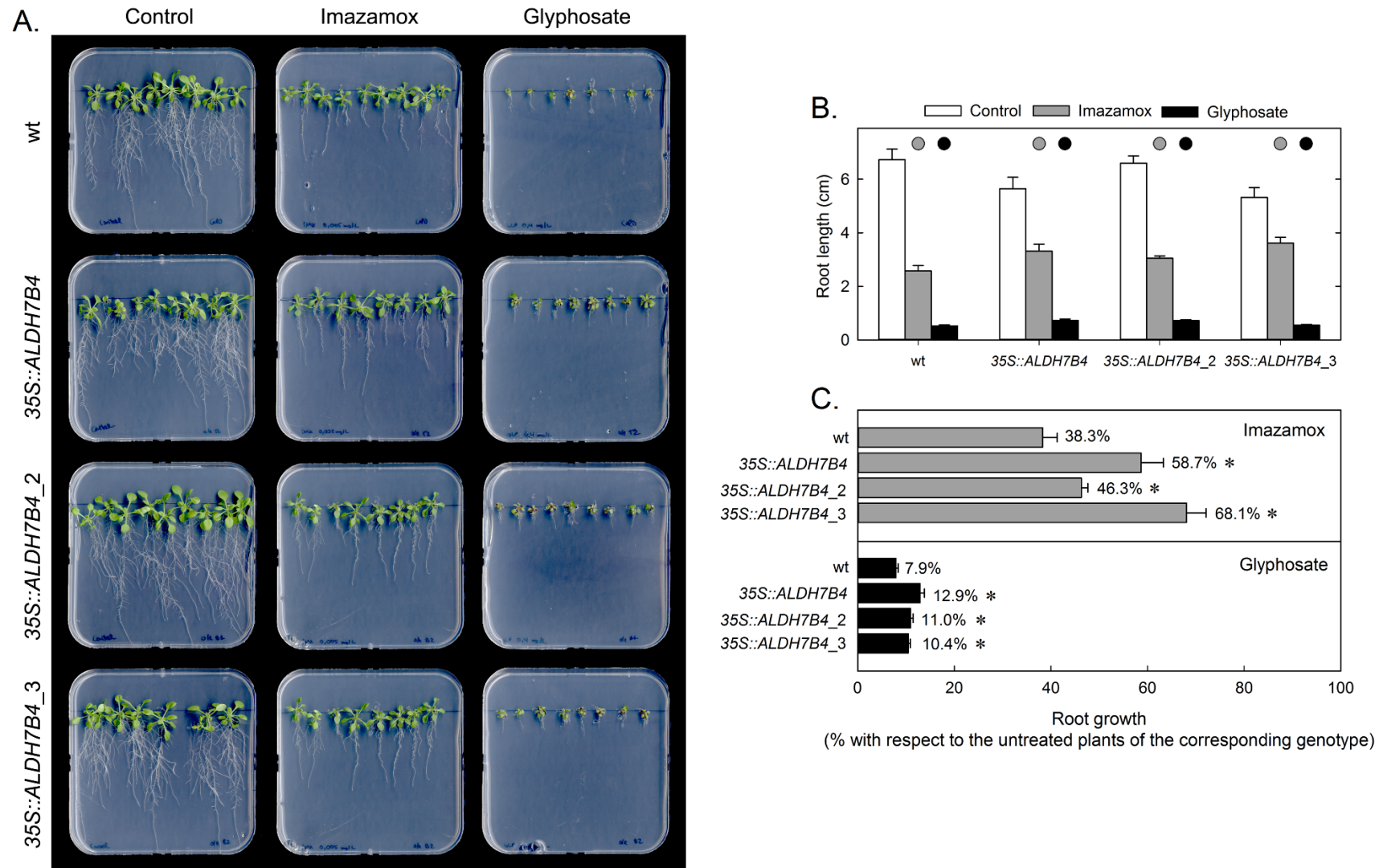
Fig. S12. As a marker of sensitivity to the herbicide, root length of seedlings was monitored in wild-type (wt) *Arabidopsis thaliana* Col-0, and three independent *ALDH7B4* overexpressing mutants, untreated (control) or treated with imazamox or glyphosate **A.** Photographs shown are representative examples of the treatments. **B**. Root length was measured in seedlings of wild-type (wt) *Arabidopsis thaliana* Col-0, and the three *35S::ALDH7B4* independent transgenic lines *(35S::ALDH7B4, 35S::ALDH7B4_2* and *35S::ALDH7B4_3)*, untreated (control) or treated with imazamox or glyphosate. In each genotype, significant variations are marked with ⬤ for differences between control and imazamox-treated plants, and with ⬤ for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$). **C.** Comparison of the inhibitory effect of each herbicide in each genotype. * indicates differences between the plants of the corresponding genotype and wild-type plants (*t*-Test, $p < 0.05$). Values represent the mean ± SE (n = 48, biological replicates).