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

Miriam Gil-Monreal[a], Ana Zabalza[a], Tagnon D. Missihoun[b,1], Peter Dörmann[b], Dorothea Bartels[b], Mercedes Royuela[a,*]

[a] Departamento Ciencias del Medio Natural, Universidad Pública de Navarra, Campus Arrosadía, E-31006 Pamplona, Spain
[b] Institute of Molecular Physiology and Biotechnology of Plants (IMBIO), University of Bonn, D-53115 Bonn, Germany

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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 *ALDH7B4* alleviates the effects of the herbicides, potentially through the detoxification of the metabolites produced in the pyruvate dehydrogenase bypass.

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 an herbicide that inhibits the activity of the enzyme acetohydroxyacid synthase (AHAS, EC 2.2.1.6) in the branched-chain amino acid biosynthesis pathway [1]. 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 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

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

* Corresponding author.
E-mail address: royuela@unavarra.es (M. Royuela).
[1] Present address: Department of Biology, Rutgers University, Camden, NJ, United States.

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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 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 25/20 °C 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 μM $CaCl_2$, 0.1 mM Na-Fe-EDTA, 50 μM KCl, 50 μM H_3BO_3, 5 μM $MnSO_4$, 1 μM $ZnSO_4$, 1 μM $CuSO_4$, and 0.1 μM $(NH_4)_6Mo_7O_{24}$. 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 GUS detection, measurement of GUS activity and for fatty acid determination.

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 Murashige and Skoog (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 half-strength MS medium (pH 5.7) (Sigma-Aldrich Co., St. Louis, MO, USA) enriched with 1% (w/v) sucrose and 0.7% (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 12/12 h day/night cycle. After 4 days in the growing chamber, when the root length was about 1 cm, seedlings were transferred to 12 × 12 cm plates containing half-strength MSmedium (pH 5.7)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 an 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)