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Fermentation and alternative oxidase contribute to the action of amino acid biosynthesis-inhibiting herbicides

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ABSTRACT

Acetolactate synthase inhibitors (ALS-inhibitors) and glyphosate (GLP) are two classes of herbicide that act by the specific inhibition of an enzyme in the biosynthetic pathway of branched-chain or aromatic amino acids, respectively. The physiological effects that are detected after application of these two classes of herbicides are not fully understood in relation to the primary biochemical target inhibition, although they have been well documented. Interestingly, the two herbicides' toxicity includes some common physiological effects suggesting that they kill the treated plants by a similar pattern despite targeting different enzymes. The induction of aerobic ethanol fermentation and alternative oxidase (AOX) are two examples of these common effects. The objective of this work was to gain further insight into the role of fermentation and AOX induction in the toxic consequences of ALS-inhibitors and GLP. For this, Arabidopsis T-DNA knockout mutants of alcohol dehydrogenase (ADH) 1 and AOX1a were used. The results found in wild-type indicate that both GLP and ALS-inhibitors reduce ATP production by inducing fermentation and alternative respiration. The main physiological effects in the process of herbicide activity upon treated plants were accumulation of carbohydrates and total free amino acids. The effects of the herbicides on these parameters were less pronounced in mutants compared to wild-type plants. The role of fermentation and AOX regarding pyruvate availability is also discussed.

Introduction

Herbicides that inhibit amino acid biosynthesis are useful tools in weed management and have been particularly successful as their biochemical target can only be synthesized by plants and microorganisms, thus reducing the probability of a toxic effect on mammals. These herbicides were developed in the early 1970s and are still of great agronomic and commercial importance. There are several types of herbicides whose target or primary site of action is the specific inhibition of enzymatic activity in biosynthetic pathways of amino acids. In this context, two important sites of herbicide action are acetolactate synthase (ALS, EC 4.1.3.18; also referred to as acetohydroxyacid synthase) and 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19), key enzymes in branched-chain and aromatic amino acid biosynthetic pathways, respectively (Duke, 1990).

ALS is the first enzyme in the biosynthesis of the three branched-chain amino acids valine, leucine and isoleucine. ALS-inhibitors include several classes of chemicals and have become one of the most widely used types of herbicides due to their wide-spectrum weed control activity, high crop selectivity, low application doses and low toxicity to mammals (Zhou et al., 2007). More than 50 commercial herbicide ingredients have ALS as their primary target. EPSPS is a key enzyme in the biosynthesis of aromatic amino acids (tyrosine, phenylalanine and tryptophan) and is inhibited by the herbicide glyphosate (GLP) (Steinrucken and Amrhein, 1980). GLP (*N*-(phosphonomethyl)glycine) is a wide-spectrum, non-selective post-emergence herbicide and is currently the most commonly used herbicide in the world, particularly since the introduction of transgenic GLP-resistant crops (Powles, 2008).

The herbicide action cannot be considered only in terms of interaction at a target site. The interaction can be viewed as the first step that is followed by a series of physiological consequences that result in death of the plant. A major limitation to understanding herbicide

Abbreviations: GABA, γ-aminobutyric acid; GLP, glyphosate; IMX, imazamox; ADH, alcohol dehydrogenase; ALS, acetolactate synthase; AOX, alternative oxidase; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; PDC, pyruvate decarboxylase.

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action is the lack of detailed knowledge of such consequences. Some possible causes of plant death as a consequence of the inhibition of biosynthetic pathways are the accumulation of toxic precursors or intermediates, end-product depletion, diversion of precursor into other products or deregulation of that pathway.

Although the specific biochemical targets of herbicides inhibiting branched or aromatic amino acid biosynthesis are well documented, the ultimate cause of plant death is not known. For both types of amino acid biosynthesis-inhibiting herbicides, the sequence of events that leads from herbicide application to plant death is still unclear. To understand the physiological effects that are involved in the lethal process after the herbicide treatment is important, as it can lead to their more rational use, and also because it can help in the development of new compounds with similar herbicidal activities but with different enzyme inhibition targets to avoid weed resistance. Several common physiological effects produced by ALS and EPSPS-inhibitors have been described in the literature, leading to the conclusion that their toxicities share certain characteristics. The common effects include growth arrest followed by the slow death of treated plants (Gruys and Sikorski, 1999; Wittenbach and Abell, 1999), a general increase in total free amino acid content (Shaner and Reider, 1986; Wang, 2001; Zulet et al., 2013a) and the accumulation of some secondary metabolites, such as quinate, a compound synthesized in a lateral branch of the shikimate pathway (Orcaray et al., 2010).

Moreover, ALS-inhibitors and GLP have been reported to impair carbon metabolism. They cause growth arrest in roots leading to an accumulation of unused carbohydrates, which in turn triggers a decrease in sink strength with a consequent accumulation of carbohydrates in the leaves (Zabalza et al., 2004; Orcaray et al., 2012). Another common effect on the roots of plants treated with ALS and EPSPS-inhibitors is the induction of fermentation and the alternative respiratory pathway; both of which are low-ATP producing pathways (Gaston et al., 2002, 2003; Zabalza et al., 2005; Orcaray et al., 2012), These metabolic impairments indicate that the effect of these herbicides on primary plant metabolism has broader physiological consequences than a lack of certain amino acids alone.

What induces the fermentation and alternative respiration in roots following ALS or EPSPS inhibition remains unknown. The induction of fermentation under aerobic conditions after ALS inhibition has been related to pyruvate availability, as pyruvate is a common substrate of both ALS and PDC (pyruvate decarboxylase, the first enzyme in ethanol fermentation, EC 4.1.1.1). Therefore ALS inhibition would mean an increase in the availability of pyruvate to be used by other enzymes, such as PDC. Moreover, pyruvate has been reported to be an allosteric activator in the activity of alternative oxidase (AOX) (Millar et al., 1993) (Vanlerberghe et al., 1995). However, induced fermentation after treatment with EPSPS-inhibiting GLP is not expected to be related to increased pyruvate availability as this herbicide does not inhibit any pyruvate-consuming enzymes, so what triggers fermentation after the application of this herbicide is unknown.

It is important to elucidate the role of induction of these two pathways (fermentation and alternative respiration) after the application of the two types of herbicides. Two, non-contradictory explanations can be considered. Firstly, the induction of these two pathways could be a plant defense mechanism that promotes better tolerance of the herbicide, and/or secondly, it could be a consequence of the herbicidal activity, thus contributing to the chemical's toxicity.

To achieve insights into the common series of consequences of amino acid biosynthesis-inhibiting herbicides we focused on the induction of fermentation and AOX. We evaluated whether the effect of the herbicides was increased or decreased by reduced fermentation activity or reduced AOX expression in mutant plants. For this purpose, fermentation, AOX, carbohydrate and amino acid content were compared in Arabidopsis lines (wild-type, *adh* and *aox1a* knockouts) treated with imazamox (IMX, an ALS-inhibitor) (Ohba et al., 1997) or GLP. Here, we show how Arabidopsis mutant plants treated with these amino acid biosynthesis-inhibiting herbicides respond differently than wild-type plants. Some of these parameters were less affected in the mutants compared to the wild-type lines, providing evidence that fermentation and AOX contribute to herbicide action.

Materials and methods

Plant material and treatment application

Arabidopsis thaliana ecotype Col-0, its alcohol dehydrogenase (*adh*, NASC N552699 (Banti et al., 2008)) and its alternative oxidase 1a (*aox1a*, SALK_084897 (Strodtkötter et al., 2009)) knockout mutants were grown in aerated hydroponic culture. Growth conditions were 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 afterwards to prevent flowering. The nutrient solution was slightly modified from (Loqué et al., 2003): 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 $(NH_4)_6Mo_7O_{24}$. When plants were approx. 8 weeks old imazamox (IMX) or glyphosate (GLP) were applied. The experiment was performed in duplicate. Throughout the course of the experiment the plants remained in the rosette vegetative phenological stage.

The two herbicides were applied to the nutrient solution as commercial formulations: 1.5 mg active ingredient L^{-1} (4.8 μM) IMX (Pulsar®40, BASF Española SA, Barcelona, Spain) and 50 mg active ingredient L^{-1} (220.8 μM) GLP (Roundup®Plus, MONSANTO Agricultura España SL, Madrid, Spain). Preliminary studies were conducted to find comparable doses of IMX and GLP.

Net carbon dioxide assimilation rates were measured from the youngest, fully expanded leaf in intact plants using a portable ADC-LCpro+ system equipped with an Arabidopsis chamber (ADC BioScientific Ltd., Herts, UK). Measurements were made in the growth chamber under growing conditions (400 ppm CO_2, 25 °C leaf temperature, 1.1 kPa VPD).

Root samples were taken at day 3 after application of the treatment. Plant material was immediately frozen in liquid nitrogen and stored at −80 °C. Some material was dried for 48 h at 75–80 °C in order to obtain the fresh weight/dry weight ratio.

ADH and PDC activities and protein immunoblot assay

Pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) activities were assayed in desalted extract as described by (Gaston et al., 2002). Total protein was isolated from roots as described by (Zabalza et al., 2005). Protein blots were performed according to standard techniques (Zabalza et al., 2009). PDC and ADH antibodies from Agrisera (Vännäs, Sweden) were used at dilutions of 1:10,000 and 1:3000, respectively. Goat Anti-Rabbit IgG HRP Agrisera (Vännäs, Sweden) was used as the secondary antibody at a dilution of 1:20,000 and bands were visualized using ECL Prime Western Blotting Reagents (GE Healthcare, Buckinghamshire, UK) and a Bio-Rad ChemiDoc Imaging system (ChemiDoc, Biorad, USA).

Real-time qRT-PCR analysis

Total RNA was extracted using the NucleoSpin RNA Plant kit (Macherey-Nagel, GMBH) according to the manufacturer's instructions. 13 μg of RNA (DNA free) was reverse transcribed into cDNA