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The pattern of shikimate pathway and phenylpropanoids after inhibition by glyphosate or quinate feeding in pea roots

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ABSTRACT

The shikimate pathway is a metabolic route for the biosynthesis of aromatic amino acids (AAAs) (*i.e.* phenylalanine, tyrosine, and tryptophan). A key enzyme of shikimate pathway (5-enolpyruvylshikimate-3-phosphate synthase, EPSPS) is the target of the widely used herbicide glyphosate. Quinate is a compound synthesized in plants through a side branch of the shikimate pathway. Glyphosate provokes quinate accumulation and exogenous quinate application to plants shows a potential role of quinate in the toxicity of the herbicide glyphosate. Based on this, we hypothesized that the role of quinate accumulation in the toxicity of the glyphosate would be mediated by a deregulation of the shikimate pathway. In this study the effect of the glyphosate and of the exogenous quinate was evaluated in roots of pea plants by analyzing the time course of a full metabolic map of several metabolites of shikimate and phenylpropanoid pathways. Glyphosate application induced an increase of the 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHPS, first enzyme of the shikimate pathway) protein and accumulation of metabolites upstream of the enzyme EPSPS. No common effects on the metabolites and regulation of shikimate pathway were detected between quinate and glyphosate treatments, supporting that the importance of quinate in the mode of action of glyphosate is not mediated by a common alteration of the regulation of the shikimate pathway. Contrary to glyphosate, the exogenous quinate supplied was probably incorporated into the main trunk from the branch pathway and accumulated in the final products, such as lignin, concomitant with a decrease in the amount of DAHPS protein.

1. Introduction

The biosynthesis of aromatic amino acids (AAAs) proceeds by way of the shikimate pathway: from phosphoenol pyruvate and erythrose-4-phosphate to chorismate (a common precursor of all the AAAs) and the specific terminal pathways that use chorismate as a substrate to synthesize phenylalanine (Phe)/tyrosine (Tyr) on one hand and tryptophan (Trp) on the other [1]. Plants synthesize a large number of specialized metabolites originating from the three AAAs (in particular, from Phe) and from several intermediates of the shikimate pathway leading to side branches, such as quinate or dehydroquinate. In land plants, very high fluxes are noted with estimates of the amount of fixed carbon passing through the pathway varying between 20 and 50% [2]. The high flow through the shikimate pathway and its complexity in plants are related to AAAs being used not only for protein building blocks but also for many secondary metabolites, such as phenylpropanoids, with diverse physiological roles [3,4]. Although the regulation of the synthesis of AAAs from chorismate has been studied extensively in plants [4], the regulation of flux through the shikimate pathway itself in plants is much less understood.

A key enzyme of the shikimate pathway: 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19), is the only known target of the widely used herbicide glyphosate [5]. Despite its widespread use in global crop production, the precise mechanisms by which glyphosate kills plants remain unclear. In general, after the target of an inhibitor has been affected, death is proposed to occur due to (1) accumulation or increased availability of the substrates of the inhibited enzymatic pathway, (2) lack of end products generated by the inhibited pathway and/or, (3) several side reactions triggered following de regulation of this pathway. Although it is not fully understood how plants actually die after inhibition of EPSPS, many plant physiological processes are affected by glyphosate and could also be associated with glyphosate toxicity [6]. Although glyphosate treatment kills plant slowly [7], carbon and nitrogen metabolism are affected as soon as 1 or 3 days after glyphosate treatment; *i.e.*, total free amino acid content increases, soluble protein content decreases [8] and carbohydrate content accumulates [9].

Several shikimate pathway intermediates are substrates for branch points leading to secondary metabolic processes. Among these, quinate can be formed in a single step reaction from the main shikimate

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pathway. Quinate is widely distributed and abundant in higher plants, particularly woody species, and may accumulate to high levels (up to 10% of the leaf dry weight) in some plants [10]. Interestingly, most of the plant which accumulates quinate do not contain a significant amount of shikimate, and the converse is also true. Both compounds exhibit a similar pattern of accumulation during an annual cycle, with a peak in spring and a decrease in summer. This biphasic pattern suggests a reserve role because these compounds could be first accumulated and then used as carbon sources for the synthesis of a wide range of phenolic compounds, such as lignins [11].

Although quinate is considered a reserve compound of the shikimate pathway; its physiological role has not been completely clarified. Indeed, quinate accumulation in leaves detected after glyphosate treatment [12] raised the question of whether quinate could mimic the action of the herbicide. The phytotoxic and metabolic effects of exogenous quinate were studied after application through the nutrient solution or leaf spraying [13]. Both treatments affected plant growth, and mimicked some physiological effects of glyphosate. This suggest that quinate plays an important role in the mode of action of glyphosate. It was hypothesized that quinate may not have a target by itself, but it would mimic the mode of action of glyphosate by entering the shikimate pathway and deregulating different processes related with this pathway. Nevertheless, that hypothesis remains to be proven.

In order to gain new insights in possible similarities of the toxicity of glyphosate and quinate, the current study compared the effect of the glyphosate and of the exogenous quinate on several metabolites and key enzymes of the shikimate and phenylpropanoid pathway. We hypothesized that the role of quinate accumulation in the toxicity of the herbicide would be mediated by a deregulation of the shikimate pathway. Therefore, we expected to observe a similar effect of both treatments on the shikimate pathway regulation.

2. Materials and methods

2.1. Plant material and treatment application

Seeds of pea *Pisum sativum* L. cv. Snap Sugar Boys (surface sterilized) were grown in vermiculite for 3 days at 26 °C in darkness prior to transfer to hydroponic tanks filled with nutrient solution and placed in a growth chamber [14]. Nutrient solution (2.7 l $tank^{-1}$) was aerated continuously (700 ml $tank^{-1}$ min^{-1}) and renewed every 3 days. At 12 days of age, the plants were divided into two groups: to one glyphosate was added and quinate to the other

Plants were grown under treatment for 3 weeks. In the group used to perform the glyphosate treatment, root samples were taken at 0, 1, 3, 7, 10, and 15 days after the onset of the treatment. The quinate study only included harvest at day 7 or 15. At harvest, samples were immediately frozen in liquid nitrogen and stored at −80 °C for analytical determinations. Some material was dried for 48 h at 75–80 °C to obtain the fresh weight/dry weight ratio.

2.1.1. Glyphosate treatment

In preliminary studies, a concentration of glyphosate to pea roots in a hydroponic system was chosen to produce a slow, robust and synchronous death of this crop plant within 20 days [9]. In the group of plants used to assess the effect of glyphosate, half of the plants were treated with glyphosate applied to the nutrient solution as a commercial formulation (isopropylamine salt, Glyfos, BayerGarden, Valencia, Spain) at a final concentration of 0.23 mM (53 mg active ingredient l^{-1}) and the other half was not treated and served as the control treatment.

2.1.2. Exogenous quinate application

Preliminary studies were also conducted to determine a quinate dose (Fluka Chem Co, WI, USA), which produced similar effects on growth arrest and lethality to those described following glyphosate treatment to the nutrient solution [12]. Based on these results, 4 mM quinate was added to the nutrient solution (768 mg l^{-1}) was finally selected as a comparable concentration for this study [13]. In this group, half of the plants were treated with 4 mM quinate and the other half was not treated.

2.2. Analytical determinations

The extraction of amino acids was performed in HCl. After protein precipitation, amino acid concentrations were measured in the supernatant using capillary electrophoresis equipped with a laser-induced fluorescence detector, as previously described [12]. Quinate content in pea roots was extracted in trichloroacetic acid (TCA) and measured using ion chromatography as previously described [12]. The determination of the content of shikimate, hydroxybenzoic acids (4-hydroxybenzoic, gentisic, vanillic and syringic acids) and hydroxycinnamic acids (*p*-coumaric, caffeic, ferulic and sinapic acids) was performed by high-performance liquid chromatography (HPLC) as described previously [12]. For anthocyanin determination, fresh tissues were homogenized in acidic methanol (0.1 N HCl) and the homogenates were centrifuged for 20 min at 20000*g*. Anthocyanin content was quantitated by measuring the difference in absorbence at 525 nm and 585 nm [15]. The lignin content was determined in pea roots according to previously described methods [16]. The PAL activity was determined in pea roots, using methods previously described [16]. DAHPS immunoblots were produced according to standard techniques. DAHPS immunoblotting was performed as described previously [16].

2.3. Statistical analysis

An unpaired Student's *t*-test was used to determine the significance between each treatment and each control plant (untreated plants) on the given day of glyphosate or quinate treatment. Each mean value was calculated using samples from single plants as biological replicates. Significant differences ($p < 0.05$) are discussed.

3. Results and discussion

The application of glyphosate or quinate to the nutrient solution of pea plants caused a rapid inhibition of plant growth [12]. Growth arrest persisted during the experimental time course, and plant death took approximately 20 days. Measurements of the plant status were performed in the roots and only for 15 days from the onset of the treatment.

3.1. Glyphosate treatment

Fig. 1 summarizes the content of specific metabolites at different time points in pea roots after glyphosate treatment. As early as 3 days from the onset of treatment accumulation of shikimate and protocatechuic were detected. Gallic acid accumulation was detected from day 7 and all accumulations increased over time. Shikimate is typically the main compound that accumulates after glyphosate, and a rapid accumulation of gallic and protocatechuic acid is also a characteristic effect of the herbicide [17–19]. This large increase in EPSP precursors was also detected in the leaves of pea plants when glyphosate was applied through the nutrient solution [12].

An important difference in the pattern of EPSP precursors was detected between leaves and roots of pea plants. No accumulation of quinate, a compound synthesized in a lateral branch of the shikimate pathway, was detected in roots although glyphosate was absorbed through this organ, while glyphosate induced an increase in the content of quinate in leaves of the same plants [12] and in other species [20,21]. Indeed, the organ where quinate is accumulated after glyphosate treatment seems to be species-dependent, as recently quinate accumulation has been reported in roots of *Lolium perenne* treated with glyphosate [22]. Two explanations can be proposed to the lack of quinate accumulation in roots of glyphosate-treated pea plants. On one hand, quinate

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