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Research article

Biochemical and molecular changes in response to aluminium-stress in highbush blueberry (*Vaccinium corymbosum* L.)

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ABSTRACT

Aluminium (Al) stress is an important factor limiting crop yields in acid soils. Despite this, very little is known about the mechanisms of resistance to this stress in woody plants. To understand the mechanisms of Al-toxicity and response in blueberries, we compared the impact of Al-stress in Al-resistant and Al-sensitive genotypes using *Vaccinium corymbosum* L. (Ericaceae) as a plant model. We investigated the effect of Al-stress on the physiological performance, oxidative metabolism and expression of genes that encode antioxidant enzymes in two *V. corymbosum* cultivars maintained hydroponically with $AlCl_3$ (0 and 100 μM). Microscopic analyses of Al-treated root tips suggested a higher degree of Al-induced morphological injury in Bluegold (sensitive genotype) compared to Brigitta (resistant genotype). Furthermore, the results indicated that Brigitta had a greater ability to control oxidative stress under Al-toxicity, as reflected by enhancement of several antioxidative and physiological properties (radical scavenging activity: RSA, superoxide dismutase: SOD and catalase: CAT; maximum quantum yield: Fv/Fm, effective quantum yield: ΦPSII, electron transport rate: ETR and non-photochemical quenching: NPQ). Finally, we analyzed the expression of genes homologous to *GST* and *ALDH*, which were identified in a global expression analysis. In the resistant genotype, the expression of these genes in response to Al-stress was greater in leaves than in roots.

1. Introduction

Among environmental stresses, Al-toxicity constitutes a major limiting factor in acid soils [1]. Al-stress in plants affects the functionality of the photosynthetic apparatus, by reducing the photochemical efficiency of PSII and restricting electron transport. Furthermore, Al-stress induces changes in the oxidative metabolism caused by an increase in the concentration of reactive oxygen species (ROS) [2,3] and alters the expression of antioxidant genes [4,5]. Plants differ in their ability to withstand Al-stress. However, the resistance mechanisms to this stress are not well understood in many species [4]. Mechanisms of Al-resistance have usually been classified as either exclusion mechanisms (avoidance), or internal tolerance, also called protoplastic tolerance [6,7]. According to Barceló and Poschenrieder [8], the exclusion of Al seems to be the most important resistance mechanism in cultivated and wild species that grow in acid soils with high concentrations of phytotoxic (Al^{3+}). Furthermore, plants have developed diverse mechanisms of antioxidant defense against Al-toxicity. These mechanisms involve antioxidant enzymes, such as superoxide dismutase (SOD, E.C.1.15.1.1), peroxidase (POD, EC 1.11.1.7), catalase (CAT, E.C.1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11), glutathione reductase (GR, EC 1.6.4.2), gluthatione *S*-transferase (GST, E.C. 2.5.1.18) and aldehyde dehydrogenases (ALDH, EC 1.2.1.3), as well as non-enzymatic compounds of low molecular weight, such as

Abbreviations: Al, aluminium; Fv/Fm, maximum quantum yield; ϕPSII, effective quantum yield; ETR, electron transport rate; NPQ, non-photochemical quenching; PPF, photosynthetic photon flux; cDNA-AFLP, complementary DNA-amplified fragment length polymorphism; TDF, transcript-derived fragment; qRT-PCR, real-time quantitative PCR.

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ascorbic acid (AsA), reduced glutathione (GSH) and phenol-like compounds such as tocopherols (α-tocopherols), flavonoids, carotenoids (β-carotene) and uric acid [9,10]. These responses engage diverse resistance mechanisms that can act at the cell and tissue levels or at the whole plant level [11]. Aluminium phytotoxicity has also been shown to cause lipid peroxidation of biomembranes [12,13]. Recent studies indicated that Al-induced ROS increased lipid peroxidation in *Glycine max* [14]. This toxicity may be associated with an augmentation in the activities of antioxidant enzymes such as SOD, CAT and GST [15,16]. Furthermore, differential expression of oxidative stress genes, which encode for SOD, GST and CAT, have been reported under Al-toxicity [17]. A strong connection between Al-stress and oxidative stress in plants has been highlighted by Darko et al. [18].

Our aim is to investigate the impact of Al-stress on the physiological performance, oxidative metabolism and expression of genes encoding antioxidant enzymes in two blueberry cultivars. For this purpose, we used an Al-resistant (Brigitta) and an Al-sensitive (Bluegold) genotype [19] and evaluated histological alterations in root tips, photochemical efficiency of PSII, total antioxidant activity as well as specific activities of key enzymes of antioxidant metabolism. Additionally, we evaluated the effect of Al-stress on the expression of two differentially expressed putative antioxidant genes which had been identified previously in a cDNA-AFLP analyses in blueberry (Inostroza-Blancheteau et al. [30]).

2. Materials and methods

2.1. Plant material and growth conditions

Two genotypes of *Vaccinium corymbosum* L. were used in this study, Brigitta (Al-resistant) and Bluegold (Al-sensitive) [19]. One year old plants of uniform size growing in a substrate of oat shell:sawdust:pine needles at a 1:1:1 proportion were selected. Forty plants were conditioned in plastic boxes filled with 18 L of Hoagland's nutrient solution for 1 week [20]. The pH of the solution was adjusted to 4.8 with 0.4 M HCl or NaOH and aerated with an aquarium pump. The Hoagland solution was changed every 2 days. Thereafter, ten plants for treatment were exposed to 0.5 mM $CaCl_2$ containing 0 and 100 μM $AlCl_3$ for 0, 2, 6, 24 and 48 h. The experiment was performed in springtime in a greenhouse with a mean temperature of 25/20 °C (day/night) and a photoperiod of 14/10 h (light/dark), with 70% relative humidity.

The photosynthetic photon flux (*PPF*) density at the plant canopy was 120 μmol m^{-2} s^{-1}. At 0, 2, 6, 24 and 48 h *in vivo* chlorophyll fluorescence parameters of PSII were determined and root and leaf samples were collected for biochemical analysis. For RNA extraction, the root apices (~3 cm) were cut and washed with distilled water, quickly frozen in liquid nitrogen and stored at −80 °C.

2.2. Histological study

The changes in root-tip ultrastructure were observed by optical microscopy. Two millimeters were removed from root tips, washed with deionized water to remove Al from the root surfaces, rapidly fixed with 3% glutaraldehyde and postfixed with 1% osmium tetroxide (both in 0.1 mol L^{-1} Na-cacodylate buffer, pH 7.2). Samples were then dehydrated in an acetone series (between 50 and 100% v/v) and embedded in Epon 812. Sections (1–2 μm) of root tips were stained with toluidine blue and finally examined by microscopy (Nikon Eclipse 80i), according to [21] with some modifications.

2.3. Chlorophyll fluorescence parameters of PSII

Leaf chlorophyll fluorescence from the second to fourth node of shoots was used to determine *in vivo* the photochemical efficiency of PSII using a portable pulse-amplitude modulated fluorimeter (FMS 2; Hansatech Instruments, King's Lynn, UK), as described by Reyes-Díaz et al. [19]. Minimal fluorescence (Fo) was determined in dark-adapted (20 min) leaves by applying a weak modulated light (0.4 μmol m^{-2} s^{-1}), and maximal fluorescence (Fm) was induced by a short pulse (0.8 s) of saturating light (9000 μmol m^{-2} s^{-1}). After 10 s, actinic light (120 μmol m^{-2} s^{-1}) was turned on to obtain fluorescence parameters during steady-state photosynthesis. Saturating pulses were applied after steady-state photosynthesis has been reached to determine maximal fluorescence in light-adapted leaves (Fm′) and steady-state fluorescence (Fs). Finally, the actinic light was turned off and a 5 s far-red (FR) pulse was immediately applied to obtain minimal fluorescence in light-adapted leaves (Fo′). Maximum quantum yield (Fv/Fm), effective quantum yield (ΦPSII), electron transport rate (ETR), and non-photochemical quenching (NPQ) were estimated as described by Genty et al. [22,23]. $Fv/Fm = (Fm - Fo)/Fm$ is the indicator of the maximum quantum yield; $\Phi PSII = (Fm' - Fs)/Fm'$ is the indicator of the effective quantum yield of PSII; $ETR = PPF \times 0.5 \times \Phi PSII \times 0.84$ [22]; $NPQ = (Fm - Fm')/Fm'$ [23].

2.4. Antioxidant enzymes activities

For extraction of antioxidant enzymes, samples of fresh leaves and roots were frozen in liquid nitrogen and stored at −80 °C until use. The extraction procedure was performed as described by Mora et al. [24]. SOD activity was determined through the photochemical inhibition of nitroblue tetrazolium (NBT) as described by Giannopolitis and Ries [25] with minor modifications [24]. CAT activity was measured by monitoring the conversion of H_2O_2 to H_2O and O_2 [26] and enzyme activity was estimated by H_2O_2 consumption for 60 s at 240 nm. All enzymatic activity values were standardized by the total protein content, as determined by Bradford [27].

2.5. Radical scavenging activity (RSA)

The RSA of roots and leaves was tested in methanolic extracts by the free 2.2 diphenyl-1-picrylhydrazyl (DPPH) method [28] with minor modifications. The absorbance was measured at 515 nm in a spectrophotometer (UNICO® 2800 UV/VIS, Spain) using Trolox as the standard.

2.6. Isolation of total RNA and cDNA synthesis

Total RNA was isolated from 500 mg of root apices of blueberry plants with the method described for woody plants by Gambiano et al. [29] with some modifications. To eliminate any contamination with genomic DNA, the total RNA was treated with RNase-free DNase I (Invitrogen) and the concentrations were measured spectrophotometrically using a NanoDrop instrument (Thermo Scientific NanoDrop TM 1000 Technologies, Wilmington, USA). The purity of the total RNA was assessed using the A260/280 and A260/230 ratios given by NanoDrop. Quality was also inspected visually following gel electrophoresis of denatured RNAs and finally adjusted to a concentration of 1.5 μg μL^{-1} for synthesis of the first strand cDNA using 200 units of Superscript II reverse transcriptase (Invitrogen) and 1 μl biotinylated oligo-dT_{25} (700 ng mL^{-1}).

2.7. Real-time quantitative PCR (qRT-PCR) analysis

In a previous study, we identified two transcript-derived fragments (TDFs) homologous to antioxidant genes [30]. *VCAL21* is homologous to *gluthatione S-transferase* (*GST*) and *VCAL68* is homologous to *aldehyde dehydrogenase* (*ALDH*). The sequences of these TDFs have been deposited in GenBank (HO054812, *VCAL21*;