UNIVERSIDAD DE LA FRONTERA
Facultad de Ingeniería, Ciencias y Administración
Programa de Doctorado en Ciencias de Recursos Naturales

IDENTIFICATION AND CHARACTERIZATION OF GENE IN RESPONSE TO ALUMINUM STRESS (Al^{3+}) IN HIGHBUSH BLUEBERRY (*Vaccinium corymbosum* L.)

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CLAUDIO ANDRES INOSTROZA-BLANCHETEAU
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Esta tesis es presentada bajo la supervisión del Director de Tesis, Dr. Patricio Arce-Johnson, del Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas de la Pontificia Universidad Católica de Chile para su aprobación por la comisión.

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A mi familia, esposa e hijos por su comprensión y compañía en los momentos difíciles…

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Abstract

Soil acidity is a serious limitation for production of many plant species because it allows the solubility of phytotoxic elements such as aluminum (Al). An excess of Al affects the physiology of plants and thus, their growth, development and geographical distribution. Some species and genotypes of plants, however, have evolved mechanisms to resist Al-toxicity. The difference in these mechanisms is the site of detoxification for this element in the plant. This may occur by a symplastic rute in the inclusion mechanism, as in the case of Al-tolerance, or by the apoplast in the exclusion mechanism. The main mechanism is exudation of an external organic acid into the rhizosphere of the roots and subsequent chelation of Al, through an organic anion Al binding (exclusion). On the other hand, the inclusion mechanism acts after the Al has entered inside the cell, where it is chelated by organic acids in the cytoplasm and organelles and then compartmentalized into the vacuole. The toxic effects are manifested earlier, such as inhibition of elongation and cell division of roots, which prevents water absorption and transport of nutrients. These are essential for cellular metabolism, resulting finally in a reduction of yield and plant quality. At the cellular level, Al disrupts the plasma membrane and organelles, leading to enzyme disorders, as well as damage to nuclear DNA level. It has been reported that when Al is joined to the plasma membrane, an increase of oxidative stress is induced by reactive oxygen species (ROS). However, plants possess sophisticated antioxidant defense mechanisms for protection against ROS. These mechanisms use antioxidant enzymes and low molecular weight molecules, such as phenolic compounds. The mechanisms of gene expression in

response to oxidative stress and to Al-resistance not well studied in root tissues of blueberry. In recent years the production of blueberries (*Vaccinium corymbosum* L.) has experienced a significant development in our country, especially in the south, where soils are characterized by high acidity and therefore high concentrations of phytotoxic aluminum (Al^{3+}). Previous studies on Al-toxicity in blueberry genotypes grown in Chile are carried out by our investigation group resulting in identification of genotypes that are resistant and sensitive to this element. Nevertheless, genes involved in molecular mechanism of response to Al toxicity in woody species such as blueberry and the variation between genotypes are unknown. The aim of the present thesis is to investigate the molecular mechanisms of Al stress in blueberry genotypes, throughout cDNA-amplified fragment length polymorphism (cDNA-AFLP) analysis, identifying genes regulated by Al in roots in two contrasting Al-resistant or sensitive genotypes of highbush blueberry (Brigitta, Al-resistant and Bluegold, Al-sensitive). Eight months old plants were grown in hydroponic Hoagland's solution for 7 days to homogenize plant nutrition. Subsequently they were transferred to a solution of calcium chloride ($CaCl_2$) supplemented with aluminum chloride ($AlCl_3$), with concentration of 0 or 100 µM Al. Samples of root tips were taken at different times of treatment with Al (0, 2, 6, 24 and 48 hours). Seventy transcript derived fragments (TDFs) were identified in response to Al-stress by using BLASTX. Thirty one of these transcripts showed significant homology with genes of known function. 12 TDFs were homologous uncharacterized genes and 27 had no significant homology with known proteins. The expression patterns of several of these genes with known function in other species were confirmed by real-time RT-PCR.

12 genes were related to cell metabolism, and 9 genes were associated to stress response and related to transcription and transport. Genes involved in signal transduction, photosynthesis and energy were also identified, suggesting that multiple processes are involved in resistance to Al as reported previously in other species. We performed molecular characterization of a potential relevant gene for Al-resistance, TDF VCAL19 homologous to calmodulin. Now named *VcCaM1* for *Vaccinium corymbosum Calmodulina 1*, this gene can be participed in the Al-resistance in blueberry. The Al stress-response genes in this work could be involved in the resistance to Al in woody plants. Additionally, we conducted an evaluation of the antioxidant system in leaves and roots evaluating: antioxidant activity (AA), superoxide dismutase (SOD), catalase (CAT) and have correlated this background to leaf-level physiological parameters such as effective quantum yield PSII (ΦPSII), potential photochemical efficiency of PSII (Fv / Fm), the non-photochemical quenching (NPQ) and electron transport rate (ETR).

Resumen

La acidez de los suelos es una seria limitante para la producción de muchas especies de plantas, ya que permite la solubilidad de elementos fitotóxicos como el aluminio (Al). El exceso de Al afecta la fisiología de las plantas y por ende su crecimiento, desarrollo y distribución geográfica. Algunas especies y genotipos de plantas, han desarrollado mecanismos para resistir la toxicidad por Al. La diferencia entre estos mecanismos radica en el sitio de detoxificación de este elemento que puede ser, vía simplasto o inclusión en el caso de la tolerancia a Al y la vía apoplasto en el mecanismo de exclusión. El principal mecanismo es el externo a través de la exudación de ácidos orgánicos en la rizosfera de la raíz y posterior quelación del Al, a través de la unión de un anión orgánico y Al (exclusión). Por otro lado, el mecanismo de inclusión, actúa una vez que el Al ha ingresado a la célula el cual es quelado por ácidos orgánicos en el citoplasma y posteriormente compartimentalizado a organelos como la vacuola. Los efectos tóxicos del Al se manifiestan tempranamente en una inhibición de la elongación y división celular de las raíces, lo que impide la absorción de agua y transporte de nutrientes, esencial para el metabolismo celular, traduciéndose finalmente en una disminución del rendimiento y calidad de la planta. A nivel celular, el Al altera el funcionamiento de la membrana plasmática y de los organelos, provocando desórdenes enzimáticos, así como también daño a nivel del ADN nuclear. Se ha reportado que al unirse el Al a la membrana plasmática, aumenta el estrés oxidativo producido por las especies de oxigeno reactivas (EROS). Sin embargo, las plantas poseen sofisticados mecanismos de defensa antioxidante para la protección contra EROS. Estos

mecanismos utilizan enzimas antioxidantes y moléculas de bajo peso molecular, como también compuestos fenólicos. Los mecanismos de expresión de genes en respuesta a estrés oxidativo y tolerancia a Al han sido poco estudiados en tejidos radicales de arándano. En estos últimos años la producción de arándano (*Vaccinium corymbosum* L.) ha experimento un importante desarrollo en nuestro país, especialmente en la zona Sur, cuyos suelos se caracterizan por una alta acidez y por ende altas concentraciones de aluminio fitotóxico (Al^{3+}). Estudios previos sobre toxicidad por Al en genotipos de arándano cultivados en Chile, realizados por nuestro grupo de trabajo, han permitido identificar genotipos resistentes y sensibles a este elemento. Actualmente, se desconocen los genes involucrados en los mecanismos moleculares de la respuesta frente a la toxicidad por Al en especies leñosas como el arándano, y la variación entre genotipos es desconocida. Es por ello, que para investigar los mecanismos moleculares del estrés por Al en arándano, realizamos un análisis de "cDNA-amplified fragment length polymorphism" (cDNA-AFLP) para identificar genes regulados por Al^{3+} en raíces de dos genotipos contrastantes a Al de arándano alto (Brigitta, resistente-Al y Bluegold, sensible-Al). Plantas de 8 meses fueron crecidas en solucion hidropónica Hoagland's, por 7 días para homogeneizar la nutrición de las plantas. Posteriormente, fueron establecidas en una solución de cloruro de calcio ($CaCl_2$) suplementada con cloruro de aluminio ($AlCl_3$), con 0 y 100 µM de Al. Las muestras de los ápices radicales fueron tomadas a diferentes tiempos de tratamiento con Al^{3+} (0, 2, 6, 24 y 48 horas). 70 fragmentos derivados de transcritos (FDTs) fueron identificados en respuesta al estrés por Al mediante BLASTX, 31 de los cuales mostró una significativa homología con

genes de función conocida. 12 FDTs fueron homólogos a genes no caracterizados y 27 no tienen una significativa homología con proteínas conocidas. Los patrones de expresión de varios de estos genes con función conocida en otras especies fueron confirmados por real-time RT-PCR. 12 genes fueron relacionados con el metabolismo celular, 9 genes asociados a respuesta a estrés y otros relacionados a transcripción y transporte. También fueron identificados genes involucrados en transducción de señales, fotosíntesis y energía, sugiriendo que múltiples procesos están involucrados en la resistencia a Al como se ha reportado previamente en otras especies. Se realizó la caracterización molecular de un gen relevante para la resistencia a Al^{3+}, FDT VCAL19 homólogo a calmodulina. Ahora llamado *VcCaM1* por *Vaccinium corymbosum Calmodulina 1*, este gen podría participar en la resistencia a Al en arándano. Los genes en respuesta a estrés por Al^{3+} en este trabajo podrían estar involucrados en la resistencia a Al en plantas leñosas. Adicionalmente, hemos realizados una evaluación del sistema antioxidante en hojas y raíces evaluando la: actividad antioxidante (AA), superóxido dismutasa (SOD), catalasa (CAT) y hemos correlacionados estos antecedentes con parámetro fisiológicos a nivel foliar, tales como: rendimiento cuántico efectivo del PSII (ΦPSII), eficiencia fotoquímica potencial del PSII (Fv/Fm), el apagamiento no-fotoquímico (NPQ) y la tasa de transporte de electrones (ETR).

Abbreviations

Al	Aluminun
Al^{3+}	Aluminum ion
ALMT1	(*aluminum-activated malate transportes 1*)
$AlCl_3$	Aluminum chloride
bp	Base pairs
cDNA-AFLP	DNA complementary to RNA-amplified fragment length polymorphism
cv	Cultivars
cDNA	DNA complementary to mRNA
CaM	Calmodulin
Ct	Threshold values
Ca^{2+}	Calcium ions
°C	Grad celsius
DNA	Deoxyribonucleic acid
DPPH	2.2 diphenyl-1-picrylhydrazyl
DNasa	Desoxirribonuclease
DTT	Ditiotreitol
ETR	Electron transport rate
Fv/Fm	Maximum quantum yield
FR	Far-red
Fo'	Minimal fluorescence
h	Hours
L	Liters
MATE	(*multidrug and toxic compound extrusion*)
M	Molar
mM	Millimolar
NPQ	Non-photochemical quenching
NBT	Nitroblue tetrazolium
nt	Nucleotide
PCR	Polymerase chain reaction
PPF	Photosynthetic photon flux
ΦPSII	Effective quantum yield
qRT-PCR	Real time quantitative PCR
RNA	Ribonucleic acid
RACE	System for rapid amplification of cDNA ends
RSA	Radical scavenging activity
ROS	Reactive oxygen species
TDF	Transcript derived fragment
µM	Micromolar
UTRs	Region untranslated
VCAL	*Vaccinium corymbosum Aluminum*
VcCaM1	(*Vaccinium corymbosum Calmodulin 1*)

Chapter I

General Introduction

Acid soils and Aluminum phytotoxicity (Al^{3+})

Soil acidity is the major growth-limiting factor for more than 40% of the world's arable land and represents a major limitation to crop production (von Uexkull and Mutert 1995; Degenhardt et al. 1998). In southern Chile, about 50% of Andean soil has acidity levels that increase the amounts of exchangeable and highly toxic Al^{3+} to the plants (Mora et al. 2009). This is also the high winter rainfall of this region, resulting in loss of the cation exchange capacity by leaching, allows rapid acidification of these soils (Mora et al. 1999). In addition, the use of acid reaction fertilizer also contributes to this process. Crop production in acid soils can be maintained neutralizing soil acidity with lime, based on calcium carbonate ($CaCO_3$), dolomite ($MgCO_3$) or a mixture of both (Hede et al. 2003). So do cations that can reach toxic in acid soils, such as aluminum (Al), which is precipitated as an oxide and non-toxic to plants. This happend because it is replaced by the calcium cation (Ca^{2+}) at its exchange site (Khan and McNeilly, 1998). Although this is a common practice in southern Chile, its effectiveness is atingent to the first centimeters of soil, and the correction of acidity in the deeper soil layers may take decades.

The first site of Al accumulation and toxicity are the root tips, evidenced in a rapid inhibition of elongation and cell division (Ma et al. 2001). This alteration difficult absorption of water and nutrients resulting finally, in declining productivity and crop quality (Zheng et al. 1998; Raman et al. 2002; Meriga et al. 2004). At a physiological level, Al affects the functioning of cell membranes, causing enzyme disorders, as well as acting on nuclear DNA (Murali Achary and Panda, 2010). Numerous evidences

concludes that plant species vary in their response to soil acidity complex, characterized by the prevalence of element such as H^+, Al^{3+} and Mn^{2+} (Bona, et al. 1991). This response may determine various mechanisms of tolerance (Delhaize and Ryan, 1995), which can be activated at cellular, tissue, whole plant levels and soil. Some plants detoxify Al in the rhizosphere through exudation of organic acids from plant roots. This efflux is located in the root tips, a highly sensitive region for Al toxicity, as it is an area of constant division and cell elongation. Organic acids, such as malate, citrate and oxalate are commonly secreted (Li et al. 2009). Organic acid levels vary among species, cultivars, or even between tissues of the same plant, growing under identical conditions. In addition, the biosynthesis and accumulation of organic acids increases dramatically in response to environmental stress (López-Bucio et al. 2000).

Gene expression in response to Al stress in plants

It is widely accepted, that the knowledge of genes expressed under stress allows the use of biotechnological tools, to improve responses to stress by transforming, susceptible species to resistant ones, through over-expression of endogenous genes or expression of foreign genes (Kwon et al. 2001). In relation to Al stress, there are several strategies to increase tolerance to this element through over-expression of genes related to the synthesis of citrate and malate, using genes from both Al tolerant plants or bacteria. Examples of these studies have been reported by de La Fuente et al. (1997) in transgenic plants of *Nicotiana tabacum* L. and *Carica papaya* L. in which they induced the synthesis of citrate by citrate synthase gene overexpression (*35S-CSb*) which resulted in increased Al tolerance of these species. In transgenic plants of *Hordeum*

vulgare L. species, overexpressed gene *ALMT1,* which is associated with the exudation of malate and resistance to Al-stress, gave this species a high level of tolerance to this element (Delhaize et al. 2004). The exudation of organic acids from root tips, activated by Al, is a mechanism used by a wide range of Al-tolerant plants (Ma et al. 2001). This mechanism has been well studied in *Triticum aestivum* L. (Delhaize et al. 1993a) and *H. vulgare* (Raman et al. 2002). In the plasma membrane of root apical cells of wheat, a gene has been identified that activates anion channels responsible for Al-tolerance (Sasaki et al. 2004). The gene encoding this membrane protein was identified as *ALMT1* (*aluminum-activated malate transporter 1*). The active heterologous expression of malate exudation in transgenic rice seedlings, into a sensitive barley, represents the first major gene identified in relation to Al tolerance in plants (Delhaize et al. 2004). Moreover, and based on the close relationship between stress induced either by deficiencies of phosphorus or Al toxicity, it has been assumed that some genes associated with Al tolerance, would also be induced by phosphorus deficiency (Ligaba et al. 2004). In *Arabidopsis thaliana* L., to prove that Al induces gene expression of *WAK1* (*cell wall-associated receptor kinase 1*) in the roots, plants were exposed for six hours to Al, after which high levels of WAK protein were identified by Western blots. This gene has been nominated as a candidate for plant defense against Al toxicity (Sivaguru et al. 2003). In wheat, Southern blot analysis allowed the identification of a small family of hybrids *TaPSS1* (*Triticum aestivum phosphatidylserine synthase 1*), and Northern blots showed that Al induces the expression of *TaPSS1* in root tips (Delhaize et al. 1999).

Another type of genes that are expressed under environmental stress, are *AtBCB* (*Arabidopsis thaliana blue copper binding*) in Arabidopsis and *NtGDI1* (*Nicotiana tabacum guanisine diphosphate 1*) of tobacco, both fundamental in controlling oxidative stress. This suggests that there are several mechanisms of resistance to Al in plants, and not only the chelation using organic acid anions (Ezaki et al. 2005). As well as genes that encode antioxidant enzymes induced by Al-stress, among these genes we can find *parB*, which codes for glutathione *S*-transferase (Ezaki et al. 1995) and *NtPox* which encodes a moderately anionic peroxidase, both in tobacco (Ezaki et al. 1996), among others. Studies in rice have shown that the superoxide dismutase gene family (SOD), is differentially regulated in response to a wide range of stimuli (Menezes et al. 2004). The role of *MnSOD* in plants has been extensively studied, due to its protective role in mitochondria, along with being involved in tolerance to environmental stresses such as cold, freezing, oxidative stress and Al toxicity (Basu et al. 2001; Baek et al. 2003; Baek et al. 2006). In transgenic Brassica plants subjected to Al toxicity and oxidative stress, an overexpression of the gene *WMnSOD* was found at a 1.5 to 2.5 fold higher than in the wild type (Basu et al. 2001). In wheat, the MnSOD transcript expression increased significantly in spring and summer wheat, in response to freezing (Baek and Skinner, 2003). There is now interest on the gene regulation *MnSOD* of plants for increased tolerance to environmental stress. However, available information on gene structure and regulatory elements is still insufficient.

From these studies it appears that overall plant response to oxidative stress induced by Al may involve some of these genes. This knowledge is still not applicable to other

species than herbaceous plants. It is completely unknown whether this behavior changes in woody species. The response of commercially important fruit species, like blueberry that develops in acid soils with high concentrations of phytotoxic Al is also unknown.

Blueberry production in Chile and the world

The Blueberry is a species native to the U.S. rather than Europe. It is named Bilberry and belongs to the family Ericaceae. The three main species are: the Rabiyette *Vaccinium ashei*, Highbush and Lowbush (Carlson, 2003). Highbush blueberry production in the United States develops in temperate regions, specifically the Northeast Pacific, in the region of the Great Lakes and the Atlantic States. In countries such as Canada, production is located in the east, characterized by low blueberry crops (Kalt et al. 2001). In Poland, there has been a great interest in growing this species, due to the benefits it brings to the health and nutrition (Skupien, 2006). In Chile, the cultivation of blueberries (*Vaccinium corymbosum*) has developed from the IV to the XII region, comprising a cultivated area of approximately six thousand hectares. Its cultivation is concentrated between the IX and X regions (Guerrero, 2006). The great impact that has had this crop worldwide is that it exhibits a wide range of biologic effects beneficial to health and human food, such as its high antioxidant capacity and anticancer properties (Head, 1998; Scalbert et al. 2005; Cho et al. 2007). Moreover, the presence of flavonoids in this crop has protective properties against heart disease (Zheng and Wang, 2003). In the soils of south-central and southern Chile, high acidity is the main chemical factor that decreases crop production, producing Al toxicity. This results in

reduced growth and plant quality (Mora et al. 2006). However, despite the great development of this species in this region, there are no studies on the oxidative stress caused by Al toxicity, nor on antioxidant gene expression induced by high concentrations of this ion, which can be used to improve more sensitive species. Therefore, it is essential to have research that may contribute to the knowledge of physiological and genetic mechanisms that determine the adaptation of this species. Chile currently exports of fresh fruit, and blueberry is the kind that delivers better value for money on return (FOB/kg) and is displayed as a crop with high economic projections (ODEPA, 2009).

Blueberry production in southern Chile has been an important development, because of the production and commercial benefits of this crop. However, this species grows in acid soils, characterized by high concentrations of phytotoxic aluminum (Al^{3+}). On the other hand, this explosive development is based on external research and agronomic development empirical, with little knowledge from the scientific point of view that can contribute to the development of well adapted genotypes with better quality, thus allowing us to extend the cultivation areas blueberry in our country and improve the agronomic management of this species. One of the options for increasing Al tolerance in blueberry cultivars of commercial importance is breeding. This requires genetic variation for tolerance in the genome of *V. corymbosum* and / or their wild relatives, so as to increase the capacity against oxidative damage to sensitive species. Because the quality and performance will depend on the normal morphological, physiological and biochemical characteristics of the crop, it is necessary to develop Al-resistant genotypes

and agronomic quality. Another alternative is the knowledge and manipulation of the expression of genes for resistance to Al, which would provide a better way to study the specific role against oxidative stress tolerance (Yu et al. 1999).

This research aims to study the expression of genes in response to Al stress in root tips of genotypes of blueberries (*V. corymbosum*) with contrasting resistance to Al, through transcript derived fragments (TDFs), obtained by cDNA-AFLP. The information of genes in this species may be useful for better understanding of the mechanisms underlying Al toxicity and resistance of blueberry, as it can provide a first approach to determine the function of these genes. Identification and characterization of key genes involved in the mechanism of resistance may be useful for genetic engineering and / or breeding to obtain cultivars that are resistant to this element in the Ericaceae family.

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Hypothesis:

- **Using a cDNA-AFLP analysis applied to an Al-sensitive and Al-resistant blueberry genotype, new genes involved in Al resistance mechanisms will be identify. The levels of expression of these genes will be higher in the Al-resistant than in the Al-sensitive genotype.**

General Objective:

- **To study the differential expression of genes in response to Al toxicity in roots of genotypes of blueberry.**

Specifics Objectives:

1. **To identify gene in response to Al-stress in roots of two blueberry genotypes with contrasting resistance.**

2. **Evaluation of the genes associated to Al-stress and characterization of the antioxidant system.**

3. **To characterize at the molecular level the most important genes expressed during treatment with Al-phytotoxic.**

Chapter II

Molecular and physiological strategies to increase aluminum resistance in plants

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Molecular and physiological strategies to increase aluminum resistance in plants

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Running Head: Aluminum stress resistance mechanisms in plants

Abstract

Aluminum (Al) toxicity is a primary limitation to plant growth on acid soils. Root meristems are the first site for toxic Al accumulation, and therefore inhibition of root elongation is the most evident physiological manifestation of Al toxicity. Plants may resist Al toxicity by avoidance (Al exclusion) and/or tolerance mechanisms (detoxification of Al inside the cells). The Al exclusion involves the exudation of organic acid anions from the root apices, whereas tolerance mechanisms comprise internal Al detoxification by organic acid anions and enhanced scavenging of free oxygen radicals. One of the most important advances in understanding the molecular events associated with the Al exclusion mechanism was the identification of the *ALMT1* gene (Al-activated malate transporter) in *Triticum aestivum* root cells, which codes for a plasma membrane anion channel that allows efflux of organic acid anions, such as malate, citrate or oxalate. On the other hand, the scavenging of free radicals is dependent on the expression of genes involved in antioxidant defenses, such as peroxidases (eg. in *Arabidopsis thaliana* and *Nicotiana tabacum)*, catalases (eg. in *Capsicum annuum*)*,* and the gene *WMnSOD* from *T. aestivum*. However, other recent findings show that reactive oxygen species (ROS) induced stress may be due to acidic (low pH) conditions rather than to Al stress. In this review, we summarize recent findings regarding molecular and physiological mechanisms of Al toxicity and resistance in higher plants. Advances have been made in understanding some of the underlying strategies that plants use to cope with Al toxicity. Furthermore, we discuss the physiological and molecular responses to Al toxicity, including genes involved in Al resistance that have been identified and

characterized in several plant species. The better understanding of these strategies and mechanisms is essential for improving plant performance in acidic, Al-toxic soils.

Keywords: Aluminum resistance, antioxidant genes, *ALMT1* gene, MATE, ROS, oxidative stress.

Introduction

Acid soils (pH<5.5 in water) represent between 30 and 40% of the arable soils in the world and around 70% of the soils that are potentially arable [1, 2]. In these acidic soils, aluminum (Al) toxicity is the primary factor limiting crop productivity [3].

Aluminum toxicity has been widely studied in herbaceous plants, particularly cereals, where inhibition of root growth has been reported as the primary symptom of exposure to Al excess [4]. These interferences with root growth impede the absorption of water and nutrients and hence result in a decrease of yield and grain quality in cereals [5]. Other important physiologic effects of Al on plant cells involve alterations of their membranes, enzymatic disorders, as well disturbances in the synthesis of the nuclear DNA [6, 7, 8].

Aluminum triggers lipid peroxidation and oxidative stress in roots due to an increase in the production of reactive oxygen species (ROS) [9, 10]. To counteract the deleterious effect of ROS, plants have developed diverse mechanisms of antioxidant defense. These mechanisms involve antioxidant enzymes and specific low-molecular-weight compounds [11, 12].

Plant species differ in their responses to acid soils. These responses include diverse resistance mechanisms at the cell, tissue or whole plant levels [13]. The Al resistance mechanisms are usually classified as exclusion (avoidance) mechanisms and internal tolerance (also called protoplastic tolerance) [14]. According to Barceló and Poschenrieder [15], the exclusion of Al appears to be the most important resistance

mechanism in cultivated and wild species that grow in acid soils with high concentrations of phytotoxic Al.

Isogenic lines of *T. aestivum* cultivated in a nutrient solution allowed the characterization of an exclusion mechanism [16]. It was found that these lines differ in Al resistance. The addition of Al stimulated the release of malate from the root apices, with the tolerant genotype excreting 5 to 10 times more organic acid anions than the sensitive genotype. This evidence suggested that the excretion of organic acid anions may be an Al resistance mechanism in *T. aestivum* [17, 18], similar to other plant species [19, 20, 21].

Most genes induced by Al appear to be involved in various stress responses, including phosphorus deficiency [22], exposure to heavy metals [23], and oxidative stress [24, 25]. Nearly twenty genes induced by Al stress have been identified in species like *T. aestivum* [26, 27], *N. tabacum* [28, 29] and *A. thaliana* [24, 30].

In this review, the responses of plants to Al toxicity and the relevant resistance mechanisms are described, with the primary focus on the expression of genes underlying the mechanisms of Al stress resistance in plants.

Soil chemistry: Al species and their relation to Al phytotoxicity

Aluminum is the most abundant metal and the third most abundant chemical element in the Earth crust [31]. Various studies highlighted the importance of different Al ionic forms in soils to elucidate Al phytotoxicity, for references see [14]. Aluminum toxicity not only depends on the total Al concentration, but also on the Al chemical forms, with Al speciation being highly dependent on the pH of the soil solution [14]. Hence, it is

necessary to identify the chemical forms of Al to estimate its biological impact as dependent on availability, physicochemical reactivity, and transport in the environment and into the food chain [32, 33]. In acid soils with pH_{water} lower than 5.0, forms of Al as hexaaquaaluminum $[Al(H_2O)_6]^{3+}$, or Al^{3+} may appear; they may react with available ligands to form additional chemical species [34]. As soil pH increases, mononuclear hydrolysis species such as $Al(OH)^{2+}$ and $Al(OH)_2^{+}$ are formed [35]. At near-neutral pH, the solid phase $Al(OH)_3$ (gibbsite) occurs, whereas at slightly alkaline conditions the amphoteric species $Al(OH)_4^{-}$ (aluminate) predominates [13]. There is a significant correlation between low pH and high concentrations of Al^{3+} in soil [36]. One of the options used to alleviate Al toxicity consists of neutralizing the soil acidity with calcium carbonate ($CaCO_3$), dolomite $CaMg(CO_3)_2$, or a mixture of both [37]. Although this is a common agricultural practice in acid soils, the effectiveness of this treatment is limited to amelioration of only the first few centimeters of the arable topsoil layer, with amelioration of deeper layers being very slow, if at all, and dependent on soil and weather conditions [38]. Another option to reduce Al toxicity is the genetic improvement of plants directed to increase their Al resistance [39].

Al toxicity and its relation to oxidative stress

The oxidative stress produced by Al causes an increase in the production of ROS such as superoxide radicals ($O_2^{\cdot-}$), hydroxyl radicals ($^{\cdot}OH$), and hydrogen peroxide (H_2O_2) [10]. The ROS may affect biological macromolecules, such as unsaturated fatty acids in the cell membrane, causing peroxidation of membrane lipids, which in turn may lead to

severe cellular damage [40, 41, 42]. Aluminum has a strong affinity to biomembranes, causing the rigidification of the membranes and the peroxidation of lipids mediated by Al-enhanced Fe^{2+} [9]. Aluminum may also be associated with phosphates and carboxyl groups and could accumulate in the membranes due to its low exchange rate. The Al accumulation causes changes in the membrane structure and function, affecting aggregation, fusion and changes in the permeability of liposomes and packaging of fatty acids of the plasma membranes [43].

The destructive effects of Al-induced ROS in plants are counteracted through antioxidant defense mechanisms, such as detoxifying cations by phytochelatins [44], and chelation by organic acid anions and amino acids [45]. When these mechanisms are saturated, Al induces damage in cells and tissues, increasing the level of lipid peroxidation, which alters the activities of antioxidant enzymes [40].

Aluminum resistance in plants is reliant on antioxidant enzymes, such as superoxide dismutase (SOD, EC 1.15.1.1), peroxidase (POD, EC 1.11.1.7), catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11), and glutathione reductase (GR, EC 1.6.4.2), as well as non-enzymatic molecules of low molecular weight, such as ascorbic acid (AsA), reduced glutathione (GSH) and phenol-like compounds such as tocopherols (α-tocopherol), flavonoids, carotenoids (β-carotene), and uric acid [10, 11, 12, 46].

Aluminum toxicity induced genes that code for enzymes SOD, POD and glutathione *S*-transferase (GST, EC 2.5.1.18), and resulted in an increase in the SOD [47, 48] and APX activities [47, 49]. Additionally, these genes were also induced by oxidative stress

[24]. Even though it has been widely reported that Al induces ROS and increases oxidative stress in plants [41, 50], recent findings show that ROS-induced stress may be due to acidic (low pH) conditions rather than to Al stress. Nevertheless, it must be taken into account that it is not possible to have Al stress without acidic conditions. However, characterization of the role of acidic vs acidic + Al-toxicity stress can be achieved using *A. thaliana* genotypes differing in resistance to acidic vs Al-stress [51].

Plant Al-resistance mechanisms

Plants that grow in acid soils with a high concentration of Al^{3+} have developed mechanisms for its detoxification (Figure 1). Aluminum resistance mechanisms have been classified into internal and external [52]. Among external mechanisms, Al resistance in plants involves the exclusion of this element from the root apex by exudation of organic acid anions into the rhizosphere. These organic anions chelate toxic Al in the rhizosphere, forming stable non-phytotoxic complexes. This mechanism could correspond to an avoidance mechanism [53, 54]. On the other hand, the Al-tolerant genotypes are also able to detoxify this element inside the cell by chelating it in the cytoplasm with organic acid anions or other organic ligands and then compartmentalizing it in organelles like the vacuole [14, 55] (Figure 1).

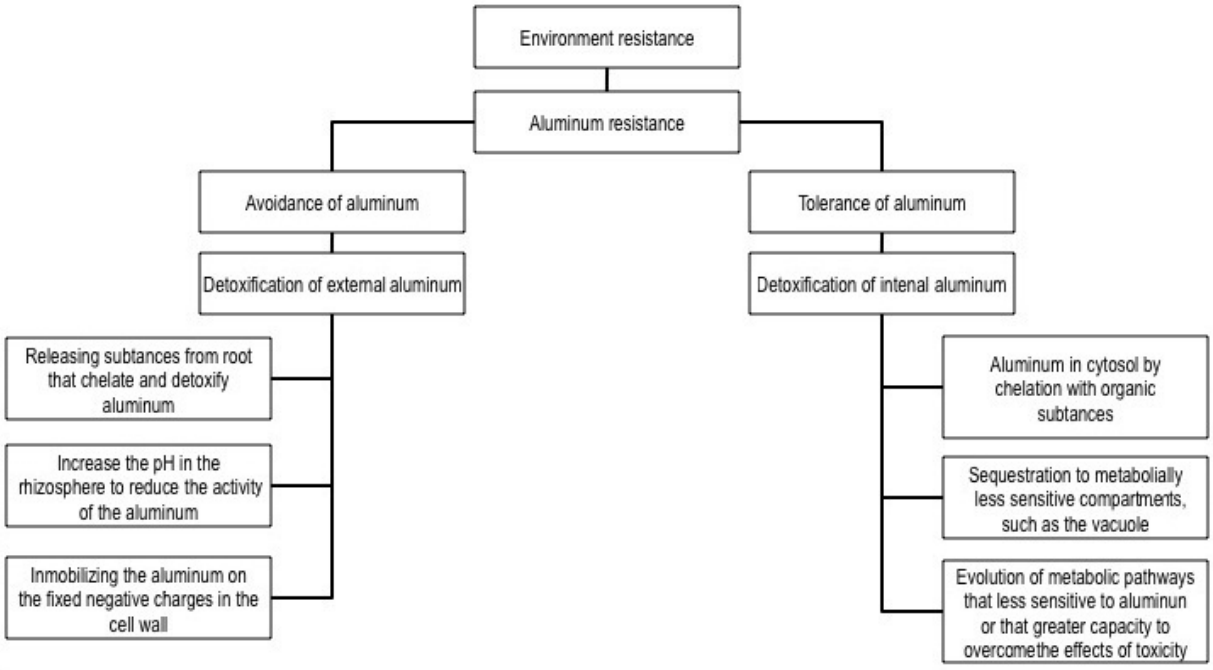

Figure 1. Theoretical diagram of the possible categories of the aluminum resistance in plants.

Aluminum toxicity can cause damage to the membranes, such as lipid peroxidation and loss of cellular compartments, the events that happen later in the process of root growth inhibition [56]. Programmed cell death (PCD) or apoptosis can also occur. In *Hordeum vulgare* roots exposed to 0.1 to 1.0 mM Al, apoptosis began to occur after 8 h of Al treatment [57]. This phenomenon was also studied *in vivo* in mitochondria of *A. Thaliana,* and a quick burst of mitochondrial ROS in Al-treated protoplasts was detected [58].

Biochemical studies indicate that Al ions have strong affinity for biomembranes [9]. This is because the plasma membrane has negative charges, such as phospholipids to which

Al binds irreversibly [59]. Aluminum induced quick production of ROS in *Zea mays,* together with rigidification of the cell wall, which could inhibit root development [60]. This was due to the strong binding of Al to the cell wall pectins concomitant with displacement of calcium (Ca^{2+}), thus causing disturbance in the cellular expansion processes [59]. Aluminum can affect the mechanisms that control organization of microtubules in the cytoskeleton and polymerization of tubulin, causing a delay in disassembly of microtubules during mitosis [61]. This can also affect the normal cell division by Al interference with the cortical actin filaments suggested to play a role in fixing the axis of cell division in the correct position [62] and the orientation of microtubules, which is closely related to cellular elongation [63].

Studying a possible role of ethylene in the initial signal transduction of Al-induced root growth responses in *Z. mays* genotypes with contrasting tolerance to Al, Gunse et al. [64] found that Al treatment did not induce ethylene production in any of the genotypes. Similar studies in *Phaseolus vulgaris* showed that Al^{3+}-induced inhibition of root growth is preceded by significant changes in cytokinin levels and enhanced ethylene evolution, suggesting that cytokinin can induce ethylene production, and that the rapid increase in cytokinin may contribute to inhibition of root-growth by affecting plant hormone homeostasis [65]. On the other hand, lesser inhibition by Al was observed in *A. thaliana* mutants defective in ethylene signaling (*etr1-3* and *ein2-1*) and auxin polar transport (*aux1-7* and *pin2*) compared with the wild type, suggesting that Al-induced ethylene production is likely to act as a signal to alter auxin distribution in roots, inhibiting their elongation [66].

The reaction centers of photosystems I and II (PSI and PSII) in chloroplast thylakoids are the major sites of ROS generation in plants [67]. However, little is known about the effects of Al stress on the functionality of the photosynthetic apparatus. Aluminum toxicity damaged functioning chloroplasts [68, 69], decreased total chlorophyll content, photosynthetic rate and CO_2 assimilation, as well as partially inhibited electron transport in PSII in some species [70, 71, 72, 73]. In leaves, it has been observed that Al-stress negatively affects absorption of light by lowering pigment concentration. Both energy dissipation and antioxidant systems in Al-stressed leaves are enhanced to protect them from photo-oxidative damage under high light [74].

In plants, Al toxicity displaces Ca^{2+} from the plasma membrane, disrupts the signaling cascades of cytosolic Ca^{2+} and blocks ion-channel pumps [75]. In addition, microscopy analysis of microtubules and microfilaments in *Z. mays* roots showed changes in the organization and stability correlated with Al toxicity [76]. In the same species, Amenós et al. [77] showed that the actin cytoskeleton and vesicle trafficking were the primary targets for Al toxicity in the root tips of sensitive genotypes. The Al uptake by plants is a slow process, and the mechanism has not yet been elucidated, although diffusion is speculated (Figure 2).

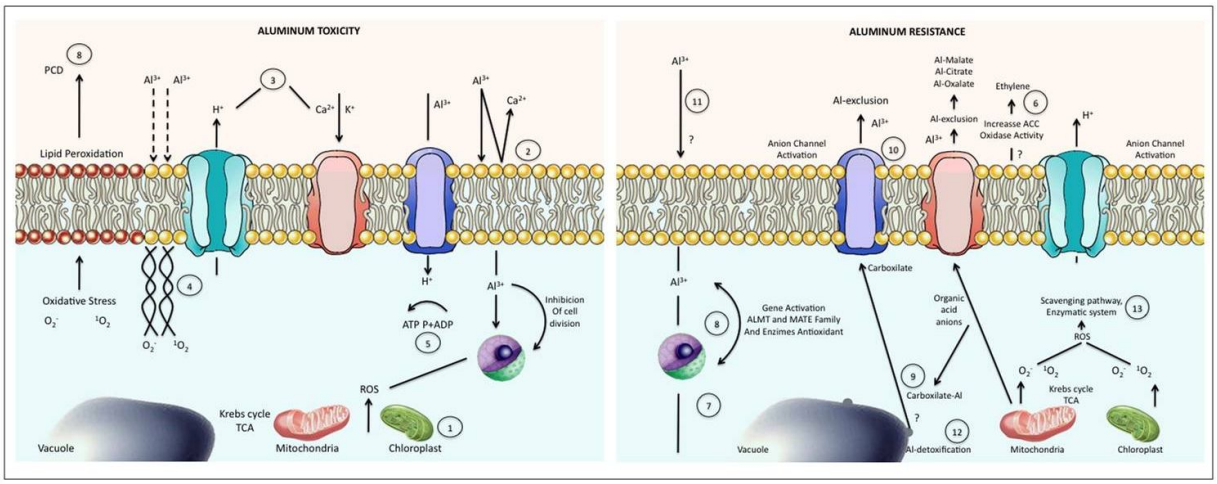

Figure 2. Proposed model of the resistance mechanisms and response to Al toxicity in plant cells. Adapted from Kochian et al. (2005) with modifications. Numbers enclosed in circles in the figure represent: 1) ROS production in chloroplasts, 2) Displaced Ca^{2+} from the plasma membrane by Al^{3+}, 3) Blockage of ion-channel pumps and primary and secondary transport across pumps and channels, 4) Disruption of cytoskeleton, 5) Inhibition of cellular division by Al^{3+}, 6) Aluminum induction of ACC (1-aminocyclopropane-1-carboxylic acid) oxidase activity, stimulating ethylene production and therefore the root growth inhibition, 7) Gene expression for transport membrane proteins and synthesis of antioxidant enzymes such as superoxide dismutase (SOD), peroxidases (POD) and glutathione-*S*-transferase (GST), 8) Programmed cell death (PCD), 9) Complexation of Al with organic acid anions, 10) exudation of organic acid anions and complexation with Al in the rhizosphere, 11) Transport of Al^{3+} across the plasma membrane, 12) Compartmentalization of Al-organic acid anion complexes in vacuole, 13) Scavenging pathway in plant cells (antioxidant enzyme system). Question marks (?) denote unknown metabolic routes.

Mechanisms of Al exclusion or avoidance

Some plant species can detoxify the rhizospheric Al through the exudation of organic acid anions [78, 79]. This exudation occurs at the root tips, which are highly sensitive to Al toxicity because of continuous cell division and elongation [59, 80]. The rates and amounts of organic acid anion exudation vary among species, cultivars and genotypes, and even among tissues [81, 82]. Tolerant genotypes exude larger amounts of organic acid anions than sensitive ones, supporting the exudation of organic acid anions as a mechanism of resistance to Al [13]. As a consequence of poor exudation of organic acid anions, sensitive *T. aestivum* genotypes show greater Al accumulation in the cortical tissue, being 5 to 10 times more than in tolerant genotypes [83].

Some organic acid anions, such as citrate, oxalate and malate, form stable complexes with Al [59, 84, 85], with formation constants decreasing in the order Al-citrate > Al-oxalate > Al-malate [59], because of Al affinity for the oxygen donor ligands [15]. Exudation of organic acid anions from the root tip cells is mediated by anion channels in the plasma membrane [84]. These anion channels are activated by Al, as demonstrated via patch-clamping in protoplasts isolated from *T. aestivum* root tips [86]. In addition, the use of inhibitors of anion channels, such as niflumic acid, support the role of these channels in exudation of organic acid anions in response to Al [87, 88, 89]. On the other hand, Al also induced exudation of certain phenolic compounds, such as catechins and quercetin, from the corn root tips. Given that these compounds form stable complexes with Al, they may contribute to Al resistance [90].

Mechanisms of Al tolerance (inclusion and internal tolerance)

Another mechanism of Al resistance acts within the cell (tolerance) [14, 91, 92]. Recent investigations have focused on plant species that can accumulate high Al concentrations in their aboveground parts (Al-hyperaccumulator plants). The term Al-hyperaccumulator refers to plants that can accumulate more than 1 g Al kg^{-1} of dry leaves [93]. Species like *Hydrangea macrophylla* can accumulate about 3 g Al kg^{-1} [94] and *Fagopyrum esculentum* up to 15 g Al kg^{-1} in their leaves when growing in acid soils [84]. In *F. esculentum*, Al captured by the root cells is internally chelated by oxalate, forming a non-phytotoxic complex of Al-oxalate in a 1:3 ratio [95, 96]. This complex is converted to Al-citrate (1:1) in the xylem [97] and then transported towards the leaf cells, where it is converted back to Al-oxalate and stored in the vacuole [59, 98, 99, 100]. Nuclear magnetic resonance (NMR) studies in *Camellia sinensis* and *H. macrophylla* showed that Al exists in leaves as Al-catequin and Al-citrate, respectively [94, 101].

Recently, it has been observed that phosphorus (P) can alleviate the toxic effect of Al in *Citrus,* facilitating the immobilization of Al in roots through increasing organic acid secretion [102], as well as preventing the inhibition of photosynthetic performance [103]. On the other hand, the symptoms of Al toxicity in leaves may resemble the symptoms of P deficiency: the mature leaves turn dark green, the stems turn purple and the leaf tips die [104]. In other cases, Al reduces the transport of calcium (Ca^{2+}) in the leaves, causing rolling of young leaves that eventually impedes the growth and development of petiole [36]. Aluminum phytotoxicity in *Lycopersicon esculentum* results in changes in

the CO_2 assimilation rate and chlorophyll content, modifying the activity of numerous enzymes [105].

It has been also reported that Al can accumulate in stems. This implies that the soluble complexes of Al are transported through the xylem and subsequently accumulate in either soluble or solid forms in the leaf vacuoles or the apoplast [15]. In experiments with labeled Al applied to accumulating and non-accumulating Al species, none of the tissues of the non-accumulating species showed evidence of high concentrations of Al. In contrast, all the Al-accumulating species had high concentrations of Al in all phloem elements and the total absence of it in the vessel members, xylem fibres and palisade parenchyma [106].

At the cellular level, binding of Al to ATP is weaker than the binding of Al to organic acid anions such as citrate or oxalate. This could indicate that organic acid anions can protect plants by chelating Al in the cytosol. The metallic anion complex could then be transported around the plant for storage [59], thus immobilizing, compartmentalizing and detoxifying Al [98, 10].

Molecular strategies for increasing Al resistance

The main mechanism of Al avoidance is associated with Al-activated exudation of organic acid anions from root apices. For example, malate is exuded in the presence of Al by *T. aestivum* [107], and citrate by *Z. mays* [108], *Secale cereale* [109, 110] and *Glycine max* [111]. Numerous studies have been carried out with the aim of identifying the cellular mechanisms involved in exudation of organic acid anions from the root

apical cells [112]. Increased synthesis of organic acid anions was related to increased resistance to the Al stress, e.g. in transgenic plants of *N. tabacum* and *Carica papaya* overexpressing the gene for citrate synthase (*35S-CSb*) [113], in *Saccharomyces cerevisiae* and *Brassica napus* overexpressing mitochondrial citrate synthase (*CS*) [114], in *Medicago sativa* transformed with *Pseudomonas aeruginosa* gene for *CS* [115], in transgenic *Nicotiana benthamiana* lines overexpressing mitochondrial citrate synthase from *Citrus junus* (*CjCS*) [116]. Similarly, transgenic *M. sativa* and *N. tabacum* (modified with malate dehydrogenase gene) had enhanced malate synthesis and greater Al resistance [117, 118]. Recently, Trejo-Tellez et al. [119] reported for the first time that the overexpression of pyruvate phosphate dikinase (PPDK, EC 2.7.9.1) in tobacco roots increased the exudation of organic acid anions, with a concomitant decrease in plant Al accumulation. Sasaki et al. [120] identified a malate transporter encoded by the gene denominated *TaALMT1* (*Triticum aestivum* aluminum-activated malate transporter 1) in Al-tolerant (ET8) and Al-sensitive (ES8) isogenic *T. aestivum* lines. *TaALMT1* is the first major gene that confers resistance to high Al concentrations in acid soils that has been transferred into plants of agricultural importance using transgenic techniques [121]. This gene encoded a member of the membrane-bound ALTM protein family [122]. The gene *TaALMT1* encodes a membrane protein that is expressed constitutively, but at a higher level in the root apices of the Al-resistant compared with the Al-sensitive *T. aestivum* line. The localization of this malate transporter was confirmed in onion and tobacco cells through analysis of transient expression of green fluorescent protein (GFP) [123]. In transgenic *H. vulgare* plants expressing *TaALMT1,* a relationship between exudation of

malate and resistance to Al stress was reported [121]. In contrast, in rice the expression of the same gene (*TaALMT1*) significantly increased Al-activated exudation of malate, but there was no increase in Al resistance [120, 124]. This could be attributed to a relatively low affinity of malate for Al compared to the higher affinities of citrate or oxalate [84, 96]. Furthermore, the small amount of malate released from root apices of Al-resistant *T. aestivum* may be insufficient for reducing the Al activity at the root surface because microorganisms are likely to decompose malate relatively quickly [124, 125].

Suppression subtractive hybridization (SSH) and microarray analysis of isogenic lines of *T. aestivum* identified genes that were differentially expressed in Al-tolerant (Chisholm-T) and Al-sensitive line (Chisholm-S) [85]. These authors reported that despite more than one thousand genes assessed, only 57 were differentially expressed during the Al treatment for 7 days. Among these genes, *ALMT1* and the genes coding for ent-kaurenoic acid oxide-1, β-glucosidase, lectin, histidine kinase and phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) were included. These genes exhibited abundant transcripts in the Al-tolerant line, mainly clustered into those that increase Al resistance (A, B, C clusters) and sensitive to Al toxicity (D, E, F clusters) in the Al-sensitive line (Figure 3). This suggests that the resistance to Al can be co-regulated by multiple genes with different functions. Recently, Furukawa et al. [126] used mapping analysis and microarrays in *H. vulgare* cultivars with differential resistance to Al (Murasakimochi-T and Morex-S) to identify a gene (*HvAACT1*) (encoding a membrane protein HVAACT1) responsible for citrate exudation, which was activated by Al in the Al-resistant cultivar. This gene belongs to the family of multidrug and toxic compound extrusion (MATE)

genes, and it was expressed constitutively in the roots of the Al-resistant cultivar. This gene is also referred to as *HvMATE1* by Wang et al. [127]. The expression of the gene *HvAACT1* correlated well with citrate exudation in 10 *H. vulgare* cultivars that differed in their resistance to Al, demonstrating that HvAACT1 could be the citrate transporter responsible for Al resistance in *H. vulgare* [126].

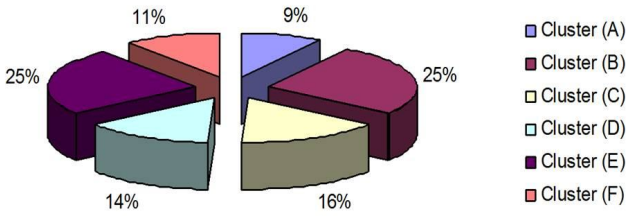

Figure 3. Distribution of functional cluster transcripts of root tissues from Al-treated Chisholm-T and Chisholm-S wheat genotypes based on their homology. The genes in clusters A, B and C may enhance Al tolerance, whereas genes in cluster D, E and F may be associated with sensitivity to Al toxicity. Data adapted from Guo et al. (2007).

Microarray analysis in *A. thaliana* identified Al-responsive genes, such as *GST*, *POD* and *chitinase* that were up-regulated by Al stress, and *Wali 3* and *Wali4* that were down-regulated by this stress [128]. On the other hand, Eticha et al. [129] SSH in *Phaseolus vulgaris* under Al treatment identified two genes of MATE family associated with the exudation of citrate. These genes were named *MATE-a* and *MATE-b* and were strongly expressed upon Al treatment. On the other hand, the exogenous application of polyamines such as putrescine, spermidine and spermine (Put, Spd, Spm) in *Crocus sativus* improved the plant performance under Al stress, with respect to control [130].

This might be attributed to lower Al content in the root tips, and subsequent less lipid peroxidation and oxidative stress. Through the overexpression of *spermidine synthase* of apple (*MdSPDS1*) in transgenic European pear *Pyrus communis*, the performance of transgenic was much better than that of wild type, indicating that Spd is implicated in elevating of Al-stress tolerance [131]. Such evidence showed that polyamines are involved in many physiological processes (e.g. cell growth and development) and contribute to stress tolerance to various environmental factors. Strategies of genetic manipulation of crop plants with genes encoding enzymes of polyamine biosynthetic pathways may enhance stress tolerance [132].

Recent studies in *Oryza sativa* have identified genes involved in Al stress. An example is *STAR1* (sensitive to Al rhizotoxicity). This gene encodes only the nucleotide-binding domain (NBD) of an ATP-binding cassette transporter (ABC) and interacts with the gene *STAR2*, a transmembrane domain protein (TMD), to form a complex implicated in Al tolerance that functions as a bacterial type ABC transporter [124, 133]. *OsSTAR1* and *OsSTAR2* are both expressed predominantly in roots and the expression of both is specifically induced by Al [134]. However, further study is needed to determine whether they confer high levels of resistance if expressed in highly Al-sensitive species.

Han et al. [135] isolated a new Al-induced gene (mitochondrial citrate synthase 1) from *O. sativa* (*OsCS1*). Several transgenic lines of *N. tabacum* in which *OsCS1* was overexpressed exhibited increased citrate efflux and higher tolerance to Al, suggesting that *OsCS1* could be a gene candidate for enhancing Al tolerance in plants.

Genes involved in antioxidant defense mechanisms under Al toxicity

A relationship exists between Al phytotoxicity and oxidative stress. The oxidative stress genes induced by Al include those encoding e.g. SOD and the Bowman-Birk protease inhibitors in *A. thaliana* [136, 24], Ser/Thr kinase, RAS-related proteins, and GTP-binding proteins in *Saccharum* spp hybrid cv N19 [137]. These genes are direct or indirect participants in signaling events in many organisms [29], and are suggested to be involved in a signaling system associated with Al stress. However, neither the precise induction of these genes by Al stress nor a common induction mechanism among various stresses has been completely clarified [138]. However, Richards et al. [24] built a cDNA library for *A. thaliana* treated with Al and found that, after induction time of at least 48 h, four genes were expressed, encoding POD, GST, blue copper-binding protein, and a protein homologous to reticulin (oxygen oxidoreductase enzyme). The first three genes are known to be induced by oxidative stress. Similarly, under Al-stress, Basu et al. [40] observed high expression of the mitochondrial gene *WMnSOD* in *T. aestivum* and *Brassica napus*, enhancing Al resistance. Hence, it has been proposed that there might be a common induction/signaling mechanism for responses to oxidative stress and Al toxicity [139].

In tobacco cells, three genes (*parA*, *parB*, and *NtPox*) were induced by Al toxicity as well as P deficiency [22, 136]. The genes *parB* and *NtPox* encode GST and POD, respectively [136]. In *A. thaliana*, the expression of two GST genes (*AtGST1* and *AtGST11*) was induced by Al at different levels [138]. Recently, studies associated with non-enzymatic antioxidant defense mechanisms in transgenic tobacco plants indicated

that manipulating the pathway of AsA biosynthesis and overexpressing dehydroascorbate reductase (DHAR, EC 1.8.5.1) resulted in high AsA levels, conferring tolerance to the Al stress [56], further confirming the link between the oxidative and the Al stress.

Directions for future research

In the last decade or so, diverse genes involved in plant responses to abiotic stresses have been identified. A range of transcription factors that regulate these genes has also been characterized, some of them regulating the transport of Al-binding organic acid anions. The identification of genes that confer resistance to Al, such as *ALMT1* and *HvAACT1,* in diverse species constitutes a crucial advance for the improvement of important agronomic species. Possible strategies for increasing Al resistance comprise overexpression of genes involved in the malate or citrate synthesis (already evaluated in various transgenic plant species) as well as the overexpression of antioxidant genes induced by Al.

The most understood Al resistance mechanism is the chelation of Al^{3+} ions with organic acid anions. Nonetheless, several lines of evidence exist that imply genes associated with oxidative stress as important players in Al resistance in plants. Thus, exudation of organic acid anions and the enhanced antioxidative defense are relevant mechanisms against Al toxicity. Future research would need to concentrate on (i) the transcriptional regulation of genes associated with biosynthesis and exudation of organic acid anions, and (ii) antioxidative defense in Al-resistant genotypes. Such work may result in

identification and characterization of new genes, which can be used to improve plant resistance to Al and possibly other toxic ions because many of the identified genes with altered expression are not specific only for Al.

It is clear that tolerance to acid soils with high concentrations of toxic Al involves complex interactions that are controlled by many genes and transcription factors. We hope this review will provoke fervent discussion and analysis that will improve our knowledge and understanding of how higher plants cope with Al stress.

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Chapter III

Identification of aluminum-regulated genes by cDNA-AFLP analysis of roots in two contrasting genotypes of highbush blueberry (*Vaccinium corymbosum* L.)

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Abstract

To investigate the molecular mechanisms of Al^{3+}-stress in blueberry, a cDNA-amplified fragment length polymorphism (cDNA-AFLP) analysis was employed to identify Al-regulated genes in roots of contrasting genotypes of highbush blueberry (Brigitta, Al^{3+}-resistant and Bluegold, Al^{3+}-sensitive). Plants grown in hydroponic culture were treated with 0 and 100 µM Al and collected at different times over 48 h. Seventy transcript-derived fragments (TDFs) were identified as being Al^{3+} responsive, 31 of which showed significant homology to genes with known or putative functions. Twelve TDFs were homologous to uncharacterized genes and 27 did not have significant matches. The expression pattern of several of the genes with known functions in other species was confirmed by quantitative relative real-time RT-PCR. Twelve genes of known or putative function were related to cellular metabolism, 9 associated to stress responses and other transcription and transport facilitation processes. Genes involved in signal transduction, photosynthetic and energy processes were also identified, suggesting that a multitude of processes are implicated in the Al^{3+}-stress response as reported previously for other species. The Al^{3+}-stress response genes identified in this work could be involved in Al^{3+}-resistance in woody plants.

Keywords:

Blueberry; Aluminum toxicity; cDNA-AFLP; qRT-PCR; Global Gene Expression

Abbreviations:

cDNA-AFLP, DNA complementary to RNA-amplified fragment length polymorphism.

TDF, transcript derived fragment.

Aluminum, Al^{3+}

Introduction

The blueberry (*Vaccinium corymbosum* L., Ericaceae) is a bush originating from North America. This plant produces a small fruit that is an important crop as it is rich in antioxidants and their consumption is beneficial for human health [1]. In Chile, the blueberry was introduced in the 1990's with excellent adaptative results in southern regions. Currently, Chile is the main blueberry-producing country in the Southern Hemisphere and the third largest producer at global level [2].

Aluminum (Al^{3+}) is the most abundant metal in the earth's crust and at high concentrations, it is a major limitation to crop productivity in acid soils, which comprise up to 50% of the world's arable lands [3]. In southern Chile, about 50% of andisol soil has acidity levels that increase the amounts of exchangeable and highly toxic Al^{3+} to the plants [4]. The major symptom of excess Al^{3+} is a rapid inhibition of root growth that is accompanied by an accumulation of this phytotoxic ion in the cell walls [5]. Al^{3+}-stressed roots thus become thick, brown and inefficient in water and nutrient uptake. Different mechanisms of Al^{3+}-resistance have been reported [3]. Of these mechanisms, exudation of Al^{3+}-chelating organic acids into the rhizosphere, such as malate, oxalate or citrate, is the most effective means to avoid Al^{3+}-toxicity in many species [6]. Some genes of Al-resistance of the ALMT (Al-activated malate transporter) and MATE family (multidrug and toxic compound extrusion), have been identified and characterizated in different species of plant [7]. These genes encode membrane proteins which mediate the exudation of organic acid anions from the root. On the other hand, when Al^{3+} crosses the cell membrane, it is chelated by these organic acids and phenolic compounds in the

cytosol [8]. It has been reported that Al^{3+} can alter cell redox homeostasis as a consequence of enhanced production of reactive oxygen species (ROS) [9]. The increase of ROS production could be involved in Al^{3+}-mediated inhibition of root growth [10] and programmed cell death caused by Al^{3+}-stress [11]. It is well known that plants have efficient enzymatic and non-enzymatic mechanisms to protect cellular components from oxidative damage caused by different stresses [12]. Research by Ezaki et al. [13], indicates that there are different processes involved in Al^{3+}-resistance in addition to chelation of Al^{3+} by organic acid anions, and other means have also been suggested in plants [14]. On the other hand, it has been reported that Al^{3+}-stress resistance varies between genotypes of the same species. For example, Al^{3+} stimulated between 5- to 10-fold more malate exudation from root apices of the Al^{3+}-tolerant isogenic lines of wheat than from the Al^{3+}-sensitive lines [15]. Global gene expression analyses have identified the genes induced by Al^{3+} in roots of *Triticum aestivum* [16], *Oryza sativa* [17], *Saccharum* spp. [18] and *Arabidopsis thaliana* [19]. However, most of these genes not only respond to Al^{3+} but also to other toxic metals [20], pathogens response [21] and oxidative stress [18]. In summary, plants have evolved several mechanisms that enable them to resist Al^{3+}-stress, and the ability to cope with Al^{3+} toxicity depends on the species and the genotype in question.

Although blueberry requires acid soils for optimum development, soluble Al^{3+} is detrimental to highbush blueberry growth [22]. However, differential responses to Al-stress have been observed between blueberry cultivars (cv). Using biochemical and

physiological parameters, the cv. Brigitta and Bluegold have been described as Al^{3+}-resistant and Al^{3+}-sensitive cultivars, respectively [23].

To understand the molecular mechanisms underlying the differential response to Al^{3+}-stress by the contrasting genotypes, we used cDNA-AFLP for identification of differentially expressed genes [24]. This technique allows the discovery of unknown genes in species, such as blueberry, where there is no information in the genomic databases. In this work, we report the identification of 70 transcript-derived fragments (TDFs) that were sequenced and classified. Their putative function in the Al^{3+}-stress response is evaluated and discussed. The identification of these genes is very important to understand the mechanisms of toxicity and Al^{3+}-resistance in blueberry and other woody perennial plant species.

Materials and methods

Plant material and growth conditions

Two genotypes of *V. corymbosum*, which are widely-used in southern Chile were used in this study; Brigitta, Al^{3+}-resistant and Bluegold Al^{3+}-sensitive [23]. Uniform 8-month-old plants (about 15 cm high) grown in solid substrate (1 peat: 1 rice husks: 1 pine needles) were provided by the Experimental Station Maquehue of the Universidad de La Frontera (Temuco, Chile). Plants were conditioned in plastic boxes filled with 18 L of Hoagland's nutrient solution for for 7 days [25]. The solution was adjusted to pH 4.8 with 0.4 M HCl or NaOH, aerated with an aquarium pump and changed every 2 days. After conditioning, saplings were transferred to a hydroponic solution of $CaCl_2$ (0.5 mM) with and without

(control) Al^{3+} supplied as $AlCl_3$ (100 µM) for 48 h. The experiment was conducted in a greenhouse with a photoperiod of 14/10 h (light/dark), at 25/20 °C (day/night), 65 to 85% relative air humidity, and photosynthetic photon flux (*PPF*) densities of 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at the plant canopy. Samples of blueberry roots (root apex to the elongation zone, ~3 cm) were collected at five time points (0, 2, 6, 24 and 48 h) after Al^{3+}-treatment, washed with distilled water and quickly placed in liquid nitrogen and stored at -80 °C until analysis.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from the root apices according to Gambiano et al. [26]. Two biological replicates were performed at each time point. To eliminate any contamination with genomic DNA, the total RNA was treated with RNAse-free DNAase I (Invitrogen). First strand cDNA was synthesized from 1.5 µg of total RNA using 200 U of Superscript II reverse transcriptase (Invitrogen) and 1 µl biotinylated oligo-dT_{25} (700 ng/ml). The double-stranded cDNA was synthesized using 50 U of *E. coli* DNA polymerase I, 15 U of *E. coli* ligase and 1.6 U of RNAse-H at 12 °C for 1 h and then at 22 °C for 1 h. The cDNA was purified using the Qiaquick PCR purification kit (Qiagen).

cDNA-AFLP analysis

The cDNA-AFLP analysis was undertaken basically as described by Bachem et al. [24] with some modifications described in Aquea et al. [27]. Selective amplification products were resolved in a 4.5% polyacrylamide sequencing gel at 120 W for 4 h and detected

by autoradiography performed in cassettes with an intensifier screen using Clear Blue X-Ray Film and stored at -80 °C. In the gel, AFLP products ranged from 100-800 bp. For each primer combination, an average of 40 bands were observed in this size window. For each combination of primers, the same TDF patterns were observed in both biological replicates.

Isolation and sequencing of TDFs

The TDFs which were differentially-expressed between genotypes were excised from the polyacrylamide gels and reamplified by PCR using 1 µl of the eluted sample as template with the same combination of primers used during the second round of amplification with the conditions described for the pre-amplification reactions. The resulting PCR products were cloned in pGEM-T EASY (Promega, Madison-Wisconsin, USA) and sequenced (Macrogen Inc., dna.macrogen.com). To identify the corresponding genes, the sequence of each TDF was searched against all sequences in the non-redundant databases using the BLASTN, BLASTX and TBLASTX algorithms the TIGR gene index (www.tigr.org) and in GENBANK (NCBI). The sequences were manually assigned to functional categories based on the analysis of scientific literature, TAIR (www.arabidopsis.org) and the Gene Ontology Consortium (www.geneontology.org).

Real-time RT-PCR analysis

Validation of the pattern of expression of 12 TDFs was confirmed by quantitative real time RT-PCR (qRT-PCR). The primers were designed using Amplifx 1.4.5 software. The list of candidate genes and their respective primer pairs are shown in Table 1. As a housekeeping gene, the expression of *metallothionein* was used, as previously described [28]. All reactions were performed in triplicate. Quantification of expression was performed using a Mx 3000 p^{TM} Real-Time PCR System (Stratagene). The PCR amplification conditions were 95 °C for 3 min, followed by 40 cycles at 95 °C for 20 s, 56 °C for 20 s, 72 °C for 30 s. The dissociation curves were generated for each reaction to ensure specific amplification. Threshold values (Ct), which represent the PCR cycle at which fluorescence passes the threshold, were generated using the MxPro^{TM} qPCR software for the Mx 3000 p^{TM} System. Gene expression data (Ct values) were employed to quantify relative gene expression using the comparative $2^{-\Delta\Delta CT}$ method [29].

Table 1. Primers used for real-time-PCR analysis of *Vaccinium corymbosum* L. *VCAL* genes.

Gene Name	Forward primer (5'-3')	Reverse primer(5'-3')
VCAL6	TCGGTCGACTCTGAAGTGCT	CATGACACGTACACGGACAAAG
VCAL19	TGCTGATGGGAATGGGACTATA	CTTGTCGAACACCCGGAAAG
VCAL25	GTGATCTGCCCAATGCAATGAACG	TGTTGCGCAGGTGCTCTGAATA
VCAL30	GCCGTCTGAATCTCCCGAGAAGTA	GGCCATGGGGATCATGAACAGTTT
VCAL32	TAGTGATCTCCAGCCGGGTCAAAT	TCAAGCACTTCTCGAGTCTCCTTC
VCAL38	TCTGGTGTGCAGGTTGCTATCT	GCGTACGGGCATGTTCACTA
VCAL47	AGTCTCCAGCGAAGGTCAAATCAC	AAGGATGGGAGGCATGTAGTCAGA
VCAL50	CTCTCTTGACACGGTGGAGATT	GTCAGCTGCATCTTGAACGGTA
VCAL59	AATTGGCCAAGGAACCGTCATC	ATGAGTCCTGAGTAACCCAGCAAC
VCAL85	TGGGTGATGTTCTCGGTGCATTGA	GATGAGTCCTGAGTAACGCGGTTT
VCAL90	GCAGTCTGTCTTCAATGCCCACTA	TTGAGCTACTTCCTCACCAAC
VCAL99	ATGCGGTCATGGGTCTGATTCAAG	TTGGTTGCCGCAGTCGATATTG

Statistical analysis

A one-way ANOVA ($P<0.05$) was carried out to evaluate differences between the treatment and genotypes, followed by a Tukey test for comparisons with a 95% confidence level.

Results

Identification of genes induced by aluminum-stress in blueberry

To identify genes responsive to Al^{3+} stress, cDNA-AFLP analysis was performed on roots of the Brigitta (Al^{3+}-resistant) and Bluegold (Al^{3+}-sensitive) cultivars subjected to Al^{3+}-stress. The differentially expressed fragments were investigated by selective amplification using 28 primer combinations. To avoid false-positive fragments, the experiment was carried out using two biological replicates. More than 1200 bands were generated and all the bands with sizes ranging from 120 to 720 bp were analyzed and compared in all five time treatments (0, 2, 6, 24 and 48 h) in both genotypes. Five different banding patterns were observed, as illustrated in Figure 1: A) TDFs that are induced in later time points; B) Non-expressed TDFs; C) TDFs that are induced in early time points; D) TDFs that are repressed and E) Constitutively-expressed TDFs. The clones corresponding to different TDFs were named as *VCAL* for *Vaccinium corymbosum Aluminum*, following by and identification number.

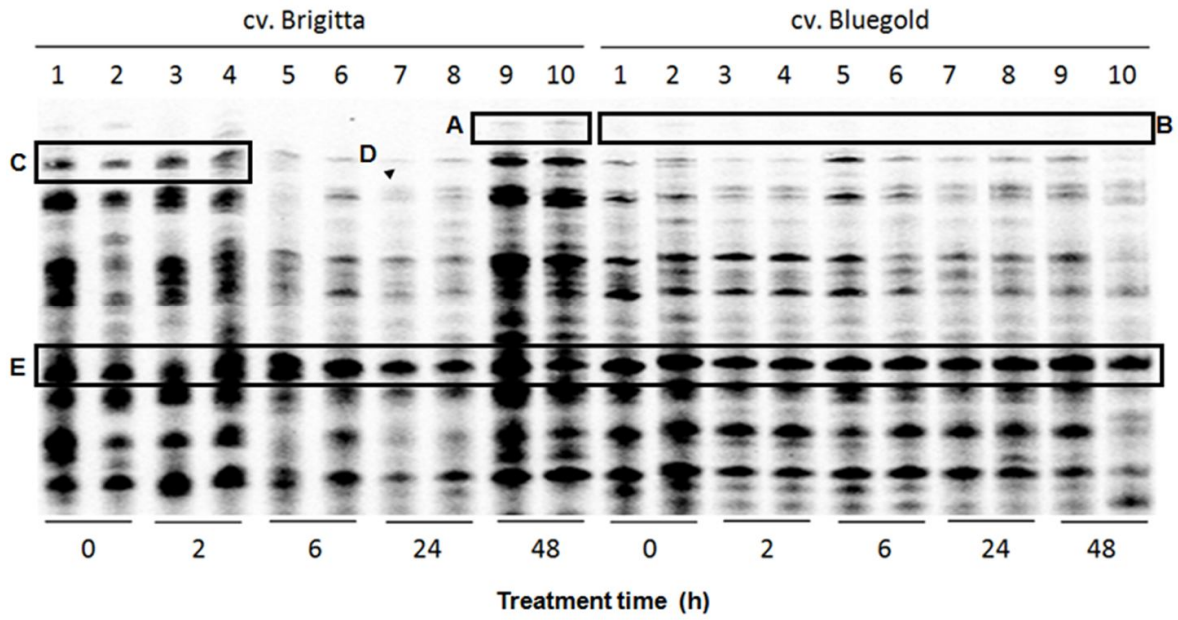


Figure 1. Autoradiogram of the cDNA-AFLP results showing the transcript derived fragments (TDFs) induced or repressed after 0, 2, 6, 24 and 48 hours of Al-treatment in two cultivars of highbush blueberry (Brigitta, Al-resistant and Bluegold, Al-sensitive). The reaction products were derived from independent non-selective pre-amplifications and generated using selective primers Bst-TC/Mse. See text for an explanation of A-E.

Sequence analysis of cDNA clones

To understand the molecular mechanism of Al^{3+}-stress in *V. corymbosum*, the TDFs were isolated for sequence and expression pattern analysis. The more abundant up-regulated and down-regulated transcripts were selected from the autoradiographic films. These *VCAL* fragments were re-amplified and sequenced and their identities were assigned using the TIGR and NCBI GenBank databases. This analysis revealed a total

of 70 unique TDF sequences. The TDF name, and the size, homology and accession numbers of the *VCAL* fragments identified are shown in Table 2. Of the sequences identified, 27 TDFs do not show homology in the databases and were classified as "no match". Forty-three TDFs show significant homology with known or putative proteins and ESTs deposited in the databases (E value $<10^{-4}$). Of these, 31 are homologous with proteins of known function and 12 to genes that code for proteins with unknown functions. Nine *VCALs* are homologous to *V. corymbosum* sequences and 2 to sequences annotated or described in plants of the Ericaceae family. The remaining 34 TDFs are homologous to genes annotated in other plant species.

The genes encoding proteins of known functions were classified in 8 potential functional categories according to the scientific literature and gene annotations from the Gene Ontology Database. Figure 2 shows the percentages of *VCALs* assigned to different functional categories. Most of the *VCALs* (38.6%) correspond to fragments

Table 2. Sequence homology of Al-regulated TDFs in blueberry, with sequences deposited in the database

TDF clone	Accession #	Length (pb)	Homology[a]	(%)[c]	BLAST score[d]
			Cellular metabolism		
VCAL1	HO054805	500	40S ribosomal protein [*Rhododendron catawbiense*] (CV015045)	75	1.7 e-53
VCAL17	HO054973	298	40S ribosomal protein [*Vaccinium corymbosum*] (CF810807)	70	3.6 e-21
VCAL5	HO054791	311	ATP citrate lyase [*Juglans hindsii* x *Juglans regia*] (EL900206)	82	2.7 e-33
VCAL9	HO054808	143	Actin-2 [*Sorghum bicolor*] (AW285316)	71	1.3 e-06
VCAL32	HO054819	169	10-hydroxygeraniol oxidoreductase [*Helianthus annuus*] (TA160144232)	75	1.5 e-08
VCAL39	HO054823	110	Protein binding protein [*Ricinus communis*] (XM002521941.1)	84	2 e-21
VCAL51	HO054827	143	RBX1-like protein [*Petunia integrifolia*] (TA476285681)	80	2.5 e-30
VCAL78	HO054837	238	Ubiquitin C variant [*Theobroma cacao*] (CA795100)	66	2.3 e-11
VCAL80	HO054839	302	Ubiquitin C variant [*Ipomoea batatas*] (TA28054120)	75	3.6 e-30
VCAL81	HO054840	252	Actin-binding [*Vaccinium corymbosum*] (TA67469266)	88	2.7 e-38
VCAL85	HO054842	376	ETO1-like protein 1 [*Malus x domestica*] (CN857381)	68	2.5 e-08
VCAL88	HO054843	552	Fructose-bisphosphate aldolase [*Vaccinium corymbosum*] (TA70469266)	99	5.2 e-127
			Stress response		
VCAL6	HO054792	191	S-adenosylmethionine decarboxylase [*Cyclamen persicum*] (AJ887644)	64	1.9 e-07
VCAL21	HO054812	193	Glutathione *S*-transferase GST 14 [*Glycine max*] (TA582483847)	77	2.3 e-15
VCAL38	HO054822	363	Vacuolar H^+-pyrophosphatase [*Prunus persica*] (AF367447.1)	82	1 e-38
VCAL68	HO054833	223	Aldehyde dehydrogenase [*Vitis vinifera*] (DQ150259.1)	83	4 e-57
VCAL90	HO054844	320	Aspartic proteinase [*Camellia sinensis*] (CV013914)	81	2.0 e-51
VCAL99	HO054849	419	Endochitinase A precursor [*Solanum lycopersicum*] (TA361774081)	81	1.2 e-35
VCAL124	HO054860	299	Putative disease resistance [*Arabidopsis thaliana*] (AB425274.1)	93	9 e-119
VCAL125	HO054861	501	Putative disease resistance [*Arabidopsis thaliana*] (AB425273.1)	85	3 e-135
VCAL163	HO054867	399	Anthranilate N-benzoyltransferase [*Euphorbia esula*] (TA127083993)	70	3.9 e-35
			Transcription		
VCAL30	HO054818	257	Histone H2B.1 [*Fragaria x ananassa*] (DV438603)	70	1.5 e-19
VCAL144	HO054866	108	Basic leucine zipper BZIP [*Arabidopsis thaliana*] (TA364323702)	78	5.9 e-08
			Transport		
VCAL25	HO054815	136	ARF-like [*Salvia miltiorrhiza*] (HM051059.1)	84	1 e-42
VCAL50	HO054826	261	Plastid acyl carrier protein [*Camellia oleifera*] (EU717697.1)	87	3 e-78
VCAL82	HO054841	201	Putative plasma membrane intrinsic [*Ricinus communis*] (TA11803988)	69	2.3 e-09
			Signal transduction		
VCAL19	HO054811	511	Calmodulin (Cam) mRNA [*Ricinus communis*] (XM002527338.1)	88	2 e-138
VCAL47	HO054825	457	Phospholipase PLDa1 [*Solanum tuberosum*] (CK860893)	64	6.5 e-22

VCAL61	HO054829	452	F-box family protein [*Populus trichocarpa*] (XP002304470.1)	74	2 e-48
			Photosynthesis and energy		
VCAL27	HO054816	528	Photosystem I subunit XI [*Rhododendron catawbiense*] (TA230257784)	87	1.9 e-86
VCAL59	HO054828	127	Peptidyl-prolyl isomerase FKBP12 [*Camellia sinensis*] (CV014093)	83	1.8 e-17
			Unknown protein		
VCAL2	HO054806	320	Unknown protein [*Capsicum annuum*] (BM063365)	66	7.7 e-23
VCAL8	HO054807	126	Unknown protein [*Vaccinium corymbosum*] (CF811488)	87	3.1 e-15
VCAL10	HO054809	212	Unknown protein [*Vaccinium corymbosum*] (CF810890)	69	4.3 e-12
VCAL18	HO054810	431	Unknown protein [*Vitis vinifera*] (TA81769266)	97	1.8 e-58
VCAL29	HO054817	305	Unknown protein [*Nicotiana tabacum*] (EB451503)	76	1.6 e-32
VCAL37	HO054821	309	Unknown protein [*Vitis vinifera*] (EE085586)	73	2.0 e-27
VCAL73	HO054835	278	Unknown protein [*Vaccinium corymbosum*] (TA76969266)	97	4.7 e-53
VCAL79	HO054838	223	Unknown protein [*Solanum tuberosum*] (DV625248)	65	1.3e-07
VCAL123	HO054859	243	Unknown protein [*Vaccinium corymbosum*] (TA90169266)	65	7.7e-10
VCAL128	HO054863	179	Unknown protein Expressed protein [*Camellia sinensis*] (TA4544442)	69	1.1e-10
VCAL129	HO054864	251	Unknown protein [*Vaccinium corymbosum*] (TA76969266)	99	7.5e-50
VCAL130	HO054865	235	Unknown protein [*Vaccinium corymbosum*] (CF810562)	73	5.3 e-19
			No match[b]		
VCAL7	HO054868	273	No match	-	-
VCAL11	HO054869	766	No match	-	-
VCAL14	HO054870	264	No match	-	-
VCAL15	HO054871	667	No match	-	-
VCAL16	HO054804	147	No match	-	-
VCAL20	HO054872	578	No match	-	-
VCAL22	HO054813	390	No match	-	-
VCAL26	HO054873	355	No match	-	-
VCAL28	HO054874	465	No match	-	-
VCAL33	HO054875	689	No match	-	-
VCAL41	HO054876	583	No match	-	-
VCAL42	HO054877	501	No match	-	-
VCAL44	HO054878	389	No match	-	-
VCAL49	HO054879	835	No match	-	-
VCAL46	HO054803	321	No match	-	-
VCAL53	HO054880	883	No match	-	-
VCAL54	HO054881	506	No match	-	-

VCAL67	HO054882	557	No match	-	-
VCAL69	HO054883	867	No match	-	-
VCAL70	HO054884	830	No match	-	-
VCAL71	HO054885	950	No match	-	-
VCAL74	HO054886	899	No match	-	-
VCAL83	HO054887	498	No match	-	-
VCAL91	HO054888	489	No match	-	-
VCAL94	HO054796	470	No match	-	-
VCAL95	HO054797	663	No match	-	-
VCAL106	HO054889	539	No match	-	-

[a]GenBank accession numbers of the sequences homologous to cDNA-AFLP fragments are in parentheses.

[b]No significant sequence homology found in the genome, EST and protein database.

[c]Percentage of similarity between VCAL and their homologue sequence.

[d]All are BLASTN scores.

without homology in databases, while the 17.1% of the VCALs is homologous to genes that codify unknown proteins. Among all of VCALs with known function, the most of them are homologous to genes involved in cellular metabolism (17.1%).

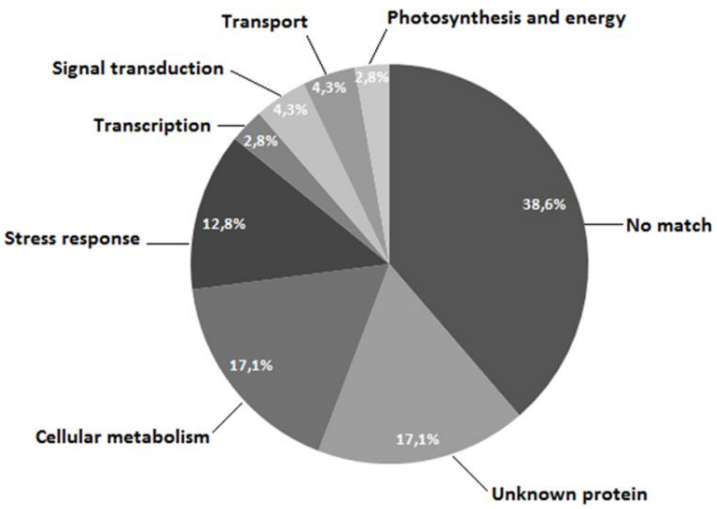
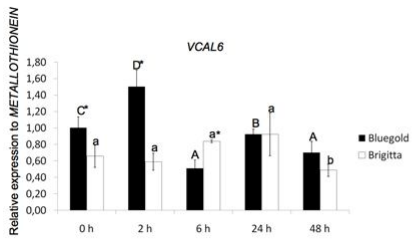


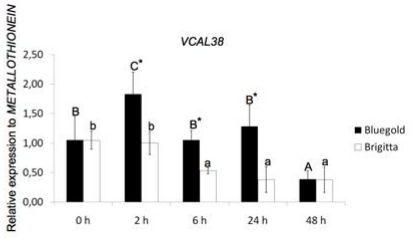
Figure 2. Distribution of differentially-expressed TDFs under Al-stress in blueberry. A total of 70 unique cDNA-AFLP fragments were grouped into eight functional categories and classified on the basis of their homology to sequences deposited in the databases.

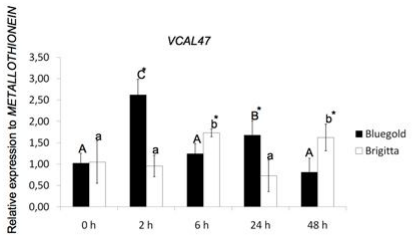
Validation of representative genes by real-time RT-PCR

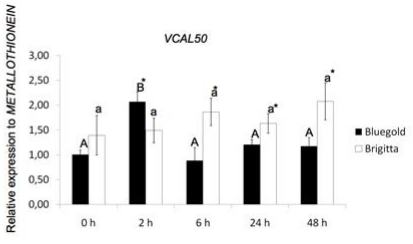
Twelve TDFs were selected to validate the results of the cDNA-AFLP analysis by real-time RT-PCR: 4 related to oxidative stress functions (*VCAL6*, *VCAL38*, *VCAL90* and *VCAL99*); 2 related to cellular metabolism (*VCAL32* and *VCAL85*), 2 related to signal transduction (*VCAL19* and *VCAL47*), 2 related to transport (*VCAL25* and *VCAL50*), the *VCAL30* homologue of histone H2B and the *VCAL59* homologue of peptidyl-prolyl

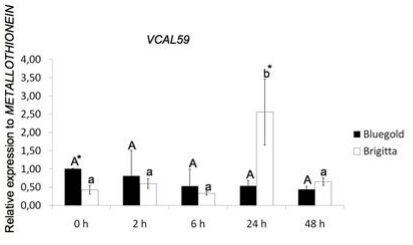
isomerase, FKBP12 (Table 1). Under our experimental conditions, four differential expression patterns were observed using qRT-PCR (Figure 3): a) TDFs induced at early time points in the sensitive genotype and then repressed at later time points (*VCAL6*, *VCAL38*, *VCAL47*and *VCAL50*); b) TDFs induced at early time points in the resistant genotype (*VCAL19*, *VCAL32* and *VCAL99*); c) TDFs which are initially strongly-repressed and then induced at later time points in the resistant genotype (*VCAL59* and *VCAL85*) and d) TDFs induced in both genotypes but with significant increases in expression levels only in the resistance genotype (*VCAL25*, *VCAL30* and *VCAL90*). The four expression patterns observed by qRT-PCR confirm the profiles observed in the cDNA-AFLP assay.

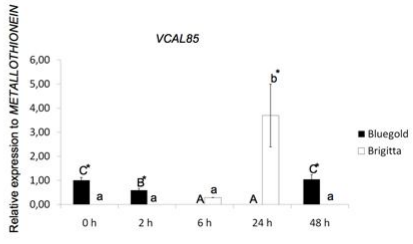

VCAL6
Relative expression to METALLOTHIONEIN
Bluegold
Brigitta
0 h
2 h
6 h
24 h
48 h

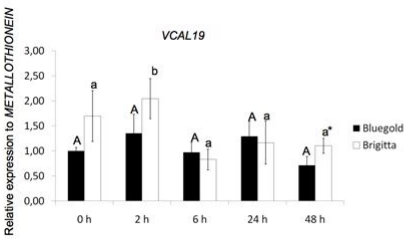

VCAL38
Relative expression to METALLOTHIONEIN
Bluegold
Brigitta
0 h
2 h
6 h
24 h
48 h

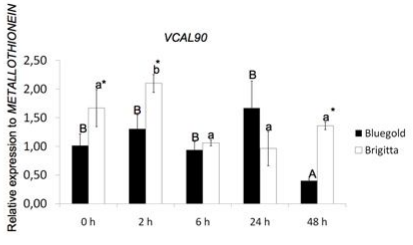

VCAL47
Relative expression to METALLOTHIONEIN
Bluegold
Brigitta
0 h
2 h
6 h
24 h
48 h

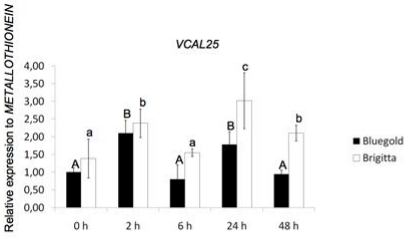

VCAL50
Relative expression to METALLOTHIONEIN
Bluegold
Brigitta
0 h
2 h
6 h
24 h
48 h

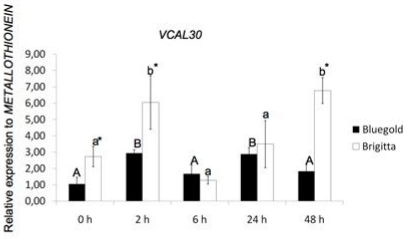

VCAL59
Relative expression to METALLOTHIONEIN
Bluegold
Brigitta
0 h
2 h
6 h
24 h
48 h

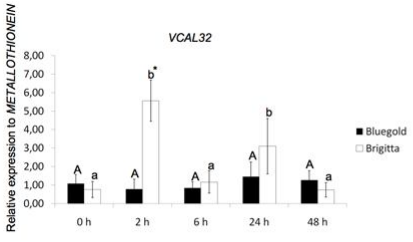

VCAL85
Relative expression to METALLOTHIONEIN
Bluegold
Brigitta
0 h
2 h
6 h
24 h
48 h


VCAL19
Relative expression to METALLOTHIONEIN
Bluegold
Brigitta
0 h
2 h
6 h
24 h
48 h


VCAL90
Relative expression to METALLOTHIONEIN
Bluegold
Brigitta
0 h
2 h
6 h
24 h
48 h


VCAL25
Relative expression to METALLOTHIONEIN
Bluegold
Brigitta
0 h
2 h
6 h
24 h
48 h


VCAL30
Relative expression to METALLOTHIONEIN
Bluegold
Brigitta
0 h
2 h
6 h
24 h
48 h


VCAL32
Relative expression to METALLOTHIONEIN
Bluegold
Brigitta
0 h
2 h
6 h
24 h
48 h

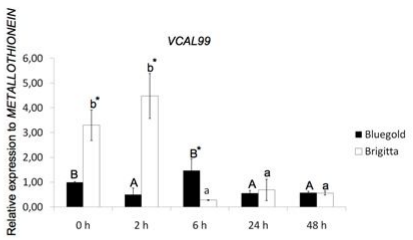

VCAL99
Relative expression to METALLOTHIONEIN
Bluegold
Brigitta
0 h
2 h
6 h
24 h
48 h

Figure 3. Expression analyses of 12 genes obtained from cDNA-AFLP fingerprinting by means of quantitative real-time PCR. Three independent biological replications were performed. All data were normalized to *metallothionein* expression levels [66]. Capital letters show significant differences in the susceptible cultivar (Bluegold) and lowercase letters show significant differences in the resistant cultivar (Brigitta). The asterisks show significant differences between cultivars with ($p < 0.05$) according to the Tukey test.

Discussion

During Al^{3+}-stress, a set of 70 differentially-expressed TDFs in blueberry were identified using cDNA-AFLP analysis. Identified transcripts were sequenced, annotated and classified into functional categories. These TDFs were the subject of this study and several of the sequences identified are putative new genes in *V. corymbosum*, which have been deposited in GenBank (Table 2). All TDFs were assigned to independent functional categories using Gene Ontology and the scientific literature. Overall, 46% of the 70 Al^{3+}-responsive genes were homologous to genes of known function and could thus be categorized in differents functions. The remaining 54% corresponded to unknown proteins and those with no match. This analysis did not identify homologous genes involved in the exclusion mechanism (ALMT1 and MATE), mainly due to the experimental strategy and the number of primers combination used. With our results we cannot rule out this mechanism in blueberry and further experiments are needed to clarify this point.

It is well known that the first target of Al-toxicity in plants is the inhibition of root growth [30]. Root growth depends on two basic developmental processes, cell division and elongation of cells in the root apical meristem, reviewed in Scheres et al. [31]. Root growth is regulated by phytohormones such as auxin, cytokinin, giberellin and ethylene [32]. Al^{3+} rapidly stimulates ethylene biosynthesis, induces a decrease in cytokinin levels and inhibits root growth in *Phaseolus vulgaris* [33]. Recently, it has been reported that Al^{3+}-induced inhibition of root elongation is mediated by ethylene and auxin in Arabidopsis [34]. Application of exogenous auxin increases root meristem size and ethylene regulates root growth by both stimulating auxin biosynthesis and by modulating the auxin transport machinery [32]. In our work, we identified *VCAL85* as an ETO1-like protein 1 which is strongly-expressed in the Al-resistant genotype after 24 h (Figure 3). ETO1 negatively regulates ethylene synthesis via its ability to target 1-aminocyclopropane-1-carboxylic acid synthase (ACS) for breakdown [35]. *VCAL51* is homologous to genes encoding RING-H2 proteins related to human RBX1. Arabidopsis RBX1 is an SCF subunit and reduced RBX1 levels result in severe defects in growth associated with a reduced auxin response [36]. *VCAL51* is expressed equally in both cultivars, but is strongly expressed soon after Al^{3+}-treatment in the cv. Brigitta that could be the result of Al^{3+}-toxicity. These results suggest that cv. Brigitta produces less ethylene and has a better auxin response in comparison to the cv. Bluegold.

In several higher plants, it has been reported that Al^{3+} could disturb cellular metabolism by disrupting Ca^{2+} homeostasis as a result of the known antagonism between Al^{3+} and Ca^{2+} [37]. Kurita et al. [38] reported that there is an interaction

between calmodulin (CaM) and Al^{3+} and suggested that Al^{3+} may affect the Ca^{2+} signaling pathway in cells. In our study, *VCAL19*, which is homologous to CaM, was induced by Al^{3+}-stress in both genotypes, but is strongly induced in the cv. Brigitta at 0-2 h (Figure 3), suggesting that it may play a role in the resistant genotype.

In *Oryza sativa*, it has been reported that putrescine accumulation is a factor causing root growth inhibition under Al^{3+}-stress [39]. The overexpression of *spermidine synthase* in European pear enhanced levels of spermidine and alleviated oxidative stress caused by Al^{3+} [40]. In blueberry, *VCAL6* is homologous to *S*-adenosylmethionine decarboxylase and is highly induced in the early hours in the Al^{3+}-sensitive genotype. This gene is involved in the synthesis of polyamines such as putrescine, spermidine and spermine, which alleviate Al^{3+} toxicity by possibly lowering the Al^{3+} content in the root tips, and subsequently reducing lipid peroxidation and oxidative stress [41].

On the other hand, *VCAL38* which is homologous to a vacuolar H^{+}-pyrophosphatase is expressed in both Brigitta and Bluegold cultivars in the initial hours (0-2 h), and expression levels subsequently fall (6, 24, 48 h). This protein plays an important role in resistance to drought and it has been suggested as a potential target for genetic engineering of root systems in crop plants [42]. A proton pump similar to the vacuolar H^{+}-ATPase (V-ATPase) was discovered in an Al^{3+}-resistant cultivar of *Triticum aestivum* [43], and has been described as a component of the Al^{3+}-stress response, with the ATP required for its activity supplied by ATP synthase [44]. The importance of this gene in blueberry requires further study

In the category transport, *VCAL25* was identified. This TDF is homologous to ADP-ribosylation factors (ARFs), a subfamily of the Ras superfamily of GTP-binding proteins that regulate diverse processes in eukaryotic cells such as signal transduction, cell proliferation, cytoskeletal organization, and intracellular membrane trafficking [45]. *VCAL25* is expressed in both genotypes, although the expression level is significantly higher in the Al^{3+}-resistant cultivar. A GDP dissociation inhibitor gene derived from tobacco (*NtGDI1*), when overexpressed in *Saccharomyces cerevisiae* confers Al^{3+} tolerance [46]. Additionally, it was proposed that overexpression of the NtGDI1 protein in Arabidopsis activates an Al-efflux system that protects against Al^{3+}-toxicity [13].

The finding that a number of the sequences isolated show no significant similarity with any sequences in the public databases is interesting. These sequences that do not have homology to those in the database could correspond to 3'UTRs, new coding sequences or non-coding intergenic sequences. Recent evidence indicates that the majority of sequences in eukaryotic genomes are transcribed [47] suggesting that thousands of novel genes and transcripts have not yet been annotated. These sequences have been defined as transcriptional forests, that is, regions of the genome that present a complex array of sense and anti-sense, coding and non-coding transcripts [48]. These results provide a new set of genes of potential interest to unravel further the molecular mechanisms of plant Al^{3+}-responses.

In summary, the cDNA-AFLP analysis allowed genes to be identified whose expression is modulated by Al^{3+} in blueberry. This study reveals that a multitude of processes are implicated in determining the response to Al^{3+} and that these processes

require the activation of different genes. Detailed characterization of several genes, including putative novel genes and genes of unknown function, which may be involved in specific processes, will help to discover the fine networks underlying heavy metal accumulation and tolerance in plants.

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Chapter IV

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

Claudio Inostroza-Blancheteau, Marjorie Reyes, Felipe Aquea, Adriano Nunes-Nesi, Miren Alberdi, Patricio Arce-Johnson

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

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Keywords: Blueberry, Aluminium stress, Antioxidant gene, Fluorescence parameters.

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.

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 impact 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 (resistance 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 nonphotochemical 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.

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), catalase (CAT, E.C.1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11), glutathione reductase (GR), gluthatione *S*-transferase (GST, E.C. 2.5.1.18) and aldehyde dehydrogenases (ALDH, EC 1.2.1.3), as well as nonenzymatic compounds of low molecular weight, such as 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-tolerant (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 impact 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. 2011).

Materials and methods

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.

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 to 100% v/v) and embedded in Epon 812. Sections (1 to 2 µm) of root tips were stained with toluidine blue and finally examined by microscopy (Nikon Eclipse 80i), according to [21] with some modifications.

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 (9,000 µmol m^{-2} s^{-1}). After 10 seconds, 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 had 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; ΦPSII = (Fm'-Fs)/Fm' is the indicator of the effective quantum yield of PSII; ETR = PPF x 0.5 x ΦPSII x 0.84 [22]; NPQ = (Fm - Fm')/Fm' [23].

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].

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.

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-dT25 (700 ng mL^{-1}).

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 Stransferase* (*GST*) and *VCAL68* is homologous to *aldehyde dehydrogenases* (*ALDH*). The sequences of these TDFs have been deposited in GenBank (HO054812, *VCAL21*; HO054833, *VCAL68*). The relative quantification of *VCAL21* and *VCAL68* expression was determined by qRT-PCR. The primers were designed using Amplified 1.4.5. The specific primers used were: VCAL21-F 5'-GAGGAAGTTGGGTCCATGAAAAT-3' and VCAL21-R 5'-CGGCGGTAACTTGTCCTTGA-3'; VCAL68-F 5'-AGGCTCCAAAGGCTTCTACATCCA-3' and VCAL68-R 5'-ACCGGGCCGAAGATTTCATCTTGT-3', which amplify 120 bp fragments of *VCAL21* and *VCAL68*. All experiments were performed with three biological replicates and two technical replicates. As a housekeeping gene, the expression of *metallothionein* was used, as previously described by Naik et al. [31] for highbush blueberry. PCR amplification was performed in a 25 µl-reaction containing 12 µl SensiMixTM Plus SYBR® (Quantace), 2 µl cDNA and 0,5 µl of each primer (10 µmol). Cycling conditions were 95 °C for 3 min followed by 40 cycles at 95 °C for 20 s, 56 °C for 20 s and 72 °C for 30 s. Dissociation curves were generated for each reaction to ensure specific amplification. Threshold values (Ct), which represent the PCR cycle at which fluorescence passes the threshold, were generated using the MxProTM qPCR

software of the Mx 3000 pTM System. Gene expression data (Ct values) were employed to quantify relative gene expression using the comparative $2^{-\Delta\Delta Ct}$ method [32].

Statistical analysis

To test significant differences in gene expression between treatments and genotypes, one-way ANOVA was performed ($P<0.05$), using Skewness, Kurtosis and Omnibus tests for normality, and the Modified-Levene Equal-Variance Test for homogeneity of variances. Statistical analyses were carried out using the NCSS software (Number Cruncher Statistical System, Kaysville, Utah, USA). When differences in the means were significant, a Tukey's test was performed with 95% confidence level.

Results

Ultrastructural changes in root tip cells caused by Al

The main target of Al toxicity in plants is the roots. Therefore, we performed histological analyses to monitor the structural alterations in root tips of an Alresistant (Brigitta) and an Al-sensitive (Bluegold) genotype of blueberry (Figure 1). The roots of the Al-tolerant genotype did not exhibit notable anatomical modifications after Al treatments (Figure 1a, b). However, the Al-sensitive genotype growing without Al possessed uniformly-stained cells, whereas in the Al-treated plants the root cells had a disintegrated peripheral region, with narrower cell walls in the central region compared with control root tips (arrows, Figure 1c, d). Thus, this experiment showed that root tip cells, particularly those

of the epidermis of a sensitive genotype subjected to an Al concentration of 100 µM, were seriously affected.

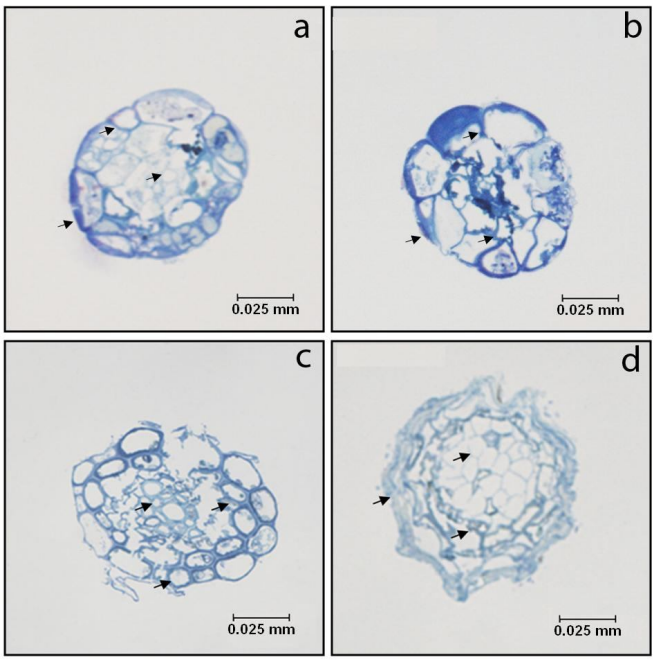

Figure 1. Effect of Al-stress on root-tip structure of Brigitta (Al-resistant) and Bluegold (Al-sensitive) blueberry genotypes, grown for 48 h in 0 and 100 µM $AlCl_3$ in 0.5 mM $CaCl_2$ solution. (a) Brigitta without Al; (b) Brigitta with Al; (c) Bluegold without Al and (d) Bluegold with Al. The arrows indicate the effects of Al on root tip cells (see text for details). Scale bars represent: 25 µm.

Fluorescence parameters of PSII

To determine the physiological impact of Al stress on different blueberry genotypes, we evaluated the *in vivo* chlorophyll fluorescence parameters over a period of 48 h. The maximum quantum yield of PSII (Fv/Fm) was close to 0.8 at the start of the experiment in plants of both genotypes (Figure 2), a figure which is typically observed in leaves of unstressed plants [23]. The plants of both cultivars subjected to Al-stress treatment did not show any difference in Fv/Fm during the first 6 h of stress (Figure 2). However, the Al-sensitive genotype experienced a significant decrease in Fv/Fm after 24 h of exposure to Al (Figure 2).

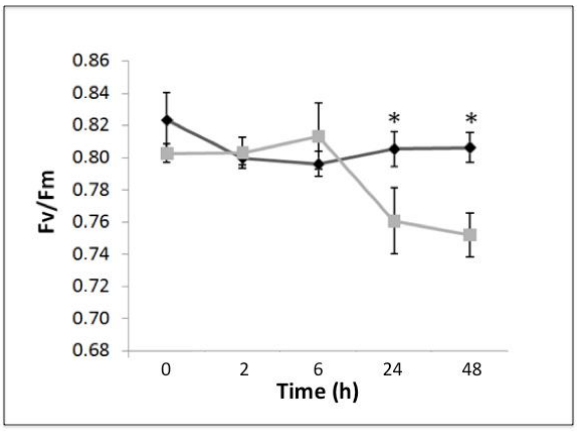

Figure 2. Effect of Al-stress on the maximum quantum yield (Fv/Fm) in leaves of Brigitta (Al-resistant, black line) and Bluegold (Al-sensitive, gray line) blueberry genotypes. The data points represent the mean ± SE of at least three replicates. The asterisks indicate significant differences between genotypes ($P < 0.05$).

To gain further insights into the effects of Al stress on photosynthesis, other fluorescence parameters (ΦPSII, ETR and NPQ) were assayed (Figure 3). In the Al-

sensitive genotype (Bluegold), a significant decrease in ΦPSII and ETR values was observed at each time point in the Al-treated plants in comparison with the 0 h controls (Figure 3a and 3c). By the end of the experiment (48 h), the sensitive genotype exhibited a significant reduction (55%) in ΦPSII after Al treatment, whereas this parameter fell by just 16% in the resistant genotype (Brigitta). Similar effects were observed in the ETR in both genotypes (Figure 3c). The NPQ, which indicates the capacity of PSII to dissipate the excess energy as heat, increased significantly in the resistant genotype with respect to the zero time point control, while in the sensitive genotype, NPQ levels diminished significantly in all time points of Al-treatment (Figure 3b).

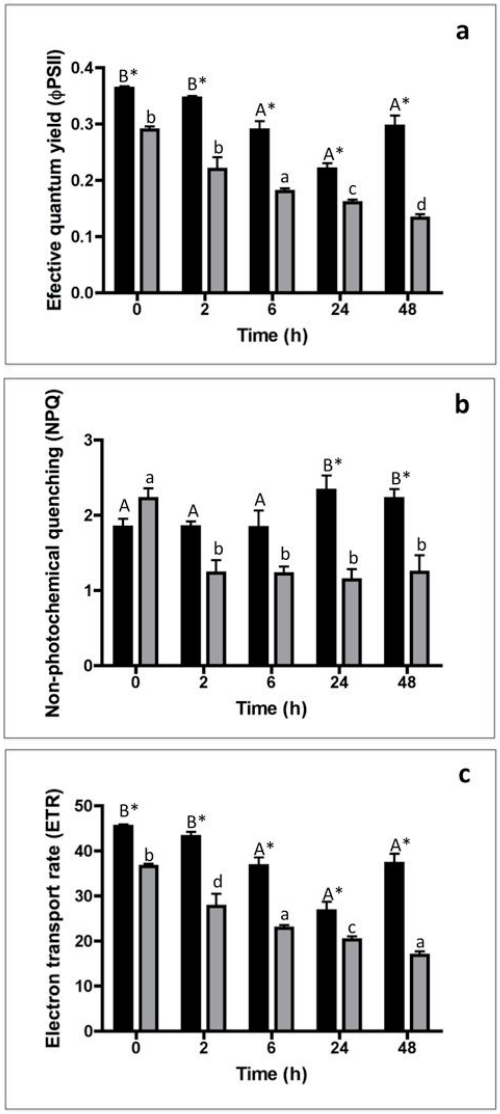

Figure 3. Effect of Al-stress on effective quantum yield (ΦPSII), electron transport rate (ETR) and non-photochemical quenching (NPQ) in leaves of Brigitta (Al-resistant, dark lines) and Bluegold (Al-sensitive, gray lines) blueberry genotypes. The data points represent the mean ± SE of at least three replicates. Different upper case letters indicate significant differences between Al-exposure times for the same genotype and treatment whereas different lower case letters indicate significant differences between Al treatment

for the same genotype and Al exposure time. The asterisks indicate significant differences between genotypes (P< 0.05).

Radical scavenging activity (RSA) and activities of antioxidant enzymes

Like other heavy metals and environmental stresses, presence of Al in the soil solution is known to promote the production of ROS [16]. Therefore, we evaluated the impact of Al stress on radical scavenging activity (RSA) in the two genotypes. Interestingly, the RSA was 2-fold higher in leaves than in roots (Figure 4). However, no differences in the RSA were found between genotypes at each time of Al-treatment, with the exceptions of leaves at 48 h (Figure 4a) and roots at 2 h (Figure 4b) where significant differences ($p<0.05$) were observed.

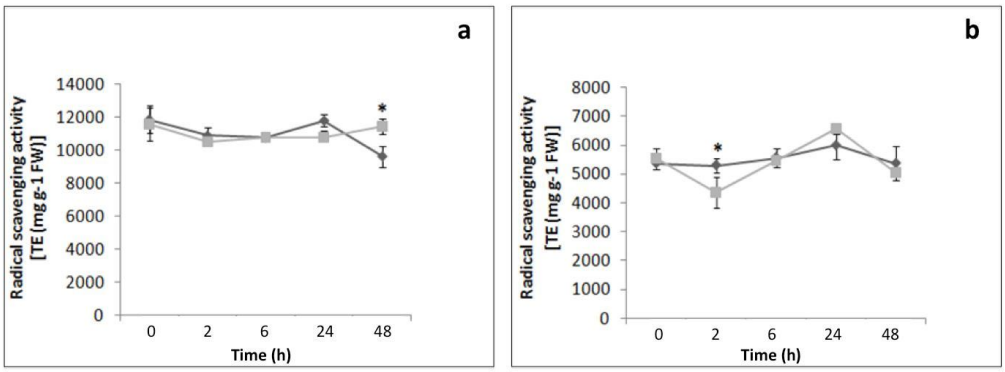

Figure 4. Effect of Al-stress on radical scavenging activity in leaves (a) and roots (b) of Brigitta (Al-resistant, black lines) and Bluegold (Al-sensitive, gray lines) blueberry genotypes. The data points represent the mean ± SE of at least three replicates. RSA was measured as Trolox equivalents (TE) in roots and leaves. The asterisks indicate significant differences between genotypes (P < 0.05).

Additionally, we determined the activity of enzymes related to oxidative stress in roots and leaves. During the time course of the experiments, the activity of SOD in both genotypes in the presence of Al showed interesting changes. The resistant Brigitta genotype exhibited a strong increase (around 4-fold) in SOD activity in leaves after 2 h of exposure, levels which were maintained until 6 h. However, after 24 h, SOD activity returned to the initial levels (Figure 5a). Interestingly, this initial increase in the activity of SOD was not observed in the sensitive genotype, in which activity remained constant (Figure 5a). However, in roots, changes in SOD activity in both genotypes were similar; activity increased by 1.7-fold after 6 h of Al-stress, before diminishing gradually until 48 h (Figure 5b). Subsequently, we decided to verify in leaves if the increase in SOD activity for the resistant genotype occurred in parallel with an increase in CAT activity. Indeed, a significant 2-fold increase in CAT activity was observed during the first 6 h of Al-treatment in the resistant genotype, before falling back to pre-treatment levels after 24 h (Figure 5c). CAT activity did not change during the time course of the experiment in leaves

of the Al-sensitive genotype (Figure 5c). Surprisingly, CAT activity in roots was higher in the resistant genotype, reaching a peak after 24 h of exposure to Al (Figure 5d). Nevertheless, a decrease in CAT activity was observed in the sensitive genotype after 2 h of Al-treatment before reaching values similar to the zero-time point control (Figure 5d).

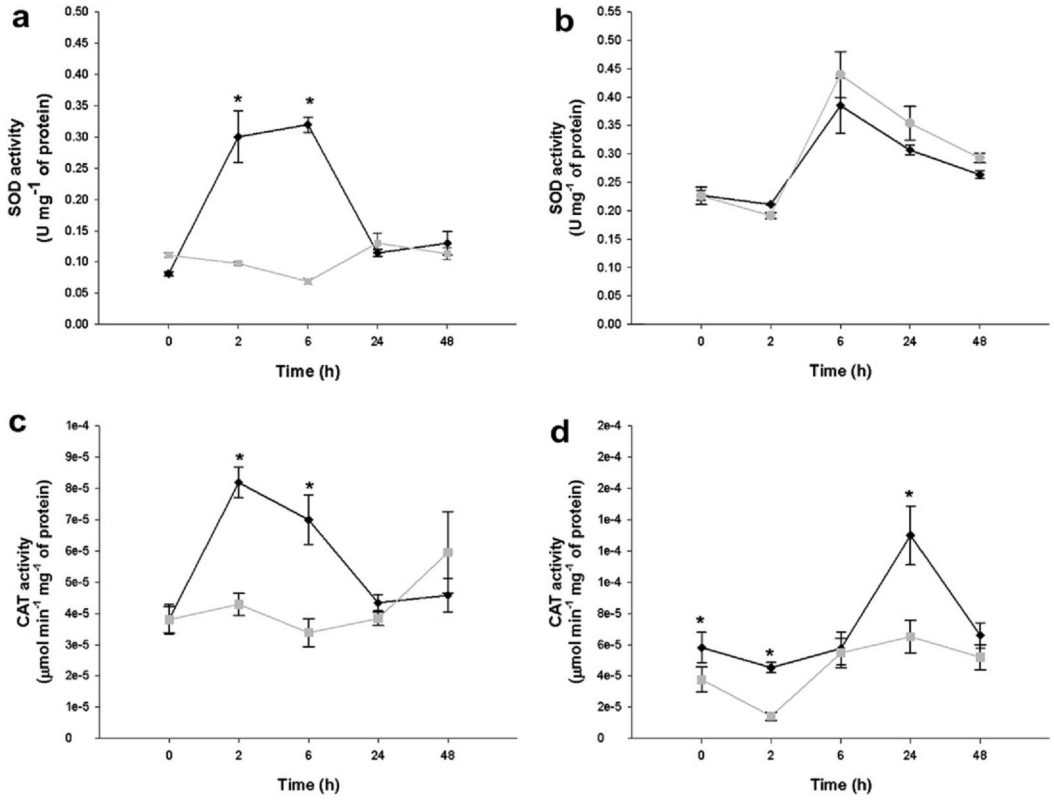

Figure 5. Effect of Al-stress on the activity of SOD in leaves (a) and roots (b), and CAT in leaves (c) and roots (d) of Brigitta (Al-resistant, black line) and Bluegold (Al-sensitive, gray lines) blueberry genotypes. Changes in enzyme activities were compared with the control (time 0 h). The data points represent the mean ± SE of at least three replicates. The asterisks indicate significant differences between genotypes ($P < 0.05$).

Expression analysis of antioxidant genes

Previously, we identified two genes homologous to *glutathione S-transferase* (*GST*) and *aldehyde dehydrogenase* (*ALDH*), here named as *VCAL21* and *VCAL68*, respectively [30]. During environmental stress, GST and ALDH have been described as two important antioxidant genes induced by ROS and lipid peroxidation in higher plants [9].

To evaluate the expression pattern under Al-stress of *VCAL21* and *VCAL68*, we performed qRT-PCR analysis (Figure 6). In this study, we confirmed the expression pattern observed by cDNA-AFLP for *VCAL21* and *VCAL68* in roots. In general terms, both genes showed different expression patterns in leaves and roots. In leaves, we detected a basal expression of both genes in control conditions (time 0 h). *VCAL21* was inhibited by the treatment in both genotypes, and there was a significant difference in their response at 2 h (Figure 6a). The resistant genotype showed greater expression of *VCAL68* in leaves after 6, 24 and 48 h of treatment in comparison to the sensitive genotype (Figure 6c). Interestingly, the expression of *VCAL21* and *VCAL68* in roots was significantly different in both genotypes (Figure 6b and 6d). Unlike the resistant genotype, a significant increase in the expression of *VCAL21* was observed after 2 h in the sensitive genotype, whereas in the resistant cultivar, the expression of *VCAL21* was higher after 24 h of Al treatment (Figure 6b). A similar pattern was also observed for *VCAL68* expression, which had higher transcript levels detected after 2 h in both genotypes, especially in Bluegold (Al-sensitive; Figure 6d). Subsequently, *VCAL68* expression peaked again after 24 h in the sensitive genotype, before falling abruptly after 48 h (Figure 6d).

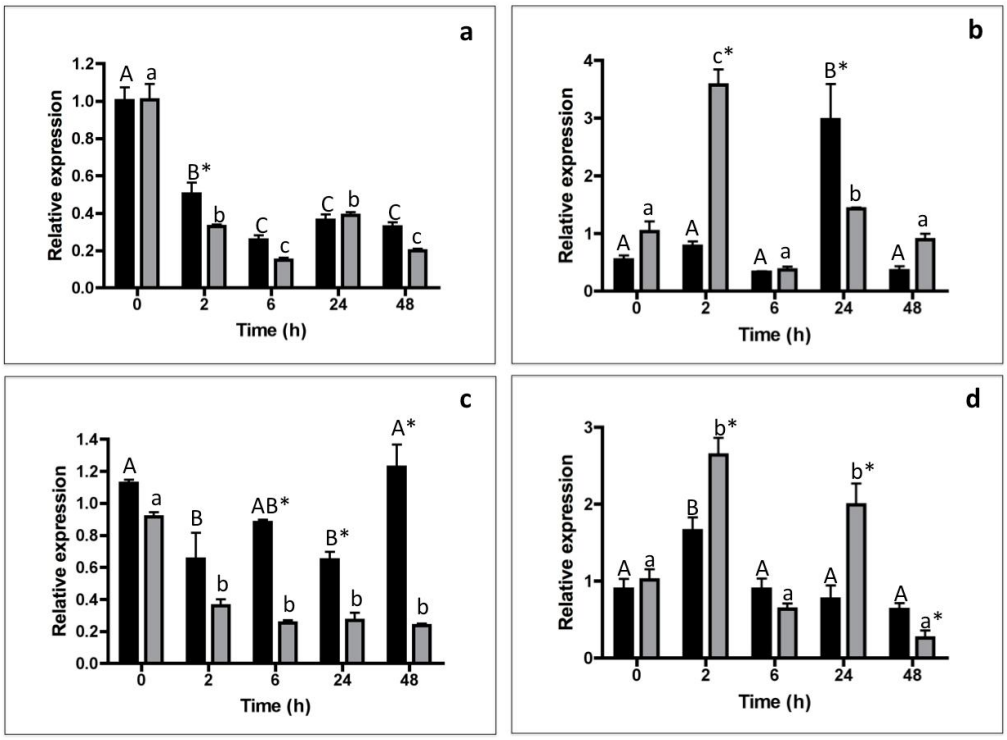

Figure 6. Effect of Al-stress on the expression of *glutathione S-transferase* (*VCAL21*) in leaves (a) and roots (b), and *aldehyde dehydrogenase* (*VCAL68*) in leaves (c) and roots (d) of Brigitta (Al-resistant, black lines) and Bluegold (Al-sensitive, gray lines) blueberry genotypes. The data points represent the mean ± SE of at least three replicates. Different upper case letters indicate significant differences between Al-exposure times for the same genotype and treatment, whereas different lower case letters indicate significant differences between Al treatment for the same genotype and Al exposure time. The asterisks indicate significant differences between genotypes ($P < 0.05$).

Discussion

This article attempts to elucidate the mechanisms underlying Al susceptibility in two blueberry genotypes with contrasting tolerance to this metal ion. For this, we adopted a multi-faceted approach, performing experiments to investigate morphological, physiological, biochemical and molecular aspects of the Al response in highbush blueberry, a very little-studied woody plant species.

Several studies have been carried out that demonstrate that Al induces oxidative stress, changes in gene expression and antioxidant responses [33, 34, 35]. Although Al itself is not a transition metal and is not able to catalyze redox reactions, it leads to a higher production of ROS, which are induced by oxidative stress in higher plants [16, 36]. On the other hand, it is known that the outermost cell layers of roots constitute a primary protection mechanism against abiotic and biotic environmental stress factors [37]. Research by Brigham et al. [38] confirmed that border cells in roots are involved in the avoidance of Al toxicity in pea. Nevertheless, it has been suggested that Al could lead to programmed cell death in roots [39, 40] and also trigger DNA damage and adaptive responses to genotoxic stress [41, 42] as a consequence of changes in the levels of ROS. We observed that Al responses are more pronounced in the layer epidermal and endodermal cells in root tips of the sensitive genotype. In maize plants, a rapid inhibition of cellular division in root tips has been observed after 5 min of exposure to Al in Alsensitive genotypes [43].

It has been proposed that Al promotes damage in the sub-apical region of the roots, leading to the separation of the rhizodermis and outer cortical layers from the inner

cortical cell layers. Additionally, this damage is related to the binding of Al to the cell wall, making this structure more rigid and less elastic [44, 45].

Analysis of chlorophyll fluorescence parameters showed that during Al exposure, Fv/Fm was in the normal range (near to 0.83) for healthy plants [23] of the two genotypes at the start of the experiment. However, the Al treatment induced a slight decrease (0.75) in the Al-sensitive Bluegold. In contrast, the Al-resistant genotype Brigitta maintained a value of 0.8 at all times. The slight decrease in Fv/Fm of Bluegold suggests some degree of disturbance of the photosynthetic apparatus under Al-stress. Furthermore, Al differentially affected ΦPSII and ETR of both genotypes, with the Bluegold genotype being more affected (Figure 3). Similar results were reported by Reyes-Díaz et al. [19, 46]. Our findings also confirm the report in leaves of *Citrus reshni* treated with aluminium, where a decrease in photochemical efficiency of PSII with respect to the untreated control was found [3]. The NPQ of the Bluegold genotype decreased with Al treatment, suggesting that thermal dissipation did not have a central role in dissipating excess excitation energy under Al treatment. Other dissipating processes such as the water-water cycle and photorespiration may be involved in the dissipation of excess energy, as found in other plants [47]. Our work confirms this assumption because when we measured the activity of antioxidant enzymes involved in the water-water cycle, we found significant increases in their activity in response to Al-stress (Figure 5). These may be up-regulated and/or activated to cope with the increased excess of excitation energy under Al stress. The activity of CAT, an enzyme involved in scavenging the bulk H_2O_2 generated by photorespiration [48], was augmented by Al in the early hours of Al-

stress in Brigitta leaves (Figure 5a) and after 24 h in roots (Figure 5b). In the Bluegold genotype, significantly greater CAT activity was only observed in the leaves after 48 h of treatment, whilst both CAT and SOD activities showed similar kinetics in Brigitta during Al stress (Figure 5c). The CAT activity in roots of both genotypes showed an increase after 2 h, decreasing gradually afterwards (Figure 5d). Another method to evaluate the stress-induced antioxidant system is to measure the DPPH-radical scavenging activity, which is a means to quantify non-enzymatic antioxidant activity [49]. There were no differences in RSA between the blueberry genotypes in the different tissues. However, it can be seen that the RSA in leaves is two-fold higher than in roots (Figure 4) and thus these results appear not to be associated with the Al-sensitivity of the Bluegold genotype. In this study, the expression of two antioxidant genes induced by Al-stress in roots and leaves was evaluated: *gluthatione S-transferase* (*VCAL21*) and *aldehyde dehydrogenase* (*VCAL68*). Both genes have been associated with the antioxidant response in higher plants [9, 34]. Several Al-induced genes, such as *GST* and *SOD*, have also been found to be induced by oxidative stress [33], and overexpression of a *GST* (*parB*) of *Nicotiana tabacum* ameliorated Al-toxicity in *Arabidopsis* [17]. Further studies showed that this gene also provided protection against oxidative stress, suggesting that Al-stress and oxidative stress are related in plants [34]. In our work, an increased expression of *GST* (*VCAL21*) was observed in leaves of Brigitta (Al-resistant) in comparison to the Bluegold genotype (Al-sensitive) after 2 h of Al-stress (Figure 6a). In roots, there was higher expression of *VCAL21* in the Bluegold genotype (Al-sensitive),

peaking after 2 h of Al-stress (Figure 6b). This suggests that Bluegold genotype (Al-sensitive) has to quickly activate some prompt mechanisms aimed at counteracting the stress, differently from the Brigitta (Al-resistant). That could be interpreting as an acclimation response of Bluegold genotype. Ezaki et al. [17] expressed the *GST* gene (*parB*) in *Arabidopsis* and found that it conferred substantial protection against Al-stress. These authors also suggested that expression of this gene is linked to both Al and oxidative stress. Lipid peroxidation is a common symptom of Al-toxicity [12], resulting in the generation of aldehydes in roots of tobacco, downstream of ROS [50]. Other studies have reported the isolation of an inducible gene encoding aldehyde dehydrogenases (ALDHs) in transgenic *Craterostigma plantagineum* and *Arabidopsis thaliana* plants conferring tolerance to heavy metals [9]. Unexpectedly, in our studies this gene (*VCAL68*) was highly induced after 2 and 24 h of exposure to Al treatment in roots of the Al-sensitive genotype (Figure 6d) whereas in leaves, there were significant changes in *VCAL68* expression after 6, 24 and 48 h of treatment in the Al-resistant genotype.

We conclude that the morphological, physiological and biochemical alterations monitored in this study contribute towards a higher Al-resistance of the Brigitta genotype. Surprisingly, at the molecular level, the expression of the two antioxidant genes evaluated in roots was more highly-induced in the Al-sensitive genotype (Bluegold) than in the resistant genotype. On the other hand, in leaves of the Al-resistant genotype, expression of both genes was induced, suggesting that these antioxidant genes may be involved in the Al-resistant mechanisms in the shoots of the plant.

However, further molecular studies should be performed to clarify the Al-resistant mechanism in blueberry.

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Chapter V

Molecular characterization of a Calmodulin *VcCaM1* gene differentially expressed under aluminum-stress in highbush blueberry

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Molecular characterization of a Calmodulin *VcCaM1* gene differentially expressed under aluminum-stress in highbush blueberry

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Introduction

In plants, calcium ions (Ca^{2+}) are important cellular second messengers involved in many biological processes in response to different stress (Poutrain et al. 2011). The changes in cytosolic Ca^{2+} are sensed by a group of Ca^{2+}-binding proteins, among these, Ca^{2+}-dependent protein kinases (CDPKs) and calmodulin (CaM) (Zhang et al. 2002). CaM plays a key role in signal transduction pathways, because it regulates a variety of cellular processes by modulation the activities of numerous target proteins (Kim et al. 2009). Between the physiological responses we can include biotic stress, gravitropism, phototropism, environmental stress, growing and development (Yang and Poovaiah, 2003; Zhang and Lu, 2003; Du and Poovaiah, 2005). Different studies performed in plants using CaM inhibitors showed that several responses to environmental stress are dependent on CaM. Recent studies have established a role of CaM proteins in cold acclimation and tolerance freezing in *Arabidopsis thaliana* (Doherty et al. 2009). On the other hand, Xu et al. (2011) identified a calmodulin gene (*OsMSR2*) involved in tolerance to drought and salinity in *Oryza sativa.* However, the roles of CaM at the physiological and molecular levels in plants have not been well clarified.

The toxicity caused by aluminum in acid soils is a main environmental stress that causes damage in plants (Kochian et al. 2005). The most obvious symptom is the rapid inhibition of root growth, having a direct effect on the ability of a plant to acquire water and nutrients (Pavlokin et al. 2009). The plants have developed mechanisms to cope with Al-toxicity. Moreover, great differences in tolerance to Al have been reported among genotypes of the same species. Over the last years, several results have shown that

plant tolerance to Al toxicity is a complex, with multigenic characteristics. However, the pathways leading to this Al-resistance are not well understood in woody plants. In the present work, we report the Identification, cloning and characterization of one calmodulin (CaM) differencialy expressed in blueberry under Al-stress. This gene cans bee participed in resistance to the Al-toxicity in blueberry.

Materials and methods

Plant material and growth conditions

The plant material and growth conditions have been described previously in Inostroza-Blancheteau et al. (2011). Briefly, the plants were conditioned in a Hoagland's solution for 7 days and subsequently placed in a solution of calcium chloride with 0 and 100 µM aluminum for 48 hours. The samples roots were collected to 0, 2, 6, 24 and 48 hours and frozen at -80 C until use.

Molecular cloning and sequence analysis

To obtain the 3' terminal region of VCAL19, 3' RACE was performed with the GeneRacerTM RACE ready cDNA Kit (Invitrogen) following the manufacturer's instructions. Total RNA was isolated from root tips and 2 µg of DNAse I-treated total RNA were reverse-transcribed using the GeneRacer oligo dT primer and Superscript II RNase H Reverse Transcriptase (Invitrogen). The PCR reaction was performed with the primer 19L (5'-TGAGTTCAAGGAGGCCTTCAGTCT-3') and the GeneRacer 3' primer. The 3' RACE product was cloned into the pGEM-T Easy vector (Promega) and

sequenced. To obtain the initial codon and 5` UTR of VCAL19, 5' RACE was subsequently performed (Gene-Racer) using specific primers. These were 19GSP1: 5'-TCGGGTATCACTTGGCCATCAT-3'; 19GSP2: 5'-CTTGTCGAACACCCGGAAAG-3'. The 5' RACE product was cloned into the pGEM-T Easy vector and sequenced. Full length VCAL19 was cloned using the primers VCAL19-F: GATATCTATCGCTCTTGAATTGC and VCAL19-R: CAGGTTTTACTCAGGACTCATCA. The cDNA sequence homology searches and comparisons were performed using BLAST-X at the National Center for Biotechnology Information (NCBI) network service (http://www.ncbi.nlm.nih.gov/blast). The protein prediction and analysis were performed using the SMART (Simple Modular Architecture Research Tool) domain (Schultz et al. 1998; Letunic et al. 2008). The software Vector NTITM advance 8 was used for alignment of multiple sequences deduced from CaM DNA sequences.

Quantitative analysis of tissue-specific expression of VcCaM gene expression in different blueberry organs.

Results

Identification of an VcCaM-like gene fragment expressed under Al-stress in blueberry

With the aim to identify genes expressed during a Al-treatment in *Vaccinium corymbosum*, transcripts expressed in two contrasting genotypes for Al-resistance in five points of treatment, were analyzed by the cDNA-AFLP procedure and compared the

expression in root tips. One of the TDFs generated that is up-regulated in Brigitta (Al-resistant) under Al-stress, was named VCAL19 for *Vaccinium corymbosum* Aluminum. This fragment was excised from the acrylamide gel, cloned and sequenced. The expression of VCAL19 was detected mostly in all point of treatments in Brigitta genotypes. By comparing the sequence with the GenBank databases using the BLAST tool, VCAL19 was identified as a homologous to Calmodulin-like gene widely described in plants (Hrabak et al. 1996; Zielinski, 2002; Hrabak et al. 2006; Xu et al. 2011).

Isolation of the full-length sequence and bioinformatics analysis of the VCAL19 homologous to VcCaM1-like gene

More than 50 types of CaM-binding proteins have been described in plants and their physiological functions are implicated in diverse aspects of cellular processes. However, the structure of these proteins is highly conserved having around 148 amino acids. VCAL19 was similarity with ORF of 150 amino acids. Alignment of CaM amino acid sequences indicates a high degree of conservation between animals and plants (more of 70% identity). Through of 5' and 3' RACE, we isolated full-length VCAL19 cDNA from roots of *V. corymbosum* (GenBank Accession Nº HO054811). Using the full cDNA sequence we searched by BLAST in NCBI databases and verified that VCAL19 is homologous to Calmodulin-like protein. Therefore, we named the gene *VcCaM1*. A schematic representation of the cDNA sequence of this gene and the encoded amino acids are shown in Fig. 2. The full-length cDNA was 790 bp in length. VcCaM cDNA had an open reading frame of 450 bp encoding a predicted protein of 150 amino acids, a

putative 5’ UTR of 115 bp upstream from the start codon and a 3’ UTR (229 bp) downstream from the stop codon. VcCaM1 is a highly conserved protein and has four domains EFH along the ORF (Fig. 1).

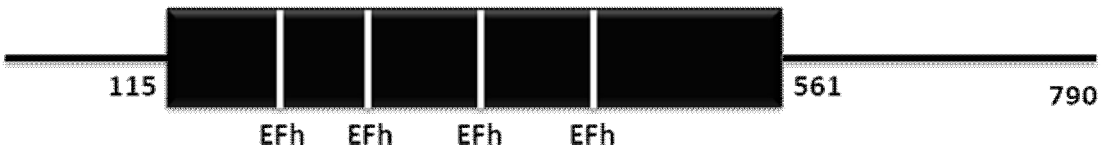

Figure 1. A schematic representation of Calmodulin cDNA showing the open reading frame (box) and putative untranslated regions (line). Numbers below the line refer to nucleotide positions. The domain EF-hands are calcium-binding motifs, are shown as EFh.

The amino acid sequence of VcCaM1 was similar in length and composition when compared with homologous proteins from other species. VcCaM1 shares 99% identity to homologues in *Daucus carota*, 98% identity to *Solanum tuberosum*, 97% identity to *Morus nigra* and *Actinidia melliana*, 96% identity to *Arabidopsis thaliana*. A detailed comparison between these proteins revealed that the VcCaM1 domain was similar to those in the homologous proteins (Fig. 2).

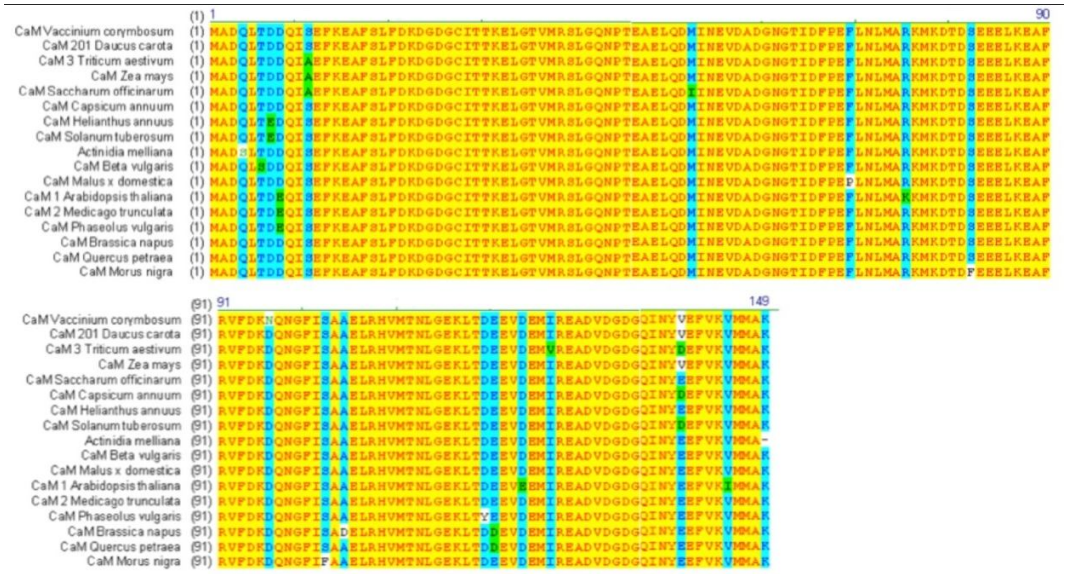

Figure 2. Alignment of the deduced amino acid sequence of VcCaM1 with *Daucus carota* (AAT73614.1), *Solanum tuberosum* (AAA62351.1), *Morus nigra* (ABS12106.1), *Actinidia melliana* (ABR21718.1), and *Arabidopsis thaliana* (NP_198594.1). The identical amino acid residues are shaded in yellow and other colors indicate different identical amino acid residues.

A phylogenetic tree of VcCaM1 with other CaM from different plants species was created by neighbor-joining using the MEGA 3.0 program (Fig. 2C). The analysis indicated that VcCaM1 is a member of the conserved CaM family, forming several subgroups with similar proteins from plants, and that this gene is more related to proteins from monocotyledonous than from dicotyledonous species.

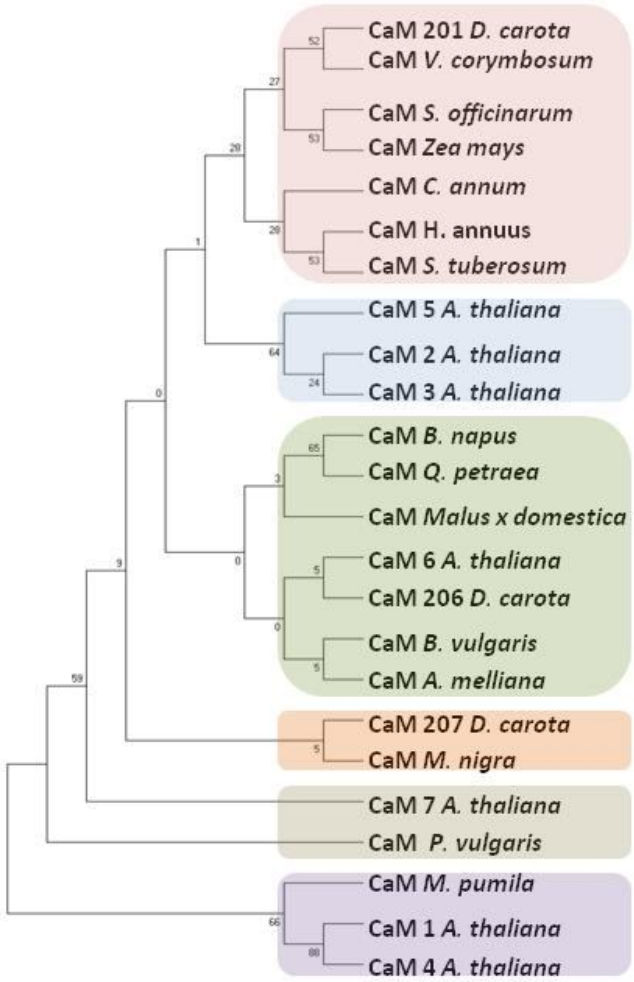

Figure 3. Phylogenetic tree analysis of VcCaM1 and other CaM proteins from other species. The tree was constructed by the neighbor-joining method with the MEGA program 3.0. Branch numbers represent the percentage of bootstrap values in 1,000 sampling replicates and the scale indicates branch lengths.

Tissue-specific expression of VcCaM1 in V. corymbosum

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General Discussion

As mentioned in the introduction, blueberry is an extremely important from the standpoint of nutritional and economic. This species has had an explosive development in our country, mainly in southern Chile. However, the edaphoclimatic conditions, this soil is characterized by high concentrations of aluminum phytotoxic (Al^{3+}) (Chapter I). The trivalent ion produces inhibition of root growth, thus interfering with the absorption of water and nutrients affecting crop productivity (Chapter II). On the other hand, little is known about the mechanisms of resistance and Al toxicity in this species at the molecular level. Therefore, in this thesis work we were interested in identifying genes that are expressed by Al stress in woody species of commercial importance and molecular characterization of new genes important for understanding the mechanism of resistance to Al in blueberry. The study used young plants in a hydroponic solution in vivo under Al stress. Through a comprehensive analysis of expression identified genes that are induced and repressed under this stress on blueberry roots of genotypes with contrasting tolerance to Al (Brigitta, Al-resistant; Bluegold, Al-sensitive) (Reyes-Díaz et al. 2009; 2010). We selected some genes that are present in different functional categories and quantified its expression pattern (Chapter III). We also evaluated the expression of two antioxidant genes identified by cDNA-AFLP supplemented by histological and biochemical experiments. Finally (Chapter IV), select and characterize a major gene could be involved in Al-resistance in blueberry. From the sequence fragments identified in the global analysis determined the complete sequence of the gene (Chapter V).

It is well known that the most obvious symptom of Al toxicity is inhibition of root growth (Doncheva et al. 2005). Affecting cell division and elongation of root tip (Scheres et al. 2002). On the other hand, there are several works has been described some phytohormones as auxin, cytokinin, giberellin and ethylene regulate this process (Wolters et al. 2009). Al^{3+} rapidly stimulates ethylene biosynthesis, induces a decrease in cytokinin levels and inhibits root growth in bean (Massot et al. 2002). Recently, it has been reported that Al^{3+}-induced inhibition of root elongation is mediated by ethylene and auxin in Arabidopsis (Sun et al. 2010). In our work, we identified *VCAL85* as an ETO1-like protein 1 which is strongly-expressed in Brigitta after 24 h. ETO1 negatively regulates ethylene synthesis via its ability to target 1-aminocyclopropane-1-carboxylic acid synthase (ACS) for breakdown (Christians et al. 2009).

Moreover, rice has been described in certain polyamines as putrescine is a factor causing root growth inhibition under Al-stress (Wang et al. 2006). However, the overexpression of *spermidine synthase* in European pear enhanced levels of spermidine and alleviated oxidative stress caused by Al (Wen et al. 2009). In blueberry, *VCAL6* is homologous to *S*-adenosylmethionine decarboxylase and is highly induced in the early hours in Bluegold. This gene is involved in the synthesis of polyamines such as putrescine, spermidine and spermine, which alleviate Al^{3+} toxicity by possibly lowering the Al^{3+} content in the root tips, and subsequently reducing lipid peroxidation and oxidative stress (Chen et al. 2008).

On the other hand, search responses about mechanisms of Al toxicity and response in blueberries; we investigated the effect of Al-stress on the physiological

performance, oxidative metabolism and expression of genes that encode antioxidant enzymes. Microscopic analyses of Al-treated root tips suggested a higher degree of Al induced morphological damage in Bluegold compared to Brigitta. On the other hand, it has been reported that epidermal root cells are the primary protection against aluminum toxicity (Brigham et al. 2001).

Furthermore, the results indicated that Brigitta had a greater ability to control oxidative stress under Al-toxicity, as reflected by enhancement of activity SOD and CAT. Studies in barley have shown that SOD might be involved in detoxification of Al after a long exposure to stress (Simonovicova et al. 2004). In roots of oat under Al stress was an increase in CAT activity increased in all three genotypes evaluated. However, this activities was higher in the resistant genotype (Pereira et al. 2011). The physiological parameter was less affected Brigitta compared with Bluegold that declined in almost all parameters evaluated. In the study of Al-stress in blueberry, we evaluated the antioxidant gene expression of two Al-induced antioxidante gene: gluthatione *S*-transferase (corresponding to *VCAL21*) and aldehyde dehydrogenase (corresponding *VCAL68*) specifically in leaves and roots under Al-stress. Both genes have been associated with the antioxidant response in higher plants (Sunkar et al. 2003; Ezaki et al. 2004). GST have been found to be differentially regulated by a variety of stimuli, including abiotic and biotic stresses, plant hormones such as auxins, cytokinins and ABA, heavy metals, GSH and hydrogen peroxide (Marrs, 1996; Sappl et al. 2009). It has been observed that Al-stress induces the *parB* gene, which codes a GST identified in tobacco (Ezaki et al. 2000). In addition, it has been described that when there's an

enhancement of the GST activity induces a reduction of lipid peroxidation conferring better resistance against Al-stress (Ezaki et al. 2000; Katsuhara et al. 2005). The results in our studies indicated that this gene is induced by Al-stress in a different degree. Gluthatione *S*-transferase gene was transiently expressed in roots treated with 100 µM Al for 48 h in roots. This gene was highly induced in Bluegold genotype (Al-sensitive) at 2 h and quickly repressed at 6 h in both genotypes. However, it was highly induced in Brigitta (Al-resistant) at 24 h. On the other hand, it was observed that the expression was more stable at different times in leaves for both genotypes, with a significant increased expression in Brigitta genotype (Al-resistant), at 2 h under Al-stress. This suggests that Bluegold genotype (Al-sensitive) has to quickly activate some prompt mechanisms aimed at counteracting the stress, differently from Brigitta (Al-resistant). This could be interpreted as an acclimation response of Bluegold genotype.

The group of Sunkar et al. 2003 reported the isolation of a heavy metals inducible gene encodes an aldehyde dehydrogenases *(ALDHs)* in transgenic *Craterostigma plantagineum* and *Arabidopsis thaliana*. The overexpression of this gene confers tolerance to heavy metals and other stresses in transgenic Arabidopsis plants (Sunkar et al. 2003). In our studies this gene (*VCAL68*) was highly-induced after 2 and 24 h of exposure to Al-treatment in roots of the Bluegold genotype (Al-sensitive). Whereas in leaves, the *VCAL68* expression was significately induced after 6, 24 and 48 h of treatment in the Brigitta genotype (Al-resistant). Summing up, both antioxidante genes were strongly expressed in leaves in the resistant genotype and, contrary to expectations, were repressed in roots. These results suggest a mechanism of

antioxidant protection in blueberry at the leaf level, to cope Al-stress. This is described and discussed in more detail in the Chapter IV.

Finally we characterize *VCAL19* homologous to calmodulin, named by us *VcCaM1*, for *Vaccinium corymbosum Calmodulin 1*. CaM interaction studies and Al, have shown that this ion is able to interact with the active site of CaM, thus affecting the homeostasis of Ca^{2+}. (Kurita et al. 2005). On the other hand, Ca^{2+} plays an essential role in the growth and development of plants (Meriño-Gergichevich et al. 2010), As a divalent cation, Ca^{2+} plays a structural role in cell walls and cell membranes. It also participates in root and stem elongation (White and Brodley, 2003). We obtained the complete sequence of this gene that presented a high similarity (99%) with *CaM 202* of carrot.

In summary, we could identify new gene expression is modulated by Al^{3+} in highbush blueberry. This study reveals that multitude of processes are implicated in determining the response to Al and that these processes require the activation of different genes. Detailed characterization of several genes, including putative novel genes and genes of unknown function, which may be involved in specific processes, will help to discover the fine networks underlying heavy metal accumulation and tolerance in woody plants.

Finally a diagrammatic model including the main responses to Al-toxicity at cellular level in leaves (model A) and roots (model B) of blueberry genotypes with contrasting resistance to Al was elaborated. The model considered also other published results mentioned in Inostroza-Blancheteau 2010, 2011). See appendix.

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General Conclusions

Through a global expression analysis we identified genes that are expressed or repressed under stress of aluminum (Al^{3+}) in bluberry. Of a total of 70 TDFs, 31 have significant homology with genes encoding known function proteins, 12 TDFs were homologous to uncharacterized genes and 27 TDFs did not have significant matches.

We have identified new genes in blueberry related to Al-stress response, which could explain the mechanism of toxicity and Al-resistance. These genes can be used as candidates for the improvement of woody plants.

Blueberry plants under Al-stress present an antioxidant system differential response in leaves and roots, suggesting greater relevance of this mechanism in leaves.

We selected, identified and cloned complete sequence of TDF VCAL19, homologous to calmodulin (CaM), now named *VcCaM1* for *Vaccinium corymbosum Calmodulin 1*. Addicionally made alignments of genetic similarity and detemine that our protein has a high similarity (99%) with *CaM 202* of *Daucus carota*. The product of this gene coud be participanting of the Al-stress resistance in blueberry plants.

Conclusiones Generales

A través de un análisis de la expresión global hemos identificado genes que son expresados o reprimidos bajo el estrés de aluminio (Al^{3+}) en arándano alto. De un total de 70 FDT, 31 FDT tienen significativa homología con genes que codifican proteínas de función conocida, 12 FDT fueron homólogas a los genes no caracterizados y FDT 27 no tienen homología en las bases de datos.

Se han identificado nuevos genes relacionados con la respuesta a estrés por Al, los que podrían explicar el mecanismo de toxicidad y resistencia a Al. Estos genes pueden ser utilizados como candidatos para el mejoramiento de plantas leñosas.

Plantas de arándano sometidas a estrés por Al, presenten una respuesta diferencial del sistema antioxidante en hojas y raíces, sugierendo una mayor relevancia de este mecanismo en las hojas.

Hemos seleccionado, identificado y clonado la secuencia completa del FDT VCAL19, homólogo a calmodulina (CaM), ahora llamada *VcCaM1* por *Vaccinium corymbosum Calmodulina 1.* Addicionally, hicimos alineaciones de similitud genética y determinan que nuestra proteína tiene una alta similitud (99%) con *CaM 202* de *Daucus carota*. El producto de este gen podría participar de la resistencia al estrés por Al en arándano.

Appendix

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RESEARCH

Identification of Aluminum-Regulated Genes by cDNA-AFLP Analysis of Roots in Two Contrasting Genotypes of Highbush Blueberry (*Vaccinium corymbosum* L.)

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Abstract To investigate the molecular mechanisms of Al^{3+}-stress in blueberry, a cDNA-amplified fragment length polymorphism (cDNA-AFLP) analysis was employed to identify Al-regulated genes in roots of contrasting genotypes of highbush blueberry (Brigitta, Al^{3+}-resistant and Bluegold, Al^{3+}-sensitive). Plants grown in hydroponic culture were treated with 0 and 100 μM Al^{3+} and collected at different times over 48 h. Seventy transcript-derived fragments (TDFs) were identified as being Al^{3+} responsive, 31 of which showed significant homology to genes with known or putative functions. Twelve TDFs were homologous to uncharacterized genes and 27 did not have significant matches. The expression pattern of several of the genes with known functions in other species was confirmed by quantitative relative real-time RT-PCR. Twelve genes of known or putative function were related to cellular metabolism, nine associated to stress responses and other transcription and transport facilitation processes. Genes involved in signal transduction, photosynthetic and energy processes were also identified, suggesting that a multitude of processes are implicated in the Al^{3+}-stress response as reported previously for other species. The Al^{3+}-stress response genes identified in this study could be involved in Al^{3+}-resistance in woody plants.

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Keywords Blueberry · Aluminum toxicity · cDNA-AFLP · qRT-PCR · Global gene expression

Abbreviations

cDNA-AFLP	DNA complementary to RNA-amplified fragment length polymorphism
TDF	Transcript derived fragment
Aluminum	Al^{3+}

Introduction

The blueberry (*Vaccinium corymbosum* L., Ericaceae) is a bush originating from North America. This plant produces a small fruit that is an important crop as it is rich in antioxidants and their consumption is beneficial for human health [1]. In Chile, the blueberry was introduced in the 1990s with excellent adaptative results in the southern regions. Currently, Chile is the main blueberry-producing country in the Southern Hemisphere and the third largest producer at global level [2].

Aluminum (Al^{3+}) is the most abundant metal in the earth's crust and at a high concentration, it is a major limitation to crop productivity in acid soils, which comprise up to 50% of the world's arable lands [3]. In southern Chile, about 50% of andisol soil has acidity levels that increase the amounts of exchangeable and highly toxic Al^{3+} to the plants [4]. The major symptom of excess Al^{3+} is a rapid inhibition of root growth that is accompanied by an accumulation of this phytotoxic ion in the cell walls [5]. Al^{3+}-stressed roots thus become thick, brown, and inefficient in water and nutrient uptake. Different mechanisms of Al^{3+}-resistance have been reported [3]. Of these mechanisms, exudation of

Al^{3+}-chelating organic acids into the rhizosphere, such as malate, oxalate, or citrate, is the most effective means to avoid Al^{3+}-toxicity in many species [6]. Some genes of Al-resistance of the ALMT (Al-activated malate transporter) and MATE family (multidrug and toxic compound extrusion) have been identified and characterized in different species of plants [7]. These genes encode membrane proteins which mediate the exudation of organic acid anions from the root. On the other hand, when Al^{3+} crosses the cell membrane, it is chelated by these organic acids and phenolic compounds in the cytosol [8]. It has been reported that Al^{3+} can alter cell redox homeostasis as a consequence of enhanced production of reactive oxygen species (ROS) [9]. The increase of ROS production could be involved in Al^{3+}-mediated inhibition of root growth [10] and programmed cell death caused by Al^{3+}-stress [11]. It is well known that plants have efficient enzymatic and non-enzymatic mechanisms to protect cellular components from oxidative damage caused by different stresses [12]. Research by Ezaki et al. [13] indicates that there are different processes involved in Al^{3+}-resistance in addition to chelation of Al^{3+} by organic acid anions, and other means have also been suggested in plants [14]. On the other hand, it has been reported that Al^{3+}-stress resistance varies between genotypes of the same species. For example, Al^{3+} stimulated between 5- and 10-fold more malate exudation from root apices of the Al^{3+}-tolerant isogenic lines of wheat than from the Al^{3+}-sensitive lines [15]. Global gene expression analyses have identified the genes induced by Al^{3+} in roots of *Triticum aestivum* [16], *Oryza sativa* [17], *Saccharum* spp. [18] and *Arabidopsis thaliana* [19]. However, most of these genes not only respond to Al^{3+} but also to other toxic metals [20], pathogens response [21], and oxidative stress [18]. In summary, plants have evolved several mechanisms that enable them to resist Al^{3+}-stress, and the ability to cope with Al^{3+} toxicity depends on the species and the genotype in question.

Although blueberry requires acid soils for optimum development, soluble Al^{3+} is detrimental to highbush blueberry growth [22]. However, differential responses to Al-stress have been observed between blueberry cultivars (cv). Using biochemical and physiological parameters, the cv. Brigitta and Bluegold have been described as Al^{3+}-resistant and Al^{3+}-sensitive cultivars, respectively [23].

To understand the molecular mechanisms underlying the differential response to Al^{3+}-stress by the contrasting genotypes, cDNA-AFLP was used for identification of differentially expressed genes [24]. This technique allows the discovery of unknown genes in species, such as blueberry, where there is no information in the genomic databases. In this study, we report the identification of 70 transcript-derived fragments (TDFs) that were sequenced and classified. Their putative function in the Al^{3+}-stress response is evaluated and discussed. The identification of these genes is very important to understand the mechanisms of toxicity and Al^{3+}-resistance in blueberry and other woody perennial plant species.

Materials and Methods

Plant Material and Growth Conditions

Two genotypes of *V. corymbosum*, which are widely used in southern Chile were used in this study: Brigitta, Al^{3+}-resistant and Bluegold Al^{3+}-sensitive [23]. Uniform 8-month-old plants (about 15 cm high) grown in solid substrate (1 peat: 1 rice husks: 1 pine needles) were provided by the Experimental Station Maquehue of the Universidad de La Frontera (Temuco, Chile). Plants were conditioned in plastic boxes filled with 18 L of Hoagland's nutrient solution for 7 days [25]. The solution was adjusted to pH 4.8 with 0.4 M HCl or NaOH, aerated with an aquarium pump and changed every 2 days. After conditioning, saplings were transferred to a hydroponic solution of $CaCl_2$ (0.5 mM) with and without (control) Al^{3+} supplied as $AlCl_3$ (100 μM) for 48 h. The experiment was conducted in a greenhouse with a photoperiod of 14/10 h (light/dark), at 25/20°C (day/night), 65–85% relative air humidity, and photosynthetic photon flux (*PPF*) densities of 120 μmol/m^2/s at the plant canopy. Samples of blueberry roots (root apex to the elongation zone, ~3 cm) were collected at five time points (0, 2, 6, 24, and 48 h) after Al^{3+}-treatment, washed with distilled water and quickly placed in liquid nitrogen and stored at −80°C until analysis.

Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from the root apices according to Gambiano et al. [26]. Two biological replicates were performed at each time point. To eliminate any contamination with genomic DNA, the total RNA was treated with RNAse-free DNAase I (Invitrogen). First strand cDNA was synthesized from 1.5 μg of total RNA using 200 U of Superscript II reverse transcriptase (Invitrogen) and 1 μl biotinylated oligo-dT_{25} (700 ng/ml). The double-stranded cDNA was synthesized using 50 U of *E. coli* DNA polymerase I, 15 U of *E. coli* ligase and 1.6 U of RNAse-H at 12°C for 1 h and then at 22°C for 1 h. The cDNA was purified using the Qiaquick PCR purification kit (Qiagen).

cDNA-AFLP Analysis

The cDNA-AFLP analysis was undertaken basically as described by Bachem et al. [24] with some modifications described in Aquea and Arce-Johnson [27]. Selective

amplification products were resolved in a 4.5% polyacrylamide sequencing gel at 120 W for 4 h and detected by autoradiography performed in cassettes with an intensifier screen using Clear Blue X-Ray Film and stored at −80°C. In the gel, AFLP products ranged from 100–800 bp. For each primer combination, an average of 40 band was observed in this size window. For each combination of primers, the same TDF patterns were observed in both biological replicates.

Isolation and Sequencing of TDFs

The TDFs which were differentially expressed between genotypes were excised from the polyacrylamide gels and reamplified by PCR using 1 µl of the eluted sample as template with the same combination of primers used during the second round of amplification with the conditions described for the pre-amplification reactions. The resulting PCR products were cloned in pGEM-T EASY (Promega, Madison-Wisconsin, USA) and sequenced (Macrogen Inc., dna.macrogen.com). To identify the corresponding genes, the sequence of each TDF was searched against all the sequences in the non-redundant databases using the BLASTN, BLASTX, and TBLASTX algorithms the TIGR gene index (www.tigr.org) and in GENBANK (NCBI). The sequences were manually assigned to functional categories based on the analysis of the scientific literature, TAIR (www.arabidopsis.org), and the Gene Ontology Consortium (www.geneontology.org).

Real-Time RT-PCR Analysis

Validation of the pattern of expression of 12 TDFs was confirmed by quantitative real time RT-PCR (qRT-PCR). The primers were designed using Amplifx 1.4.5 software. The list of candidate genes and their respective primer pairs are shown in Table 1. As a housekeeping gene, the expression of *metallothionein* was used, as previously described [28]. All reactions were performed in triplicate. Quantification of expression was performed using a Mx 3000 p^{TM} Real-Time PCR System (Stratagene). The PCR amplification conditions were 95°C for 3 min, followed by 40 cycles at 95°C for 20 s, 56°C for 20 s, and 72°C for 30 s. The dissociation curves were generated for each reaction to ensure specific amplification. Threshold values (Ct), which represent the PCR cycle at which fluorescence passes the threshold, were generated using the MxPro^{TM} qPCR software for the Mx 3000 p^{TM} System. Gene expression data (Ct values) were employed to quantify relative gene expression using the comparative $2^{-\Delta\Delta CT}$ method [29].

Statistical Analysis

A one-way ANOVA ($P < 0.05$) was carried out to evaluate differences between the treatment and genotypes, followed by a Tukey test for comparisons with a 95% confidence level.

Results

Identification of Genes Induced by Aluminum-Stress in Blueberry

To identify genes responsive to Al^{3+} stress, cDNA-AFLP analysis was performed on roots of the Brigitta (Al^{3+}-resistant) and Bluegold (Al^{3+}-sensitive) cv subjected to Al^{3+}-stress. The differentially expressed fragments were investigated by selective amplification using 28 primer combinations. To avoid false-positive fragments, the experiment was carried out using two biological replicates. More than 1200 bands were generated, and all the bands with sizes ranging from 120 to 720 bp were analyzed and

Table 1 Primers used for real-time-PCR analysis of *Vaccinium corymbosum* L. *VCAL* genes

Gene Name	Forward primer (5′–3′)	Reverse primer(5′–3′)
VCAL6	TCGGTCGACTCTGAAGTGCT	CATGACACGTACACGGACAAAG
VCAL19	TGCTGATGGGAATGGGACTATA	CTTGTCGAACACCCGGAAAG
VCAL25	GTGATCTGCCCAATGCAATGAACG	TGTTGCGCAGGTGCTCTGAATA
VCAL30	GCCGTCTGAATCTCCCGAGAAGTA	GGCCATGGGGATCATGAACAGTTT
VCAL32	TAGTGATCTCCAGCCGGGTCAAAT	TCAAGCACTTCTCGAGTCTCCTTC
VCAL38	TCTGGTGTGCAGGTTGCTATCT	GCGTACGGGCATGTTCACTA
VCAL47	AGTCTCCAGCGAAGGTCAAATCAC	AAGGATGGGAGGCATGTAGTCAGA
VCAL50	CTCTCTTGACACGGTGGAGATT	GTCAGCTGCATCTTGAACGGTA
VCAL59	AATTGGCCAAGGAACCGTCATC	ATGAGTCCTGAGTAACCCAGCAAC
VCAL85	TGGGTGATGTTCTCGGTGCATTGA	GATGAGTCCTGAGTAACGCGGTTT
VCAL90	GCAGTCTGTCTTCAATGCCCACTA	TTGAGCTACTTCCTCACCAAC
VCAL99	ATGCGGTCATGGGTCTGATTCAAG	TTGGTTGCCGCAGTCGATATTG

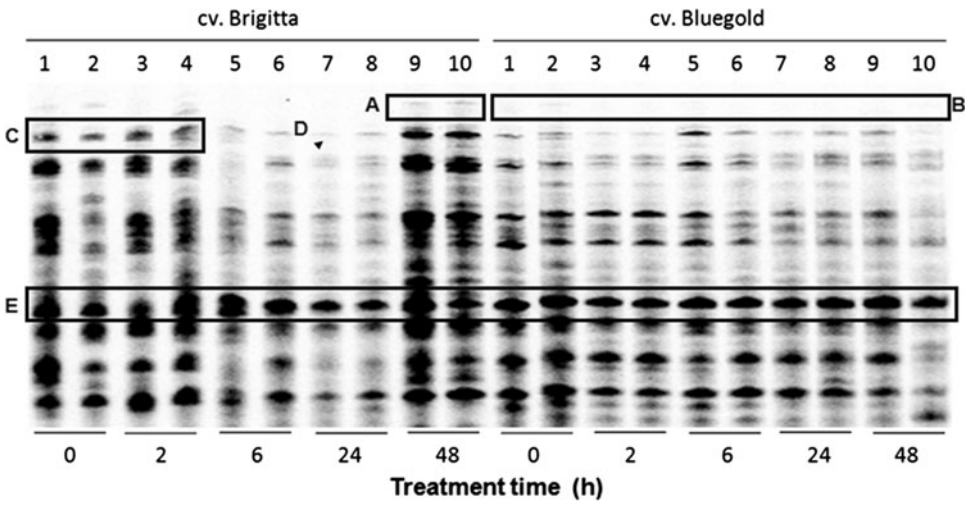

Fig. 1 Autoradiogram of the cDNA-AFLP results showing the transcript derived fragments (TDFs) induced or repressed after 0, 2, 6, 24, and 48 h of Al^{3+}-treatment in two cultivars of highbush blueberry (Brigitta, Al^{3+}-resistant and Bluegold, Al^{3+}-sensitive). The reaction products were derived from independent non-selective pre-amplifications and generated using selective primers Bst-TC/Mse. See text for an explanation of *a–e*

compared in all the five time treatments (0, 2, 6, 24, and 48 h) in both genotypes. Five different banding patterns were observed, as illustrated in Fig. 1 (a) TDFs that are induced in later time points; (b) Non-expressed TDFs; (c) TDFs that are induced in early time points; (d) TDFs that are repressed, and (e) Constitutively expressed TDFs. The clones corresponding to different TDFs were named as *VCAL* for *Vaccinium corymbosum Aluminum*, followed by an identification number.

Sequence Analysis of cDNA Clones

To understand the molecular mechanism of Al^{3+}-stress in *V. corymbosum*, the TDFs were isolated for sequence and expression pattern analysis. The up-regulated and down-regulated transcripts which were more abundant were selected from the autoradiographic films. These *VCAL* fragments were re-amplified and sequenced, and their identities were assigned using the TIGR and NCBI GenBank databases. This analysis revealed a total of 70 unique TDF sequences. The TDF name, the size, the homology, and the accession numbers of the *VCAL* fragments identified are shown in Table 2. Of the sequences identified, 27 TDFs do not show homology in the databases and were classified as "no match." Forty-three TDFs show significant homology with known or putative proteins and ESTs deposited in the databases (E value $<10^{-4}$). Of these, 31 are homologous with proteins of known function and 12 to genes that code for proteins with unknown functions. Nine *VCALs* are homologous to *V. corymbosum* sequences and two to sequences annotated or described in plants of the Ericaceae family. The remaining 34 TDFs are homologous to genes annotated in other plant species.

The genes encoding proteins of known functions were classified in eight potential functional categories according to the scientific literature and gene annotations from the Gene Ontology Database. Figure 2 shows the percentages of *VCALs* assigned to different functional categories. Most of the *VCALs* (38.6%) correspond to fragments without homology in databases, while the 17.1% of the VCALs is homologous to genes that codify unknown proteins. Among all of VCALs with known function, the most of them are homologous to genes involved in cellular metabolism (17.1%).

Validation of Representative Genes by Real-Time RT-PCR

Twelve TDFs were selected to validate the results of the cDNA-AFLP analysis by real-time RT-PCR: four related to oxidative stress functions (*VCAL6*, *VCAL38*, *VCAL90* and *VCAL99*); two related to cellular metabolism (*VCAL32* and *VCAL85*); two related to signal transduction (*VCAL19* and *VCAL47*), two related to transport (*VCAL25* and *VCAL50*); and the *VCAL30* homologue of histone H2B and the *VCAL59* homologue of peptidyl-prolyl isomerase, FKBP12 (Table 1). Under the experimental conditions, four differential expression patterns were observed using qRT-PCR (Fig. 3): (a) TDFs induced at early time points in the sensitive genotype and then repressed at later time points (*VCAL6*, *VCAL38*, *VCAL47,* and *VCAL50*); (b) TDFs induced at early time points in the resistant genotype (*VCAL19*, *VCAL32,* and *VCAL99*); (c) TDFs which are initially strongly repressed and then induced at later time points in the resistant genotype (*VCAL59* and *VCAL85*); and (d) TDFs induced in both genotypes but with significant increases in expression levels only in the resistance genotype (*VCAL25*, *VCAL30,* and *VCAL90*). The four expression patterns observed by qRT-PCR confirm the profiles observed in the cDNA-AFLP assay.

Table 2 Sequence homology of Al-regulated TDFs in blueberry, with sequences deposited in the database

TDF clone	Accession #	Length (bp)	Homology[a]	(%)[c]	BLAST score[d]
Cellular metabolism					
VCAL1	HO054805	500	40S ribosomal protein [*Rhododendron catawbiense*] (CV015045)	75	1.7 e-53
VCAL17	HO054973	298	40S ribosomal protein [*Vaccinium corymbosum*] (CF810807)	70	3.6 e-21
VCAL5	HO054791	311	ATP citrate lyase [*Juglans hindsii* × *Juglans regia*] (EL900206)	82	2.7 e-33
VCAL9	HO054808	143	Actin-2 [*Sorghum bicolor*] (AW285316)	71	1.3 e-06
VCAL32	HO054819	169	10-hydroxygeraniol oxidoreductase [*Helianthus annuus*] (TA160144232)	75	1.5 e-08
VCAL39	HO054823	110	Protein binding protein [*Ricinus communis*] (XM002521941.1)	84	2 e-21
VCAL51	HO054827	143	RBX1-like protein [*Petunia integrifolia*] (TA476285681)	80	2.5 e-30
VCAL78	HO054837	238	Ubiquitin C variant [*Theobroma cacao*] (CA795100)	66	2.3 e-11
VCAL80	HO054839	302	Ubiquitin C variant [*Ipomoea batatas*] (TA28054120)	75	3.6 e-30
VCAL81	HO054840	252	Actin-binding [*Vaccinium corymbosum*] (TA67469266)	88	2.7 e-38
VCAL85	HO054842	376	ETO1-like protein 1 [*Malus x domestica*] (CN857381)	68	2.5 e-08
VCAL88	HO054843	552	Fructose-bisphosphate aldolase [*Vaccinium corymbosum*] (TA70469266)	99	5.2 e-127
Stress response					
VCAL6	HO054792	191	*S*-adenosylmethionine decarboxylase [*Cyclamen persicum*] (AJ887644)	64	1.9 e-07
VCAL21	HO054812	193	Glutathione *S*-transferase GST 14 [*Glycine max*] (TA582483847)	77	2.3 e-15
VCAL38	HO054822	363	Vacuolar H^+-pyrophosphatase [*Prunus persica*] (AF367447.1)	82	1 e-38
VCAL68	HO054833	223	Aldehyde dehydrogenase [*Vitis vinifera*] (DQ150259.1)	83	4 e-57
VCAL90	HO054844	320	Aspartic proteinase [*Camellia sinensis*] (CV013914)	81	2.0 e-51
VCAL99	HO054849	419	Endochitinase A precursor [*Solanum lycopersicum*] (TA361774081)	81	1.2 e-35
VCAL124	HO054860	299	Putative disease resistance [*Arabidopsis thaliana*] (AB425274.1)	93	9 e-119
VCAL125	HO054861	501	Putative disease resistance [*Arabidopsis thaliana*] (AB425273.1)	85	3 e-135
VCAL163	HO054867	399	Anthranilate *N*-benzoyltransferase [*Euphorbia esula*] (TA127083993)	70	3.9 e-35
Transcription					
VCAL30	HO054818	257	Histone H2B.1 [*Fragaria* × *ananassa*] (DV438603)	70	1.5 e-19
VCAL144	HO054866	108	Basic leucine zipper BZIP [*Arabidopsis thaliana*] (TA364323702)	78	5.9 e-08
Transport					
VCAL25	HO054815	136	ARF-like [*Salvia miltiorrhiza*] (HM051059.1)	84	1 e-42
VCAL50	HO054826	261	Plastid acyl carrier protein [*Camellia oleifera*] (EU717697.1)	87	3 e-78
VCAL82	HO054841	201	Putative plasma membrane intrinsic [*Ricinus communis*] (TA11803988)	69	2.3 e-09
Signal transduction					
VCAL19	HO054811	511	Calmodulin (CaM) mRNA [*Ricinus communis*] (XM002527338.1)	88	2 e-138
VCAL47	HO054825	457	Phospholipase PLDa1 [*Solanum tuberosum*] (CK860893)	64	6.5 e-22
VCAL61	HO054829	452	F-box family protein [*Populus trichocarpa*] (XP002304470.1)	74	2 e-48
Photosynthesis and energy					
VCAL27	HO054816	528	Photosystem I subunit XI [*Rhododendron catawbiense*] (TA230257784)	87	1.9 e-86
VCAL59	HO054828	127	Peptidyl-prolyl isomerase FKBP12 [*Camellia sinensis*] (CV014093)	83	1.8 e-17
Unknown protein					
VCAL2	HO054806	320	Unknown protein [*Capsicum annuum*] (BM063365)	66	7.7 e-23
VCAL8	HO054807	126	Unknown protein [*Vaccinium corymbosum*] (CF811488)	87	3.1 e-15
VCAL10	HO054809	212	Unknown protein [*Vaccinium corymbosum*] (CF810890)	69	4.3 e-12
VCAL18	HO054810	431	Unknown protein [*Vitis vinifera*] (TA81769266)	97	1.8 e-58
VCAL29	HO054817	305	Unknown protein [*Nicotiana tabacum*] (EB451503)	76	1.6 e-32
VCAL37	HO054821	309	Unknown protein [*Vitis vinifera*] (EE085586)	73	2.0 e-27
VCAL73	HO054835	278	Unknown protein [*Vaccinium corymbosum*] (TA76969266)	97	4.7 e-53
VCAL79	HO054838	223	Unknown protein [*Solanum tuberosum*] (DV625248)	65	1.3e-07
VCAL123	HO054859	243	Unknown protein [*Vaccinium corymbosum*] (TA90169266)	65	7.7e-10
VCAL128	HO054863	179	Unknown protein Expressed protein [*Camellia sinensis*] (TA4544442)	69	1.1e-10

Table 2 continued

TDF clone	Accession #	Length (bp)	Homology[a]	(%)[c]	BLAST score[d]
VCAL129	HO054864	251	Unknown protein [*Vaccinium corymbosum*] (TA76969266)	99	7.5e-50
VCAL130	HO054865	235	Unknown protein [*Vaccinium corymbosum*] (CF810562)	73	5.3 e-19
No match[b]					
VCAL7	HO054868	273	No match	–	–
VCAL11	HO054869	766	No match	–	–
VCAL14	HO054870	264	No match	–	–
VCAL15	HO054871	667	No match	–	–
VCAL16	HO054804	147	No match	–	–
VCAL20	HO054872	578	No match	–	–
VCAL22	HO054813	390	No match	–	–
VCAL26	HO054873	355	No match	–	–
VCAL28	HO054874	465	No match	–	–
VCAL33	HO054875	689	No match	–	–
VCAL41	HO054876	583	No match	–	–
VCAL42	HO054877	501	No match	–	–
VCAL44	HO054878	389	No match	–	–
VCAL49	HO054879	835	No match	–	–
VCAL46	HO054803	321	No match	–	–
VCAL53	HO054880	883	No match	–	–
VCAL54	HO054881	506	No match	–	–
VCAL67	HO054882	557	No match	–	–
VCAL69	HO054883	867	No match	–	–
VCAL70	HO054884	830	No match	–	–
VCAL71	HO054885	950	No match	–	–
VCAL74	HO054886	899	No match	–	–
VCAL83	HO054887	498	No match	–	–
VCAL91	HO054888	489	No match	–	–
VCAL94	HO054796	470	No match	–	–
VCAL95	HO054797	663	No match	–	–
VCAL106	HO054889	539	No match	–	–

[a] GenBank accession numbers of the sequences homologous to cDNA-AFLP fragments are in parentheses

[b] No significant sequence homology found in the genome, EST and protein database

[c] Percentage of similarity between VCAL and their homologue sequence

[d] All are BLASTN scores

Discussion

During Al^{3+}-stress, a set of 70 differentially expressed TDFs in blueberry were identified using cDNA-AFLP analysis. Identified transcripts were sequenced, annotated and classified into functional categories. These TDFs were the subject of this study, and several of the sequences identified are putative new genes in *V. corymbosum*, which have been deposited in GenBank (Table 2). All TDFs were assigned to independent functional categories using Gene Ontology and the scientific literature. Overall, 46% of the 70 Al^{3+}-responsive genes were homologous to genes of known function and could thus be categorized into different functions. The remaining 54% corresponded to unknown proteins and those with no match. This analysis did not identify homologous genes involved in the exclusion mechanism (*ALMT1* and *MATE*), mainly because of the experimental strategy and the number of primers combination used. Based on the results of this study, this mechanism in blueberry cannot be ruled out, and further experiments are needed to clarify this point.

It is well known that the first target of Al-toxicity in plants is the inhibition of root growth [30]. Root growth depends on two basic developmental processes: cell division and elongation of cells in the root apical meristem, reviewed in Scheres et al. [31]. Root growth is regulated by

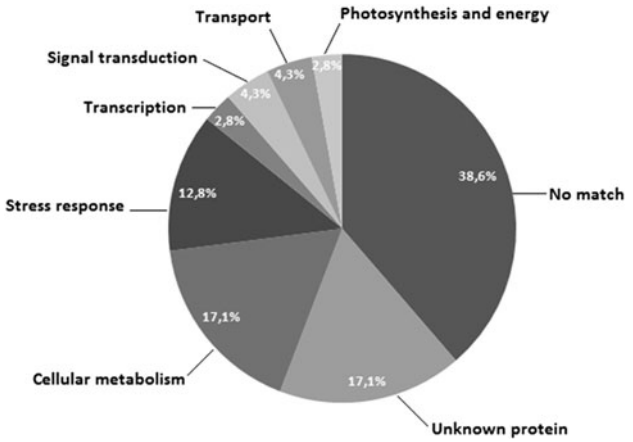

Fig. 2 Distribution of differentially expressed TDFs under Al^{3+}-stress in blueberry. A total of 70 unique cDNA-AFLP fragments were grouped into eight functional categories and classified on the basis of their homology to sequences deposited in the databases

phytohormones such as auxin, cytokinin, gibberellin, and ethylene [32]. Al^{3+} rapidly stimulates ethylene biosynthesis, induces a decrease in cytokinin levels, and inhibits root growth in *Phaseolus vulgaris* [33]. Recently, it has been reported that Al^{3+}-induced inhibition of root elongation is mediated by ethylene and auxin in Arabidopsis [34]. Application of exogenous auxin increases root meristem size, and ethylene regulates root growth by both stimulating auxin biosynthesis and by modulating the auxin transport machinery [32]. In this study, *VCAL85* as an ETO1-like protein 1 was identified, which is strongly expressed in the Al^{3+}-resistant genotype after 24 h (Fig. 3). ETO1 negatively regulates ethylene synthesis via its ability to target 1-aminocyclopropane-1-carboxylic acid synthase (ACS) for breakdown [35]. *VCAL51* is homologous to genes encoding RING-H2 proteins related to human RBX1. Arabidopsis RBX1 is an SCF subunit, and a reduced RBX1 levels result in severe defects in growth associated with a reduced auxin response [36]. *VCAL51* is expressed equally in both cultivars, but is strongly expressed soon after Al^{3+}-treatment in the cv. Brigitta that could be the result of Al^{3+}-toxicity. These results suggest that cv. Brigitta produces less ethylene and has a better auxin response in comparison to the cv. Bluegold.

In several higher plants, it has been reported that Al^{3+} could disturb cellular metabolism by disrupting Ca^{2+} homeostasis as a result of the known antagonism between Al^{3+} and Ca^{2+} [37]. Kurita et al. [38] reported that there is an interaction between calmodulin (CaM) and Al^{3+} and suggested that Al^{3+} may affect the Ca^{2+} signaling pathway in cells. In our study, *VCAL19*, which is homologous to CaM, was induced by Al^{3+}-stress in both genotypes, but is strongly induced in the cv. Brigitta at 0–2 h (Fig. 3), suggesting that it may play a role in the resistant genotype.

In *Oryza sativa*, it has been reported that putrescine accumulation is a factor causing root growth inhibition under Al^{3+}-stress [39]. The overexpression of *spermidine synthase* in European pear enhanced levels of spermidine and alleviated oxidative stress caused by Al^{3+} [40]. In blueberry, *VCAL6* is homologous to *S*-adenosylmethionine decarboxylase and is highly induced in the early stages in the Al^{3+}-sensitive genotype. This gene is involved in the synthesis of polyamines, such as putrescine, spermidine, and spermine, which alleviate Al^{3+} toxicity by possibly lowering the Al^{3+} content in the root tips, and subsequently reducing lipid peroxidation and oxidative stress [41].

On the other hand, *VCAL38* which is homologous to a vacuolar H^+-pyrophosphatase is expressed in both Brigitta and Bluegold cultivars in the initial hours (0–2 h), and expression levels subsequently fall (6, 24, 48 h). This protein plays an important role in resistance to drought and it has been suggested as a potential target for genetic engineering of root systems in crop plants [42]. A proton pump similar to the vacuolar H^+-ATPase (V-ATPase) was discovered in an Al^{3+}-resistant cultivar of *Triticum aestivum* [43], and has been described as a component of the Al^{3+}-stress response, with the ATP required for its activity supplied by ATP synthase [44]. The importance of this gene in blueberry requires further study

In the category transport, *VCAL25* was identified. This TDF is homologous to ADP-ribosylation factors (ARFs), a subfamily of the Ras superfamily of GTP-binding proteins that regulate diverse processes in eukaryotic cells such as signal transduction, cell proliferation, cytoskeletal organization, and intracellular membrane trafficking [45]. *VCAL25* is expressed in both genotypes, although the expression level is significantly higher in the Al^{3+}-resistant cultivar. A GDP dissociation inhibitor gene derived from tobacco (*NtGDI1*), when overexpressed in *Saccharomyces cerevisiae* confers Al^{3+} tolerance [46]. In addition, it was proposed that overexpression of the NtGDI1 protein in Arabidopsis activates an Al-efflux system that protects against Al^{3+}-toxicity [13].

The finding that a number of the sequences isolated show no significant similarity with any sequences in the public databases is interesting. These sequences that do not have homology to those in the database could correspond to 3′UTRs, new coding sequences, or non-coding intergenic sequences. Recent evidence indicates that the majority of sequences in eukaryotic genomes are transcribed [47] suggesting that thousands of novel genes and transcripts have not yet been annotated. These sequences have been defined as transcriptional forests, that is, regions of the genome that present a complex array of sense and anti-sense, coding, and non-coding transcripts [48]. These results provide a new set of genes of potential interest

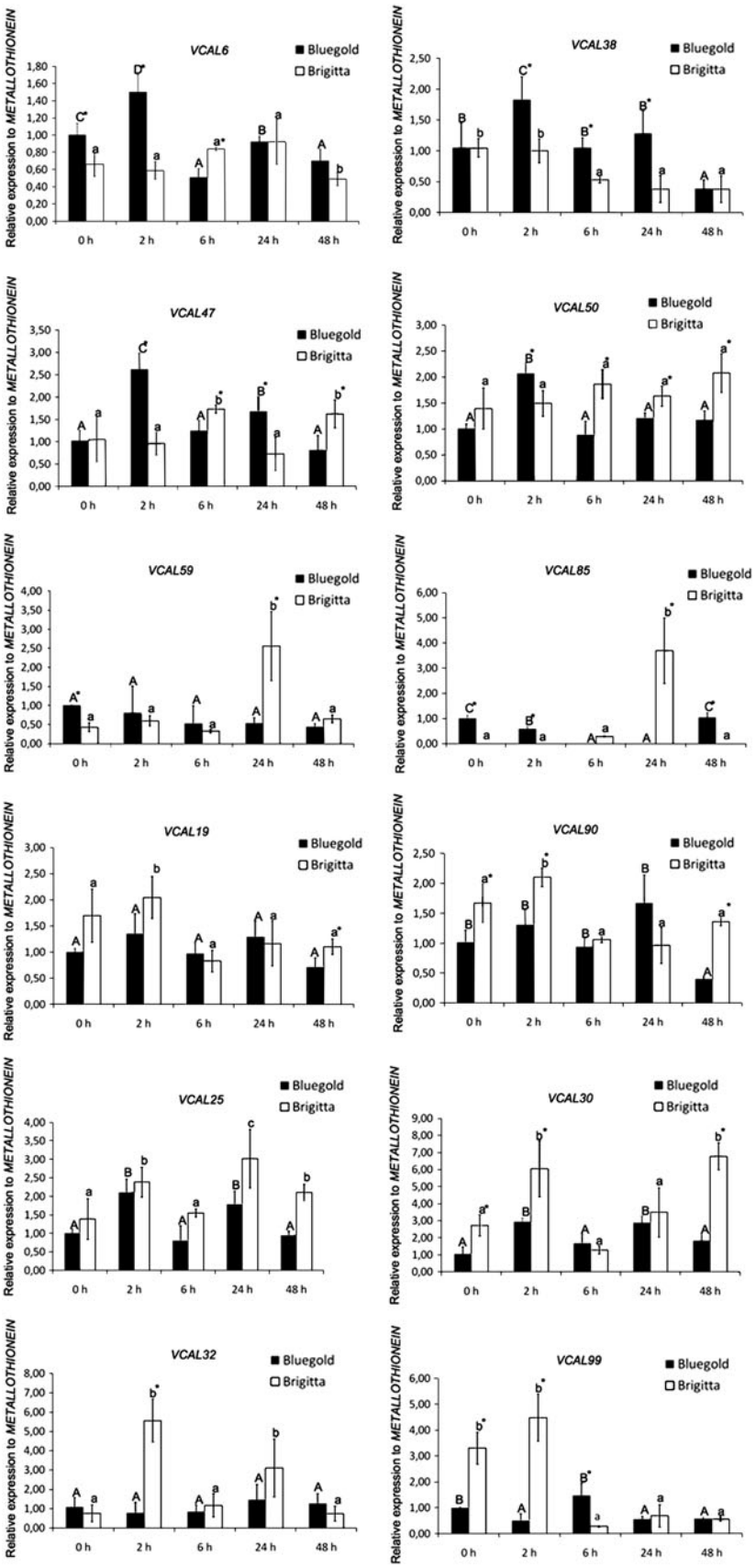

Fig. 3 Expression analyses of 12 genes obtained from cDNA-AFLP fingerprinting by means of quantitative real-time PCR. Three independent biological replications were performed. All data were normalized to *metallothionein* expression levels [28]. *Capital letters* show significant differences in the susceptible cultivar (Bluegold) and *lowercase letters* show significant differences in the resistant cultivar (Brigitta). The *asterisks* show significant differences between cultivars with ($P < 0.05$) according to the Tukey test

to unravel further the molecular mechanisms of plant Al^{3+}-responses.

In summary, the cDNA-AFLP analysis allowed genes to be identified whose expression is modulated by Al^{3+} in blueberry. This study reveals that a multitude of processes are implicated in determining the response to Al^{3+} and that these processes require the activation of different genes. Detailed characterization of several genes, including putative novel genes and genes of unknown function, which may be involved in specific processes, will help us to discover the fine networks underlying heavy metal accumulation and tolerance in plants.

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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.

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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*;

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HO054833, *VCAL68*). The relative quantification of *VCAL21* and *VCAL68* expression was determined by qRT-PCR. The primers were designed using Amplified 1.4.5. The specific primers used were: VCAL21-F 5′-GAGGAAGTTGGGTCCATGAAAAT-3′ and VCAL21-R 5′-CGGCGGTAACTTGTC CTTGA-3′; VCAL68-F 5′-AGGCTCCAAAGGCTT CTACATCCA-3′ and VCAL68-R 5′-ACCGGGCCGAAGATTTCATCTTGT-3′, which amplify 120 bp fragments of *VCAL21* and *VCAL68*. All experiments were performed with three biological replicates and two technical replicates. As a housekeeping gene, the expression of *metallothionein* was used, as previously described by Naik et al. [31] for highbush blueberry. PCR amplification was performed in a 25 μl-reaction containing 12 μl SensiMix™ Plus SYBR® (Quantace), 2 μl cDNA and 0.5 μl of each primer (10 μmol). Cycling conditions were 95 °C for 3 min followed by 40 cycles at 95 °C for 20 s, 56 °C for 20 s and 72 °C for 30 s. Dissociation curves were generated for each reaction to ensure specific amplification. Threshold values (Ct), which represent the PCR cycle at which fluorescence passes the threshold, were generated using the MxPro™ qPCR software of the Mx 3000 p™ System. Gene expression data (Ct values) were employed to quantify relative gene expression using the comparative $2^{-\Delta\Delta Ct}$ method [32].

2.8. Statistical analysis

To test significant differences in gene expression between treatments and genotypes, one-way ANOVA was performed ($P < 0.05$), using Skewness, Kurtosis and Omnibus tests for normality, and the Modified-Levene Equal-Variance test for homogeneity of variances. Statistical analyses were carried out using the NCSS software (Number Cruncher Statistical System, Kaysville, Utah, USA). When differences in the means were significant, a Tukey's test was performed with 95% confidence level.

3. Results

3.1. Ultrastructural changes in root tip cells caused by Al

The main target of Al-toxicity in plants is the roots. Therefore, we performed histological analyses to monitor the structural alterations in root tips of an Al-resistant (Brigitta) and an Al-sensitive (Bluegold) genotype of blueberry (Fig. 1). The roots of the Al-tolerant genotype did not exhibit notable anatomical modifications after Al treatments (Fig. 1a and b). However, the Al-sensitive genotype growing without Al possessed uniformly-stained cells, whereas in the Al-treated plants the root cells had a disintegrated peripheral region, with narrower cell walls in the central region compared with control root tips (arrows, Fig. 1c and d). Thus, this experiment showed that root tip cells, particularly those of the epidermis of a sensitive genotype subjected to an Al concentration of 100 μM, were seriously affected.

3.2. Fluorescence parameters of PSII

To determine the physiological impact of Al-stress on different blueberry genotypes, we evaluated the *in vivo* chlorophyll fluorescence parameters over a period of 48 h. The maximum quantum yield of PSII (Fv/Fm) was close to 0.8 at the start of the experiment in plants of both genotypes (Fig. 2), a figure which is typically

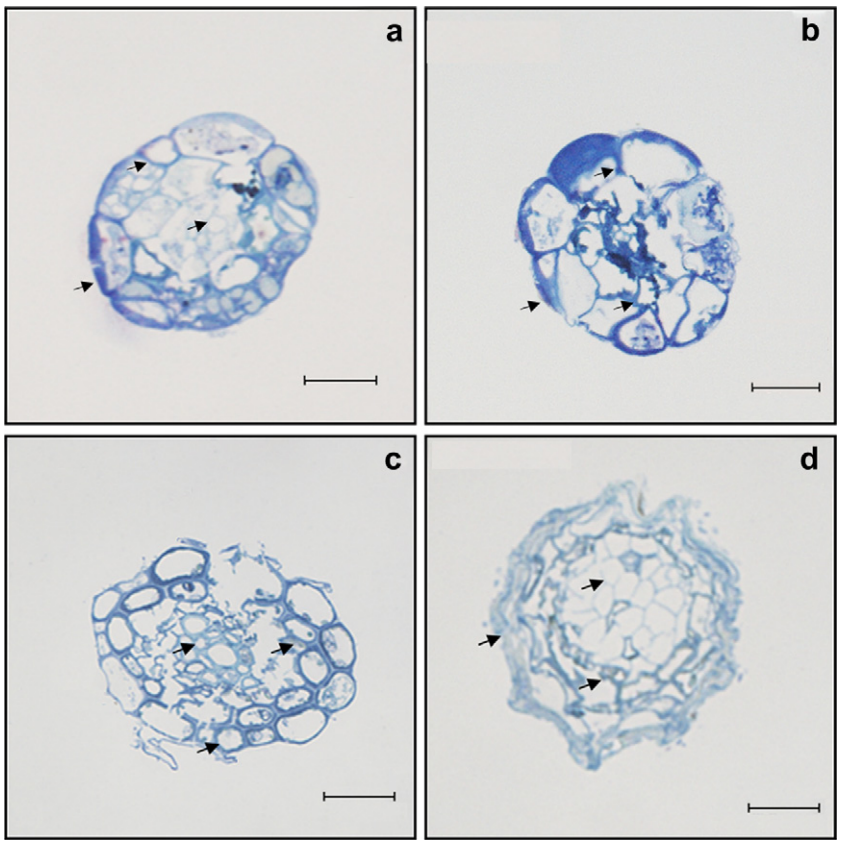

Fig. 1. Effect of Al-stress on root-tip structure of Brigitta (Al-resistant) and Bluegold (Al-sensitive) blueberry genotypes, grown for 48 h in 0 and 100 μM $AlCl_3$ in 0.5 mM $CaCl_2$ solution. (a) Brigitta without Al; (b) Brigitta with Al; (c) Bluegold without Al and (d) Bluegold with Al. The arrows indicate the effects of Al on root tip cells (see text for details). Scale bars represent: 25 μm.

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observed in leaves of unstressed plants [23]. The plants of both cultivars subjected to Al-stress treatment did not show any difference in Fv/Fm during the first 6 h of stress (Fig. 2). However, the Al-sensitive genotype experienced a significant decrease in Fv/Fm after 24 h of exposure to Al (Fig. 2).

To gain further insights into the effects of Al-stress on photosynthesis, other fluorescence parameters (ΦPSII, ETR and NPQ) were assayed (Fig. 3). In the Al-sensitive genotype (Bluegold), a significant decrease in ΦPSII and ETR values was observed at each time point in the Al-treated plants in comparison with the 0 h controls (Fig. 3a and c). By the end of the experiment (48 h), the sensitive genotype exhibited a significant reduction (55%) in ΦPSII after Al treatment, whereas this parameter fell by just 16% in the resistant genotype (Brigitta). Similar effects were observed in the ETR in both genotypes (Fig. 3c). The NPQ, which indicates the capacity of PSII to dissipate the excess energy as heat, increased significantly in the resistant genotype with respect to the zero-time point control, while in the sensitive genotype, NPQ levels diminished significantly in all time points of Al treatment (Fig. 3b).

3.3. Radical scavenging activity (RSA) and activities of antioxidant enzymes

Like other heavy metals and environmental stresses, presence of Al in the soil solution is known to promote the production of ROS [16]. Therefore, we evaluated the effect of Al-stress on radical scavenging activity (RSA) in the two genotypes. Interestingly, the RSA was 2-fold higher in leaves than in roots (Fig. 4). However, no differences in the RSA were found between genotypes at each time of Al treatment, with the exceptions of leaves at 48 h (Fig. 4a) and roots at 2 h (Fig. 4b) where significant differences ($p < 0.05$) were observed.

Additionally, we determined the activity of enzymes related to oxidative stress in roots and leaves. During the time course of the experiments, the activity of SOD in both genotypes in the presence of Al showed interesting changes. The resistant Brigitta genotype exhibited a strong increase (around 4-fold) in SOD activity in leaves after 2 h of exposure, levels which were maintained until 6 h. However, after 24 h, SOD activity returned to the initial levels (Fig. 5a). Interestingly, this initial increase in the activity of SOD was not observed in the sensitive genotype, in which activity remained constant (Fig. 5a). However, in roots, changes in SOD activity in both genotypes were similar; activity increased by 1.7-fold after 6 h of Al-stress, before diminishing gradually until 48 h (Fig. 5b). Subsequently, we decided to verify in leaves if the increase in SOD activity

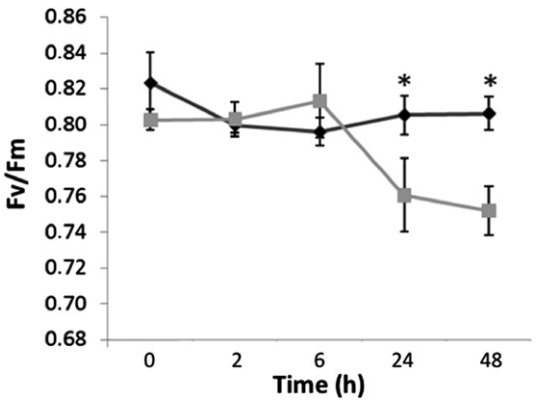

Fig. 2. Effect of Al-stress on the maximum quantum yield (Fv/Fm) in leaves of Brigitta (Al-resistant, black line) and Bluegold (Al-sensitive, gray line) blueberry genotypes. The data points represent the mean ± SE of at least three replicates. The asterisks indicate significant differences between genotypes ($P < 0.05$).

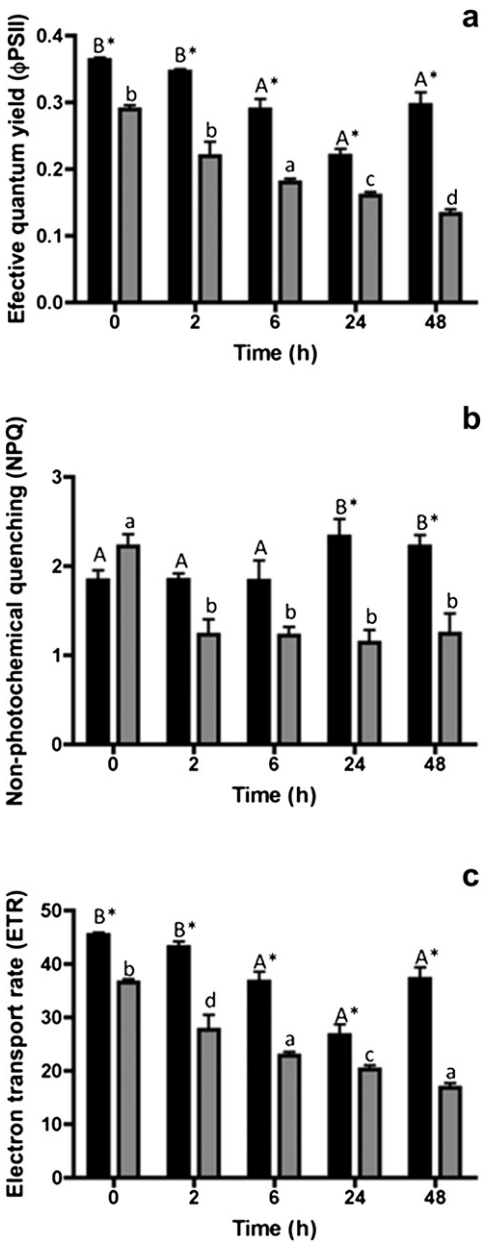

Fig. 3. Effect of Al-stress on effective quantum yield (ΦPSII), electron transport rate (ETR) and non-photochemical quenching (NPQ) in leaves of Brigitta (Al-resistant, dark bars) and Bluegold (Al-sensitive, gray bars) blueberry genotypes. The data points represent the mean ± SE of at least three replicates. Different upper case letters indicate significant differences between Al-exposure times for the same genotype and treatment whereas different lower case letters indicate significant differences between Al treatment for the same genotype and Al-exposure time. The asterisks indicate significant differences between genotypes ($P < 0.05$).

for the resistant genotype occurred in parallel with an increase in CAT activity. Indeed, a significant 2-fold increase in CAT activity was observed during the first 6 h of Al treatment in the resistant genotype, before falling back to pre-treatment levels after 24 h (Fig. 5c). CAT activity did not change during the time course of the experiment in leaves of the Al-sensitive genotype (Fig. 5c). Surprisingly, CAT activity in roots was higher in the resistant genotype, reaching a peak after 24 h of exposure to Al (Fig. 5d). Nevertheless, a decrease in CAT activity was observed in the sensitive genotype after 2 h of Al treatment before reaching values similar to the zero-time point control (Fig. 5d).

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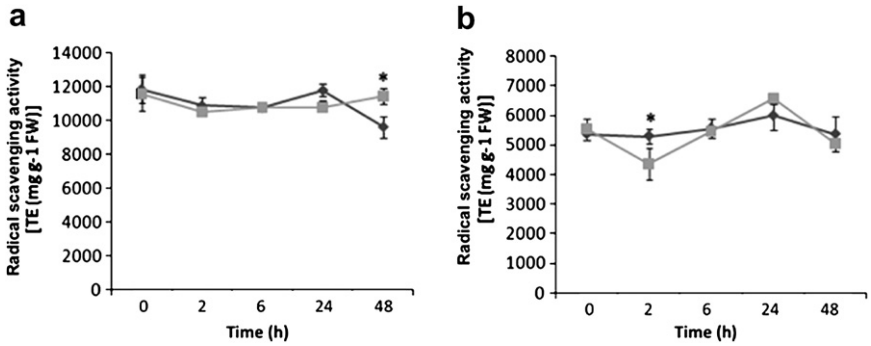

Fig. 4. Effect of Al-stress on radical scavenging activity in leaves (a) and roots (b) of Brigitta (Al-resistant, black lines) and Bluegold (Al-sensitive, gray lines) blueberry genotypes. The data points represent the mean ± SE of at least three replicates. RSA was measured as Trolox equivalents (TE) in roots and leaves. The asterisks indicate significant differences between genotypes ($P < 0.05$).

3.4. Expression analysis of antioxidant genes

Previously, we identified two genes homologous to *glutathione S-transferase* (*GST*) and *aldehyde dehydrogenase* (*ALDH*), here named as *VCAL21* and *VCAL68*, respectively [30]. During environmental stress, GST and ALDH have been described as two important antioxidant genes induced by ROS and lipid peroxidation in higher plants [9]. To evaluate the expression pattern under Al-stress of *VCAL21* and *VCAL68*, we performed qRT-PCR analysis (Fig. 6). In this study, we confirmed the expression pattern observed by cDNA-AFLP for *VCAL21* and *VCAL68* in roots. In general terms, both genes showed different expression patterns in leaves and roots. In leaves, we detected a basal expression of both genes in control conditions (time 0 h). *VCAL21* was inhibited by the treatment in both genotypes, and there was a significant difference in their response at 2 h (Fig. 6a). The resistant genotype showed greater expression of *VCAL68* in leaves after 6, 24 and 48 h of treatment in comparison to the sensitive genotype (Fig. 6c). Interestingly, the expression of *VCAL21* and *VCAL68* in roots was significantly different in both genotypes (Fig. 6b and d). Unlike the resistant genotype, a significant increase in the expression of *VCAL21* was observed after 2 h in the sensitive genotype, whereas in the resistant cultivar, the expression of *VCAL21* was higher after 24 h of Al treatment (Fig. 6b). A similar pattern was also observed for *VCAL68* expression, which had higher transcript levels detected after 2 h in both genotypes, especially in Bluegold (Al-sensitive; Fig. 6d). Subsequently, *VCAL68* expression peaked again after 24 h in the sensitive genotype, before falling abruptly after 48 h (Fig. 6d).

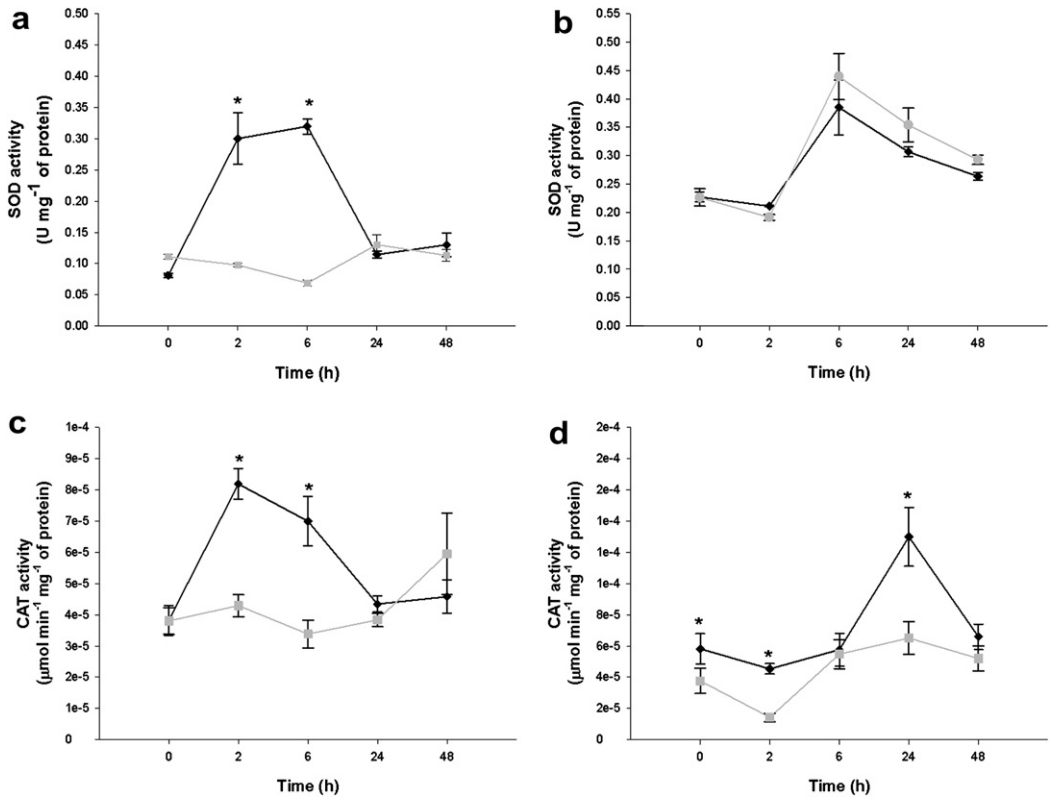

Fig. 5. Effect of Al-stress on the activity of SOD in leaves (a) and roots (b), and CAT in leaves (c) and roots (d) of Brigitta (Al-resistant, black line) and Bluegold (Al-sensitive, gray lines) blueberry genotypes. Changes in enzyme activities were compared with the control (time 0 h). The data points represent the mean ± SE of at least three replicates. The asterisks indicate significant differences between genotypes ($P < 0.05$).

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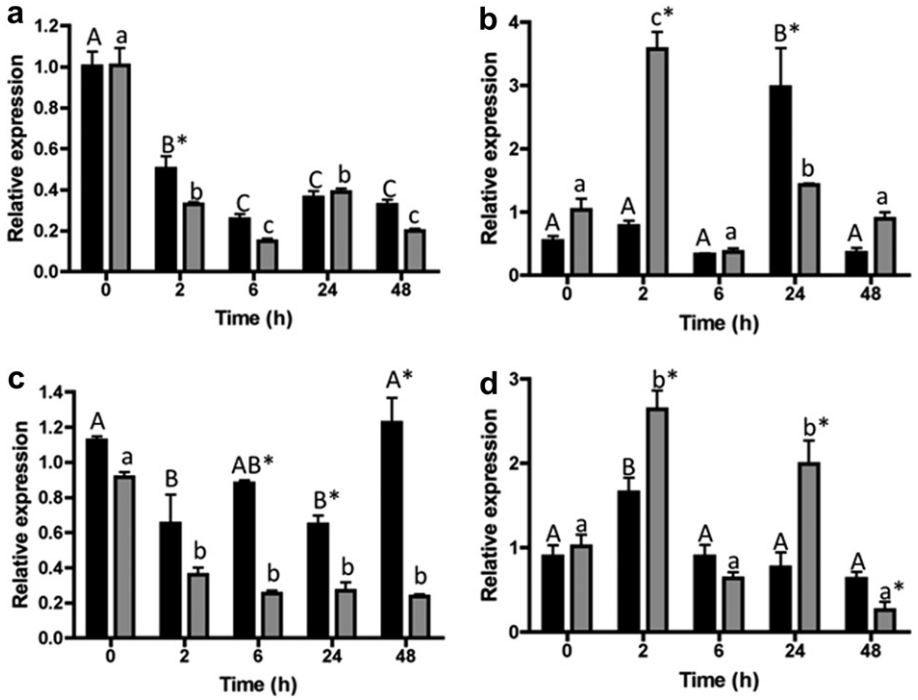

Fig. 6. Effect of Al-stress on the expression of *glutathione S-transferase* (*VCAL21*) in leaves (a) and roots (b), and *aldehyde dehydrogenase* (*VCAL68*) in leaves (c) and roots (d) of Brigitta (Al-resistant, black bars) and Bluegold (Al-sensitive, gray bars) blueberry genotypes. The data points represent the mean ± SE of at least three replicates. Different upper case letters indicate significant differences between Al-exposure times for the same genotype and treatment, whereas different lower case letters indicate significant differences between Al treatment for the same genotype and Al-exposure time. The asterisks indicate significant differences between genotypes ($P < 0.05$).

4. Discussion

This article attempts to elucidate the mechanisms underlying Al susceptibility in two blueberry genotypes with contrasting tolerance to this metal ion. For this, we adopted a multi-faceted approach, performing experiments to investigate morphological, physiological, biochemical and molecular aspects of the Al response in highbush blueberry, a very little-studied woody plant species.

Several studies have been carried out that demonstrate that Al induces oxidative stress, changes in gene expression and antioxidant responses [33–35]. Although Al itself is not a transition metal and is not able to catalyze redox reactions, it leads to a higher production of ROS, which are induced by oxidative stress in higher plants [16,36]. On the other hand, it is known that the outermost cell layers of roots constitute a primary protection mechanism against abiotic and biotic environmental stress factors [37]. Research by Brigham et al. [38] confirmed that border cells in roots are involved in the avoidance of Al-toxicity in pea. Nevertheless, it has been suggested that Al could lead to programmed cell death in roots [39,40] and also trigger DNA damage and adaptive responses to genotoxic stress [41,42] as a consequence of changes in the levels of ROS. We observed that Al responses are more pronounced in the layer epidermal and endodermal cells in root tips of the sensitive genotype. In maize plants, a rapid inhibition of cellular division in root tips has been observed after 5 min of exposure to Al in Al-sensitive genotypes [43].

It has been proposed that Al promotes damage in the sub-apical region of the roots, leading to the separation of the rhizodermis and outer cortical layers from the inner cortical cell layers. Additionally, this damage is related to the binding of Al to the cell wall, making this structure more rigid and less elastic [44,45].

Analysis of chlorophyll fluorescence parameters showed that during Al-exposure, Fv/Fm was in the normal range (near to 0.83) for healthy plants [23] of the two genotypes at the start of the experiment. However, the Al treatment induced a slight decrease (0.75) in the Al-sensitive Bluegold. In contrast, the Al-resistant genotype Brigitta maintained a value of 0.8 at all times. The slight decrease in Fv/Fm of Bluegold suggests some degree of disturbance of the photosynthetic apparatus under Al-stress. Furthermore, Al differentially affected ΦPSII and ETR of both genotypes, with the Bluegold genotype being more affected (Fig. 3). Similar results were reported by Reyes-Díaz et al. [19,46]. Our findings also confirm the report in leaves of *Citrus reshni* treated with Al, where a decrease in photochemical efficiency of PSII with respect to the untreated control was found [3]. The NPQ of the Bluegold genotype decreased with Al treatment, suggesting that thermal dissipation did not have a central role in dissipating excess excitation energy under Al treatment. Other dissipating processes such as the water–water cycle and photorespiration may be involved in the dissipation of excess energy, as found in other plants [47]. Our work confirms this assumption because when we measured the activity of antioxidant enzymes involved in the water–water cycle, we found significant increases in their activity in response to Al-stress (Fig. 5). These may be up-regulated and/or activated to cope with the increased excess of excitation energy under Al-stress. The activity of CAT, an enzyme involved in scavenging the bulk H_2O_2 generated by photorespiration [48], was augmented by Al in the early hours of Al-stress in Brigitta leaves (Fig. 5a) and after 24 h in roots (Fig. 5b). In the Bluegold genotype, significantly greater CAT activity was only observed in the leaves after 48 h of treatment, whilst both CAT and SOD activities showed similar kinetics in Brigitta during Al-stress (Fig. 5c). The CAT activity in roots of both genotypes showed an increase after 2 h, decreasing gradually afterward (Fig. 5d). Another method to evaluate the stress-induced antioxidant system is to measure the DPPH-radical scavenging activity, which is a means to quantify non-enzymatic antioxidant activity [49]. There were no differences in RSA between the blueberry

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genotypes in the different tissues. However, it can be seen that the RSA in leaves is two-fold higher than in roots (Fig. 4) and thus these results appear not to be associated with the Al-sensitivity of the Bluegold genotype. In this study, the expression of two antioxidant genes induced by Al-stress in roots and leaves was evaluated: *gluthatione S-transferase* (*VCAL21*) and *aldehyde dehydrogenase* (*VCAL68*). Both genes have been associated with the antioxidant response in higher plants [9,34]. Several Al-induced genes, such as *GST* and *SOD*, have also been found to be induced by oxidative stress [33], and overexpression of a *GST* (*parB*) of *Nicotiana tabacum* ameliorated Al-toxicity in *Arabidopsis* [17]. Further studies showed that this gene also provided protection against oxidative stress, suggesting that Al-stress and oxidative stress are related in plants [34]. In our work, an increased expression of *GST* (*VCAL21*) was observed in leaves of Brigitta (Al-resistant) in comparison to the Bluegold genotype (Al-sensitive) after 2 h of Al-stress (Fig. 6a). In roots, there was higher expression of *VCAL21* in the Bluegold genotype (Al-sensitive), peaking after 2 h of Al-stress (Fig. 6b). This suggests that Bluegold genotype (Al-sensitive) has to quickly activate some prompt mechanisms aimed at counteracting the stress, differently from the Brigitta (Al-resistant). That could be interpreting as an acclimation response of Bluegold genotype. Ezaki et al. [17] expressed the *GST* gene (*parB*) in *Arabidopsis* and found that it conferred substantial protection against Al-stress. These authors also suggested that expression of this gene is linked to both Al and oxidative stress. Lipid peroxidation is a common symptom of Al-toxicity [12], resulting in the generation of aldehydes in roots of tobacco, downstream of ROS [50]. Other studies have reported the isolation of an inducible gene encoding aldehyde dehydrogenases (ALDHs) in transgenic *Craterostigma plantagineum* and *Arabidopsis thaliana* plants conferring tolerance to heavy metals [9]. Unexpectedly, in our studies this gene (*VCAL68*) was highly-induced after 2 and 24 h of exposure to Al treatment in roots of the Al-sensitive genotype (Fig. 6d) whereas in leaves, there were significant changes in *VCAL68* expression after 6, 24 and 48 h of treatment in the Al-resistant genotype.

We conclude that the morphological, physiological and biochemical alterations monitored in this study contribute toward a higher Al-resistance of the Brigitta genotype. Surprisingly, at the molecular level, the expression of the two antioxidant genes evaluated in roots was more highly-induced in the Al-sensitive genotype (Bluegold) than in the resistant genotype. On the other hand, in leaves of the Al-resistant genotype, expression of both genes was induced, suggesting that these antioxidant genes may be involved in the Al-resistant mechanisms in the shoots of the plant. However, further molecular studies should be performed to clarify the Al-resistant mechanism in blueberry.

Acknowledgments

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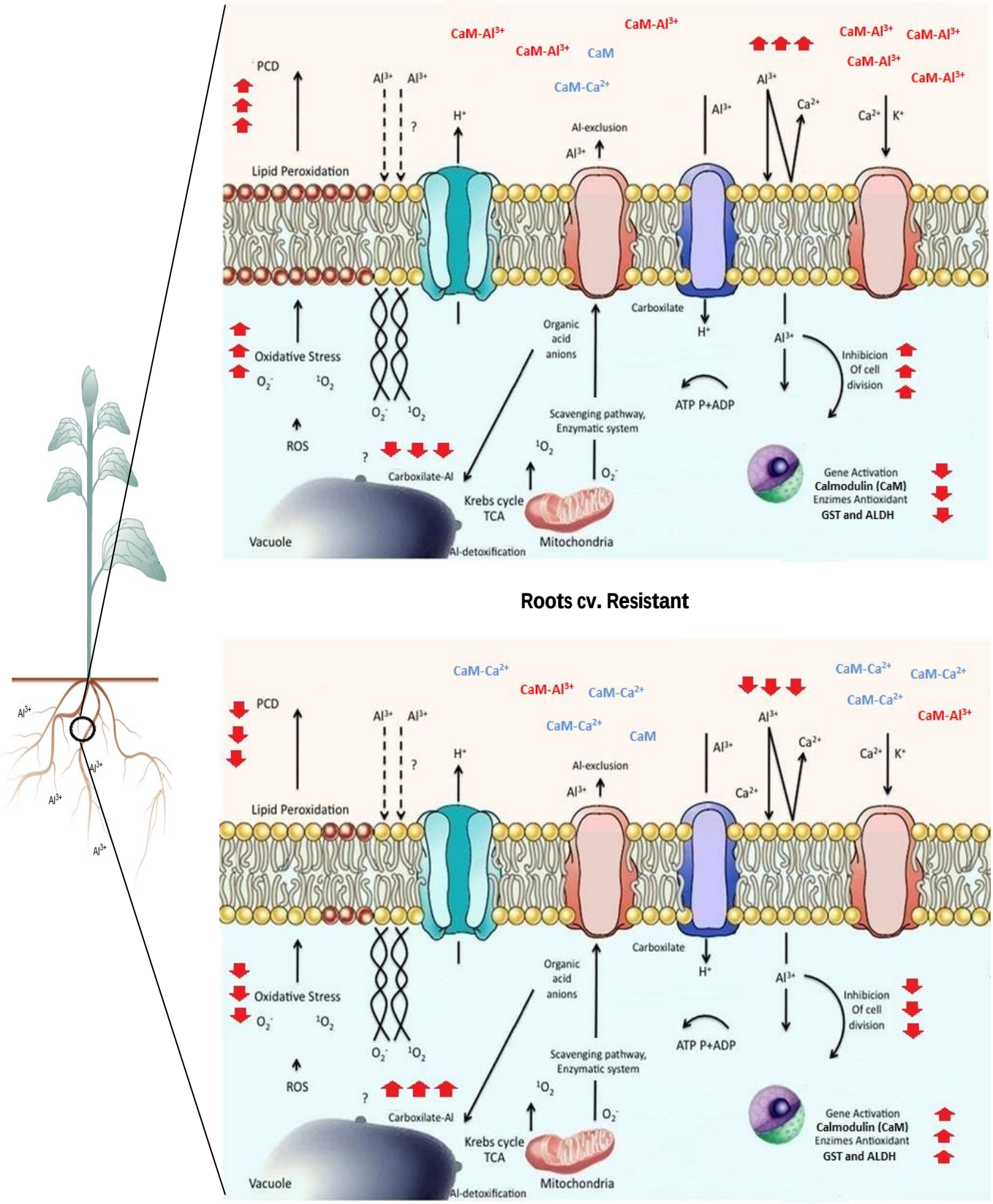
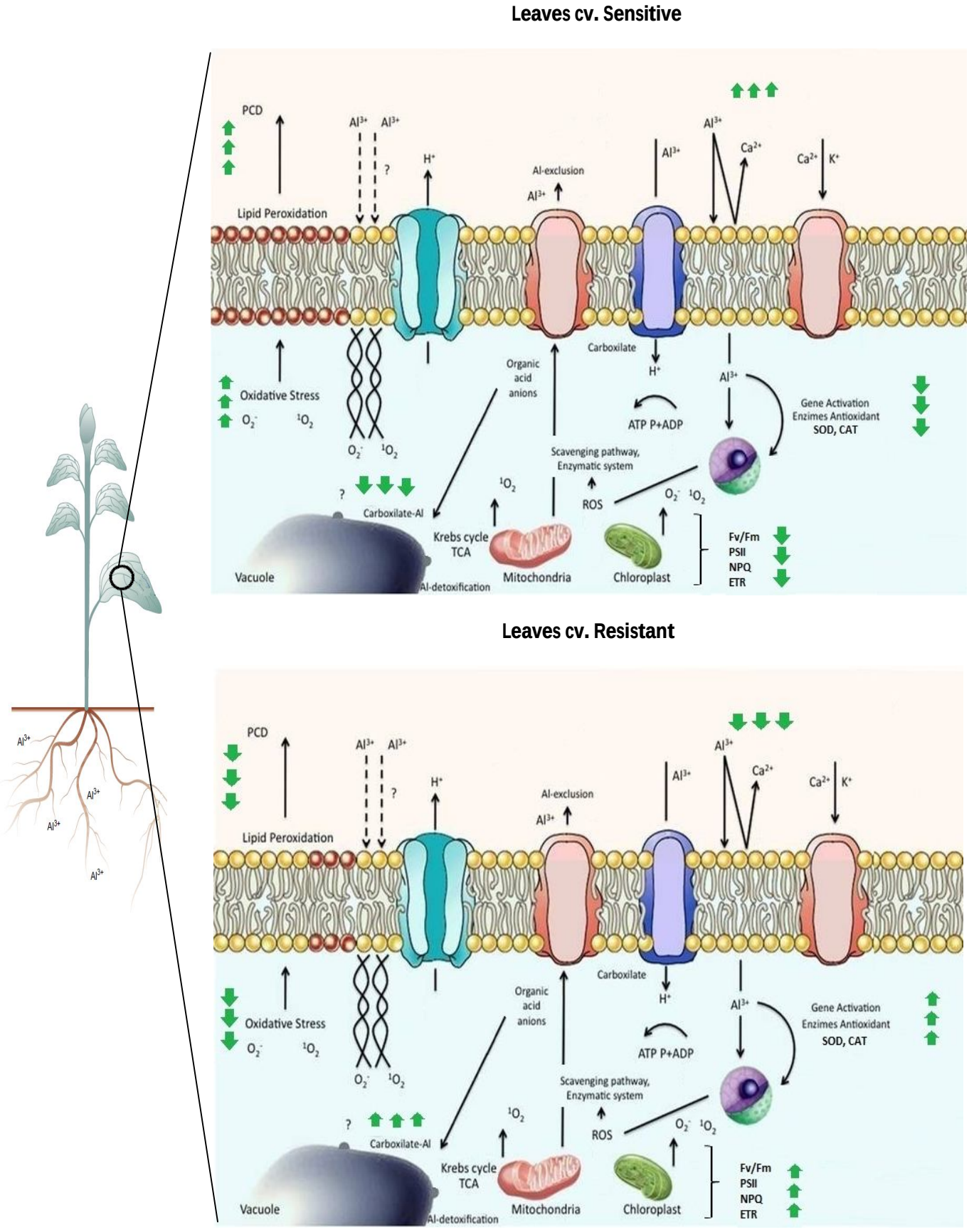


Model A: Diagrammatic representation at cellular levels of response to Al-toxicity on leaves of blueberry genotypes (Bluegold, Al-sensitive: at the top) and (Brigitta, Al-resistant: below), under controlled laboratory conditions. These models represent: Plasma membrane structure, with transmembrane proteins involved in transport processes, nucleus and subcellular organelles (chloroplast, mitochondria and vacuole) are represented. Unknown Al pathways are indicated segmented arrows. The big red arrows represent the performance state of the corresponding processes or metabolic events.



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