Cellular and ultrastructural alterations of Arabidopsis thaliana roots in response to exogenous trans-aconitic acid

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Abstract
In this work, the responses of Arabidopsis thaliana (L.) Heynh to trans-aconitic acid (TAA) were investigated. A. thaliana was grown in the presence of TAA in a concentration range of 400–1200 µM for 7 or 15 days. Changes in the morphoanatomy, cellular ultrastructure, and micromorphology of the roots were evaluated by light and transmission electron (TEM) microscopy. At concentrations below 1000 µM, TAA reduced the length of the primary roots, but induced an early appearance of lateral roots and root hairs. At a concentration of 1200 µM, TAA suppressed the growth of seedlings. The images of longitudinal sections of root tips of seedlings treated with IC50 of TAA (684 µM) revealed a reduced elongation zone with an increased differentiation zone. TEM images showed an increase in the number and volume of vacuoles, an increase in vesicles containing electron-dense material derived from plasmalemma, and electron-dense granules attached to the cell wall. Trans-aconitic acid induced an early differentiation of A. thaliana seedlings suggesting an interference in the auxin action. Changes in the cellular ultrastructure may represent vacuolar and extracellular accumulation of TAA, to remove excess TAA in the cytosol and mitochondria. An inhibition of aconitase and the chelation of intracellular cations may have contributed to cytotoxicity of TAA at 1200 µM concentration.

Keywords Organic acid · Auxin · Weed · Root system · Seedling · Crop protection

Introduction

Trans-aconitic acid (TAA) [(E)-1-propene-1,2,3-tricarboxylic acid], a natural isomer of the tricarboxylic acid cycle (TCA) intermediate cis-aconitate, occurs in nature in sugar-containing plants, such as sugar cane (Saccharum officinarum L.), wheat (Triticum aestivum L.) (Thompson et al. 1997), maize (Zea mays L.) (Brauer and Teel 1982), and sweet sorghum (Sorghum bicolor L.) (Klasson 2017). Trans-aconitic acid is also present in the forage species Urochloa sp. (Voll et al. 2004; Brum et al. 2009) and in medicinal plants, such as Asarum europaeum (Krogh 1971). It is also produced by bacteria of the genus Pseudomonas (Yuhara et al. 2015).

Trans-aconitic acid is synthesized by the interconversion between cis-aconitate and TAA, mediated by aconitate isomerase in both microbes and plants (Klinman and Rose 1971; Thompson et al. 1990), and by the dehydration of citric acid in plants catalyzed by citrate dehydratase (Brauer and Teel 1981, 1982). Although closely related to the TCA cycle, TAA is an effective inhibitor of mitochondrial and cytosolic aconitase and thus it is compartmentalized in vacuoles (Saffran and Prado 1949; Eprintsev et al. 2015).

The role of TAA in plants is not clear, but there is evidence that it acts as an antifeedant, (Katsuhara et al. 1993), in resistance against diseases (Kidd et al. 2001; Rémus-Borel et al. 2006) and in aluminum resistance (Kidd et al. 2001; Wenzl et al. 2002; Mariano and Keltjen 2003). The role of TAA and its methylated derivative as a phytoalexin has also been suggested based on its antifungal activity against...
Cladosporium cucumerinum (Rémus-Borel et al. 2006), in Si amendment, and in pathogen resistance in wheat plants (Rémus-Borel et al. 2009). A nematicidal effect of TAA synthesized by Bacillus thuringiensis has been suggested by Du et al. (2017). These bioactivities suggest a great potential of TAA and its methylated derivative in crop protection.

Because TAA can be extracted with high yields and purity from sugar cane molasses, which contains 0.5–3.0% of TAA (Dorman et al. 2015), its use for industrial purposes has been proposed, such as in the production of biodegradable polyesters (Dorman et al. 2015). However, studies on the effects of exogenous TAA on plants are scarce.

Herbicidal action has been suggested based on the suppressive action on the emergence of some weeds including Ipomoea grandifolia (Dammer) O’Donnell, Bidens pilosa L., Euphorbia heterophylla L., and Sida rhombifolia L. (Voll et al. 2010; Foletto et al. 2012). Phytotoxicity of exogenous TAA in the soybean has been reported by Coelho-Bortolo et al. (2017). These bioactivities suggest a great potential of TAA in the soybean has been reported by Coelho-Bortolo et al. (2017) but at higher doses of TAA than those reported (Foletto et al. 2012). Phytotoxicity of exogenous TAA (Foletto et al. 2012).

Alterations in seedling growth by phytotoxicants added to the soil are generally associated with changes in the root system, which can be observed in the cellular ultrastructure. Arabidopsis thaliana (L.) Heynh., an herbaceous plant of the Brassicaceae family, has been widely used as a model plant for studies on the regulation of root development, the responses to nutritional changes, and the effects of natural and synthetic compounds (Pang and Meyerowitz 1987; Williamson et al. 2001; Rahman et al. 2002; Müller and Schmidt 2004; Petersson et al. 2009; Pelagio-Flores et al. 2012; Kellermeier et al. 2014; Sánchez-Moreiras et al. 2018).

In the context of the potential utilization of TAA for agricultural purposes, in this work we investigated the mode of action of exogenous TAA by examining changes in morphology, cellular ultrastructure, and micromorphology in roots of A. thaliana seedlings.

Materials and methods

Plant material and growth conditions

Seeds of A. thaliana (L.) Heynh., ecotype Columbia (Col-0), were surface-sterilized with 50% ethanol (3 min) and 0.5% sodium hypochlorite (3 min), both in 0.01% Triton, and washed three times with distilled water. Subsequently, the seeds were vernalized in 0.1% agar (w/v) at a temperature of 4°C, for 48 h, and then transferred to the top of square Petri dishes (100×15 mm) containing 0.8% agar (w/v) supplemented with macro- and micronutrients (Murashige–Skoog basal medium, M5519 Sigma-Aldrich) and 1% sucrose (w/v); the pH was adjusted to 6.0.

The stock solutions of trans-aconitic acid were dissolved in distilled water, adjusted to pH 7.0 with KOH, then added to the agar at the proportion of 0.1% (v/v) to obtain the final concentrations of 400, 600, 800, 1000 and 1200 µM. For each concentration, 24 seeds were sown per square dish under a laminar flow hood, the dishes were sealed with “Leukopor” (BSN Medical) and placed upright in a growth chamber for promoting geotropism, at a temperature of 22°C, a photoperiod of 8 h light (120 µmol/m²s), and a relative humidity of 55%. After 15 days, the primary root length was measured and the dose–response curve was used to calculate the IC₅₀ for root growth inhibition. Seedlings treated with IC₅₀ TAA for 7 and 15 days were randomly selected to photograph the whole root structure in an Olympus SZX9 stereoscopic microscope.

Light and transmission electron microscopy (TEM) of longitudinal sections of Arabidopsis roots

In total, 40 roots of A. thaliana grown in the absence (control) or presence of the IC₅₀ of TAA for 7 and 15 days were used for microscopic studies. The apical meristems, approximately 0.5 cm, were cut, immersed in 0.1 M cacodylate buffer (pH 7.2) with 5% glutaraldehyde fixative, and incubated for 4 h. Subsequently, three washes of 4 h each were performed with 0.1 M cacodylate buffer (pH 7.2). After washing, the samples were immersed in 0.1 M cacodylate buffer with 2% osmium tetroxide for 3 h and in 10% acetone with 2% uranyl for 1 h. For dehydration, the samples were immersed in increasing dilutions of acetone: 50% acetone (2×30 min), 75% acetone (2×1 h), 80% acetone (2×1 h), 95% acetone (2×1 h), and 100% acetone (2×2 h). After this, impregnation was started in the rotor with Spurr resin: 1:3 Spurr in acetone (3×2 h), 2:2 Spurr in acetone (3×2 h), and 3:1 Spurr in acetone (2×2 h + 3×1 h); all dehydration and impregnation steps were carried out at 4°C.

Inclusion in Spurr resin was performed in the rotor at room temperature. Subsequently, the samples were placed in resin molds and heated in an oven at 60°C for 2 days. Semi-fine cuts of 0.7 µm and ultrathin cuts of 50–70 nm were done for light and electron microscopy, respectively.

The semi-fine sections were stained with toluidine blue and observed under a Nikon Eclipse 800 light microscope attached to a Nikon DS-U2 digital camera with the NIS-Elements D 2.30 SP1 software.

The ultrathin sections were collected on copper grids of 100 and 200 “mesh” and contrasted with uranyl acetate (2%) for 30 min and with lead citrate (Reynolds 1963) for 12 min (2-min washes with ultrapure water were done after each
Ultrathin sections were observed with a TEM JEOL JEM-1010 (100 kV) (Peabody, MA, USA) equipped with an Orius-CCD digital montage plug-in camera (Gatan Inc., Gatan, CA, USA) and a Gatan Digital micrograph software (Gatan, Inc.).

**Statistical analysis**

All the experiments were carried out in a completely randomized design with five replications for dose–response curve. The data were expressed as mean ± standard error (S.E.) and analyzed using analysis of variance (ANOVA). Significant differences between means were identified by Duncan’s multiple range test, and $P \leq 0.05$ was adopted as the minimum criterion of significance. The IC$_{50}$ values were calculated by numerical interpolation through a cubic spline function of GraphPad Prism 5 software. Statistical analyses were performed using the Statistic™ software package.

**Results**

**Root morphology**

Representative images of *A. thaliana* seedlings grown in the absence (control) or presence of TAA at a concentration range of 400–1200 µM for 15 days are shown in Fig. 1. *Trans*-aconitic acid significantly altered root morphology, inducing a reduction of primary root length along with an increase in the number of lateral roots. At the highest concentration of 1200 µM, the development of both aerial parts and roots was extremely reduced compared with the untreated seedlings. Figure 2 shows the dose-dependent reduction in the length of primary roots with an IC$_{50}$ of 684.31 µM.

Stereoscopic microscope images of root tips from 7- and 15-day-old untreated seedlings (Fig. 3a, c, e, g) show well-characterized differentiation and elongation zones, with a progressive development of root hairs in the differentiation zone.
zones. Seedlings treated with IC50 TAA (684 µM) for 7 days exhibited a precocious initiation of lateral roots and a reduction in the number and length of root hairs (Fig. 3b, d). Different from the root tip of control seedlings, TAA-treated seedlings exhibited bulges of root hairs near the root tip (Fig. 3d). After 15 days of treatment a highly branched root system with an abundance of long hairs in the lateral roots was observed (Fig. 3f, h). The root tip exhibited asymmetric rows of cells and ectopic root hairs, which were longer compared with those found at the 7th day of treatment (Fig. 3b, d).

The longitudinal sections of roots observed by light microscopy (Fig. 4) showed that treatment with TAA did not cause significant changes in the tissue organization in the root cap, elongation, and differentiation zones when compared with the control (Fig. 4a, c) with symmetric rows of cells. There was, however, a clear change in the division pattern of root zones, with a relative reduction in the elongation zone and a consequent increase in the differentiation zone. These findings were observed on the 7th day (Fig. 4b) and on the 15th day of treatment with TAA (Fig. 4d).

**Root cell ultrastructure**

Comparison of the ultrastructural analysis of root tip cell structure and organization in seedlings of *A. thaliana* grown for 7 or 15 days in the absence (Figs. 5, 6) or presence of TAA (Figs. 7, 8) revealed modifications in different cell organelles, particularly in vacuoles and
mitochondria, and the appearance of electron dense materials in intracellular vesicles or in the cell corners. In both 7- and 15-day-old seedlings, an increased number of vacuoles containing dense granular material were observed in the cytosolic space. In the interspace between cell wall and plasma membrane, it was found a high number of vesicles, indicating increased secretory activity. In addition, cell corners appeared swollen compared to the control and rich in electron-dense deposits.

Increased numbers of mitochondria with variable shapes were observed in 7-day-old TAA-treated cells, including large and extended mitochondria, characteristic of dividing

Fig. 5 TEM micrographs of root tip cells from a 7-day-old A. thaliana seedling (Control), showing a complete cell with centrally located nucleus (a, c), nucleus with one or two nucleoli (a, b), a number of small vacuoles (a, c), mitochondria of various shapes (a–c), vesicles (d) amyloplasts (b), endoplasmic reticulum (d, e), Golgi apparatus (d, e), cell wall with plasmodesmata (e). AP Amyloplasts, CC cell corners, CW cell wall, ER endoplasmic reticulum, G Golgi apparatus, M mitochondria, Nue nucleoli, N nucleus, V vacuoles, Ve vesicles, P plasmodesmata

Magnification
(a, b) 1200 x; (c) 2500 x;
(d, e) 6000 x
mitochondria. In 15-day-old TAA-treated cells, it was also found enlarged mitochondria with irregular shapes, but no evidence of broken mitochondria or significant changes in their cristae stromal translucency. In both 7- and 15-day-old TAA-treated seedlings no significant change was observed in the cell format, their nuclei, and in the endoplasmic reticulum and the Golgi apparatus when compared with untreated ones.

**Fig. 6** TEM micrographs of root tip cells from *A. thaliana* (Control) grown for 15 days, showing features similar to those of 7-day-old root tip cells regarding the format of complete cell, nuclei (a–c), the number and shapes of mitochondria and vacuoles (a–c), endoplasmic reticulum (d, e), Golgi apparatus (d, e), cell wall with plasmodesmata (c), and amyloplasts (a). AP Amyloplasts, CW cell wall, ER endoplasmic reticulum, G Golgi apparatus, M mitochondria, Nue nucleoli, N nucleus, V vacuoles, Ve vesicles, P plasmodesmata

Magnification

(a, b) 1200 x; (c) 2500 x;
(d) 6000 x; (e) 10000 x
Discussion

This study revealed that the exogenous application of TAA at a concentration range of 400–1200 µM exerted significant changes in the development of A. thaliana seedlings, with a pronounced alteration of root architecture. Despite a substantial reduction in primary root length, the TEM images of root tips of seedlings grown in the presence of TAA at

![Fig. 7 TEM micrograph of root tip cells of A. thaliana grown for 7 days in the presence of IC_{50} trans-aconitic acid, showing a complete cell with nucleus not significantly different from that of untreated ones (a), amyloplasts (a), increased number of mitochondria and vacuoles (a–d), presence of cell phragmoplast (b), mitochondria with various sizes (e), including a huge and extended mitochondria (d), swollen cell corners with accumulation of electron-dense deposits (e), numerous vesicles in the plasma membrane/cell wall interspace (d, e), vacuoles containing dense granulose material and some of them with irregular shapes (c, d). AP Amyloplasts, CC cell corners, CW cell wall, ER endoplasmic reticulum, M mitochondria, Nue nucleoli, N nucleus, V vacuoles, Ve vesicles, Ph phragmoplast, P plasmodesmata

Magnification

(a) 1200 x; (b) 2000 (c) 2500 x;
(d) 6000 x; (e) 10000 x
the IC$_{50}$ concentration (684 µM) did not reveal structural
disorganization of the cell layers or the presence of hyper-
trophic or ruptured cells, alterations normally found under
cell death conditions (Burgos et al. 2004; Ishii-Iwamoto
et al. 2012; Diaz-Tielas et al. 2012). Apparently, there was
no cellular energy deficit, since the growth of aerial parts of
*Arabidopsis thaliana* seedlings grown in the presence of 400–800 µM
TAA was not significantly altered. However, a predominance

**Fig. 8** TEM micrograph of root tip cells of *A. thaliana* grown for
15 days in the presence of IC$_{50}$ trans-aconitic acid, showing a com-
plete cell with an increased number of mitochondria (a, b), presence
of amyloplasts (a), and no significant differences in the nuclei (b, c)
compared with those from untreated cells, enlarged vacuoles contain-
ing electron-dense material (b, e), enlarged mitochondria with irregu-
lar shapes (c, d), membrane-bound structures containing granulose
material (d, e), swollen cell corners with accumulation of electron-
dense deposits (be); vesicles in the plasma membrane/cellwall inter-
space containing granulose material (d, e). AP Amyloplasts, CC cell
corners, CW cell wall, ER endoplasmic reticulum, G Golgi apparatus,
M mitochondria, Nue nucleoli, N nucleus, V vacuoles, Ve vesicles

Magnification

(a, b) 1200 x; (c) 2500 x;
(d) 6000 x; (e) 10000 x
of cytotoxic effects was observed at the highest dose of 1200 µM TAA, with a substantial reduction in the development of both roots and aerial parts of the seedlings.

The morphological and ultrastructural changes of A. thaliana seedlings at lower doses of TAA are, most probably, reflecting the direct effects of TAA and the adaptive responses of cells to higher TAA accumulation in the cells. TAA caused a reduction of the distance between the meristematic zone and the zone of lateral roots formation, with precocious appearance of lateral roots and root hairs just above the root apex. These findings suggest a premature exit of cells from the meristematic zone, probably due to the loss of quiescent center identity, an action similar to that induced by farnesin in A. thaliana (Araniti et al. 2017). These changes suggest an interference in the homeostasis of hormones that act in an integrated way in the development of the root system, including auxins, cytokinins, abscisic acid, ethylene and gibberellins (Clouse and Sasse 1998; Depuydt and Hardtke 2011; Ubeda-Tomás et al. 2012; Pacifici et al. 2015; Araniti et al. 2017).

It seems that TAA interfered with the development of A. thaliana as many natural and artificial substances that act in a similar mode to the exogenous auxins. In general, these auxinic compounds induce elongation of primary roots in low concentrations and/or inhibition in higher concentrations, promote the growth of stems, lateral/adventitious roots, and root hair, and facilitate root gravitropism (Casimiro et al. 2003; Benková et al. 2003; Laskowski et al. 2006; Pelagio-Flores et al. 2012; Pacurar et al. 2014; Wang et al. 2016). The regulatory actions of auxin on root development depend on the reactions of the synthesis, conjugation, transport, signaling, and/or metabolism (Diekmann et al. 1995; Friml et al. 2003; Cheng et al. 2007), defining the effective concentrations of auxins in different zones of the primary roots along its longitudinal axis (Swarup et al. 2005; Verbelen et al. 2006; Wang et al. 2016). The auxins can be protonated or deprotonated depending on the pH and these state of protonation alter their affinity to transporters or receptors and the diffusability across membranes (Swarup et al. 2005; Verbelen et al. 2006; Hachiya et al. 2014; Wang et al. 2016). It seems plausible that changes in the pH of different parts of root tissues play a role in the mode of action of TAA in A. thaliana. As a low molecular organic acid, the exogenous TAA may penetrate and accumulate in many parts of the root tissues, changing the local pH. By crossing the cell plasma membrane in the undissociated form, TAA can lead to a decrease in the internal cell pH when protons are internally released, which can also result in TAA accumulation in the dissociated form (Piper et al. 2001; Klasson 2017). Thus, the effects of TAA on root development may be, partly at least, mediated by changes in IAA distribution, an action secondary to TAA-induced changes in the pH in root tissues.

Access of TAA to various tissues and cell compartments was evidenced by TEM images, showing an increase in the vacuoles and membrane vesicles in TAA-treated seedlings. It is known that organic acids such as malate and citrate are accumulated in the vacuole, reaching concentrations 1 to 10-fold higher than in the cytosol, a process favored by low vacuolar pH values (Osmond 1976; Chang and Roberts 1991; Gout et al. 1993). The increase in the number and volume of vacuoles, along with the deposition of dense materials observed in TEM images, may represent a mechanism to prevent excessive TAA in the cytosol and mitochondria, forcing it into a limited area. Besides accumulation in vacuoles, TEM images showed vesicles derived from the invaginations of plasmalemma also containing dense materials and electron-dense granules attached to the cell wall. These findings suggest active apoplastic and symplastic movements of TAA in the roots of A. thaliana, changing the pH of various tissue compartments.

The accumulation of large amounts of TAA in the vesicles and/or in the cell wall was possibly favored because of its ability to chelate intracellular cations such as Ca²⁺, Mn²⁺, Mg²⁺, or Fe³⁺. This property is the basis of the exudation of many organic acids such as malate, malonate, citrate, and aconitate from the roots to the soil to favor nutrient acquisition and to protect plants against metal detoxification (Jones 1998). The binding of TAA with magnesium is also the cause of the grass tetany syndrome in ruminants (Thompson et al. 1997). It seems that electron-dense particles, seen in vacuoles, vesicles and cell walls were derived from insoluble salts of TAA with cellular cations. This accumulation of substances outside cells usually represents a protective mechanism of the cells to foreign or self-produced substances, a phenomenon that has been observed in the protection against poisoning ions such as cadmium (Khan et al. 1984) and lead (Phang et al. 2011).

The vacuolar and cell wall accumulation of TAA may play a role in the tolerance of A. thaliana to TAA at concentrations lower than 1000 µM. By preventing the circulation of excessive free TAA in the cytosol and mitochondria, the inhibition of mitochondrial and cytosolic aconitase could be prevented (Saffran and Prado 1949; Epriptsev et al. 2015). This assumption is in accordance with the lack of signs of metabolic disturbances in TAA-treated seedlings. Under an energy deficit condition, numerous disturbances in the cellular ultrastructure are expected, such as in chalcone-treated A. thaliana roots (Diaz-Tielas et al. 2012), in which the cells become irregular, swollen, and deformed, and the different zones cannot be longer distinguished in the apical meristem. Besides not exhibiting such alterations, many signs of energy-dependent processes were observed in TAA-treated cells, including an active cell and mitochondria division seen in TEM images of root tips and the development of numerous lateral roots.
The mechanisms of intra- and extracellular accumulation of TAA must be kinetically controlled and dependent on exogenous concentrations of TAA. Possibly, at a higher concentration of 1200 µM, these mechanisms reached saturation as the development of A. thaliana was strongly inhibited. Under this condition, free TAA may reach concentrations inhibitory to aconitase. The inhibition of mitochondrial aconitase by TAA is higher than that of cytosolic aconitase, and thus, the citrate may be directed to isocitrate formation in the cytosol, disturbing TCA functioning (Eprintsev et al. 2015). Magnesium is an essential cofactor for aconitase activity, and the binding of free magnesium by TAA also contributes to aconitase inhibition (Blair 1969). Other enzymes dependent on Mg2+ or other cations, such as Ca2+, may also be affected by TAA at a higher concentration. Therefore, besides mitochondrial energy metabolism, a systemic metabolic dysfunction could be expected.

The equilibrium between cellular protection and cytotoxicity against exogenous TAA likely depends on the species. In Glycine max L., Coelho-Bortolo et al. (2018) observed alterations in root growth at TAA concentrations higher than 2000 µM and alteration of photosynthetic parameters only at concentrations higher than 7500 µM. In the weed species I. grandifolia, TAA inhibits seedling growth in a concentration range of 30–300 µM (Foletto et al. 2012), i.e., well above the cytotoxic concentrations in Glycine max. TAA seems to interfere with weeds at concentrations well below those toxic to crops.

Conclusions

The data presented here reveal that the roots of A. thaliana are highly sensitive to exogenous TAA. The alterations in the root morphology and ultrastructure were suggestive of adaptive responses to exclude free TAA from the cytosolic and mitochondrial space, thus preserving cell metabolism. The movements of TAA from the external medium into the cells probably changed the pH along the primary root, affecting the actions of auxins, as suggested by the early appearance of lateral roots and root hairs. At concentrations higher than 1000 µM, the adaptive responses seem not be longer effective leading to cytotoxic effects. Besides contributing to an understanding of the mode of exogenous TAA action on initial seedling growth, the results of the present work highlight the potential of TAA and also of cover plants rich in TAA, such as sweet sorghum and Urochloa, to reduce weed infestations. A decrease in weed competitiveness due to the actions of a natural compound can contribute to minimize crop yield losses and to decrease the use of synthetic herbicides.

Author contributions statement AMS-M, MJR and ELI-I contributed to the study conception and design. Material preparation, data collection and analysis were performed by KAK-C and MSM. The first draft of the manuscript was written by ELI-I and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

Conflict of interest Author Adela María Sánchez-Moreiras has received research grants from Spanish Ministry of Economy and Competitiveness (Project number AGL2013-41281-R). Kátia Aparecida Kern-Cardoso has received a doctorate-sandwich scholarship from the Coordination for the Improvement of Higher Education Personnel (CAPES).

Ethics approval Not applicable.

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