

Effect of transporters on the secretion of phytochemicals by the roots of *Arabidopsis thaliana*

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Abstract Root exudation, the process by which plants secrete compounds into the soil, is becoming accepted as a communicative process that determines organismal interactions in the rhizosphere. However, the mechanistic processes involved in the root exudation of phytochemicals have not been elucidated; traditionally, exudation has been regarded as a passive process. There is evidence that transporters in plants (and other organisms) have been involved in the movement of chemicals across different membranes. Here, we describe the involvement of different transporters in root exudation of phytochemicals by employing a pharmacological approach. We used a range of concentrations of several compounds known to inhibit different transporters, including potassium cyanide, orthovanadate, quinidine, glibenclamide, nifedipine and verapamil, to examine the effects of transporter inhibition on root exudation profiles in *Arabidopsis*. Generally, the exudation profile of phenolic compounds in 18-day-old

plants shows more than 15 major phytochemicals. In contrast, the inhibitors listed above caused differences in the secretion of specific compounds. For instance, nifedipine and verapamil completely inhibited the exudation of the phytochemicals with molecular masses of 142 and 294, respectively. These results highlight that root exudation of phytochemicals is an active process controlled at the biochemical level and that different transporters may be involved in this root-specific mechanism.

Keywords *Arabidopsis thaliana* · Exudates · Inhibitors · Phytochemicals · Transporters

Abbreviations

MATE	Multidrug and toxic compound extrusion
MDR/PGP	Multidrug resistance protein/P-glycoprotein
RT	Retention time

Introduction

The phytochemicals present in the root exudates of plants mediate several types of communication processes in the rhizosphere such as root–root, root–microbe and root–insect interactions (Walker et al. 2003a; Bais et al. 2004), and some of these interactions, such as the legume–rhizobium symbiosis, are of extreme agricultural importance (Colebatch et al. 2002). Despite the importance of root exudation of phytochemicals to agriculture and ecology, the genes and networks that control this process have not been studied; and in fact classical knowledge about root exudation regards this occurrence as a passive process

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controlled by the concentration gradient of compounds (Phillips et al. 2004).

Current literature suggests that phytochemicals are transported from the site of synthesis to the site of storage by vesicles or specialized organelles (Grotewold 2001). For example, it has long been known that specific steps of the isoquinoline alkaloid biosynthetic pathway are sequestered in alkaloid vesicles, and that pathway intermediates must traffic from one subcellular compartment to another by mechanisms that prevent their free diffusion in the cytosol (Grotewold 2001). Thus, similar mechanisms could operate in roots in such a way that vesicles present in root epidermal or cap cells could fuse to the cell membrane to release their components as root exudates.

Evidence in other systems indicates that membrane transporters may be involved in the root secretion of phytochemicals. The ABC superfamily of membrane transporters found in animals, plants, fungi and bacteria participate in the transport of compounds across the membrane and protect cells from xenobiotics (Holland et al. 2003; Dean and Annilo 2005). In plants, members of this gene family have been involved in the transport of phytochemicals across different membranes. Among the compounds that are mobilized by these transporters are: glutathione-conjugates (Martinoia et al. 1993; Lu et al. 1998; Tommasini et al. 1998), chlorophyll catabolites (Lu et al. 1998; Tommasini et al. 1998), antifungal terpenoids (Jasinski et al. 2001), auxins (Noh et al. 2001; Geisler et al. 2005), flavones (Klein et al. 2000), berberine (benzylisoquinoline alkaloid) (Shitan et al. 2003; Otani et al. 2005), peptides (Kispal et al. 1999), carbohydrates, lipids (Zolman et al. 2001), inorganic acids, steroids (Forestier et al. 2003), tropane alkaloids (hyoscyamine and scopolamine) (Goossens et al. 2003), anthocyanins (Goodman et al. 2004), wax precursors (Pighin et al. 2004) and xenobiotics (Baerson et al. 2005), among others.

The plasma membrane in plant cells also has other types of transporters that could be involved in the movement of phytochemicals, although this function has not yet been conclusively demonstrated. For instance, a family of putative secondary transporters unique to plants and microbes is MATE (multidrug and toxic compound extrusion). The MATE family is thought to encode efflux pumps (Brown et al. 1999). Pumps of this family utilize an electrochemical potential of Na^+ across the cytoplasmic membrane as the driving force with one exception, where the electrochemical potential is provided by H^+ (He et al. 2004). Also, plants have three distinct membrane H^+ -pumps capable of generating pH gradients (Sze et al. 1999). One member of this group, the vacuolar H^+ -ATPase (V-ATPase) complex, acidifies

the vacuole and other intracellular trafficking compartments, and it has been found that this type of pump is required for endocytic and secretory trafficking in *Arabidopsis* (Dettmer et al. 2006), as well as for root growth.

Combining this extensive literature on transporters with recent reports on root–microbe interactions showing that pathogens can alter the secretion of phytochemicals (Bais et al. 2005), and thus that this process is biologically and biochemically controlled, we developed a pharmacological approach to test the effect of transporter inhibitors on root secretion of phytochemicals.

Materials and methods

Plant material and growth conditions of *Arabidopsis thaliana* in sterilized planting mix

Wild-type *Arabidopsis thaliana* ecotype Columbia (Col-0) seeds (Lehle Seeds, Round Rock, TX, USA) were surface-sterilized using sodium hypochlorite (3%, v/v) for 2 min followed by three washes with sterile distilled water, and germinated on solidified Murashige and Skoog (MS) (Caisson Laboratories, Inc; Rexburg, ID, USA) basal medium (Murashige and Skoog 1962) in a growth chamber (Percival Scientific, Perry, IO, USA) at 25°C, with a photoperiod of 16 h light and 8 h dark.

Inhibition assays

For inhibition assays, 7-day-old seedlings were transferred to 12- or 6-well culture plates (Fisher Co., Hampton, NH, USA) each holding 2 or 5 ml of liquid MS basal media containing 3% (w/v) sucrose, incubated on an orbital shaker at 90 rpm, and illuminated under cool white fluorescent light ($45 \mu\text{mol m}^{-2} \text{s}^{-1}$) with a photoperiod of 16 h light/8 h dark at $25 \pm 2^\circ\text{C}$ for 11 days prior to the addition of inhibitors. The inhibitors were added when the plants were 18 days old. Stock solutions (10 mM) in water for sodium azide, potassium cyanide, sodium orthovanadate, and in DMSO for nifedipine, quinidine, glibenclamide, verapamil, as well as cyclosporin A 1 mM in methanol (all the chemicals were from Sigma Chemical Co., St. Louis, MO, USA) were prepared and filter-sterilized. Plantlets 18 days old were washed with sterile water and transferred to 12- or 6-well culture plates (Fisher Co., Hampton, NH, USA); each well held 2 or 4 ml, respectively of liquid MS basal media containing 3% sucrose and the appropriate amount of inhibitor. The doses and times for each inhibitor are described in the legends of each figure. The plates were incubated in a shaker at 90 rpm and $25 \pm 2^\circ\text{C}$. The effect of each

inhibitor on growth was assessed by fresh weight, and the analysis of the exudates was performed in each case by high-performance liquid chromatography (HPLC) analysis combined with mass spectroscopy (see below).

Medium extraction

To examine rhizosecreted phytochemicals from *A. thaliana* roots, liquid media samples from in vitro-grown Arabidopsis plants with well-differentiated roots were collected from two 6- or 12-well plates to make a final volume of 45–48 ml, filtered through a nylon syringe filter of pore size 0.22 μm (Pall Life Sciences, East Hill, NY, USA, Cat. PN 4612 or Nalagene, Rochester, NY, USA, Cat. 195-2520) to remove any cellular debris and concentrated by freeze-drying (Labconco, Kansas City, MO, USA) to remove water; the concentrate was dissolved in 5 ml double distilled water and extracted with ethyl acetate (Fisher Scientific) 1:1 volume, three times. The ethyl acetate fractions were pooled together and air-dried. The final concentrate was dissolved in 500 μl of absolute methanol (Fisher Scientific) and analyzed by HPLC. The same procedure was followed for each treatment.

High-performance liquid chromatography and mass spectroscopy analyses of root exudates

Extracts from the media were injected into an HPLC system. Compounds in the root exudates were chromatographed by gradient elution on a 150 mm \times 4.6 mm reverse phase, C_{18} column (Dionex Co., Sunnyvale, CA, USA). The chromatographic system (Dionex Co., Sunnyvale, CA, USA) consisted of two P680 pumps connected to an ASI-100 automated sample injector. The injected samples (30 μl) were detected at 280 nm with a UV-vis detector (Dionex Co., Sunnyvale, CA, USA). Mass determination of the peaks was made with an MSQ-MS detector system (Thermo Electron Co., Waltham, MA, USA) operating in the negative ion mode.

A second chromatographic system (Dionex Co., Sunnyvale, CA, USA) was used that consisted of P580 pumps connected to an ASI-100 automated sample injector. The injected samples (30 μl) were subjected to a broad-range wavelength scan between 190 and 800 nm using a PDA-100 photodiode array variable UV-vis detector (Dionex Co., Sunnyvale, CA, USA)

A gradient was applied for all separations with a flow rate of 1 ml min^{-1} . The gradient was as follows: 0–10 min, 90.0% water and 10% methanol; 10–60 min, 10.0–90% (v/v) methanol, 90–10% (v/v) water; 60–70 min, 90.0% (v/v) methanol and 10% (v/v) water.

Retention times and peak heights of ten commercially purchased compounds (Sigma Chemical Co.,

St. Louis, MO, USA) identified previously in the Arabidopsis root exudates (Walker et al. 2003b) were used to determine a compound's possible presence in root exudates and to calculate the concentrations of these compounds in root exudates on the third day following inhibition. The compounds were analyzed in the presence or absence of MS medium or root exudates in order to determine the efficiency of the process of extraction.

To determine the efficiency of phytochemical extraction using the above-described methodology, we applied the following chemical standards to 45 ml MS or 45 ml MS containing Arabidopsis root exudates: *p*-hydroxybenzamide, butanoic, vanillic (1), syring, *p*-coumaric (2), ferulic, *o*-coumaric, *t*-cinnamic acid (4) and 3-indolepropionic acids, as well as methyl-*p*-hydroxybenzoate (3). The efficiency of extraction was the same in both conditions (Figs. 1, 2 supplementary). In all the cases, the absorbance was measured at 280 nm and the height of the peaks shows the difference in the molar extinction coefficient (ϵ) of every standard.

Results

Arabidopsis growing conditions

Plants grown in 6- or 12-well plates were sampled and weighed every 2 or 3 days for 33 days as described in 'Sect. Materials and methods' (Fig. 1). Plants in both cases followed a characteristic sigmoidal pattern of biomass accumulation. In both cases, the lag phase could be as long as 14 days; however, after 15 days the plants in the 6-well plates developed very fast, during the linear and logarithmic phases, until they reached an average of more than 600 mg plant^{-1} by day 21 until the end of the growth cycle. The plants in the 12-well plates only reached 65% of the weight of the plants grown in the 6-well plates.

Effect of the inhibitors on plant growth

The inhibitors tested were chosen for their ability to inhibit different transport processes. If the exudation of phytochemicals is mediated by ABC transporters, ATP must play a central role and inhibition of ATP production must modify the exudation process. Sodium azide and potassium cyanide inhibit ATP synthesis (Lew and Spanswick 1984). Sodium orthovanadate is an inhibitor of the membranal ATPases (Cantley Jr et al. 1978; Willsky et al. 1984) and it is able to inhibit all the ABC transporters in a general way (Horio et al. 1988; Urbatsch et al. 1995). Some channel inhibitors, such as

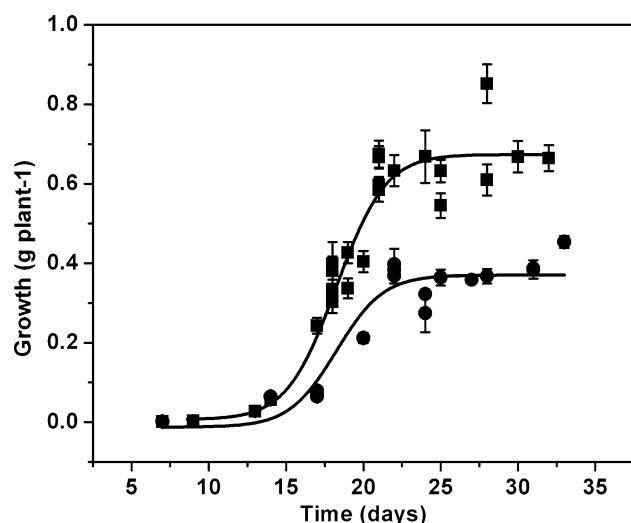


Fig. 1 Growth of *Arabidopsis* plants in 6- (filled square) and 12-well plates (filled circle). Seeds were surface-sterilized and germinated on solidified MS basal medium in a growth chamber at 25°C, with a photoperiod of 16 h light and 8 h dark. When the plantlets were 7 days old, they were transferred to plates and incubated on an orbital shaker at 90 rpm, and illuminated under cool white fluorescent light with a photoperiod of 16 h light/8 h dark at 25 ± 2°C until the end of the growth cycle. Each point is the average of data from at least 12 plants and three independent repetitions

verapamil and nifedipine (both calcium channel blockers) (Gottesman and Pastan 1993; Boumendjel et al. 2005), quinidine (potassium channel blocker) (Horio et al. 1988), cyclosporin A (Liminga et al. 1994) and glibenclamide (potassium channel blocker), and some types of inhibitors of the sulfonylurea receptor (Golstein et al. 1999), are also able to inhibit some ABC transporters.

The inhibitors used in this work exhibited a wide spectrum of effects on the plants' growth depending on the concentrations used in the assays. When the plants were grown in the 12-well-plates and sodium azide was added to the MS medium, it produced, after 5 days, an inhibition of the plants' growth with an LD₅₀ of 150 μM (Fig. 3 supplementary). With doses as low as 100 μM the plants showed minor symptoms of chlorosis on the leaves (Fig. 4 supplementary). On the other hand, potassium cyanide did not significantly change the growth of the plants until doses over 350 μM; for example, 375 μM produced only an 18% decrease in the weight of the plants. The LD₅₀ for KCN was over 400 μM, and the plants looked healthy; however, the plants were most resistant to sodium orthovanadate since the LD₅₀ was determined to be close to 1 mM and even at this concentration they resembled the plants grown in the liquid MS medium alone. At 250 μM of sodium azide, potassium cyanide and sodium orthovanadate, the percentages of growth inhibition were

82.3, 18.3 and 37.9%, respectively. Most specific inhibitors did not affect growth even at high concentrations in either 6- (Fig. 5 supplementary) or 12-well plates (Fig. 6 supplementary), and the plants developed normally (Fig. 7 supplementary). Glibenclamide was the only inhibitor that decreased the growth of the plants (Fig. 6 supplementary) and the plants under this treatment showed symptoms of chlorosis (Fig. 7 supplementary). The effect of the inhibitors in general was time-dependent, a fact that we used to determine their effect on plant growth as a function of incubation time (Fig. 2). After 72 h the only inhibitors that decreased plant growth were potassium cyanide, by 31.2%, and glibenclamide, by 18.2%. All the other inhibitors tested, including verapamil, did not change the growth of the plants in the 6-well plates and the plants grew as well as the controls did. We decided to use potassium cyanide, sodium orthovanadate, quinidine, glibenclamide, nifedipine and verapamil as potential inhibitors of root exudation because none of them modified in a meaningful way the growth of the plants (Figs. 2, 3, 7 supplementary).

Effect of the inhibitors on the secretion of phytochemicals

To potentially limit root exudation by inhibiting the function of ABC transporters, potassium cyanide and

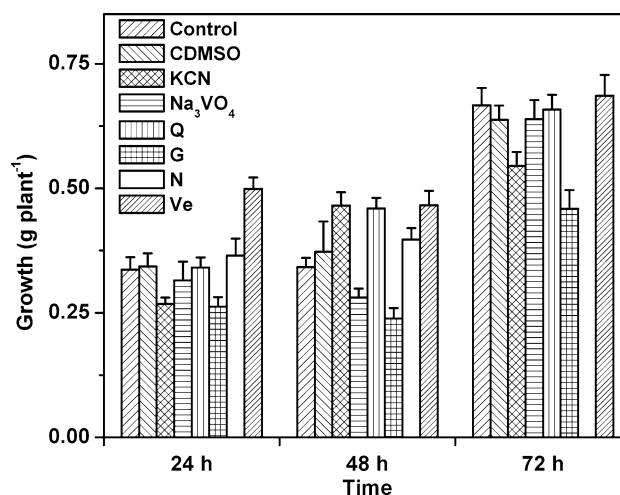


Fig. 2 Growth of *Arabidopsis* plants in the presence of potassium cyanide, sodium orthovanadate, quinidine (Q), glibenclamide (G), nifedipine (N) and verapamil (Ve). Eighteen-day-old plants were transferred to 6-well plates with 4 ml of MS medium containing 100 μM of inhibitor and incubated for the indicated time on an orbital shaker at 90 rpm, and illuminated under cool white fluorescent light with a photoperiod of 16 h light/8 h dark at 25 ± 2°C. Each bar is the average of data from at least 12 plants and three independent repetitions. The value for N at 72 h was not determinate

sodium orthovanadate were used at 100 and 500 μM concentration, and quinidine, glibenclamide, nifedipine and verapamil were used at 100 μM . All exudation profiles were compared with respective controls.

The exudation profile of compounds of the control samples showed, after 3 days of incubation, approximately 16 major peaks (Fig. 3, control) with retention times from 15.2 to 55.4 min and molecular masses from 142 to 442. When 100 μM of potassium cyanide and sodium orthovanadate were included in the MS medium, they caused a decrease in the exudation of nine and seven peaks, respectively (Figs. 3, 4). This inhibition is expected if the secretion of these compounds is mediated by ATP. In contrast, if these inhibitors do not affect the secretion of phytochemicals this process would be ATP-independent. The compounds for which exudation was inhibited comprise the entire range of molecular masses and retention times; they include the four major exudate peaks of Arabidopsis roots, which are peaks 3, 4, 5 and 8 with retention times of 31.25, 34.00, 34.59 and 41.84 min, respectively. The molecular masses corresponding to these peaks are 142, 142, 224 and 224, respectively and their maximum wavelengths are 264.2, 275, 289.5 and 290 nm, respectively (Table 1).

Sodium orthovanadate inhibited the exudation of seven compounds secreted by Arabidopsis roots (Fig. 4); these seven chemicals are also among those inhibited by potassium cyanide. Within the seven peaks inhibited by sodium orthovanadate are three of the major compounds secreted by Arabidopsis plants:

Table 1 Retention times, molecular masses and maximum wavelength of the most abundant compounds in exudates from Arabidopsis roots. The molecular masses were determined from the data in the MS/HPLC chromatograms. The maximum wavelength was determined with a PDA-100 photodiode array variable UV-vis detector

Peak	Retention time (min)	Molecular mass	Maximum wavelength (nm)
1	25.57	442	299.0
2	26.28	224	279.2
3	31.25	142	264.2
4	34.00	142	275.0
5	34.59	224	289.5
6	35.62	142	ND
7	36.46	224	ND
8	41.84	224	290.0
9	55.40	294	278.3
10	27.61	126	293.5
11	39.64	118	ND
12	43.07	134	ND
13	43.33	130	ND
14	45.50	148	ND
15	52.52	224	279.7

ND not detectable under the experimental conditions used

peaks 3, 4 and 8. It is interesting to note that the exudation of peaks 2 and 5 which are inhibited by potassium cyanide is not inhibited by orthovanadate (Fig. 4).

The use of the inhibitors potassium cyanide and sodium orthovanadate at different doses led to the conclusion that the inhibition of the secretion was dose-dependent (Fig. 5). The exudation of some of the compounds was dramatically inhibited by large amounts of

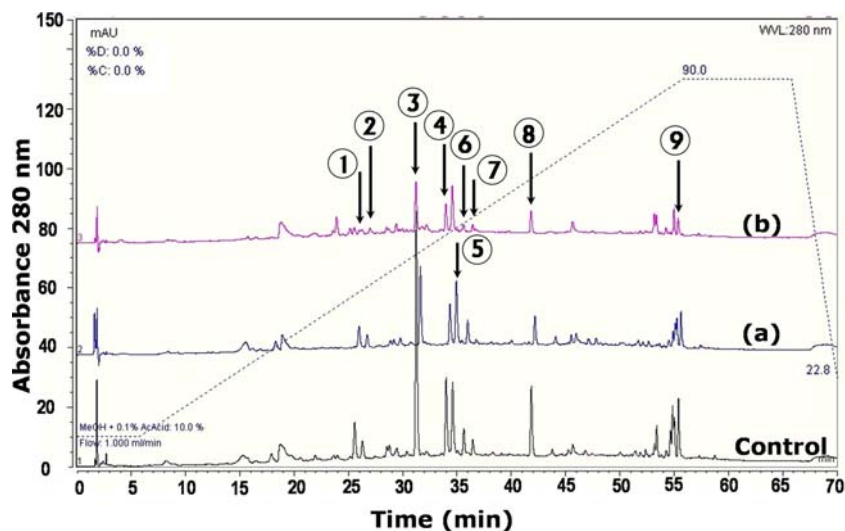


Fig. 3 Profile of Arabidopsis root exudates in the presence of **a** 100 μM or **b** 500 μM of potassium cyanide. Eighteen-day-old plants were transferred to 6-well plates with 4 ml of MS medium with the inhibitors and incubated for 3 days on an orbital shaker at 90 rpm, and illuminated under cool white fluorescent light with

a photoperiod of 16 h light/8 h dark at $25 \pm 2^\circ\text{C}$. The samples were extracted and analyzed as described in 'Sect. Materials and methods'. The retention times and molecular masses are listed in Table 1. The numbers identify the peaks the exudation of which is affected by the inhibitor

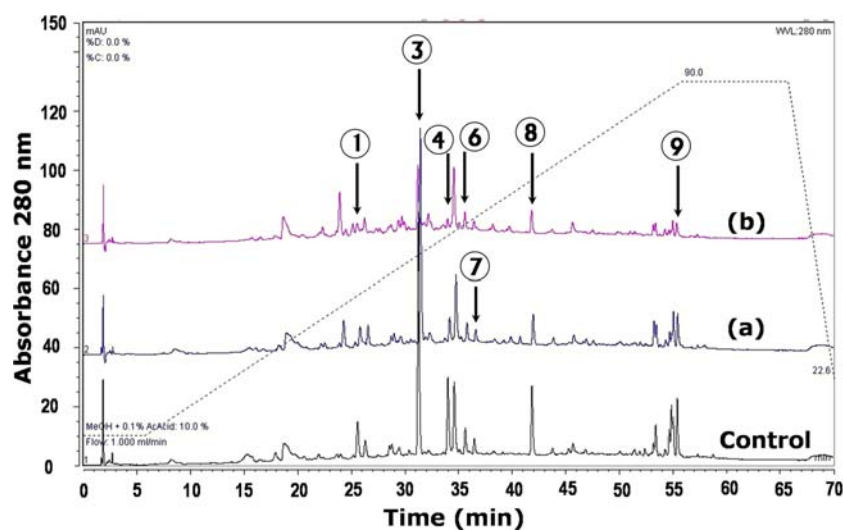


Fig. 4 Profile of Arabidopsis root exudates in the presence of **a** 100 μM or **b** 500 μM of sodium orthovanadate. Eighteen-day-old plants were transferred to 6-well plates with 4 ml of MS medium with the inhibitors and incubated for 3 days on an orbital shaker at 90 rpm, and illuminated under cool white fluorescent light with

a photoperiod of 16 h light/8 h dark at $25 \pm 2^\circ\text{C}$. The samples were extracted and analyzed as described in ‘Sect. Materials and methods’. The retention times and molecular masses are listed in Table 1. The numbers identify the exudation profile peaks affected by the inhibitor

inhibitors; i.e., when 500 μM potassium cyanide was used peaks 1 and 3 were inhibited more than 80%; peaks 2, 4, 6, 8 and 9, 70% or more. The other two peaks, 2 and 7, were inhibited 50%. Sodium orthovanadate at 500 μM did not inhibit the exudation of peaks 2 and 5 (Figs. 4, 5). The inhibition of exudation of peaks 6 and 7 was 38 and 49%. In addition, the exudation of peaks 3, 4, 8 and 9 was inhibited by 73, 88, 67 and 78%, respectively (Fig. 5).

The channel blockers quinidine, glibenclamide, nifedipine and verapamil caused the inhibition of eight, six, five and two peaks, respectively (Fig. 6; Table 2). Some of these peaks (3 and 9) are the same as those

inhibited by potassium cyanide or sodium orthovanadate.

After 24 h of treatment, quinidine (100 μM) inhibited, in some cases up to 100%, the exudation of seven peaks with retention times between 27.61 and 55.4 min and molecular masses between 118 and 294 (Table 2).

Peak 15 was not inhibited by quinidine, glibenclamide, nifedipine or verapamil (100 μM) even after 48 h of treatment (Table 2). Verapamil (100 μM) after 48 h inhibited the exudation of this compound by 6.9%. The exudation of some of the compounds secreted by Arabidopsis roots was inhibited totally, like those

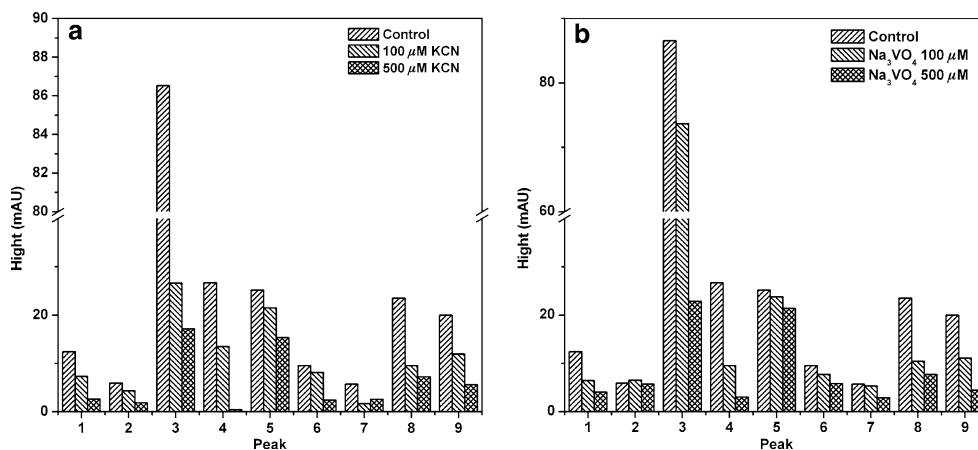


Fig. 5 Height of the major peaks from the exudates of Arabidopsis roots in the presence of **a** potassium cyanide or **b** sodium orthovanadate. Eighteen-day-old plants were transferred to 6-well plates with 4 ml of MS medium with the inhibitors and incubated

for 3 days on an orbital shaker at 90 rpm, and illuminated under cool white fluorescent light with a photoperiod of 16 h light/8 h dark at $25 \pm 2^\circ\text{C}$. The samples were extracted and analyzed as described in ‘Sect. Materials and methods’

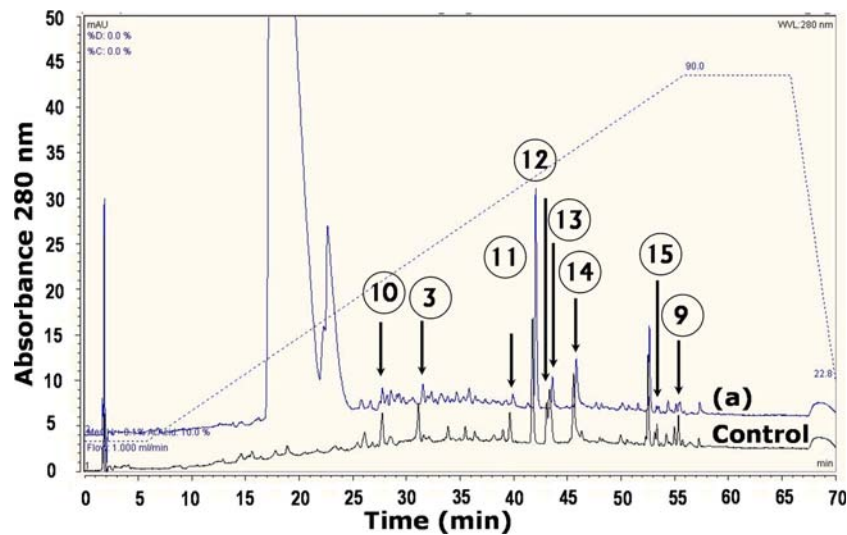


Fig. 6 Profile of Arabidopsis root exudates in the presence of a 100 μ M of quinidine. Eighteen-day-old plants were transferred to 6-well plates with 4 ml of MS medium with the inhibitor or 1% of DMSO (control) and incubated for 24 h on an orbital shaker at 90 rpm, and illuminated under cool white fluorescent light with a

photoperiod of 16 h light/8 h dark at $25 \pm 2^\circ\text{C}$. The samples were extracted and analyzed as described in ‘Sect. Materials and methods’. The retention times and the molecular masses are listed in Table 1. The numbers identify the exudation profile peaks affected by the inhibitor

Table 2 Retention times, molecular masses and inhibition of the exudation of compounds by Arabidopsis roots 24 h after quinidine, glibenclamide, nifedipine and verapamil were added to MS medium. The molecular masses were determined from the data in the MS/HPLC chromatograms

Peak	Retention time (min)	Molecular mass	Inhibition (%)			
			Quinidine	Glibenclamide	Nifedipine	Verapamil
3	31.25	142	51.5	0	100	0
9	55.40	294	60.6	50.8	86.5	100
10	27.61	126	41.0	0	100.0	0
11	39.64	118	60.1	33.8	0	0
12	43.07	134	100.0	100.0	100.0	0
13	43.33	130	40.1	28.5	50.7	0
14	45.50	148	29.3	32.6	0	0
15	52.52	224	4.4	10.6	0	4.4

compounds in the peaks 12 (quinidine, glibenclamide and nifedipine); 3 and 10 (nifedipine); and 9 and 16 (verapamil). The two peaks with retention times of 17.56 and 22.64 (Fig. 6) correspond to the inhibitor quinidine.

Discussion

In order to examine if transporters are involved in the secretion of chemicals by Arabidopsis roots we compared the exudation profiles of Arabidopsis roots in the presence and absence of some substances that deplete the ATP pool or inhibit the membrane P-ATPases, as well as some channel blockers which also inhibit ABC transporters.

Previously it has been shown that P-type H⁺-ATPase isoforms found in Arabidopsis membranes (Palmgren and Christensen 1994) and P-glycoproteins are strongly inhibited by vanadate (Urbatsch et al. 1995; Taguchi et al. 1997). Vanadate and potassium cyanide also inhibit the uptake of berberine in *Cjmdr1*-injected oocytes (Shitan et al. 2003). In our case both potassium cyanide and sodium orthovanadate inhibited root exudation by Arabidopsis roots in a dose-dependent way (Figs. 3, 4, 5). These data suggest that the exudation process is ATP-dependent and that either a primary or secondary active transporter could be involved in the secretion of phytochemicals into the rhizosphere by Arabidopsis roots.

The dose-dependent inhibition observed in the time-course experiments using vanadate and cyanide

(Fig. 2; Table 1) also support the conclusion of an active transporter being involved in *Arabidopsis* root exudation of phytochemicals.

It has been shown that orthovanadate causes moderate alkalization after it is added to *Lycopersicon peruvianum* cell cultures (Schaller and Oecking 1999), leading to a possible link between proton fluxes across the plasma membrane and the secretion of compounds into the rhizosphere. However, in our case alkalization did not occur, since the pH value of the medium, during the culture period of the treatments, varied from 5.8 to 6.3 in plants treated with orthovanadate and from 5.8 to 6.2 for the controls.

It is possible that a chemiosmotic model can describe the efflux driven by a plasma membrane H^+ gradient of phytochemicals by *Arabidopsis* roots. However, such a situation could be possible only if the chemicals are weak acids (with pK_a s in the acid range) and the pH of the apoplast is acid. When 18-day-old *Arabidopsis* plants are cultured in MS medium at a low pH (from 3.7 to 5) we found that in only 3 days they were able to restore the pH to values around 6 (data not shown). These data suggest that it is more probable that the secretion of phytochemicals occurs through a primary transporter. Nevertheless, we cannot discard the possibility that in the microenvironment of the apoplast, around the cell wall, the concentration of protons is enough to drive the efflux of phytochemicals.

Another possibility is that the secreted phytochemicals are stored in the vacuole; if this is the case, it is possible that the secretion of the compounds is mediated by active secondary transport systems that require the V-ATPase and vacuolar pyrophosphatase for maintenance of a proton gradient across the tonoplast.

Several compounds which inhibit the activity of various transporters were examined by addition to the medium at the concentrations of 100 or 500 μM of each inhibitor. Glibenclamide, quinidine, nifedipine and verapamil are inhibitors of channel blockers and of ABC transporters (Wigler 1996; Boumendjel et al. 2005) that function as a drug efflux pump in human cancer cells (Ueda et al. 1987). In plants the berberine uptake by a vacuolar P-glycoprotein is inhibited by nifedipine and quinidine in a dose-dependent manner (Sakai et al. 2002). Verapamil, nifedipine and glibenclamide inhibit berberine uptake in *Cjmdr1*-injected oocytes (Shitan et al. 2003) and it has been shown that an ABC-type efflux-transporter is functioning in *Thalictrum minus* suspension cultures (Terasaka et al. 2003). Geisler et al. (2005) used MDR/PGP inhibitors cyclosporin A and verapamil to inhibit the efflux of auxin in PGP 1 transformed yeast. By studying the hypersensitive response of *A. thaliana* to salt stress, glibenclamide was used to

analyze the response of the *atmrp5-2* mutant (Lee et al. 2004). As shown in Table 2, the secretion of some of the phytochemicals produced by *Arabidopsis* was inhibited by these compounds, suggesting that a primary transporter might be involved in their exudation into the medium. The inhibition is very selective: each inhibitor causes a decrease in a few different compounds, while the rest of the profile remains the same. This selectivity suggests that probably different transporters are involved in each compound's secretion. However, some phytochemicals, such as taxol, indole alkaloids and flavonoids, have been shown to be substrates for yeast Pdr5p-mediated multidrug transport (Kolaczkowski et al. 1996), suggesting that in some cases unrelated compounds can be targeted by the same transporter.

The increase in secretion of some peaks (Fig. 6) observed at 100 μM quinidine could be the result of a plasmatic membrane leak because of the strong inhibition of the transport system; however, this explanation appears improbable since there is a clear inhibition of eight other peaks. Since the transport processes of solutes across the vacuolar and cellular membranes of higher plants are complex, and many membrane proteins of divergent classes are involved (Martinoia et al. 2000) and all of the mammalian ABC transporters characterized that are localized in the plasma membrane show excretion activity for substrates [e.g., P-glycoprotein and members of the multidrug-resistance-related protein subfamily in animal cells efflux anticancer drugs from the cytosol (Glavinas et al. 2004)], it is possible that the increase in secretion of these two peaks may be an indirect effect of the inhibitor.

Glibenclamide inhibited the secretion of six peaks. This compound inhibits the potassium channels and some ATP transporters in animal cells (Golstein et al. 1999). Recently it has been shown that glibenclamide, in the presence of NaCl, inhibits the growth of the roots of the mutant *atmrp5-2* grown in NaCl alone (Lee et al. 2004). The Na^+ -dependent reduction of root growth of the wild-type plant in the presence of glibenclamide was partially restored by diazoxide, a known K^+ -channel opener that reverses the inhibitory effects of sulfonylureas in animal cells. However, we cannot discard the possibility of an indirect effect of this compound with MATE transporters or the possible indirect effect on vacuolar, pH-dependent transport (Frangne et al. 2002).

In summary, although further molecular and genetics studies are needed to determine the nature of the transporter molecules that are exporting endogenous compounds into the rhizosphere, our present findings suggest that *Arabidopsis* root secretion is ATP-dependent and that primary transporters could be involved in the secretory mechanisms of the roots. Thus, it is

possible to infer that specific transporters may be responsible for the secretion of specific compounds or group of phytochemicals.

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