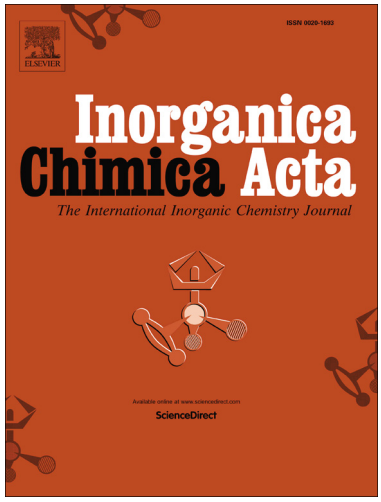

Research paper

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PII: S0020-1693(17)30334-1
DOI: http://dx.doi.org/10.1016/j.ica.2017.05.019
Reference: ICA 17588

To appear in: *Inorganica Chimica Acta*

Received Date: 3 March 2017
Revised Date: 8 May 2017
Accepted Date: 10 May 2017

Please cite this article as: M.B. Patel, S. Negin, A. Stavri, G.W. Gokel, Supramolecular Cation Transporters Alter Root Morphology in the *Arabidopsis Thaliana* Plant, *Inorganica Chimica Acta* (2017), doi: http://dx.doi.org/10.1016/j.ica.2017.05.019

Supramolecular Cation Transporters Alter Root Morphology in the *Arabidopsis Thaliana* Plant

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Submitted to *Inorganica Chimica Acta*
March 3, 2017 Revised May 5, 2017

Version printed: Thu, May 11, 17 at 11:42

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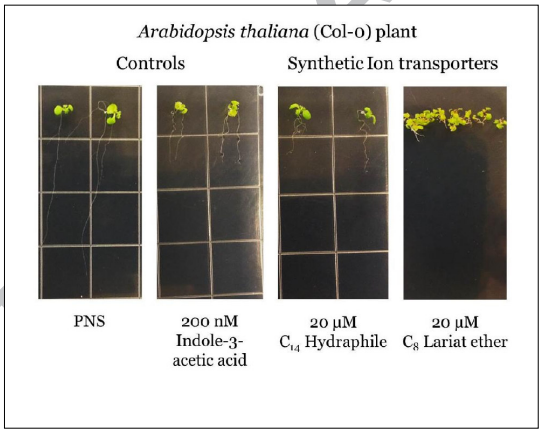
Abstract

Bibracchial (two-armed) 4,13-diaza-18-crown-6 lariat ether and *tris*(macrocycle) hydraphile synthetic amphiphiles alter root morphology in *Arabidopsis thaliana* plants. The effect on root structure and growth depends both on the hydraphile spacer chain length and lariat ether side chain length as well as the concentration of compound in the growth medium. In some cases a correlation to ion transport activity was apparent, but such a correlation is not always manifested. Surprisingly, planar bilayer conductance (BLM) studies showed that lariat ethers and lariat ether amides both exhibited well controlled membrane activity. Pore formation in soybean asolectin membranes occurred readily and the pores were stable and sustained. Low concentrations of active hydraphiles and lariat ethers altered the primary:lateral root density ratio, generally increasing it. The transporter-mediated alterations in lateral root density were suggestive of the activity of plant auxins such as indole-3-acetic acid and 2,4-dichlorophenoxyacetic acid, which are known to depend on cytosolic potassium ion concentrations. The hypothesis that the compounds interfere with the auxin pathway was tested and discounted by using auxin-resistant *A. thaliana* mutants. Rather than functioning directly as auxin mimics, ionophores affect the ion gradients producing an auxin-like effect on root development.

Graphical Abstract



Keywords:
Arabidopsis thaliana
Auxin
Hydraphile
Ion channel
Ionophore
Lariat ether
Planar bilayer conductance
Plant growth
Pore formation
Root morphology

Introduction

Crown ethers have been known and extensively studied for more than four decades. [1] A spectacular surge in new structures followed Pedersen's seminal discovery of synthetic alkali metal binders. [2] [3] [4] Macrocycles having varied sizes and donor groups form a range of stable and selective complexes with metal and organic cations. [5] Numerous crown ethers have been reported to transport cations through bulk liquid [6] and bilayer membranes. [7] A series of dibenzo-18-crown-6 [8] and *N,N'*-dialkyldiaza-18-crown-6 ethers or lariat ethers [9] are known to transport Na^+ and K^+. In addition, the biological activity of some crown ethers has been known for decades, but such studies have mostly focused on toxicity. [10] Toxicity studies against various microbes, for example, have been extensive [11] and are ongoing. There has also been considerable interest in developing synthetic ion channels of various structural types, including membrane-spanning and self-assembled variants.[12]

The increase in complexity and sophistication of crown ether structures [13] and their ability to mimic such natural ionophores as valinomycin, [14] has led to *in vivo* applications to mimic biological processes. [15] [16] Several conventional crown ethers and their derivatives have shown antiproliferative/antitumor properties. [17] The *N,N'*-dialkyldiaza-18-crown-6 lariat ether compounds that we have studied show varied biological activity including toxicity to diverse microbes.[18] The lariat ethers show toxicity to Gram negative *Escherichia coli*, Gram positive *Bacillus subtilis*, and to the primary eukaryotic yeast *Saccharomyces cerevisiae*. Although very few complex organism studies, especially with plants, have been reported, it is known that benzo-15-crown-5, at low concentrations, transports K^+ ions in wheat roots. [19] In other work, it was found that benzo-18-crown-6 reduced potassium efflux, transport, and uptake in onion root segments. [20] The leaves of the Asian day lily (*Commelina communis*) were studied in the presence of benzo-18-crown-6. [21] Differences were observed in effects on the upper (abaxial) and lower (adaxial) leaf surfaces that correlated to macrocycle administration. The authors also noted that the crown had a similar effect on stomata as observed with the plant auxin phenylacetic acid.

Many of the synthetic channel compounds that we call hydraphiles are biologically active. [22][23][24][25] Our observation of some similarity in biological effect on *Arabidopsis thaliana* between lariat ethers and hydraphile compounds was surprising. We expected lariat ethers that function as ion carriers to be much less active than hydraphiles, which are synthetic ion channels (pore-formers). We expanded our preliminary work on hydraphiles [26] to include lariat ethers that show effects on plant growth that are remarkably similar to those exhibited by hydraphiles. We have hypothesized mechanisms for this biological activity either in terms of ion binding and transport or direct involvement in an auxin (hormone) regulated pathway. We report here clear planar bilayer conductance evidence for lariat ether pore formation and we examine possible auxin-like behavior.

Results and Discussion

Higher plants exhibit diverse root architectures [27] and elastic root development. These properties are important for the extraction of water, macronutrients, and micronutrients from the environment and for plant anchorage. [28] *A. thaliana* is the best characterized plant in terms of genetic, hormonal, and nutritional control of lateral

root development. [29] It typically grows with a single primary root from which the lateral roots extend. Extracellular potassium ion concentration and ion transport have been reported to affect the development of *Arabidopsis* roots. [30] We thus initially hypothesized that lariat ethers and hydraphiles, as ion transporters, might affect growth and root architecture by altering ion concentrations. This seemed reasonable in light of our previous findings of the effect of hydraphiles on root structure. [26]

Compounds used in the study. The work described here used two types of synthetic amphiphiles: hydraphiles [31] and *N,N'*-disubstituted-4,13-diaza-18-crown-6 two-armed lariat ethers. [32] [33] The three hydraphiles (previously reported [25]) are identical to each other except for the spacer chain lengths, which are octylene (C_8), tetradecylene (C_{14}), and hexadecylene (C_{16}) respectively in compounds **1**-**3**. Each lariat ether side chain is attached to a macroring nitrogen atom. In compounds **4**-**8**, the side chain is an *n*-alkyl group and in **9**-**13**, the alkyl group is linked *via* an amide. Two additional compounds were used in this study. They are the natural and synthetic plant auxins indoleacetic acid (IAA) **14** and 2,4-dichlorophenoxyacetic acid (2,4-D), **15**. The structures are shown in Figure 1.

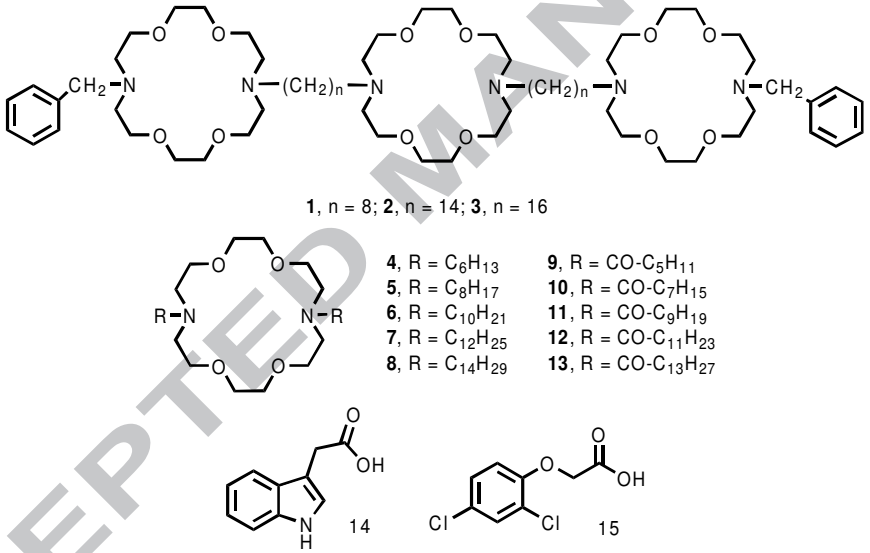
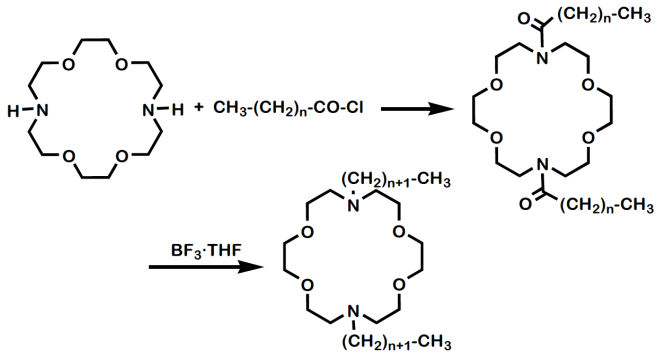


Figure 1. Structures of hydraphiles **1**-**3**, lariat ethers of various chain lengths, without (compounds **4**-**8**) or with (compounds **9**-**13**) amide residues. Indole-3-acetic acid (**14**) and 2,4-dichlorophenoxyacetic acid (**15**) are known plant auxins.

We have recently reported an improved preparation of benzyl hydraphiles **1**-**3**. [34] It was used to prepare the hydraphiles reported here. The synthesis of the lariat ether amides (**9**-**13**) was accomplished by diacylation of the diazacrown with the appropriate acid chloride. Part of each amide product was reduced using $LiAlH_4$ or BH_3•THF, affording the *n*-alkyl analogs, **4**-**8**. The sequence is shown in Scheme 1 below and details of the syntheses and products are recorded in the Experimental Section.



Scheme 1. Synthesis of lariat ether amides and their reduction to dialkyl lariat ethers.

Plant growth. *A. thaliana* Col-0, which is the most common strain of this plant, was used as the test organism. After sterilizing, the seeds were plated on plant nutrient media including 0.5% sucrose, ("PNS") and which contained 0.6% agar. Each experiment involved 20-30 plants and each experiment was repeated at least three times (minimum 60-90 plants for each data point). The primary root length for each plant was measured 11 days after initial incubation and the data obtained are reported herein below in millimeters. A dissecting microscope was used to assist in counting the number of lateral roots emerging from the primary root of each plant. We found that certain hydraphiles and lariat ethers required addition of DMSO to the media for solubility reasons. It was therefore necessary to confirm that the amount of cosolvent added was inconsequential because DMSO can affect membrane permeability [35] [36] and biological activity [37] at higher concentrations. The average primary root length observed was 42 ± 8 mm when *A. thaliana* was grown on PNS media. In the presence of 0.2 volume-% DMSO, the primary root length of *A. thaliana* was 38 ± 4 mm, which is within experimental error of PNS alone.

We next conducted experiments to confirm the known effects [38] of the auxins IAA (**14**) and 2,4-D (**15**) on root development in *A. thaliana*. The results are recorded in Table 1. Here, the lateral root density is defined as [(number of lateral roots)/(primary root length in mm)]. Thus, the first line in Table 1, for PNS media alone, gives a lateral root density of 6.9/42 = 0.16.

Table 1. Effect of **14** and **15** on *A. thaliana* root development

A. thaliana (Col-0) plant	Primary root length (mm)	Decrease in primary root length (%)	Number of lateral roots	Lateral root density
PNS media	42 ± 8	not applicable	6.9 ± 0.2	0.16

DMSO (0.2%)	38 ± 6	10	6.3 ± 0.1	0.16
IAA (**14**) 200 nM	7.6 ± 2.3	67	3.7 ± 1.3	0.48
IAA (**14**) 1 μM	2 ± 1	85	1.9 ± 1.4	0.91
2,4-D (**15**) 200 nM	3.1 ± 0.1	93	3.7 ± 0.3	1.19
2,4-D (**15**) 1 μM	1.6 ± 0.3	96	1.8 ± 0.6	1.14

Indoleacetic acid (IAA, **14**) showed, as expected for this known auxin, [39] a concentration dependent effect both on primary root length and lateral root density. When IAA was administered at a concentration of 200 nM, the primary root length was 7.6 ± 2.3 mm and the lateral root density was 0.48. However, when [IAA] = 1 μM, the primary root length decreased by 85% and lateral root density increased to 0.91. In the presence of 2,4-D (**15**), the primary root lengths at concentrations of 200 nM and 1 μM were, respectively, 3.1 ± 0.1 mm and 1.6 ± 0.3 mm. The lateral root density at both concentrations was ~1.1. The synthetic auxin 2,4-D has been reported to alter root morphology at concentrations as low as 100 nM, [38] so no major change was expected or observed between 200 nM and 1 μM. These control studies gave results corresponding to those reported by others.

Effect of hydraphiles on *A. thaliana* root morphology. We have previously reported that hydraphiles **1-3**, when added to PNS media at a concentration of 20 μM and 50 μM, affected the root architecture of *A. thaliana*.[26] After 11 days, the primary root lengths were diminished with a corresponding increase in lateral root density (Supplementary table 1). The change in root architecture by hydraphiles showed an apparent dependence on the ability of these compounds to transport ions. [40] [41] It is obvious from the concentrations used that the natural auxin IAA (**14**) and its synthetic mimic 2,4-D (**15**) are substantially more active than the hydraphiles. The amphiphiles reported here are structurally unrelated to **14** or **15** and were tested because they were expected to influence ion transport rather than to be auxin mimics.

It seemed possible that the presence of hydraphiles in the growth medium could affect *A. thaliana* seed germination. We therefore added varying concentrations of benzyl C_{16} hydraphile, **3**, during germination. The results, shown in the graph of Figure 2, suggest that germination is indifferent to the presence of **3** in the concentration range 0.5-50 μM. Error bars in the graph have been omitted for clarity.

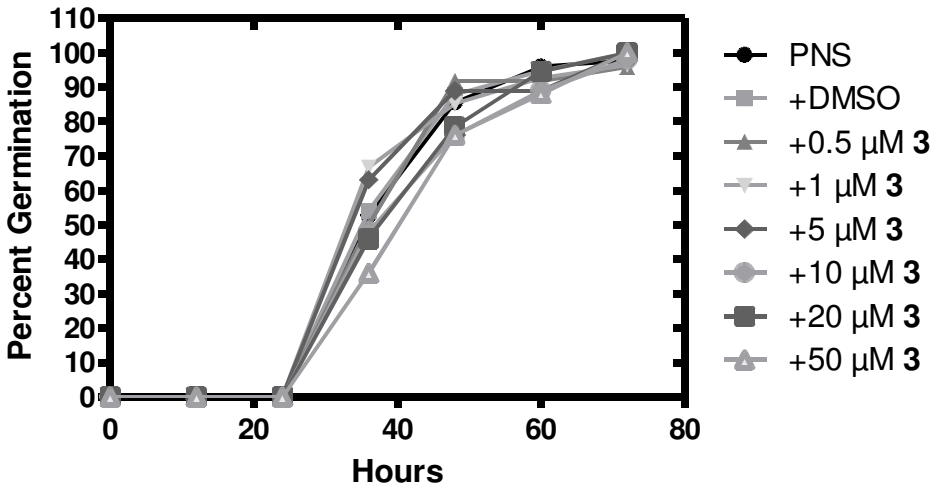
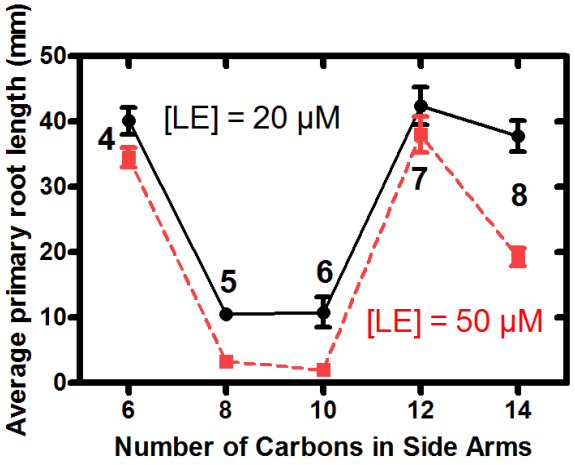


Figure 2. Germination of *A. thaliana* in the absence or presence of 0.2 volume-% DMSO or 0.5-50 µM **3.** Each point represents the average of plants grown from 17-25 seeds in each case. PNS is plant growth media plus sucrose. Error bars have been deleted for clarity.

The fact that hydraphiles **2** and **3** that are known to be effective pore-formers were more active and more toxic than poor ion-transporter **1**, suggested the importance of a pore formation mechanism. The simpler, but related, lariat ethers [42] are generally known to be ion carriers, [43] but not pore formers. We therefore explored their activity as a probe of mechanism and toxic effect.

Effect of lariat ethers on *A. thaliana* root morphology. The *N,N'*-disubstituted 4,13-diaza-18-crown-6 compounds shown in Figure 1 as **4-13** were incorporated into *A. thaliana* growth media at the indicated concentrations. Figure 3 shows the effect of *n*-alkyl lariats **4** to **8** on the primary root lengths of *A. thaliana* plants. Each lariat ether (**4-8**) was tested at concentrations of 20 µM (solid line) and 50 µM (dotted line). The abscissa records the number of CH_2 groups in the side arms of **4** to **8**. The ordinate



shows the average primary root length in mm.

Figure 3. Effect of lariat ethers (compounds **4**-**8**) at 20 μM (solid line) and 50 μM (dotted line) on primary root length. Each data point represents the average of 3 to 4 trials each with 18-23 plants tested per experiment (total sample 54-92 plants). The error bars represent the standard error in each experiment.

We did not expect any significant effect of lariats on plant morphology. However, if any effect was witnessed, we anticipated that it would parallel the ion transport ability known for these compounds. In fact, the only substantial effects observed at 20 μM were for **5** (C_8) and **6** (C_{10}). At 20 μM concentrations, the primary root lengths in the presence of **4**, **7** (C_{12}), and **8** (C_{14}) were similar to those of PNS control (42 ± 8 mm). At 50 μM, the primary root lengths in the presence of **4**, **7**, and **8** were 34 ± 2 mm, 38 ± 3 mm, and 21 ± 2 mm, respectively. The effects of compounds **4**-**7** at either 20 μM or 50 μM were similar, but not identical. A surprise was that **8** had essentially no effect on plant growth at 20 μM, but showed a dramatic decrease in primary root length at 50 μM.

The graph of Figure 4 plots the effect of **4**-**8** on *A. thaliana* lateral root density as a function of lariat side chain length. When administered at a concentration of 20 μM, the lateral root densities for **4**, **7**, and **8** were 0.16 ± 0.01, 0.15 ± 0.02, and 0.2 ± 0.02, respectively. At 50 μM, the corresponding values were similar to those observed at 20 μM and little different from those apparent in PNS alone (0.16). As was the case with primary root length, **5** and **6** showed a significant increase in lateral root density at 20 μM. The variation was similar, but amplified, at 50 μM.

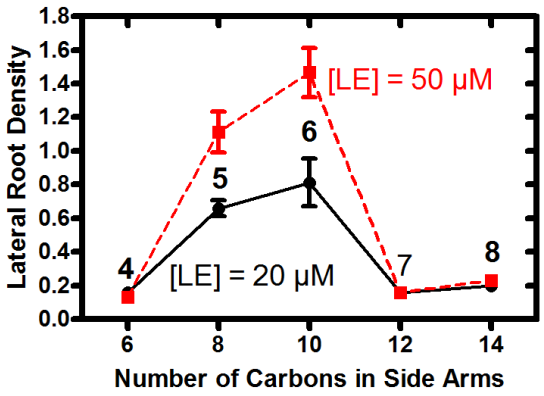


Figure 4. Effect of lariat ethers (compounds **4**-**8**) at 20 μM (solid line) and 50 μM (dotted line) on the lateral root density of *A. thaliana*. Each data point represents the average of 3 to 4 trials each with 18-23 (total 54-92) plants tested per experiment. The error bars represent the standard error in each experiment.

The difference in lariat ether activity observed at 20 μM and 50 μM prompted us to examine the concentration dependence of C_8 (**5**) and C_{10} (**6**) lariat ethers on primary root length and lateral root density. The lateral root densities observed for C_8 lariat at

concentrations of 5, 15, 25, 35, 45, and 50 μM were, 0.16, 0.20, 0.42, 0.62, 0.80, 1.17, and 1.43, respectively. For the C_{10} lariat ether at the same concentrations, the lateral root densities were 0.56, 0.77, 1.16, 1.20, 1.40, and 1.77. The zero concentration point serves as a control and includes 0.2% (v/v) of DMSO in the PNS media. The increase in lateral root density over this concentration range is essentially linear (Supplementary Figure 1) for both compounds (R^2 = 0.96 for **5**; R^2 = 0.95 for **6**).

Assessment of lariat ether effects on *A. thaliana* germination and plant growth. As observed in the hydraphile study (see Figure 2), no change in seed germination was observed over time in the presence of 50 μM C_8 lariat ether (**5**), 50 μM C_{10} lariat ether (**6**), 50 μM C_8 lariat ether amide (**10**) and 50 μM C_{10} lariat ether amide (**11**) (Supplementary Figure 2). These observations comport with the hypothesis that both hydraphiles and lariat ethers affect plant root development and not germination.

Figure 5 shows a comparison of *A. thaliana* growth in the presence of active lariat ethers **5** and **6**, IAA, and 2,4-D. Di-*n*-octyl lariat ether ([**5**] = 20 μM) decreased primary root length by 76% and increased lateral root density to 0.67 (4-fold). At 20 μM, *A. thaliana* plants in the presence of C_8 lariat ether, **5**, appeared on visual inspection to be healthier than in the presence of C_{10} lariat ether, **6**. No discoloration or chlorosis of the leaves was observed. At a concentration of 50 μM of C_8 (**5**) and C_{10} (**6**) lariat ethers, plant growth was stunted and appeared to be similar to that observed when 1 μM 2,4-D was present.

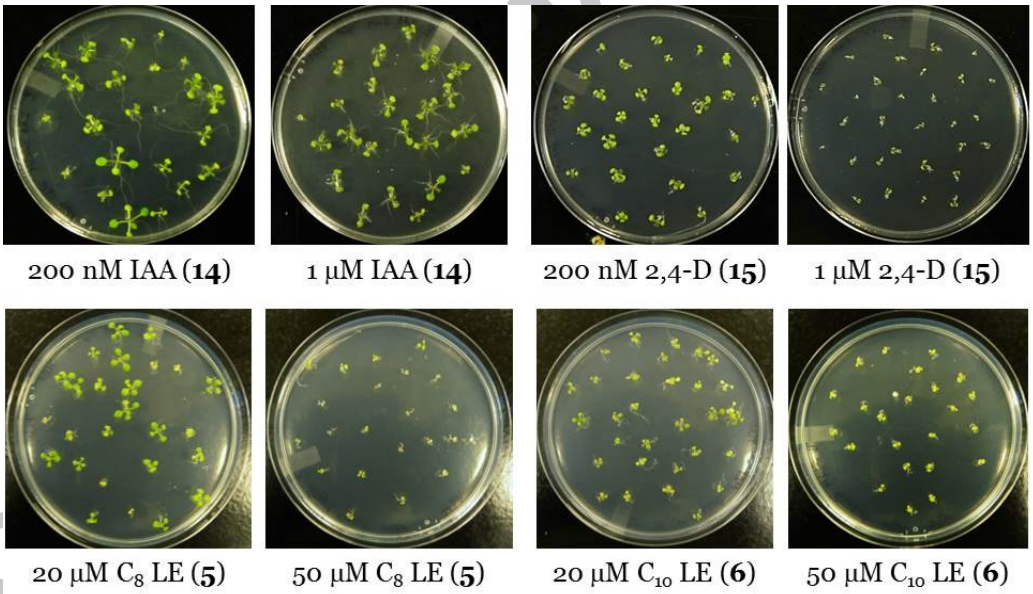


Figure 5. Comparison of plant development in the presence of IAA (**14**) and 2,4-D (**15**) (top row) and C_8 lariat ether (**5**), C_{10} lariat ether (**6**) (bottom row). Plants were grown in a Petri dish containing PNS media in the presence of **5**, **6**, **14** or **15**.

Effect of lariat ether amides on the growth and morphology of *A. thaliana* roots. The side arms of compounds **9-13** have the same number of carbon atoms as **4-8**, but both linkages to diaza-18-crown-6 in the former group are amidic. Amides are inherently less flexible than the corresponding amines. [44] The macroring nitrogen atoms of lariat ether amides are poorer donors that the nitrogen atoms of the corresponding alkyl diazalariat ethers. [45] Any dependence of *A. thaliana* primary root length on crown-ether cation binding, rather than transport, would be expected to show results distinct from those observed for lariat ethers or hydraphiles. Figure 6 compares the effect of lariat ethers (circles) to that of lariat ether amides (squares) at 50 μM concentration, on primary root length. Each data point is the average of 2 trials; each trial involved 18-23 plants. Data for compounds **4-8** may be found in Figure 3. No effect on lateral root density was observed in *A. thaliana* when **9-13** were present in PNS media at 50 μM.

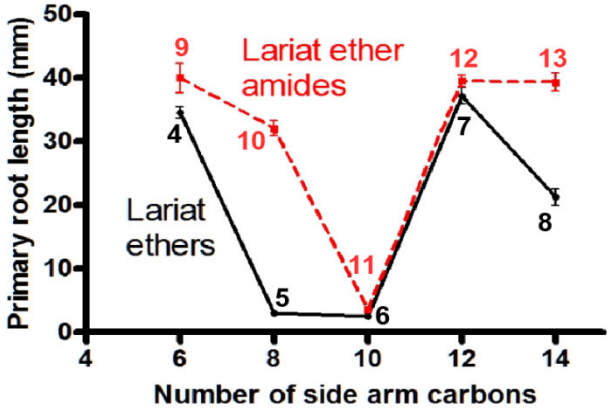


Figure 6. The change in primary root length for lariat ethers (circles, **4-8**) and lariat ether amides (squares, **9-13**) at a concentration of 50 μM.

The C_{10} lariat ether amine (**6**) and amide (**11**) had a similar effect on the *A. thaliana* primary root length. These compounds decreased the primary root length by ~95 %. Surprisingly, **6** increased the lateral root density by ~9-fold but no change was observed with the amide (**11**).

Probe of macrocyle effect on the auxin pathway. The plant hormones IAA and 2,4-D affect both growth and ion transport. The similarity in overall effect of C_8 and C_{10} lariat ethers to IAA and 2,4-D on *A. thaliana* root architecture suggested that lariat ethers might be involved in the auxin signaling pathway. To evaluate this hypothesis, we tested C_{10} lariat ether against two auxin resistant *A. thaliana* mutants: *aux 1-7* [46] and *axr 1-12*. [47] In *aux 1-7*, a mutated permease-like *Aux1* protein significantly reduces the rate of carrier mediated auxin uptake. [48] *Axr1* encodes a mutated subunit of a heterodimeric RUB (related to ubiquitin) activating enzyme that is unable to degrade the Aux/IAA transcriptional repressors. Both *aux 1-7* and *axr 1-12* have defects in root gravitropism and lateral root formation. Lateral root densities of Col-0, *aux 1-7* and *axr 1-12* plants were determined in the presence and absence of 50 μM C_{10} lariat ether (**6**), 200 μM IAA and 200 μM 2,4-D . The results are recorded in Table 2.

Table 2. Assessment of auxin properties in genetically modified *A. thaliana* plants

	Primary root length (mm)	Number of lateral roots	Lateral root density
Col-0 (wild type)			
PNS	49.2 ± 2.2	7.4 ± 0.8	0.15 ± 0.01
200 nM IAA	7.3 ± 0.7	3.3 ± 0.2	0.51 ± 0.05
200 nM 2,4-D	3.1 ± 0.2	3.7 ± 0.3	1.3 ± 0.1
50 μM C_{10} LE (**6**)	1.5 ± 0.1	1.6 ± 0.2	1.2 ± 0.2
Aux 1-7			
PNS	57.3 ± 1.6	5.4 ± 0.5	0.1 ± 0.01
200 nM IAA	28.2 ± 0.9	4.6 ± 0.5	0.16 ± 0.01
200 nM 2,4-D	23.8 ± 1.6	17.8 ± 1.7	0.74 ± 0.06
50 μM C_{10} LE (**6**)	1.6 ± 0.2	2.3 ± 0.3	1.43 ± 0.14
Axr 1-12			
PNS	57.7 ± 1.4	0.78 ± 0.3	0.01 ± 0.05
200 nM IAA	14.1 ± 0.8	0.92 ± 0.2	0.07 ± 0.02
200 nM 2,4-D	27.3 ± 0.9	2.1 ± 0.4	0.08 ± 0.01
50 μM C_{10} LE (**6**)	1.3 ± 0.1	1.6 ± 0.1	1.38 ± 0.17

The important observations from table 2 are as follows. In the genetically modified *aux 1-7* and *axr 1-12* mutants, similar effects to those apparent in Col-0 were observed when either of the known auxins IAA or 2,4-D was present. The changes in the primary root lengths and lateral root densities, in the presence of IAA or 2,4-D, were much smaller for mutants. This contrasts dramatically with the presence of **6**. The effect of C_{10} lariat ether (**6**) on the root development was the same in the wild type Col-0, the auxin transport mutant (*aux 1-7*), and transcriptional repressor mutant (*axr 1-12*). Thus, C_{10} lariat ether is not recognized in the auxin uptake pathway nor does it affect the transcription repressor of AUX/IAA. It is well known that primary and lateral root development is modulated by various molecular pathways and environment signals. [49] Lariat ethers may affect such molecular pathways as auxin biosynthesis in addition to or instead of auxin transport or transcriptional repression. More likely, these ionophores alter potassium homeostasis in root cells, which in turn, is known to alter auxin response and root development. [50]

Pore formation by lariat ethers and lariat ether amides. Several reports of crown ethers affecting potassium concentration or transport in plants appeared in the 1980s. [18] [19] [20] We therefore hypothesized that the diazalariat ethers transport potassium ions through plant root cells, which results in downstream changes in either an auxin dependent or independent pathway. Having dispelled the auxin dependent

pathway hypothesis, the similarity in effect on plants between hydraphiles and lariat ethers led us to query the possibility that lariat ethers were behaving in a fashion similar to hydraphiles in membranes. Very recent results have suggested that at least some lariat ethers may stack and form viable pores. In a study by Barboiu, Fyles, and their coworkers, an H-bond, urea-based network led to a stacked channel. More recently, Liu and coworkers have reported pore formation from benzo-18-crown-6-derived lariat ethers that incorporate various switching elements.

In our own recent work, [51] we found that co-administration of either C_8 or C_{11} lariat ether with the antibiotics rifampicin or tetracycline showed enhanced potency against the DH5α strain of *E. coli*. These experiments were done by determining the minimum inhibitory concentration (MIC) of the lariat ether and then using concentrations of it in the non-toxic ranges of ~$^1/_6$ to $^2/_3$ [MIC]. The enhancements observed ranged from 4-fold to 48-fold compared to the antimicrobial toxicity in the absence of lariat ethers.

The dialkyl lariat ethers that are the focus of this report lack hydrogen bonding or stacking elements and were not expected to form stable pores. The surprising behavior of lariats that we observed is shown in the traces below, and parallels that results of the studies noted above. The data shown in Figure 7 were obtained by planar bilayer voltage clamp studies. We have recently reported planar bilayer conductance data for both C_8 and C_{11} lariat ethers. [51] We also noted there that C_{10} lariat showed activity, but that C_{12} did not. Figure 7 also shows traces for C_{10} lariat ether and for C_8 and C_{10} lariat ether amides. The latter could potentially form a hydrogen bonded stack leading to pores. To our knowledge, though, there has been no previous report of pore formation by dialkyl lariat ether amides.

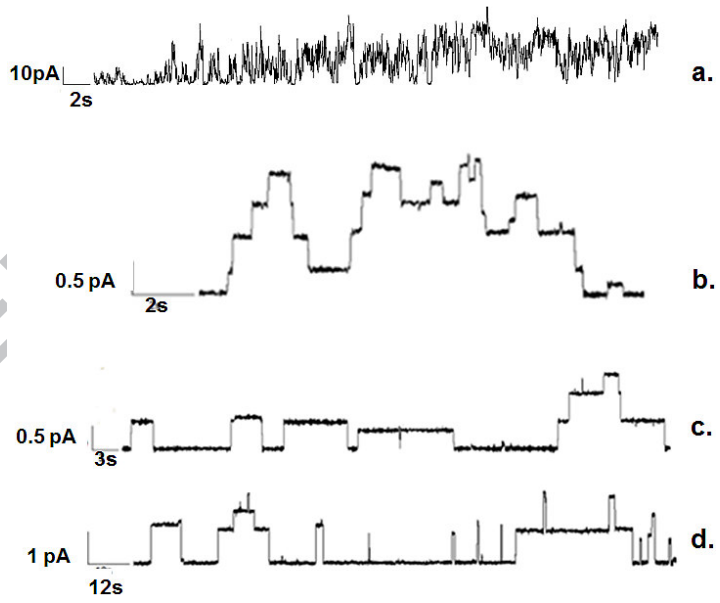


Figure 7. Planar bilayer conductance (BLM) study of C_6 (**4**), C_{10} (**6**) lariat ethers and C_8 (**10**) and C_{10} (**11**) lariat ether amides in asolectin membranes. (a) The C_6 lariat ether (**4**) trace showed only membrane disruption at 7 μM and 50 mV potential (scale: 10pA, 2 s). (b) The C_{10} lariat ether trace was obtained at a concentration of 12 μM and an applied potential of 50 mV (scale: 0.5 pA, 2 s). This was observed at all

concentrations of **4** and applied potentials surveyed. (c) The C_8 lariat amide (**10)** was determined at 3 μM concentration and 30 mV applied potential (scale: 0.5 pA, 3 s). (d) The C_{10} lariat ether amide (**11**) trace was obtained at 7 μM and 50 mV (scale: 1 pA, 12 s). Membrane: soybean asolectin.

Figures 3 and 4 show that among the lariat ethers surveyed, C_8 (**5**) and C_{10} (**6**) are the most active and C_6 (**4**) shows no effect either on lateral root density or primary root length. The lariat ether having C_6 side arms (**4**, top panel of Figure 7), showed only membrane disruption at all concentrations and applied potentials studied. The applied potential for C_8 and C_{10} in both cases was 50 mV but the concentrations for **5** and **6** were 3 μM and 12 μM, respectively. The greater number of multiple channel openings apparent for C_{10} may reflect this higher concentration, at least in part. In either event, the openings are regular and continuous channel behavior was sustained for up to an hour. The trace shown for C_{10} represents only 15 s. The amide traces illustrate longer durations: C_8 ~60 s and C_{10} ~100 s.

Three major conductance states (in picoSiemens, pS) were observed for C_8 (**5**) lariat ether: 11 pS, 17 pS, and 23 pS. [51] Multiple openings are observed for these states. We infer that C_{10} lariat ether is even more active as a pore-former because the multiple openings are more numerous. The two principal open states for **6** are 15 pS and 20 pS. The chain length dependence observed for lariat ether activity with *A. thaliana* paralleled previous results obtained with bacteria. [18]

Since the presence of amide chains, R-CO<N18N>CO-R, in **9**-**13** reduce the donicity of nitrogen [31] and the flexibility of the overall compound, we anticipated poorer biological activity. [45] In contrast, C_7-CO<N18N>CO-C_7 (**10**) showed a modest effect on *A. thaliana*, but C_9-CO<N18N>CO-C_9 (**11**) was as biologically active as the saturated analog lariat **6**. The ability of **11** to form stable pores was not expected. Surprisingly, both the C_8 and C_{10} amides formed stable, ion-conducting pores in soybean asolectin membranes.

Figure 7 (above) shows the traces obtained from a planar bilayer voltage clamp (BLM) study of C_8 (**10**, top) and C_{10} lariat (**11**, bottom) amides. The data for C_8 lariat were obtained at a concentration of 3 μM and an applied potential of 30 mV. The concentration and potential for C_{10} lariat amide were 7 μM and 50 mV. The traces shown span approximately 1 min (top) and 3.5 min (bottom). These pores remained stable within the bilayer for an hour or more, suggesting substantial stability. Both traces show unmistakable open-close behavior and multiple channel openings. Although both of these compounds are biologically active in *A. thaliana*, plant boundary layers and extracted asolectin bilayers are sufficiently different to preclude attempting to directly correlate activity with pore formation. Notwithstanding, it seems clear that both **10** and **11** can form pores, which presumable affect K^+ transport in the plants.

Both **10** (C_8) and **11** (C_{10}) exhibit two stable conductance states. For C_8 lariat amide **10**, they are 14 and 21 picoSiemens (pS), respectively. For C_{10} lariat amide **11**, they are 20 and 24 pS. Multiple openings are apparent in both cases. Studies of ion binding by lariats such as C_{10} (**6**) with its counterpart diamide (**11**) show much reduced ion binding. Strong binding is presumably inimical to rapid ion transport so the presence of amides is not problematic. Indeed, the lariat ethers may well be protonated in the biological milieu and the positive charges within the macrocycle would likely impede ion

transport. Even so, the recorded conductances are similar in both cases.

Conclusion

The present study examines the effect of known ion transporters – hydraphiles and lariat ethers – on the growth, development, and morphology of *A. thaliana*. Several of the compounds studied engendered significant morphological changes, notably in primary root length and lateral root density. The initial hypothesis that variations in ionophore complexation strength or transport rates could account for the effects was not supported. The effect of these ionophores was auxin-like, but a study of two genetically modified *A. thaliana* plants showed that the mechanism of action was not based on auxin uptake or transcription repressor of auxin pathways.

Disruption of cation gradients in plant root cells is also known to affect plant growth and development. Indeed, auxin induced growth is dependent both on extracellular potassium ion gradients and the presence of potassium channels in plant plasma membranes. Stress signaling caused by these synthetic amphiphiles could also affect root development. [52] This may lead to the failure of cell elongation and root development. In addition, at least five K^+ transport proteins have been characterized in *A. thaliana* roots. These channel proteins regulate K^+ uptake or efflux in the root cap, the epidermis of the primary and lateral roots, the root tip, or the root-hypocotyl junction. Hirch, *et al.* reported [53] that plant nutrition and growth depends on the AKT1 channel, which functions as an inward rectifying potassium channel. Thus, a range of proteins could be affected by synthetic ionophore-altered cation gradients. We speculate that the auxin signaling cascade induced by potassium channels may be disrupted by the synthetic ionophores studied here. Alternately, the synthetic ionophores reported here may interfere with the regulation of existing protein channels.

Lariat ethers and lariat ether amides affect the growth of *A. thaliana* and, surprisingly, show clear evidence for well-behaved pore formation in soybean asolectin membranes. The biological effects observed are similar to those of herbicide 2,4-D, which has been hypothesized to act as a potassium carrier and increase ion transport. [54] Very recently, silver nanoparticles were reported to show an effect similar to that of hydraphiles and lariat ethers on *A. thaliana* primary root length. This change in root morphology by silver nanoparticles was related to pore formation in plant membranes (56 ± 9 pS). [52] Alternatively, these ionophores (hydraphiles and lariat ether) may function in an auxin independent pathway. Alkamides such as *N*-isobutyldecanamide are known to decrease primary root length and to increase lateral root density at high concentrations. [55] Synthetic ionophores were effective against auxin transport and transcription repression mutants. This may suggest a simple alkamide-like auxin-independent pathway. In any event, the pore forming ability of these compounds is unprecedented and will no doubt require extensive future study.

Acknowledgment. We thank the National Science Foundation for a grant (CHE-1307324) that, in part, supported this work. We are also grateful to Dr. Bethany Zolman and Gretchen Spiess for valuable discussions and technical assistance.

Experimental Section

Plant nutrient media and plating seeds. *A. thaliana* Columbia-0 (Col-0) seeds (>20), the most common strain of this plant, were sterilized and plated on plant nutrient and 0.5% sucrose (PNS) media [56] [57] containing 0.6% agar. To make 500 mL PN media, 485 mL doubly distilled H_2O was first added to an autoclave beaker. The following salts and nutrients were added to a beaker and mixed well: 5 mL of 250 mM of KPO_4 (pH 5.5), 2.5 mL of 1M KNO_3, 1 mL of 1M $MgSO_4$, 1 mL of 1M $Ca(NO_3)_2$, 0.5 mL of Ferric EDTA (1000×) and 0.5 mL of Micronutrients (1000×). [49] [50] In a 1 L autoclaved bottle, 0.6% (3 g) of bacteriological agar was added, followed by 500 mL solution prepared above. The media was autoclaved using the Liquid-30 program. 5 mL of 50% sterile sucrose was added to PN media (55 °C) to make PNS media containing 0.5% sucrose.

The types of plates, compounds, and concentrations to be used were confirmed while the media cooled to 55 °C. Sterile centrifuge tubes (50 mL) were used to measure 29.94 mL PNS media. All the compounds were dissolved in DMSO and 60 µL of its desired concentration was added to the PNS media, mixed by inverting the tube and poured in the plate. IAA (Sigma-Aldrich) and 2,4-D (Sigma-Aldrich) were dissolved in autoclaved MilliQ water. For DMSO control 60 µL DMSO was added to PNS media before it solidified. For PNS control, 30 mL of PNS media was used. Plates were incubated at room temperature for 2 hours before plating seeds on them.

Seeds were sterilized before plating them on the PNS media. *A. thaliana* Col-0 seeds were added to sterile 1.5 mL micro-centrifuge tube. Sterilizing solution (500 µL, 30% bleach + 0.01% Triton X-100) was added to the seeds, vortexed for 3 seconds and incubated at room temperature for 8 minutes. After 8 minutes, sterilizing solution was removed and 1 mL autoclaved MilliQ water was added. Seeds were briefly vortexed, allowed to settle, and the water was removed. The seeds were washed two more times with autoclaved MilliQ water. Seeds were finally suspended in 200 µL of sterile 0.1% agar. These seeds were plated (> 20 seeds per plate) on PNS media using sterilized Pasteur pipettes. Seeds were not allowed to touch each other or the boundaries of the plates. The plates were covered with surgical tape and incubated using an Intellus Environment Controller, under continuous white light at 23 °C. IAA was incubated for the same time and temperature under yellow light. [58]

Germination measurements. Col-0 seeds were plated on PNS media containing compounds **1-13** and incubated using an Intellus Environment Controller at 23 °C. The number of Col-0 seeds germinated were observed under dissecting microscope for all the plates. Here, radicle protrusion was scored every 12 hours for 3 days. Percentage of seeds germinated was calculated for every reading and graphed as percent of seeds germinated vs. time (in hours). The plates were returned to the incubator for root development.

Primary root length measurements and number of lateral roots. *A. thaliana* Col-0 seeds were incubated under white light (except IAA) for 11 days. At 11 days, the agar around each plant was disturbed and the plant was removed from the agar using sterile forceps. The primary root length for each plant was measured (data reported in millimeters) using a ruler. The plant was then transferred to a petri dish containing

water, and examined by using a dissecting microscope. All the lateral roots, including emerging and developed roots, were counted. The lateral roots emerging from the primary root were recorded. The average and standard errors (used for error bars) for 3-4 trials (~16-23 seeds each) was calculated. Each root length and lateral root density value reported here is the average of values observed for ~60 plants.

Synthesis of lariat ether and lariat ether amides.

Dibenzyl hydraphile C_8, **1,** was prepared as described in reference 34.

Dibenzyl hydraphile C_{14}, **2,** was prepared as described in reference 34.

Dibenzyl hydraphile C_{16}, **3,** was prepared as described in reference 34.

***N,N'*-Di-*n*-hexyl-4,13-diaza-18-crown-6, 4**, was prepared in a fashion identical to that described for **5,** as described below. The product was isolated in 91% yield.

***N,N'*-Di-*n*-octyl-4,13-diaza-18-crown-6, 5**, was prepared as previously reported[17] except that the product was crystallization from acetone (yield: 87%). The crystals gradually became a colorless oil on standing at room temperature.

***N,N'*-Di-*n*-decyl-4,13-diaza-18-crown-6, 6**, was prepared as reported previously,[17] except that the product was purified by crystallization from acetone (yield: 86%).

***N,N'*-Di-*n*-dodecyl-4,13-diaza-18-crown-6, 7**, was prepared (yield: 82%) as reported in detail in reference 17.

***N,N'*-Di-*n*-tetradecyl-4,13-diaza-18-crown-6, 8**, was obtained in 83% yield after alkylation of *N,N'*-diaza-18-crown-6 with 1-bromotetradecane by using the general procedure reported in reference 17, mp 54 °C.

***N,N'*-Di-1-oxohexyl-4,13-diaza-18-crown-6, 9**, was prepared by the reaction of hexanoic acid with thionyl chloride, followed by reaction with 4,13-diaza-18-crown-6 as described for **7** in reference 41. The product was isolated in 91% yield as a colorless solid.

***N,N'*-Di-1-oxooctyl-4,13-diaza-18-crown-6, 10**, was prepared by the reaction of hexanoic acid with thionyl chloride, followed by reaction with 4,13-diaza-18-crown-6 as described for **7** in reference 47. The product was isolated in 90% yield as a white solid.

***N,N'*-Di-1-oxodecyl-4,13-diaza-18-crown-6, 11**, was prepared (yield: 92%) as reported in reference 17.

***N,N'*-Di-1-oxododecyl-4,13-diaza-18-crown-6, 12**, was prepared (yield 92%) as reported in reference 17.

***N,N'*-Di-1-oxotetradecyl-4,13-diaza-18-crown-6, 13**, was prepared by the reaction of hexanoic acid with thionyl chloride, followed by reaction with 4,13-diaza-18-crown-6

as described for **7** in reference 47. The product was isolated in 91% yield as a white solid.

Indoleacetic acid, IAA, 14, and 2,4-Dichlorophenoxyacetic acid, 2,4-D, **15**, were purchased from Sigma-Aldrich and used as received.

Planar bilayer voltage clamp studies. Planar lipid bilayer studies were conducted by using a Warner BC-525D bilayer clamp apparatus. The membrane was formed by painting the 200 μM orifice with a *n*-decane solution of asolectin (from soybean) in *n*-decane (25 mg mL^{-1}). This membrane blocks theonly connection between two buffer-filled compartments containing KCl (450 mM) buffer solution (HEPES, 10 mM, pH 7). The lariat ether was dissolved in DMSO and added to the one side of the membrane to achieve the desired concentration. The records of current flow at the specified potentials were filtered with a 4-pole Bessel filter (100 Hz) and digitized at a 1 kHz sampling interval per signal by using Clampex 9.2 (Axon instruments). Data analysis was performed with Clampfit 9.2 (Axon instruments).

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Supramolecular Cation Transporters Alter Root Morphology in the *Arabidopsis Thaliana* Plant

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Highlights:

- The synthetic ion transporters – hydraphiles and lariat ethers – engendered significant morphological changes, notably in primary root length and lateral root density of *Arabidopsis thaliana* plant.
- The change in root morphology was dependent on length and concentration of the synthetic ionophores used.
- Lariat ethers and lariat ether amides affect the growth of *A. thaliana* and, surprisingly, show clear evidence for well-behaved pore formation in soybean asolectin membranes.
- A study of two genetically modified *A. thaliana* plants showed that the mechanism of action was not based on auxin uptake or transcription repressor of auxin pathways. The change in root morphology may be associated with the pore forming and ion transporting ability of these compounds.