Silver Ions Increase Auxin Efflux Independently of Effects on Ethylene Response

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Silver nitrate and aminooxyvinylglycine (AVG) are often used to inhibit perception and biosynthesis, respectively, of the phytohormone ethylene. In the course of exploring the genetic basis of the extensive interactions between ethylene and auxin, we compared the effects of silver nitrate (AgNO₃) and AVG on auxin responsiveness. We found that although AgNO₃ dramatically decreased root indole-3-acetic acid (IAA) responsiveness in inhibition of root elongation, promotion of DR5-β-glucuronidase activity, and reduction of Aux/IAA protein levels, AVG had more mild effects. Moreover, we found that that silver ions, but not AVG, enhanced IAA efflux similarly in root tips of both the wild type and mutants with blocked ethylene responses, indicating that this enhancement was independent of ethylene signaling. Our results suggest that the promotion of IAA efflux by silver ions is independent of the effects of silver ions on ethylene perception. Although the molecular details of this enhancement remain unknown, our finding that silver ions can promote IAA efflux in addition to blocking ethylene signaling suggest that caution is warranted in interpreting studies using AgNO₃ to block ethylene signaling in roots.

INTRODUCTION

The phytohormones auxin and ethylene regulate many aspects of plant growth and development. Auxin directs embryonic patterning, root and stem elongation, lateral organ development, and leaf expansion, whereas ethylene modulates fruit ripening, senescence, seed germination, abscission, and stress responses (reviewed in Davies, 2004). Ethylene synthesis is controlled by the activity of the rate-limiting 1-aminocyclopropane-1-carboxylic acid synthase (ACS) enzymes. Several ACS proteins, such as ACS5/ETHYLENE OVERPRODUCER2 (ETO2), are posttranscriptionally regulated by the ETO1 E3 ubiquitin ligase. Loss-of-function eto1 mutations and gain-of-function acs5/eto2 mutations confer ethylene overproduction, which results in short hypocotyls in dark-grown seedlings and short roots in light-grown seedlings (Figure 1A; reviewed in Chae and Kieber, 2005). Ethylene is perceived by transmembrane histidine kinase receptors that use a copper cofactor for ethylene binding (reviewed in Benavente and Alonso, 2006). ETHYLENE INSENSITIVE2 (EIN2) acts downstream of ethylene perception and is required for ethylene response (Alonso et al., 1999).

Extensive crosstalk exists between ethylene and auxin at the levels of synthesis, signaling, and transport. Ethylene stimulates synthesis of the auxin indole-3-acetic acid (IAA) (Ruzicka et al., 2007; Swarup et al., 2007), and auxin stimulates ethylene synthesis by increasing ACS transcription (reviewed in Yang and Hoffman, 1984; Tsuehika and Theologis, 2004). Many ethylene signaling mutants are also auxin resistant, and many auxin signaling mutants are also ethylene resistant (Stepanova et al., 2007), suggesting that some facets of auxin response require ethylene response and some aspects of ethylene response require auxin response. In further support of this interconnection, mutants with decreased IAA synthesis are mildly ethylene resistant (Stepanova et al., 2005, 2008). In addition to effects on synthesis and signaling, ethylene affects auxin transport. Ethylene inhibits polar auxin transport in cotton (Gossypium hirsutum) stem sections and pea (Pisum sativum) epicotyls (Burg and Burg, 1966; Morgan and Gausman, 1966) and increases acropetal and basipetal [³H]-IAA transport in Arabidopsis thaliana roots (Negi et al., 2008). Moreover, root ethylene responses require basipetal auxin transport (Ruzicka et al., 2007).

Two compounds commonly used to differentiate between blocking ethylene biosynthesis and response are aminooxyvinylglycine (AVG) and silver nitrate (AgNO₃). AVG, an inhibitor of pyridoxal phosphate-mediated reactions, decreases ethylene production (Adams and Yang, 1977) by inhibiting ACS activity (Yang and Hoffman, 1984). The 1976 discovery that silver ions block ethylene responses (Beyer, 1976) has led to extensive use of AgNO₃ for both agronomic and research purposes. Ag⁺ is thought to occupy the copper binding site of ethylene receptors and interact with ethylene but inhibits the ethylene response (Rodriguez et al., 1999; Zhao et al., 2002; Binder et al., 2007). Either silver or AVG can restore eto1-1 root and hypocotyl elongation (Figure 1A; Guzman and Ecker, 1990). In this study, we present evidence suggesting that AgNO₃ promotes IAA efflux in roots and that this promotion acts independently of AgNO₃ effects in blocking ethylene response.

RESULTS AND DISCUSSION

A point mutation of E1-CONJUGATING ENZYME-RELATED1 (ECR1) confers both indole-3-propionic acid resistance and
Figure 1. AgNO₃ Dampens IAA Responses in Roots.

(A) Silver nitrate and AVG are similarly effective in restoring eto1 mutant phenotypes. Wild-type (Col-0), eto1-1, and eto2-1 seedlings were grown on medium supplemented with various concentrations of AgNO₃ or AVG. Hypocotyls were measured 4 d after transfer of 1-d-old seedlings to the dark (top panels). Primary roots of 8-d-old seedlings were measured after growth under continuous white light (bottom panels). Error bars represent SE (n = 12).

(B) Root elongation inhibition response of the wild type (Col-0), aux1-7, ein2-1, and eir1-1 to IAA and 2,4-D in the presence and absence of AgNO₃ or AVG. Primary root lengths of 8-d-old seedlings grown under continuous yellow-filtered light on mock (ethanol)-supplemented medium or medium supplemented with 600 nM IAA or 100 nM 2,4-D with or without 5 μM AgNO₃ or 10 μM AVG are shown. Error bars represent SE (n ≥ 12).

(C) Silver nitrate decreases IAA-induced DR5-GUS expression. Eight-day-old light-grown wild-type (Col-0) seedlings carrying the DR5-GUS transgene (Ulmasov et al., 1997) were mock treated or treated with 1 μM IAA for 2 h in medium lacking or containing 10 μM AgNO₃ or 10 μM AVG and then stained for GUS activity. Bar = 0.5 mm.

(D) Silver nitrate decreases IAA-induced IAA28myc degradation. Ten-day-old wild-type (Col-0) seedlings carrying the IAA28myc construct (Strader et al., 2008a) were treated for 10 min with the indicated combinations of IAA (top panel), 2,4-D (bottom panel), AgNO₃, and AVG in liquid media. Anti-myc and anti-HSC70 antibodies were used to detect IAA28myc and HSC70 (loading control), respectively, on immunoblots of protein prepared from roots of treated seedlings.
certain ethylene overproduction phenotypes (Woodward et al., 2007). Blocking ethylene response with AgNO₃ was required to reveal that ethylene was resistant not only to indole-3-propionic acid but also to IAA (Woodward et al., 2007). Intriguingly, much higher IAA levels were required to inhibit wild-type root elongation in the presence of AgNO₃ (Woodward et al., 2007), suggesting that part of the reduced root elongation in response to exogenous IAA might be attributable to the inhibitory effects of ethylene synthesized following IAA treatment.

We further explored this hypothesis by examining the impact of pharmacological ethylene blockers on auxin responsiveness of the wild type, the auxin influx mutant aux1 (Marchant et al., 1999), the ethylene response mutant ein2 (Alonso et al., 1999), and the auxin efflux mutant eir1 (Luschning et al., 1998). We assayed root elongation inhibition responses to IAA and the auxin compound 2,4-D in the presence and absence of AVG or AgNO₃. We expected that either blocking ethylene production with AVG or blocking ethylene response with AgNO₃ would similarly reduce auxin responsiveness. Instead, we found that AgNO₃ blocked wild-type IAA response more effectively than AVG (Figure 1B). AgNO₃ and AVG were similarly effective in restoring hypocotyl and root elongation to the auxin efflux mutant eto1-1 (Figure 1A), suggesting that both were active in our conditions, and similarly reduced wild-type root elongation inhibition in response to 2,4-D (Figure 1B). We concluded that the interaction between AgNO₃ and IAA is different from that of AgNO₃ and 2,4-D.

We expected that AgNO₃ and AVG would not affect the auxin responsiveness of ein2 because ein2 is completely ethylene insensitive (Alonso et al., 1999) and presumably would not respond to ethylene produced in response to auxin treatment. Indeed, ein2 resistance to the auxin analog 2,4-D was unaffected by AgNO₃ or AVG (Figure 1B). However, AgNO₃, but not AVG, reduced ein2 response to IAA (Figure 1B), suggesting that AgNO₃ decreased IAA response independently of ethylene response. Similarly, the diminished IAA sensitivity of eir1/pin2 in the presence of AgNO₃ but not AVG (Figure 1B) suggested that AgNO₃ did not reduce IAA sensitivity by enhancing PIN2-mediated IAA efflux. Although nearly insensitive to IAA at the concentration tested, aux1 also displayed further decreased IAA sensitivity in the presence of AgNO₃ (Figure 1B), suggesting that AgNO₃ reduced IAA sensitivity without reducing AUX1-mediated IAA influx.

To determine the site in the IAA response pathway that was impacted by silver ions, we examined two auxin-responsive reporters, DR5-β-glucuronidase (GUS), which monitors auxin-responsive transcription (Ulmasov et al., 1997), and IAA28myc, which monitors auxin-stimulated protein degradation (Strader et al., 2008a). We found that AgNO₃ greatly reduced the ability of IAA to induce the DR5-GUS transcriptional reporter in roots tips (Figure 1C), whereas AVG had only a mild effect (Figure 1C), suggesting that AgNO₃ dampened auxin response upstream of auxin-responsive gene expression. Because Aux/IAA proteins repress auxin-induced gene expression, we examined the effect of AgNO₃ and AVG on IAA-induced IAA28myc destabilization in roots. A 10-min IAA treatment is sufficient to dramatically decrease IAA28myc levels (Figure 1D; Strader et al., 2008a). We found that 10 μM AVG had no apparent effect on IAA-induced instability of IAA28myc in this assay (Figure 1D). By contrast, including AgNO₃ with the applied IAA reduced the IAA effects on IAA28myc levels in a dose-dependent manner (Figure 1D), suggesting that AgNO₃ acted upstream of Aux/IAA destabilization. Because the interaction between AgNO₃ and IAA is different from that of AgNO₃ and 2,4-D (Figure 1B), we also examined the effect of AgNO₃ or AVG on 2,4-D-induced IAA28myc destabilization. We found that neither inhibitor had an apparent effect on 2,4-D-induced stability of IAA28myc (Figure 1D). Because Aux/IAA destabilization is very rapid and because the Aux/IAA protein itself forms a part of the IAA receptor complex (Tan et al., 2007), these data suggested that the AgNO₃ effect on IAA28myc levels was not solely due to the ability of silver ions to block ethylene signaling.

Because our data suggested that the effect of silver ions on root IAA responsiveness was upstream of the known signaling components, we examined the effect of AgNO₃ on [³H]-IAA accumulation in an excised root tip assay (Ito and Gray, 2006; Strader et al., 2008b; Strader and Bartel, 2009). We found that AgNO₃, but not KNO₃ (Figure 2A) or AVG (Figure 2B), dramatically decreased [³H]-IAA accumulation following a 1-h incubation. We also examined the effect of AgNO₃ and AVG on [³H]-IAA accumulation in aux1-7, ein2-1, and eir1-1 root tips. We found that aux7-7, as previously reported (Strader et al., 2008a), accumulated less [³H]-IAA than the wild type in this assay, consistent with the role of AUX1 in IAA uptake. Like the wild type, aux1 root tips accumulated less [³H]-IAA in the presence of AgNO₃ (Figure 2B), suggesting that AgNO₃ does not reduce [³H]-IAA accumulation by blocking ethylene signaling. We also tested mutants defective in various proteins implicated in auxin efflux and found that eir1, pen3, mdr1, mdrl, pp1, and pgp1 mdr1 root tips responded like the wild type to AgNO₃ by accumulating less [³H]-IAA (Figures 2C and 2D), suggesting that AgNO₃ does not reduce [³H]-IAA accumulation by increasing IAA efflux by EIR1/PIN2, PEN3/PDR8/ABCG36, MDR1/ABCBI9, MDR4/ABCBI4, or PGP1/ABCBI.

During the 1-h labeling period, the amount of [³H]-IAA accumulated within root tips reflects the balance of [³H]-IAA influx and efflux. To separate influx from efflux, we incubated root tips in [³H]-IAA for 1 h, rinsed the root tips thoroughly with buffer, and then incubated the root tips for additional time in a large buffer volume (to minimize reuptake of effluxed [³H]-IAA) with or without 10 μM AgNO₃. We found that the effect of AgNO₃ when provided only during an efflux period (Figure 2E) was similar to its effects when provided continually (Figures 2A to 2D), suggesting that AgNO₃ reduced [³H]-IAA accumulation by increasing IAA efflux. We also found that the effect of AgNO₃ on [³H]-IAA efflux was dose dependent in a 1-h efflux period (Figure 2F). To determine whether auxin transport inhibitors could alter AgNO₃-responsive [³H]-IAA efflux, we examined the effects of including 1-N-naphthylphthalamic acid (NPA) or 2,3,5-triiodobenzoic acid (TIBA) during the efflux period. We found that inclusion of NPA or TIBA did not affect the accumulation of [³H]-IAA with or without AgNO₃ in this assay (Figure 2G).
Figure 2. AgNO₃ Promotes [³H]-IAA Efflux from Root Tips.

(A) Root tips of Col-0 (Wt) seedlings were incubated for 1 h in uptake buffer containing 25 nM [³H]-IAA, 25 nM [³H]-IAA and 10 µM AgNO₃, or 25 nM [³H]-IAA and 10 µM KNO₃, rinsed three times with uptake buffer, and then removed and analyzed by scintillation counting.

(B) Root tips of Col-0 (Wt), aux1-7, ein2-1, and eir1-1 seedlings were incubated for 1 h in uptake buffer containing 25 nM [³H]-IAA, 25 nM [³H]-IAA and 10 µM AgNO₃, or 25 nM [³H]-IAA and 10 µM AVG, rinsed three times with uptake buffer, and then removed and analyzed by scintillation counting.

(C) Root tips of Col-0 (Wt), pen3-4, etr1-1, and ers2-1 seedlings were incubated for 1 h in uptake buffer containing 25 nM [³H]-IAA or 25 nM [³H]-IAA and 10 µM AgNO₃, rinsed three times with uptake buffer, and then removed and analyzed by scintillation counting.

(D) Root tips of Col-0 (Wt), mdr1-3, mdr4-1, pgg1-100, Ws-2 (Wt), and pgg1-1 mdr1-1 (in the Wassilewskija background) seedlings were incubated for 1 h in uptake buffer containing 25 nM [³H]-IAA or 25 nM [³H]-IAA and 10 µM AgNO₃, rinsed three times with uptake buffer, and then removed and analyzed by scintillation counting.

(E) Root tips of Col-0 (Wt), aux1-7, ein2-1, and eir1-1 seedlings were incubated for 1 h in 80 µL uptake buffer containing 25 nM [³H]-IAA, rinsed three times, incubated for an additional 30 or 60 min in 400 µL buffer with or without 10 µM AgNO₃ or 10 µM AVG, and then removed and analyzed by scintillation counting.

(F) Root tips of Col-0 (Wt) seedlings were incubated for 1 h in 80 µL uptake buffer containing 25 nM [³H]-IAA, rinsed three times, incubated for an additional 1 h in 400 µL buffer containing 0, 0.5, 1, 5, or 10 µM AgNO₃, and then removed and analyzed by scintillation counting.

(G) Root tips of Col-0 (Wt) seedlings were incubated for 1 h in 80 µL uptake buffer containing 25 nM [³H]-IAA, rinsed three times, incubated for an additional 30 min in 400 µL buffer containing mock (ethanol), 100 µM NPA, or 100 µM TIBA, and then removed and analyzed by scintillation counting. For all experiments, data are from six replicates of assays with five root tips (5-mm sections) of 8-d-old light-grown seedlings of each genotype. Error bars represent SE.

(H) A model for the effects on AgNO₃ and AVG on auxin and ethylene signaling. IAA is transported into cells by AUX1 and related transporters and via diffusion through the membrane and is removed by effluxers such as EIR1/PIN2 and the ABCB proteins. In the cell, IAA stimulates the degradation of Aux/IAA proteins to relieve repression of auxin-responsive transcription (reviewed in Woodward and Bartel, 2005), leading to various responses, including induction of ACS transcription and ethylene production (reviewed in Yang and Hoffman, 1984; Tsuchisaka and Theologis, 2004). ACS activity can be blocked with AVG (Yang and Hoffman, 1984), and signaling by ethylene receptors, such as ETR1 and ERS2, can be blocked with Ag⁺ (Rodriguez et al., 1999; Zhao et al., 2002; Binder et al., 2007). EIN2 is required for ethylene signaling and acts downstream of ethylene perception (Alonso et al., 1999). The double-headed gray arrow represents extensive crosstalk between auxin and ethylene pathways. Solid black arrows depict signaling, dashed black arrows depict hormone synthesis, and dotted black arrows represent transport. Silver ions appear to stimulate IAA efflux independently of known IAA efflux components, such as EIR1/PIN2 and the ABCB proteins. Whether the Ag⁺-stimulated IAA efflux is transporter mediated or results from an effect on membrane permeability is unknown.
Our data suggest that, in addition to blocking ethylene responsiveness, silver ions can promote IAA efflux and thereby reduce accumulation of applied IAA in roots. This observation could explain why AgNO₃ reduces the efficacy of IAA treatment in root elongation inhibition (Figure 1B), gene expression (Figure 1C), and Aux/IAA degradation (Figure 1D) bioassays. The AgNO₃ promotion of IAA efflux appears to be independent of AUX1, EIN2, and known auxin efflux proteins (Figure 2) and thus may be mediated by an unidentified auxin effluxer or an effect on membrane permeability. Although the molecular details of this enhancement remain unknown, our finding that silver ions can impact IAA efflux independent of effects on ethylene signaling suggest that caution is warranted in interpreting studies using AgNO₃ to dissect the extensive interactions between ethylene and auxin pathways in roots.

METHODS

Plant Materials and Phenotypic Assays

The Columbia (Col-0) accession of Arabidopsis thaliana was used as the wild type for all experiments, unless noted otherwise. Surface-sterilized seeds (Last and Fink, 1988) were plated on PN (plant nutrient medium; Haughn and Somerville, 1988) supplemented with 0.5% (w/v) sucrose (PNS) and solidified with 0.6% (w/v) agar and grown under continuous illumination at 22°C. Hormone stocks were dissolved in ethanol; AgNO₃ and AVG stocks were dissolved in water.

For hypocotyl elongation assays, seeds were plated on media supplemented with various concentrations of AgNO₃ or AVG. After 1 d in the light, plates were wrapped with aluminum foil and incubated for an additional 4 d in the dark before lengths of hypocotyls were measured. For AgNO₃- or AVG-responsive root elongation assays, seedlings were grown for 8 d under continuous illumination on media supplemented with various concentrations of AgNO₃ or AVG, and the lengths of primary roots were measured.

For auxin-responsive root elongation assays, seedlings were grown for 8 d under continuous illumination through yellow long-pass filters (yellow 2208, 1/8-inch-thick Plexiglass; A&C Plastics) to slow indolic compound breakdown (Stasinopoulos and Hangarter, 1990) on PNS with the indicated auxin, AgNO₃, or AVG concentrations, and the lengths of primary roots were measured.

For GUS activity assays, 8-d-old light-grown seedlings were removed from PNS medium and floated in liquid PN supplemented with the indicated concentrations of KNO₃, AgNO₃, or AVG containing radiolabeled IAA (20 Ci mmol⁻¹; American Radiolabeled Chemicals). After 1 h at room temperature, root tips were rinsed with three changes of uptake buffer supplemented with the indicated concentrations of KNO₃, AgNO₃, or AVG and then removed and analyzed by scintillation counting in 3 mL of Cytoscint scintillation cocktail (Fisher Scientific).

For auxin retention assays, root tips were incubated as described above. After 1 h at room temperature, root tips were rinsed with three changes of uptake buffer and placed in 400 µL fresh uptake buffer supplemented with the indicated concentrations of KNO₃, AgNO₃, AVG, NPA, or TIBA. After 30 or 60 min incubation at room temperature, root tips were removed and analyzed by scintillation counting.

Accession Numbers

The Arabidopsis Genome Initiative locus identifiers of genes used in this study are as follows: AUX1, At2g38120; EIN2, At1g503280; ERF1/PIN2, At5g57090; ERF2, At2g04310; ERF1, At3g51770; ERF2/ACSS, At5g65800; ERF1, At1g66340; MDR1/ABC19, At3g28860; MDR4/ABC4, At2g47000; PEN3/PDR8/ABC36, At1g59870; and PGP1/ABC1, At2g36910.

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