# MATE Transporter-Dependent Export of Hydroxycinnamic Acid Amides[OPEN]

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**The ability of *Arabidopsis thaliana* to successfully prevent colonization by *Phytophthora infestans*, the causal agent of late blight disease of potato (*Solanum tuberosum*), depends on multilayered defense responses. To address the role of surface-localized secondary metabolites for entry control, droplets of a *P. infestans* zoospore suspension, incubated on Arabidopsis leaves, were subjected to untargeted metabolite profiling. The hydroxycinnamic acid amide coumaroylagmatine was among the metabolites secreted into the inoculum. In vitro assays revealed an inhibitory activity of coumaroylagmatine on *P. infestans* spore germination. Mutant analyses suggested a requirement of the *p*-coumaroyl-CoA:agmatine *N*4-*p*-coumaroyl transferase ACT for the biosynthesis and of the MATE transporter DTX18 for the extracellular accumulation of coumaroylagmatine. The host plant potato is not able to efficiently secrete coumaroylagmatine. This inability is overcome in transgenic potato plants expressing the two Arabidopsis genes *ACT* and *DTX18*. These plants secrete agmatine and putrescine conjugates to high levels, indicating that DTX18 is a hydroxycinnamic acid amide transporter with a distinct specificity. The export of hydroxycinnamic acid amides correlates with a decreased ability of *P. infestans* spores to germinate, suggesting a contribution of secreted antimicrobial compounds to pathogen defense at the leaf surface.**

## INTRODUCTION

*Arabidopsis thaliana* is not a host plant for *Phytophthora infestans*, the causal agent of late blight disease of *Solanum tuberosum* (Kamoun, 2001). This nonhost resistance depends on efficient defense mechanisms at the cell periphery as well as on postinvasion defense responses (Lipka et al., 2005; Westphal et al., 2008; Kopischke et al., 2013; Geissler et al., 2015). An extracellular chemical defense mechanism, identified in genetic screens, involves the myrosinase PEN2, which metabolizes indole glucosinolates to release tryptophan-derived antimicrobial metabolites. These are postulated to be transported out of the cell by the ABC transporter PEN3 to the site of attempted penetration (Bednarek, 2012). Compromised PEN2 or PEN3 functions result in enhanced penetration success of the oomycete (Lipka et al., 2005; Kobae et al., 2006; Stein et al., 2006), suggesting that the export of specific indole derivatives is required for the first layer of nonhost resistance.

Hydroxycinnamic acid amides (HCAAs) are major phytoalexins in leaves of potato, a host plant of *P. infestans* (Keller et al., 1996; Schmidt et al., 1998). Both infection with the oomycete and treatment with elicitors lead to the accumulation of HCAAs such as coumaroyltyramine and feruloyltyramine (Schmidt et al., 1998). These metabolites are assumed to act as antimicrobial and as cell wall reinforcing compounds. The potato enzyme catalyzing the biosynthesis of coumaroyltyramine is a member of the General Control Non-Repressed Protein5-related *N*-acyl transferase superfamily (Schmidt et al., 1999). By contrast, the Arabidopsis enzyme catalyzing the biosynthesis of coumaroylagmatine, *p*-coumaroyl-CoA:agmatine *N*4-*p*-coumaroyl transferase (ACT), belongs to the class of BAHD enzymes, which was named after the first characterized enzymes of this family, benzylalcohol *O*-acetyltransferase, anthocyanin *O*-hydroxycinnamoyltransferase, *N*-hydroxycinnamoyl/benzoyltransferase, and deacetylvindoline 4-*O*-acetyltransferase (D'Auria, 2006; Muroi et al., 2009). Thus, sequence-unrelated enzymes are present in Arabidopsis and potato, which display similar enzyme activities.

Despite detailed studies on the biosynthesis of secondary metabolites, less is known about their subcellular localization and transport within and out of the cell. Multidrug and toxin extrusion (MATE) transporters are membrane-localized proteins that transport metabolites, toxins, or xenobiotics across membranes, presumably in exchange for $H^+$ or $Na^+$ ions. There are 58 MATE transporter genes in Arabidopsis, only a few of which have been functionally characterized. The MATE transporter Enhanced Disease Susceptibility 5 is required for the accumulation of salicylic acid, an important signaling compound in the pathogen defense response of plants (Nawrath et al., 2002). The transporter
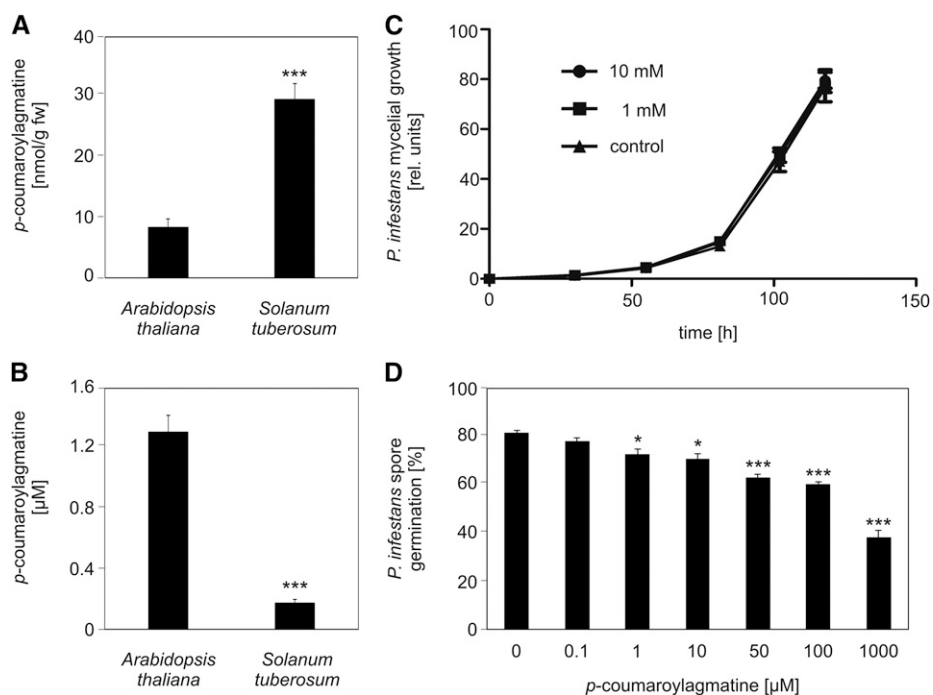
**Figure 1.** Coumaroylagmatine Accumulates Extracellularly in Arabidopsis, But Not in Potato, and Inhibits *P. infestans* Spore Germination in Vitro.

**(A)** and **(B)** Coumaroylagmatine content of leaves **(A)** and in droplets of the inoculum **(B)** from Arabidopsis and potato plants 24 h after inoculation with *P. infestans*. Data shown were obtained in at least five independent experiments (Arabidopsis, $n \geq 107$; potato, $n = 56$). Error bars represent SE. Significance analysis of differences was performed by *t* test, ***P < 0.001. fw, fresh weight.

**(C)** Mycelial growth inhibition assays. Coumaroylagmatine was added to GFP-expressing *P. infestans* (isolate Cra208m2) mycelium at the indicated concentrations. GFP fluorescence was determined at the time points indicated (control, $n = 5$; 1 mM, $n = 10$; 10 mM, $n = 9$). Data shown are representative for at least three independent experiments.

**(D)** Spore germination inhibition assay. Coumaroylagmatine was added to a *P. infestans* zoospore suspension ($10^5/mL^{-1}$) at the indicated concentrations. After 24 h, the samples were evaluated microscopically for spore germination. Data are derived from two independent experiments ($n = 4$). Significance analysis of differences was performed by *t* test, *P < 0.05 and ***P < 0.001.

is localized at the chloroplast envelope and transports salicylic acid from the chloroplast to the cytosol (Serrano et al., 2013; Yamasaki et al., 2013). The Arabidopsis MATE transporter Activated Disease Susceptibility 1 acts as a negative regulator of plant disease resistance (Sun et al., 2011). In addition, other plant MATE transporters have been shown to transport secondary metabolites, such as the tonoplast-localized anthoMATE1 of grapevine (*Vitis vinifera*), which transports acylated anthocyanins into the vacuole (Gomez et al., 2009), or MATE2, an essential transporter for proanthocyanidin biosynthesis in *Medicago truncatula* (Zhao et al., 2011).

In this study, the nonhost interaction of Arabidopsis and *P. infestans* was characterized at the metabolite and transcript level. Metabolite profiling identified the antimicrobial compound coumaroylagmatine, whose biosynthesis is catalyzed by ACT, as the major secreted hydroxycinnamic acid amide. Functional analyses showed that the MATE transporter DETOXIFICATION18 (DTX18) is required for export of coumaroylagmatine to the plant surface, suggesting that biosynthesis and secretion of coumaroylagmatine is part of the chemical defense activated in the nonhost resistance response of Arabidopsis against *P. infestans*. Gain of function experiments in potato show that DTX18 mediates

export of specific HCAAs, leading to an enhanced capacity to inhibit *P. infestans* spore germination.

## RESULTS

### Coumaroylagmatine Accumulates Extracellularly in Response to Inoculation by *P. infestans* in Arabidopsis, but Not in Potato

To determine the extent of extracellular chemical defense in the nonhost resistance response of Arabidopsis to *P. infestans* infection, untargeted metabolite profiling using UPLC-electrospray ionization-quadrupole time-of-flight-mass spectrometry (UPLC-ESI-QTOF-MS) was performed (Böttcher et al., 2008). Arabidopsis plants (Col-0) were drop-inoculated with a *P. infestans* zoospore solution. The droplets and the inoculated leaves were collected separately 1 d after inoculation and subjected to metabolite analysis. In both inoculated leaves and droplets of the inoculum, the HCAA coumaroylagmatine was detectable (Figures 1A and 1B). Interestingly, *P. infestans*-infected potato leaves contained significantly higher levels of coumaroylagmatine compared with Arabidopsis; however, in droplets
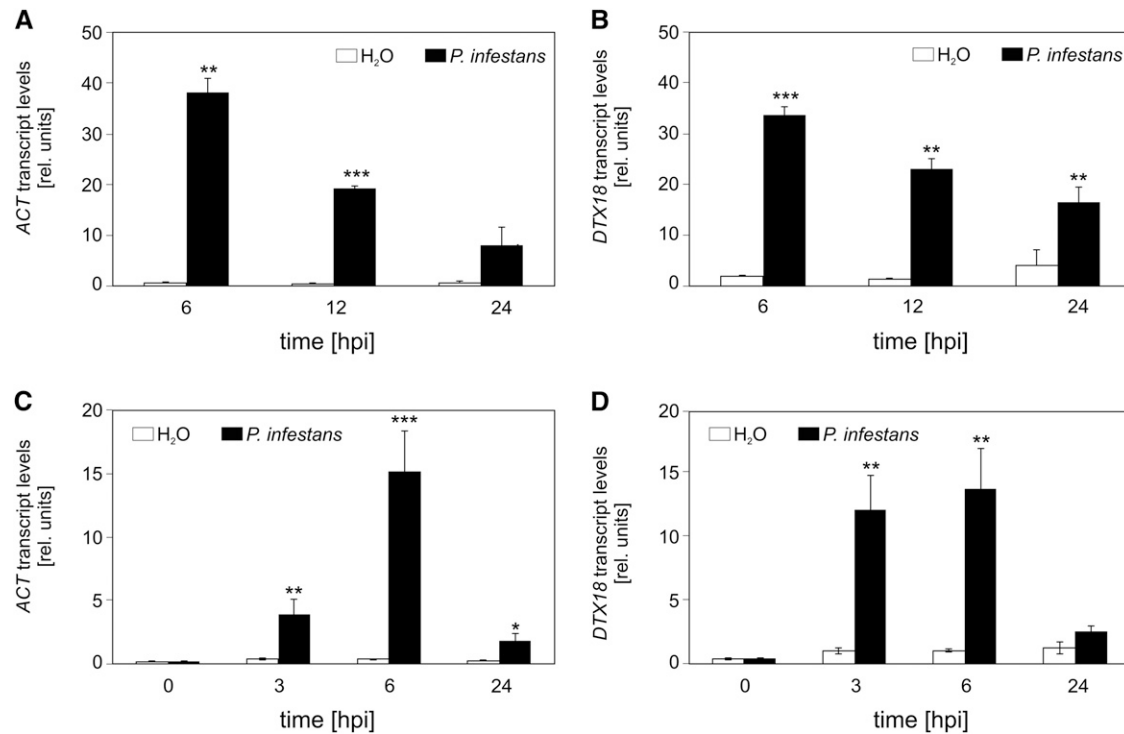
**Figure 2.** *P. infestans*-Induced Expression of *ACT* and *DTX18* in Arabidopsis.

**(A)** and **(B)** Microarray analyses. *ACT* **(A)** and *DTX18* **(B)** expression in response to inoculation with *P. infestans* (black bars) or water (white bars). Data were obtained from AtGenExpress (www.arabidopsis.org; *n* = 3). hpi, hours after inoculation.

**(C)** and **(D)** Time course of *ACT* **(C)** and *DTX18* **(D)** expression in response to inoculation with *P. infestans*. qRT-PCR was performed with RNA isolated from *P. infestans*-inoculated Arabidopsis (black bars) or water-treated plants (white bars) at the time points indicated. At-*PP2A* transcript levels were used for standardization. Data are derived from three independent experiments (*n* > 5). Error bars represent SE. Significance analysis of differences was performed by *t* test, *P < 0.05, **P < 0.01, and ***P < 0.001.

isolated from potato plants, only very low levels of coumaroylagmatine were detectable (Figures 1A and 1B). This observation suggests that both host and nonhost plants accumulate coumaroylagmatine intracellularly but that only Arabidopsis is able to export coumaroylagmatine to the leaf surface efficiently.

## Coumaroylagmatine Inhibits Cyst Germination but Not Mycelial Growth of *P. infestans*

The pathogen-induced accumulation of coumaroylagmatine in Arabidopsis leaves might indicate a role of this HCAA in defense against the oomycete. To address this, synthesized coumaroylagmatine was tested for antioomycete activity against *P. infestans* in an in vitro assay (Prost et al., 2005) that determines fluorescence of the GFP-expressing *P. infestans* isolate Cra208m2 as an indicator for oomycete biomass (Si-Ammour et al., 2003). Even at concentrations of 10 mM, coumaroylagmatine did not affect mycelial growth of *P. infestans* (Figure 1C). Since extracellular coumaroylagmatine might influence early stages of *P. infestans* infection prior to penetration, spore germination was analyzed microscopically in the presence and absence of coumaroylagmatine. Germination of *P. infestans* zoospores was inhibited in a dose-dependent manner, with 50% inhibition at a concentration of 1 mM coumaroylagmatine (Figure

1D). These observations suggest that only extracellular coumaroylagmatine might act as an antimicrobial compound against *P. infestans*, when spores encounter the compound during germination on the cell surface.

## ACT Is Responsible for the *P. infestans*-Induced Accumulation of Coumaroylagmatine

Microarray analyses with RNA from *P. infestans*-inoculated Col-0 plants (AtGenExpress; www.arabidopsis.org; www.genevestigator.com) were analyzed for genes that were activated in Arabidopsis specifically after inoculation with *P. infestans*, but not in the interaction with bacterial pathogens. One of these genes, *At5g61160*, encodes ACT (Muroi et al., 2009). The microarray data demonstrated that *ACT* transcript levels were more than 60-fold higher in *P. infestans*-inoculated leaves compared with those of water-treated plants (Figure 2A). The gene *At3g23550*, annotated to encode the MATE transporter DTX18 (Li et al., 2002), was coexpressed with the *ACT* gene (Figure 2B). More detailed qRT-PCR analyses revealed that *ACT* and *DTX18* are expressed within 3 h after inoculation with *P. infestans* (Figures 2C and 2D).

Leaves of homozygous plants of the *act* T-DNA insertion line SALK_097380 were inoculated with *P. infestans* and analyzed by untargeted metabolite profiling using UPLC-ESI-QTOF-MS
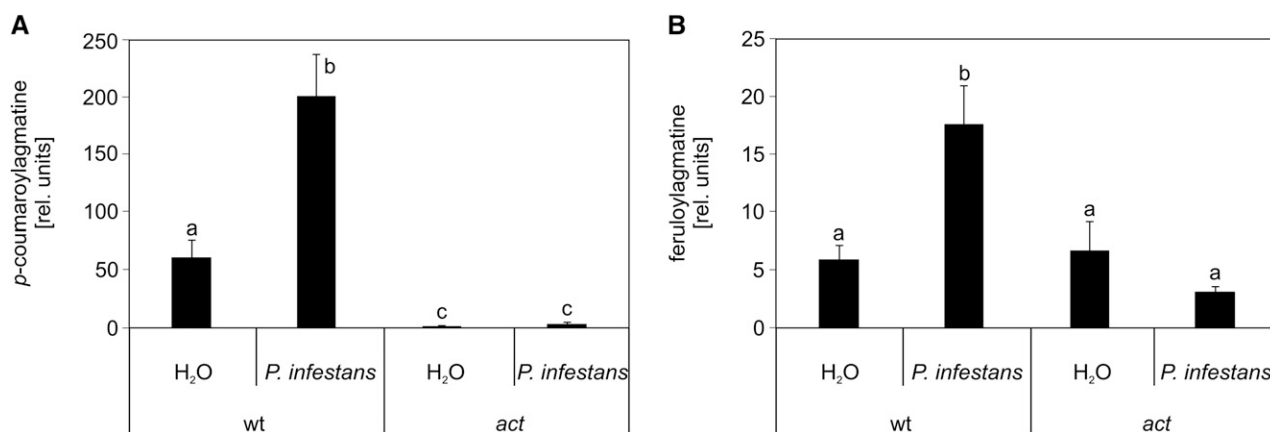
**Figure 3.** Reduced Levels of HCAAs in the *act* Mutant.

Coumaroylagmatine **(A)** and feruloylagmatine **(B)** levels were determined by UPLC-ESI-QTOF-MS in methanolic extracts of leaves of wild-type and *act* lines 24 h after inoculation with *P. infestans* or water. Data are derived from four independent experiments (wild type, water, $n = 27$; *P. infestans*, $n = 95$; *act*, water, $n = 12$; *P. infestans*, $n = 23$). Error bars represent SE. Letters indicate statistically different values (two-way ANOVA, $P < 0.05$).

(Böttcher et al., 2008). Under these conditions, coumaroylagmatine and feruloylagmatine were highly decreased in inoculated leaves (Figure 3). These compounds accumulated in wild-type plants in response to inoculation with *P. infestans*. In the *act* mutant, coumaroylagmatine levels were close to the detection limit in both water-treated and *P. infestans*-inoculated leaves (Figure 3A). Feruloylagmatine levels in the *act* mutant were similar to those in water-treated wild-type leaves, regardless of the treatment (Figure 3B). These results suggest that the *P. infestans*-induced accumulation of coumaroylagmatine and feruloylagmatine is mediated by ACT.

To characterize ACT enzyme activity, the coding region of *At5g61160* was cloned into the bacterial expression vector pDEST17. The recombinant protein was purified from extracts of isopropyl β-D-1-thiogalactopyranoside-treated bacteria via a HisTrap HP column (Supplemental Figure 1). Recombinantly expressed protein converted *p*-coumaroyl-CoA and feruloyl-CoA with agmatine as amine acceptor (Table 1). Feruloyl-CoA as donor showed better affinity but produced lower turnover velocity with agmatine.

### The Coexpressed Gene *At3g23550* Encodes a MATE Transporter

*At3g23550* is highly coexpressed with *ACT* (Figure 2; www.genevestigator.com). The gene is annotated to encode the MATE transporter DTX18 (Li et al., 2002). The encoded protein of 469 amino acids is predicted to contain 12 transmembrane domains (http://www.enzim.hu/hmmtop/) and to be localized to the plasma membrane (http://aramemnon.botanik.uni-koeln.de/).

To address the function of DTX18, homozygous plants of the T-DNA insertion line SALK_062231 and of the GABI-KAT line GK411D06 (Kleinboelting et al., 2012) were obtained (Figures 4A and 4B). In contrast to wild-type plants, *DTX18* transcripts were below the detection limit 6 h after inoculation with *P. infestans* (Figure 4C). Methanolic extracts of *P. infestans*-infected leaves contained similar levels of coumaroylagmatine as *P. infestans*-infected leaves of wild-type Arabidopsis plants (Figure 4D), suggesting that *dtx18* lines retain

the ability to biosynthesize coumaroylagmatine. However, coumaroylagmatine was absent in *P. infestans* droplets incubated on the leaf surface of both *dtx18* mutant lines (Figure 4E). Thus, the disruption of the *DTX18* gene resulted in an inability to secrete coumaroylagmatine. These results suggest that the MATE transporter encoded by *At3g23550* is required for export of coumaroylagmatine.

### *DTX18* Expression in Potato Results in Extracellular Accumulation of HCAAs

The major phytoalexins of potato are HCAAs such as coumaroyl- and feruloyltyramine, which are exported to the apoplast and incorporated into the cell wall upon pathogen infection (Keller et al., 1996; Schmidt et al., 1999). The low extracellular levels of coumaroylagmatine in potato in response to *P. infestans* infection (Figure 1B) might be due to a missing or inefficient export system for this compound. To assess whether DTX18 can confer the ability to secrete coumaroylagmatine to potato, transgenic plants were generated which expressed either *ACT* alone or both *ACT* and *DTX18* under the control of the 35S promoter. *Agrobacterium tumefaciens*-mediated leaf disk transformation resulted in the regeneration of independently transformed plants, which were tested for the presence of the transgenes by DNA gel blot analyses. Expression of the transgenes was demonstrated by qRT-PCR for four individual lines each for *ACT* (B, H, M, and Z) and *ACT-DTX18* transgenic plants (D, K, N, and X; Figures 5A and 5B).

**Table 1.** Enzymatic Parameters of Arabidopsis ACT Purified after Expression in *Escherichia coli*

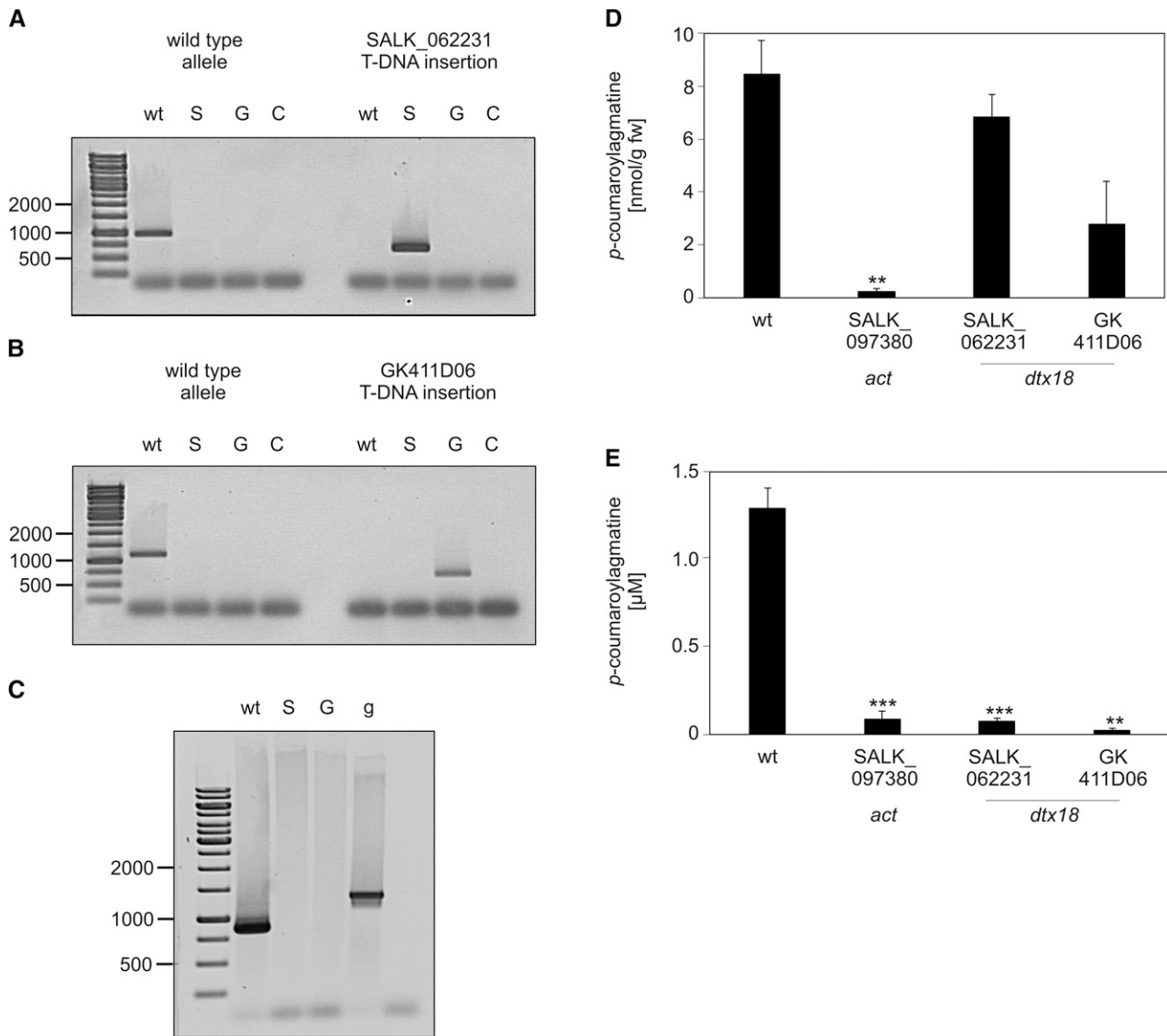| Donor | $K_m$ (μM) | $V_{max}$ (nkat/mg) | $V_{max}/K_m$ |
|---|---|---|---|
| *p*-coumaroyl-CoA | $1.13 \pm 0.12$ | $11.0 \pm 0.29$ | 9.7 |
| Feruloyl-CoA | $0.61 \pm 0.01$ | $4.9 \pm 0.16$ | 8.2 |
| Acceptor[a] | | | |
| Agmatine | $0.45 \pm 0.16$ | $6.06 \pm 0.27$ | 24.4 |

[a]With *p*-coumaroyl-CoA as acyl donor.

**Figure 4.** *dtx18* Knockout Lines Are Unable to Secrete *p*-Coumaroylagmatine.

**(A)** and **(B)** Characterization of T-DNA insertion lines of *DTX18*. DNA was isolated from leaves of wild-type plants or the T-DNA insertion lines SALK_062231 and GK_411D06. PCR was performed using specific primers for the alleles from the wild type, SALK_062231 (S), or GK_411D06 (G). C, water control.
**(C)** Loss of *DTX18* gene expression. RT-PCR using DTX18-specific primers was performed using RNA from leaves of wild-type plants and the T-DNA insertion lines SALK_062231 (S) and GK_411D06 (G). Genomic DNA (g) was used as a control.
**(D)** and **(E)** Reduced extracellular levels of coumaroylagmatine in *dtx18* mutant lines. Coumaroylagmatine levels were determined in leaves **(D)** and in droplets of the inoculum **(E)** from Arabidopsis wild-type and mutant plants (SALK_062231 and GK_411D06) 24 h after inoculation with *P. infestans*. Data shown were obtained in at least three independent experiments (wild type, $n \geq 106$; *act*, $n = 21$; SALK_062231, $n = 47$; GK_411D06, $n = 8$). Significance analysis of differences was performed by *t* test, *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. fw, fresh weight.

While *ACT*-expressing potato plants did not show morphological alterations, *ACT-DTX18*-expressing plants were smaller, had crinkled leaves, and developed spontaneous necroses at later development stages (Figure 5C).

Metabolite analyses revealed enhanced levels of coumaroylagmatine in leaf disks of *P. infestans* inoculation sites in all lines, with highest levels detected in *ACT-DTX18*-expressing lines (Figures 6A and 6B). However, infection droplets on *ACT*-expressing

plants contained very low levels of coumaroylagmatine, comparable to those of wild-type and empty vector plants (Figure 6C). Thus, despite significantly higher levels of coumaroylagmatine in the leaves, coumaroylagmatine was not secreted in the *ACT*-expressing lines. These results suggest that coumaroylagmatine levels are not the limiting factor for export in potato. By contrast, extracellular accumulation of coumaroylagmatine was observed for all four *ACT-DTX18*-expressing lines, reaching more than 40 µM for
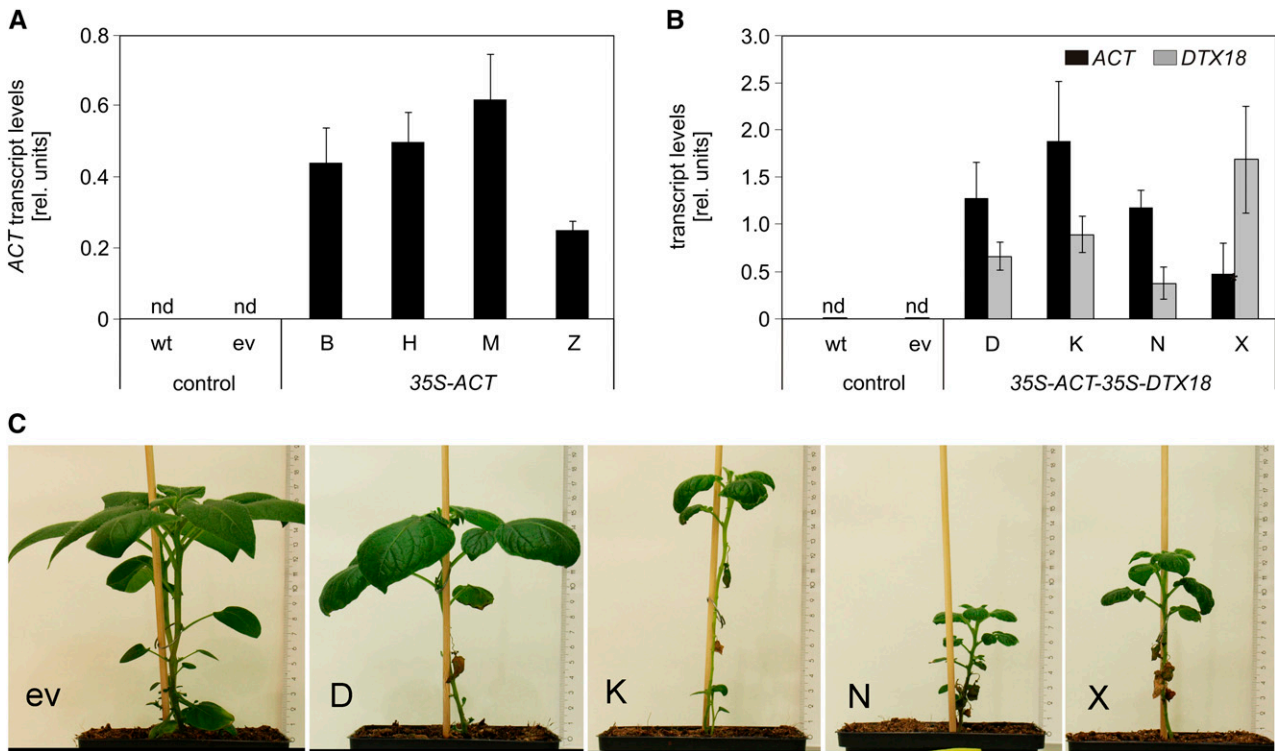
**Figure 5.** Expression of *ACT* and *DTX18* in Transgenic Potato Plants.

**(A)** and **(B)** qRT-PCR analysis for *ACT* (black bars) or *DTX18* expression (gray bars) was performed with RNA from leaves of wild-type and transgenic potato plants carrying an empty vector construct (ev) or expressing *35S-ACT* (lines B, H, M, and Z) **(A)** or *35S-ACT-35S-DTX18* (lines D, K, N, and X) **(B)**. Expression of *EF1a* was used as a reference. Data are derived from two independent experiments (**[A]**: wild type, *n* = 7; empty vector, *n* = 5; B, M, H, and Z, *n* = 3; **[B]**: wild type, *n* = 8; empty vector, *n* = 6; D, K, N, and X, *n* = 3). Error bars represent sᴇ. nd, not detectable.
**(C)** Phenotype of *35S-ACT-35S-DTX18* potato plants.

line N (Figure 6D). Compared with the extracellular coumaroylagmatine levels in Arabidopsis, roughly 25-fold higher levels were obtained in *ACT-DTX18*-expressing potato plants. These observations suggest that expression of *DTX18* is required and sufficient for export of coumaroylagmatine.

Untargeted metabolite profiling revealed additional differences in the metabolite pattern of control, *ACT*, and *ACT-DTX18* plants in response to *P. infestans* infection. Among the differentially accumulating compounds (Supplemental Figure 2), other HCAAs were identified based on tandem mass spectrometry analyses (Supplemental Figure 3). In addition to coumaroylagmatine, *ACT*-expressing plants displayed more than 10-fold higher levels of coumaroylputrescine and coumaroylspermidine, as well as a 3-fold increase in feruloylspermidine and a 2-fold increase in caffeoylputrescine (Figure 7A). Despite the increased intracellular levels of these compounds, no changes in their extracellular abundance were observed (Figure 7B). Apparently, intracellular levels of these HCAAs are not limiting for their export. By contrast, when *DTX18* was expressed in addition to *ACT* in potato, the intra- and extracellular HCAA patterns changed dramatically. Intracellular levels of coumaroylagmatine, coumaroylputrescine, caffeoylputrescine, as well as coumaroyl- and feruloylspermidine were 10-fold to more than 300-fold higher than in *P. infestans*-infected control plants (Figure 8A). Importantly, *ACT-DTX18* plants secreted high amounts of

feruloyl- and caffeoylagmatine, in addition to coumaroylagmatine, as well as the respective putrescine conjugates into the inoculum (Figure 8B). Interestingly, despite 100- and 50-fold enhanced intracellular levels of coumaroyl- and feruloylspermidine, respectively (Figure 8A), these compounds were not detectable in the inoculum. Moreover, the extracellular abundance of coumaroyl- and feruloyltyramine was not enhanced in *ACT-DTX18*-expressing plants (Figure 8B). These results indicate that the transporter protein DTX18 selectively exports putrescine- and agmatine-conjugated hydroxycinnamic acids, but no tyramine nor spermidine HCAA derivatives.

### Secretion of HCAAs in Potato Contributes to Defense against *P. infestans*

The efficient secretion of the HCAAs in *ACT-DTX18* potato plants raised the question of whether these plants display enhanced resistance against *P. infestans*. Pathogen biomass was determined in infected leaves by qPCR with primers directed at highly repetitive sequences in the *P. infestans* genome (Judelson and Tooley, 2000). Significantly reduced growth of *P. infestans* compared with control plants was observed upon infection of three of the four *ACT-DTX18*-expressing potato lines, but not in any of the *ACT*-expressing plants (Figures 9A and 9B). Since the transgenic line with the highest level of extracellular
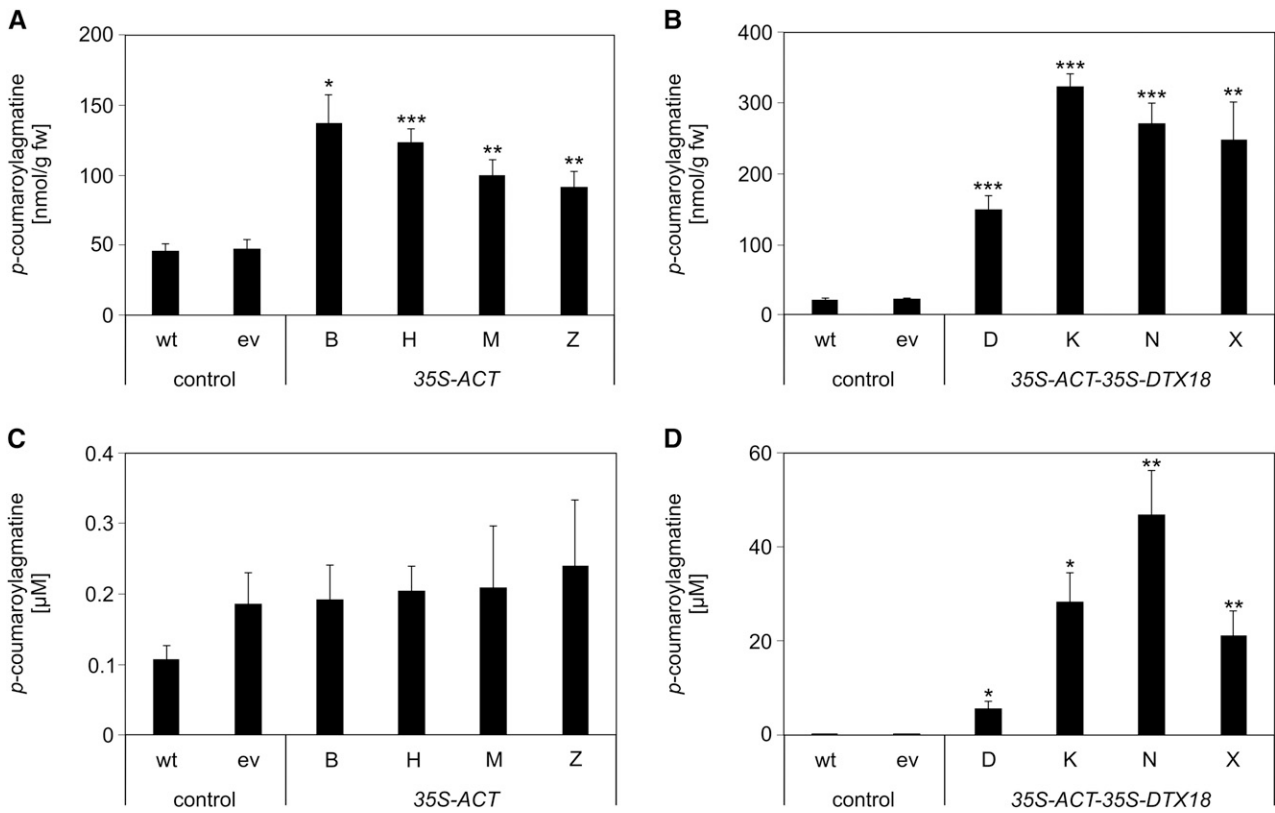
**Figure 6.** Characterization of *ACT*- and *ACT-DTX18*-Expressing Potato Plants.

Coumaroylagmatine levels were determined in leaves (**[A]** and **[B]**) and in droplets of the inoculum (**[C]** and **[D]**) from wild-type and transgenic potato plants carrying an empty vector construct (ev) or *35S-ACT* (lines B, H, M, and Z) (**[A]** and **[C]**) or *35S-ACT-35S-DTX18* (lines D, K, N, and X) (**[B]** and **[D]**). Significance analysis of differences was performed by *t* test, *P < 0.05, **P < 0.01, and ***P < 0.001.

**(A)** Two independent experiments; wild type, empty vector, *n* = 10; B, *n* = 5; H, M, and Z, *n* = 6.

**(B)** Three independent experiments; wild type, empty vector, *n* = 18; D2, *n* = 7; K3, *n* = 6; N3, *n* = 7; X2, *n* = 8.

**(C)** Two independent experiments; wild type, empty vector, *n* = 10; lines B, H, M, and Z, *n* = 6.

**(D)** Three independent experiments; wild type, empty vector, *n* = 18; D2, *n* = 7; K3, *n* = 5; N3, *n* = 6; X2, *n* = 7.

coumaroylagmatine did not show significantly reduced pathogen biomass (Figure 9B) and since *ACT-DTX18*-expressing plants displayed major changes in morphology compared with control and *ACT*-expressing plants (Figure 5C), reduction of pathogen biomass might be caused by secondary effects.

Therefore, to assess the importance of secretion of HCAAs, the inoculation droplets were collected from transgenic and control plants after 24 h, sterile filtrated, and incubated with a zoospore suspension of *P. infestans*. Microscopy analyses of spore germination revealed a slight, but highly significant, inhibitory effect of droplets collected from all four transgenic *ACT-DTX18*-expressing lines compared with those from wild-type and empty vector plants (Figure 9C). These observations point to a contribution of DTX18-mediated secretion of HCAAs to defense against *P. infestans*.

## DISCUSSION

This study has identified coumaroylagmatine as a secondary metabolite that accumulates extracellularly on Arabidopsis leaves in response to *P. infestans* inoculation. Loss- and gain-of-function experiments in Arabidopsis and potato, respectively, indicate that the MATE transporter DTX18 is required and sufficient for the export of the HCAA, coumaroylagmatine, to the leaf surface.

HCAAs are biosynthesized via the phenylpropanoid pathway and have been reported to act as antimicrobial compounds against bacteria, such as *Staphylococcus aureus* and *Streptococcus pyogenes* (Georgiev et al., 2013). Antifungal effects of different HCAAs were tested using *Saccharomyces cerevisiae*, *Trichoderma beigelii*, and *Candida albicans* (Lee et al., 2004), as well as mycorrhizal fungi (Grandmaison et al., 1993). A limited number of reports exists for the effect of HCAAs on phytopathogenic fungi, such as the cereal fungal pathogen *Fusarium culmorum* (Fattorusso et al., 1999), the causal agent of brown rot in stone fruit, *Monilinia fructicola* (Venis, 1969), and the Arabidopsis pathogen *Alternaria brassicicola* (Muroi et al., 2009). Interestingly, while coumaroylagmatine inhibited both spore germination and hyphal growth in *A. brassicicola* (Muroi et al., 2009), only spore germination was affected for *P. infestans* (Figures 1C and 1D).
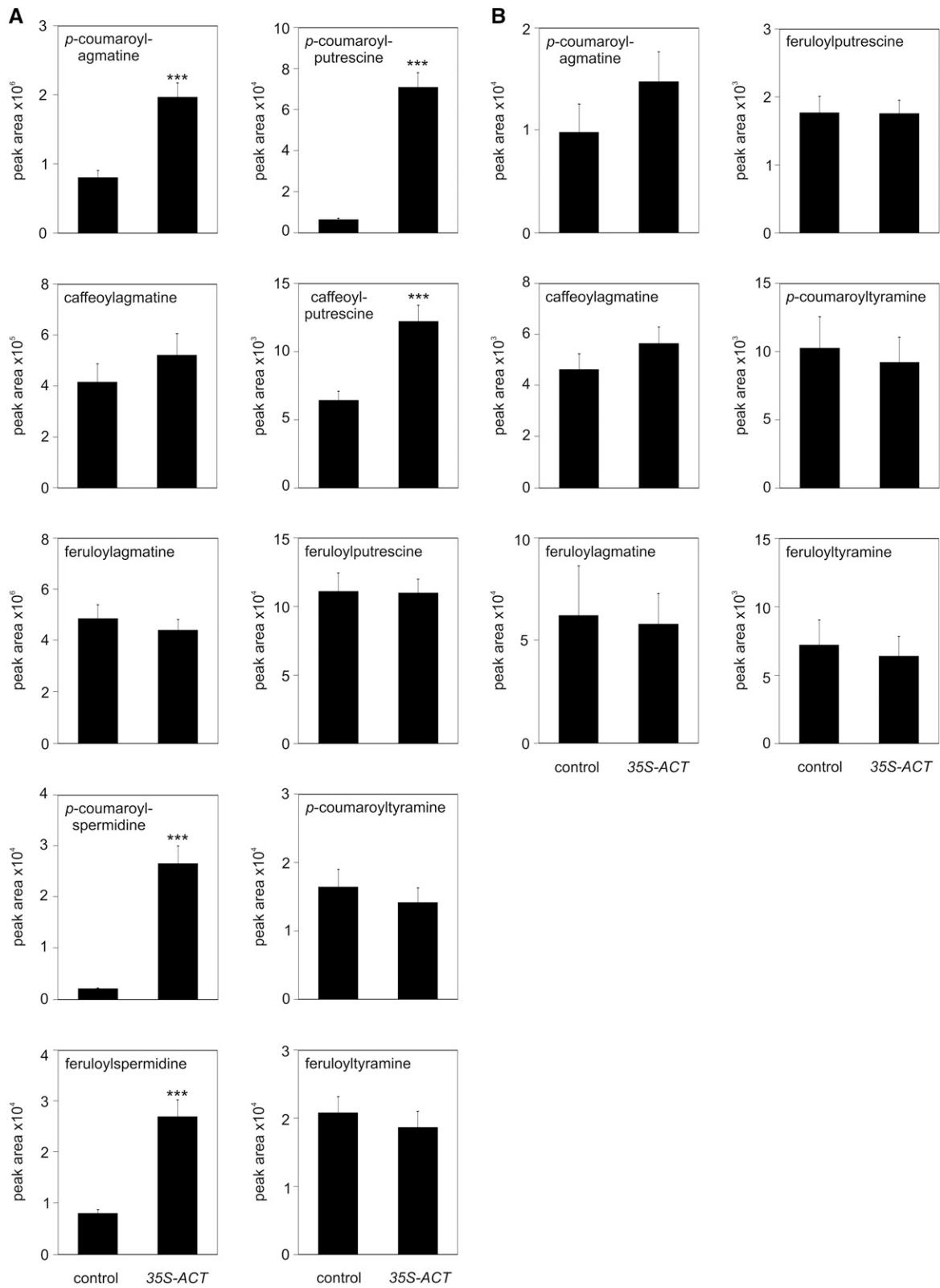
**Figure 7.** Expression of *ACT* Leads to Alterations in HCAA Patterns in *P. infestans*-Infected Potato Leaves.

HCAA levels were determined in leaves **(A)** and in the inoculum **(B)** 24 h after infection of leaves of control (wild-type and empty vector-carrying plants) or *35S-ACT* plants (lines B, H, M, and Z). Data are derived from three independent experiments (**[A]**: $n \geq 32$, except for spermidine derivatives, $n \geq 15$; **[B]**: $n \geq 32$, except for feruloyltyramine, $n \geq 20$, and caffeoylagmatine, $n \geq 8$). Significance analysis of differences was performed by *t* test, *P < 0.05, **P < 0.01, and ***P < 0.001.
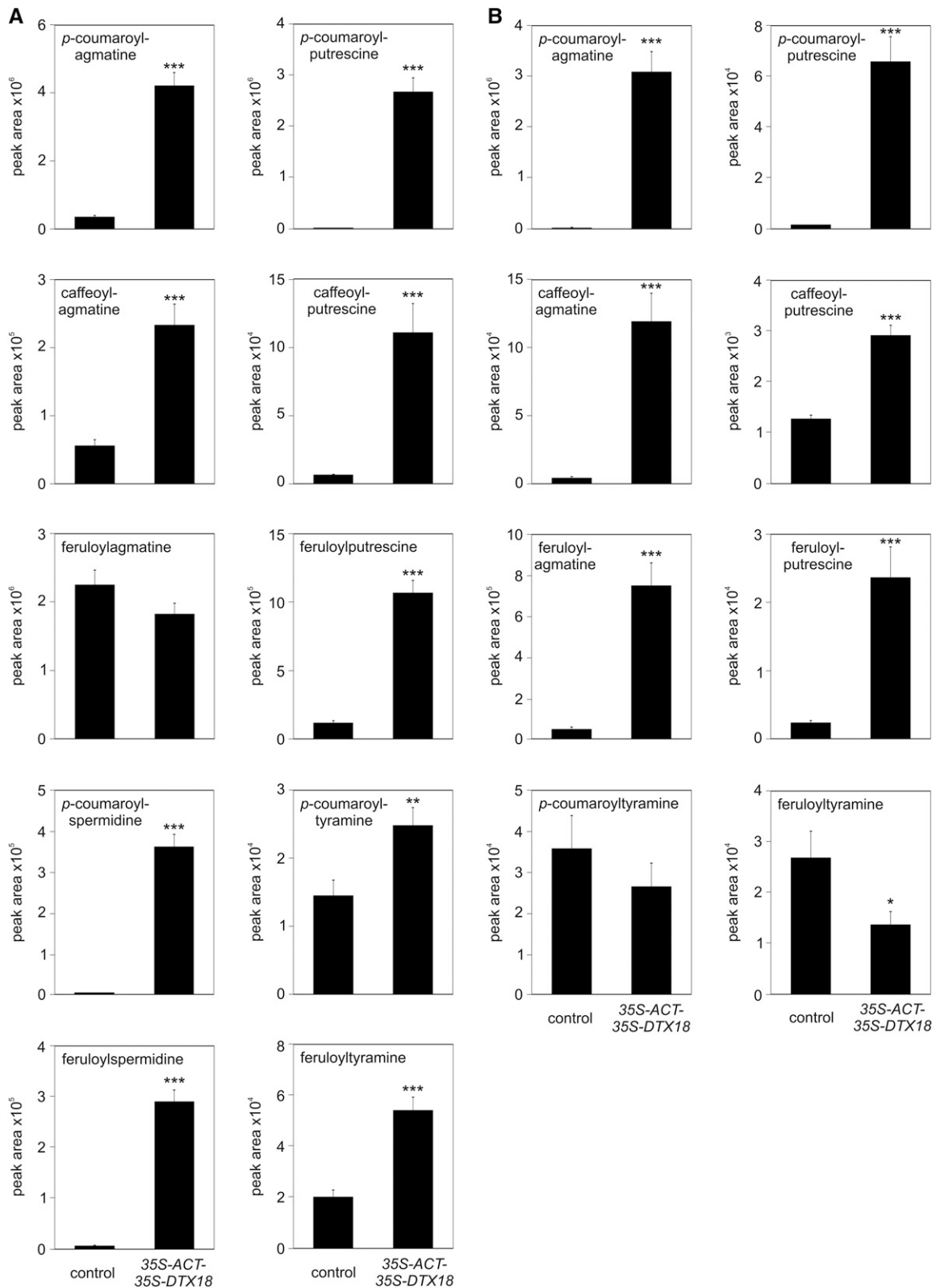
**Figure 8.** Expression of *ACT* and *DTX18* Leads to Extracellular Accumulation of HCAAs in *P. infestans*-Infected Potato Leaves.

HCAA levels were determined in leaves **(A)** and in the inoculum **(B)** 24 h after infection of leaves of control (wild-type and empty vector-carrying plants) or *35S-ACT-35S-DTX18*-expressing plants (lines D, K, N, and X). Data are derived from three independent experiments (**[A]**: *n* ≥ 44; **[B]**: *n* ≥ 36, except for feruloyltyramine and caffeoylputrescine, *n* ≥ 24). Significance analysis of differences was performed by *t* test, *P < 0.05, **P < 0.01, ***P < 0.001.

**A**



**B**



**C**



The finding that coumaroylagmatine inhibits spore germination, but not mycelial growth, of the oomycete *P. infestans* implies that an inhibitory effect of this compound requires its presence at early stages of the infection process and, thus, its occurrence on the plant surface. While this is the case for the nonhost plant Arabidopsis, potato is apparently not able to secrete coumaroylagmatine efficiently (Figure 1B). Other HCAAs, such as coumaroyl- and feruloyltyramine, accumulate extracellulary in elicitor-treated cultured potato cells (Schmidt et al., 1998), suggesting the existence of a functional export system for these HCAAs in potato. Apparently, coumaroylagmatine cannot be transported by this system, not even if intracellular levels are increased (Figures 6A and 6C). This suggests that the existing HCAA export system in potato has a distinct specificity for HCAAs containing tyramine but not agmatine (Figure 10).

Our results strongly suggest that DTX18 is a transporter for coumaroylagmatine. First, loss-of-function experiments revealed no extracellular accumulation of coumaroylagmatine in two independent Arabidopsis *dtx18* mutants, despite wild-type levels of coumaroylagmatine in leaf extracts (Figures 4D and 4E). Second, the inability of potato plants to efficiently export coumaroylagmatine is overcome by expression of *DTX18* in transgenic plants (Figure 6D). The extracellular coumaroylagmatine levels obtained in plants expressing both *ACT* and *DTX18* is more than 100-fold higher than those in transgenic lines expressing only *ACT*, and, interestingly, even higher than those in Arabidopsis (Figure 1B).

Due to the different HCAA repertoire of potato, additional potential substrates of DTX18 could be postulated. Untargeted metabolite profiling of *ACT-DTX18*-expressing plants revealed high levels of extracellular coumaroyl, feruloyl, and caffeoyl conjugates of agmatine and putrescine. By contrast, tyramine and spermidine conjugates apparently were not exported by DTX18, since their extracellular levels were not altered or not detectable in *DTX*-overexpressing plants, although higher intracellular abundances were observed. Therefore, it was possible to assign a specificity to DTX18 as a HCAA transporter of agmatine and putrescine conjugates. Alternatively, the increased export of coumaroylagmatine by DTX18 might induce other metabolic mechanisms like the activation of an endogenous transporter for putrescine conjugates. Since putrescine conjugates of hydroxycinnamic acids were not detectable in Arabidopsis, analysis of the *dtx18* mutant cannot clarify this issue. Similarly, whether DTX18 is able to transport sinapoyl derivatives cannot be
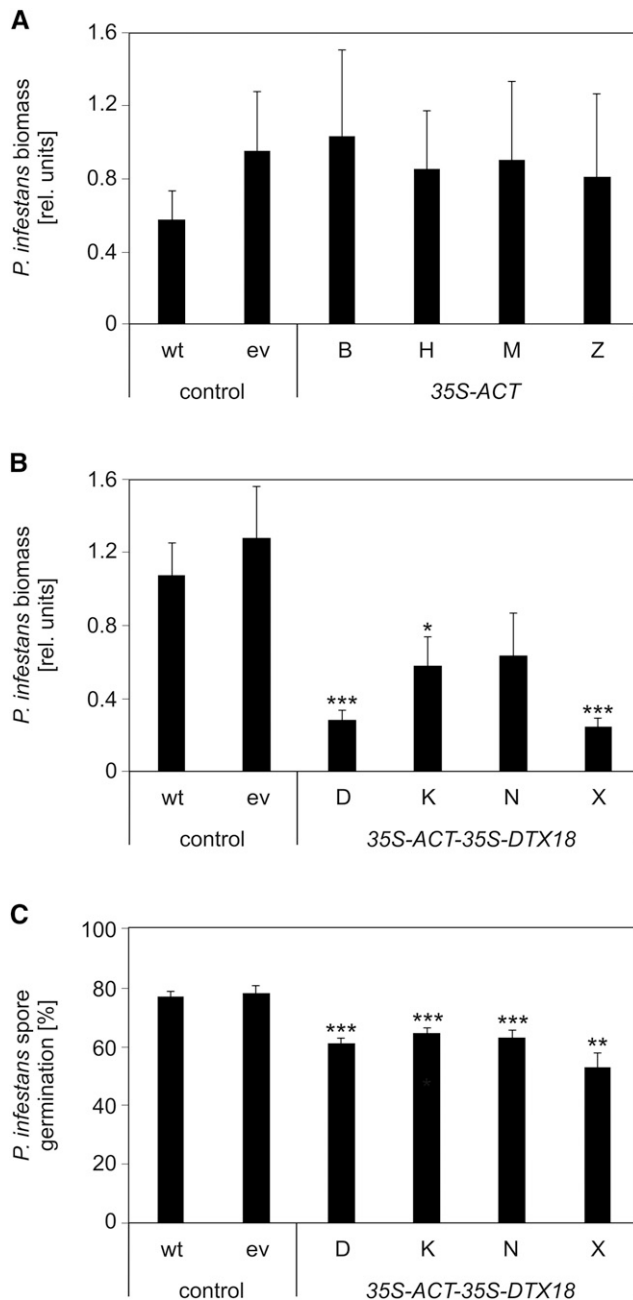
**Figure 9.** Defense of *ACT* and *ACT-DTX18*-Expressing Potato Plants against *P. Infestans*.

**(A)** and **(B)** Determination of pathogen biomass. Wild-type and transgenic potato plants carrying an empty vector construct (ev), *35S-ACT* (lines B, H, M, and Z) **(A)**, or *35S-ACT-35S-DTX18* (lines D, K, N, and X) **(B)** were inoculated with *P. infestans*. Pathogen biomass was determined 3 d after inoculation. Error bars represent SE. Significance analysis of differences was performed by *t* test, *P < 0.05 and ***P < 0.001. P values for comparison of the *ACT-DTX18*-expressing lines: D-K, 0.098; D-N, 0.17; D-X, 0.57; K-N, 0.84; K-X, 0.06; N-X, 0.13.
**(A)** Three independent experiments; wild type, empty vector, *n* = 24; B, H, M, and Z, *n* = 12.

**(B)** Five independent experiments; wild type, *n* = 35; empty vector, *n* = 43; D, *n* = 17; K, *n* = 13; N, *n* = 10; X, *n* = 18.
**(C)** Spore germination inhibition assay. *P. infestans* inoculum incubated on wild-type and transgenic potato plants carrying an empty vector construct (ev) or expressing *35S-ACT-35S-DTX18* (lines D, K, N, and X) were collected after 24 h, sterile filtrated, and added to a zoospore suspension of *P. infestans*. Inhibitory activity was determined by microscopy analyses. Data were obtained in three independent experiments (*n* = 9). At least 1300 spores were evaluated for each line. Significance analysis of differences was performed by *t* test, *P < 0.05, **P < 0.01, ***P < 0.001.
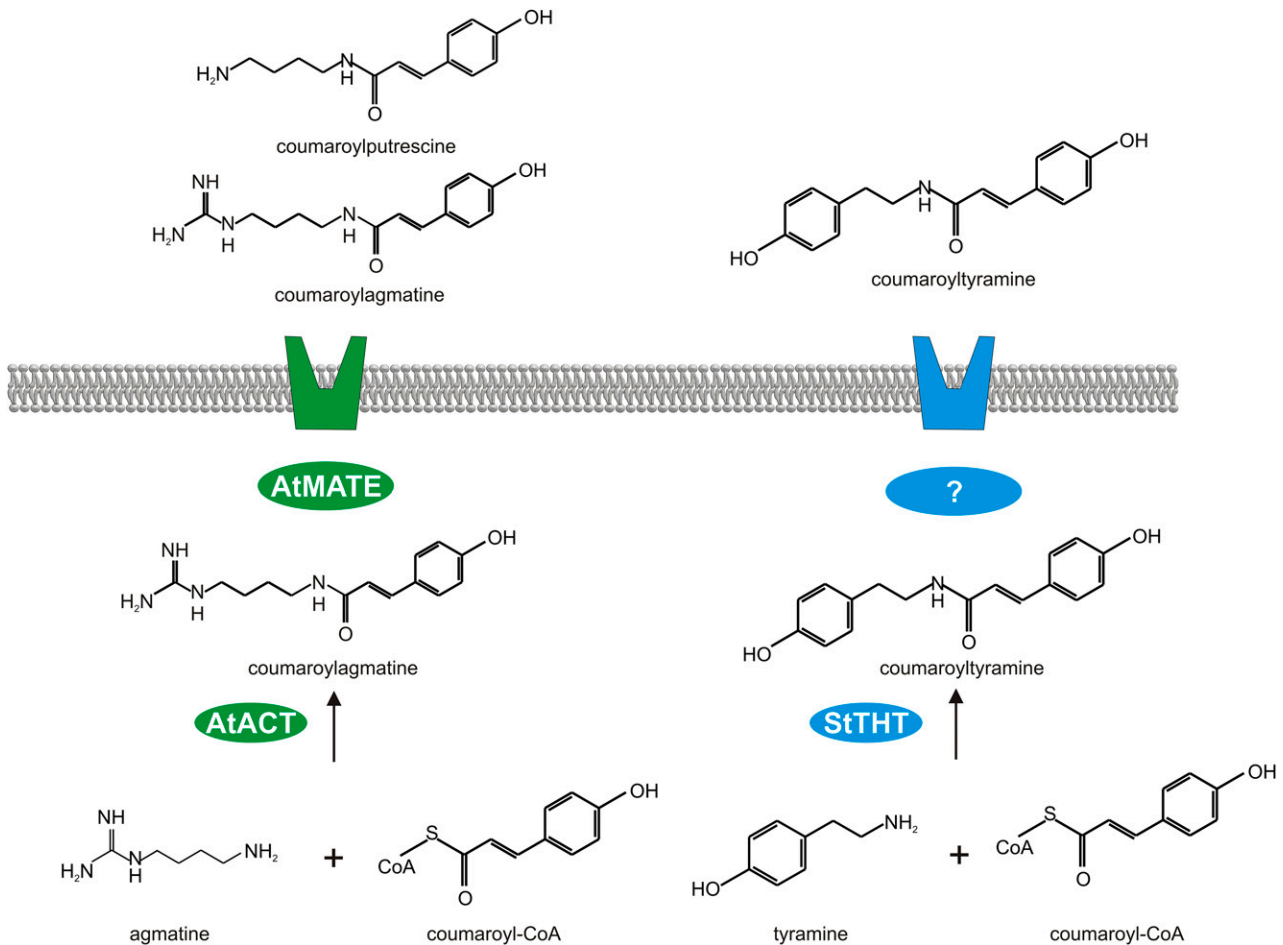
**Figure 10.** Hypothetical Model of DTX18-Mediated Coumaroylagmatine Secretion.

Potato responds to infection by *P. infestans* with the biosynthesis of coumaroyl- and feruloyltyramine by THT (Schmidt et al., 1998) and their export by an unknown transport system. The extracellular accumulation of novel HCAAs in transgenic potato plants expressing *DTX18* from Arabidopsis, predicted to encode a plasma membrane-localized MATE transporter, suggests that DTX18 is able to export coumaroyl, caffeoyl, and feruloyl conjugates of both agmatine and putrescine.

addressed here, since these compounds were not detectable in leaf extracts or the inoculum droplets.

Both the expression of the biosynthetic enzyme and the transporter resulted in major alterations in intracellular HCAA levels. Consistent with ACT's ability to take not only agmatine but also putrescine as an acceptor (Muroi et al., 2009), *ACT*-expressing potato plants contained higher levels of coumaroyl- and caffeoyl-putrescine. The enhanced presence of spermidine conjugates, moreover, suggests that ACT is also able to use this amine as an acceptor. By contrast, the expression of a maize (*Zea mays*) putrescine hydroxycinnamoyl transferase in rice (*Oryza sativa*; Wen et al., 2014) led to increased levels of agmatine and putrescine conjugates, as observed in *ACT*-expressing potato plants, but unchanged or even decreased amounts of spermidine conjugates.

Even more drastic changes in intracellular HCAA levels were observed in *ACT-DTX18*-expressing potato plants. Compared with *ACT*-expressing plants, nearly all analyzed HCAAs were present at higher levels in *ACT-DTX18* lines. The selective removal of specific

HCAAs from the intracellular metabolic system apparently leads to enhanced biosynthesis and accumulation of HCAAs in the cell. Most strikingly, coumaroyl- and feruloylspermidine levels in *ACT-DTX18*-expressing plants are 100-fold higher than in control plants, but this does not correlate with any export of these compounds. Feedback mechanisms might occur as a response to the depletion of agmatine and putrescine conjugates, resulting in the accumulation of spermidine derivatives, which are biosynthesized by either ACT or a yet unidentified enzyme.

Interestingly, the expression of the transporter, but not of the biosynthetic enzyme, leads to major morphological alterations in transgenic plants, since the phenotype of *ACT*-expressing plants does not differ from that of wild-type plants. Possibly, the export of HCAAs from the cell leads to a depletion of metabolites of the phenylpropanoid and other pathways, which are important for cell growth and development. Moreover, since HCAAs are incorporated into cell walls (Schmidt et al., 1998; Burhenne et al., 2003), distinct mechanical cell wall properties and expansion

flexibility might be affected, which could explain the smaller leaves of the transgenic plants. Alternatively, secondary effects such as membrane destabilization due to high DTX18 expression might be responsible for the alterations in plant morphology. Developmental defects have also been observed in Arabidopsis upon overexpression of other MATE transporters, such as Os-MATE1, Os-MATE2 (Tiwari et al., 2014), and ZRZ (Burko et al., 2011), indicating the potential importance of proper localization of secondary metabolites.

The secretion of antimicrobial secondary metabolites is considered to be of importance for control of pathogen entry. In Arabidopsis, currently unidentified indole derivatives, produced by the degradation of indole glucosinolates via the PEN2 pathway (Bednarek, 2012), are believed to be transported out of the cell by the ABC transporter PEN3. Lack of transport results in enhanced penetration rates of several filamentous pathogens, implying that the occurrence of these indolic compounds in the apoplast contributes to penetration resistance (Stein et al., 2006). Similarly, the *Nicotiana plumbaginifolia* ABC transporter Np-PDR1 has been suggested to be responsible for the secretion of the antifungal terpenoid sclareol (Jasiński et al., 2001). Transgenic plants with reduced Np-*PDR1* expression displayed an enhanced susceptibility to infection by *Botrytis cinerea* (Stukkens et al., 2005). In our study, we correlate the secretion of the antimicrobial compound coumaroylagmatine to significantly reduced growth of *P. infestans* in three out of four transgenic potato lines expressing Arabidopsis *ACT* and *DTX18* (Figure 9B). Moreover, all four lines secreted coumaroylagmatine to levels that were inhibitory to *P. infestans* spore germination in vitro (Figure 9C). These results suggest the importance of secreted secondary metabolites for pathogen entry control by inhibiting early pathogen development.

## METHODS

### Plant Growth Conditions and Infection Experiments

The *Arabidopsis thaliana* T-DNA insertion mutants SALK_097380, SALK_062231, and GK411D06 were obtained from NASC (Alonso et al., 2003; Kleinboelting et al., 2012). Arabidopsis plants were grown on steam-sterilized soil in a mix with vermiculite (3:1) in a phytochamber with 8 h light ($\sim$150 $\mu$E m$^{-2}$ s$^{-1}$) at 22°C and 16 h dark at 20°C. Plants were inoculated by placing 10 $\mu$L droplets of a *Phytophthora infestans* zoospore suspension ($5 \times 10^5$ mL$^{-1}$) onto the adaxial side of the leaves. Subsequently, plants were placed in a phytochamber with 16 h light ($\sim$150 $\mu$E m$^{-2}$ s$^{-1}$, metal halide lamp HQI-T; Osram) at 20°C and 8 h dark at 18°C. Potato plants (*Solanum tuberosum* cv Désirée) derived from sterile explants were grown in soil in a phytochamber with 16 h of light (140 $\mu$E) at 20°C and 60% humidity for 4 weeks. Plants were inoculated by placing 10-$\mu$L droplets of a *P. infestans* zoospore suspension ($10^5$ mL$^{-1}$) onto the abaxial side of the leaves. Leaves were subsequently covered with plastic bags to ensure high humidity.

### *P. infestans* Inhibition Assays

Spore germination and mycelial growth inhibition were assayed as described (Prost et al., 2005; Eschen-Lippold et al., 2009). Spore germination inhibition assays were performed at 15°C.

### Expression Analyses

RNA was isolated from potato plants or *P. infestans*-inoculated Arabidopsis plants as described previously (Halim et al., 2004). cDNA was synthesized after DNase I digestion (RNase-free DNase Set; Qiagen) using RevertAid H Minus first-strand cDNA synthesis kit (Thermo Fischer Scientific) according to the manufacturer's instructions. Quantitative PCR was performed with Maxima Probe qPCR MasterMix (Thermo Fischer Scientific) and analyzed with an Mx3000P qPCR system (Agilent). The following probes and primers were used for amplification: Roche Universal Probe Library probe #131 (5′-ACCACCAG-3′) for *ACT* (*At5g61160*) with the primers 5′-CGCTGCTGATTTTAGGAACC-3′ and 5′-CACAA-GTCCCGAAGTATGTCAC-3′; Roche Universal Probe Library probe #15 (5′-GAGCAGGA-3′) for *DTX18* (*At3g23550*) with the primers 5′-GGTAGCTATATGCGTCAACACG-3′ and 5′-CCTTCTTTGCGCCTTTTA-CA-3′; Roche Universal Probe Library probe #29 (5′-GGCAGAAG-3′) for the housekeeping gene *AtPP2A* A3 (*At1g13320*) with the primers 5′-GACCGGAGCCAACTAGGAC-3′ and 5′-AAAACTTGGTAACTTTTC-CAGCA-3′; and Roche Universal Probe Library probe #162 for potato *EF1α* with the primers 5′-CACTGCCCAGGTCATCATC-3′ and 5′-GTCGAGCACTGGTGCATATC-3′. Relative transcript levels were determined using MxPro qPCR software (Agilent).

### Expression of ACT in *Escherichia coli* and Enzyme Activity Assays

The coding region of *ACT* was amplified from Col-0 genomic DNA using the primers 5′-CACCATGGCCGTTAAAGGTGATCAAG-3′ and 5′-GACCGTACGTTTTCAGTTTCCAATC-3′, cloned into pENTR, and subsequently transferred to the vector pDEST17 by LR recombination (Life Technologies). Recombinant protein was produced after induction with isopropyl β-D-1-thiogalactoside and purified from extracts of *pDEST17-ACT*-carrying Rosetta gami cells (Novagen) via HisTrap HP columns (GE Healthcare) with the ÄKTA explorer 100 protein chromatography system (GE Healthcare) according to the manufacturer's instructions. ACT activity assays and kinetic measurements were performed in a standard assay reaction mixture containing 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 5 mM substrate (acceptor), 2 $\mu$g purified recombinant enzyme, and 40 $\mu$M CoA donor. The assay was incubated for 30 min at 30°C in the dark and the reaction stopped by adding 52.5 $\mu$L 50% trichloroacetic acid. For each substrate concentration, three samples were analyzed in parallel. Substrate affinities ($K_m$) and velocities ($V_{max}$) were calculated using SigmaPlot 10 (Systat Software).

### Liquid Chromatography-Mass Spectrometry Measurements

Arabidopsis and potato plants were inoculated with 30 droplets of 10 $\mu$L each of a *P. infestans* zoospore suspension ($5 \times 10^5$ and $1 \times 10^5$ spores mL$^{-1}$, respectively). After 24 h, droplets were removed, pooled, and immediately frozen in liquid nitrogen. Droplet samples were thawed and centrifuged for 10 min at 21,000g at 4°C and the supernatants were directly transferred to HPLC vials. Methanolic extracts were prepared from inoculated leaves of Arabidopsis or from potato leaf disks covering the inoculation sites as described (Böttcher et al., 2009).

Chromatographic separations were performed as described (Landgraf et al., 2014) with the following modifications: The binary gradient was applied at a flow rate of 150 $\mu$L/min for 0 to 1 min, isocratic 95% A (water/formic acid, 99.9/0.1 [v/v]), 5% B (acetonitrile/formic acid, 99.9/0.1 [v/v]); 1 to 5 min, linear from 5 to 25% B; 5 to 10 min, linear to 95% B; 10 to 12 min, isocratic 95% B; 12 to 15 min, isocratic 5% B.

Eluting compounds were detected from *m/z* 80 to 1000 using a micrOTOF-Q II hybrid quadrupole time-of-flight mass spectrometer (Bruker Daltonics) equipped with an Apollo II electrospray ion source in positive and negative ion mode using the following instrument settings: nebulizer gas, nitrogen, 1.4 bar; dry gas, nitrogen, 6 L min$^{-1}$, 190°C; capillary, –5000 V; end plate offset, −500 V; funnel 1 RF, 200 V; funnel 2 RF, 200 V; in-source CID energy, 0 V; hexapole RF, 100 V; quadrupole ion energy, 5 eV; collision gas, nitrogen; collision energy, 7 eV; collision RF 150/350 V (timing 50/50); transfer time, 70 $\mu$s; prepulse storage, 5 $\mu$s; pulser frequency, 10 kHz;

spectra rate, 3 Hz. Mass spectra were acquired in centroid mode. Mass calibration of individual raw data files was performed on lithium formate cluster ions obtained by automatic infusion of 20 μL 10 mM lithium hydroxide in isopropanol/water/formic acid, 49.9/49.9/0.2 (v/v/v) at a gradient time of 12 min using a diverter valve.

Raw data files were converted to mzData format using the vendor-specific software CompassXport (http://www.bdal.de/) and processed using the XCMS package (http://bioconductor.org/packages/release/bioc/html/xcms.html). XCMS settings for processing liquid chromatography-mass spectrometry data with findPeaks.centWave() were: prefilter = (3200); snthr = 5; ppm = 25; peak width = (5,12); scan range (50, 1020). For alignment group density function with parameters minfrac = 0.75 and bw = 5, mzwid = 0.05, max = 20 was used.

Identification of the compounds was based on manual interpretation of tandem mass spectra. For comparison with different mass spectra databases, the open source metabolite identification software MetFrag (http://msbi.ipb-halle.de/MetFrag/MetFragIPB.iface) was used. A synthetic standard of *p*-coumaroylagmatine was used for identification and quantification.

## Generation of *35S-ACT* and *35S-ACT-35S-DTX18* Transgenic Potato Plants

The coding regions of *ACT* and *DTX18* (including the first intron) were cloned into the binary vector pICH52292 via Golden Gate Cloning (Weber et al., 2011). Potato plants were transformed and analyzed by DNA gel blots as described (Feltkamp et al., 1995; Schmidt et al., 1999).

## Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: At5g61160 (*ACT*) and At3g23550 (*DTX18*).

## Supplemental Data

**Supplemental Figure 1.** Purification of Recombinant ACT.

**Supplemental Figure 2.** HPLC Chromatograms of Leaves and Droplets of Control and *ACT*- and *ACT-DTX18*-Expressing Potato Plants.

**Supplemental Figure 3.** MS/MS of Hydroxyl Cinnamic Acid Derivatives.

## AUTHOR CONTRIBUTIONS

M.D., T.L., L.E.-L., E.B., and A.M. performed research. S.M., C.B., and K.G. analyzed data. B.D. and S.R. designed research. S.R. wrote the article.

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