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Acquired immunity of transgenic torenia plants overexpressing agmatine coumaroyltransferase to pathogens and herbivore pests

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We investigated the ability of transgenic torenia (Scrophulariaceae) plants to resist fungi and arthropod herbivores. *Torenia hybrida* cv. Summerwave Blue was manipulated to produce Arabidopsis agmatine coumaroyltransferase (AtACT). This catalyses the last step in the biosynthesis of hydroxycinnamic acid amides (HCAAs) which function in defence. Transgenic plants accumulated substantial HCAAs, predominantly *p*-coumaroylagmatine, and the HCAAs were isomerized from the *trans*-form to the *cis*-form *in planta*. The transgenic line, accumulated the highest amount of endogenous HCAAs (CouAgm at 32.2 μM and total HCAAs at 47.5 μM) and this line was resistant to the necrotrophic fungus, *Botrytis cinerea*. There was no resistance, however, in their wild-type progenitors or in other transgenic lines accumulating low HCAA amounts. In contrast, the transformants were not significantly resistant to three representative herbivores, *Frankliniella occidentalis*, *Aphis gossypii*, and *Tetranychus ludeni*.

Hydroxycinnamic acid amides (HCAAs) are antifungal substances, some of which have been shown to serve as phytoalexins synthesized *de novo* by plants in response to infection by incompatible pathogens[1]. HCAAs have been observed in several plant species, including members of the Poaceae[1], Solanaceae[2] and Brassicaceae[3]. They play roles in acquired immunity by suppressing the elongation of hyphae[4–6] and limiting the entry of pathogens by being deposited in cell walls causing physical blockage or increasing leaf toughness[2,7,8]. HCAA-derived polymers have been reported as components of suberin (a highly hydrophobic substance that prevents water from penetrating the tissue) that has accumulated in mechanically wounded potato tubers[9].

HCAAs are formed by the condensation of various biogenic amines with hydroxycinnamic acids derived from phenylalanine via the phenylpropanoid pathway. The genes involved in HCAA biosynthesis was first isolated from elicited cell cultures of carnation as anthranilate *N*-hydroxycinnamoyl/benzoyltransferase (HCBT), which catalyses the condensation reaction between hydroxycinnamoyl-/benzoyl-CoA and anthranilate to form dianthramides[10]. *In vitro*, benzoyltransferase shows narrow substrate specificity for anthranilate but accepts a variety of aromatic acyl-CoAs[10]. Agmatine coumaroyltransferase (ACT), which catalyses the biosynthesis of antifungal hydroxycinnamoylagmatine derivatives, was isolated from etiolated barley seedlings[11]. Both native and recombinant ACT was highly specific for agmatine as an acyl acceptor and had the highest specificity for *p*-coumaroyl-CoA among various acyl donors[11]. ACT is classified in the BAHD acyltransferase family[12] and is predicted to locate in the cytosol[11]. It was recently isolated from Arabidopsis, in which it mainly formed *p*-coumaroylagmatine (CouAgm) (81.2% of total product) and minor amounts of other HCAA products (feruloylagmatine [FerAgm, 3.7%], *p*-coumaroylputrescine [CouPtr, 14.6%] and feruloylputrescine [FerPtr, 0.5%])[3] (**Fig. 1**). An *AtACT* T-DNA insertion line was deficient in accumulation of all the above HCAA products and was much more susceptible to *Alternaria brassicicola* infection than wild-type (WT) plants[3].

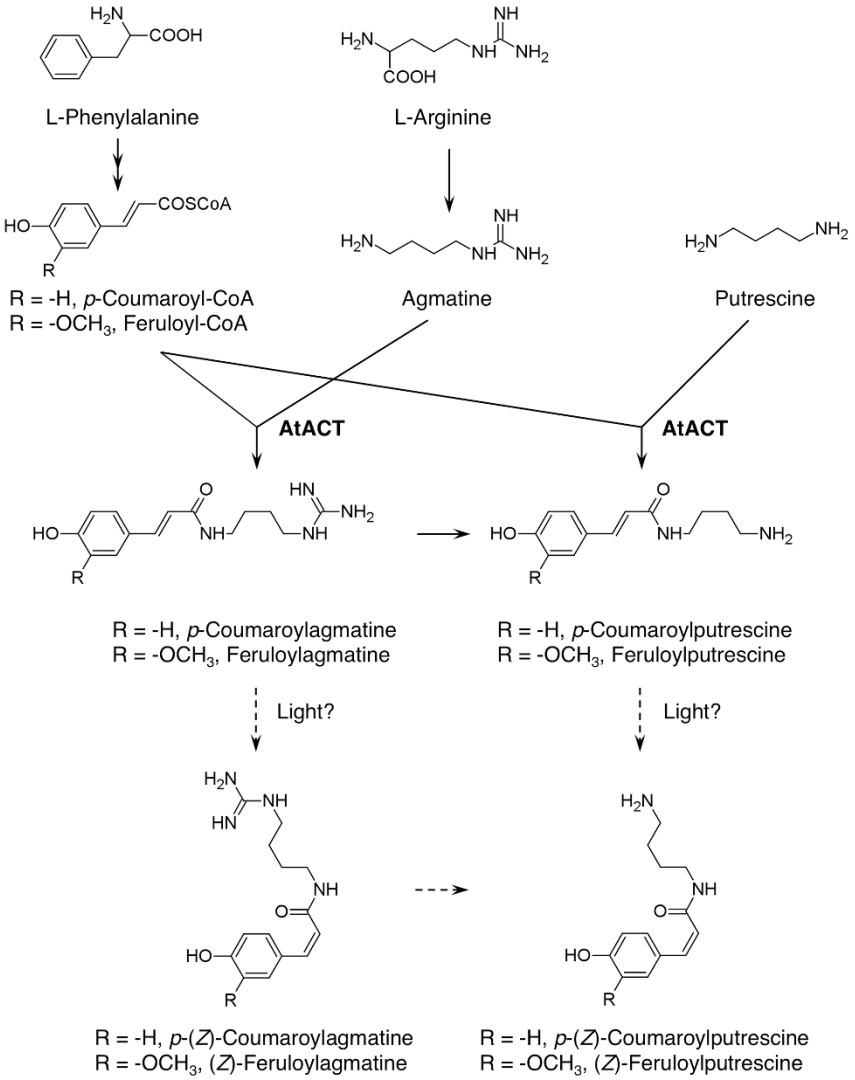

Figure 1 | **Pathways of HCAA biosynthesis.** The catalytic function of Arabidopsis agmatine coumaroyltransferase (AtACT) is shown.

Torenia is an annual or perennia plant in the Scrophulariaceae, and is grown as an ornamental summer bedding plant. Hybrids have been produced in the last few decades, resulting in various floral colours ranging from white with yellow throats to blue, cobalt, lavender and violet. Torenia is also an experimental plant with several useful characteristics, such as ease of genetic transformation, differentiability of adventitious structures, applicability for *in vitro* fertilization, and small genome size, comparable with that of Arabidopsis[13]. Since a simple and efficient transformation system has been established for torenia, various transformation studies targeting modification of ornamental characteristics have been conducted[13]. To assess the ability of HCAA compounds to control pests and diseases of torenia, we produced transgenic plants which constitutively express the *AtACT* gene and tested their defences against pathogens and arthropod herbivores.

Results

Generation of transgenic torenia plants producing HCAAs constitutively. Results of four representative lines (called ThACT) are presented (**Fig. 2**). CouAgm accumulated in leaves at between 2.6 (ThACT2) and 32.2 nmol g^{-1} FW (ThACT4), whereas WT and GUS-transgenic control plants showed no detectable accumulation. The other HCAA compounds were accumulated in only trace amounts, peaking in ThACT4 at 8.3 nmol CouPtr g^{-1} FW (17.6% of the total HCAA product), 6.8 nmol FerAgm g^{-1} FW (14.3%) and 0.2 nmol FerPtr g^{-1} FW (0.4%). The *cis*-isomers of all the HCAAs in the transgenic torenia leaves were present in much higher amounts than those from the native AtACT protein and from recombinant protein produced in *Escherichia coli* and fungus-infected Arabidopsis leaves[3]. The *trans*-isomers of phenylpropanoid products are likely *cis*-isomerized by light[14].

All the ThACT lines exhibited trans-gene (*AtACT*) expression in the leave, whereas WT and GUS-transgenic control plants did not (**SI, Supp. Fig. 1**).

Immunity to fungi. ThACT4 showed resistance to *Botrytis cinerea*, with clearly smaller lesions than on WT and the GUS-transgenic control leaves 48 h after infection ($F = 3.710$, $df = 5$, $P < 0.01$, ANOVA followed by Tukey-Kramer HSD test, $P < 0.05$; **Fig. 3a**). However, the three other lines were not significantly more resistant than WT and the GUS-transgenic control leaves, although they had smaller lesions than did the WT and the GUS-transgenic control leaves ($P > 0.05$). Higher concentrations of CouAgm (180 and 1800 μM) significantly inhibited the germination and development of conidial germ tubes (0 *vs* 180 μM or 1800 μM; χ^2-test weighted by the Bonferroni correction ($df = 1, P < 0.05/n = 0.008, n = 6$: the number for all combinations of the 4 groups; **Fig. 3b**). But 36 μM, which approximated the endogenous level in ThACT4, was

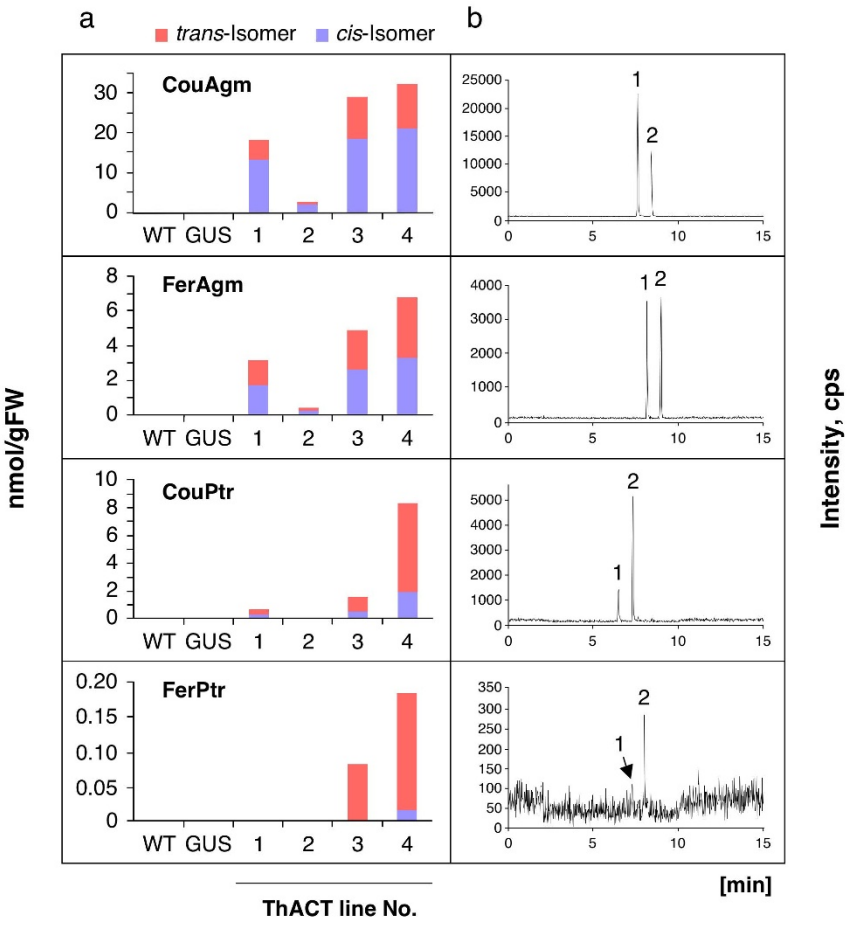

Figure 2 | Accumulation of HCAAs in the leaves of wild-type (WT), GUS-transgenic control and ThACT plants. (a) Means of *trans*- and *cis*-isomers of HCAA products in leaves ($n = 4$). (b) Representative HPLC-MS/MS profiles of HCAAs. Peaks 1 and 2 correspond to *trans*- and *cis*-isomers. The monitored mass transitions (Q1/Q3) were *m/z* 277.2/147.2 for CouAgm, 307.2/177.2 for FerAgm, 235.2/147.2 for CouPtr, and 265.2/177.2 for FerPtr.

ineffective (0 *vs* 36 μM; χ^2-test, $df = 1$, $P = 0.106$; **Fig. 3b**). Nevertheless, there seemed to be fewer well grown germ tubes (>21 μm) than in the control.

Resistance to arthropod herbivores. All three herbivores survived and reproduced equally well on WT and on the lines, ThACT3 and ThACT4 (*Frankliniella occidentalis* survival: GLM-test, $\chi^2 = 2.697$, $df = 2$, $P = 0.26$, **Fig. 4a**; oviposition: ANOVA, $F = 0.663$, $df = 2$, $P = 0.518$, **Fig. 4b**; *Aphis gossypii* survival: $\chi^2 = 2.604$, $df = 2$, $P = 0.272$; nymphs: $F = 0.119$, $df = 2$, $P = 0.888$; *Tetranychus ludeni* survival: GLM-test, $\chi^2 = 3.245$, $df = 2$, $P = 0.197$, see **SI, Supp. Fig. 2a**; oviposition: $F = 0.663$, $df = 2$, $P = 0.518$, see **SI, Supp. Fig. 2b**).

Discussion

Botrytis is a common fungal disease of torenia leaves[15]. The constitutive accumulation of HCAAs in ThACT4 leaves (CouAgm at 32.2 μM; total HCAAs at 47.5 μM) suppressed *B. cinerea* lesion development, and the addition of extra CouAgm showed a dose effect at 180 and 1800 μM (**Fig. 3b**). Similar levels were detrimental to *Monilinia fructicola*, in which 200 μM CouAgm decreased the germination of spores by 24%, and 410 μM by 78%[16]. Moreover, CouAgm at >1000 μM inhibited the germination of spores and the elongation of hyphae of *Alternaria brassicicola*[3]. It is noteworthy that the endogenously produced CouAgm in ThACT4 was much less but nevertheless conferred tolerance to *B. cinerea* (**Figs. 2a and 3a**). Arabidopsis leaves inoculated with *A. brassicicola* accumulated CouAgm at 85 nmol g^{-1} FW after 3 days, but the same level applied exogenously was not sufficiently anti-fungal[3]. The difference might be explained by potential synergism with other HCAAs that accumulate, as seen with resveratrol, a phytoalexin with antioxidant activities, and curcumin, another phytochemical antioxidant, which showed great synergistic potential in antioxidant activity[17]. Thus, low levels of CouAgm in leaves may control fungi more effectively in the presence of synergists. In addition, since HCAAs, including CouAgm, have been found to be localized in cell wall fractions in various plant species[3,18,19], a rich store of HCAAs in cell walls might be detrimental to fungal development.

Although ThACT3 leaves predominantly accumulated CouAgm at 29.9 μM, an amount comparable to that in ThACT4 (**Fig. 2a**), they were not clearly resistant to the fungi (**Fig. 3a**). This was probably due to an insufficient, below threshold, accumulation of endogenous total HCAAs, albeit a sufficient accumulation of CouAgm, in ThACT3 leaves. CouPtr was clearly accumulated much less in ThACT3 than in ThACT4 (**Fig. 2a**). It should be assessed, therefore, whether these HCAA products, except CouAgm, sustain anti-fungal activity.

In contrast to their anti-fungal activity, the HCAAs in the transgenic plants had no significant effects on the arthropod herbivores. Little is known about the effects of HCAAs on arthropod herbivores. However, HCAAs of tyramine and dopamine[20] and phenolic acid derivatives and HCAAs of serotonin dimers[21,22] have shown notable antioxidant activity. Since these HCAAs decreased membrane lipid peroxidation levels and induced plant defence responses[23,24], we expected that the HCAAs in the ThACT lines would affect the

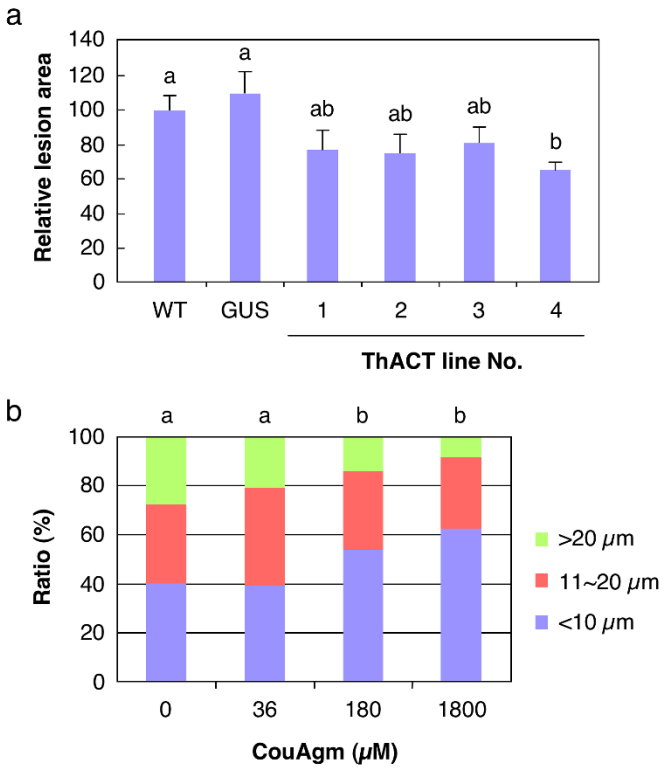

Figure 3 | Inhibitory effects of HCAAs on *B. cinerea* development. (a) *B. cinerea* lesions on the leaves of wild-type (WT), GUS-transgenic control and ThACT plants 48 h after inoculation. Means (± > + SE) of relative lesion areas with different letters are significantly different ($n = 47-77$; ANOVA followed by Tukey-Kramer HSD test, $P < 0.05$). (b) Dose effect of exogenous CouAgm on germination of *B. cinerea* conidia. The conidia were grouped by germ tube length (0–10, 11–20, and >21 μm) to evaluate how well they grew. Means with different lower-case letters are significantly different ($n = 222-302$, χ^2-test weighted by the Bonferroni correction, $df = 1$, $P < 0.05/n = 0.008$, $n = 6$: the number for all combinations of the 4 groups).

herbivores. Instead, the transgenic plants had no detrimental effects on *F. occidentalis*, *A. gossypii* or *T. ludeni* (**Fig. 4** and see **SI, Supp. Fig. 2**). Nevertheless, the HCAAs might affect bacterial and fungal pathogens transmitted via *F. occidentalis* and *A. gossypii*[25,26], indirectly affecting these herbivores.

Remarkable advances in torenia research in recent years have made this garden plant notable as a model plant for genetic engineering aimed at studying ornamentation[27,28] and pest control[29]. Future trials using transgenic torenia plants will aim at providing new insights into plant engineering, pest control and ecology.

Methods

Generation of transgenic torenia plants. We generated transgenic plants that constitutively biosynthesize HCAAs. We prepared gene constructs encoding Arabidopsis agmatine coumaroyltransferase (AtACT), which catalyses the last step in the biosynthesis of the HCAAs (Fig. 1), downstream of the constitutive 35S cauliflower mosaic virus (CaMV) promoter, and generated a set of independent transgenic torenia lines.

The full-length coding region of *AtACT* (At5g61160) was inserted into the *GFP* reporter gene site of the binary vector pSMABR35SsGFP, in which the selectable marker gene *bar* was replaced by the kanamycin resistance gene *nptII*[30]. The resulting plasmid, pSKAN-*AtACT*, was transformed into *Agrobacterium tumefaciens* strain EHA101 by electroporation. Torenia was transformed as described[31]. The *A. tumefaciens* strain carrying the binary plasmid pBI121 (containing the *uidA* gene coding for β-glucuronidase [GUS]), was transformed as a control. In brief, leaf fragments of *in vitro*–grown torenia plants (*Torenia hybrida* = *T. fournieri* × *T. concolor* cv. Summerwave Blue) were cocultured with *Agrobacterium* for 7 days at 22°C in the dark. Kanamycin-resistant shoots were regenerated by culturing on medium containing 300 mg L^{-1} kanamycin at 25°C under a 16-h photoperiod. Since torenia is a heterozygous plant developed by a combination of extensive hybridization and mutation breeding, it is difficult to obtain homozygous lines[13]. So the resulting transgenic plants (T_0) were maintained *in vitro* by subculturing every month until use, under sterile conditions in a plant box containing 1/2 Murashige and Skoog medium[32] supplemented with 3% sucrose and 0.2% gellan gum in a growth chamber at 25°C (160 μE m^{-2} s^{-1} with a 16-h photoperiod).

Plants and pests. Plants were maintained under sterile conditions as above. To prepare potted plants, we rooted herbaceous cuttings in soil, one to a plastic pot, in a climate-controlled room (25°C, 80 μE m^{-2} s^{-1}, 16-h photoperiod) for 3 to 5 weeks until use.

For inoculation with *B. cinerea* (strain *IuRy-1*), a suspension of conidia (5 μL, 10^5 mL^{-1} in 2.5% glucose) was placed on the center of upper surface of a leaf, and the plants were covered with a plastic sheet to hold the humidity. The plants were placed in the dark at 25°C for 48 h. Leaf lesions <1.0 mm^2 were not included in the analysis as these were caused by unsuccessful colonization.

Western flower thrips (*F. occidentalis*) were maintained on both pollen (of *C. sinensis* > *Camellia sinensis*) and seeds (of *Vicia faba*) in rearing cages in a climate-controlled room (22°C, 60 ± 10% relative humidity [RH], 16-h photoperiod)[33]. Female adults 3–7 days after emergence were used for experiments. Cotton aphids (*Aphis gossypii* >*A. gossypii*) were reared on eggplants (*Solanum melongena* cv. Senryo-nigou) in a climate-controlled room (15 > 22°C, 60 ± 10% RH, 13 > 16-h photoperiod). Female adults 3–5 days after emergence were used for experiments. Spider mites (*T. ludeni*) were reared on kidney bean plants (*Phaseolus vulgaris* cv. Nagauzuramame) in a climate-controlled room (22°C, 60 ± 10% RH, 16-h photoperiod). Adult female mites 3–7 days after the final moulting were used for experiments.

Chemical analysis. A piece of torenia leaf (25–50 mg fresh weight) was extracted with 10 volumes of absolute methanol at 25°C for 24 h. HCAAs were analysed by high-performance liquid chromatography (HPLC) – tandem mass spectrometry on an API 3000 chromatograph (Applied Biosystems, Foster City, CA, USA) in multiple reaction monitoring mode with a solvent gradient of 0.01% trifluoroacetic acid (TFA)–acetonitrile (95:5, v/v) at 0 min to 0.01% TFA–acetonitrile (70:30, v/v) at 10 min (column, Mightysil RP-18 GP 3 μm [Kanto Chemical, Tokyo, Japan], 2 mm × 100 mm; flow rate, 0.2 mL min^{-1}; temperature, 40°C). The monitored mass transitions (Q1/Q3) were *m/z* 277.2/147.2 for CouAgm, 307.2/177.2 for FerAgm, 235.2/147.2 for CouPtr, and 265.2/177.2 for FerPtr.

Quantitative reverse transcription (RT)-PCR. Total RNA was isolated from leaf tissues using a Qiagen RNeasy Plant Mini Kit and an RNase-Free DNase Set (Qiagen > Qiagen, Hilden, Germany) following the manufacturer's protocol. First-strand cDNA was synthesized using a PrimeScript RT reagent Kit (Takara, Japan > Takara, Otsu, Japan), and 0.5 μg of total RNA at 37°C for 15 min. Real-time PCR was carried out on an Applied Biosystems 7300 Real-Time PCR System using Power SYBR Green Master Mix (Applied Biosystems), cDNA (1 μl from 10 μl of each RT product pool), and 300 nM primers. The following protocol was followed: initial polymerase activation 2 min at 50°C, and 10 min at 95°C, 40 cycles of 15 s at 95°C, and 60 s at 60°C. PCR conditions were chosen by comparing threshold values in a dilution series of the RT product, followed by non-RT template control and non-template control for each primer pair. Relative RNA levels were calibrated and normalized with the level of *ACT3* (AB330989) mRNA. The primers used were as follows: *AtACT* (5′-TCGGGACTTGTGTCTTAGCC-3′ and 5′-CCCGTTTACGACCAACAACT-3′) and *ACT3* (5′-CAACTGCAGAGCGTGAAATC-3′ and 5′-ATCATCGATGGCT-GGAAAG-3′).

Effects of CouAgm on germination of *B. cinerea* conidia. *B. cinerea* grown on potato sucrose agar was exposed to black light (Panasonic FL10BL-B [10W]) for 1 week to promote the formation of conidia. Conidia were collected in sterilized water, filtered through one layer of facial tissue, centrifuged at 500 ×*g* for 3 min, and washed twice with sterilized water. The conidia were suspended at 2.6 × 10^6 mL^{-1} in malt extract broth containing 0.1% agar and a known concentration of CouAgm. The suspension (0.1 mL) was spread on an agar-coated plastic plate (1.1 cm^2, 1.1 mm thickness), and incubated at 25°C for 4 h in dark at 100% RH. Germination was observed on three independent plates.

Effects on resistance to arthropod herbivores. Leaf discs 14 mm in diameter, cut with a biopsy punch, were placed upside down on agar (2.7%), one per well of a 24-well plate (Nalge Nunc International, Tokyo, Japan).

An adult female of *F. occidentalis* was introduced onto each leaf disc on a fine paintbrush. Each plate was covered with a micro-plate seal (UC-500; Ina Optica, Osaka, Japan), in which tiny holes for air circulation had been made with a fine injection needle, and placed in a climate-controlled room (25°C, 60% ± 10% RH, 16-h photoperiod). After 3 days, survivors and eggs were counted under a binocular microscope (MZ160 microscope with TL5000 Ergo light base with automatic aperture; Leica, Tokyo, Japan). We analysed 44 independent leaf discs from each transgenic line and WT plants.

Adult females of *A. gossypii* were similarly introduced. After 3 days' incubation as above, the survivors and nymphs were counted under the microscope. We analysed 40 independent leaf discs from each transgenic line and WT plants.

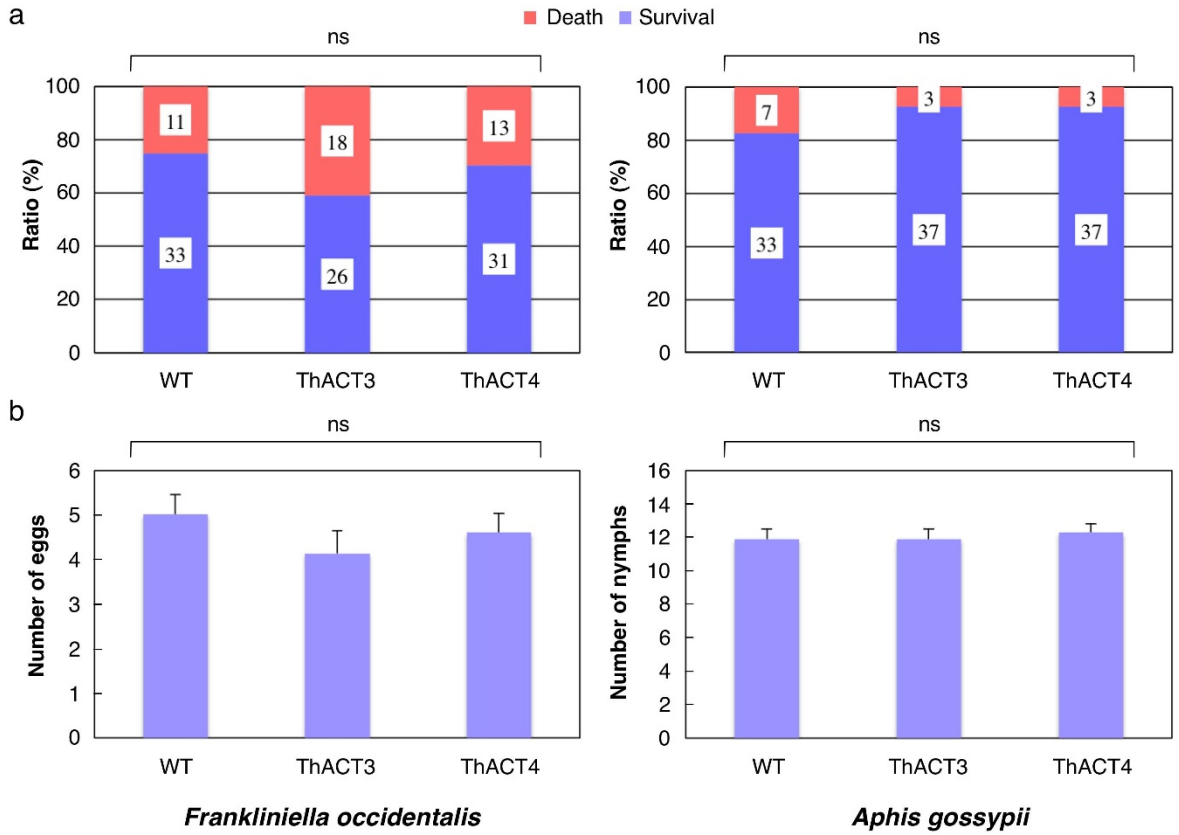

Figure 4 | Effect of HCAAs on arthropod herbivores. (a) Survival rate of adult female thrips (*F. occidentalis*) and aphids (*A. gossypii*) on the leaves of wild-type (WT), ThACT3 and ThACT4 lines 3 days after inoculation. ns, $P > 0.05$, GLM-test. (b) Total number of eggs or nymphs (means ± > + SE) produced during the inoculation period. ns, $df = 2$, $P > 0.05$, ANOVA.

Adult females of *T. ludeni* were introduced onto leaf discs placed upside down on water-soaked cotton wool in a Petri dish (9 cm diameter, 1.7 cm deep). After 3 days' incubation as above, the survivors and eggs were counted under the microscope. We analysed 30 independent leaf discs from the transgenic lines and WT plants.

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Author contributions

AM, KM, TS, GA designed the study; AM, KM, TS, HK, RO, MN, GA, performed the experiments and data analyses; AM, KM, TS, GA wrote the manuscript; AI supervised the study.

Additional information

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ERRATUM: Acquired immunity of transgenic torenia plants overexpressing agmatine coumaroyltransferase to pathogens and herbivore pests

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There are several typographical errors in this article. In the legends of Figures 3 and 4, "(± > + SE)" should read "(+ SE)". In the Methods, Plants and Pets subsection, "(of *C. sinensis* > *Camellia sinensis*)" should read "(of *Camellia sinensis*)"; "(*Aphis gossypii* >*A. gossypii*)" should read "(*A. gossypii*)"; "(15 > 22°C, 60 ± 10% RH, 13 > 16-h photoperiod)" should read "(22°C, 60 ± 10% RH, 16-h photoperiod)". In the Quantitative reverse transcription (RT)-PCR subsection, "(Qiagen > Qiagen, Hilden, Germany)" should read "(Qiagen, Hilden, Germany)" and "(Takara, Japan > Takara, Otsu, Japan)" should read "(Takara, Otsu, Japan)".