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Chrysanthemum expressing a linalool synthase gene ‘smells good’, but ‘tastes bad’ to western flower thrips

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Summary
Herbivore-induced plant volatiles are often involved in direct and indirect plant defence against herbivores. Linalool is a common floral scent and found to be released from leaves by many plants after herbivore attack. In this study, a linalool/nerolidol synthase, FaNES1, was overexpressed in the plastids of chrysanthemum plants (Chrysanthemum morifolium). The volatiles of FaNES1 chrysanthemum leaves were strongly dominated by linalool, but they also emitted small amount of the C11-homoterpeno, (3E)-4,8-dimethyl-1,3,7-nonatriene, a derivative of nerolidol. Four nonvolatile linalool glycosides in methanolic extracts were found to be significantly increased in the leaves of FaNES1 plants compared to wild-type plants. They were putatively identified by LC-MS-MS as two linalool–malonyl–hexoses, a linalool–pentose–hexose and a glycoside of hydroxy–linalool. A leaf-disc dual-choice assay with western flower thrips (WFT, Frankliniella occidentalis) showed, initially during the first 15 min of WFT release, that FaNES1 plants were significantly preferred. This gradually reversed into significant preference for the control, however, at 20–28 h after WFT release. The initial preference was shown to be based on the linalool odour of FaNES1 plants by olfactory dual-choice assays using paper discs emitting pure linalool at similar rates as leaf discs. The reversal of preference into deterrence could be explained by the initial nonvolatile composition of the FaNES1 plants, as methanolic extracts were less preferred by WFT. Considering the common occurrence of linalool and its glycosides in plant tissues, it suggests that plants may balance attractive fragrance with ‘poor taste’ using the same precursor compound.

Introduction
Plant volatiles play important roles in plant–insect interactions (Maffei et al., 2011; Pichersky and Grishenzo, 2002; Schoonhoven et al., 2005), and those induced by herbivore attack are often found to have roles in direct defence by repelling herbivores, and indirect defence by attracting predators or parasitoids (Clavijo McCormick et al., 2012; Dicke and Baldwin, 2010; Dicke and Van Loon, 2000; Paré and Tumlinson, 1999). Herbivore-induced plant volatiles are usually dominated by monoterpene and sesquiterpenes (Degenhardt et al., 2003), and enhanced emissions of these terpenes have led to improved plant defence against herbivores (Dudareva and Pichersky, 2008; Turlings and Ton, 2006).

Linalool is a monoterpene alcohol with a sweet fragrance occurring in the floral scent of a wide variety of plants (Kamatou and Viljoen, 2008). It is also reported to be induced in different plants by damage of a variety of herbivore species, suggesting that linalool may have a role in direct or indirect plant defence against several herbivores. For example, it is induced in crucapple damaged by Japanese beetles (Loughrin et al., 1995), cotton and peanut damaged by beet armyworms (Cardoza et al., 2002; Paré and Tumlinson, 1997), maize damaged by caterpillars (Turlings et al., 1998), Nicotiana attenuata damaged by caterpillars, leaf bugs or flea beetles (Kessler and Baldwin, 2001), spruce damaged by white pine weevils (Miller et al., 2005), lima bean damaged by caterpillars (Mithöfer et al., 2005) or spider mites (Dicke et al., 1990), and tobacco damaged by western flower thrips (WFT) or caterpillars (Delphia et al., 2007). Constitutive high emission of linalool has been engineered in several transgenic plant species by overexpressing the linalool synthase gene (Aharoni et al., 2003; Aharoni et al., 2006; Lavy et al., 2002; Lewinsohn et al., 2001; Yang, 2008). Among these, linalool-emitting Arabidopsis were found to be less attractive to aphids and diamondback moths than wild-type plants, but it remained unclear whether linalool or linalool derivatives were responsible for the observed effects (Aharoni et al., 2003; Yang, 2008).

In the context of a plant, the expression of monoterpenes alcohol synthase genes often results in the synthesis of an array of additional volatile and nonvolatile derivatives resulting from conversions by endogenous enzymes. Those compounds may also, or exclusively, be accountable for the observed effects on plant defence. Linalool synthase overexpressing plants have been found, for example, to emit, apart from linalool, also linalool oxide, hydroxylinalool or hydroxy-dihydrolinalool (Lewinsohn et al., 2001; Lavy et al., 2002; Aharoni et al., 2003; Aharoni et al., 2006). By contrast, transgenic petunia expressing linalool synthase did not produce any volatile linalool or derivative, but instead efficiently converted linalool to a nonvolatile glycoside (Lücke et al., 2001). The glycosides of linalool and hydroxylinalool were also reported in Arabidopsis and potato overexpressing linalool synthase (Aharoni et al., 2003; Aharoni et al., 2006). Transgenic plants overexpressing a similar linear monoterpeno alcohol geraniol by means of a geraniol synthase produced both volatile and nonvolatile derivatives of geraniol such as geranial (volatile), geranic acid (volatile), geranyl acetate...
Expression of the linalool synthase gene in chrysanthemum

Chrysanthemum genotype 1581 was transformed with a linalool/nerolidol synthase gene from strawberry (Aharoni et al., 2004), FaNES1, under the control of the Rubisco small subunit promoter (Outchkourov et al., 2003). The FaNES1 protein was targeted to the plastids by fusion with a plastid-targeting signal. Wild-type plants were used as control. Transcript levels of FaNES1 in cuttings of two T0 transgenic lines, line 28 and 37, were determined by quantitative RT-PCR and found to be similar, ranging from 963 to 1107, relative to the household gene actin (Figure 1a). FaNES1 chrysanthemum plants were slightly shorter and lighter in leaf colour compared with wild-type plants (Figure 1c). This kind of phenotype was observed before for FaNES1 Arabidopsis and potato plants (Aharoni et al., 2003; Aharoni et al., 2006) and it could due to insufficient availability of isoprenoid precursors for other essential metabolites such as carotenoids, chlorophylls and gibberellins (Aharoni et al., 2003).

Results

Expression of the linalool synthase gene in chrysanthemum

Volatile behaviour were analysed. volatile metabolites were analysed by GC- and LC-MS, and the chrysanthemum plants. Constitutively expressed volatile and non-volatile metabolites were analysed by GC- and LC-MS, and the FaNES1 plants, but not found in wild-type plants (Figure 1b). Besides linalool, an acyclic C11-homoterpene, (3E)-4,8-dimethyl-1,3,7-nonatriene (DMNT), was also found to be emitted from FaNES1 plants and not from wild-type plants (Figure 2d,e). The peak area (total ion current) of DMNT was 30- to 70-fold smaller than that of linalool in transgenic plants.

GC-MS analysis of volatile compounds

Volatile compounds were collected from the headspace of cut leaves at half height of the plants. Linalool, the primary product of FaNES1, was strongly dominant among the detected volatiles (Figure 2a–c). The linalool emission was quantified to be 1.41 to 1.82 μg/g FW in FaNES1 plants, but not found in wild-type plants (Figure 1b). Besides linalool, an acyclic C11-homoterpene, (3E)-4,8-dimethyl-1,3,7-nonatriene (DMNT), was also found to be emitted from FaNES1 plants and not from wild-type plants (Figure 2d,e). The peak area (total ion current) of DMNT was 30- to 70-fold smaller than that of linalool in transgenic plants.

LC-MS analysis of nonvolatile compounds

As linalool could also be stored in the form of glycosides, we analysed the nonvolatile metabolites in transgenic (n = 6) and control (n = 3) plants. Aqueous methanol extracts from young leaves were prepared and analysed by accurate mass LC-MS in negative mode (Figure 3a,b). To reveal differential compounds, the LC-MS profiles of transgenic plants and control plants were compared in an untargeted manner using MetAlign followed by multivariate mass spectra reconstruction (MMSSR) clustering of extracted signals, as described in Experimental procedures.

In total, 8968 mass signals were extracted, which grouped into 301 clusters of different metabolites. Among all 8968 masses, 2482 masses (i.e. 28%, distributed in 80 clusters) showed at least twofold intensity difference (P < 0.05) between transgenic and control plants. More masses were found to be significantly increased (2312 masses, distributed in 74 clusters) than decreased (170 masses, distributed in six clusters) in the transgenic plants. Differential masses with a signal intensity higher than 5000 (i.e. about 500-fold higher than noise, 18 masses, distributed in four clusters) were subsequently analysed by LC-MS/MS (Table 1, Figures 3 and S1). These compounds had signals of 22 625–33 651 and were 8–457 times higher expressed than in control plants. According to their MS/MS spectra, all these compounds were putatively identified as derivatives of linalool and hydroxy-linalool: two different types of linalool–malonyl–glucose, a linalool–pentose–glucose and a glycoside of hydroxy–linalool.

Figure 1  Expression level of FaNES1 and its effect on linalool emission and phenotype of chrysanthemum plants. (a) expression level of FaNES1 relative to the expression level of actin gene in wild-type or transgenic chrysanthemum plants. Expression levels of the reference gene, actin gene, were set to 1. Transgene expression levels were determined by quantitative RT-PCR. Wt, wild type; T, transgenic. Error bars indicate SE from 3 biological replicates. (b) linalool emission of cut leaves of wild-type and transgenic chrysanthemum plants. Error bars indicate SE from 3 biological replicates. (c) phenotype of wild-type chrysanthemum plant (Wt-1) and transgenic chrysanthemum plants T28-1 and T37-1.
Effects of FaNES1 plants on thrips behaviour

Leaves at similar leaf stage were picked from plants of transgenic line 37 or line 39 and used to study the effects of FaNES1 plants on WFT behaviour. The results of repeated dual-choice assays showed that WFT were significantly deterred by FaNES1 plants 20–28 h after WFT release with 65%–70% of WFT settling on wild-type leaf discs (Figure 4a). However, we also noticed that in

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Effects of volatile emissions of FaNES1 plants on thrips attraction

To test whether volatile cues determined the initial attraction, we assayed the choice of WFT by placing individual insects on a wire separating two leaf discs and scoring their choice for either leaf when they left the wire. In this way we compared their response to olfactory cues from wild-type and FaNES1 chrysanthemum leaves. This assay demonstrated that WFT preferred the odour from FaNES1 chrysanthemum leaves (Figure 4c). As linalool was the major compound in the volatile profile of FaNES1 chrysanthemum leaves, we also tested whether pure linalool dissolved in paraffin oil was attractive to WFT and found that both 10% and 0.1% linalool in paraffin oil were similarly significantly attractive (Figure 4c). The trend towards preference for wild-type plants could already be observed after four hours, but was not yet significant in this replication ($P = 0.173$).

Discussion

In this study, we aimed to introduce resistance to WFT into chrysanthemum by genetically engineering production of the monoterpene alcohol linalool in the aerial parts of the plant. A linalool/nerolidol synthase, FaNES1, was expressed in the plastids of chrysanthemum plants and resulted in linalool emissions and accumulation of several forms of linalool glycosides. We observed that WFT during the first 15 min significantly preferred these FaNES1 plants, but in the next 24 h gradually changed their preference to the wild type. We were interested to test the hypothesis that volatile emissions from FaNES1 chrysanthemum dominated by linalool were attractive to WFT, and the nonvolatiles dominated by linalool glycosides were deterrent. These two opposing forces of attraction and deterrence could possibly result in the observed gradual change from attraction to deterrence. To prove the basic premise of simultaneous attraction and deterrence, we needed to dismiss the possibility of an induced effect. For example, endogenous defence mechanisms could be induced earlier or more strongly in the transgenic plant by the higher initial damage of thrips resulting from the linalool-induced attraction. Experiments were therefore designed to score thrips behaviour in dual-choice assays, which did not allow for these alternative induced defence hypotheses.

Firstly, we tested the olfactory response of WFT on leaf discs. Olfactory bioassays typically avoid all direct physical and visual contact of the insect with the plant substrate. Our olfactory dual-choice assay fulfilled the first requirement, but did not fully exclude a role for visual cues. This point was relevant as the plants expressing linalool were slightly lighter in colour if you compared them next to each other. However, during the assays, the insects would walk back and forth on the metal wire before deciding...
where to go. This behaviour is typical of insects trying to observe concentration gradients of volatiles. Besides, a direct way to prove that linalool was the major factor for WFT to choose FaNES1 plants was by taking the pure compound in the same assay at transgenic chrysanthemum leaves. The presence of the thrips on either leaf disc was visually recorded 0.25, 1, 2, 4, 20 and 28 h post-thrips release. The x-axis represents $10^{\log}$-transformed time data. Asterisks indicate significant differences to the control ($* P < 0.05; ** P < 0.01$). Error bars indicate SE ($n = 120$ per treatment). (b) dual-choice assays of WFT on plant nonvolatile contents from wild-type or transgenic chrysanthemum leaves. The thrips behaviour was recorded by video. In a period of 40 min, thrips spent significantly more time underneath the wild-type plant nonvolatile contents after 24 h ($P = 0.047$). Mean ± SE; for transgenic line 37: 0, 4 and 24 h measured with $n = 10$, 6 and 10, respectively; for wild-type plant: 0, 4 and 24 h measured with $n = 20$, 18 and 16, respectively. (c) effect of transgenic chrysanthemum plant and linalool on the olfactory response of WFT. Solvent, paraffin oil used to make 10% or 0.1% linalool. Ten microlitre solvent, 10% or 0.1% linalool was applied on filter paper. Asterisks indicate significant differences of the choices between odour sources ($n = 60$ per treatment; $* P < 0.05$). Wt, wild type; T, transgenic. The dashed line indicates 50% level of the y-axis.

Figure 4 Dual-choice assays of western flower thrips (WFT) on different food or odour sources. (a) dual-choice assays of WFT on wild-type versus transgenic chrysanthemum leaves. The presence of the thrips on either leaf disc was visually recorded 0.25, 1, 2, 4, 20 and 28 h post-thrips release. The x-axis represents $10^{\log}$-transformed time data. Asterisks indicate significant differences to the control ($* P < 0.05; ** P < 0.01$). Error bars indicate SE ($n = 120$ per treatment). (b) dual-choice assays of WFT on plant nonvolatile contents from wild-type or transgenic chrysanthemum leaves. The thrips behaviour was recorded by video. In a period of 40 min, thrips spent significantly more time underneath the wild-type plant nonvolatile contents after 24 h ($P = 0.047$). Mean ± SE; for transgenic line 37: 0, 4 and 24 h measured with $n = 10$, 6 and 10, respectively; for wild-type plant: 0, 4 and 24 h measured with $n = 20$, 18 and 16, respectively. (c) effect of transgenic chrysanthemum plant and linalool on the olfactory response of WFT. Solvent, paraffin oil used to make 10% or 0.1% linalool. Ten microlitre solvent, 10% or 0.1% linalool was applied on filter paper. Asterisks indicate significant differences of the choices between odour sources ($n = 60$ per treatment; $* P < 0.05$). Wt, wild type; T, transgenic. The dashed line indicates 50% level of the y-axis.

from leaves of plants that had not been in contact with thrips resulted in a similar deterrence as was observed on the leaf discs after 24 h. Also in this video-monitored assay, only the data at 24 h were significant, suggesting that thrips take some time to avoid these compounds, possibly due to an induced physiological response in thrips itself. ‘Bad taste’ therefore refers to any directly or indirectly sensed nonvolatiles affecting their feeding behaviour to the extent that they switch food source. These results make it likely that the compounds present in these plant nonvolatile contents dominated by the four linalool glycosides are responsible for the deterrence. Direct proof of this relationship will require purification of these compounds and direct feeding choice assays. It will be interesting then to also test thrips performance (e.g. oviposition, WFT growth rate) in nonchoice assays and to investigate the mechanism of action.

In other transgenic plants overexpressing different linalool/nerolidol synthase genes, linalool and nerolidol were reported to be stored as glycosides as well (Aharoni et al., 2003; Aharoni et al., 2006; Lücke et al., 2001). The glycone was determined in linalool synthase expressing petunia as glucose (Lücke et al., 2001). In our previous study of transgenic maize expressing a geraniol synthase, which also produces a monoterpene alcohol, the glycone was determined as malonyl–glucose (Yang et al., 2011). In this study, several glycosides of linalool or hydroxy–linalool were putatively identified by LC-MS/MS. The linalyl–glucopyranoside, reported as the only glycoside of linalool in the linalool synthase expressing petunia, was not detected as the major linalool glycoside in FaNES1 chrysanthemum. Among the major linalool glycosides listed in Table 1, two glycosides showed the same molecular mass and mass spectrum. They could be isomers and they were identified as linalool conjugated to malonyl–glucose by comparing their mass spectra to that of geranyl-6-O-malonyl-β-D-glucopyranoside, which was identified by NMR in geraniol synthase expressing maize. Another linalool glycoside was putatively identified as linalool conjugated to a pentose-glucose. Such a glycoside has been found to be naturally present in raspberry fruit as S-(−)-linalool 3-O-α-L-arabinopyranosyl-(1→6)-β-D-glucopyranoside (Pabst et al., 1991). A glycoside of hydroxy–linalool was also putatively identified in FaNES1 chrysanthemum. The hydroxy–linalool glycosides have also been reported in FaNES1 Arabidopsis and potato; however, the glycone parts were not determined (Aharoni et al., 2003; Aharoni et al., 2006).

Terpene glycosides are regarded as transport and storage forms of terpenes in plant tissues, and they have been recognized to play important roles as precursors of terpene release (Winterhalter et al., 1997). They may be involved in indirect plant resistance against insects by releasing terpene volatiles as signal compounds attracting predators and parasitoids upon attack by herbivores, or they may be directly toxic to the herbivores (Pankoke et al., 2010; Zou and Cates, 1997). As linalool was attractive to WFT, we propose that the major glycosides stored in FaNES1 chrysanthemum may explain the deterrence against WFT. It is interesting to note that a study of the natural distribution of linalool and its glycosides in several linalool-emitting plants showed that linalool glycosides accumulated much more in flowers than in leaves, and that linalool emission was only detected from flowers (Raguso Robert and Pichersky, 1999). Attraction of pollinators by emitted linalool and parallel deterrence of co-attracted herbivores by stored linalool glycosides may therefore represent an intricate tactic of flowers to balance ‘attractive smell’ with ‘poor taste’ to optimize seed yields using the same precursor compound.
Experimental procedures

Plant materials

The linalool/nerolidol synthase gene from strawberry (Aharoni et al., 2004), FaNES1, driven by the rubisco small subunit promoter from chrysanthemum (Outchkoourov et al., 2003), was cloned into ImpactVector1.1 (www.impactvector.com) and introduced into chrysanthemum plants (Chrysanthemum morifolium Ramat.) cv. 1581. The N terminus of FaNES1 was fused to the plastidic targeting signal derived from FvNES1 to direct FaNES1 from cytosol to the plastids (Aharoni et al., 2004). Wild-type chrysanthemum plants were used as control. Plants were grown in a greenhouse at 25 ± 2 °C under long day conditions (16-h-light/8-h-dark photoperiod).

Two T0 transgenic plant lines 28 and 37 producing the highest levels of linalool (Figure S2) and a wild-type control line were introduced into chrysanthemum plants (Chrysanthemum morifolium Ramat.) cv. Sunny Casa in a greenhouse under a photoperiod of L16:D8 at 25 ± 2 °C. In this study, only adult female thrips were used. All bioassays were conducted in a climate room at 20–22 °C with a L16:D8 photoregime as described by Yang et al. (2012). Chrysanthemum flowers do not emit linalool (Manjunatha et al., 1998), and thus, WFT would not be affected in the olfactory choice assays in this study.

Thrips dual-choice assays with leaf discs

A population of WFT, Frankliniella occidentalis, was mass-reared on flowering chrysanthemum (Chrysanthemum morifolium Ramat.) cv. Sunny Casa in a greenhouse under a photoperiod of L16:D8 at 25 ± 2 °C. In this study, only adult female thrips were used. All bioassays were conducted in a climate room at 20–22 °C with a L16:D8 photoregime as described by Yang et al. (2012). Chrysanthemum flowers do not emit linalool (Manjunatha et al., 1998), and thus, WFT would not be affected in the olfactory choice assays in this study.

Leaf discs from wild-type chrysanthemum plants were used as control discs, and leaf discs from plants of transgenic line 37 or line 39 were used as test discs. Twelve replicates were used in this experiment. The number of WFT on each leaf disc was recorded 0.25, 1, 2, 4, 20 and 28 h after the release of the WFT. At each time point, a two-tailed Wilcoxon signed rank test was used to assess the significance of the differences in the mean number of WFT between test and control. The plants of line 39 emitted slightly lower amount of linalool than plants of lines 37 and 28 (Figure S2).

Thrips dual-choice assay based on olfactory cues

To dissect the component of thrips host choice based on olfactory cues only, a metal wire (0.5 mm diameter, ~2.5 cm long) was placed between two leaf discs (1.6 cm diameter) embedded, abaxial side up, on a 1.5% (w/v) agar bed in a Petri dish (7 cm diameter). One of the leaf discs was from wild-type chrysanthemum plants as control disc and the other leaf disc was from transgenic plants of line 37 as test disc. The metal wire was not in contact with any of the leaf discs, and there was about 0.5 cm distance between the end of the metal wire and the leaf disc. Every time, one ice-anaesthetized thrips was released in the middle of the metal wire. Once the thrips became active, it walked along the metal wire without going off it. After one or a couple of rounds of walking, the thrips would finally leave the wire at either end and walk towards the leaf disc of choice. The...
number of thrips reaching either leaf disc was recorded. Every pair of leaf discs was assayed with 10 individual thrips, and this experiment was replicated with six pairs of leaf discs. More than 90% WFT made their choices within 2 min in this assay. A two-tailed Wilcoxon signed rank test was used to assess the significance of the differences in the mean number of WFT between test and control.

In the experiment checking the olfactory response of thrips to the linalool standard, WFT were given choices between filter papers (~1.5 cm²) applied with 10 µL paraffin oil or with 10 µL 10% or 0.1% linalool dissolved in paraffin oil,

**Thrips dual-choice assay with leaf nonvolatile contents**

Female WFT (3 weeks old) were starved overnight. Six wells of 24-well plates with some pollen added were inoculated with 3 WFT per well and sealed with stretched parafilm. On top of the parafilm, two droplets (30 µL) of plant nonvolatile contents were added (Figure S3). Preference of WFT for the plant nonvolatile contents from either wild-type or transgenic plants was determined by placing the plate on top of a light source and video recording the behaviour for 40 min at the start, and after 4 and 24 h post-WFT release. The plant nonvolatile contents were prepared by freeze-drying the methanol extracts used for the LC-MS analysis and then redissolving the residue in MQ water. The chemical concentration in the plant nonvolatile contents is comparable to that in leaves, assuming the water content of leaves is 90%. These plant nonvolatile contents were also analysed by LC-MS to check whether the residue was dissolved well in MQ water.

The set-up for the video tracking was shown in Figure S3. The footage was analysed live, using EthoVision 8.5xt software. Droplets of wild-type and transgenic line 37 were interpreted as ‘zone 1’ and ‘zone 2’, respectively, accounting for left or right preference. WFT were only in focus and detected when walking on the parafilm ceiling of the wells. For data analyses, time spent in either zone was averaged per arena (across all observed WFT). Misinterpreted subject detection by EthoVision (e.g. subject detected was not a WFT, but an artefact) was regarded as artefacts and filtered out if their critical reading of the manuscript, Ric de Vos for his help in LC-MS analysis and Yury Tikunov for his help in using MMSR approach. This research was supported by Technology Top Institute Green Genetics of the Netherlands (grant no. 1C001RP) and the Technology Foundation of the Netherlands Organization for Scientific Research (NWO) (grant STW10989, Perspectief Programme ‘Learning from Nature’).

**Conflict of interest**

The authors have no conflict of interest to declare.

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**References**


Supporting information

Additional Supporting information may be found in the online version of this article.

**Figure S1** Scheme of collision-induced MS/MS-fragmentation of mass 401 eluting at 47.79 and 46.07 min (a, putatively identified as linalool–malonyl–glucose), mass 447 eluting at 37.46 min (b, putatively identified as linalool–pentose–hexose) and mass 459 eluting at 36.15 min (c, putatively identified as hydroxy–linalool–hexose conjugate to a molecule with formula of C7H14O3, such as hydroxyheptanoic acid).

**Figure S2** Linalool emission of cut leaves of wild-type and different transgenic chrysanthemum plants. Wt, wild type; T, transgenic. The data represented the average emission levels from 3 leaves of the same plant. Error bars indicate SE (*n* = 3).

**Figure S3** Experimental setup of the video assay. Screenshot of 6 wells as observed by EthoVision 8.5. Wells have a diameter of 1.6 cm and are composed of 2 zones that represent Wild type (zone 1) and T37 (zone 2) droplets (30 μL). In the Wt vs Wt experiment, both zones represent Wild type. Red, blue and green dots/lines are the positions of thrips (max. 3 thrips per well) in the last 20 s of recording.