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Spider mite tetranins elicit different defense responses in different host habitats

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SUMMARY

Spider mites (*Tetranychus urticae*) are a major threat to economically important crops. Here, we investigated the potential of tetranins, in particular Tet3 and Tet4, as *T. urticae* protein-type elicitors that stimulate plant defense. Truncated Tet3 and Tet4 proteins showed efficacy in activating the defense gene pathogenesis-related 1 (*PR1*) and inducing phytohormone production in leaves of *Phaseolus vulgaris*. In particular, Tet3 caused a drastically higher Ca^{2+} influx in leaves, but a lower reactive oxygen species (ROS) generation compared to other tetranins, whereas Tet4 caused a low Ca^{2+} influx and a high ROS generation in the host plants. Such specific and non-specific elicitor activities were examined by knockdown of *Tet3* and *Tet4* expressions in mites, confirming their respective activities and in particular showing that they function additively or synergistically to induce defense responses. Of great interest is the fact that *Tet3* and *Tet4* expression levels were higher in mites on their preferred host, *P. vulgaris*, compared to the levels in mites on the less-preferred host, *Cucumis sativus*, whereas *Tet1* and *Tet2* were constitutively expressed regardless of their host. Furthermore, mites that had been hosted on *C. sativus* induced lower levels of *PR1* expression, Ca^{2+} influx and ROS generation, i.e., Tet3- and Tet4-responsive defense responses, in both *P. vulgaris* and *C. sativus* leaves compared to the levels induced by mites that had been hosted on *P. vulgaris*. Taken together, these findings show that selected tetranins respond to variable host cues that may optimize herbivore fitness by altering the anti-mite response of the host plant.

Keywords: ***Cucumis sativus*, elicitor, *Phaseolus vulgaris*, spider mite (*Tetranychus urticae*), tetranin.**

INTRODUCTION

To induce defense responses in host plants, plants detect and utilize specific herbivore-derived elicitors. The oral secretions of various (mostly plant-chewing) phytophagous arthropods have been found to contain several types of elicitors (Arimura, 2021; Snoeck et al., 2022): they include fatty acid-amino acid conjugates (FACs; e.g., volicitin [*N*-(17-hydroxylinolenoyl)-L-glutamine]) (Alborn et al., 1997; Spiteller & Boland, 2003; Yoshinaga et al., 2010) and polysaccharides (Bricchi et al., 2012; Uemura et al., 2020) from lepidopteran larvae, disulfooxy fatty acids (caeliferins) from the American bird grasshopper (*Schistocerca americana*) (Alborn et al., 2007), and peptides derived from chloroplastic ATP synthase γ-subunit (cATPC) proteins (inceptin-related peptides) from the fall armyworm (*Spodoptera frugiperda*) (Schmelz et al., 2006).

The characterization of elicitors from small, plant-sucking herbivores has only recently become possible, largely due to the availability of a large genome/RNA-seq database and cutting-edge research in plant-herbivore interactions. This breakthrough has allowed the identification of salivary gland proteins (SGPs) responsible for eliciting responses. For example, a mucin-like salivary protein (NIMLP) was characterized in the salivary glands of the piercing-sucking insect *Nilaparvata lugens* (Shangguan et al., 2018). Moreover, the small N-terminal subunit of vitellogenins of the planthopper

N. lugens (NlVgN) has been shown to trigger defense responses in rice during feeding or oviposition by the insect (Zeng et al., 2023).

In spider mites (*Tetranychus urticae* and *T. evansi*), not only elicitors but also effectors, including Te16, TePDI, Tu/Te28, and Tu/Te84, were identified in the salivary glands (Cui et al., 2023, 2024; Villarroel et al., 2016). Other SGPs, namely tetranins (Tet1 and Tet2), have also been characterized as protein elicitors in *T. urticae*. These tetranins induce Ca^{2+} influx and plasma membrane depolarization, potentially activating jasmonate (JA) and salicylate (SA) biosynthesis (Iida et al., 2019). This subsequently triggers direct and indirect defense responses in common bean (*Phaseolus vulgaris*) leaves. The two tetranin proteins are notable for the fact that they are functionally similar but structurally different. Accordingly, tetranins, which are categorized on the basis of their functionality rather than their structural features, differ from other well-known elicitors such as FACs (Spiteller & Boland, 2003) and inceptin-related peptides (Schmelz et al., 2007), which consist of structurally similar molecules. Notably, tetranin proteins show no similarity to proteins from other arthropods, except those within the family Tetranychidae. Therefore, it can be concluded that tetranin proteins have evolved uniquely within the Tetranychidae lineage.

In addition to the *in silico* screening methods for SGPs described above, proteomic analysis with *in vivo* bioassays led to the identification of 95 SGPs secreted by *T. urticae* (Jonckheere et al., 2016). It became clear that most of SGPs could not be considered in the initial phase of our tetranin screening due to the lack of comprehensive bioinformatics information (Iida et al., 2019), providing us with a valuable opportunity to discover additional potential tetranin candidates. In other words, we propose the possibility that plants are sensitive not only to a few SGPs (e.g., Tet1 and Tet2), but also to different SGPs as elicitors, resulting in multiple arrays of defense responses against *T. urticae* and, in the case of their having independent and/or coordinated actions, this could achieve a strong anti-mite strategy. Therefore, in the present study, we were motivated to search for novel tetranins among the candidates. As a result, we observed that two new tetranin members, namely Tet3 and Tet4, contribute to the intracellular signaling dynamics of plant defense responses. These members can act independently or cooperate with Tet1 and Tet2 to induce defense in *P. vulgaris*, a preferred host, and *Cucumis sativus*, a less-preferred host. More importantly, we show that the different expression levels of *Tet3* and *Tet4* in *T. urticae*, induced by different host cues, significantly affect the threshold of plant defense capabilities against *T. urticae*. Based on our results, we discuss whether the wide host range and varying preferences of *T. urticae* for different host plants with different levels of resistance (Giménez-Ferrer et al., 1994) are influenced by and/or result from tetranin levels in the mites.

RESULTS

Isolation of new tetranins

To evaluate the tetranin-like activity of 18 SGPs, they were transiently expressed in leaves of the host plant *P. vulgaris*. We then assessed the mortality of adult female mites on *P. vulgaris* leaves expressing each of the SGPs (Figure 1A). Of the 18 SGPs tested, expression of SGP7 and SGP12 in *P. vulgaris* leaves resulted in significantly higher mite mortality than expression of the control plasmid. Consequently, we identified SGP7 and SGP12 as novel tetranins and named them Tet3 and Tet4, respectively.

Relevance of sequences and unknown properties of tetranin proteins

BLAST searches of the amino acid sequences of the truncated tetranin proteins showed identity scores below 12% for all pairwise comparisons (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Figure S1). In addition, Tet4 showed similarity to genes in *T. urticae*, including two uncharacterized *T. urticae* proteins (Tetur32g02297 and Tetur44g00291) with identity scores of 89% and 87%, respectively. Furthermore, it showed the highest degree of similarity to SGP11 among all SGPs analyzed in this study (60% identity; Figure S1). On the other hand, Tet3 shared 73% and 57% identity with the uncharacterized *T. evansi* protein LOC107363530 and the *Panonychus citri* protein LOC128397275, respectively.

Despite functional and structural analyses using InterPro (https://www.ebi.ac.uk/interpro/), Tet3 and Tet4 remain uncharacterized. Therefore, we conclude that tetranins, including Tet3 and Tet4 with unknown functions, are likely to be exclusive to the family Tetranychidae.

The innate activity of Tet3 and Tet4

To investigate the elicitor activity of Tet3 and Tet4 secreted by *T. urticae* in *P. vulgaris* hosts, we attempted to knockdown *Tet3* and *Tet4* expressions in the *T. urticae* body using RNA interference (RNAi) methods. As a result, the level of *Tet3* expression in *T. urticae* reared on *P. vulgaris* leaves expressing *Tet3* RNAi (T3-Ri) was 76% of that in control *T. urticae* (Figure S2). Knockdown of the *Tet4* expression in *T. urticae* similarly reared on leaves expressing *Tet4* RNAi (T4-Ri) was more effective, resulting in a *Tet4* expression level that was 43% of that in control *T. urticae* (Figure S2). Adult females of T3-Ri and T4-Ri showed more ovipositions on *P. vulgaris* leaves than control *T. urticae* (Figure 1B), indicating that both T3-Ri and T4-Ri exhibit higher performance. This was consistent with the expression level of the defense gene

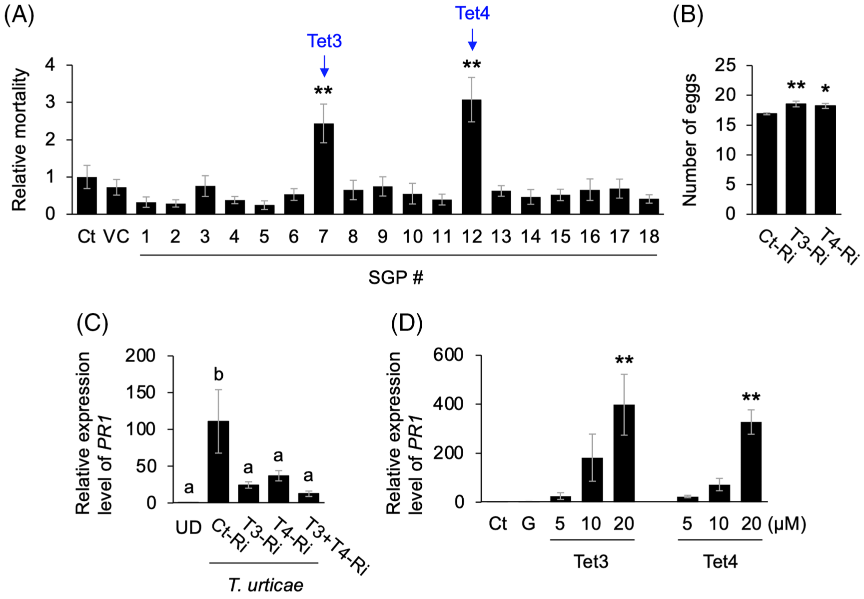

Figure 1. Screening of new members of the tetranins.

(A) Adult female mites were incubated on discs of *Phaseolus vulgaris* leaves transiently expressing truncated salivary gland proteins (SGPs) or vector control (VC) using the agroinfiltration system. Leaf discs infiltrated with MES buffer served as control (Ct). Mean lethal counts were determined relative to those for control discs.

(B) Number of eggs laid by adult female *Tetranychus urticae* that had been incubated on *Tet3* or *Tet4* RNAi-expressing leaves (T3-Ri and T4-Ri, respectively) or control RNAi-expressing leaves (Ct-Ri) for 2 days.

(C) Expression levels of genes encoding pathogenesis-related protein 1 (*PR1*) in leaves undamaged (UD) or damaged by Ct-Ri, T3-Ri, T4-Ri or *T. urticae* that had been incubated on *Tet3* + *Tet4* RNAi-expressing leaves (T3 + T4-Ri) .

(D) *PR1* expression levels were measured in leaves 24 h after application of GFP control (G), Tet3, or Tet4 proteins at concentrations of 5, 10, or 20 μM following mechanical damage. Untreated leaves served as an additional control (Ct). Data represent means and standard errors [$n = 11$–12 for (A), $n = 7$–9 for (B), and $n = 6$ for (C, D)]. For (A), data marked with asterisks are significantly different from those of VC, based on an ANOVA with Dunnett's contrasts (**$P < 0.01$). For (B, D) data marked with an asterisk(s) are significantly different from those of Ct-Ri (B) or GFP (D), based on an ANOVA with Holm's sequential Bonferroni *post hoc* test (**$P < 0.01$; *$0.01 \le P < 0.05$). For (C), means indicated by different small letters are significantly different based on an ANOVA with *post hoc* Tukey's HSD ($P < 0.05$).

pathogenesis-related 1 (*PR1*) in *P. vulgaris* leaves damaged by these T3-Ri mites and T4-Ri mites, which was induced to only 22.1 and 33.3% of that in *P. vulgaris* leaves damaged by control *T. urticae*, respectively (Figure 1C). Furthermore, when leaves were damaged by *T. urticae* that had been reared on leaves expressing both T3-Ri and T4-Ri, the expression level of *PR1* was only 11.3% of that in leaves damaged by control *T. urticae*, suggesting that Tet3 and Tet4 may act additively or synergistically on *PR1* activation.

To further investigate the role of tetranins in the defense response of *P. vulgaris* leaves, the recombinant Tet3 and Tet4 proteins were applied at different doses to mechanically damaged (MD) leaves of *P. vulgaris*. After 24 h, we observed that the *PR1* expression levels were significantly upregulated in leaves treated with Tet3 or Tet4 proteins, especially at a concentration of 20 μM each, compared to the levels in leaves treated with the control protein green fluorescent protein (GFP) (Figure 1D). Notably, Tet3 proteins at a concentration of 20 μM upregulated the expression of *PR3*, another defense gene in leaves, but Tet4 proteins did not (Figure S3). Therefore, we focused on *PR1* as a responsive defense gene for the tetranin response of plants in this research.

Differential action of the tetranins in the defense system of the plant

Based on the above results, we subsequently evaluated the response of all tetranin proteins at 20 μM on several defense responses in *P. vulgaris* leaves to compare the potential function of the tetranin members. First, we evaluated the levels of endogenous phytohormones such as JA, SA, and abscisic acid (ABA), which are important properties in activating plant defenses against spider mites (Alba et al., 2015; Ament et al., 2004; Ozawa et al., 2000). The data showed an increase in JA accumulation in leaves after application of MD with all the tetranin proteins in leaves after 3 h, compared to that in leaves treated with a GFP control (Figure 2A). In addition, an increase in SA and ABA accumulation was observed compared to leaves treated with GFP when MD + Tet3 or MD + Tet4 were applied.

Activation of JA and SA signaling in spider mite-damaged plants triggers the release of volatile organic compounds (VOCs), which attract predators such as *Phytoseiulus persimilis* (Arimura et al., 2009). Therefore, we next evaluated the efficacy of tetranins in promoting VOC emission in *P. vulgaris*. As hypothesized, application of

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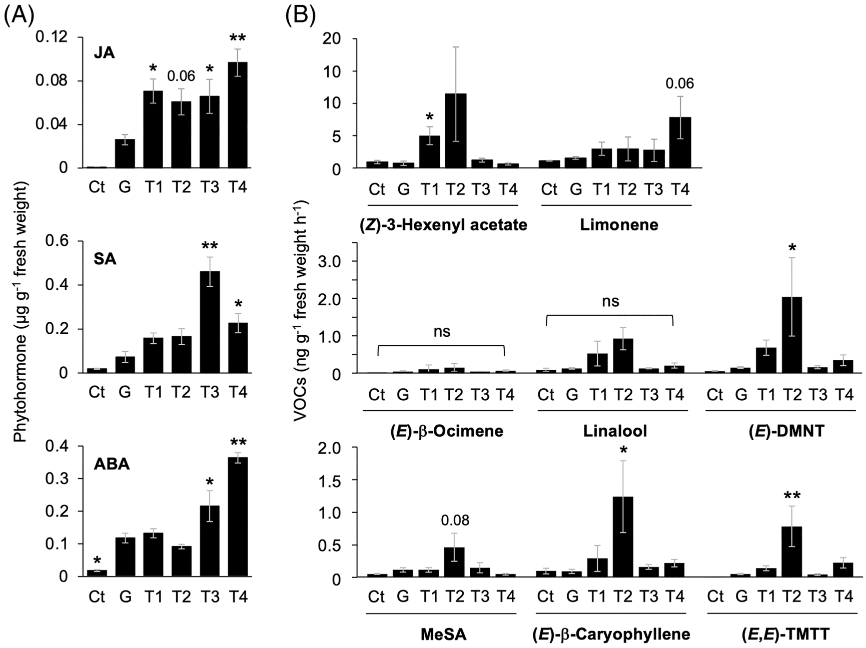

Figure 2. Response of tetranin members on phytohormone and volatile organic compound (VOC) profiles.
(A) Endogenous accumulation levels of phytohormones (jasmonic acid [JA], salicylic acid [SA], and abscisic acid [ABA]) were examined in *Phaseolus vulgaris* leaves 3 h after mechanical damage with GFP control (G) or tetranin proteins (Tet1, Tet2, Tet3, and Tet4) (20 µM).
(B) VOC release from *P. vulgaris* plants was quantified between 24 and 27 h after application of mechanical damage using G or tetranin proteins (20 µM). Untreated leaves served as an additional control (Ct) in all cases. Data represent means and standard errors ($n = 6$). An Asterisk(s) indicates significant differences compared to the GFP control as determined by ANOVA with Holm's sequential Bonferroni *post hoc* test (**$P < 0.01$; *$0.01 \leq P < 0.05$). The mean values shown with *P* values are marginally different from those of GFP. ns, not significant ($P \geq 0.05$). (*E*)-DMNT, (*E*)-4,8-dimethyl-1,3,7-nonatriene; (*E*,*E*)-TMTT, (*E*,*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene; MeSA, methyl salicylate.

MD + Tet2 protein to *P. vulgaris* leaves significantly enhanced the emission of VOCs, including compounds such as (*E*)-4,8-dimethyl-1,3,7-nonatriene [(*E*)-DMNT], methyl salicylate (MeSA), (*E*)-β-caryophyllene, and (*E*,*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene [(*E*,*E*)-TMTT] (Figure 2B). In contrast, Tet1 and Tet4 showed partial involvement in promoting the emission of (*Z*)-3-hexenyl acetate and limonene, respectively, while Tet3 had no effect on VOC emission.

Finally, we investigated the effect of tetranins in early defense signaling. In particular, the activation of Ca^{2+} channels and the generation of reactive oxygen species (ROS) are essential early signaling responses to herbivory and elicitors in cells (Gandhi et al., 2021; Kloth & Dicke, 2022; Zebelo & Maffei, 2015). In this context, we observed a remarkable increase in intracellular Ca^{2+} levels upon introduction of Tet3 protein into *P. vulgaris* leaves compared to those in leaves with introduction of a GFP control (Figure 3A). However, the effects of other tetranin proteins on Ca^{2+} influx were much weaker. Introduction of Tet1, Tet2, and Tet4 but not Tet3, by contrast, resulted in a significant increase in ROS generation, compared to the GFP control (Figure 3A). Furthermore, T3-Ri mites and T4-Ri mites (see above), respectively, showed defective activation of Ca^{2+} influx and ROS generation in leaves compared to control mites, indicating that Tet3 and Tet4 are predominantly responsible for the respective cascade of early signaling responses (Figure 3B). Furthermore, both the levels of Ca^{2+} influx and ROS generation were more drastically impaired in leaves when damaged by mites that had been reared on leaves expressing both T3-Ri and T4-Ri than those in leaves damaged by control *T. urticae*. This suggested that Tet3 and Tet4 act additively or synergistically to induce these early defense signals.

Nature of tetranins in various plant hosts

In addition to *P. vulgaris*, we investigated the defense responses induced by tetranin proteins in several host plants. When applied to mechanically damaged leaves of *C. sativus*, all tetranin proteins significantly increased *PR1* expression levels (Figure 4A). This response was not observed when applied to damaged leaves of *Solanum lycopersicum* and *Zea mays*, while only Tet2 showed an increase in *PR1* expression levels in *Brassica rapa* leaves.

Next, to determine if *C. sativus* leaves had the same ability of responsiveness to tetranin proteins as *P. vulgaris*, we measured intracellular Ca^{2+} levels. As we expected, Tet1, Tet2, and especially Tet3 caused an increase

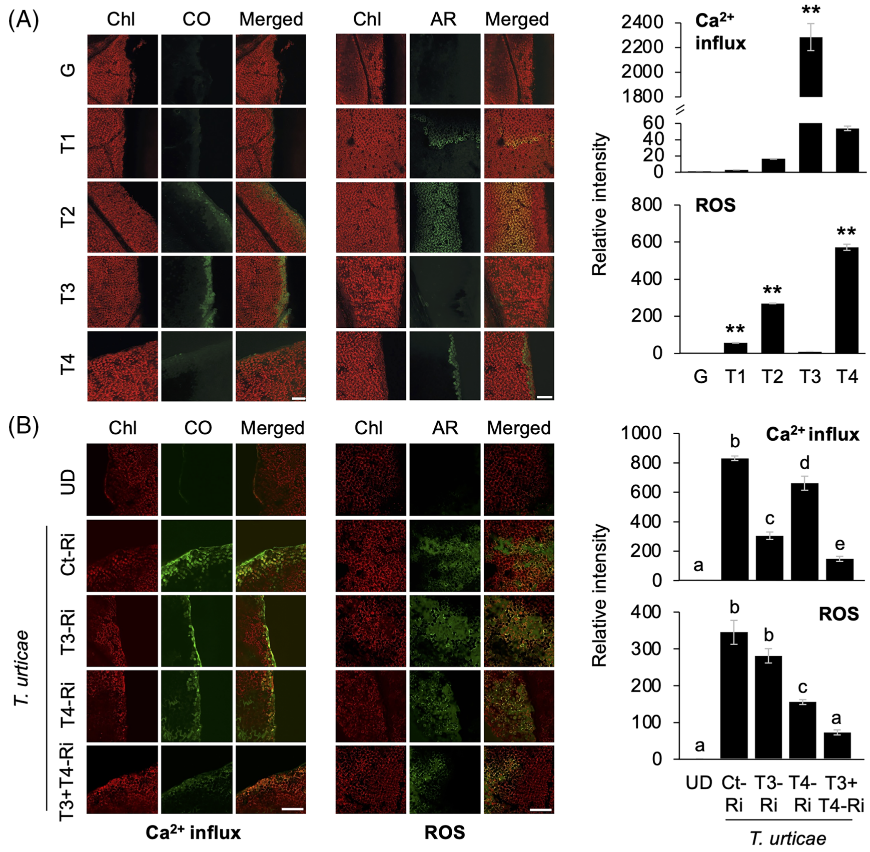

Figure 3. Ca^{2+} influx and of reactive oxygen species (ROS) generation in *Phaseolus vulgaris* leaves in response to tetranin proteins and spider mites knocking down tetranins.

(A) Leaves were subjected to mechanical damage by application of a GFP control (G) or Tet proteins (Tet1, Tet2, Tet3, and Tet4) (20 μM) for 30 min.

(B) Similarly, intracellular Ca^{2+} levels and intracellular/intercellular ROS levels were measured in leaves damaged for 2 h by *Tetranychus urticae* that had been incubated for 3 days on *Tet3* or *Tet4* RNAi-expressing leaves (T3-Ri and T4-Ri, respectively), *Tet3* + *Tet4* RNAi-expressing leaves (T3 + T4-Ri) or on control RNAi-expressing leaves (Ct-Ri). Undamaged leaves served as control (UD). Representative false-color image reconstructions of fluorochemical intracellular Ca^{2+} and intracellular/intercellular ROS in leaves are shown in the left panels. Scale bars are 200 μm. Data in the right panels represent means and standard errors of the relative quantification levels ($n = 3$). For (A), data marked with asterisks are significantly different from those of the GFP control, based on an ANOVA with Holm's sequential Bonferroni *post hoc* test (**$P < 0.01$). For (B), means indicated by different small letters are significantly different based on ANOVA with *post hoc* Tukey's HSD ($P < 0.05$). AR, Amplex Red fluorescence; Chl, chlorophyll fluorescence; CO, Calcium Orange fluorescence.

compared to a GFP control (Figure 4B; Figure S4). In addition, the intracellular and intercellular levels of ROS in *C. sativus* increased in response to Tet1, Tet2, and Tet4, but not Tet3, compared to the GFP control (Figure 4B; Figure S4), as similarly shown in *P. vulgaris* (Figure 3). It should be noted, however, that the response levels for both Ca^{2+} influx and ROS generation were lower in *C. sativus* than in *P. vulgaris* (Tables S3–S6). More importantly, *Z. mays*, which showed a lack of tetranin responsiveness of the *PR1* expression (Figure 4A), also showed significantly lower responses (Figure 4B; Figure S4; Tables S3–S6).

Effect of host habitat on expression levels of *tetranin* genes and the resulting host defense responses

We hypothesized that different hosts affect not only the functional properties of tetranins but also the endogenous levels of tetranins in the mites. We, therefore, compared the mites' preferred host, *P. vulgaris*, with the less preferred but tetranin-responsive host, *C. sativus*. When *T. urticae* reared on *P. vulgaris* leaves (referred to as Pv-mite) were transferred to *C. sativus* leaves (referred to as Cs-mite) for five generations, a significant decrease in oviposition activity was observed in Cs-mite (Figure 5A). Furthermore, Cs-mite showed significantly lower expression levels of *Tet3* and *Tet4* genes, but not *Tet1* and *Tet2* genes (Figure 5B). Two-way analysis of variance (ANOVA) analysis revealed a significant interaction between mite sources and tetranin gene expression levels ($P = 0.003$ and $P < 0.0001$, respectively, Table S7), indicating that host preference plays a role in influencing endogenous tetranin levels in the mites.

Cs-mite was much less able to induce defense responses on *PR1* expression and early cellular signaling (i.e., intracellular Ca^{2+} levels and intracellular/intercellular

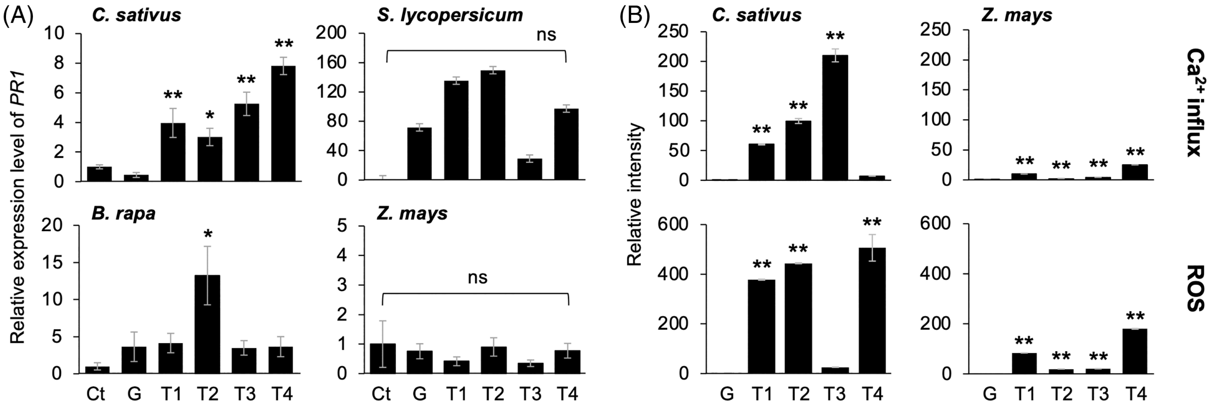

Figure 4. Defense reactions in leaves of different plants in response to tetranin proteins.
(A) Expression levels of *PR1* genes in leaves of *Cucumis sativus*, *Brassica rapa*, *Solanum lycopersicum*, and *Zea mays* at 24 h after mechanical damage (MD) with application of a GFP control (G) or tetranin proteins (Tet1, Tet2, Tet3, and Tet4) (20 μM). Untreated leaves were used as an additional control (Ct).
(B) Intracellular Ca^{2+} levels and intracellular/intercellular reactive oxygen species (ROS) levels in leaves 30 min after MD with application of each protein (20 μM). Representative false-color image reconstructions are shown in Figure S4. Data represent means and standard errors [$n = 5–6$ for (A) and $n = 3$ for (B)]. Data marked with an asterisk(s) are significantly different from those of the GFP control based on ANOVA with Holm's sequential Bonferroni *post hoc* test ($^{**}P < 0.01$; $^{*}0.01 \le P < 0.05$). ns, not significant ($P \ge 0.05$).

ROS levels) in both *P. vulgaris* and *C. sativus* leaves compared to those with Pv-mite (Figure 5C–E; Figure S5). In contrast to these results, the effect of the Pv-mite and Cs-mite on the emission levels of VOCs was marginal or not significantly different ($P \ge 0.05$, Figure 5F; Figure S6). Taken together, the low levels of *Tet3* and *Tet4* expression in Cs-mite were found to ultimately result in attenuated defense responses excluding VOC induction in plants.

DISCUSSION

In this study, the two SGPs secreted by *T. urticae*, tetur10g00100 and tetur32g00050, identified by an omics approach in Jonckheere et al. (2016), were characterized as novel tetranin members (i.e., Tet3 and Tet4) that additively or synergistically activate host plant defense responses and, predictably, in concert with previously characterized tetranins, Tet1 and Tet2. In contrast, the omics approach did not detect Tet1 or Tet2 in the secretions, suggesting that these SGPs may not be abundant in *T. urticae* salivary secretions, unlike Tet3 and Tet4. In this context, *Tet1* and *Tet4* have been shown to be expressed specifically in the salivary glands, whereas *Tet2* is expressed not only in the salivary glands, but also in other tissues (Iida et al., 2019; Jonckheere et al., 2016). There is no available evidence about the tissue localization of *Tet3* expression, but this gene should undoubtedly be highly expressed in the salivary glands, given the fact that it is secreted in large quantities outside the mite (Jonckheere et al., 2016).

Indeed, the actual amount and localization of tetranins after their secretion into host plants remain to be determined. *T. urticae* inflict damage to mesophyll cells through their stylet, which they insert between epidermal pavement cells or through stomatal openings. This feeding process results in various detrimental effects on the cells, such as plasmolysis, collapse, emptiness, or coagulation (Bensoussan et al., 2016). During this process, various oral factors, including tetranins, should be secreted into the damaged cells, either trapped within them or potentially released into the extracellular layer when the cells are disrupted. Since tetranins were found to promote the early phase of defense signaling (i.e., the Ca^{2+} channel opening and oxidative burst) (Figures 3 and 4B), it is possible that their activity occurs not only in the intracellular space but also in the apoplast, whereas intracellular Ca^{2+} influx is not restricted to the plasma membrane (Pirayesh et al., 2021; Schonknecht, 2013). These possibilities for functional localization would be consistent with the fact that tetranins produced by agroinfiltration of the SGPs without signal peptides (for possible intracellular space) or application of recombinant proteins (for possible extracellular space) functionally exhibit their significant elicitor functions in both intracellular and extracellular spaces (e.g., Figure 1). Alternatively, extracellular tetranins could be re-transported into the cell and function similarly to the endocytosis-mediated entry of HARP1, an effector from the oral secretions of the cotton bollworm (*Helicoverpa armigera*) (Yan et al., 2023). In this context, the *T. evansi* SGP elicitor Te16 has been shown to be expressed in the cytoplasm of *Nicotiana benthamiana* leaf cells, and the elicitor is capable of inducing defense responses, including ROS generation, callose deposition, and other JA-related responses (Cui et al., 2024). Taken together, these facts indicate that SGPs that are either secreted into the cell or transported from the extracellular space, or both, are potentially capable of acting as intracellular elicitors. Nevertheless, the tetranins may not be stable long enough in

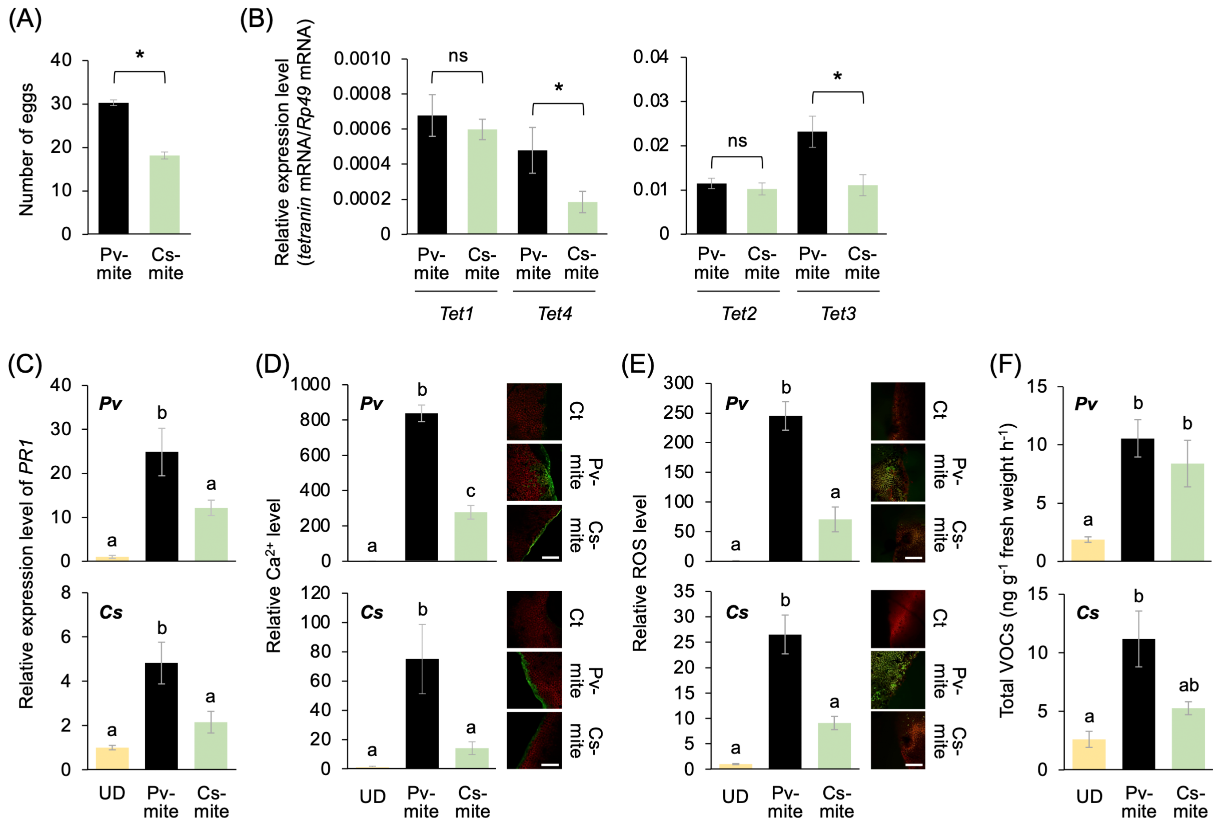

Figure 5. Influence of tetranin gene expression variability in *Tetranychus urticae* on host plant defense with a focus on preferred and less-preferred plant hosts.
(A) Number of eggs laid by adult female *T. urticae* hosted on *Phaseolus vulgaris* (*Pv*) (Pv-mite) and *Cucumis sativus* (*Cs*) (Cs-mite) for 3 days.
(B) Tetranin gene expression levels in Pv-mite and Cs-mite.
(C) *PR1* expression levels in leaves of *Pv* and *Cs* plants after 24 h of damage by Pv-mite or Cs-mite and undamaged plants (UD).
(D, E) Intracellular Ca^{2+} levels (D) and intracellular/intercellular reactive oxygen species (ROS) levels (E) in leaves of *Pv* and *Cs* plants after 24 h of damage by Pv-mite or Cs-mite and undamaged plants. The representative merged false-color image reconstructions of the fluorochemicals are shown in the right panels of each graph. Scale bars indicate 200 μm. See Figure S5 for more details.
(F) Total volatile organic compounds (VOCs) released from undamaged *Pv* or *Cs* plants were compared with those damaged by Pv-mite or Cs-mite for 24–27 h.
Data represent means and standard errors [$n = 6$ for (A), $n = 9$–10 for (B), $n = 6$ for (C), $n = 3$ for (D, E) and $n = 6$–8 for (F)]. For (A, B), data are significantly different between Pv-mite and Cs-mite, based on a Student's *t*-test (*$0.01 \le P < 0.05$). For (C–F), means indicated by different small letters are significantly different based on ANOVA with *post hoc* Tukey's HSD ($P < 0.05$). ns, not significant ($P \ge 0.05$).

host cells, probably due to their degradation as foreign proteins, e.g., by selective autophagy (Guo et al., 2023), as immunoblot analysis of *T. urticae*-damaged leaf samples using Tet3 and Tet4 antibodies did not yield visible signals (Figure S7); however, this interpretation is highly dependent on the strength of antibody affinity to the antigen and other factors.

Notably, four members of the tetranins exhibit a complex nature of both specific and non-specific elicitor activity in host plants (Table 1). For example, in the early phase of defense signaling, Tet3 consistently caused a strong Ca^{2+} influx but less ROS generation in both *P. vulgaris* and *C. sativus* leaves (Figures 3 and 4B). In contrast, Tet1, Tet2, and Tet4 are likely more responsible for the oxidative burst, suggesting that the respective tetranin members are differentially responsible for early cellular signaling. These responses are thought to be sequentially triggered by herbivore oral factors and other biotic stresses via Ca^{2+} signaling through ion channels and ROS generation through NADPH/RBOHD crosstalk (Kloth & Dicke, 2022; Marcec & Tanaka, 2021; Snoeck et al., 2022; Tyagi et al., 2022). However, there are some exceptions; for example, Ca^{2+} influx induced by the *Phytophthora sojae* β-glucan elicitor was not necessarily required for ROS generation in soybean cell suspension cultures (Mithöfer et al., 2001). Thus, tetranins may act as a β-glucan.

In the mid- and late phases of defense signaling, all tetranins are responsible for *de novo* biosynthesis of JA, and Tet3 and Tet4 are relatively responsible for SA and ABA biosynthesis. However, it has also been shown that SA accumulation is significantly increased by application of Tet1 and Tet2 (Iida et al., 2019), suggesting that all members of the tetranins are more or less involved in induced SA biosynthesis. This prediction is consistent with the fact

Table 1 Characteristics of various plant defense responses induced by tetranins

	Ca^{2+} influx	ROS	JA	SA	ABA	*PR1*	VOCs	Remarks
Tet1	−	+	+	−	−	+	−	
Tet2	−	+	+	−	−	+	+	
Tet3	+	−	+	+	+	+	−	Host-dependent expression
Tet4	−	+	+	+	+	+	−	Host-dependent expression

The specific activity of tetranins in different defense responses is indicated by "+" for high and "−" for low. Since these only indicate the relative activity among four tetranin members, "−" does not mean that there is no activity.
ABA, abscisic acid; JA, jasmonate; ROS, reactive oxygen species; SA, salicylate; VOCs, volatile organic compounds.

that the expression of *PR1*, an SA-responsive gene (Javed et al., 2024), is induced by the application of these tetranins. Intriguingly, Tet3 and Tet4, which are strong inducers of JA and SA accumulation in *P. vulgaris* leaves (Figure 2A), do not have the ability to induce VOC emission. Instead, Tet2 is most likely responsible for VOC emission (Figure 2B), which plays a crucial role as an infochemical for indirect defense by attracting predatory mites such as *P. persimilis* (Iida et al., 2019). However, it should be noted that not all VOC emissions induced in *P. vulgaris* by *T. urticae* damage are induced by the application of Tet2, so it is likely that other uncharacterized members of the elicitor family are involved in *T. urticae*-induced VOC biosynthesis in addition to Tet2.

There is no doubt that several other elicitors and effectors of *T. urticae* act to coordinate the defense response in host plants. Given the polyphagous nature of the mite, which allows it to feed on an extensive range of over 1100 plant species (Jeppson et al., 1975), the allocation of the efficacy of these oral factors may be key to host adaptation and susceptibility. In addition to these oral factors, Ojeda-Martinez et al. (2021) showed that *T. urticae* egg extracts also exhibit elicitor activities—specifically, they influence the regulation of Ca^{2+} influx, ROS burst, JA- and ethylene-responsive gene expression, as well as VOC and glucosinolate biosynthesis in *Arabidopsis thaliana*. Moreover, Tu28 and Tu84 have been characterized as effectors that suppress defense responses in tomato (Villarroel et al., 2016). Similarly, SGPs 1, 2, and 5 may also act as potential effectors that cause a marginal decrease in *T. urticae* mortality in *P. vulgaris* leaves ($P \geq 0.05$, ANOVA with Dunnett's contrasts Figure 1A). Presumably, these elicitors and effectors function synergistically in *T. urticae*-infested plants, as shown among the tetranin members (Figures 1C and 3B).

Of great interest is the fact that the expression of the selective tetranin genes in *T. urticae* is variable depending on the host plant, as shown by *Tet3* and *Tet4*, which were expressed at high levels in *T. urticae* hosted on their preferred host, *P. vulgaris*, compared to the less-preferred host, *C. sativus*. Such different levels of the selective tetranin genes result in anti-mite response regulated predominantly by Tet3 and Tet4, including Ca^{2+} influx, ROS generation and *PR1* expression induction, but not VOC emissions regulated by Tet2 (Figure 5). In this context, it has been shown that the transfer of mites from *P. vulgaris* to non-preferred hosts such as *A. thaliana* or tomato results in transcriptional upregulation of 24% of genes, many of which are involved in digestion, detoxification, and xenobiotic transport (Grbic et al., 2011), likely leading to negative feedback regulation of *de novo* biosynthesis of SGPs. Taken together, these findings suggest that spider mites can survive on less-preferred or non-preferred hosts, where nutritional limitations or toxins affect their fitness, by activating the most effective genes, which, in turn, leads to reduced expression of SGP genes. Alternatively, it cannot be excluded that their reduction is strategic, thereby weakening defensive traits in host plants. It also makes sense that the distribution of oral factors in herbivores is strongly influenced by their host. For example, Yoshinaga et al. (2010) showed that the quality of the diet consumed by *Spodoptera litura* affects the composition of hydroxylated and non-hydroxylated compounds of FACs. Similarly, fall armyworm (*S. frugiperda*) larvae feeding on either cowpea or spinach plants produce different inceptin sequences in their oral secretions that affect *de novo* ethylene production in cowpea leaves (Schmelz et al., 2007).

In conclusion, we present two novel members of the tetranins, Tet3 and Tet4, as spider mite elicitors that stimulate both specific and non-specific plant defense signaling. These tetranins respond to variable host cues that may optimize herbivore fitness by altering the anti-mite status of the host plant. However, we emphasize that the evolutionary logic of tetranins and functional regulation remains to be elucidated: it is highly possible that tetranins are functional in mites for a reason other than elicitors in host plants.

EXPERIMENTAL PROCEDURES

Plants

Common bean (*Phaseolus vulgaris* L. cv. Nagauzuramame), cucumber (*Cucumis sativus* L. cv. Suyo), *Brassica rapa* L. var. *perviridis* (Natsurakuten), tomato (*Solanum lycopersicum* L. cv.

Odoriko), and maize (*Zea mays* L. cv. Royal Dent) plants were grown in soil in climate-controlled rooms for up to 2–3 weeks at 24 ± 1°C, with a photoperiod of 16 h and illumination of 80 μmol m^{-2} sec^{-1}.

Mites

Tetranychus urticae Koch (Acari: Tetranychidae) was reared under controlled conditions (see above). Detached leaf discs of approximately 25 cm^2 were obtained from *P. vulgari*s and placed on water-soaked cotton in petri dishes (90 mm diameter, 14 mm depth) at a constant temperature of 24 ± 1°C. Small (~1 cm^2) discs containing approximately 20 mites and eggs were transferred to fresh discs every 2 weeks. To culture mites on *C. sativus*, we followed the same method except using a different host plant and repeating the process for at least five generations. Adult female mites 10 days after oviposition were used for all major assays, except for RNAi analysis where adult female mites 7 days after oviposition were used.

Agrobacterium tumefaciens-mediated transient expression (agroinfiltration) in leaves and spider mite mortality assays

To identify new members of the tetranins, we focused on 95 SGPs characterized by Jonckheere et al. (2016). We then excluded putative enzymes and proteins previously analyzed by Iida et al. (2019) as our target, resulting in 25 SGP targets. Using mRNA obtained from adult female mites, we successfully amplified the cDNAs of 18 genes (Table S1). The corresponding SGP proteins were predicted to be truncated in the expressed cells, so we used the SignalP 4.0 program (Petersen et al., 2011) to determine which cDNAs lacked a signal peptide sequence at the N-terminus (Table S1).

To obtain the 18 SGP gene clones, 150 female *T. urticae* were homogenized in liquid nitrogen, and then the total RNA was then isolated and purified using Sepasol®-RNA I Super G (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's protocol. Using the 0.5 μg of total RNA and ReverTra Ace (Toyobo, Osaka, Japan), the open reading frame (ORF) of each SGP cDNA fragment without the signal peptide sequence was synthesized and amplified using KOD-Plus-Neo DNA polymerase (Toyobo) and primers listed in Table S1. The cDNA was then cloned into Gateway™ (GW) pDONR™/Zeo (Thermo Fisher Scientific, Waltham, MA, USA) by GW cloning and further transferred into binary vector pMDC32 (2× cauliflower mosaic 35S promoter::GW region::*nopaline synthase* [*NOS*] terminator). The plasmid, pMDC32-SGPs 1–18, or pMDC32, was transformed into *A. tumefaciens* strain EHA105 by electroporation. After overnight cultivation in 5 ml Luria-Bertani (LB) medium containing kanamycin and spectinomycin, the cells were cultivated in 30 ml of LB medium containing kanamycin and spectinomycin for 2–3 h before harvesting by centrifugation and resuspension in 10 mM MES buffer (pH 5.6) and 10 mM $MgCl_2$. The bacterial suspensions, adjusted to an OD_{600} of 0.6 and washed twice with MES buffer, were supplemented with acetosyringone to a final concentration of 150 μM before infiltration into one of the primary leaves of the individual *P. vulgaris* plants (500 μl) using a needleless syringe to obtain an infiltrated area of approximately 3 cm^2.

After a single day of bacterial infiltration, five-leaf discs (1.8 cm^2 each) were prepared from the affected area and 10 adult female mites were placed on each disc, in a 90 mm diameter plastic petri dish with wet cotton as a substrate. The petri dishes were then incubated for 48 h at 24 ± 1°C, under a 16-h photoperiod (80 μmol m^{-2} sec^{-1}). The number of dead mites on each disk was then counted when they did not respond to brushing. The mean of the dead mites from the five discs was taken as a single sample unit, with 11–12 independent sample units for the final replicate analysis. The data are presented as a normalization of mite mortality compared to the control treatment on the same day. This normalization helps to account for any discrepancies in the control data obtained on different test days.

RNAi

To construct the RNAi clones, cDNA fragments of *Tet3* (Tet3-Ri [200 bp]) and *Tet4* (Tet4-Ri [200 bp]) were amplified by PCR using cDNA clones (see above). The primers are listed in Table S2. The amplified fragments were then inserted into the GW vector pANDA (maize ubiquitin-1 gene promoter::GW region::GUS linker::GW region::*NOS* terminator) (Miki & Shimamoto, 2004). The constructed plasmids and the original pANDA plasmid (Ct-Ri) were transformed into *A. tumefaciens* strain EHA105 by electroporation.

For agroinfiltration, see details in the above section. Each *A. tumefaciens* line (Tet3-Ri, Tet4-Ri or Ct-Ri; 500 μl) or a mixture of Tet3-Ri and Tet4-Ri lines (250 μl + 250 μl) was infiltrated into one of the primary leaves of individual *P. vulgaris* plants. After 24 h, a leaf disc (25 cm^2) was cut and placed on wet filter paper for 24 h and 150 adult female mites (7 days post-oviposition) were released onto the leaf disc for an additional 2 or 3 days.

For RNA analysis, one leaf disc (1.8 cm^2) was challenged with 10 mites and sampled after 24 h for RNA extraction. For the determination of intracellular Ca^{2+} levels and intracellular/intercellular ROS levels as well as mite oviposition assays, the methods are described below.

Recombinant tetranin proteins

The ORF of each tetranin cDNA fragment without the signal peptide coding sequence or GFP was subcloned into the GW pDEST™15 vector (Thermo Fisher Scientific) (Table S1 and see Iida et al., 2019 for Tet1/2 and GFP cloning). The resulting vectors (pDEST15-Tet1/2/3/4) were transformed into *Escherichia coli* BL21-CodonPlus(DE3) (Thermo Fisher Scientific) to produce each of the recombinant tetranin proteins fused to glutathione-*S*-transferase (GST) at the N-terminus. Transformed bacterial strains were grown to an OD_{600} of 0.5 at 37°C in 2 l of LB medium with ampicillin at 100 μg ml^{-1}. Cultures were induced with a final concentration of 0.002% or 0.0002% (w/v) L-arabinose and incubated overnight at 18°C. Cells were pelleted by centrifugation and resuspended in 50 ml of GST binding buffer (20 mM NaH_2PO_4, 150 mM NaCl, 2 mM dithiothreitol, 5 mM EDTA, and 1 mM benzylsulfonyl fluoride [pH 7.0]). The resuspended cells were sonicated and cell debris was removed by centrifugation. Soluble proteins in the supernatant were purified using GSTrap FF (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's protocol. The purified proteins were finally concentrated with 20 mM HEPES buffer (pH 7.0) using an Amicon Ultra 4 ml centrifugal filter (Merck Millipore, Darmstadt, Germany).

Application of recombinant proteins with MD to leaves

MD was performed by rolling a stainless steel tracing wheel (Clover Mfg. Co., Ltd., Osaka, Japan) on mature leaves of potted plants (with 2-mm intervals between the MD spots). In total, about 50 MD spots were applied to one of the leaves for gene expression and hormone analysis. Otherwise, for VOC analysis, two primary leaves of *P. vulgaris* were each treated with approximately

50 MD spots. The recombinant tetranin or GFP proteins (0.1–20 μM) dissolved in 20 mM HEPES buffer (pH 7.0) were applied to the MD spots (approximately 1 μl per spot) immediately after MD application, and the plants were kept at 24 ± 1°C with a photoperiod of 16 h (80 μmol m^{-2} sec^{-1}).

RNA extraction, cDNA synthesis, and quantitative PCR

Approximately 100 mg of leaf tissue or approximately 150 adult female mites were homogenized in liquid nitrogen, and total RNA was isolated and purified using TRI-REAGENT® RNA/DNA/Protein Isolation Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo) and 0.5 μg of total RNA incubated first at 37°C for 5 min for the DNase reaction and second at 37°C for 15 min for the reverse transcriptase reaction. Real-time PCR was performed using a CFX Connect Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA) with THUNDERBIRD SYBR qPCR Mix (Toyobo) and gene-specific primers (Table S2). The following protocol was used: initial polymerase activation: 60 sec at 95°C; 40 cycles of 15 sec at 95°C and 30 sec at 60°C; and then melting curve analysis preset by the instrument was performed. Relative expression abundances were determined after normalization of raw signals with the expression abundance of *P. vulgaris* actin gene (*PvACT*), *C. sativus* actin gene (*CsACT*), and *T. urticae* actin gene (*TuACT*). To quantify tetranin RNA levels, calibration curves were generated using pDEST15-Tet1/2/3/4 as reference DNA, and tetranin RNA levels relative to the ribosomal protein 49 gene (*Rp49*) (Morales et al., 2016) were estimated according to the method in Ronnestad et al. (2010). Samples or data were discarded if abnormal quantification cycle (Cq) values were obtained.

Determination of intracellular Ca^{2+} levels and intracellular/intercellular ROS levels

Calcium Orange dye (stock solution in DMSO, Molecular Probes, Leiden, The Netherlands) was diluted to a final concentration of 5 μM in 5 mM MES-Na buffer (pH 6.0) and applied to attached leaves of *P. vulgaris* and *C. sativus* leaves as described in detail in Barbero et al. (2021). Fluorescence intensity in leaves treated with recombinant tetranin proteins was measured using a Leica TCS SP5 (Leica Microsystems Srl, Milan, Italy) multiband confocal laser scanning microscope (CLSM), which eliminated the need to separate the leaf from the plant for measurement. Five microliters of a 20 μM solution of GFP or tetranin proteins were applied to the leaves and the subsequent calcium signature was observed after an incubation period of 30 min, according to the method described previously (Brosset et al., 2021).

Similarly, leaves of potted plants were treated with 5 μl of 20 μM of the respective solutions of GFP or tetranin proteins after incubation with the dye 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red; Thermo Fisher Scientific) as described in a previous study (Brosset et al., 2021). The Amplex® Red reagent was then used in combination with horseradish peroxidase to detect hydrogen peroxide (H_2O_2) released from the leaves, with the reagent being dissolved in 50 mM MES buffer (pH 6.0) containing 0.5 mM calcium sulfate to a final concentration of 50 μM. After treatment, leaves were observed using a Leica TCS SP2 (Leica Microsystems Srl) microscope according to the method described in the previous study (Brosset et al., 2021). For calcium and ROS images, the parameters were set to 1024 × 1024 pixels, 8 bits per sample, and one sample per pixel. Analysis of the images generated by the FluoView software (Olympus, Tokyo, Japan) was performed using the NIH image software as previously demonstrated (Mithöfer et al., 2009).

To quantify the fluorescence levels in leaves challenged with *T. urticae*, small leaf discs (0.25 cm^2) obtained from primary leaves of *P. vulgaris* and *C. sativus* were placed on water-soaked cotton in petri dishes. Five adult female mites were then incubated on each leaf disc and removed after 2 h of incubation. The leaf discs were then incubated in the dark with 50 μl of either Calcium Orange dye (5 μM, see above) to assess Ca^{2+} changes or Amplex Red (50 μM, see above) to detect ROS for 30 min. Finally, the leaf discs were mounted on the stage of an Olympus FV3000 (Olympus) multiband CLSM for analysis. Image analysis was performed as described above.

Phytohormone analysis

Harvested leaves (60–100 mg fresh weight) were immediately frozen in liquid nitrogen and homogenized using five 2.3 mm zirconia beads in a 2 ml screw-capped microtubes (Sarstedt, Tokyo, Japan) containing 1 ml of ethyl acetate solvent spiked with deuterated internal standards (10 ng d3-JA, 20 ng d4-SA, and 10 ng d6-ABA). Phytohormone analysis was performed according to the previously described method (Tzin et al., 2017) with slight modifications.

VOC analysis

A potted *P. vulgaris* plant whose two primary leaves were treated with MD (approximately 50 MD spots per leaf) + Tet1, Tet2, Tet3, Tet4, or GFP protein solution (20 μM each) for 24 h (see above). Alternatively, 100 adult *T. urticae* females were placed on the primary leaves of *P. vulgaris* of a potted single plant for 24 h. VOCs from the potted plants were collected in a glass container (2 l) using Tenax 60/80 (Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany) in a laboratory room (24 ± 1°C, under light conditions) for 3 h. Clean air passed through the charcoal filter was drawn into the glass bottle, and VOCs from the headspace of the bottle were collected at a flow rate of 100 ml min^{-1}. *n*-Tridecane (100 ng), infiltrated on a piece of filter paper (1 cm^2) was added to the glass container as an internal standard at the beginning of the VOC collection. The collected volatile compounds were identified and quantified by gas chromatography–mass spectrometry according to the method described previously (Rim et al., 2017).

Mite oviposition assays

Three leaf discs (1.8 cm^2) were collected from a single *P. vulgaris* or *C. sativus* plant. An adult female *T. urticae* (10 days post-oviposition) was then transferred to each of the leaf discs in a plastic petri dish (90 mm diameter) which had been filled with wet cotton. The dish was incubated at 24 ± 1°C for 2 or 3 days, and the total number of eggs laid was counted. The mean of the three disks from the plant was calculated and used as a single independent replicate, and the final replicate analyses were performed with six independent samples. We did not use data from any mites that died during the assays.

Anti-tetranin antibody preparation and Western blot analysis

Antibodies (anti-Tet3 and anti-Tet4) were obtained by immunizing rabbits with the corresponding peptide antigens (YRRGNRRFR-DRADYYASRC and CGDFGGESYYDVHDYEF; Cosmo Bio, Tokyo Japan) and evaluated by Western blot analysis (Figure S7). Total proteins from 600 *T. urticae* adult female mites were extracted with 60 μl of Laemmli sodium dodecyl sulfate (SDS) sample buffer. The following solutions of extracted proteins (Tet3: 5 μl, Tet4: 7 μl) and *E. coli*-synthesized recombinant proteins (5 ng) were run on a 15% SDS-polyacrylamide gel electrophoresis (PAGE) gel and then transferred to a PVDF membrane (Bio-Rad). The anti-tetranin antibody was used as the primary antibody, and unlabeled anti-rabbit IgG (#3678, Cell Signaling Technology, Danvers, MA, USA) and horseradish peroxidase (HRP)-conjugated anti-mouse IgG (#7076) were used as the secondary and tertiary antibodies, respectively. The membrane was then immobilized with Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore Ltd.) and signals were detected using an ImageQuant LAS-4000 imaging system (GE Healthcare).

Statistical analyses

For pairwise analyses, we used Student's *t*-test. For multiple-sample analyses, we performed one-way ANOVA with Holm's sequential Bonferroni *post hoc* test or Tukey's *post hoc* honestly significant difference (HSD) for multiple-sample analyses using the multiple-sample comparison program (http://astatsa.com/OneWay_Anova_with_TukeyHSD/) and one-way ANOVA with Dunnett's contrasts using GraphPad Prism (GraphPad Software Inc, Boston, MA, USA). In addition, we performed two-way ANOVA with Tukey's HSD or Student's *t*-test as *post hoc* analyses for multiple-sample analyses using JMP software (ver. 16; SAS Institute Inc., NC, USA).

AUTHOR CONTRIBUTIONS

YE, MT, TU, KT, YD, and GiA planned and designed the research. YE, MT, TU, KT, YD, RO, SB, MEM, TS, and IG. performed experiments and analyzed data, etc. GiA wrote the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

SUPPORTING INFORMATION

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

Figure S1. Phylogenetic trees of deduced amino acid sequences of tetranins and other SGPs analyzed using MEGAX (https://www.megasoftware.net/). Tetranin proteins are highlighted in red.

Figure S2. *Tet3* and *Tet4* expression levels in *Tetranychus urticae* that had been incubated with Tet3 and Tet4 RNAi-expressing leaves (T3-Ri and T4-Ri, respectively) or control RNAi-expressing leaves of *Phaseolus vulgaris* (Ct-Ri).

Figure S3. *PR3* expression levels were measured in *Phaseolus vulgaris* leaves 24 h after application of GFP control (G), Tet3, or Tet4 proteins at concentrations of 5, 10, or 20 μM following mechanical damage.

Figure S4. The representative false-color image reconstructions of fluorochemical intracellular Ca^{2+} and reactive oxygen species (ROS) in leaves of *Cucumis sativus* or *Zea mays* plants at 30 min after MD with application of each tetranin protein or GFP control protein (20 μM).

Figure S5. The representative false-color image reconstructions of fluorochemical intracellular Ca^{2+} and reactive oxygen species (ROS) in leaves of *Phaseolus vulgaris* or *Cucumis sativus* plants, either undamaged (UD) or damaged by *T. urticae* hosted on *P. vulgaris* (Pv-mite) and *C. sativus* (Cs-mite), for 24 h.

Figure S6. Volatile organic compounds released by *Phaseolus vulgaris* (Pv) or *Cucumis sativus* (Cs) plants, either undamaged (UD) or damaged by *T. urticae* hosted on *P. vulgaris* (Pv-mite) and *C. sativus* (Cs-mite), for 24–27 h.

Figure S7. Immunoblotting of tetranin proteins.

Table S1. *Tetranychus urticae* putative secretory gland proteins (SGPs) focused on this study.

Table S2. Primers.

Table S3. A comprehensive analysis of the effect of tetranins on intracellular Ca^{2+} levels in *Phaseolus vulgaris*, *Cucumis sativus*, and *Zea mays* plants, using two-way ANOVA.

Table S4. Analysis of the effect of tetranins on intracellular Ca^{2+} levels in *Phaseolus vulgaris* (*Pv*), *Cucumis sativus* (*Cs*), and *Zea mays* (*Zm*) plants.

Table S5. A comprehensive analysis of the effect of tetranins on a reactive oxygen species burst in *Phaseolus vulgaris*, *Cucumis sativus*, and *Zea mays* plants, using two-way ANOVA.

Table S6. Analysis of the effect of tetranins on a reactive oxygen species burst in *Phaseolus vulgaris* (*Pv*), *Cucumis sativus* (*Cs*), and *Zea mays* (*Zm*) plants.

Table S7. A comprehensive analysis of tetranin gene expression in different mite sources using two-way ANOVA.

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