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# The phytoplasmal virulence factor TENGU causes plant sterility by downregulating of the jasmonic acid and auxin pathways

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**Despite plants infected by pathogens are often unable to produce offspring, it remains unclear how sterility is induced in host plants. In this study, we demonstrate that TENGU, a phytoplasmal virulence peptide known as a dwarfism inducer, acts as an inducer of sterility. Transgenic expression of TENGU induced both male and female sterility in *Arabidopsis thaliana* flowers similar to those observed in double knockout mutants of *auxin response factor 6* (*ARF6*) and *ARF8*, which are known to regulate floral development in a jasmonic acid (JA)-dependent manner. Transcripts of *ARF6* and *ARF8* were significantly decreased in both *tengu*-transgenic and phytoplasma-infected plants. Furthermore, JA and auxin levels were actually decreased in *tengu*-transgenic buds, suggesting that TENGU reduces the endogenous levels of phytohormones by repressing *ARF6* and *ARF8*, resulting in impaired flower maturation. TENGU is the first virulence factor with the effects on plant reproduction by perturbation of phytohormone signaling.**

Phytoplasmas (class *Mollicutes*, genus *Candidatus* Phytoplasma) are among the smallest bacterial plant pathogens transmitted by insect vectors. Phytoplasmas induce drastic malformation of plants such as witches' broom, dwarfism, phyllody (the transformation of floral organs into leaf-like structures), virescence (the greening of floral organs), and flower sterility[1,2]. Because phytoplasma-infected plants are often unable to produce offspring, phytoplasmas have been described as turning plants into zombies[3].

To date, four phytoplasmal effectors have been functionally characterized[4–7]. A virulence effector, phytoplasma tengu-su inducer (TENGU), was first identified from onion yellows phytoplasma (OY)[4]. The detection of TENGU in apical meristem tissue indicated that TENGU might be transported from the phloem sieve elements into neighboring tissues[4]. Expression of TENGU in *Arabidopsis thaliana* and *Nicotiana benthamiana* induced dwarfism and witches' broom symptoms and downregulated multiple auxin responsive genes, suggesting that disruption of the auxin signaling pathway might be the cause of these symptoms[4].

In *A. thaliana*, the maturation of both stamens and gynoecia is regulated by AUXIN RESPONSE FACTOR (ARF) 6 and ARF8[8,9]. Recent studies indicated that ARF6 and ARF8 act in a partially redundant manner and regulate anther dehiscence by inducing jasmonic acid (JA) production or by decreasing JA breakdown[9,10]. In addition, auxin has been implicated in the floral-bud development process almost through flower maturation[11].

Plant sterility caused by plant pathogens is an important symptom that has a significant impact on crop production[12]. Plants infected by many *Ca.* Phytoplasma species, such as '*Ca.* Phytoplasma solani', '*Ca.* P. oryzae', and '*Ca.* P. asteris' also have sterile flowers[13–15]. Some of these phytoplasmas induce virescence and phyllody in infected floral organs, which can lead to sterility. For example, *Ca.* P. solani-infected tomato plants exhibit drastic malformations of the stamen and carpels, which causes sterility[14,16]. By contrast, some phytoplasma strains belonging to '*Ca.* P. asteris' and '*Ca.* P. oryzae' induce sterility in both male and female flowers without floral malformations such as virescence and phyllody in particular host plants[4,13,15,17]. Despite these intriguing observations, how such sterility symptoms are induced in phytoplasma-infected plants remains unclear.

Here, we demonstrate that TENGU, a phytoplasmal virulence factor that causes dwarfism, also induces both male and female sterility without floral malformations in *A. thaliana*. TENGU significantly affects the expression

of flower maturation genes and alters JA and auxin syntheses in flowers, indicating that TENGU causes developmental defects that lead to sterility through modulation of the two phytohormones.

## Results

**TENGU arrests flower development.** We previously reported that both OY-infected and transgenic *A. thaliana* plants constitutively expressing TENGU exhibited dwarfism or witches' broom phenotypes[4]. In this study, we found that *tengu*-transgenic plants also showed sterility.

Whereas no difference in morphology was apparent between most wild-type Col-0 and *tengu*-transgenic lines during the vegetative stage (Fig. 1a), the majority of *tengu*-transgenic plants exhibited sterile phenotypes of varying degrees upon flowering. Approximately 37% of the *tengu*-transgenic plants exhibited severe sterility with closed flower buds and no seed production (Fig. 1, d and h). In the flowers of these severe-sterile lines of a 35S::*tengu* plant, anthers of infertile buds failed to dehisce and release pollen grains and stigmatic papillae in the carpels were somewhat shorter than those of wild-type flowers (Fig. 1, l, n, and p). By contrast, ~57% of the *tengu*-transgenic plants showed mild sterility with many opened flowers but extremely low seed production, as reported previously[4] (Fig. 1, e and i). The stigmatic papillae were as long as wild-type flowers, and anthers released pollen grains (Fig. 1, n, q, and m). In all OY-infected plants, flowers exhibited severe sterility with undehisced anthers and immature gynoecia, similar to severe-sterile 35S::*tengu* flowers (Fig. 1, c, g, k, and o).

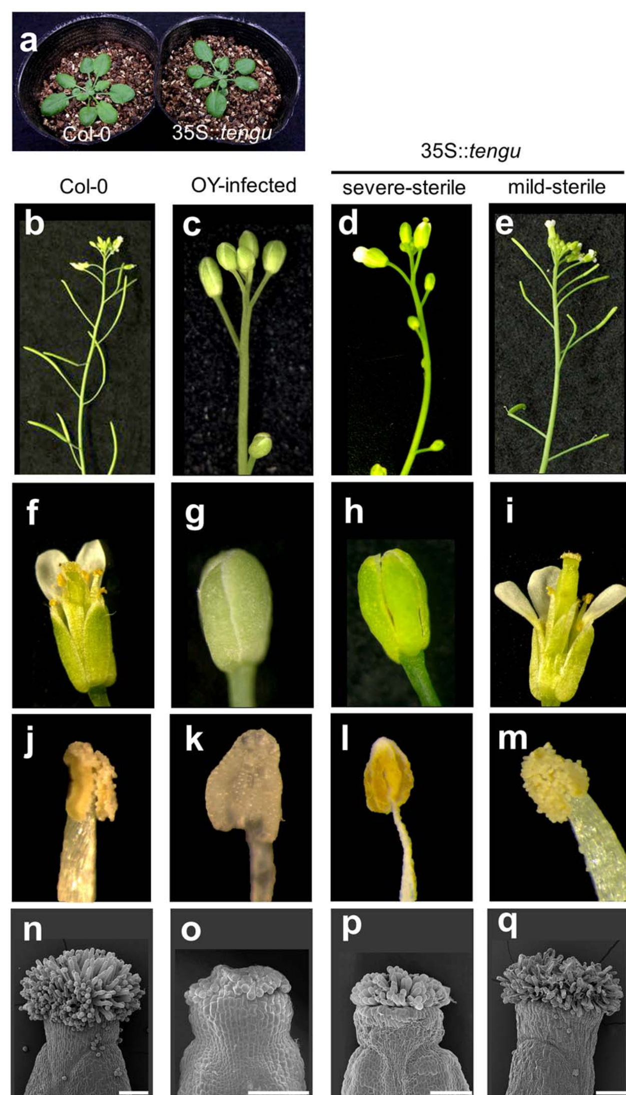


**Figure 1 | Sterility in phytoplasma-infected and *tengu*-transgenic plants.** (a), 27-day-old wild-type (Col-0) and 35S::*tengu* plants. (b–e), Inflorescence stems of Col-0 (b), onion yellows phytoplama (OY)-infected (c), and 35S::*tengu* plants exhibiting severe sterility (d) and mild sterility (e). (f–i), Flowers of Col-0 (f), OY-infected (g), and 35S::*tengu* plants exhibiting severe sterility (h), and mild sterility (i). (j–m), Anther development in mature Col-0 (j), OY-infected (k), and 35S::*tengu* plants exhibiting severe sterility (l) and mild sterility (m). (n–q), Scanning electron micrographs of apices of gynoecia of mature Col-0 (n), OY-infected (o), 35S::*tengu* plants exhibiting severe sterility (p), and mild sterility (q). Scale bars are 100 μm.

After self-crossing of 35S::*tengu* plants with severe sterility, the siliques of pistils of 35S::*tengu* did not elongate and almost all 35S::*tengu* fruits with severe sterility were seedless (Fig. 2a, upper panel). After self-crossing of 35S::*tengu* plant with mild sterility, the pistils frequently produced shorter siliques (Fig. 2a, middle panel). Among 171 fruits from 35S::*tengu* plants with mild sterility, 108 (63.2%) were fecund and 63 (36.8%) were infertile. In addition, *tengu*-transgenic pistils were pollinated with wild-type pollens using reciprocal hand-pollination (data not shown). Pollen grains from mild-sterile *tengu*-transgenic flowers were smaller with exine wall sculpturing (Fig. 2b), indicating that 35S::*tengu* plants showing mild sterility are male sterile.

To evaluate the effect of TENGU expression on the severity of sterility among transformants, we examined the levels of *tengu* mRNA in 35S::*tengu* plants using quantitative reverse transcription polymerase chain reaction (qRT-PCR). The level of *tengu* transcripts in 35S::*tengu* plants with severe sterility was significantly higher than that in 35S::*tengu* plants with mild sterility (Fig. 2c), indicating that the severity of sterility in the transgenic plants is determined in a TENGU-dependent manner. In subsequent studies, we used the 35S::*tengu* plants with severe sterility to investigate TENGU function.

**Altered expression of auxin response factor genes in TENGU transgenic flowers.** Two paralogous ARF genes, *ARF6* (*At1g30330*) and *ARF8* (*At5g37020*), are known to promote flower maturation[8,9,18]. Developmental abnormalities in *tengu*-transgenic plants were very similar to those observed in double knockout mutants of *ARF6* and *ARF8,* including the production of infertile closed buds, resulting from arrested flower development[9] (Fig. 1). To determine whether *ARF6* and *ARF8* are involved in the severe sterility phenotypes, we examined *ARF6* and *ARF8* expression levels in OY-infected and severe-sterile *tengu*-transgenic flowers using qRT-PCR analysis. Notably, the mRNA levels of both *ARF6* and *ARF8* significantly decreased in both the OY-infected and *tengu*-transgenic flowers (Fig. 3), suggesting that TENGU represses *ARF6* and *ARF8* gene expression. Despite the fact that transcripts of *ARF6* and *ARF8* are targeted by *microRNA (miR) 167* for cleavage[18], the levels of *miR167* were only slightly higher in the OY-infected and *tengu*-transgenic plants than in wild-type plants (see Supplementary Fig. S1 online), suggesting that the repression of *ARF6* and *ARF8* expression in the OY-infected and *tengu*-transgenic plants is not due solely to increased *miR167* transcription.

**Altered expression of floral maturation genes in TENGU transgenic flowers.** The expression of a gene encoding a putative enzyme involved in the jasmonate biosynthesis pathway, *LOX2* (*At3g45140*), is repressed in the flowers of *ARF6* or *ARF8* mutant plants[10]. Expression of the *MYB21* (*At3g27180*) and *MYB24* (*At5g40350*) genes is upregulated by JA and induced downstream of *ARF6* and *ARF8* expression[10]. Therefore, we examined the expression levels of these genes in OY-infected and *tengu*-transgenic flowers using qRT-PCR analysis. In *tengu*-transgenic plants, the level of *LOX2* mRNA significantly decreased (Fig. 4a). The level of *LOX2* also slightly decreased in the OY-infected plants compared with wild-type plants (Fig. 4a). Levels of *MYB21* and *MYB24* mRNAs were

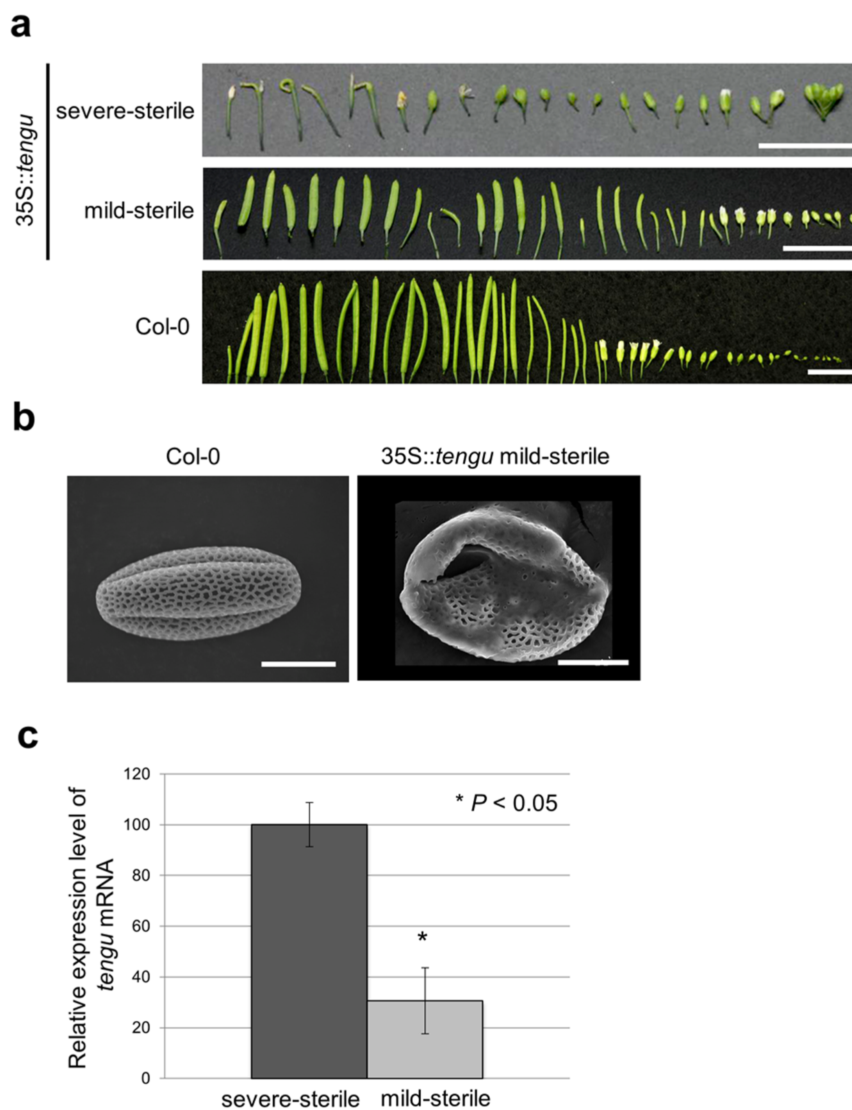


**Figure 2** | **Phenotypes of *tengu*-transgenic plants.** (a), Flowers and fruits of Col-0, and 35S::*tengu* plants exhibiting severe sterility, and mild sterility. Flower buds and fruits from a single inflorescence are arranged from youngest to oldest. Scale bars are 1 cm. (b), Scanning electron micrographs of pollen grains of wild-type and 35S::*tengu* flowers exhibiting mild sterility. Scale bars are 10 μm. (c), Relative *TENGU* gene expression in 35S::*tengu* plants exhibiting severe sterility and mild sterility. The mRNA levels were determined using quantitative RT-PCR, normalized to an internal *Actin2* gene control. Data are represented as the means of biological triplicates. Error bars represent the standard error of the mean. * indicates a significant difference (Student's *t*-test; $P < 0.05$) from 35S::*tengu* plants exhibiting severe sterility.

significantly lower in *tengu*-transgenic and OY-infected plants than in wild-type plants (Fig. 4b). The transcriptional repression of JA-related genes in OY-infected and *tengu*-transgenic plants were consistent with the previously observed expression patterns in *ARF6* and/or *ARF8* mutant plants.

**Reduction of endogenous phytohormone levels in TENGU transgenic flowers.** Given the very low JA concentration in flowers of the double knockout mutant of *ARF6* and *ARF8*[9,19], we examined whether TENGU downregulates JA synthesis. We determined the *cis*-JA and active jasmonoyl-isoleucine (JA-Ile) levels in wild-type and *tengu*-transgenic buds. The levels of both *cis*-JA and JA-Ile were significantly lower in *tengu*-transgenic buds than in wild-type buds (Fig. 5, a and b). By contrast, the *cis*-JA and JA-Ile levels in seedlings were not significantly different between *tengu*-transgenic and wild-type plants (Fig. 5, d and e), consistent with the high expression level of *ARF6* and *ARF8* in flowers[20]. A significant decrease in the total levels of *cis*-JA and JA-Ile in *tengu*-transgenic buds indicated that TENGU expression reduced JA synthesis.

TENGU was suggested previously to interfere with auxin signaling or biosynthesis pathways in *A. thaliana*[4]. Auxin presumably induces ARF6 and ARF8 activity by destabilizing auxin/indole-3-acetic acid (Aux/IAA) transcriptional repressor proteins[10,21,22]. To investigate whether TENGU affects the auxin production, we measured the IAA levels in wild-type and *tengu*-transgenic buds. The IAA levels were significantly lower in *tengu*-transgenic buds than in wild-type buds (Fig. 5c), suggesting that TENGU also reduced the endogenous levels of auxin.

## Discussion

Our data indicate that TENGU is an effector protein with pleiotropic effects on at least two phytohormones, auxin and JA, resulting in plant sterility. In this study, we demonstrated that transgenic expression of the phytoplasmal effector TENGU induced developmental abnormalities in Arabidopsis flowers similar to those in double knockout mutants of *ARF6* and *ARF8*, and that both *ARF6* and *ARF8* transcripts were significantly diminished in *tengu*-transgenic plants (Figs. 1-3). We further showed that JA and JA-Ile levels

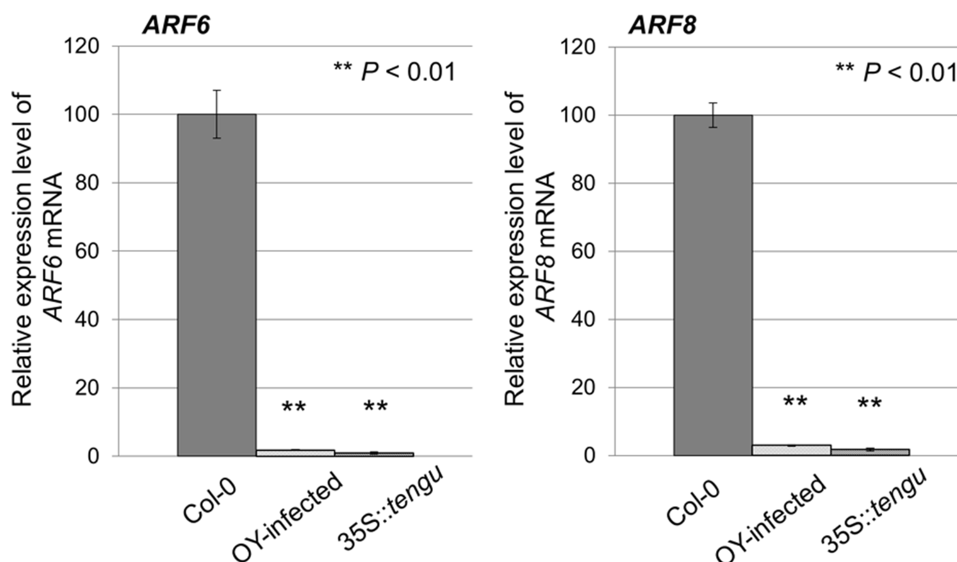


**Figure 3 | Relative expression of *ARF* genes.** Relative expression of *ARF6* and *ARF8* genes in OY-infected and *tengu*-transgenic plants. The mRNA levels were determined using qRT-PCR, normalized to an internal *Actin2* gene control. Data are represented as the means of biological triplicates. Error bars represent the standard error of the mean. Asterisk indicates significant differences (Student's *t*-test; $**P < 0.01$) from the wild-type (Col-0).

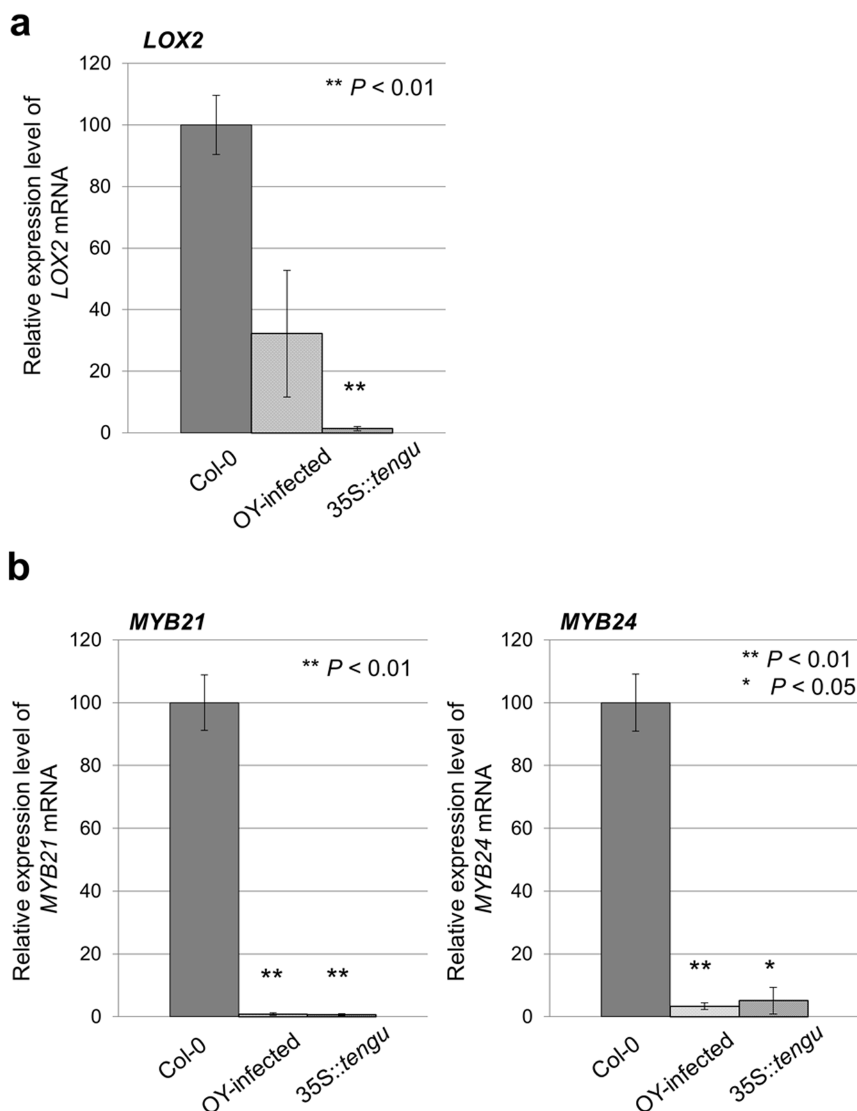


**Figure 4 | Relative expression of floral maturation genes.** Relative expression of *LOX2* (a), *MYB21*, and *MYB24* (b) genes in OY-infected and 35S::*tengu* transgenic plants. The mRNA levels were determined using qRT-PCR. Data are represented as the means of biological triplicates. Error bars represent the standard error of the mean. Asterisks indicate significant differences (Student's *t*-test; $**P < 0.01$; $*P < 0.05$) from the wild-type (Col-0).

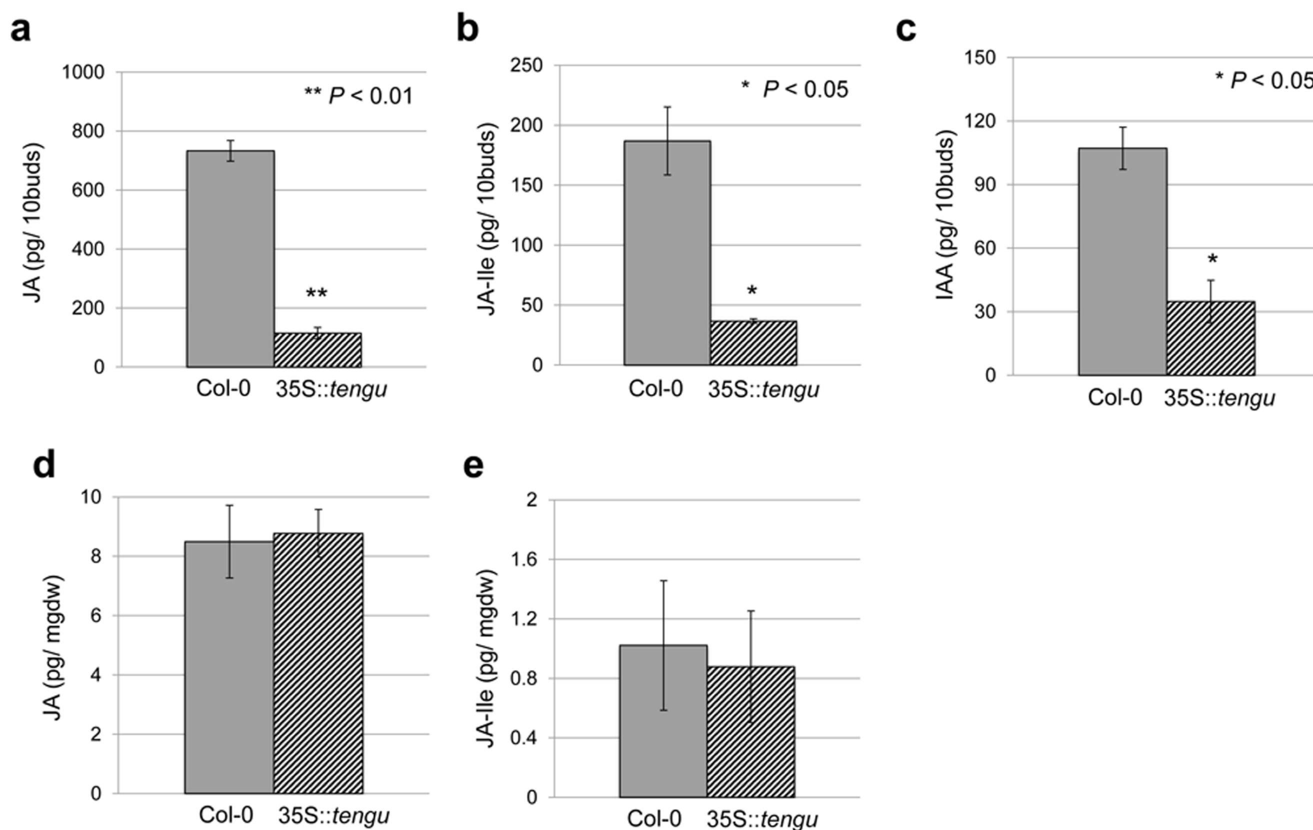


**Figure 5 | Quantification of the endogenous hormone levels.** Quantification of the endogenous phytohormone levels in buds (a–c) and in 10-day-old seedlings (d and e). (a and d), *cis*-JA, (b and e), JA-Ile, and (c), IAA. All values represent the means of four biological replicates ($n = 10$ buds). Error bars represent the standard error of the mean. Asterisks indicate significant differences (Student's *t*-test; $**P < 0.01$; $*P < 0.05$) between the wild-type and *tengu*-transgenics.

were significantly decreased in buds of *tengu*-transgenic plants (Fig. 5).

Since the IAA levels significantly decreased in the *tengu*-transgenic plants (Fig. 5c), repression of the auxin signaling pathway by TENGU may also contribute to plant sterility (Fig. 6). Understanding the roles of auxin in plant reproduction is quite limited, however, increased IAA content was shown to alter male development in tobacco[11] and auxin was shown to contribute to the coordination of male and female gametophytes in orchids[23]. Moreover, recent studies demonstrated that some *A. thaliana* mutants deficient in auxin biosynthesis genes exhibited infertile phenotypes[24]. TENGU is thought to downregulate early auxin-responsive genes[4], suggesting that repression of the auxin signaling pathway by TENGU may play a role in flower sterility.

Based on these observations, although it remains unclear the molecular interaction between TENGU peptide and its unknown target, we can formulate some hypothesis on the targets of TENGU. TENGU may target the auxin biosynthesis and/or signaling pathways resulting in the repression of *ARF6* and *ARF8* expression and the reduction of JA production. Auxin is presumed to induce ARF6 and ARF8 activity[10,21,22]. Another interesting possibility is that TENGU might recognize or interact with an unknown regulatory factor of ARF6 and ARF8 and finally interfere with the auxin signaling pathway. Recently, one of the Arabidopsis auxin biosynthesis genes *YUCCA9* was shown to be upregulated by JA[25], indicating that the JA pathway is associated with auxin homeostasis. Further studies are required to establish whether TENGU directly or indirectly represses *ARF6* and *ARF8* expression via reduced auxin production.

We demonstrated that *cis*-JA and JA-Ile levels were decreased in *tengu*-transgenic plants (Fig. 5). Moreover, both of the phytoplasmal effectors TENGU and SAP11, a secreted protein from the aster yellows strain witches' broom (AY-WB) phytoplasma, repressed JA biosynthesis in transgenic *A. thaliana*[26,27]. In plants, the JA signaling pathway plays a central role in regulating defense responses to insects[28]. Yang *et al.* (2008) demonstrated that the tomato yellow leaf curl China virus (TYLCCNV)-encoded protein βC1 suppresses a subset of JA-responsive genes, which contribute to accelerate the increase the insect vector population in TYLCCNV-infected plants[29]. This indicated that the manipulation of JA levels by plant pathogens might contribute to enhanced insect vector reproduction. In fact, SAP11 leads to reduced JA biosynthesis and an increase in the colonization ability of the AY-WB insect vector[27]. Based on the inhibition of JA synthesis by TENGU, our results suggest that phytoplasmas reduces the endogenous JA levels via effectors to attract their insect vectors.

Note that two phytoplasmal effectors regulate JA biosynthesis in a different manner. SAP11 destabilizes the TCP transcription factors (TEOSINTEBRANCHED, CYCLOIDEA, PROLIFERATION FACTOR 1 AND 2) that lead to the repression of JA biosynthesis. By contrast, we demonstrated in this study that the TENGU-induced reduction in JA synthesis is mediated by ARF6 and ARF8, but not by TCPs. TENGU-transgenic plants did not have serrated leaves caused by the repression of TCPs in SAP11-transgenic plants[27] (Fig. 1a). Moreover, ectopic expression of SAP11 in *A. thaliana* led to decreased JA synthesis only after wounding[27]. By contrast, ectopic expression of TENGU reduced the constitutive production of JA and JA-Ile even in healthy plants, and is not likely to be involved in wound-induced pathways (Fig. 5). Thus, the three different effectors βC1, SAP11, and TENGU decrease the JA levels through different mechanisms.

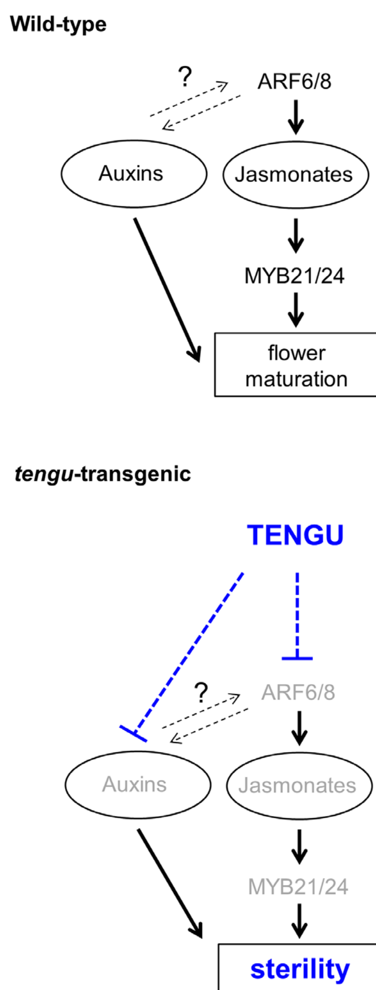


**Figure 6 | A model for TENGU-induced reduction of the endogenous levels of JA and auxin.** Black arrows represent mRNA, protein, or phytohormone products. Blue dashed T-bars indicate the suggested molecular events leading to the repression of *ARF* gene expressions and auxin synthesis. Gray letters represent negative effects of TENGU on each part of the floral maturation pathway.

JA and salicylic acid (SA) are important signaling molecules involved in various aspects of plant defense responses and the JA- and SA-mediated signaling pathways are mutually antagonistic[30]. Microarray analysis revealed that during phytoplasma infection, a marker gene of the SA signaling pathway, *pathogenesis-related proteins 1* (*PR-1*), was strongly induced in phytoplasma-infected grapevine, suggesting that SA is involved in the response to phytoplasma infection[31]. However, the expression of *PR-1* was not significantly induced in *tengu*-transgenic plants[4], indicating that repression of JA synthesis in *tengu*-transgenic flowers is not likely the result of antagonism between SA and JA.

We demonstrated that endogenous auxin content decreased in *tengu*-transgenic plants (Fig. 5), consistent with previously reported microarray data[4]. Similarly, several effectors from the well-studied pathogenic bacterium *Pseudomonas syringae* pv. *tomato* DC3000 have been shown to modulate phytohormone levels[32]. *P. syringae*-induced *miR393* negatively regulates mRNAs encoding auxin receptors thereby reducing auxin signaling, which is thought to antagonize the SA pathway[33]. By contrast, as discussed above, the reduction in endogenous auxin levels in *tengu*-transgenic plants appears to be independent of the SA signaling pathway, suggesting that TENGU may control the endogenous levels of the two phytohormones independent of SA.

Most of phytoplasma species make host plants unable to produce seeds by inducing flower sterility, phyllody, and virescence. In this study, we demonstrated that phytoplasma-induced male and female sterility is caused by perturbation of the JA and auxin signaling pathways. Disruption of JA synthesis may be beneficial for phytoplasmas by attracting more insect vectors and increasing the colonization of phytoplasmas themselves. However, it can also result in plant sterility, which may be disadvantageous to the prosperity of phytoplasmas. Therefore, TENGU may balance the advantages of insect attraction and the disadvantages of plant sterility to maximize the fitness of phytoplasmas that can survive in both insects and plants to live in. Similarly, severe phyllody leads to prolonged plant life span and efficient colonization by insect vectors[5,6]. Therefore, sterility of flowers and phyllody appear to be common features of the manipulation of plants by phytoplasmas to attract insect vectors.

In conclusion, we demonstrated the phytoplasmal effector TENGU induces plant sterility. TENGU causes not only morphological changes during vegetative growth, but also developmental defects upon flowering by modulating of endogenous two phytohormone levels, JA and auxin.

## Methods

**Plant Material and Growth Conditions.** *A. thaliana* accession Col-0 and transgenic plants were grown and maintained under a 15 h light/9 h dark photoperiod at 23°C. To inoculate *A. thaliana* with onion yellows phytoplasma (OY), plants with four to five rosette leaves were covered with plastic and mesh cages, and seven OY-infected leafhoppers (*Macrosteles striifrons*) were released into each cage and then removed after seven days. Infection with OY was confirmed in all inoculated plants by PCR. For phenotypic analysis, we used multiple lines of *tengu*-transgenic *A. thaliana* exhibiting sterility. We scored fruits that elongated to greater than 6.0 mm in length as fertile because plants that produce fruits that elongate to greater than 5.5–6.0 mm in length are scored as fertile in *A. thaliana*[8].

**Scanning Electron Microscopy (SEM).** Carpels and pollen grains were fixed in 0.2 M phosphate buffered 4% glutaraldehyde at pH7.2 overnight at 4°C, and then dehydrated through an ethanol series. The dehydrated specimens were immersed in isoamyl acetate. They were then critical point-dried under carbon dioxide (JCPD-5; JEOL, Tokyo, Japan), sputter-coated with platinum-palladium (Hitachi E-1030; Hitachi, Tokyo, Japan), and examined with a scanning electron microscope (SEM; Hitachi S-4000; Hitachi) at 10 kV.

**RNA Extraction and Northern Blot Analysis.** Total RNA was extracted from plants using ISOGEN reagent (Nippon Gene, Tokyo, Japan). Northern blot analysis of miRNAs was performed as described previously[34]. A primer specific to the *miR167* (5′-TAGATCATGCTGGCAGCTTCA-3′) was digoxigenin (DIG)-labeled using a DIG Oligonucleotide Tailing Kit, 2nd Generation (Roche Diagnostics, Mannheim, Germany) and used as a probe for northern blot hybridization.

**Gene Expression Analysis.** Real-time PCR assays were performed as described previously[35] with minor modifications. Fold changes were calculated using the expression of a housekeeping *ACTIN2* gene as an internal control. Three biological replicates were performed for each experiment. The *Actin2*[36] mRNA levels showed only minor variations between wild-type and *tengu*-transgenic inflorescence stems, similar to those of two previously used housekeeping genes, *U-box domain containing protein* and *EF-1α*[27,37]; therefore only the data normalized against the *Actin2* gene are presented in this study.

The gene-specific primer sequences (5′-3′) are as follows: qTENGU_F, TGATGATATTGAAAACGTGATAACTC; qTENGU_R, GCCCTTTTGCAATA-AATCTTG; qAtARF6_F, GTGGGATCGAGGACTCCAATC; qAtARF6_R, CCCG-ACGTATCAAGTCTCGG; qAtARF8_F, TCAAGAACTGATTGCAAGGGATC; and qAtARF8_R, GAGATGCCGTTTGGGCTG. *LOX2*, *MYB21*, and *MYB24* genes were amplified using primers described previously[10].

**Quantification of Phytohormones.** Phytohormones were prepared and quantified according to the method of Tokuda *et al.* (2013)[38]. IAA, JA, and JA-Ile were extracted and purified using solid-phase extraction. The stable isotope-labeled compounds $D_2$-IAA (Sigma-Aldrich, St Louis, MO, USA) and $D_2$-JA (Tokyo Kasei, Tokyo, Japan) were used as internal standards. $^{13}C_6$-JA-Ile was synthesized as described previously[39].

For quantification of phytohormones, we used stage 12-13 wild-type buds and *tengu*-transgenic infertile buds. To measure phytohormone levels simultaneously, 10 buds were lyophilized using a TissueLyser (Qiagen, Venlo, The Netherlands). Phytohormones were extracted and analyzed using a liquid chromatography-electrospray ionization-tandem mass spectrometry (LC/ESI/MS/MS; Agilent 6410; Agilent Technologies, Santa Clara, CA, USA) as described previously[40] and quantified using the MassHunter vB.03.01 spectrometer software (Agilent Technologies).

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## Author contributions

N.M., M.H., A.H., H.K., K.M., K.K., A.Y., Y.K. and S.N. designed research; N.M., A.H. and Y.T. performed research; N.M. and K.O. analyzed data; and N.M., M.H. and Y.Y. wrote the paper. All authors discussed the results and commented on the manuscript.

## Additional information

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