PLANT SCIENCE

Peptide signaling for drought-induced tomato flower drop

S. Reichardt[1], H.-P. Piepho[2], A. Stintzi[1]*, A. Schaller[1]*

The premature abscission of flowers and fruits limits crop yield under environmental stress. Drought-induced flower drop in tomato plants was found to be regulated by phytosulfokine (PSK), a peptide hormone previously known for its growth-promoting and immune-modulating activities. PSK formation in response to drought stress depends on phytaspase 2, a subtilisin-like protease of the phytaspase subtype that generates the peptide hormone by aspartate-specific processing of the PSK precursor in the tomato flower pedicel. The mature peptide acts in the abscission zone where it induces expression of cell wall hydrolases that execute the abscission process. Our results provide insight into the molecular control of abscission as regulated by proteolytic processing to generate a small plant peptide hormone.

The abscission of leaves, flowers, and fruits is a regulated process that is indispensable for both vegetative and reproductive plant development. Premature abscission of reproductive organs, however, reduces fruit set and crop productivity. Early flower drop is observed in many plant species when resources are limited and under conditions of environmental stress. Drought and heat cause premature flower and fruit drop, a problem likely to be exacerbated by global warming (*1*–*3*). Here we explore the function of a subtilisin-like protease in activating a small peptide that acts as a signal for flower abscission in tomato plants.

Insight into the regulatory mechanisms of organ abscission has been obtained mainly in two model systems: flower drop in tomato and the abscission of floral organs in *Arabidopsis* (*4*, *5*). The abscission of tomato flowers occurs at an abscission zone in the fruit stem (the pedicel; Fig. 1A) and is controlled by plant hormones. During undisturbed flower and fruit development, basipetal auxin transport results in a constant supply of auxin that keeps the abscission zone inactive to prevent abscission. When auxin flow is reduced upon fruit maturation, the abscission zone is sensitized to the action of ethylene (*5*, *6*), and ethylene signaling is then required to trigger abscission (*7*, *8*). In *Arabidopsis*, the abscission of sepals, petals, and stamens is delayed but not blocked in ethylene-insensitive mutants, indicating that ethylene controls the timing but is not indispensable for abscission in this system (*9*). Abscission of *Arabidopsis* flower organs rather depends on a small peptide that is proteolytically released from the INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) precursor by subtilisin-like serine proteinases (subtilases) (*10*, *11*). The mature IDA peptide activates a receptor complex comprising one of the two redundant receptor kinases HAESA or HAESA-like 2 and SERK co-receptors, to trigger a mitogen-activated protein (MAP) kinase cascade regulating the expression of hydrolytic and cell wall–modifying enzymes necessary for the breakdown of the pectin-rich middle lamella and cell separation (*12*–*16*). The IDA signaling pathway also mediates the environmentally controlled shedding of cauline leaves in *Arabidopsis* (*17*). By contrast, the molecular mechanisms for stress-induced abscission of flowers and fruits still are poorly understood.

To understand how peptide signaling is involved in stress-induced flower and fruit drop in tomato, we generated transgenic plants overexpressing different subtilases as candidate peptide precursor–processing proteases (*11*). Premature abscission of flowers was observed in plants overexpressing phytaspase 2 (*Sl*Phyt2, *Solyc*04g078740; Fig. 1, A and B, and fig. S1). When these plants were exposed to drought stress (fig. S2), flower drop increased to 70% as compared to 50% in the wild type (Fig. 1C). Flower drop reached only 20 to 30% in transgenic plants silenced for *SlPhyt2* expression (Fig. 1C and fig. S3), resulting in increased fruit set in *SlPhyt2* knockdown lines as compared to overexpressors and wild type (fig. S4). The extent of flower drop correlated with *Sl*Phyt2 expression and activity in knockdown and overexpressing lines (Fig. 2, A and B, and fig. S3), implying a function for *SlPhyt2* in drought-induced abortion of flower and fruit development in tomato. Indeed, *SlPhyt2* expression was induced in response to drought stress in flower pedicels proximal to the abscission zone and in the leaf vasculature (Fig. 2, C and D).

We further analyzed *Sl*Phyt2 function in an inflorescence explant bioassay. Removal of the auxin source by cutting off the flower triggers abscission in this system (*6*). Flower removal induced expression of *SlPhyt2* in the proximal pedicel (Fig. 3A) before the onset of abscission (Fig. 3C). Pedicel abscission was faster than normal in *SlPhyt2*-overexpressing plants and delayed in knockdown plants (Fig. 3C). The data mirror the drought-induced flower-drop phenotype observed in transgenic overexpressing and knockdown plants (Fig. 1). Thus, flower abscission is limited by *SlPhyt2* expression.

Next, we asked how *Sl*Phyt2 functions in relation to auxin and ethylene. We analyzed expression of early auxin-dependent genes that lead to the acquisition of ethylene sensitivity and activation of the abscission zone (*IAA3*, *ERF4*, *TPRP*) (*6*). Also included were regulatory genes in the late ethylene response (*ERT10*, *PK7*) (*6*), and tomato abscission-related polygalacturonase (*TAPG4*) as one of the

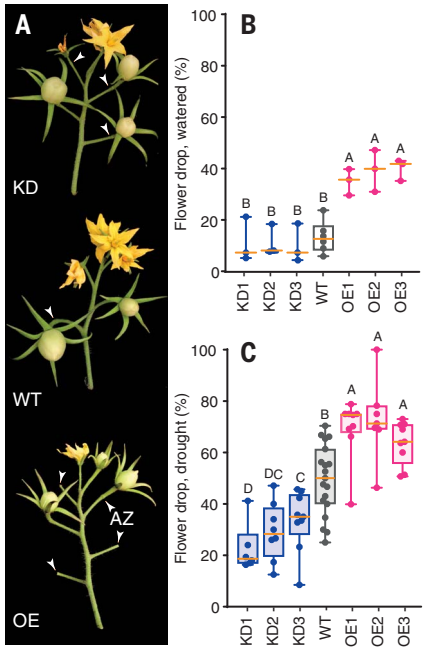

Fig. 1. Flower drop is enhanced under drought stress in a *Sl*Phyt2-dependent manner. (**A**) Inflorescence phenotype of *SlPhyt2*-silenced (knockdown, KD) and overexpressing plants (OE) compared to wild type (WT). OE plants abscise flowers prematurely at the pedicel abscission zone (AZ, arrowhead). (**B** and **C**) Flower drop was analyzed in KD (blue), OE (magenta), and WT (gray) plants under well-watered (B) and drought conditions (C). Flower drop was scored repeatedly until fruit set and is shown as the percentage of abscised flowers of all flowers or fruits per inflorescence (values for individual inflorescences are given in the raw data and statistics supplement). Each data point represents one experimental plant, showing the mean abscission value of all inflorescences on this plant [three plants for each of the transgenic lines and 6 wild-type plants in (A); at least 6 plants for the transgenic lines and 19 wild-type plants in (B)]. Total number of flowers and abscised flowers per genotype are given in the raw data supplement. Data were analyzed by fitting a generalized linear mixed model (GLMM). Genotype least squares means with a common letter are not significantly different (α = 0.05).

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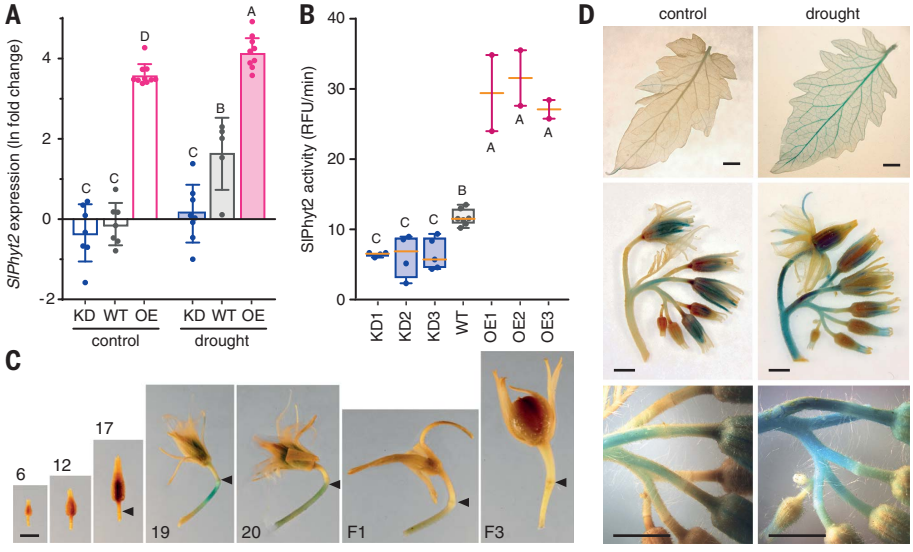

Fig. 2. *SlPhyt2* expression is induced by drought stress. (**A**) *SlPhyt2* expression is induced in leaves of drought-stressed (color-shaded bars) WT plants (gray) and overexpressing plants (magenta) but not in knockdown plants (blue); bars show mean expression levels ± SD as ln fold change relative to the watered WT control (open bars). (**B**) Phytaspase activity in cell wall extracts of leaves from drought-stressed plants is reduced in knockdown and increased in overexpressing plants as compared to wild type. Means (A) and medians (B) with no letter in common are significantly different (*t* test). (**C**) Histochemical staining of $SlPhyt2_{pro}$::GUS activity in the proximal pedicel of developing tomato flowers; numbers indicate developmental stages according to (*30*). Arrowheads mark the abscission zone. Early abscission was typically observed between flower stages 18 and 20. (**D**) GUS staining in leaves and inflorescences of control and drought-stressed plants. Scale bars in (C) and (D) represent 5 mm.

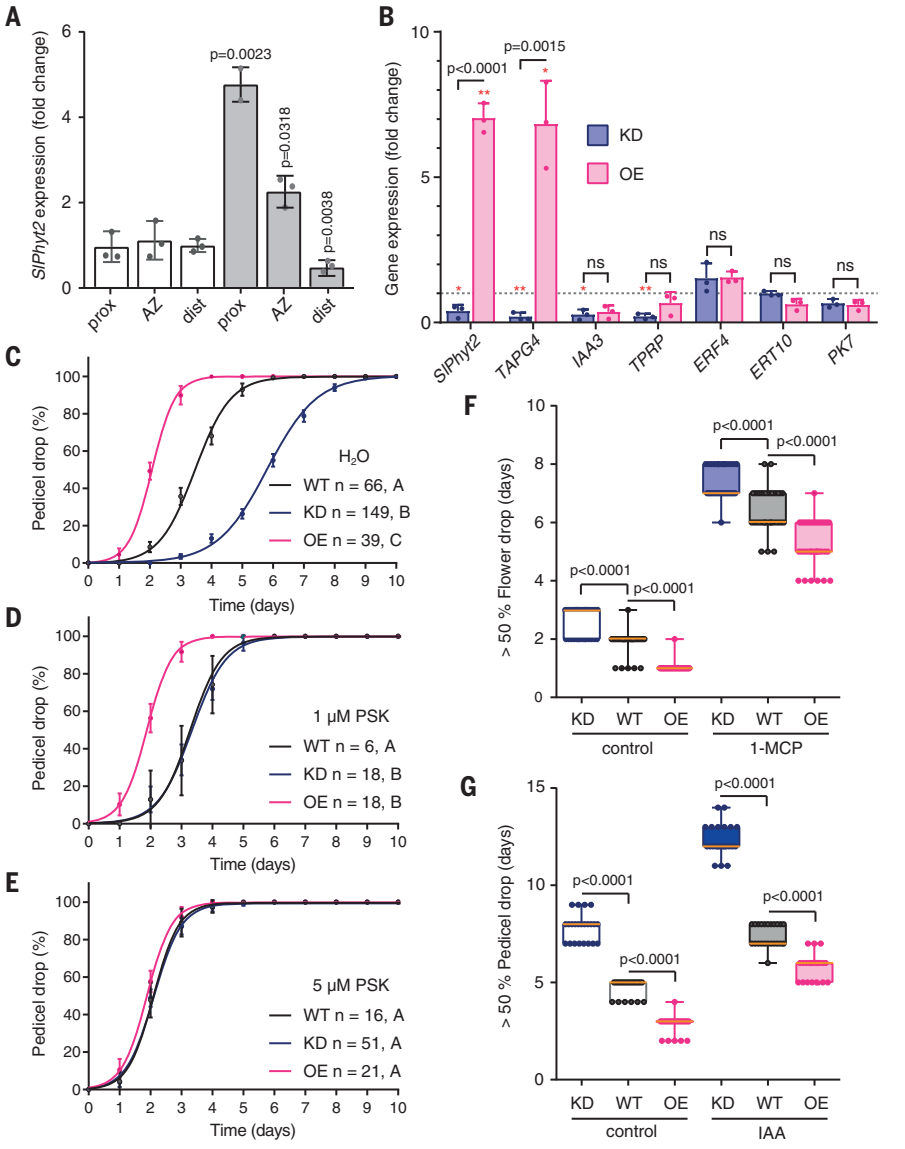

Fig. 3. Abscission is regulated by *Sl*Phyt2 and PSK in an auxin- and ethylene-independent manner. (**A**) Induction of *SlPhyt2* expression in the proximal (prox) compared to the distal (dist) pedicel after flower removal analyzed by quantitative polymerase chain reaction (qPCR) before (open bars, control) and 14 hours after flower removal (gray bars) normalized to abscission zone (AZ) control (mean ± SD; *P* values mark significant differences to the abscission zone control; *t* test). (**B**) qPCR analysis of *SlPhyt2* expression in abscission zone + 5 mm of the flanking pedicel compared to *TAPG4* and phytohormone response markers. Gene expression in knockdown (blue) and overexpressing plants (magenta) is shown relative to wild type normalized to 1 (dashed line; mean ± SD). Red asterisks mark significant differences between transgenic plants and wild type (*t* test). *P* values are shown for significant differences between knockdown and overexpressing plants (*t* test). (**C** to **E**) Pedicel abscission assayed over time for *SlPhyt2* knockdown, overexpressing, and WT plants in a detached-flower bioassay (error bars indicate 95% confidence interval; *n* = number of inflorescences analyzed; drop curves marked by the same letter are not significantly different at $\alpha = 0.05$). (C) Control in H_2O; (D) 1 µM PSK; and (E) 5 µM PSK. (**F** and **G**) Bioassay for abscission in knockdown, overexpressing, and WT inflorescence explants showing the time (day) until >50% of pedicels had abscised. (F) 1-MCP–treated inflorescences compared to controls in ambient air. (G) 50 µM indole-3-acetic acid (IAA)-treated inflorescences compared to solvent-treated controls; Mann Whitney test based on ranks.

executors of abscission (*6*). *TAPG4* transcript levels correlated with higher expression of *SlPhyt2* in the abscission zone and pedicel of overexpressing plants and with reduced expression in knockdown plants as compared to wild type (Fig. 3B). Expression of early auxin- and late ethylene-dependent genes was unaffected in the transgenic lines (Fig. 3B). Likewise, there was no difference in ethylene emission or concentration of the ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid) in *SlPhyt2* knockdown and overexpressing plants as compared to the wild type (fig. S5). Furthermore, treatment with the ethylene antagonist 1-MCP (1-methylcyclopropene) or resupply of auxin to the cut surface of the pedicel delayed abscission to a similar extent in the transgenic lines and wild-type plants, although the differences in the rate of abscission between knockdown, overexpressing, and wild-type plants persisted (Fig. 3, F and G, and figs. S6 and S7). *TAPG4* expression may thus be controlled by *Sl*Phyt2 in an auxin- and ethylene-independent manner, possibly by a peptide hormone.

To identify the hypothetical peptide, we analyzed the substrate specificity of *Sl*Phyt2. We added to this subtilase a C-terminal hexa-His tag, expressed the construct in *Nicotiana benthamiana*, and purified the tagged protein from cell wall extracts by metal chelate affinity chromatography (fig. S8A) and gel filtration. Substrate specificity of the recombinant protein was analyzed in a proteomics assay (Proteomics Identification of Cleavage Sites, PICS) (*18*, *19*). Using a substrate library of more than 10,000 peptides, we found that *Sl*Phyt2 was selective for Asp in P1 (the position immediately upstream of the scissile bond) and showed a preference for hydrophobic amino acids both upstream and downstream of the cleavage site (in P2, P3, and P2′; Fig. 4A). The precursors of known peptide hormones were scanned for this recognition motif, resulting in the identification of two candidate *Sl*Phyt2 substrates: the precursor of systemin, an 18–amino acid peptide involved in the wound response and herbivore defense signaling in tomato (*20*), and the precursor of phytosulfokine (PSK), a disulfated pentapeptide that regulates plant growth (*21*). Although prosystemin is processed by *Sl*Phyt2 in an Asp-specific manner in vitro (*22*), we did not observe any defect in wound signaling or herbivore defense in *SlPhyt2* knockdown plants (fig. S9), excluding prosystemin as a physiologically relevant *SlPhyt2* substrate in vivo. We therefore addressed the possibility that *Sl*Phyt2 is responsible for maturation of PSK as a signal for pedicel abscission in tomato.

There are eight genes in the tomato genome encoding precursors of PSK, all having Asp in P1 upstream of the conserved PSK sequence, and hydrophobic amino acids in P2 (Leu) and P2′ (Ile; Fig. 4B). Several of these genes (*SlPSK1, SlPSK4*, and *SlPSK6*) are expressed in abscission zones with highest expression levels for *SlPSK1* (fig. S10A). We found that expression of *SlPSK1* and *SlPSK6* is coinduced with *SlPhyt2* by drought stress (fig. S10B). A synthetic, extended PSK peptide comprising the disulfated PSK pentapeptide [(sY)I(sY)TQ] and five additional precursor-derived amino acids at its N terminus (EAHLD) was cleaved by *Sl*Phyt2 in an Asp-specific manner, releasing mature PSK in vitro (Fig. 4C and fig. S11). Substitution of the cleavage-site Asp by Ala rendered the PSK precursor peptide resistant to proteolytic cleavage by *Sl*Phyt2, indicating that Asp is required for cleavage site recognition and processing (Fig. 4D and fig. S11).

Mature PSK induced pedicel abscission in the inflorescence explant bioassay in a dose-dependent manner. At 5 μM PSK, the response was saturated and indistinguishable in PSK-treated knockdown, wild-type, and overexpressing plants (Fig. 3, C to E). PSK treatment also induced expression of *TAPG2* and *TAPG4* and down-regulated the expression of genes that maintain the abscission zone in an inactive state (Fig. 4E). The data verify PSK as a signal for pedicel abscission in tomato and suggest a role for *Sl*Phyt2 in precursor processing and PSK maturation in vivo. The requirement of *Sl*Phyt2 for PSK biogenesis was confirmed in the detached flower bioassay. In wild-type inflorescences, the N-terminally extended PSK precursor peptide induced pedicel abscission, whereas the protease-resistant variant of PSK was inactive (Fig. 4F and fig. S12). In *Sl*Phyt2-deficient knockdown plants, both resistant and cleavable precursor peptides were inactive, indicating that the cleavage site Asp and *Sl*Phyt2 are both required for biogenesis of the PSK abscission signal (Fig. 4F and fig. S12).

Drought stress–induced coexpression of *SlPhyt2* and *PSK* precursor genes in the pedicel, Asp-dependent cleavage of the precursor peptide by recombinant *Sl*Phyt2 in vitro, flower-drop in *SlPhyt2* overexpressors, and the inability of *SlPhyt2*-silenced plants to respond to PSK precursor peptides confirm *Sl*Phyt2 as the subtilase processing the PSK precursor into active PSK peptide. Unlike other plant proteases known to convert precursors into active peptide growth factors (*11*, *23*, *24*), *Sl*Phyt2 has a regulatory function in signal biogenesis. We propose (model, fig. S13) that stress-induced

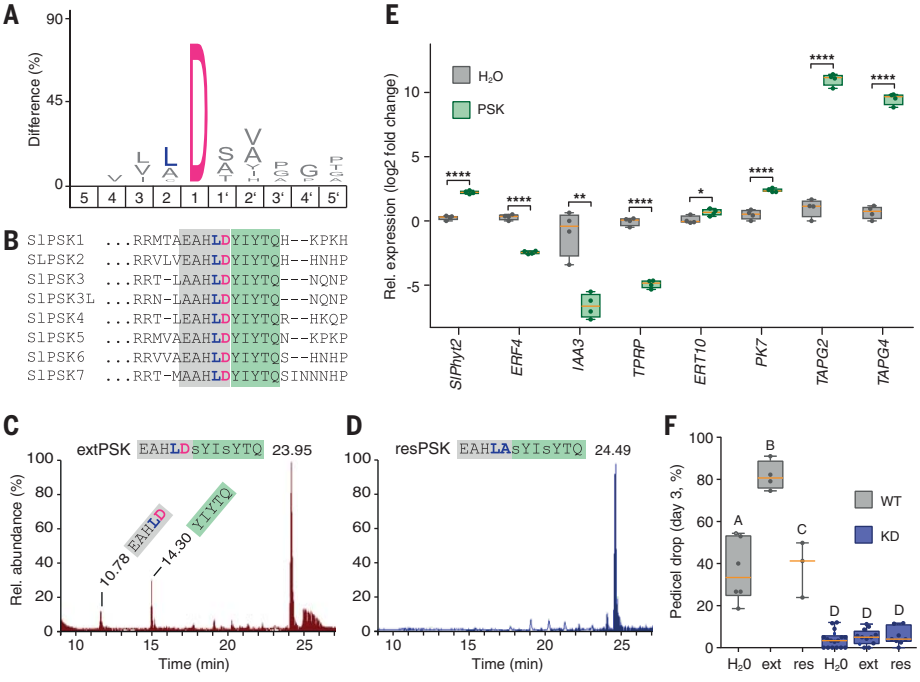

Fig. 4. Formation of PSK as an abscission signal depends on Asp-specific cleavage of the precursor by *Sl*Phyt2 in vivo. (**A**) iceLogo showing amino acid residues preferred by *Sl*Phyt2 upstream (positions 1 to 5) and downstream (positions 1′ to 5′) of the cleavage site. (**B**) C terminus of the eight PSK precursors encoded in the tomato genome; sequence of the PSK peptide highlighted in green. (**C** and **D**) Ion chromatograms showing cleavage products generated by *Sl*Phyt2 from N-terminally extended PSK (extPSK) (C) and a phytaspase-resistant D-to-A variant of the same peptide (resPSK) (D). (**E**) qPCR expression analysis of *TAPG* and phytohormone response marker genes in abscission zones of PSK-treated (5 μM; green) compared to control inflorescences (gray). (**F**) Abscission bioassay showing the percentage of pedicel drop on day 3 after flower removal in WT (gray) and knockdown inflorescences (blue) treated with extPSK (ext), resPSK (res) or water (H_2O). Treatments sharing no letter are significantly different (*t* test). Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

flower drop is controlled by the subtilase *Sl*Phyt2, expressed in the pedicel for PSK production. PSK then drives abscission by induction of cell wall hydrolases in the abscission zone.

Thus, we add the peptide PSK to the suite of known abscission signals. Developmental abscission of ripe fruits is controlled by the phytohormones auxin and ethylene, and possibly by the IDA peptide (*25*). Premature flower drop in response to environmental stress, an event of agricultural concern, is triggered by PSK in tomato. How PSK interferes with auxin and ethylene-mediated regulation of abscission zone activity remains to be investigated. IDA is unlikely to contribute to stress-induced flower drop. There is no phytaspase cleavage site in tomato IDA precursors. Also, expression of the five *IDA* precursor genes is very low in abscission zones (fig. S10A) and unresponsive to drought stress (fig. S10B). PSK is known for its growth-promoting activity (*21*, *26*, *27*), which may be as relevant to abscission as PSK-induced cell separation. Enlargement of abscission zone cells provides the shear force for organ detachment after hydrolysis of the middle lamella (*4*, *25*). The induction of cell expansion and expression of cell wall hydrolases by the PSK peptide thus may both contribute to the execution of abscission. Although PSK is found in both monocots and dicots, phytaspases, the subtype of subtilases that includes *Sl*Phyt2, are less broadly distributed. An expanded phytaspase clade is found in the nightshade family (the Solanaceae, including tomato and potato) and a few other eudicot families (Ranunculaceae, Fabaceae, Lamiaceae, and Phrymaceae) but is absent from other families (e.g., Brassicaceae) (*18*, *28*, *29*). Whether PSK-mediated regulation of abscission is restricted to the phytaspase-bearing lineages or is more widely distributed in flowering plants remains an open question.

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SUPPLEMENTARY MATERIALS

science.sciencemag.org/content/367/6485/1482/suppl/DC1
Materials and Methods
Figs. S1 to S13
References (*31*–*49*)
Raw Data and Statistics

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Fruit abscission in response to drought

Plants faced with drought, or simply not quite enough water, may be more likely to drop their fruit prematurely. Reichardt *et al.* found that a small signaling peptide hormone, phytosulfokine, which was previously known for its ability to regulate plant cell growth, also drives fruit abscission. Processed, and thus activated, by a subtilisin-like protease, phytosulfokine in turn drives expression of the hydrolases that degrade the cell walls in the abscission zone, leading to dropped fruit.

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