Recognition of floral homeotic MADS domain transcription factors by a phytoplasmal effector, phyllogen, induces phyllody

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Received 27 December 2013; revised 14 February 2014; accepted 19 February 2014; published online 6 March 2014.
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SUMMARY

Plant pathogens alter the course of plant developmental processes, resulting in abnormal morphology in infected host plants. Phytoplasmas are unique plant-pathogenic bacteria that transform plant floral organs into leaf-like structures and cause the emergence of secondary flowers. These distinctive symptoms have attracted considerable interest for many years. Here, we revealed the molecular mechanisms of the floral symptoms by focusing on a phytoplasma-secreted protein, PHYL1, which induces morphological changes in flowers that are similar to those seen in phytoplasma-infected plants. PHYL1 is a homolog of the phytoplasmal effector SAP54 that also alters floral development. Using yeast two-hybrid and in planta transient co-expression assays, we found that PHYL1 interacts with and degrades the floral homeotic MADS domain proteins SEPALLATA3 (SEP3), APETALA1 (AP1) and CAULIFLOWER (CAL). This degradation of MADS domain proteins was dependent on the ubiquitin–proteasome pathway. The expression of floral development genes downstream of SEP3 and AP1 was disrupted in 35S::PHYL1 transgenic plants. PHYL1 was genetically and functionally conserved among other phytoplasma strains and species. We designate PHYL1, SAP54 and their homologs as members of the phyllody-inducing gene family of ‘phyllogens’.

Keywords: floral development, MADS domain proteins, floral quartet model, Arabidopsis, phytoplasma, phyllody.

INTRODUCTION

Plants display a diverse range of disease symptoms upon pathogen infection. The major cause of pathogen-induced abnormal plant development is disruption of signaling mechanisms mediated by plant hormones such as jasmonates, auxin, abscisic acid and gibberellins (Grant and Jones, 2009). Plant defense responses are also tightly associated with the development of disease symptoms. Necrotrophic pathogens alter plant defense responses by secreting toxins that cause plant cell death, allowing the pathogens to absorb nutrients from dead cells (Lorang et al., 2007). Plant viruses disrupt plant development by expressing RNA silencing suppressors that inactivate endogenous small RNAs that are responsible for the proper regulation of organ development (Dunoyer et al., 2004).

Phytoplasmas (class Mollicutes, genus Phytoplasma) are cell wall-less plant-pathogenic bacteria that induce various developmental abnormalities in plants, including dwarfism, ‘witches’ broom’ (proliferation of small branches resulting in a characteristic bushy look), ‘purple top’ (purple coloration of leaves and stems), phyllody (formation of leaf-like structures instead of floral organs), virescence (green coloration of floral organs), and a loss of floral meristem determinacy (emergence of secondary flowers) (Hogenhout et al., 2008). Phytoplasmas are transmitted from plant to plant by insect vectors, mainly leaf-hoppers and psyllids, and cause devastating yield losses in diverse crops (Maejima et al., 2014). Despite their economic importance, phytoplasmas remain one of the most poorly characterized plant pathogens, primarily due to a
lack of methods for in vitro culture, gene delivery and mutagenesis (Firrao et al., 2007).

Many plant-pathogenic bacteria modulate host responses by delivering effector proteins into host cells via the type III secretion system (Abramovitch et al., 2006). While the phytoplasma genome lacks homologs of the type III secretion system (Oshima et al., 2004), effector proteins are secreted from phytoplasmas via the Sec translocation system and function directly in the cytoplasm of host cells (Hogenhout and Loria, 2008). Although phytoplasmas have none of the known effector genes found in other phytopathogenic bacteria, genes for approximately 50 putative secreted proteins have been identified in the phytoplasma genome (Sugio et al., 2011). Some of these proteins have been shown to function as effectors that cause abnormal plant morphology. A small secreted peptide (TENGU) encoded by Onion Yellows phytoplasma (Phytoplasma asteris, OY strain; mild-symptom line) was identified as a phytoplasma effector that affects plant morphology (Hoshi et al., 2009). The expression of TENGU in plants inhibits auxin-related pathways, thereby resulting in a dwarf, bushy phenotype similar to the tenu-su symptoms seen in phytoplasma-infected plants. SAP11, an effector of Aster Yellows Witches Broom phytoplasma (‘Ca. P. asteris’, AY–WB strain), induces crinkled leaves and a bushy morphology, and enhances insect vector fitness by blocking jasmonic acid biosynthesis in plants (Sugio et al., 2011). Recently, an AY–WB effector, SAP54, was reported to cause morphological changes in Arabidopsis thaliana flower organ development that were similar to characteristic symptoms seen in phytoplasma-infected plants (Maclean et al., 2011). Although the molecular mechanisms remain to be determined, it was assumed that effectors secreted by phytoplasmas interfere with the function of genes involved in flower development.

Flower development in A. thaliana involves a sequence of steps: (i) transition from the vegetative phase to the reproductive phase (formation of an inflorescence meristem), (ii) establishment and maintenance of floral meristem identity (stages 1 and 2), and (iii) development of floral organs (after stage 3) (Alvarez-Buylla et al., 2010). In step (iii), the identity of each floral organ is determined by a specific combination of floral homeotic genes constituting the ABCE model (Pelaz et al., 2000; Honma and Goto, 2001; Theissen and Saedler, 2001). Most of these homeotic genes encode members of the MADS domain family of transcription factors, of which the class E SEPALATA3 (SEP3) and class A APETALA1 (AP1) genes have critical roles. In step (iiii), they repress transcription of flowering-time genes including SHORT VEGETATIVE PHASE (SVP), SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1) and AGAMOUS-LIKE 24 (AGL24), which are involved in steps (i) and (ii) (Gregis et al., 2008; Imrink et al., 2012). The repression of the flowering-time genes contributes to prevention of the outgrowth of ectopic secondary flowers (Liu et al., 2007). Subsequently, SEP3 and AP1 up-regulate the class B genes APETALA3 (AP3) and PISTILLATA (PI) (Ng and Yanovsky, 2001; Wu et al., 2012), which are required for specifying petals and stamens, thereby inducing floral organ development.

Phytoplasmas cause distinct changes in floral morphology, including phyllody, virescence and loss of floral meristem determinacy. Although previous studies have shown that expression levels of the floral development genes were altered in phytoplasma-infected plants (Pracros et al., 2006; Cettul and Firrao, 2011; Himeno et al., 2011; Su et al., 2011), the molecular mechanisms underlying these changes in floral morphology remain poorly understood.

In this study, we identified a homolog of SAP54 from OY–W phytoplasma (‘Ca. P. asteris’, OY strain; wild-type line) called PHYL1 that also induces phyllody-like floral abnormalities. We found that PHYL1 interacts with and induces ubiquitin–proteasome-dependent degradation of the MADS domain proteins SEP3, AP1 and CAULIFLOWER (CAL), leading to inhibition of their functions. We also found that PHYL1 homologs from other phytoplasma species retain the ability to interact with and induce degradation of MADS domain proteins, suggesting a role for degradation of MADS domain proteins in the changes in floral morphology generally caused by phytoplasma infection.

RESULTS

The phytoplasma effector PHYL1 induces phyllody-like symptoms, and its N-terminal region is required for the activity

Periwinkle Catharanthus roseus and petunia Petunia x hybrida plants infected with OY–W phytoplasma show characteristic floral symptoms such as phyllody, virescence and loss of floral meristem determinacy (Figure S1) (Shiomi et al., 1996; Himeno et al., 2011). To screen for phytoplasma effectors that induce these floral symptoms, we identified putative secreted proteins encoded in the OY–W genome based on in silico sequence analysis (Hoshi et al., 2009), and produced transgenic A. thaliana plants that constitutively express each of the candidate proteins. During the screening, a putative secreted protein, SAP54, from AY–WB phytoplasma was shown to be an effector that is responsible for changes in floral morphology (Maclean et al., 2011). In the putative secreted proteins in the OY–W genome, we found a SAP54 homolog that shares a high level of amino acid identity (88%) (GenBank accession number AB812838).

Transgenic A. thaliana plants expressing the homolog exhibited phyllody-like morphological changes in their flowers (Figure 1b–d and Figure S2a–f). We designated the encoding gene phytoplasmal effector causing phyllody symptoms 1 (PHYL1). The PHYL1 gene encodes a 125
amino acid protein with a 34 amino acid signal peptide at its N-terminus (Figure 1a), which was predicted to be secreted from phytoplasma cells as a 91 amino acid, mature 10.6 kDa protein. Quantitative real-time RT-PCR analysis showed that the expression level of PHYL1 was approximately threefold higher in plants than in insects (Figure S3), suggesting a significant role for PHYL1 in infected plants. All floral organs in the 35S::PHYL1 transgenic plants were severely affected: sepals were converted into leaf-like organs covered with stellate trichomes, and petals were completely virescent (Figure 1b); the anthers failed to dehisce, and the pistil reverted to an inflorescence stem (Figure 1b). Moreover, a loss of floral meristem determinacy was observed. Several secondary flowers formed at the top of the pistil and from the axils of each leaf-like sepal (Figure 1c,d and Figure S2a), with a phenotype almost identical to that of the primary flower (Figure S2b). Some lines had undifferentiated inflorescence meristems and leaf-like sepals (Figure S2c,d). Scanning electron microscopy analysis showed that secondary floral organs formed in the swollen carpels (Figure S2e), and that tertiary flowers formed in the secondary flowers (Figure S2f).

In contrast to the dramatic morphological changes in the flowers, the flowering time and vegetative-stage phenotypes in the 35S::PHYL1 transgenic plants were similar to those in wild-type A. thaliana plants (Figure S4). The PHYL1 gene was confirmed to be expressed in each transgenic line, and was relatively highly expressed in line 10 (Table S1).

Strikingly, all ten lines of transgenic plants expressing PHYL1Δ8, which lacks the N-terminal eight amino acids of the secreted form of PHYL1, displayed a floral phenotype that was indistinguishable from that of wild-type plants (Figure 1e,f), whereas transgenic plants expressing the PHYL1 N-terminal deletion mutant series PHYL1Δ3 to PHYL1Δ7 showed floral morphological changes similar to those of the 35S::PHYL1 lines, including sepals covered with stellate trichomes (Figure 1g and Figure S2g-j). These results demonstrate an important role for the eighth amino acid residue in the N-terminus of the mature PHYL1 protein in induction of morphological changes in flowers.

**PHYL1 interacts with MADS domain proteins**

We found that the characteristic floral phenotypes observed in 35S::PHYL1 transgenic A. thaliana plants (e.g. phyllody, virescence and loss of floral meristem determinacy) were similar to those observed in loss-of-function mutants of the MADS box genes SEP1-3 (Pelaz et al., 2000) and AP1 (Irish and Sussex, 1990; Theissen and Saedler, 2001). Moreover, several 35S::PHYL1 lines also exhibited a cauliflower-like appearance (Figure S2c,d), which is a characteristic phenotype of an ap1 cal double mutant (Bowman et al., 1993; Ditta et al., 2004). Therefore, we first compared the expression levels of SEP3, AP1 and CAL in wild-type and three 35S::PHYL1 lines [using another MADS box class C gene, AG (Bowman et al., 1989), as a control], but did not observe any significant difference in their levels of mRNA accumulation (Figure S5). We hypothesized that these proteins may be functionally inactivated via physical interaction with PHYL1. We used a yeast two-hybrid (Y2H) assay to test for interactions of PHYL1 with the MADS domain proteins SEP3, AP1 and CAL, as well as WUS, a homeodomain-containing (non-MADS domain) transcription factor that is required for shoot and floral meristem integrity (Ikeda et al., 2009). Yeast cells expressing BD-fused PHYL1 (BD-PHYL1) and AD-fused SEP3, CAL or AP1 (AD-SEP3, AD-CAL or AD-AP1) grew on selective medium, indicating that PHYL1 interacted with these three MADS domain proteins (Figure 2). Similarly, growth was also observed when BD-fused PHYL1Δ8 (BD-PHYL1Δ8) was expressed with either AD-SEP3, AD-CAL or AD-AP1 in yeast, indicating that the N-terminal eight amino acids of PHYL1 are not important for its interaction with these proteins.
MADS domain proteins. In contrast, no growth was observed in yeast expressing BD-PHYL1 with AD-WUS (Figure 2). AD-fused SAP54, a homolog of PHYL1, also interacted with BD-SEP3, BD-AP1 and BD-CAL, but not with BD-WUS. These results show that PHYL1 interacts specifically with SEP3, AP1 and CAL.

**PHYL1 inhibits the function of MADS domain proteins**

To investigate whether PHYL1 interferes with the function of MADS domain proteins SEP3 and AP1, we examined the expression of genes that are positively regulated (class B genes AP3 and PI) or negatively regulated (flowering time genes SOC1, SVP and AGL24) by SEP3 and AP1 (Ng and Yanofsky, 2001; Gregis et al., 2008; Immink et al., 2012; Wu et al., 2012). Our quantitative RT-PCR analysis using RNA extracted from inflorescence apices of wild-type and 35S::PHYL1 transgenic plants revealed that both of the class B genes were significantly down-regulated in the 35S::PHYL1 transgenic plants compared with the wild-type plants (Figure 3a). In contrast, expression of SVP and SOC1 was significantly up-regulated in the 35S::PHYL1 transgenic plants, but expression of AGL24 did not differ from that in the wild-type plants (Figure 3a). These results suggest that the ability of SEP3 and AP1 to regulate the expression of downstream genes is impaired in the 35S::PHYL1 transgenic plants.

As previous studies have shown that MADS domain proteins SEP3 and AP1 localize to nuclei and interact to form SEP3–AP1 and SEP3–AG heterodimeric complexes that control differentiation of distinct floral organs (Immink et al., 2009; Smacznia et al., 2012), we next examined the effect of PHYL1 on interactions between MADS domain proteins. To address these interactions in planta, we performed a bimolecular fluorescence complementation (BiFC) assay. For the BiFC assay, the N-terminal half of YFP (NYF) and the C-terminal half of YFP (CYF) were fused to the MADS domain proteins SEP3, AP1 and AG. As a control experiment, we used the basic leucine zipper transcription factor bZIP63, which homodimerizes in the nuclei (Figure 3b, left) (Walter et al., 2004). Consistent with previous results (Immink et al., 2009; Smacznia et al., 2012), fluorescent signals were observed in the cell nuclei when NYF–SEP3 and AP1–CYF were co-expressed in *Nicotiana benthamiana* leaves, indicating that the MADS domain proteins SEP3 and AP1 interact with each other in the nuclei (Figure 3c, left). Interestingly, when PHYL1 was co-expressed with NYF–SEP3 and AP1–CYF, the fluorescence was significantly decreased (Figure 3c,e, middle). Expression of PHYL1, but not of GUS, also reduced the nuclear fluorescence signals observed when NYF–SEP3 and CYF–AG were co-expressed (Figure 3d, left and middle, and Figure 3e, bottom), but did not affect the formation of bZIP63 homodimers (Figure 3b, middle, and Figure 3e, top). PHYL1Δ8 did not affect the formation of BiFC complexes in any experiment (Figure 3b–d, right, and Figure 3e). These results suggest that PHYL1 interferes with the interaction between MADS domain proteins in planta.

**PHYL1 induces degradation of MADS domain proteins**

The above results suggest that PHYL1 targets MADS domain proteins to inhibit their functions in flower development, which results in induction of phyllody-like phenotypes. However, it remains unclear how PHYL1 interferes with the normal functions of these proteins.

To address this question, we examined whether PHYL1 expression affects the accumulation and subcellular localization of MADS domain proteins. We transiently expressed YFP-fused MADS domain proteins in *N. benthamiana* leaves by agroinfiltration in combination with either control GUS protein, PHYL1 or PHYL1Δ8, and monitored their accumulation and subcellular localization by confocal microscopy. As expected, YFP-fused MADS domain proteins SEP3 and AP1, as well as bZIP63, which served as a positive control, localized to the nucleus when GUS was co-expressed (Figure 4a–c, left). YFP-fused CAL localized in the cytoplasm (Figure 4d, left). While co-expression with PHYL1 did not affect the accumulation or subcellular localization of YFP-fused bZIP63, it significantly reduced the fluorescence derived from YFP–SEP3, YFP–AP1 and YFP–CAL (Figure 4, second left). In contrast, co-expression with

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**Figure 2.** PHYL1, and its N-terminal eight amino acid deletion mutant, interacts with MADS domain proteins SEP3, AP1 and CAL in yeast. The MADS domain proteins SEP3, AP1 and CAL, as well as WUS, fused to the GAL4 activation domain (AD), were expressed in combination with SAP54, PHYL1 and PHYL1Δ8 fused to the GAL4 DNA-binding domain (BD) in yeast strain AH109. As negative controls, each AD-fused host factor was co-expressed with empty BD vector. Yeast cells harboring the AD and BD vectors were cultured to an absorbance of 0.1 at 600 nm. Aliquots (10 μL) of these cells were plated on selective medium lacking leucine and tryptophan (-LW), or lacking leucine, tryptophan and histidine (-LWHi). The plates were incubated for 3 days at 30°C.
Figure 3. PHYL1 inhibits MADS domain protein functions.
(a) Quantitative real-time RT–PCR analysis of expression levels of genes downstream of SEP3 and AP1 (AP3, PI, SOC1, SVP and AGL24) in wild-type and 35S::PHYL1 transgenic plants. The expression level of the genes in wild-type plants was set as the reference. Error bars represent the standard deviation. Asterisks indicate statistically significant differences between wild-type and 35S::PHYL1 transgenic plants (*P < 0.05, **P < 0.01).
(b–d) BIFC assays showing that PHYL1 interferes with the interaction between the MADS domain proteins. Agrobacterium cultures (OD600 = 1.0) expressing the N- and C-terminal YFP-fused proteins and either GUS (from pCAMBIA1301, GenBank accession number AF234297), PHYL1 or PHYL1Δ8 were mixed at a ratio of 1:1:10. YFP fluorescence was detected 36 h after co-expression of (b) bZIP63–NYF and bZIP63–CYF, (c) NYF–SEP3 and AP1–CYF, and (d) NYF–SEP3 and CYF–AG, with GUS, PHYL1 or PHYL1Δ8. Scale bar = 200 μm.
(e) Quantitative analysis of the number of nuclear-localized BIFC signals in (b–d). The number of signals was quantified using a leaf area of 2.4 mm². Error bars represent the standard deviation. Asterisks indicate statistically significant differences compared with GUS (*P < 0.05, **P < 0.01).

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PHYL1Δ8 did not affect the accumulation and subcellular localization of any of the tested MADS domain proteins (Figure 4b–d, third left). Western blots also showed that SEP3, AP1 and CAL levels were reduced upon co-expression with PHYL1 (Figure 4b–d, right). These results indicate that PHYL1 induces the degradation of and binds with some MADS domain proteins, including SEP3 and AP1. These results also demonstrate that the N-terminal eight

Figure 4. Transient expression of PHYL1 induces degradation of MADS domain proteins.
(a–d) Accumulation and subcellular localization of transiently expressed YFP-fused Arabidopsis nuclear protein bZIP63 (a) and YFP-fused MADS domain proteins SEP3, AP1 and CAL (b–d). Agrobacterium cultures (OD$_{600}$ = 1.0) expressing YFP-fused proteins and either GUS, PHYL1 or PHYL1Δ8 were mixed at a ratio of 1:10, and infiltrated into N. benthamiana leaves. YFP fluorescence was observed 36 h after expression. Scale bar = 200 μm. Accumulation of YFP-fused proteins was evaluated by immunoblotting using an anti-GFP antibody (a–d, right).
amino acids of PHYL1 are essential for inducing degradation of MADS domain proteins, although they are not involved in the interaction between these proteins.

PHYL1-induced degradation of MADS domain proteins was mediated by the ubiquitin–proteasome pathway

The ubiquitin–proteasome pathway is a major pathway for selective protein degradation in many eukaryotes including plants (Schwechheimer and Schwager, 2004). The MADS domain and K domain of MADS domain proteins share considerable levels of amino acid homology and contain several lysine residues (Figure S6) that may serve as potential ubiquitin acceptor sites in the ubiquitin–proteasome pathway (Ravid and Hochstrasser, 2008). We thus explored the possibility that PHYL1-induced degradation of MADS domain proteins was mediated by the ubiquitin–proteasome pathway. As shown in Figure 4(b), the fluorescence derived from YFP-SEP3 was significantly decreased when PHYL1 was co-expressed (Figure 5a, left). However, application of the proteasome inhibitor clasto-lactacystin β-lactone (Reichel and Beachy, 2000; Gillespie et al., 2002) restored the fluorescence of YFP-SEP3 upon co-expression with PHYL1 (Figure 5a, right). To determine whether SEP3 was ubiquitinated when PHYL1 was co-expressed, proteins immunoprecipitated by an anti-GFP antibody were detected by immunoblotting with an anti-ubiquitin antibody. Expression and immunoprecipitation of YFP-fused bZIP63 and SEP3 were confirmed by immunoblotting with the anti-GFP antibody (Figure 5b, top and middle). As expected, a characteristic smear of high-molecular-weight protein bands indicative of ubiquitination was detected by the anti-ubiquitin antibody in the anti-GFP immunoprecipitates of N. benthamiana leaves co-expressing YFP-SEP3 and PHYL1, but not in those of leaves expressing YFP-SEP3 alone (Figure 5b, bottom). It was considered that PHYL1 is not responsible for the major band at 80 kDa in the lane loaded with immunoprecipitated bZIP63-YFP (Figure 5b, bottom, asterisk), because the band was observed with or without the expression of PHYL1 (Figure S7, asterisk). Hence, these

Figure 5. PHYL1-induced degradation of MADS domain proteins is mediated by the ubiquitin–proteasome pathway.

(a) Confocal images of N. benthamiana leaf epidermis cells transiently expressing YFP-SEP3 and PHYL1 following infiltration with dimethylsulfoxide (DMSO) (left) or 20 μM of the proteasome inhibitor clasto-lactacystin β-lactone (right). Agrobacterium cultures expressing YFP-SEP3 and PHYL1 were mixed at a ratio of 1:1, and infiltrated into N. benthamiana leaves. DMSO or clasto-lactacystin β-lactone were infiltrated 1 day after co-infiltration of YFP-SEP3 and PHYL1, and YFP fluorescence was observed 12 h after clasto-lactacystin β-lactone or DMSO infiltration. Scale bar = 200 μm.

(b) Immunoprecipitation of bZIP63-YFP or YFP-SEP3 using an anti-GFP antibody, and detection of ubiquitinated proteins using an anti-ubiquitin antibody. Agrobacterium cultures expressing YFP-fused proteins and either PHYL1 or buffer were mixed at a ratio of 1:1 and infiltrated into N. benthamiana leaves. Thirty-six hours after expression, total proteins were extracted from N. benthamiana leaves transiently co-expressing bZIP63-YFP and PHYL1 (left lane), YFP-SEP3 alone (middle lane) or YFP-SEP3 and PHYL1 (right lane), and immunoprecipitation (IP) was performed using an anti-GFP antibody. Total proteins (upper panel) and immunoprecipitates (middle and lower panels) were subjected to SDS-PAGE and immunoblotting (IB) using anti-GFP antibody (upper and middle panels) and anti-ubiquitin antibody (lower panel), as indicated.
results indicate that PHYL1-induced degradation of the MADS domain protein SEP3 is mediated in a ubiquitin–proteasome-dependent manner.

**PHYL1 homologs also induce degradation of MADS domain proteins**

Like the OY phytoplasma used in this study, a variety of other phytoplasmas commonly induce phyllody-like symptoms in infected plants (Lee et al., 2004; Bertaccini and Duduk, 2009). To explore the possibility that these symptoms may be induced by a mechanism similar to that in OY phytoplasma, we attempted to identify PHYL1 homologs from other phytoplasma species and to analyze their function. Previous reports have shown that all known phytoplasma genes homologous to PHYL1 and SAP54 are present in potential mobile units (Jomantiene et al., 2007; Saccardo et al., 2012). This prompted us to screen for PHYL1 homologs in potential mobile units of many other phytoplasma genomes using PCR/Southern blot hybridization. PCR amplification of potential mobile units using a primer pair used in the previous study (Jomantiene et al., 2007) followed by Southern blot hybridization with a PHYL1-specific probe identified 16 PHYL1 homologs from 16 phytoplasmas belonging to four distinct candidate species: 13 from Ca. P. asteris and one from each of Ca. P. pruni, Ca. P. trifolii, and Ca. P. phoenicium. Alignment of the amino acid sequences of PHYL1, SAP54, the homologs identified in this study and homologs available in the National Center for Biotechnology Information database, revealed high levels of amino acid identity (over 85% in the secreted region; Figure 6a). We noted that a PHYL1 homolog from peach yellows phytoplasma (PYR) contained a frameshift mutation that led to a premature termination codon and loss of approximately 30 amino acids from the C-terminus.

To examine whether PHYL1 homologs function similarly to PHYL1, we performed a Y2H assay to test for the interaction with SEP3 and a transient co-expression assay with SEP3, against the PHYL1 homologs of CA –76, CP, PEY and PYR, which contain amino acid differences compared with PHYL1 of OY – W. The Y2H assay demonstrated that PHYL1 homologs CA –76, CP and PEY interacted with SEP3 (Figure 6b) and induced a decrease in YFP–SEP3 fluorescence when co-expressed in N. benthamiana leaves (Figure 6c). These results indicate that PHYL1 homologs have the ability to interact with and induce degradation of MADS domain proteins. In contrast, PYR neither interacted with SEP3 in the Y2H assay nor induced degradation of SEP3 (Figure 6b,c), suggesting that the C-terminal region (approximately 30 amino acids) of PHYL1 homologs plays an important role in these functions.

**DISCUSSION**

A phytoplastmal phyllody-inducing protein, phyllogen, degrades floral homeotic MADS domain proteins

This study provides evidence that the phytoplasma-unique floral symptoms of ‘phyllody’ are caused by a small, phytoplasma-secreted protein, PHYL1, through ubiquitin–proteasome-mediated degradation of floral homeotic MADS domain proteins. Homologs of the PHYL1 gene are exclusively found and highly conserved in members of the genus Phytoplasma (Figure 6a), and demonstrated the same degradation activity as PHYL1 (except for PYR, a truncated mutant) (Figure 6c). Among flowering plants, the ABCE class MADS domain proteins and their functions appear to be largely conserved (Litt and Kramer, 2010; Smaczniak et al., 2012). The class A and class E proteins, which are targeted by PHYL1 (Figure 4b,c), play especially important roles in both floral organ and floral meristem determinacy (Irish and Sussex, 1990; Pelaz et al., 2000). The conservation of PHYL1 homologs among phytoplasmas and of ABCE class MADS domain proteins among flowering plants strongly suggests that PHYL1 and its homologs are determinants of phyllody symptoms. We propose that PHYL1, SAP54 and their homologs comprise a phyllody-inducing gene (phyllogen) family. The fact that symptom-inducing genes such as PHYL1 and TENGU are conserved in phytoplasmas (this study; Sugawara et al., 2013) reinforces the hypothesis that phytoplastmal symptoms such as phyllody and witches’ broom have important implications for phytoplasma survival (Hoshi et al., 2009).

**Phyllogen targets a wide range of MADS domain proteins for degradation, leading to phyllody-like phenotypes**

PHYL1 induced degradation of at least three MADS domain proteins (SEP3, AP1 and CAL) in planta (Figure 4). These
Phylogen degrades floral MADS domain proteins

(a)

| Ca. P. asteris | GY-W | ED | A-AY | NY-A | CA76 | AVUT | FrG | AY192 |
|---------------|------|----|------|------|------|------|-----|-------|
| RV            | YF   | YF | YF   | YF   | YF   | YF   | YF  | YF    |
| GLAW          | YF   | YF | YF   | YF   | YF   | YF   | YF  | YF    |
| GLAW          | YF   | YF | YF   | YF   | YF   | YF   | YF  | YF    |
| LEO           | YF   | YF | YF   | YF   | YF   | YF   | YF  | YF    |
| PYR           | YF   | YF | YF   | YF   | YF   | YF   | YF  | YF    |
| CP            | YF   | YF | YF   | YF   | YF   | YF   | YF  | YF    |
| Ca. P. prunii | SP1† | YF | YF   | YF   | YF   | YF   | YF  | YF    |
| Jr1†††        | YF   | YF | YF   | YF   | YF   | YF   | YF  | YF    |
| Ca. P. trifoli | CP6  | YF | YF   | YF   | YF   | YF   | YF  | YF    |
| Ca. P. phoenicium | CPY | YF | YF   | YF   | YF   | YF   | YF  | YF    |

(b)

| AD | CA-76 | CP | PEY | PYR |
|----|-------|----|-----|-----|
| SEP3 | empty |    |     |     |
| BD  |       |    |     |     |

(c)

| YFP-SELP | CA-79 | CP |
|----------|-------|----|
| PEY      |       |    |
| PYR      |       |    |

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proteins are members of the two distinct but closely related sub-families: SEP3 belongs to the SEP sub-family, and AP1 and CAL belong to the SQUA sub-family (Smaczniak et al., 2012). As the amino acid sequences are highly conserved in each sub-family, it is likely that phyllogen widely induces degradation of MADS domain proteins of these sub-families. For example, Arabidopsis harbors four SEP sub-family genes, SEP1–4, and a well-studied SQUA sub-family gene, FRUITFULL (FUL). Therefore, it is possible phyllogen targets these proteins as well as SEP3, AP1 and CAL.

The roles of SEP1–4, AP1 and CAL in floral development have been previously clarified by analyses of mutants. Fluorescences of ap1 mutants produce leaf-like sepals and secondary flowers in the axils of the first-whorl organs (Bowman et al., 1993; Gregis et al., 2006). Although cal single mutants cannot be distinguished from wild-type plants, ap1 cal double mutants develop cauliflower-like inflorescences (Bowman et al., 1993). Due to the functional redundancy exhibited by SEP1-4, single mutations of these genes produce few morphological changes. However, the sep1 sep2 sep3 triple mutant shows conversion of all floral organs into green sepal-like organs, and a loss of meristem determinacy in the center of the flower (Pelaz et al., 2000). Moreover, the sep1 sep2 sep3 sep4 quadruple mutant exhibits conversion of all floral organs into leaves and an increased loss of floral meristem determinacy (Ditta et al., 2004). The floral phenotypes of these mutants are similar to those of 35S::PHYL1 plants, suggesting that PHYL1-induced degradation of the MADS domain proteins may be a major cause of the phylloidy-like phenotypes of 35S::PHYL1 plants (Figure 1b–d and Figure S2a–f). Further evidence in support of this idea comes from a previous study of knockdown of all MADS domain proteins targeted by PHYL1 using an artificial microRNA, amir-mads–2, which mainly targets and efficiently silences SEP1–4, AP1, CAL and FUL MADS box genes (Schwab et al., 2006). Plants expressing the microRNA produced flowers with green leaf-like organs in the outer two whorls and new inflorescence shoots in the fourth whorl, and developed secondary flowers. These floral phenotypes were similar to those of 35S::PHYL1 plants. The morphological similarities between plants expressing either amir-mads–2 or PHYL1 strongly suggest a link between PHYL1-induced degradation of MADS domain proteins, including SEP1–4, AP1, CAL and FUL, and the phylloidy-like phenotypes.

The molecular mechanism of phylloidy symptoms

The compromised expression pattern of downstream genes regulated by SEP3 and AP1 (Figure 3a) indicates that the activity of SEP3 and AP1 as transcription factors is impaired in 35S::PHYL1 plants. Like SEP3 and AP1, products of the class B genes AP3 and PI are also essential components of protein complexes that control floral organ identity as postulated by the floral quartet model (Theissen and Saedler, 2001; Smaczniak et al., 2012). It is therefore reasonable to expect that their down-regulation (Figure 3a) affects normal floral development, leading to the abnormal floral phenotypes of the 35S::PHYL1 plants. On the other hand, although expression of the class C gene AG was down-regulated at floral meristems when SEP1-4 were knocked out (Liu et al., 2009), there was no significant difference in the AG expression level between wild-type and PHYL1-expressing plants (Figure S6). A constant expression pattern of the AG gene similar to the 35S::PHYL1 plants was also observed in plants expressing amiR-mads–2, despite the microRNA-mediated silencing of SEP1–4 (Schwab et al., 2006). These data may indicate that both PHYL1 and amiR-mads–2 also target other MADS domain protein(s) that negatively control the expression of AG.

Figure 7 shows a model for the molecular mechanism of the phylloidy symptoms induced by phytoplasma infection. Phytoplasma expresses and secretes phyllogens into plant cells. The phyllogen binds to the class A and E proteins AP1 and SEP1–4, respectively, and induces degradation of them (Figure 4b,c), leading to down-regulation of the class B genes AP3 and PI (Figure 3a). According to the floral quartet model, floral organ identity is determined by organ-specific combinational quaternary complexes consisting of SEP–AP1, SEP–AG and AP3–PI heterodimers of ABCE class MADS domain transcription factors (Theissen and Saedler, 2001; Smaczniak et al., 2012). Degradation of the class A and E proteins results in a significant reduction in the frequency of formation of the SEP3–AP1 and SEP3–AG heterodimers (Figure 3c–e). Likewise, down-regulation of the class B genes decreases the frequency of AP3–PI heterodimerization. As a consequence of these molecular effects, the first whorl, in which all components of the quartet are degraded, exhibits a severe leaf-like phenotype, and the second whorl, in which two members of the quartet are degraded and the others are down-regulated, exhibits a moderate degree of leaf-like phenotype. The third whorl (in which one of the quartet is degraded and two are down-regulated) is less affected, and shows a weak leaf-like phenotype without drastic morphological changes. The fourth whorl (in which two of the quartet are degraded) is moderately affected, and forms a secondary flower similar to that of sep triple and quadruple mutants (Pelaz et al., 2000; Ditta et al., 2004). Although the molecular mechanisms underlying floral organ development are not yet fully understood, this model explains the characteristic floral phenotype of 35S::PHYL1 plants really well.

In addition to the leaf-like floral organs, flowers of 35S::PHYL1 plants often formed new inflorescence shoots and ectopic secondary flowers from the axils of leaf-like sepals (Figure 1c and Figure S2a). We also attribute these floral reversion phenotypes to the degradation of AP1 and SEP3
proteins. Indeed, the flowering-time genes SOC1, SVP and AGL24, which induce formation of ectopic secondary flowers (Liu et al., 2007), are negatively regulated by AP1 and SEP3 (Gregis et al., 2008; Immink et al., 2012). The significant induction of SOC1 and SVP in the inflorescence apices of 35S::PHYL1 plants (Figure 3a) suggests dysfunction of AP1 and SEP3 and its involvement in the floral reversion phenotypes.

Role of phyllogen in degradation of MADS domain proteins

In this study, we found that PHYL1-mediated degradation of SEP3 is dependent on the ubiquitin–proteasome pathway (Figure 5a). The fact that SEP3 was polyubiquitinated in the presence of PHYL1 (Figure 5b) indicates that PHYL1 directly or indirectly ubiquitinates SEP3, leading to its degradation by the proteasome. In the ubiquitin–proteasome pathway, E3 ubiquitin ligases are key factors that determine substrate specificity (Vierstra, 2009). Our finding that phyllogen interacted with the MADS domain proteins in the Y2H assay (Figures 2 and 6b) suggests that phyllogen may perform an E3 ubiquitin ligase-like function. Indeed, the Y2H assay (Figures 2 and 6b) and in planta transient expression assay (Figures 4 and 6c) using PHYL1 deletion mutants indicated that the N- and C-terminal regions of phyllogen play critical but distinct roles in targeting of MADS domain proteins: the N-terminal region is involved in induction of degradation, whereas the C-terminal region is involved in binding to the target proteins. It is likely that the N-terminal region of phyllogen recruits the ubiquitination machinery, while the C-terminus binds to the MADS domain proteins. The
ubiquitin attachment site in substrate proteins is commonly a lysine side chain (Ravid and Hochstrasser, 2008), and the targeted MADS domain proteins possess several lysine residues (Figure S6). In order to unravel the molecular mechanisms underlying phylogen-induced degradation of MADS domain proteins, identification of the ubiquitination machinery complex involved, as well as ubiquitinated lysine residue(s) in the MADS domain proteins, will be required.

**EXPERIMENTAL PROCEDURES**

**Biological materials**

The phytoplasma strains and PHYL1 homologs used in this study are listed in Table S2. The ‘Ca. Phytoplasma asteris’ OY strain wild-type line (OY–W) was isolated in Saga Prefecture, Japan (Shiomi et al., 1998). OY–W phytoplasma was maintained in the plant host garland chrysanthemum (Chrysanthemum coronarium) using a leafhopper vector, Macrosteles striifrons, as previously described (Oshima et al., 2001). A plant codon-optimized SAP54 gene was custom-synthesized by Eurofins MWG Operon (http://www.operon.com/). A plant infected with strain ED (Kakizawa et al., 2006) was kindly provided by T. Shiomi (Department of Biological Safety, National Institute for Agro-Environmental Sciences, Ibaraki, Japan). T. Usugi (Plant Protection Division, National Agricultural Research Center, Ibaraki, Japan), and N. Nishimura and T. Tsuchizaki (Department of Agribusiness, Koibuchi College of Agriculture and Nutrition, Ibaraki, Japan). Samples of DNA from other phytoplasma strains were kindly provided by A. Bertaccini (DipSA, University of Bologna, Bologna, Italy). The Arabidopsis plants (Arabidopsis thaliana ecotype Col-0) and Nicotiana benthamiana plants were maintained in growth chambers with a 16 h light/8 h dark cycle at 23°C and 25°C, respectively.

**Transgenic plants**

A plant-codon-optimized PHYL1 gene (Figure S8) and its N-terminally truncated mutants (PHYL1Δ3 to PHYL1Δ8) were cloned into binary pFAST02 (Inplanta Innovations Inc., http://www.inplanta.jp/en.html) under the control of the CaMV 35S promoter. Agrobacterium tumefaciens strain EHA105 was then transformed with the constructs, and A. thaliana plants were transformed using the floral-dip method as described previously (Hoshi et al., 2009). T1 seeds of the transgenic plants were collected by fluorescence stereomicroscopy. The expression of PHYL1 was confirmed by RT–PCR.

**Yeast two-hybrid assays**

Y2H assays were performed using the Matchmaker GAL4 Two-Hybrid System 3 (Clontech, http://www.clontech.com/) as described by Yamaji et al. (2006). cDNA of each MADS domain protein was amplified from total RNA of Arabidopsis Col-0 using the primer pairs listed in Table S3. The coding regions of the cDNAs encoding each protein (excluding the signal peptide of PHYL1) were cloned into pGADT7 and pGBK7 (Clontech). Co-transformants of the yeast strain AH109 harboring appropriate constructs based on pGADT7 and pGBK7 were selected on minimal synthetic dextrose medium lacking leucine and histidine. For evaluation of protein interaction, the yeast co-transformants were plated on selective medium lacking tryptophan, leucine and histidine.

**RNA extraction and qRT–PCR**

Total RNA from inflorescence apices containing stage 1-10 floral buds from 35S::PHYL1 transgenic lines and wild-type A. thaliana plants was extracted using isogen (Nippon Gene, http://nippongene.com/index/english/e/index.html). The total RNA was treated with DNase I (TaKaRa, http://www.takara-bio.com), and reverse-transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, http://www.appliedbiosystems.com) according to the manufacturer’s instructions. Quantitative RT–PCR assays were performed using the Thermal Cycler Dice real-time system (TaKaRa) and the SYBR Premix Ex Taq (TaKaRa). Primers used for the quantification of gene expression are listed in Table S3. The expression levels of the target genes were normalized to that of the actin gene. Analyses were performed on four biological replicates.

**Fluorescence microscopy**

Agrobacterium cells containing the constructs were adjusted to an absorbance at 600 nm of 1.0, mixed in ratios described in the corresponding figure legends, and infiltrated into N. benthamiana leaves as described by Senshu et al. (2009). Confocal imaging was performed 36 h after agroinfiltration using a Leica TCS SP5 confocal microscope (http://www.leica-microsystems.com/). Constructs for expression of a YFP-fused MADS domain protein were created using pEarleyGate101 or pEarleyGate 104 binary vectors (Earley et al., 2006) and Gateway technology (Invitrogen, http://www.lifetechnologies.com). For BiFC, pEarleyGate101 was modified to generate pEarleyGate-CBiFC-N and pEarleyGate-CBiFC-C, carrying the N- and C-terminal halves of the YFP gene, respectively. The pEarleyGate 104 binary vector was also modified to generate pEarleyGate–NYF and pEarleyGate–CYF. The cDNAs of MADS domain proteins were introduced into these BiFC vectors using Gateway technology. Stock solution (2.0 mM) of Clasto-lactacystin β-lactone (EMD Millipore, http://www.emdmillipore.com/) was prepared in dimethyl sulfoxide (DMSO) and diluted in water to produce a final concentration of 20 μM.

**Protein extraction, immunoprecipitation and immunoblot analysis**

Thirty-six hours after agroinfiltration of YFP-fused protein, the inoculated N. benthamiana leaves were collected and homogenized in RIPA buffer (Yamaji et al., 2006) containing Complete Mini protease inhibitor (Roche, http://www.roche.com) and 0.1% 2-mercaptoethanol. The homogenate was centrifuged at 12,000 g for 10 min at 4°C to remove cell debris, and the supernatant was incubated for 60 min at 4°C with anti-GFP antibody (#18114460, Roche) and for 60 min at 4°C with Protein G-Sepharose 4 Fast Flow (GE Healthcare, http://www3.gehealthcare.com). The resin was collected by centrifugation at 12,000 g for 20 sec at 4°C, and washed five times with a 20-fold volume of RIPA buffer for 1 min at 4°C. Proteins were eluted from the resin complex by boiling with SDS sample buffer. The proteins were separated by SDS-PAGE, blotted onto a polyvinylidene difluoride membrane, and detected using anti-GFP antibody and anti-ubiquitin antibody (anti-UBQ11, Agrisera, http://www.agrisera.com).

**PCR/Southern blot hybridization**

Potential mobile units of phytoplasmas were PCR-amplified using the primary primers listed in Table S3. The amplicons were used for Southern blotting analysis as described previously (Oshima et al., 2001). The probe for PHYL1 was prepared from the OY–W genome
using the primers listed in Table S3 and a PCR DIG probe synthesis kit (Roche). The amplicons with positive signals were purified and sequenced.

ACKNOWLEDGMENTS

We are grateful to A. Bertaccini (DipSA, University of Bologna, Bologna, Italy) for providing phytoplasma DNA samples. This work was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (category ‘S’ of Scientific Research Grant 25221201), the Funding Program for Next Generation World-Leading Researchers (project GS005) initiated by the Council for Science and Technology Policy, and the Program for the Promotion of Basic Research Activities for Innovative Bioscience (PROBRAIN).

SUPPORTING INFORMATION

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

Figure S1. Floral symptoms of periwinkle and petunia plants infected with OY-W phytoplasma.

Figure S2. Floral phenotypes of PHYL1 over-expressing Arabidopsis plants.

Figure S3. Quantitative expression analysis of the phytoplasma effector gene PHYL1 in plant and insect hosts.

Figure S4. Phenotype of the 35S::PHYL1 transgenic plants in the vegetative stage.

Figure S5. PHYL1 does not affect the expression levels of floral homeotic MADS box genes.

Figure S6. Amino acid sequence alignment and functional domains of MADS domain proteins used in this study.

Figure S7. PHYL1-independent ubiquitination of the bZIP63-YFP protein.

Figure S8. Optimization of the nucleotide sequence of PHYL1 for in planta expression.

Table S1. Expression level of the PHYL1 gene in the Arabidopsis 35S::PHYL1 transgenic lines.

Table S2. Phytoplasma strains and cloned PHYL1 homologs.

Table S3. Primers used for yeast two-hybrid, quantitative RT-PCR and PCR/Southern blot analyses.

REFERENCES

Abramovitch, R.B., Anderson, J.C. and Martin, G.B. (2006) Bacterial elicitation and evasion of plant innate immunity. Nat. Rev. Mol. Cell Biol. 7, 601–611.

Alvarez-Buylla, E.R., Benitez, M., Corvera-Poire, A. et al. (2010) Flower development. Arabidopsis Book, 8, e0127.

Bertaccini, A. and Duduk, B. (2009) Phytoplasma and phytoplasma diseases: a review of recent research. Phytopathol. Mediterr. 48, 355–378.

Bowman, J.L., Smyth, D.R. and Meyerowitz, E.M. (1989) Genes directing flower development in Arabidopsis. Plant Cell, 1, 37–52.

Bowman, J.L., Alvarez, J., Weigel, D., Meyerowitz, E.M. and Smyth, D.R. (1993) Control of flower development in Arabidopsis thaliana by APETALA1 and interacting genes. Development, 119, 721–743.

Cetul, E. and Ferrao, G. (2011) Development of phytoplasma-induced flower symptoms in Arabidopsis thaliana. Physiol. Mol. Plant Pathol. 76, 204–211.

Ditta, G., Pinyopich, A., Robles, P., Pelaz, S. and Yanoisky, M.F. (2004) The SEP4 gene of Arabidopsis thaliana functions in floral organ and meristem identity.Curr. Biol. 14, 1935–1940.

Dunoyer, P., Lecellier, C.H., Parizotto, E.A., Himber, C. and Voinnet, O. (2004) Probing the microRNA and small interfering RNA pathways with virus-encoded suppressors of RNA silencing. Plant Cell, 16, 1235–1250.

Earley, K.W., Haag, J.R., Pontes, O., Oppel, K., Juehne, T., Song, K. and Pikaard, C. (2006) Gateway-compatible vectors for plant functional genomics and proteomics. Plant J. 45, 616–629.

Firrao, G., Garcia-Chapa, M. and Marzachi, C. (2007) Phytoplasmas: genetics, diagnosis and relationships with the plant and insect host. Front. Bioi. 12, 1353–1375.

Gillespie, T., Boevink, P., Haupt, S., Roberts, A.G., Toth, R., Valentine, T., Chapman, S. and Opara, K.J. (2002) Functional analysis of a DNA-shuffled movement protein reveals that microtubules are dispensable for the cell-to-cell movement of Tobacco mosaic virus. Plant Cell, 14, 1207–1222.

Grant, M.R. and Jones, J.D.G. (2009) Hormone (d)isbalance moulds plant health and disease. Science, 324, 750–752.

Gregis, V., Sessa, A., Colombo, L. and Kater, M.M. (2005) AGAMOUS-LIKE24 and SHORT VEGETATIVE PHASE, and APETALA1 redundantly control AGAMOUS during early stages of flower development in Arabidopsis. Plant Cell, 17, 1373–1382.

Gregis, V., Sessa, A., Colombo, L. and Kater, M.M. (2005) AGAMOUS-LIKE24 and SHORT VEGETATIVE PHASE determine floral meristem identity in Arabidopsis. Plant J. 56, 891–902.

Himeno, N., Neriya, Y., Minato, N., Miura, C., Sugawara, K., Ishii, Y., Yamaji, Y., Kakizawa, S., Oshima, K. and Namba, S. (2011) Unique morphological changes in plant pathogenic phytoplasma-infected petunia flowers are related to transcriptional regulation of floral homeotic genes in an organ-specific manner. Plant J. 72, 571–579.

Hogenhout, S.A. and Loria, R. (2008) Virulence mechanisms of Gram-positive plant pathogenic bacteria. Curr. Opin. Plant Biol. 11, 449–456.

Hogenhout, S.A., Oshima, K., Ammar, E.-D., Kakizawa, S., Kingdom, H. and Namba, S. (2008) Phytoplasmas: bacteria that manipulate plants and insects. Mol. Plant Pathol. 9, 403–423.

Honma, T. and Goto, K. (2001) Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. Nature, 409, 525–529.

Hoshi, A., Oshima, K., Kakizawa, S., Ishii, Y., Ozeki, J., Hashimoto, M., Komatsu, K., Kagiwada, S., Yamaji, Y. and Namba, S. (2009) A unique virulence factor for proliferation and dwarfism in plants identified from a phytopathogenic bacterium. Proc. Natl. Acad. Sci. USA, 106, 6416–6421.

Ikeda, M., Mitsuda, N. and Ohme-Takagi, M. (2009) Arabidopsis WUSCHEL is a bifunctional transcription factor that acts as a repressor in stem cell regulation and as an activator in floral patterning. Plant Cell, 21, 3493–3505.

Immini, R.G.H., Tonaco, I.A.N., de Folter, S., Shchennikova, A., van Dijk, A.D.J., Busscher-Lange, J., Borst, J.W. and Angelent, G.C. (2009) SEPALLATA3: the ‘glue’ for MADS box transcription factor complex formation. Genome Biol. 10, R24.

Immini, R.G.H., Pose, D., Ferrario, S. et al. (2012) Characterization of SOC1’s central role in flowering by the identification of its upstream and downstream regulators. Plant Physiol. 160, 433–449.

Irish, V.F. and Sussex, I.M. (1990) Function of the apetala-1 gene during Arabidopsis floral development. Plant Cell, 2, 741–753.

Jomantien, R., Zhao, Y. and Davis, R.E. (2007) Sequence-variable mosaics: composites of recurrent transposition characterizing the genomes of phylogenetically diverse phytoplasmas. DNA Cell Biol. 26, 557–564.

Kakizawa, S., Oshima, K., Jung, H.-Y., Suzuki, S., Nishigawa, H., Arashida, R., Miyata, S., Ugaki, M., Ichino, H. and Namba, S. (2006) Positive selection acting on a surface membrane protein of the plant-pathogenic phytoplasmas. J. Bacteriol. 188, 3424–3428.

Lee, I.M., Gundersen-Rindal, D.E., Davis, R.E., Bottner, K.D., Marcone, C. and Seemuller, E. (2004) ‘Candidatus Phytoplasma asteris’, a novel phytoplasma taxon associated with aster yellows and related diseases. Int. J. Syst. Evol. Microbiol. 54, 1037–1040.

Litt, A. and Kramer, E.M. (2010) The ABC model and the diversification of floral organ identity. Semin. Cell Dev. Biol. 21, 129–137.

Liu, C., Zhou, J., Bracha-Drori, K., Yalogovsky, S., Ito, T. and Yu, H. (2007) Specification of Arabidopsis floral meristem identity by repression of flowering time genes. Development, 134, 1901–1910.

Liu, C., Xi, W., Shen, L., Tan, C. and Yu, H. (2009) Regulation of floral patterning by flowering time genes. Dev. Cell, 16, 711–722.

Lorang, J.M., Sweat, T.A. and Wolpert, T.J. (2007) Plant disease susceptibility conferred by a ‘resistance’ gene. Proc. Natl Acad. Sci. USA, 104, 14681–14686.

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MacLean, A.M., Sugio, A., Makarova, O.V., Findlay, K.C., Grieve, V.M., Toth, R., Nicolaïsen, M. and Hogenhout, S.A. (2011) Phytoplasma effector SAP54 induces undetermined leaf-like flower development in Arabidopsis plants. *Plant Physiol.* 157, 831-841.

Maejima, K., Oshima, K. and Namba, S. (2014) Exploring the phytoplasmas, plant pathogenic bacteria. *J. Gen. Plant Pathol.* doi: 10.1007/s10327-014-0512-8.

Ng, M. and Yanofsky, M.F. (2001) Activation of the Arabidopsis B class homeotic genes by APETALA1. *Plant Cell*, 13, 739-753.

Oshima, K., Shiomi, K., Kuboyama, T., Sawayanagi, T., Nishigawa, H., Kakizawa, S., Milya, S., Ugaki, M. and Namba, S. (2001) Isolation and characterization of derivative lines of the onion yellows phytoplasma that do not cause stunting or phloem hyperplasia. *Phytopathology*, 91, 1024-1029.

Pelaz, S., Ditta, G.S., Baumann, E., Wisman, E. and Yanofsky, M.F. (2000) B and C floral organ identity functions require SEPALLATA MADS-box genes. *Nature*, 405, 200-203.

Pracros, P., Renaudin, J., Eveillard, S., Mouras, A. and Hernould, M. (2006) Tomato flower abnormalities induced by stolbur phytoplasma infection are associated with changes of expression of floral development genes. *Mol. Plant Microbe Interact.* 19, 62-68.

Reid, T. and Hochstrasser, M. (2000) Diversity of degradation signals in the ubiquitin-proteasome system. *Nat. Rev. Mol. Cell Biol.*, 9, 679-689.

Reichel, C. and Beachy, R.N. (2000) Degradation of tobacco mosaic virus movement protein by the 26S proteasome. *J. Virol.*, 74, 3330-3337.

Saccardo, F., Martini, M., Palmano, S., Ermacora, P., Scortichini, M., Loi, N. and Firrao, G. (2012) Genome drafts of four phytoplasma strains of the ribosomal group 16SrIII. *Microbiology*, 158, 2895-2914.

Schwab, R., Osowski, S., Riester, M., Warthmann, N. and Weigel, D. (2006) Highly specific gene silencing by artificial microRNAs in Arabidopsis. *Plant Cell*, 18, 1121-1133.

Schwuchheimer, C. and Schwager, C. (2004) Regulated proteolysis and plant development. *Plant Cell Rep.*, 23, 353-364.

Senshu, H., Ozeki, J., Komatsu, K., Hashimoto, M., Hatada, K., Aoyama, M., Kagiwada, S., Yamaji, Y. and Namba, S. (2009) Variability in the level of RNA silencing suppression caused by triple gene block protein 1 (TGBp1) from various potexviruses during infection. *J. Gen. Virol.*, 90, 1014-1024.

Shiom, T., Tanaka, M., Wakiya, H. and Zenbayashi, R. (1996) Occurrence of Welsh onion yellows. *Ann. Phytopathol. Soc. Jpn.*, 62, 258-260 (in Japanese with English abstract).

Smaczniak, C., Immling, R.G.H., Angenent, G.C. and Kaufmann, K. (2012) Developmental and evolutionary diversity of plant MADS-domain factors: insights from recent studies. *Development*, 139, 3081-3098.

Su, Y.T., Chen, J.C. and Lin, C.P. (2011) Phytoplasma-induced floral abnormalities in *Catharanthus roseus* are associated with phytoplasma accumulation and transcript repression of floral organ identity genes. *Mol. Plant Microbe Interact.* 24, 1502-1512.

Sugawara, K., Homma, Y., Komatsu, K., Himeno, M., Oshima, K. and Namba, S. (2013) The alteration of plant morphology by small peptides released from the proteolytic processing of the bacterial peptide TENGU. *Plant Physiol.* 162, 2005-2014.

Sugio, A., Kingdom, H.N., MacLean, A.M., Grieve, V.M. and Hogenhout, S.A. (2011) Phytoplasma protein effector SAP11 enhances insect vector reproduction by manipulating plant development and defense hormone biosynthesis. *Proc. Natl Acad. Sci. USA*, 108, E1254-E1263.

Theissen, G. and Saedler, H. (2001) Plant biology: floral quartets. *Nature*, 409, 469-471.

Vierstra, R.D. (2009) The ubiquitin-26S proteasome system at the nexus of plant biology. *Nat. Rev. Mol. Cell Biol.*, 10, 385-397.

Walter, M., Chaban, C., Schütte, K. et al. (2004) Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. *Plant J.*, 40, 425-438.

Wu, M.F., Sang, Y., Bezhani, S., Yamaguchi, N., Han, S.K., Zhenteng Li, Z., Su, Y., Slewinski, T.L. and Wagner, D. (2012) SWI2/SNF2 chromatin remodeling ATPases overcome polycomb repression and control floral organ identity with the LEAFY and SEPALLATA transcription factors. *Proc. Natl Acad. Sci. USA*, 109, 3576-3581.

Yamaji, Y., Kobayashi, T., Hamada, K., Sakurai, K., Yoshii, A., Suzuki, M., Namba, S. and Hibi, T. (2006) In vivo interaction between Tobacco mosaic virus RNA-dependent RNA polymerase and host translation elongation factor 1A. *Virology*, 347, 100-108.