

1 **Running head:** Alteration of Pi starvation responses by SAP11

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12 **Transgenic Plants that Express the Phytoplasma Effector SAP11 Show**
13 **Altered Phosphate Starvation and Defense Responses**

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42 **ABSTRACT**

43 Phytoplasmas have the smallest genome among bacteria and lack many essential genes
44 required for biosynthetic and metabolic functions, making them unculturable, phloem-limited
45 plant pathogens. In this study, we observed that transgenic *Arabidopsis thaliana* expressing
46 the secreted effector protein SAP11_{AYWB} of the Aster Yellows phytoplasma strain Witches'
47 Broom (AY-WB) shows an altered root architecture, similarly to the disease symptoms of
48 phytoplasma-infected plants, by forming hairy roots. This morphological change is paralleled
49 by an accumulation of cellular Pi and an increase in the expression levels of Pi
50 starvation-induced genes and miRNAs. In addition to the Pi starvation responses, we found
51 that SAP11_{AYWB} suppresses salicylic acid-mediated defense responses and enhances the
52 growth of a bacterial pathogen. These results contribute to an improved understanding of the
53 role of phytoplasma effector SAP11 and provide new insights for understanding the
54 molecular basis of plant–pathogen interactions.

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57 INTRODUCTION

58 Nutrients are essential for the growth of both host cells and pathogens. Host cells obtain
59 nutrients for their own metabolism, but they also provide a favorable environment for
60 pathogens to exploit. To defend against the growth of pathogens, host cells can develop a
61 strategy to sequester key nutrients from the pathogens (Radtke and O’Riordan, 2006; Hood
62 and Skaar, 2012). However, nutritional deficiencies also impair immune responses and alter
63 the host susceptibility to pathogens. In general, successful pathogens have evolved various
64 ways of manipulating host nutrition by modulating the uptake and utilization of nutrients in
65 order to satisfy their requirements. Global transcriptome analysis has been shown that
66 intracellular bacteria, such as *Listeria monocytogenes*, can upregulate genes required for
67 nutritional stresses in mammalian cells (Chatterjee et al., 2006). In the case of *Xanthomonas*
68 *oryzae*, the transcriptional activator-like type III effector pthXol can activate the expression
69 of *OsSWEET11* in controlling cellular sugar efflux (Chen et al., 2010). Thus, it is not
70 surprising that nutritional immunity is a complex process, and nutrition is an important factor
71 in regulating the interaction between a host and a pathogen.

72 Recently, a study on huanglongbing disease through small RNA profiling analysis revealed
73 that the infection of *Candidatus Liberibacter*, an intracellular bacterial pathogen, specifically
74 induced miR399 in citrus plants (Zhao et al., 2013). MiR399 is induced by phosphate (Pi)
75 starvation and has the ability to regulate the homeostasis of Pi by repressing the expression of
76 *PHOSPHATE 2 (PHO2)* (Aung et al., 2006; Chiou et al., 2006). *PHO2* encodes a ubiquitin
77 E2 enzyme required for the degradation of PHT1 Pi transporters and PHOSHATE1 (PHO1)
78 involved in Pi uptake and translocation Pi starvation signaling (Liu et al., 2012; Huang et al.,
79 2013). The Pi deficiency symptom associated with huanglongbing disease implies that the
80 availability of Pi is an important factor for the disease development caused by *Candidatus*

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81 *Liberibacter*. Indeed, the application of Pi reduces the disease symptoms of huanglongbing
82 (Zhao et al., 2013).

83 Pi is required for the synthesis of nucleic acids and phospholipids, and it also plays an
84 important function in energy metabolism and signaling transduction (Shen et al., 2011).
85 Although Pi is an essential nutrient for plant growth and development, the amount of
86 available Pi in the soil is limited (Raghothama, 1999; Jain et al., 2012). As a result, plants
87 have evolved a way to improve their Pi acquisition by forming mutualistic symbiosis with
88 arbuscular mycorrhizal (AM) fungi (Bucher et al., 2009; Ohkama-Ohtsu and Wasaki, 2010).
89 Colonization by AM fungi can modify the plants' root architecture and enhance the uptake of
90 mineral nutrients, in particular Pi (Gu et al., 2011; Volpe et al., 2013). Interestingly, several
91 studies also reported that AM fungi reduce the disease symptoms caused by phytoplasmas
92 (Lingua et al., 2002; Romanazzi et al., 2009; Kamińska et al., 2010; Sampò et al., 2012).

93 Similar to *Candidatus Liberibacter*, phytoplasmas are intracellular bacterial pathogens and
94 depend on sap-feeding insects for transmission (Lee et al., 2000). Phytoplasmas cause various
95 disease symptoms, including witches' broom, phyllody, virescence, bunchy roots, and
96 stunting and yellowing of plants (Hogenhout et al., 2008). Owing to the significant reduction
97 in the genome of phytoplasmas, they cannot be cultured *in vitro* and are restricted to phloem
98 sieve cells with a rich growth environment (Oshima et al., 2004; Bai et al., 2006). Although
99 phytoplasmas depend on their plant host for survival, they are able to manipulate the plant's
100 physiology through their secreted effectors (Sugio et al., 2011a).

101 Recent studies have shown that the protein 11 (SAP11_{AYWB}) secreted by the Aster Yellows
102 phytoplasma strain Witches' Broom (AY-WB) contains a nuclear localization signal (NLS)
103 for nuclear-targeting outside phloem cells (Bai et al., 2008). *Arabidopsis* plants that
104 overexpress SAP11_{AYWB} display crinkled leaves that resemble transgenic plants
105 overexpressing miR319 (Sugio et al., 2011b). MiR319 has been shown to target transcripts of

TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR1 (TCP) genes, which are involved in the control of cell proliferation in leaf morphogenesis and the biosynthesis of jasmonate (JA) (Ori et al., 2007; Schommer et al., 2008). Although SAP11_{AYWB} has not been shown to interfere with miRNA expression, it has been reported that it can destabilize TCP transcription factors through direct interaction (Sugio et al., 2011b). Because TCPs can directly regulate the expression of *LIPOXGENASE 2 (LOX2)*, a key component in the biosynthesis of JA, destabilization of TCPs leads to a decrease in JA (Schommer et al., 2008). In agreement with the findings that JA plays an important role in defense responses against insect herbivores, impairment of JA biosynthesis in SAP11_{AYWB}-overexpressing plants leads to an increase in the progeny of *Macrostelea quadrilineatus* (Kessler et al., 2004; Sugio et al., 2011b).

In this study, we have shown that the expression of phytoplasma effector SAP11_{AYWB} in *Arabidopsis* triggers phosphate starvation responses and suppresses plant defense responses against a bacterial pathogen. These findings advance our understanding of the molecular mechanism underlying the disease symptoms elicited by the secreted effectors and provide new insights into the interaction between host plants and phytoplasmas.

RESULTS

Expression of SAP11_{AYWB} Alters miRNA Accumulation Involved in Pi Starvation and Auxin Responses in *Arabidopsis*

The phenotypical similarities in the leaf morphologies of SAP11_{AYWB}-overexpressing plants (35S::*SAP11_{AYWB}*) and miR319a-overexpressing (35S::*miR319a*) plants (Sugio et al., 2011b) prompted us to examine whether the expression of SAP11_{AYWB} can regulate the accumulation of miRNAs in host cells (Fig. 1A). Expression of SAP11_{AYWB} was confirmed by using the anti-SAP11_{AYWB} antibody (Fig. 1B). Then, total RNA was extracted from plants

grown on half-strength Murashige and Skoog (1/2× MS) medium for small RNA northern blotting assays. Here, we compared the accumulation of miR159, miR160, miR164, miR167, miR319, miR399, and miR393 in *Arabidopsis* wild-type (WT) Col-0 and 35S::SAP11_{AYWB} transgenic lines. Interestingly, the accumulation of miR319 and miR159, miR164, and miR167 was not affected by the expression of SAP11_{AYWB} (Fig. 1C). However, a decrease of miR393 and an induction of miR399 and miR160 were detected in 35S::SAP11_{AYWB} transgenic lines (Fig. 1C). MiR399 plays a key role in regulating Pi starvation responses, whereas miR393 and miR160 are involved in auxin responses. In generally, auxin is regulated upon Pi depletion in order to change root architecture with the aim of increasing Pi uptake (Péret et al., 2011). Thus, auxin has been shown to play an important role in stimulating root architecture during Pi starvation responses (Nacry et al., 2005; Jain et al., 2007; Pérez-Torres et al., 2008). Because miR393 and miR160 are involved in auxin responses, it is possible that those miRNAs have indirect effects on the root architecture alterations required for Pi uptake. Our results suggest that Pi starvation and auxin responses are altered in 35S::SAP11_{AYWB} transgenic plants.

Pi Starvation Responses and Pi Homeostasis Are Altered in SAP11 Transgenic *Arabidopsis* Plants

To investigate whether Pi starvation responses have been altered in 35S::SAP11_{AYWB} transgenic plants, the expression levels of Pi starvation-induced genes were examined using quantitative RT-PCR (qRT-PCR). RNA samples were extracted from seedlings grown in 1/2× MS (Pi-sufficient) medium, and typical Pi starvation-induced genes such as *INDUCED BY PHOSPHATE STARVATION 1 (IPS1)*, *IPS2*, *SPX DOMAIN GENE 1 (SPX1)*, *SPX3*, *PHOSPHATE STARVATION-INDUCED GENE 2 (PS2)*, *PS3*, and *PHOSPHATE TRANSPORTER 1;4 (PHT1;4)* were examined. *IPS1*, *IPS2*, *SPX1*, *SPX3*, *PS2*, *PS3*, and

PHT1;4 were all elicited in *35S::SAP11_{AYWB}* transgenic plants, in comparison with WT plants (Fig. 2A). To examine the levels of cellular Pi, *Arabidopsis* seedlings grown in hydroponic solution were collected. A higher amount of Pi was detected in the aerial parts of *35S::SAP11_{AYWB}* transgenic plants grown in 0.01 mM KH_2PO_4 (Pi-deficient) and 0.25 mM KH_2PO_4 (Pi-sufficient) hydroponic solutions than in WT plants (Fig. 2B). These results indicate that Pi starvation responses and Pi homeostasis are altered in *35S::SAP11_{AYWB}* transgenic plants.

Expression of *SAP11_{AYWB}* Reduces the Accumulation of Anthocyanin and Alters the Root Architecture in *Arabidopsis*

Pi is a major limiting factor for the growth and development of plants. Thus, plants have evolved a series of morphological and physiological modifications triggered by Pi deficiency, such as an increase in root/shoot ratio, proliferation of lateral roots, and accumulation of anthocyanin (Rouached et al., 2010). To examine the effects of Pi deficiency, variations in the development of *35S::SAP11_{AYWB}* transgenic lines grown on 0.01 mM and 0.25 mM KH_2PO_4 medium were compared. As a control, WT plants showed a significant reduction in plant size and displayed red/purple color in leaf when grown on 0.01 mM KH_2PO_4 medium (Fig. 3A). Compared to WT plants, *35S::SAP11_{AYWB}* transgenic plants showed a growth-inhibiting phenotype on both 0.01 mM and 0.25 mM KH_2PO_4 media (Fig. 3A). However, the accumulation of anthocyanin was strongly reduced in *35S::SAP11_{AYWB}* transgenic plants grown on 0.01 mM KH_2PO_4 medium (Fig. 3B). We further investigated the root architecture of *35S::SAP11_{AYWB}* transgenic plants. Compared to WT plants, *35S::SAP11_{AYWB}* transgenic plants grown on both 0.01 mM and 0.25 mM KH_2PO_4 media showed a clear inhibition of primary roots, a significant proliferation of adventitious roots, and an elongation of lateral roots (Fig. 3C). Taken together, the molecular alterations associated with the expression of

SAP11_{AYWB} were correlated with the morphological changes observed in the 35S::*SAP11*_{AYWB} transgenic plants.

Genome-Wide Identification of Differentially Expressed mRNAs Altered by the Expression of SAP11_{AYWB} in *Arabidopsis*

To examine the genome-wide expression profile of mRNAs in response to the expression of SAP11_{AYWB} in *Arabidopsis*, the 35S::*SAP11*_{AYWB} transgenic line #13 with higher expression of SAP11_{AYWB} was selected for a comparative transcriptome analysis (Fig. 4A). Here, total RNA from 35S::*SAP11*_{AYWB} transgenic seedlings and WT seedlings grown on 1/2× MS medium were extracted for next-generation sequencing. In summary, a total of 26.2 million reads mapped to the *Arabidopsis* genome were generated after quality trimming from two cDNA libraries (Supplemental Table S1). To identify differentially expressed genes altered by SAP11_{AYWB}, the expression levels of mapped genes from two samples were quantified and analyzed using DESeq, a differential gene expression data analysis program (Anders and Huber, 2010). A total of 395 differentially expressed genes ($p \leq 0.01$) were identified in the 35S::*SAP11*_{AYWB} transgenic plants. To narrow down the differentially expressed genes, a criterion of $p < 0.001$ was selected, and 163 differentially expressed genes were identified in the 35S::*SAP11*_{AYWB} transgenic plants (Supplemental Data S1). Among them, 59 genes were upregulated and 104 genes were downregulated.

Functional annotations on the 59 upregulated genes revealed that a total of 18 Pi starvation-induced genes, including *MYB DOMAIN PROTEIN 90* (*MYB90*), *IPS1*, *GLUCOSE-6-PHOSPHATE/PHOSPHATE TRANSLOCATOR 2* (*GPT2*), *SULFATE TRANSPORTER 1;3* (*SULTR1;3*), *SPX3*, *MONOGALACTOSYLDIACYLGLYCEROL SYNTHASE 3* (*MGD3*), *BETA-AMYLASE 5* (*BAM5*), *AT5G09570*, *PS2*, *PHOSPHOETHANOLAMINE/PHOSPHOCHOLINE PHOPHATASE 1* (*PEPC1*),

206 *AT5G20790*, *AT1G23110*, *IPS2*, *SPX1*, *PHOSPHOLIPASE D P2 (PLDP2)*, *PS3*,
 207 *SENESCENCE-RELATED GENE 3 (SRG3)*, and *PURPLE ACID PHOSPHATASE 17*
 208 (*PAP17*), were highly elicited by *SAP11_{AYWB}* (Table 1). Notably, a total of 7 Pi
 209 starvation-induced genes were found in the top 10 list of *SAP11_{AYWB}*-elicited genes. Further
 210 comparison revealed that more than 20% of the *SAP11_{AYWB}*-elicited genes ($p \leq 0.001$)
 211 overlapped with the differentially expressed genes (more than 2-fold upregulation) identified
 212 by Muller et al. (2007) and Liu et al. (2011) under Pi starvation conditions (Fig. 4B). In
 213 addition to the Pi starvation responses, a small subset of genes involved in sugar metabolic
 214 processes and iron-deficiency responses were also elicited by *SAP11_{AYWB}* (Table 1). To
 215 evaluate the quality of the RNA-Seq data, *SAP11_{AYWB}*-elicited genes were randomly selected
 216 for validation by using the qRT-PCR method. The differentially expressed genes, including
 217 *GPT2*, *BAM5*, *MGD3*, *PLDP2*, *SWEET1*, *MYB90*, *TPPI*, *SRG3*, *CYTOCHROME P450*
 218 *FAMILY 707 SUBFAMILY A POLYPEPTIDE 2 (CYP707A2)*, and *FERRIC REDUCTION*
 219 *OXIDASE 5 (FRO5)*, were all elevated in the *35S::SAP11_{AYWB}* transgenic plants (Fig. 4C).
 220 Taken together, these results indicate that Pi starvation-induced genes are highly upregulated
 221 by the expression of *SAP11_{AYWB}* in *Arabidopsis*.

222 223 **Genome-Wide Identification of Differentially Expressed miRNAs Altered by the** 224 **Expression of *SAP11_{AYWB}* in *Arabidopsis***

225 To investigate the genome-wide expression profile of miRNAs in response to the
 226 expression of *SAP11_{AYWB}* in *Arabidopsis*, RNA samples were collected from seedlings grown
 227 on 1/2× MS medium. Two small RNA libraries from *35S::SAP11_{AYWB}* transgenic line #13 and
 228 WT plants were constructed for deep-sequencing. In summary, a total of 34.5 million reads
 229 mapped to the *Arabidopsis* genome were generated after quality trimming from two cDNA
 230 libraries (Supplemental Table S2). Among them, about 9.3 million reads were mapped to

Arabidopsis miRNAs deposited in miRBase (Kozomara and Griffiths-Jones, 2011). The expression levels of known miRNAs in two samples were normalized, and the differentially expressed miRNAs with fold-change >2 or <1/2 were identified (Fig. 5; Supplemental Data S2). Compared to WT plants, 35S::*SAP11*_{AYWB} transgenic plants showed significant upregulation of miR160, miR2111, miR394, miR399, miR5639, miR5655, miR827, miR850, and miR863. In contrast, miR172, miR396, miR5020, miR5629, miR5645, and miR5648 showed significant downregulation in the 35S::*SAP11*_{AYWB} transgenic plants. Among them, miR2111, miR399, and miR827 have been shown to be upregulated by Pi starvation (Hsieh et al., 2009; Pant et al., 2009) and miR399 and miR827 play important roles in regulating Pi homeostasis (Liu et al., 2012; Huang et al., 2013; Lin et al., 2013); miR160 and miR396 are required for regulating the auxin signaling response and cell proliferation during leaf and root development (Gutierrez et al., 2009; Pulido and Laufs, 2010; Debernardi et al., 2012; Bazin et al., 2013); miR172 has been shown to regulate the transition from the vegetative to reproductive phases (Aukerman and Sakai, 2003; Wu et al., 2009); and miR394 is required for the regulation of leaf morphology and shoot meristem stem-cell maintenance (Song et al., 2012; Knauer et al., 2013). However, miR5020b, miR5629, miR5639, miR5645d, miR5648, miR5655, and miR850 are newly identified miRNAs with unknown functions.

We further evaluated the results of small RNA sequencing by using small RNA northern blotting. Here, several miRNAs were randomly selected for validation, and the results were consistent with the genome-wide miRNA expression profiling (Fig. 4D). Moreover, we investigated the expression level of *PHO2* in 35S::*SAP11*_{AYWB} transgenic line #13, because miR399 has been shown to downregulate *PHO2* by directly targeting its transcripts (Aung et al., 2006; Chiou et al., 2006). Consistent with the upregulation of miR399, a significant downregulation of *PHO2* was observed in 35S::*SAP11*_{AYWB} transgenic line #13 (Fig. 4E). Taken together, these results indicate that miRNAs involved in Pi-starvation and

auxin-signaling pathways are altered by the expression of SAP11_{AYWB} in *Arabidopsis*.

Role of MiR319a in SAP11_{AYWB}-Triggered Pi Starvation Responses

Although SAP11_{AYWB} did not interfere with the accumulation of miR319 (Fig. 1C and 4D), the crinkled leaves that appeared in 35S::*SAP11_{AYWB}* transgenic plants resembled those in the 35S::*miR319a* transgenic plants (Fig. 1A and 6A), which prompted us to examine whether miR319a has an effect on the SAP11_{AYWB}-triggered Pi starvation responses. We investigated the accumulation of miR159, miR164, miR319, miR393, and miR399 in 35S::*miR319a* transgenic plants grown on 1/2× MS medium. An increase in the amount of miR159, miR164, and miR399 was observed with the overexpression of miR319 in *Arabidopsis* (Fig. 4D). However, the amount of increase for miR399 was much lower in 35S::*miR319a* transgenic plants than in 35S::*SAP11_{AYWB}* transgenic line #13. Consistently, the expression levels of Pi starvation-induced genes were much lower in 35S::*miR319a* transgenic plants, in comparison with 35S::*SAP11_{AYWB}* transgenic line #13 (Fig. 4C and E). We further investigated whether miR319a has an effect on the root architecture. Compared to 35S::*SAP11_{AYWB}* transgenic line #13, 35S::*miR319a* transgenic plants grown on 0.01 mM KH₂PO₄ medium showed no proliferation of adventitious roots and strong inhibition of primary roots (Fig. 6B). The root architecture in 35S::*miR319a* transgenic plants was more similar to that in WT plants (Fig. 6B). Because SAP11_{AYWB} does not affect miR319 expression levels and the root architecture of miR319 transgenic plants are more similar to wild type, it is unlikely that miR319 is involved in the SAP11_{AYWB}-triggered Pi starvation responses. However, the transcript levels of Pi starvation-induced genes and miR399 were lightly upregulated in 35S::*miR319a* transgenic plants. Therefore, TCP transcripts targeted by miR319 may have minimal contribution to the phosphate starvation responses.

SAP11_{AYWB}-Triggered Pi Starvation Responses Are Mainly Dependent on *PHR1*

Molecular mechanisms underlying Pi starvation responses remain largely unknown (Rouached et al., 2010). Only a few transcription factors have been identified as important players in the Pi-starvation signaling pathway. Among them, *PHOSPHATE STARVATION RESPONSE 1 (PHR1)* that encodes a MYB transcription factor plays a key role in responding to the Pi deficiency in *Arabidopsis* (Rubio et al., 2001). However, there was no significant difference in the transcript level of *PHR1* between WT and SAP11_{AYWB} transgenic plants (Fig. 4E). When we analyzed the potential cis-regulatory elements on the Pi-starvation induced genes regulated by SAP11_{AYWB} using PlantPAN, a navigator for plant promoter analysis (Chang et al., 2008), we found that 14 out of 18 Pi-starvation induced genes contain *PHR1* binding sites in their promoter regions (Table 3). Thus, to understand whether the *PHR1*-dependent pathway contributes to the SAP11_{AYWB}-triggered Pi starvation responses, 35S::*SAP11_{AYWB}* was introduced into a *phr1* mutant. With the expression of SAP11_{AYWB}, 35S::*SAP11_{AYWB}/phr1* transgenic plants showed a wavy-margin phenotype in the leaves (Fig. 7A). However, the Pi starvation-induced genes, including *IPS1*, *IPS2*, *SPX1*, *SPX3*, *PS2*, *PS3*, and *PHT1;4*, were all suppressed when SAP11_{AYWB} was expressed under a *phr1* mutant background (Fig. 7B). These results indicate that *PHR1* is required for SAP11_{AYWB}-triggered Pi starvation responses.

SAP11_{AYWB} Transgenic Plants Show Altered Hormone Synthesis and Signaling and Are More Susceptible to the Bacterial Pathogen *Pseudomonas Syringae*

RNA-Seq analysis of the downregulated genes in 35S::*SAP11_{AYWB}* #13 transgenic line revealed that a subset of genes, including *ELICITOR-ACTIVATED GENE 3-1 (ELI3-1)*, *At3g05730* (defensin-like family protein), *PATHOGENESIS-RELATED GENE 1 (PR1)*, and *LOX2*, were highly suppressed (Table 2). The RNA-seq data was validated using the

qRT-PCR method (Fig. 8A and B). The downregulation of *LOX2* is consistent with the previous studies reported by Sugio et al. (2011b). Interestingly, *LOX2* is involved in JA synthesis, whereas *PR1* and *ELI3-1* are salicylic acids (SA)-responsive genes (Kiedrowski et al., 1992; Spoel et al., 2003). These results suggest that *SAP11_{AYWB}* not only interferes with JA synthesis but also suppresses SA-signaling responses. We found that *SAP11_{AYWB}* repressed the expression of miR393, an Flg22-induced miRNA that participates in basal resistance against bacterial pathogens (Navarro et al., 2006). Thus, repression of miR393 and *PR1* in 35S::*SAP11_{AYWB}* transgenic plants prompted us to investigate whether the expression of *SAP11_{AYWB}* can suppress innate immunity against bacterial pathogens in *Arabidopsis*. To this end, we monitored the expression levels of genes involved in the SA-mediated defense-signaling network under the treatment of 1 mM SA. Genes involved in SA biosynthesis (*EDS5* and *SID2*), SA accumulation (*FMO1*, *WIN3*, and *PAD4*), as well as the standard marker genes for SA-mediated response (*PR1* and *AIG1*), were all repressed in 35S::*SAP11_{AYWB}* #13 transgenic line (Fig. 8A). We further investigated the effect of *SAP11_{AYWB}* on the multiplication of *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pto* DC3000) in *Arabidopsis*. In this study, we used the 35S::*SAP11_{AYWB}* #12 and #29 transgenic lines for bacterial inoculation assays because the leaves of 35S::*SAP11_{AYWB}* #13 were too crinkled and small for inoculation. Compared with WT plants, both 35S::*SAP11_{AYWB}* #12 and #29 transgenic lines showed an increase in bacterial growth (Fig. 8C). Taken together, these results indicate that the expression of *SAP11_{AYWB}* suppresses the host's innate immunity and makes the plants more susceptible to a bacterial infection.

DISCUSSION

SAP11_{AYWB} has been shown to mediate the destabilization of *Arabidopsis* TCPs, which leads to the downregulation of *LOX2*, a key component in the biosynthesis of JA (Schommer

et al., 2008; Sugio et al., 2011b). In this study, our RNA-Seq analysis also showed a significant decrease in *LOX2* transcripts in *35S::SAP11_{AYWB}* transgenic plants (Table 2). In addition, we showed that the expression of miR319 was not changed in the *SAP11_{AYWB}*-overexpressing plants (Fig. 1C and 4D). These data are in agreement with previous published results showing that *SAP11_{AYWB}* degrades TCPs (Sugio et al., 2011b), and suggest that the *SAP11_{AYWB}*-mediated suppression of TCPs' activities probably occurs only through post-translational regulation. In contrast to miR319, a significant increase in the amount of miR399, miR2111, and miR827, which have been shown to be upregulated by Pi starvation (Hsieh et al., 2009; Pant et al., 2009), was observed in *35S::SAP11_{AYWB}* transgenic plants (Fig. 1C; Supplemental Data S2). Among them, miR399 and miR827 play positive roles in regulating Pi uptake and translocation (Chiou et al., 2006; Liu et al., 2012; Huang et al., 2013; Lin et al., 2013). Consistent with this, *35S::SAP11_{AYWB}* transgenic plants accumulated a higher amount of Pi in cells and displayed reduced symptoms in the Pi-deficient condition, e.g., a lower level of anthocyanin accumulation (Fig. 2B and 3B). These phenomena were paralleled by an increase in the transcript levels of Pi starvation-induced genes, e.g., *IPS1*, *IPS2*, *SPX1*, *SPX3*, *PS2*, *PS3*, and *PHT1;4* (Fig. 2A).

Although Pi homeostasis is altered in *35S::SAP11_{AYWB}* transgenic plants, the molecular mechanism of *SAP11_{AYWB}*-triggered Pi starvation responses remains unclear and has yet to be clarified. In this study, we show that the Pi starvation responses triggered by *SAP11_{AYWB}* are mainly dependent on the pathway regulated by *PHR1* (Fig. 7B). *PHR1*, a MYB transcription factor, plays a crucial role as a central hub in the transcriptional activation of genes involved in Pi transport and translocation, root architecture remodeling, anthocyanin accumulation, and sugar metabolic processes (Busto et al., 2010; Chiou and Lin, 2011). Thus, more than 75% of the Pi-starvation induced genes regulated by *SAP11_{AYWB}* contain the potential *cis*-regulatory elements for *PHR1* binding in the promoter region (Table 3). These results

might provide some information in elucidating the functional relationships among SAP11_{AYWB} and Pi starvation responses. In *Arabidopsis*, there are 24 TCPs divided into two classes based on sequence similarities (Aguilar-Martinez et al., 2007). Recently, Sugio et al. (2011b) showed that class II CIN-TCPs were selectively destabilized by SAP11_{AYWB}. Class II CIN-TCPs contains 8 members, but only 5 of them can be targeted by miR319 (Ori et al., 2007). In this study, we found that the transcript levels of Pi starvation-induced genes and miR399 were not changed significantly in 35S::*miR319a* transgenic plants (Fig. 4C to E), suggesting that miR319-mediated regulation may have minimal contribution to the SAP11_{AYWB}-triggered Pi starvation responses. Nevertheless, because only 5 transcripts out of 8 CIN-TCPs are targeted by miR319, involvement of other class II CIN-TCP members in Pi starvation responses cannot be excluded. On the other hand, whether the class II CYC/TB1-TCPs, the potential targets of SAP11_{AYWB}, is also involved in Pi starvation responses of 35S::*SAP11_{AYWB}* transgenic plants requires further investigation.

Phytoplasmas have the smallest genome among bacteria, and many essential genes required for standard metabolic functions are lost, including ATP synthase subunits (Oshima et al., 2004; Chung et al., 2013). Thus, phytoplasmas might uptake essential substances from the cytoplasm of surrounding host cells. Here, we showed that overexpression of SAP11_{AYWB} could trigger Pi starvation responses and alter the Pi homeostasis in host cells (Fig. 2A and B). Pi is an essential nutrient required for various basic biological functions, e.g., synthesis of nucleic acids and phospholipids, regulation of energy metabolism, and signal transduction cascades (Raghothama, 1999; Shen et al., 2011). Although the molecular mechanism of SAP11_{AYWB}-triggered Pi starvation responses remains elucidated, it might alter the physiology of plant cells into a metabolically rich environment, which might be able to facilitate the growth of phytoplasmas.

Pi deficiency is typically accompanied with the accumulation of Fe and sugar (Jain et al.,

2007; Ward et al., 2008; Rouached et al., 2010). Similarly, our RNA-Seq analysis uncovers transcriptomic variations not only in genes involved in Pi starvation responses, but also in genes related to sugar metabolic processes and iron-deficiency responses, e.g., *GPT2*, *BAM5*, *TPPI*, *SWEET1*, *bHLH100*, *bHLH38*, and *bHLH39* (Table 1). These molecular changes might play a role in the cross-regulation between Pi starvation, sucrose signaling, and Fe responses. Because sugar and Fe are also essential nutrients for the growth and development of pathogens (Chen et al., 2010; Hood and Skaar, 2012), upregulation of genes involved in sugar metabolic processes and iron-deficiency responses might provide a better environment for the growth of phytoplasmas in plant cells.

The importance of Pi in the development of disease symptoms could be revealed in huanglongbing disease, in which an increase in the expression levels of miR399 and Pi starvation-induced genes was observed in citrus plants infected by *Candidatus Liberibacter* (Zhao et al., 2013). Noticeably, the disease symptoms of *Candidatus Liberibacter*-infected citrus were reduced after the application of Pi. In the case of phytoplasmas, although the correlation between Pi and the disease symptoms remains unclear, the disease symptoms in phytoplasma-infected plants could be reduced by the application of AM fungi (Lingua et al., 2002; Romanazzi et al., 2009; Kamińska et al., 2010; Sampò et al., 2012). AM fungi are symbiotic organisms that are able to enhance the uptake of Pi through upregulation of Pi starvation responses in host plants (Branscheid et al., 2010; Gu et al., 2010; Volpe et al., 2013). Thus, it will be interesting to explore whether the application of Pi could reduce the phytoplasma-mediated disease symptoms in the future. Although it is reasonable to assume that the availability of Pi in host cells might affect the disease symptoms in phytoplasma-infected plants, other factors might alter the disease symptoms as well. For example, the plant responses elicited by AM fungi, such as SA- and JA-defense responses (Gutjahr and Paszkowski, 2009), might participate in counteracting the colonization of

406 phytoplasmas, leading to the reduction of disease symptoms.

407 In addition to the regulation of Pi uptake, we found that the expression of SAP11_{AYWB} is
408 able to suppress host's innate immunity by repressing and delaying the SA-mediated defense
409 responses (Table2; Fig. 8A). As a result, SAP11_{AYWB}-overexpressing plants become more
410 susceptible to bacterial infections (Fig. 8C). Because miR393 plays an important role in
411 modulating plant defense responses against bacterial pathogens through the repression of
412 auxin signaling pathway (Navarro et al., 2006; Katiyar-Agarwal and Jin, 2010), the changes
413 of host's innate immunity in 35S::*SAP11*_{AYWB} transgenic plants might correlate with the
414 downregulation of miR393. However, the role of class II TCPs in 35S::*SAP11*_{AYWB} transgenic
415 plants remains unclear. The suppression of host's innate immunity might be possible to be an
416 indirect consequence caused by the SAP11_{AYWB}-mediated destabilization of TCPs.

417 Root architecture remodeling induced by Pi starvation requires the regulation of auxin
418 signaling (Nacry et al., 2005; Jain et al., 2007; Pérez-Torres et al., 2008; Péret et al., 2011). In
419 35S::*SAP11*_{AYWB} transgenic plants, the formation of adventitious roots, elongation of lateral
420 roots, and reduction of primary roots was associated with the differential expression of
421 miRNAs involved in auxin signaling (Fig. 3C and 6B; Supplemental Data S2). These
422 miRNAs include miR160, miR393 and miR396, which target transcripts encoding the auxin
423 response factors, auxin receptors, and growth-regulating factors, respectively (Mallory et al.,
424 2005; Parry et al., 2009; Debernardi et al., 2012). Although the molecular changes in auxin
425 signaling triggered by SAP11_{AYWB} remain largely unknown, the phenotypic alterations in root
426 architecture resemble to the disease symptoms appeared in the roots of several
427 phytoplasma-infected plants (Del Serrone et al., 2001; Lee et al., 2006).

428 Our findings provide useful information for the activities of SAP11_{AYWB} in plant immune
429 responses and suggest that the expression of SAP11_{AYWB} not only suppresses the JA signaling
430 responses against insect vectors but also downregulates the SA-mediated defense responses

against bacterial pathogens. These studies not only contribute to the understanding of molecular mechanisms underlying the interaction between phytoplasmas and plants, but also provide new insights into development of the strategy in controlling phytoplasma diseases.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis ecotype Col-0 was used for the generation of transgenic plants, and bacterial inoculation. *Nicotiana benthamiana* was used for agroinfiltration. Plants were grown at 21°C in a semicontrolled walk-in chamber with a 16:8-h photoperiod for *Agrobacterium* transformation and Pi measurement; a 12:12-h photoperiod was used for SA treatment and bacterial inoculation.

Generation of Transgenic *Arabidopsis* Plants

A synthetic DNA template with the codon-optimized version of *SAP11*_{AYWB} was used for PCR amplification. A DNA fragment encoding *SAP11*_{AYWB} without the signal peptide was amplified using AccuPrime pfx DNA polymerase (Invitrogen) and subcloned into the pBA002 vector under the control of the *cauliflower mosaic virus* (*CaMV*) 35S promoter. After verification by DNA sequencing, plasmid DNAs were introduced into *Agrobacterium tumefaciens* strain ABI by using the freeze-thaw method and transformed into *Arabidopsis* by using the floral-dip method. To obtain homozygous transgenic lines expressing *SAP11*_{AYWB}, seeds were screened on 1/2× MS medium containing Basta (25 µg ml⁻¹) and carbenicillin (100 µg ml⁻¹) and examined with western blotting using specific anti-SAP11 antibodies.

Root Architecture Investigation and Anthocyanin Measurement

Arabidopsis seeds were germinated on 1/2× MS medium with 1% sucrose and 1% agar.

After incubation for 3 days, seedlings were transferred to the half-strength modified Hoagland nutrient solution containing 1% sucrose and 1% agar with the indicated concentration of KH_2PO_4 . For root architecture investigation, seedlings were placed vertically to allow root growth along the surface of the agar, and the root architecture was observed at 14 days after germination. For anthocyanin measurement, seedlings were harvested at 13 days after germination and the anthocyanin content was determined as described with modifications (Saijo et al., 2009). Briefly, seedlings were homogenized in the extraction buffer (Propanol:HCl:H₂O = 18:1:81) and immersed into boiling water for 1.5 minutes. After centrifugation, the supernatant was collected for measuring the absorbance at 535 and 650 nm. The relative anthocyanin amount was calculated by the equation: $(A_{535}-2A_{650})/\text{fresh weight (g)}$.

Pi Measurement

Pi contents were measured according to the method described by Chiou et al. (2006). Briefly, *Arabidopsis* seeds were germinated on agar plate with half-strength modified Hoagland medium containing 0.25 mM KH_2PO_4 for 11 days. The seedlings were then transferred to hydroponic culture. Before Pi starvation treatment, seedlings were grown on 0.25 mM KH_2PO_4 . Fresh tissues were homogenized with extraction buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 100 mM NaCl, 1 mM-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride) and then mixed with 1% glacial acetic acid. After centrifugation at $13,000 \times g$ for 5 min, Pi assay solution (0.35% NH_4MoO_4 , 0.86 NH_2SO_4 , and 1.4% ascorbic acid) was added to the supernatant and then incubated at 42°C for 30 min. Pi content was measured using Tecan Infinite 200 PRO at A_{820} .

Small RNA Northern Blotting

For miRNA analysis, 12 µg of total RNA was fractionated on a 15% polyacrylamide gel containing 8 M urea and then transferred to a Hybond-N⁺ membrane (GE Biosciences). Oligonucleotide probes were end-labeled with [γ -³²P]ATP by using T4 polynucleotide kinase (New England Biolabs). Hybridization was performed overnight at 42°C with the ULTRAHyb-Oligo hybridization buffer (Ambion). Signals were detected using autoradiography.

Antibody Production and Western Blotting

To generate recombinant proteins, a PCR product encoding SAP11_{AYWB} without the signal peptide was subcloned into the SUMO-pET vector to generate an N-terminal His-SUMO fusion construct. After verification by DNA sequencing, plasmid DNA was introduced into *Escherichia coli* BL21 (DE3) cells, and His-SUMO-SAP11_{AYWB} protein was purified using Ni²⁺-NTA resin (Qiagen), according to the manufacturer's instruction. After being cleaved with *Ulp1* to remove the His-SUMO tag, recombinant SAP11_{AYWB} protein was purified for antibody production in a rabbit. The polyclonal antibody against SAP11_{AYWB} was obtained by affinity purification using a polyvinylidene difluoride membrane as a coupling matrix. To detect SAP11_{AYWB} in *Arabidopsis* transgenic plants, total extracts were prepared by directly adding 2.5× SDS sample buffer into plant samples after grinding. Amersham ECL western blotting reagents were used for the reactions, and the chemiluminescence signals were captured using an ImageQuant LAS 4000 mini (GE Healthcare).

RNA-Seq Analysis

To perform the RNA-Seq analysis, 3-week-old 35S::*SAP11*_{AYWB} transgenic plants grown in 1/2× MS medium were used, and next-generation sequencings were performed using Hiseq 2000 (Illumina) with total RNA samples extracted by the RNeasy plant mini kit (Qiagen). For

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transcriptome analysis, sequence reads were aligned using CLC bio, and gene expression levels were normalized as reads per kilobase of exon model per million mapped reads (RPKM). To identify differentially expressed genes in 35S::*SAP11_{AYWB}* transgenic plants, DEseq (Anders and Huber, 2010) analysis was performed, and differentially expressed genes with $P < 0.05$ were selected for further analysis.

qRT-PCR

For qRT-PCR analysis, *Arabidopsis* total RNA was extracted using the Trizol reagent (Invitrogen), and complementary DNAs were synthesized using Superscript III first strand synthesis supermix (Invitrogen), according to the manufacturer's instructions. The RNA expression analyses were performed using the KAPA SYBR fast qPCR kit (Kapa Biosystems) and Illumina Eco real-time PCR system. The expression levels of the selected genes were determined by normalizing to the reference gene *Actin2*. Experiments were repeated at least three times.

SA Treatments and Bacterial Inoculations

To examine the immune responses in 35S::*SAP11_{AYWB}* transgenic plants, 4- to 5-week-old plants grown in the soil were treated with 1 mM SA with foliar sprays. After spraying, the aerial parts of the plants were harvested at indicated times and frozen immediately in liquid nitrogen. For bacterial inoculations, *Pseudomonas syringae* pv. *tomato* DC3000 (*Pto* DC3000) was cultured in NY broth (3 g of beef extract, 5 g of peptone, and 3 g of yeast extract in 1 L of water) at 28°C. The 4- to 5-week-old 35S::*SAP11_{AYWB}* transgenic plants were inoculated with the bacterial suspension (5×10^5 CFU ml⁻¹). The inoculated plants were maintained at 21°C, and bacterial populations were determined at indicated time intervals by using NY agar plates containing rifampicin (50 µg ml⁻¹). Experiments were repeated at least three times.

531

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536

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733 **Close TJ, Roose M, Yokomi RK, Folimonova S, Vidalakis G, Rouse R, Bowman KD,**
734 **Jin H** (2013) Small RNA profiling reveals phosphorus deficiency as a contributing factor
735 in symptom expression for Citrus Huanglongbing disease. *Mol Plant* **6**: 301-310
736

FIGURE LEGENDS

Figure 1. Expression of *SAP11_{AYWB}* regulates the accumulation of miRNAs involved in Pi- and auxin-signaling responses in *Arabidopsis*. (A) Comparison of leaf morphologies between WT (Col-0) and transgenic plants carried *SAP11_{AYWB}* or miR319a driven by *CaMV* 35S promoter. Scale bar: 8 mm. (B) Examination of the translated product of *SAP11_{AYWB}* by western blotting using specific antibody against *SAP11_{AYWB}*. Asterisk indicates the cross-reacting band appeared in all samples. Anti-tubulin was used for loading control. (C) Comparisons of the expression levels of miRNAs in WT and 35S::*SAP11_{AYWB}* transgenic lines by small RNA northern blotting. The RNAs staining bands were used as a loading control. The values of band intensities were measured using ImageJ.

Figure 2. *SAP11_{AYWB}* elicits the expression of Pi starvation-induced genes and increases the accumulation of Pi in *Arabidopsis*. (A) The mRNA levels of genes triggered by Pi starvation responses in WT (Col-0) plants and 35S::*SAP11_{AYWB}* transgenic lines were examined by quantitative RT-PCR (qRT-PCR), and normalized to *Actin2*. The relative expression levels of each gene in WT plants were set at 1. (B) The levels of Pi concentration in the aerial parts of WT and 35S::*SAP11_{AYWB}* transgenic lines grown in hydroponic solutions with 0.01 mM or 0.25 mM KH_2PO_4 were measured. Statistically significant differences were determined using Student's *t* test (* $p < 0.05$, ** $p < 0.005$ for transgenic plants versus the WT).

Figure 3. Expression of *SAP11_{AYWB}* in *Arabidopsis* alters the phenotypes in anthocyanin accumulation and root architecture. Comparisons of plant size (A), anthocyanin accumulation (B), and root morphology (C) between WT (Col-0) plants and 35S::*SAP11_{AYWB}* transgenic lines grown on horizontal (A) or vertical (C) plates containing 0.01 mM or 0.25 mM KH_2PO_4 .

Statistically significant differences were determined using Student's *t* test (* $p < 0.05$, ** $p < 0.005$ for transgenic plants versus the WT). Scale bars: 15 mm. FW: fresh weight.

Figure 4. Comparison of gene expression profiles. (A) Comparison of the expression levels of SAP11_{AYWB} in *Arabidopsis* 35S::*SAP11*_{AYWB} transgenic lines by western blotting using specific antibodies against SAP11_{AYWB}. Asterisk indicates the cross-reacting band appeared in all samples. (B) Venn diagrams show the comparisons of SAP11_{AYWB}-elicited genes ($p < 0.001$) with the Pi starvation response (PSR) genes (up-regulation, 2-fold higher). PSR (I) indicates the data set collected by Muller et al. (2007) from leaf samples in response to Pi starvation. PSR (II) and PSR (III) indicate the data set collected by Liu et al. (2011) from leaf and root samples, respectively, in response to Pi starvation. (C) The mRNA levels of differentially expressed genes identified in the 35S::*SAP11*_{AYWB} transgenic line #13 were examined by qRT-PCR, and normalized to *Actin2*. The relative expression levels of each gene in WT (Col-0) plants were set at 1. (D) Comparisons of the expression levels of miRNAs by small RNA northern blotting. The RNAs staining bands were used as a loading control. (E) The mRNA levels of genes involved in Pi starvation responses were examined by qRT-PCR, and normalized to *Actin2*. The relative expression levels of each gene in WT (Col-0) plants were set at 1. Statistically significant differences were determined using Student's *t* test (* $p < 0.05$, ** $p < 0.005$ for transgenic plants versus the WT). The values of band intensities were measured using ImageJ.

Figure 5. Comparison of miRNA expression profiles. Scatter plot shows the related expression levels of known miRNAs in *Arabidopsis* WT plants (X-axis) and 35S::*SAP11*_{AYWB} transgenic line #13 (Y-axis), in which the scales indicate the expression intensity of normalized miRNAs. Red points refer to miRNAs whose expression levels were 2-fold

higher in 35S::*SAP11_{AYWB}* transgenic line #13 as compared with WT plants. Green points refer to miRNAs whose expression levels were 2-fold lower in 35S::*SAP11_{AYWB}* transgenic line #13 as compared with WT plants. Blue points refer to miRNAs whose expression levels were not higher or lower than 2-fold in the 35S::*SAP11_{AYWB}* transgenic line #13 as compared with WT plants.

Figure 6. Phenotypic comparisons in leaf morphology and root architecture between 35S::*miR319a* and 35S::*SAP11_{AYWB}* transgenic plants. (A) Leaf morphologies of *Arabidopsis* seedlings grown on 1/2× MS medium. Scale bar: 8 mm. (B) Root architectures of *Arabidopsis* seedlings grown on the vertical plate containing 0.01 mM KH_2PO_4 . Scale bar: 15 mm.

Figure 7. *PHR1* is required for *SAP11_{AYWB}*-triggered Pi starvation responses in *Arabidopsis*. (A) Leaf morphologies of *Arabidopsis* transgenic plants overexpressing *SAP11_{AYWB}* in the background of a *phr1* mutant. The expression level of *SAP11_{AYWB}* was detected by western blotting using specific antibodies against *SAP11_{AYWB}*. Asterisk indicates the cross-reacting band appeared in all samples. Scale bar: 8 mm. (B) The mRNA levels of genes involved in Pi starvation responses among WT (Col-0), *phr1* mutant, and 35S::*SAP11_{AYWB}/phr1* transgenic plants were examined by qRT-PCR, and normalized to *Actin2*. The relative expression levels of each gene in WT plants were set at 1. Statistically significant differences were determined using Student's *t* test (* $p < 0.05$, ** $p < 0.005$ for WT versus *phr1*; + $p > 0.05$ for *phr1* versus 35S::*SAP11_{AYWB}/phr1*).

Figure 8. *SAP11_{AYWB}* suppresses the innate immunity against bacterial pathogens in *Arabidopsis*. RNA samples were extracted from SA-treated WT (Col-0) and 35S::*SAP11_{AYWB}*

812 transgenic plants harvested at indicated times. The mRNA levels of genes involved in
813 SA-mediated defense signaling network (A) and JA-signaling responses (B) were examined
814 by qRT-PCR, and normalized to *Actin2*. The relative expression levels of each gene in WT
815 plants without SA treatment were set at 1. (C) Bacterial growth of *Pseudomonas syringae* pv.
816 *tomato* DC3000 (*Pto* DC3000) in WT and 35S::*SAP11*_{AYWB} transgenic plants were measured
817 to examine the effects of *SAP11*_{AYWB} on the resistance of *Arabidopsis* against bacterial
818 pathogens. Hand-infiltrated leaves were collected at the indicated times for measuring the *in*
819 *planta* growth of *Pto* DC3000. Statistically significant differences were determined using
820 Student's *t* test (* $p < 0.05$, ** $p < 0.005$ for transgenic plants versus the WT).

821

822 TABLES

Table 1. Classification and annotation of the differentially expressed genes (<i>p</i> < 0.001) elicited by SAP11 _{AYWB}							
Category		AGI ^b No.	Base Mean ^a		Fold chang e ^c	Short description	Reference ^d
Rank	Name		WT	35S:: <i>SAP11</i> AYWB			
Pi starvation response							
1 st	<i>MYB90</i>	At1g66390	1.11	60.23	54.14	Myb domain protein 90	B, D
2 nd	<i>IPS1</i>	At3g09922	18.91	620.26	32.8	Induced by phosphate starvation 1	
3 rd	<i>GPT2</i>	At1g61800	7.79	175.29	22.51	Glucose-6-phosphate/phosphate translocator 2	B, D
5 th	<i>SULTR1;3</i>	At1g22150	5.56	82.7	14.87	Sulfate transporter 1;3	A, C
6 th	<i>SPX3</i>	At2g45130	5.56	79.11	14.22	SPX domain gene 3	B, C, D
8 th	<i>MGDC</i>	At2g11810	30.04	374.86	12.48	Monogalactosyldiacylglycerol synthase type C	A, B, C, D
10 th	<i>BAM5</i>	At4g15210	13.35	161.81	12.12	Beta-amylase 5	A, B, C
11 th		At5g09570	7.79	89.89	11.54	Cox19-like CHCH family protein	C
14 th	<i>PS2</i>	At1g73010	67.86	737.13	10.86	Phosphate starvation-induced gene 2	A, B, C, D
15 th	<i>PEPC1</i>	At1g17710	46.72	468.34	10.02	PhosphoEthanolamine/PhosphoCholine phosphatase 1	A, B, C, D
21 th		At5g20790	84.55	747.01	8.84	Unknown protein	A, B, C, D
24 th		At1g23110	28.92	232.82	8.05	Unknown protein	A
35 th	<i>IPS2</i>	At5g03545	199.13	1141.65	5.73	Induced by phosphate starvation 2	
36 th	<i>SPX1</i>	At5g20150	284.78	1629.77	5.72	SPX domain gene 1	A, B, C, D
45 th	<i>PLDP2</i>	At3g05630	73.42	317.32	4.32	Phospholipase D P2	A, B, C, D
48 th	<i>PS3</i>	At3g47420	543.98	2238.34	4.11	Phosphate starvation-induced gene 3	A, B, C, D
52 th	<i>SRG3</i>	At3g02040	327.05	1253.11	3.83	Senescence-related gene 3	A, B, C, D
56 th	<i>PAP17</i>	At3g17790	263.65	944.78	3.58	Purple acid phosphatase 17	A, B, C, D
Sugar metabolic processes							
3 rd	<i>GPT2</i>	At1g61800	7.79	175.29	22.51	Glucose-6-phosphate/phosphate translocator 2	
10 th	<i>BAM5</i>	At4g15210	13.35	161.81	12.12	Beta-amylase 5	
16 th	<i>TPPI</i>	At5g10100	13.349	133.04	9.97	Trehalose-6-phosphate phosphatase I	
44 th	<i>SWEET1</i>	At1g21460	350.42	1552.46	4.43	Nodulin MtN3 family protein	
Iron-deficiency responses							
20 th	<i>bHLH100</i>	At2g41240	20.02	191.47	9.56	Basic helix-loop-helix protein 100	
25 th	<i>bHLH38</i>	At3g56970	32.26	249.01	7.72	Basic helix-loop-helix protein 38	
29 th	<i>bHLH39</i>	At3g56980	35.6	251.7	7.07	Basic helix-loop-helix protein 39	
^a Base mean: the number of reads divided by the size factor (normalized constant) of sample.							
^b AGI: <i>Arabidopsis</i> Genome Initiative							
^c Fold change: 35S:: <i>SAP11</i> _{AYWB} base mean/WT base mean							
^d Reference: Pi starvation-induced genes (> 2 fold change) reported by Muller et al. (2007) (A), liu et al. (2011) (B: shoot, C: root) and Morcuende et al. (2007) (D).							

823

Table 2. Classification and annotation of the top 10 differentially expressed genes ($p < 0.001$) suppressed by $SAP11_{AYWB}$

Category		AGI ^b No.	Base Mean ^a		Fold change ^c	Short description
Rank	Name		WT	35S::SAP11 _{AYWB}		
1 st		At3g16670	814.29	0.89	914.9	Pollen Ole e 1 allergen and extensin family protein
2 nd	<i>ELI3-1</i>	At4g37980	1311.55	2.69	487.6	Elicitor-activated gene 3-1
3 rd		At3g05730	2143.65	8.09	264.9	Defensin-like (DEFL) family protein
4 th	<i>PR1</i>	At2g14610	150.17	0.89	168.7	Pathogenesis-related gene 1
5 th		At2g29300	518.39	5.393	96.1	NAD(P)-binding Rossmann-fold superfamily protein
6 th		At3g19620	109.01	1.79	60.9	Glycosyl hydrolase family protein
7 th		At4g12900	58.95	1.79	32.9	Gamma interferon responsive lysosomal thiol (GILT) reductase family protein
8 th	<i>LOX2</i>	At3g45140	2688.74	86.29	31.2	Lipoxygenase 2
9 th		At2g29290	80.09	3.59	22.3	NAD(P)-binding Rossmann-fold superfamily protein
10 th		At2g32160	75.64	3.59	21.1	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein

^a Base mean: the number of reads divided by the size factor (normalized constant) of sample.

^b AGI: *Arabidopsis* Genome Initiative

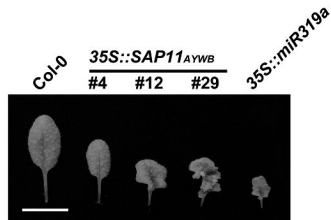
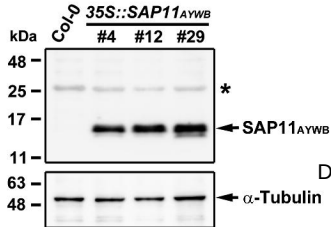
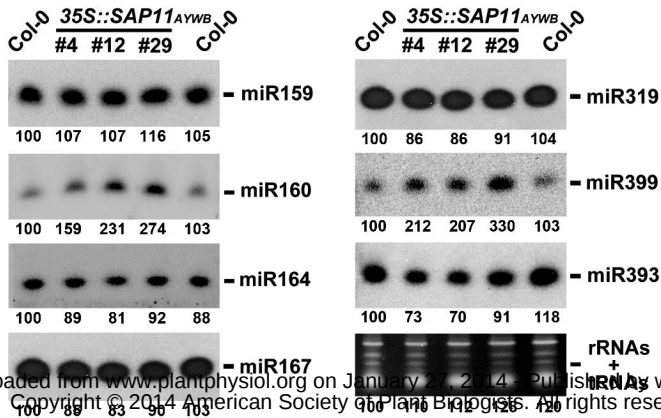
^c Fold change: WT base mean/35S::SAP11_{AYWB} base mean

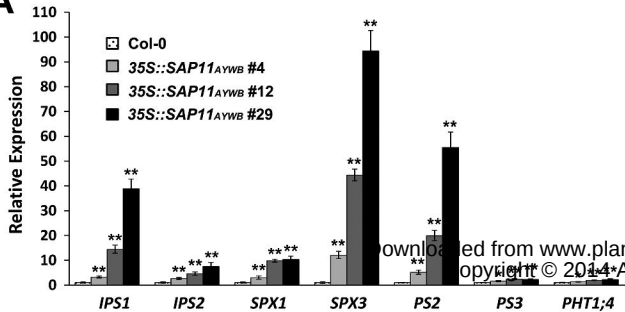
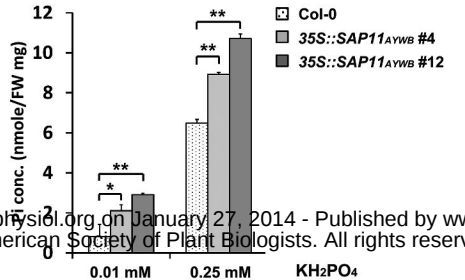
Table 3. The potential sequence of PHR1-binding site found at the upstream region of Pi-starvation induced genes regulated by SAP11_{AYWB}

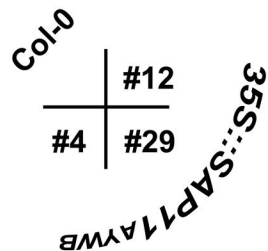
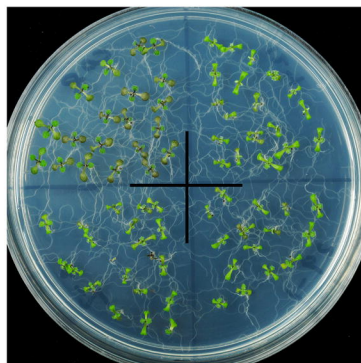
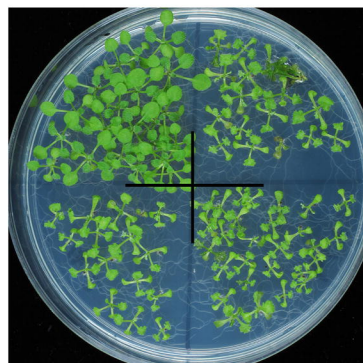
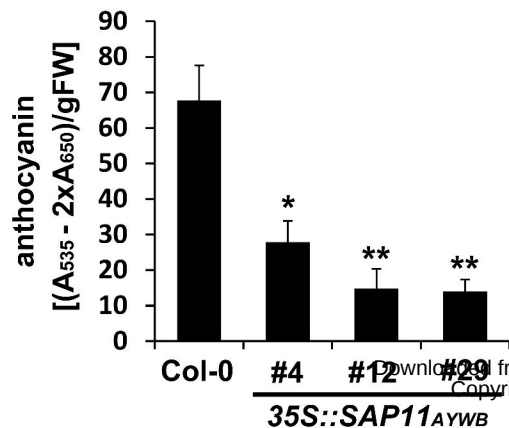
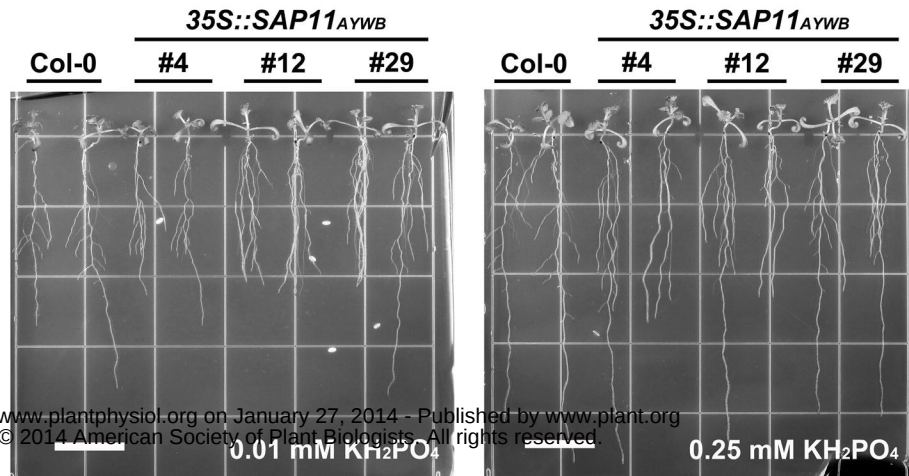
Gene	AGI ^a number	Sequence (Position ^b)
<i>IPS1</i>	At3G09922	GCATATTC (-581); GCATATTC (-547)
<i>GPT2</i>	At1G61800	GTATATTC (-125)
<i>SULTR1;3</i>	At1G22150	GGATATTC (-439)
<i>SPX3</i>	At2G45130	GAATATGC (-212); GCATATCC (-89)
	At5G09570	GGATATAC (-1656); GAATATTC (-142)
<i>PS2</i>	At1G73010	GGATATTC (-693); GAATATTC (-308)
<i>PEPC1</i>	At1G17710	GAATATTC (-765); GAATATTC (-370)
	At5G20790	GAATATGC (-1325)
	At1G23110	GAATATGC (-346); GAATATGC (-171)
<i>IPS2</i>	At5G03545	GTATATGC (-687); GCATATTC (-149)
<i>SPX1</i>	At5G20150	GAATATTC (-824); GGATATTC (-67); GAATATTC (-43)
<i>PLDP2</i>	At3G05630	GAATATTC (-698); GGATATTC (-664); GCATATAC (-635); GCATATAC (-244)
<i>SRG3</i>	At3G02040	GTATATGC (-334); GAATATTC (-137); GAATATCC (-73); GAATATTC (-73)
<i>PAP17</i>	At3G17790	GAATATCC (-131)

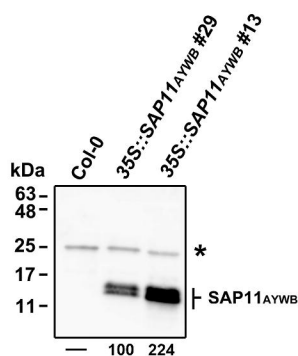
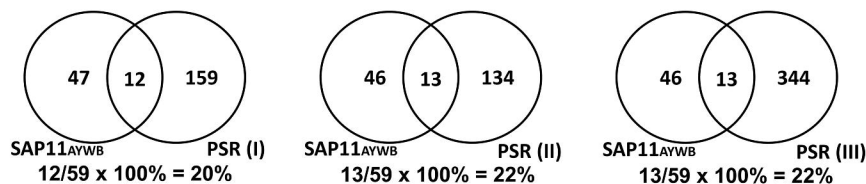
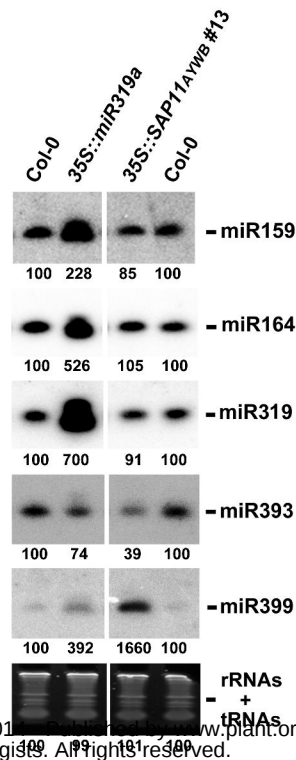
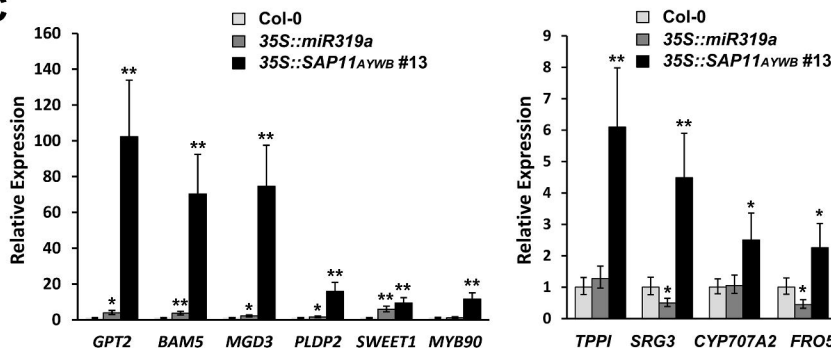
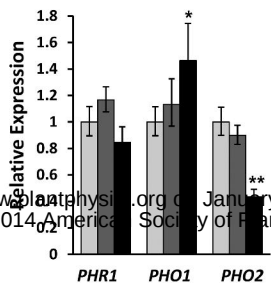
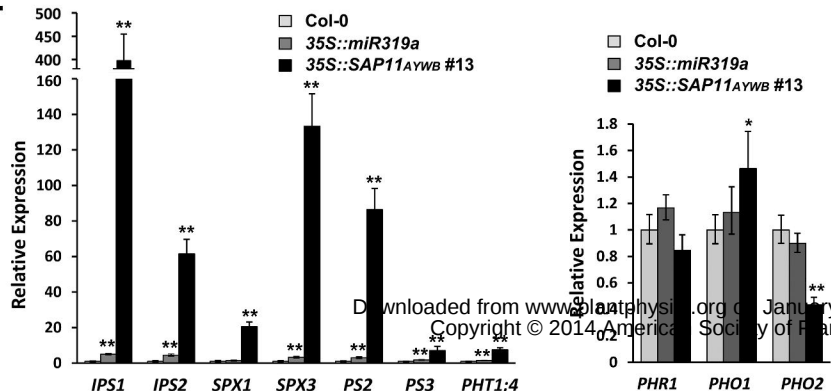
^a AGI: *Arabidopsis* Genome Initiative

^b Position: the position in the 5'-upstream region is given with respect to the ATG start codon.

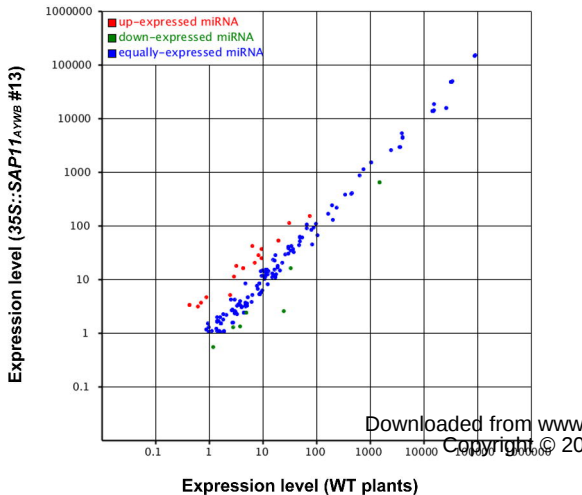
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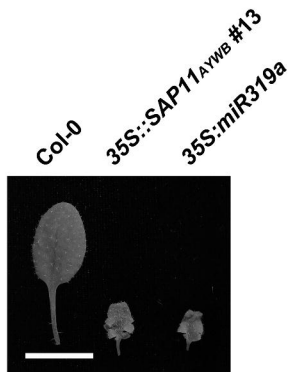
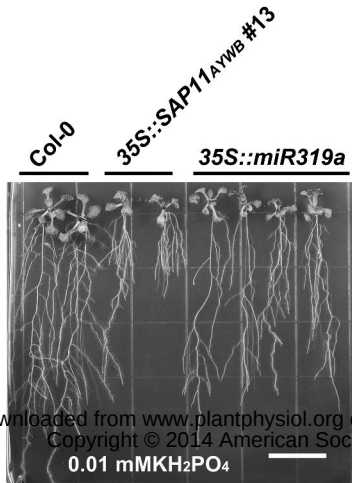
A**B**

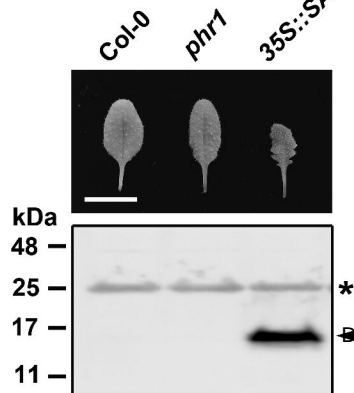
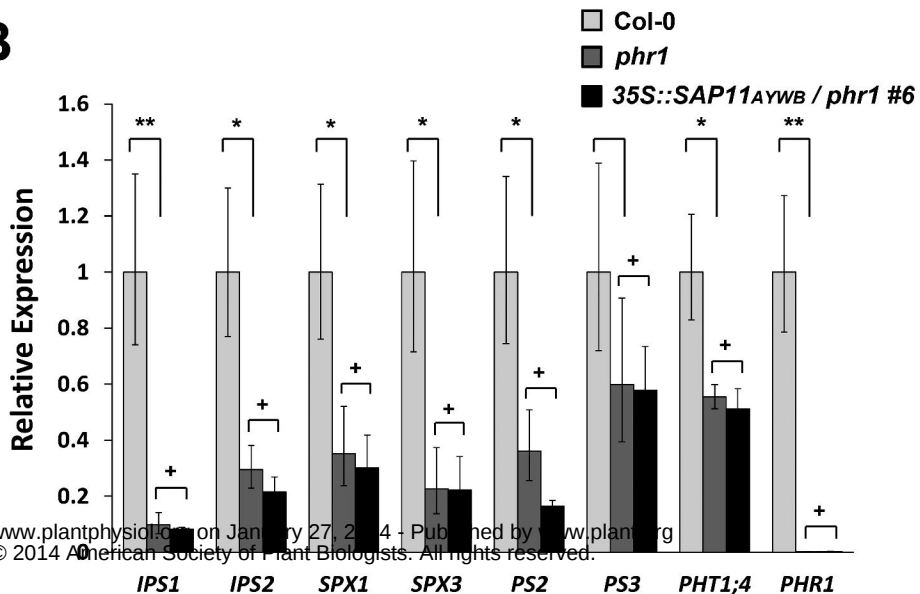
A0.01 mM KH_2PO_4 0.25 mM KH_2PO_4 **B****C**

A**B****D****C****E**

Scatter plot

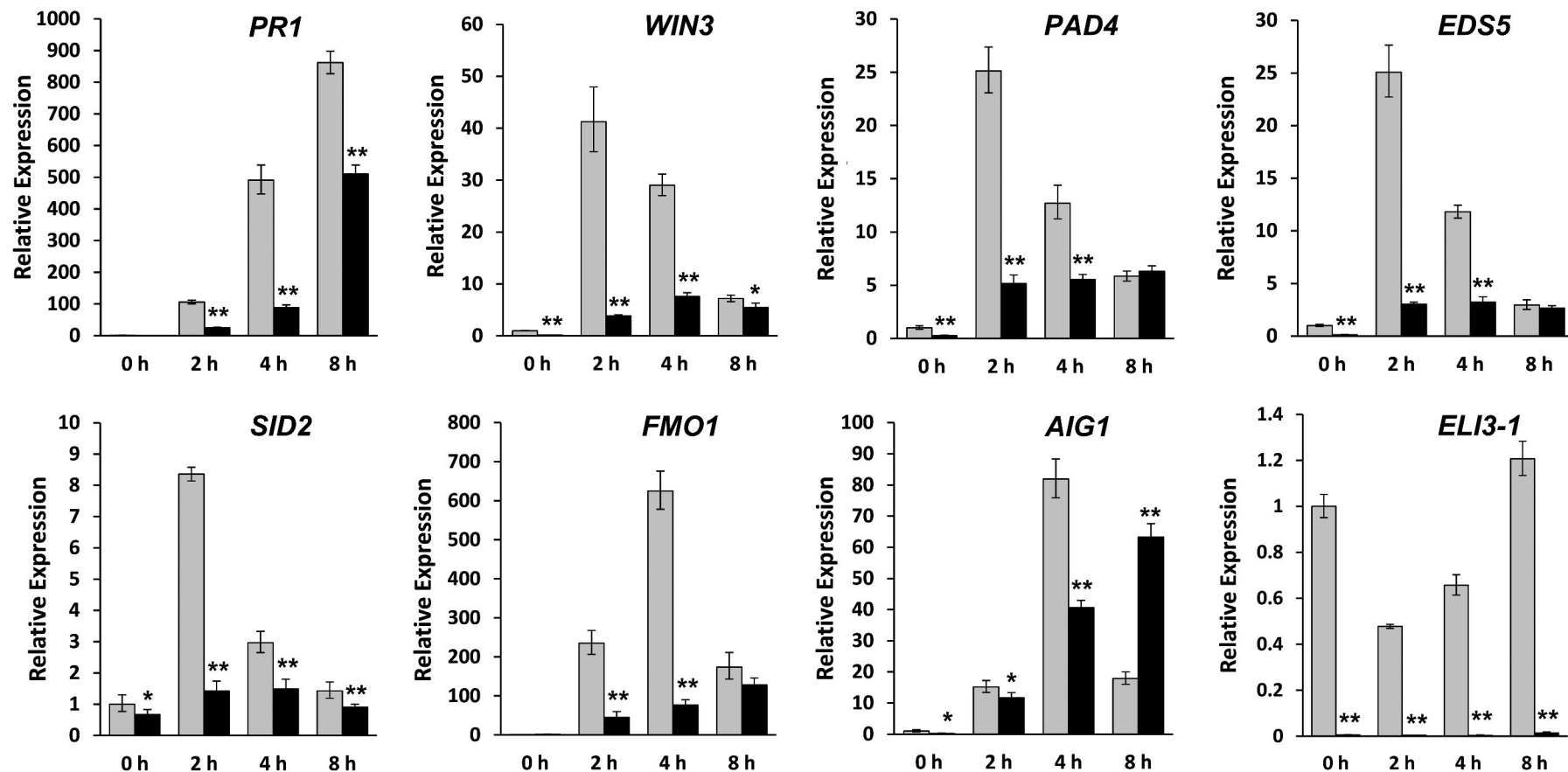


A**B**

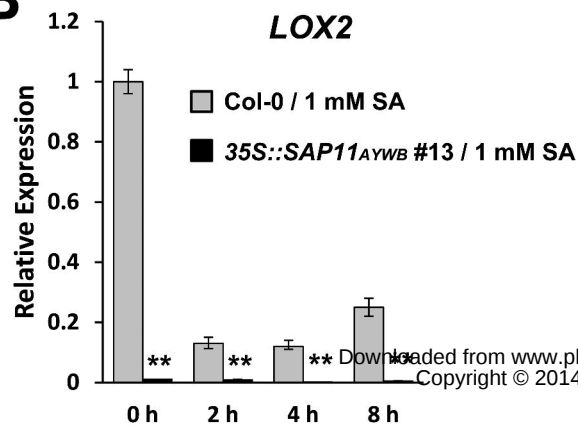
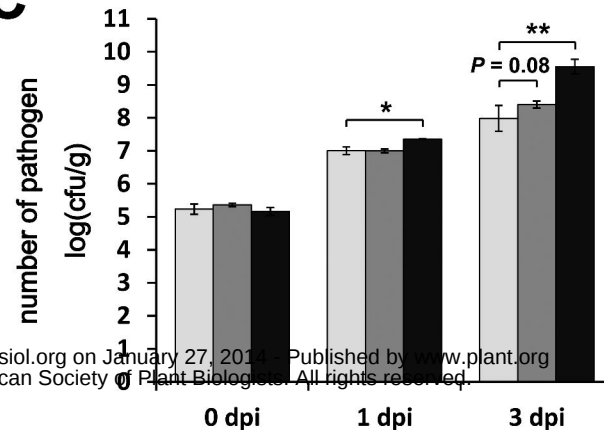
A**B**

A

Col-0 / 1 mM SA

35S::SAP11_{AYWB} #13 / 1 mM SA**B***LOX2*

Col-0 / 1 mM SA

35S::SAP11_{AYWB} #13 / 1 mM SA**C**

Col-0 / Pto DC3000

35S::SAP11_{AYWB} #12 / Pto DC300035S::SAP11_{AYWB} #29 / Pto DC3000