Ca14-3-3 Interacts With CaWRKY58 to Positively Modulate Pepper Response to Low-Phosphorus Starvation

Jinsen Cai1,2,3, Weiwei Cai1,2,3, Xueying Huang1,2,3, Sheng Yang1,2,3, Jiayu Wen1,2,3, Xiaqin Xia1,2,3, Feng Yang1,2,3, Yuanyuan Shi1,2,3, Deyi Guan1,2,3 and Shuilin He1,2,3*

1National Education Ministry Key Laboratory of Plant Genetic Improvement and Comprehensive Utilization, Fujian Agriculture and Forestry University, Fuzhou, China, 2Key Laboratory of Applied Genetics of Universities in Fujian Province, Fujian Agriculture and Forestry University, Fuzhou, China, 3Agricultural College, Fujian Agriculture and Forestry University, Fuzhou, China

Low-phosphorus stress (LPS) and pathogen attack are two important stresses frequently experienced by plants in their natural habitats, but how plant respond to them coordinately remains under-investigated. Here, we demonstrate that CaWRKY58, a known negative regulator of the pepper (Capsicum annuum) response to attack by Ralstonia solanacearum, is upregulated by LPS. Virus-induced gene silencing (VIGS) and overexpression of CaWRKY58 in Nicotiana benthamiana plants in combination with chromatin immunoprecipitation (ChIP) and electrophoretic mobility shift assays (EMSA) demonstrated that CaWRKY58 positively regulates the response of pepper to LPS by directly targeting and regulating genes related to phosphorus-deficiency tolerance, including PHOSPHATE STARVATION RESPONSE1 (PHR1). Yeast two-hybrid assays revealed that CaWRKY58 interacts with a 14-3-3 protein (Ca14-3-3); this interaction was confirmed by pull-down, bimolecular fluorescence complementation (BiFC), and microscale thermophoresis (MST) assays. The interaction between Ca14-3-3 and CaWRKY58 enhanced the activation of PHR1 expression by CaWRKY58, but did not affect the expression of the immunity-related genes CaNPR1 and CaDEF1, which are negatively regulated by CaWRKY58 in pepper upon Ralstonia solanacearum inoculation. Collectively, our data indicate that CaWRKY58 negatively regulates immunity against Ralstonia solanacearum, but positively regulates tolerance to LPS and that Ca14-3-3 transcriptionally activates CaWRKY58 in response to LPS.

Keywords: Capsicum annuum, CaWRKY58, Ca14-3-3, CaPHR1, phosphorus deficiency

INTRODUCTION

Plants are confronted with fluctuating ecological environments and are often exposed to biotic or abiotic stresses. Therefore, they have evolved sophisticated defense mechanisms to perceive stress, and to initiate and translate signaling pathways into appropriate defense responses that involve massive transcriptional reprogramming via transcription factors (Moore et al., 2011).
To maximize fitness, plant growth, development, and responses to environmental cues must be tightly and coordinately regulated, which requires extensive cross-talk among plant responses to stresses, growth, and development (Fujita et al., 2006; Xia et al., 2015; Li et al., 2020). A single transcription factor is often involved in regulating several apparently disparate processes (Rushton et al., 2010), but the molecular details underlying this coordination often remain poorly understood.

Low-phosphorus stress (LPS) is one of the most important abiotic stresses experienced by plants, due to the requirement of phosphorus (P) for plant growth and development, but its limited availability in natural soils (Shen et al., 2011). Under this selection pressure, plants have evolved strategies to maximize its availability and to adapt to LPS. Massive transcriptional reprogramming activated by LPS has frequently been observed in plant species (Lan et al., 2013; Oono et al., 2013; O’Rourke et al., 2013; Fan et al., 2014; Deng et al., 2018; Xue et al., 2018). Many genes are transcriptionally activated by LPS, leading to enhanced P acquisition and utilization; these include genes that encode proteins such as Pi transporters (Miao et al., 2009; Remy et al., 2012; Ayadi et al., 2015; Liu et al., 2018a; Zhang et al., 2019), H(+)-ATPase (Yuan et al., 2017), phosphate transporter PHT4:6 (Hassler et al., 2012), PHOSPHATE STARVATION RESPONSE 1 (PHR1; Motte and Beeckman, 2017; Wang et al., 2019), protein kinases (Lan et al., 2013; Lei et al., 2014), purple acid phosphatases (Lan et al., 2013; Liu et al., 2018c), SPX (Zhang et al., 2016; Liu et al., 2018b; Osorio et al., 2019), and PHOSPHATE1 (PHO1; Hamburger et al., 2002). The processes involved in LPS responses include alterations in root architecture (Jain et al., 2007; Peret et al., 2011, 2014; Lopez-Arredondo et al., 2014; Postma et al., 2014; Strock et al., 2018), modification of the soil chemistry surrounding roots (Lopez-Arredondo et al., 2014) and the activation of metabolism to efficiently use phosphorus (Plaxton and Tran, 2011; Lopez-Arredondo et al., 2014). The transcription of many transcription factor genes is altered in response to LPS; these transcription factors act as positive or negative modulators of the response and include members of the MYB (Khan et al., 2014; Zhou et al., 2017; Wang et al., 2019), WRKY (Wang et al., 2014; Su et al., 2015; Dai et al., 2016), JAZ (Jasmonate-ZIM domain) (Aparicio-Fabre et al., 2013), bHLH (Valdes-Lopez and Hernandez, 2008b), zinc-finger (Devaiah et al., 2007b; Ding et al., 2016), AP2/ERF (Ramaiah et al., 2014), CCAAT box-binding (NF-Y; Qu et al., 2015), and auxin response factor (ARF; Shen et al., 2013) families, as well as PHR1 (Motte and Beeckman, 2017; Wang et al., 2019). Some of these transcription factors might be modulated in a context-dependent manner by interacting with other regulators, such as SPX domain-containing proteins (Zhong et al., 2018) and WRKY proteins (Zhou et al., 2017). In addition, the response of plants to phosphorus deficiency is closely related to other biological processes, such as the response to NO3− (Medici et al., 2015), Fe starvation (Dai et al., 2016), cold stress (Dai et al., 2016), S, Fe, Zn (Briat et al., 2015), K (Wang et al., 2002) and in particular, to plant immunity (Hiruma et al., 2016; Luo et al., 2019). The silencing of TaPT29-6A, a Pi transporter that plays a major role in Pi uptake from soil by roots, significantly increased the susceptibility of wheat plants to biotrophic, hemi-biotrophic, and necrotrophic pathogens (Zhang et al., 2019). Furthermore, the levels of secondary metabolites involved in plant immune reactions, including benzoxazinoids and flavonoids, differed significantly in plants grown under Pi-deficient conditions (Luo et al., 2019), indicating extensive crosstalk between plant response to phosphorus-deficiency stress and pathogen attack. However, the underlying mechanism for this crosstalk remains largely uncharacterized.

The WRKY proteins constitute one of the largest transcription factor families in many species (Eulgem et al., 2000), and WRKY family members have been implicated in diverse biological processes and particularly in plant immunity, by specifically targeting the conserved cognate W-box within the promoter regions of their target genes (Eulgem, 2006; Eulgem and Somssich, 2007; Pandey and Somssich, 2009; Ishihama and Yoshioka, 2012; Buscaill and Rivas, 2014; Birkenbihl et al., 2017). WRKY transcription factors, including AtWRKY6 (Bakshi et al., 2015; Decker et al., 2017; Ye et al., 2018), WRKY42 (Sunar et al., 2015), WRKY45 (Wang et al., 2014), and WRKY75 in Arabidopsis (Devaiah et al., 2007a; Encinas-Villarejo et al., 2009), GbWRKY1 in cotton (Zhang et al., 2012) and OsWRKY74 in rice (Dai et al., 2016) function in response to LPS. The importance of WRKY transcription factors in the coordination of biological processes might reflect their flexible and diverse regulatory mechanisms, which are achieved by interactions with an array of proteins such as VQ proteins, MAPks, chromatin remodeling proteins, calmodulin proteins, 14-3-3 proteins, WRKY or other transcription factors (Chi et al., 2013). However, the function of the majority of WRKY transcription factors in the plant LPS response and other biological processes has not been extensively characterized.

The 14-3-3 proteins are highly conserved eukaryotic proteins characterized by a conserved central core flanked by divergent regions at the N and C termini. These regions can be subdivided into two distinct groups: the ε group, and the plant-specific non-ε group. The 14-3-3 proteins act as molecular scaffolds or chaperones by physically interacting with target proteins via phosphorylated motifs containing phosphoserine residues of (R/K)XX(pS/pT)XP, (R/K)XXX(pS/pT)XP, and pS/pT-X1-2-CO2H, in which pS and pT denote a phosphoserine and a phosphothreonine, respectively (Ormancey et al., 2017). The target proteins include H(+)-ATPase (Xu et al., 2016), nitrate reductase (Xu et al., 2016), ubiquitin ligase (Lu et al., 2016), protein kinases such as CDPks (Ito et al., 2014), and transcription factors such as AtWR11 (Kong and Ma, 2018), PIF7 (Huang et al., 2018), and bZIP (Takeo and Ito, 2017; Luang et al., 2018). The interaction of 14-3-3 proteins with other proteins alters the activity, stability, subcellular localization, or composition of the protein complex and this regulates physiological processes in plants that include metabolism, transport, growth, development, and stress responses (Ormancey et al., 2017). The expression of several 14-3-3 proteins is affected in planta in response to phosphorus deprivation (Cao et al., 2007) and these proteins have been implicated in plant responses to phosphorus deficiency (Cao et al., 2007; Ding et al., 2012; Xu et al., 2012a,b; Li et al., 2017; Yuan et al., 2017; Zhang et al., 2018).
They function by modulating H+ efflux through affecting Arabidopsis plasma membrane H+-ATPase2 (AHA2) or AHA7 (Li et al., 2017), regulating leaf carbon allocation, increasing phloem sucrose transport to promote root growth, or activating root plasma membrane H(+)−ATPases to release more protons under phosphorus deficiency (Xu et al., 2012a,b). However, knowledge concerning the role of 14-3-3 proteins and the molecular basis for the plant response to phosphorus deficiency remain elusive.

Pepper (Capsicum annuum L.) is a member of the solanaceae and is an agriculturally important vegetable crop. It is mainly distributed or planted in uplands that contain soil-borne pathogens, including Ralstonia solanacearum, and that confront plants with other stresses such as LPS, which frequently cause growth retardation and yield loss (Forster et al., 1998). The application of phosphite, which causes phosphorus deficiency and growth retardation in pepper plants, significantly reduces the incidence of Phytophthora crown rot (Forster et al., 1998), indicates potential crosstalk between responses to pathogen attack and LPS. Notably, the WRKY protein CaWRKY58 was previously shown to negatively regulate the response of pepper to Ralstonia solanacearum (Wang et al., 2013). Here, we report that CaWRKY58 is transcriptionally induced by LPS and positively regulates the response of pepper to LPS by directly targeting PHR1 and physically interacting with 14-3-3.

MATERIALS AND METHODS

Pepper and N. benthamiana Plant Cultivation and Phosphorus-Deficiency Treatment

Pepper (inbred line HN42) and N. benthamiana plants were cultivated as described previously (Cheng et al., 2017) before growth in hydroponic culture. The roots of plants at the seven-leaf stage were cleared and plants were transferred from soil to nutrient solution containing sufficient phosphorus (0.83 mM Ca2+, 0.75 mM Mg2+, 0.75 mM SO42−, 15.8 mM Fe2+, 10.3 μM Mn2+, 4.2 μM Zn2+, 43.5 μM B3, and 2.14 μM Cu2+). After 7 days, the plants were transferred to the above-mentioned hydroponic culture solution in which 0.83 mM HPO42− was replaced with 0.08 mM HPO42- to provide a LPS treatment. The plants were harvested at 7 dpt (days post treatment) and 30 dpt, to measure root system architecture (RSA) and root phosphorus content, respectively.

Vector Construction

The full-length open reading frames (ORFs) of CaWRKY58 and Ca14-3-3 were cloned into the entry vector pDONR207 by BP reaction. After confirmation by sequencing, the ORFs were recombined into the destination vectors pEarleyGate103 [for C-terminal green fluorescent protein (GFP) fusions], pDEST-15 (for N-terminal GST fusions), pDEST17 (for N-terminal 6 × His fusions and expression in Escherichia coli) by LR reactions, using Gateway cloning (Invitrogen, Carlsbad, CA, United States). To construct vectors for VIGS-mediated gene silencing, a specific 300–400 bp fragment within the 3' UTRs of CaWRKY58 or CaPHR1 was amplified with specific primer pairs, using genomic DNA from pepper accession Zunla-1 as a template. The specificity of the amplicon was confirmed by BLAST search against the pepper genome. The PCR amplicon was cloned into pDONR207 by BP reaction and the identity of the clones was confirmed by sequencing before further subcloning into the PYL279 (pTRV2) vector, to generate pTRV2:CaWRKY58, or pTRV2:CaPHR1. All vectors were introduced into Agrobacterium tumefaciens strain GV3101.

Virus-Induced Gene Silencing

Agrobacterium tumefaciens GV3101 cells containing pTRV1, pTRV2:00, pTRV2:Ca LPS, pTRV2:CaWRKY58, or pTRV2:CaPHR1 were grown overnight in LB medium supplemented with appropriate antibiotics and were then pelleted and resuspended to a final cell density of OD600 = 0.8 in infiltration medium (10 mM MES, 10 mM MgCl2, 200 μM acetylsyringone, and pH5.4). GV3101 cells harboring pTRV1 were mixed with cells containing pTRV2:00, pTRV2:CaPDS, pTRV2:CaWRKY58 or pTRV2:CaPHR1 in a 1:1 ratio and infiltrated into the cotyledons of 2-week-old pepper seedlings. The plants were then placed in a growth chamber at 16°C in the dark for 56 h, and were then transferred to a growth room at 25°C and 60% humidity, with a light intensity of 60–70 μmol photons m−2 s−1 and a 16-h light/8-h dark photoperiod, until the plants infiltrated with pTRV:CaPDS exhibited a bleached phenotype, indicative of successful silencing of the PHYTOENE DESATURASE gene. Around 36 pepper plants for each gene every time, and the experiment was replicated three times.

Construction of Transgenic N. benthamiana Lines Overexpressing CaWRKY58

Transgenic N. benthamiana plants were generated by leaf transformation as described previously (Dang et al., 2013), using the plant expression vector pEarleyGate103-CaWRKY58. Transgenic lines (T0) were selected with 0.04% BASTA and 20 BASTA-resistant T0 plants were further confirmed by PCR and RT-PCR using a CaWRKY58-specific primer pair (Supplementary Table S1). Seeds of T1 lines were harvested from BASTA-resistant and self-pollinated T0 plants; similarly, seeds of T2 lines were harvested from BASTA-resistant and self-pollinated plants of T1 lines. Plants of T1 lines were used for future analysis after confirmation by RT-PCR using CaWRKY58-specific primer pair and western blotting using an anti-GFP antibody.

Measurement of Total P and Pi Content in Roots of Pepper and N. benthamiana

Measurement of Pi concentration and plant total P content was performed as described previously (Namamori et al., 2004; Zhou et al., 2008; Wu et al., 2011). The Pi concentration was

1http://peppersequence.genomics.cn/page/species/blast.jsp
normalized to fresh weight (FW) and the total P content was normalized to dry weight. Six biological replicates were performed in the experiment.

**Transient Expression of CaWRKY58 or Ca14-3-3**
The plant expression vector pEarleyGate103-CaWRKY58 (Ca14-3-3) was transformed into GV3101 cells, which were infiltrated into leaves of pepper or *N. benthamiana*, following the method described previously (Guan et al., 2018). Leaves were harvested at indicated time points for further experiments, including subcellular protein localization and total RNA extraction.

**Prokaryotic Expression of Fusion Proteins in E. coli**
To purify soluble CaWRKY58-6 × His and Ca14-3-3-GST fusion proteins, a pDEST17 plasmid harboring the full-length ORF of CaWRKY58 or the pDEST-15 vector containing the full-length ORF of Ca14-3-3 was introduced into *E. coli* strain BL21. Expression of the fusion proteins was induced by the addition of 1 mM (final concentration) isopropyl β-D-1-thiogalactopyranoside (IPTG) at 20°C for 12 h. The presence of the soluble proteins in the supernatant of the *E. coli* BL21 cell lysate was confirmed by SDS-PAGE gel electrophoresis.

**Electrophoretic Mobility Shift Assay Analysis**
Electrophoretic mobility shift assay (EMSA) was performed as described previously (Heravi and Altenbuchner, 2014; Shen et al., 2020), using CaWRKY58-6 × His purified from *E. coli* strain BL21 and the promoter fragments containing the wild-type or mutated W-box obtained from Cy5-labeled probes (CaPHR1-P<sup>95</sup>, mCaPHR1-P<sup>95</sup>) or Cy5-nonlabeled probes (CaPHR1-P; Supplementary Table S1). Before incubation with CaWRKY58-6 × His, CaPHR1-P<sup>95</sup> and CaPHR1-P were mixed in different ratios to assay their competitive interaction with CaWRKY58-6 × His. The EMSA blot image was generated with Odyssey CLX (LI-COR). The experiment was replicated three times.

**Yeast Two-Hybrid Assay**
The yeast two-hybrid assay was performed as described previously (Liu et al., 2015), the ORF of CaWRKY58 was cloned into the pDONR201 (satellite vector) and transferred into the pDEST32 (destination vector) for the yeast two-hybrid screen. The yeast two-hybrid library screening was performed according to the manufacturer's instructions (Invitrogen).

**Bimolecular Fluorescence Complementation Assay**
The ORF of CaWRKY58 and Ca14-3-3 were cloned into the entry vector pDONR201 first, then the ORF of CaWRKY58 was recombined into destination vector pEYFP-N1 (CaWRKY58-YFP<sup>95</sup>) and the ORF of Ca14-3-3 was recombined into destination vector pEYFP-C1 (Ca14-3-3-YFP<sup>95</sup>). The constructs were transformed into the *Agrobacterium tumefaciens* strain GV3101, respectively. The GV3101 cells containing the two vectors were mixed in a 1:1 ratio and were infiltrated into *N. benthamiana* leaves. The cell layers of infiltrated leaves were visualized by microscopy (Leica, Germany) at 48 hours post infiltration (hpi). The experiment was replicated three times.

**Microscale Thermophoresis**
The interaction between CaWRKY58 and Ca14-3-3 was confirmed by microscale thermophoresis (MST; Zillner et al., 2012). For this, Ca14-3-3-GFP, or GFP isolated by immunoprecipitation with an anti-GFP antibody from pepper leaves transiently overexpressing Ca14-3-3-GFP or GFP were used as targets, and CaWRKY58-6 × His was used as a ligand. Protein-protein interactions between Ca14-3-3-GFP or GFP, and CaWRKY58-6 × His, were measured using 20 nM Ca14-3-3-GFP or GFP. The CaWRKY58-6 × His solution was diluted to a concentration range from 1.0E<sup>-10</sup> to 1.0E<sup>-3</sup> mM and the CaWRKY58-6 × His fusion protein was incubated with the labeled protein for 10 min in interaction buffer. The samples were then loaded into Monolith NT.115 Capillaries (Cat. MO-K002, NanoTemper Technologies, Germany) using 50% IR laser power and an LED excitation source, with λ = 470 nm at ambient temperature. The K<sub>d</sub> values were calculated for interactions between Ca14-3-3-GFP and CaWRKY58-6 × His or GFP and CaWRKY58-6XHis using NanoTemper Analysis 1.2.20 software (Zillner et al., 2012; Papageorgiou et al., 2016). The experiment was replicated three times.

**Pull-Down Assay and Immunoblotting**
The physical interaction between CaWRKY58 and Ca14-3-3 was confirmed by a pull-down assay. Ca14-3-3-GST fusion protein isolated from *E. coli* was incubated with GSH magnetic beads (Beaver, Suzhou, China) for 1 h, and CaWRKY58-GFP isolated from pepper leaves that transiently overexpressed CaWRKY58-GFP was added to the mixture and incubated for 1 h. After washing and elution, an appropriate amount of 1 M tris-HCl (pH 8.0) solution was added to adjust the pH. The protein was examined by immunoblotting with anti-GFP and anti-GST antibodies (Guan et al., 2018). The experiment was replicated three times.

**Quantitative Real-Time RT-PCR**
A BIO-RAD Real-time PCR system (Foster City, CA, United States) and SYBR Premix Ex Taq II system (TaKaRa, Dalian, China) were used to quantify gene expression. Total RNA extraction and real-time RT-PCR were carried out as described previously (Dang et al., 2013; Cai et al., 2015; Guan et al., 2018). The specific primer pairs used are listed in Supplementary Table S1. The relative transcript level of each sample was normalized to that of *CaACTIN* (GQ339766). Six biological replicates were performed in the experiment.
Chromatin Immunoprecipitation Analysis
Chromatin immunoprecipitation (ChIP) was performed as described previously (Guan et al., 2018). Briefly, CaWRKY58-GFP was transiently overexpressed in pepper leaves via agroinfiltration, and the DNA-protein complexes were isolated, sheared into fragments of 300–500 bp in length and immunoprecipitated using an anti-GFP antibody. The DNA was purified and used as a template for PCR and qPCR. The specific primer pairs of the fragment containing W-box in the promoter of CaPHR1 and CaNPR1 are listed in Supplementary Table S1. The experiment was replicated three times.

Ralstonia solanacearum Infection
The Ralstonia solanacearum (FJC100301) cultivation and inoculation was performed as described previously (Dang et al., 2013; Shen et al., 2020). The pepper root was harvested at 24 h post inoculation for transcript level detection of related genes.

RESULTS

CaWRKY58 Is Upregulated by LPS in Pepper Plants
CaWRKY58 is constitutively expressed in pepper plants, but is downregulated by Ralstonia solanacearum infection (RSI) and negatively regulates the response to RSI (Wang et al., 2013). To test the potential role of CaWRKY58 in other plant biological processes, we quantified the CaWRKY58 transcript level in pepper plants challenged with several different stresses (salinity, heat stress, and drought), including LPS. Expression of CaWRKY58 was upregulated by LPS at 3 and 5 dpt (Figure 1A), indicating that it might be involved in the LPS response.

Ectopic Overexpression of CaWRKY58 Significantly Enhanced the Tolerance of Nicotiana benthamiana to LPS
To further explore the role of CaWRKY58 in the response to LPS in pepper, N. benthamiana plants were generated that transgenically overexpressed CaWRKY58. Twenty T0 transgenic plants were produced, and the corresponding T1 and T2 lines were produced. Three of the resulting lines with high levels of CaWRKY58 expression were selected for further study. Transgenic and control plants were subjected to LPS, and their RSA and root Pi content were quantified. The three transgenic N. benthamiana lines exhibited an enhanced RSA and enhanced root Pi content compared with control plants (Figures 1B, C, Supplementary Figure S1), suggesting that overexpression of CaWRKY58 enhanced the tolerance of N. benthamiana to LPS.

Silencing CaWRKY58 Compromised the Tolerance of Pepper Plants to LPS
To test the potential role of CaWRKY58 in the response of pepper plants to LPS, we generated CaWRKY58-silenced pepper plants via virus-induced gene silencing (VIGS) using a vector described previously (Wang et al., 2013). The efficiency and specificity of CaWRKY58 silencing were assayed, and the transcript level of CaWRKY58 in TRV:CaWRKY58 pepper plants was approximately 20–30% that in control plants, the transcript level of CaWRKY65, whose sequence is more highly similar to that of CaWRKY58 than other WRKY members in pepper, was not significantly affected by CaWRKY58 silencing (Supplementary Figure S2), indicating that silencing was specific for CaWRKY58. The CaWRKY58-silenced pepper plants were subjected to LPS, and the RSA of TRV:CaWRKY58 and wild-type control plants was enlarged upon LPS treatment compared with that in non-limiting phosphorus conditions, and the RSA of TRV:CaWRKY58 plants under LPS was significantly reduced compared with that of control plants (Figures 2A, C). In parallel, the Pi content was analyzed and was significantly lower in TRV:CaWRKY58 plants than in wild-type plants (Figure 2C). Collectively, these data indicate that CaWRKY58 positively regulates the response of pepper plants to LPS treatment.

CaWRKY58 Directly Targets CaPHR1 in Roots of Pepper Plants Under LPS
Given that WRKY proteins function mainly by binding the conserved W-box in promoters of their target genes, and CaWRKY58 regulates the response of pepper plants to LPS, we hypothesized that CaWRKY58 positively regulates the response to LPS by binding to the W-box in the promoters of genes related to LPS tolerance. To identify the potential target genes of CaWRKY58 in response to LPS, the promoters of potential LPS-responsive genes were screened for the presence of a W-box, which was identified in the promoter of CaPHR1 (LOC107863907) in the pepper genome. CaPHR1 is the ortholog of PHR1, and PHR1 is a MYB transcription factor that positively regulates the response of different plant species to LPS (Nilsson et al., 2007; Valdes-Lopez et al., 2008a; Ren et al., 2012; Ruan et al., 2015; Pacak et al., 2016; Xue et al., 2017). To assess whether CaPHR1 is regulated by CaWRKY58 in response to LPS, the level of CaPHR1 expression was quantified in the leaves of pepper plants that transiently overexpressed CaWRKY58 and was significantly higher than that in the leaves of control plants. However, the expression of CaPHO2 (the ortholog of PHOSPHATE2 in pepper) was not significantly different to that in control plants (Figure 3A). By contrast, LPS significantly upregulated CaPHR1, whereas silencing CaWRKY58 by VIGS significantly blocked this upregulation in leaves (Figure 3B). To elucidate the role of CaPHR1 in the response to LPS, CaPHR1 was silenced by VIGS and the silenced plants were subjected to LPS. A reduced RSA was observed in TRV:CaPHR1 plants compared to in the wild-type plants at 7 dpt, which was similar to that in TRV:CaWRKY58 plants (Figures 2B, C). These results indicate that CaWRKY58 positively regulates the response to LPS by regulating the expression of CaPHR1.

To analyze whether CaPHR1 is directly targeted by CaWRKY58, the ability of CaWRKY58 to bind the CaPHR1 promoter was studied by EMSA using CaWRKY58-6 × His purified from E. coli and the promoter of CaPHR1 (CaPHR1-P5), which contains a W-box. We detected a mobility shift when CaWRKY58-6 × His
was incubated with excess CaPHR1-P (CaPHR1-P<sup>wt</sup>), but no mobility shift was detected when CaWRKY58-6 × His was incubated with excess CaPHR1-P containing a mutated W-box (mCaPHR1-P<sup>wt</sup>; Figures 3C,D). In parallel, a ChIP-PCR assay showed that CaWRKY58-GFP was enriched at the W-box containing CaPHR1 promoter but not at the W-box containing
CaNPR1 promoter (mCaPHR1-P; Figure 3E). These results indicate that CaWRKY58 directly bind to the promoter of CaPHR1.

**Ca14-3-3 Interacts With CaWRKY58**

The function of WRKY transcription factors is often modulated by other regulatory proteins via protein-protein interactions (Chi et al., 2013). To identify the potential interacting partners of CaWRKY58, a yeast two-hybrid assay was performed using CaWRKY58 as a bait. Among the 25 positive clones, a 14-3-3 protein (LOC107867389) was identified. Because the deduced amino-acid sequence contained a conserved 14-3-3 domain and exhibited a high sequence identity to 14-3-3 proteins (Supplementary Figure S3), we named this protein Ca14-3-3. The expression level of Ca14-3-3 was significantly enhanced by LPS treatment (Figure 4A). The interaction between CaWRKY58 and Ca14-3-3 was confirmed by a pull-down assay by incubating Ca14-3-3-GST purified from E. coli with proteins isolated from pepper leaves that transiently overexpressed CaWRKY58-GFP.
Following purification with GST magnetic beads, the protein complex that included Ca14-3-3-GST was immunoblotted with an anti-GFP antibody, which detected the presence of CaWRKY58-GFP. As expected, this demonstrated that Ca14-3-3 interacted with CaWRKY58 (Figure 4B). In parallel, the interaction between 14-3-3 and CaWRKY58 was further confirmed by bimolecular fluorescence complementation (BiFC) in N. benthamiana leaves. The leaves were analyzed at 48 hpi and YFP fluorescence was detected in the nucleus of epidermal cells of leaves co-expressing CaWRKY58 and Ca14-3-3 fusion proteins, but no fluorescence was observed in control leaves (Figure 4C). In addition, we purified CaWRKY58-6 × His from E. coli soluble extracts. Purified CaWRKY58-6 × His fusion protein was serially diluted and mixed with a constant amount of Ca14-3-3-GFP before performing an MST assay, which allowed the dissociation constant (Kd) of the CaWRKY58-6 × His-Ca14-3-3-GFP complex to be calculated. The CaWRKY58-6 × His-Ca14-3-3-GFP complex produced a clear binding curve, with a Kd of 2.0971E-7 M (Figure 4D).

Collectively, these data indicate that CaWRKY58 and Ca14-3-3 physically interact with each other.

The Transcriptional Activation of CaPHR1 by CaWRKY58 Is Enhanced by Interaction Between CaWRKY58 and Ca14-3-3

Because CaWRKY58 interacts with Ca14-3-3 and directly transcriptionally upregulates CaPHR1 during the response of
pepper to LPS, we hypothesized that Ca14-3-3 might affect the function of CaWRKY58 in this process. To test this hypothesis, we analyzed the effect of transient co-overexpression of CaWRKY58 and Ca14-3-3 in leaves of pepper plants on the transcription of CaPHR1, by qRT-PCR. The transcript level of CaPHR1 was promoted by transient overexpression of CaWRKY58 alone, and this promotion was enhanced by the additional transient overexpression of Ca14-3-3, although the
overexpression of Ca14-3-3 alone did not significantly enhance the transcription of CaPHR1. By contrast, the expression of CaPHO2 was not affected by either transient overexpression of CaWRKY58 or its transient overexpression together with Ca14-3-3 (Figure 4F). To test whether binding of the CaPHR1 promoter by CaWRKY58 was enhanced by the interaction of Ca14-3-3 with CaWRKY58; ChIP-qPCR assay was performed on chromatin isolated from leaves of pepper plants that either transiently overexpressed CaWRKY58-GFP alone, or CaWRKY58-GFP and Ca14-3-3-GFP together. The chromatin was sheared into fragments 300–500 bp in length and was immunoprecipitated with an anti-GFP antibody, and DNA derived from the immunoprecipitated chromatin was used as template for ChIP-qPCR using a primer pair specific for the W-box within the CaPHR1 promoter. The W-box sequence was significantly enriched by transient co-overexpression of Ca14-3-3-FLAG and CaWRKY58-GFP (Figure 4E). Consistent with this, the transcript level of CaPHR1 in plants that transiently co-expressed CaWRKY58-GFP and Ca14-3-3-GFP was significantly higher than that in plants that expressed CaWRKY58-GFP or Ca14-3-3-GFP alone (Figure 4F). These data indicate that the transcriptional regulation of CaPHR1 by CaWRKY58 is enhanced by Ca14-3-3.

Genes Related to Immunity or LPS Tolerance Are Regulated by CaWRKY58 in a Context-Dependent Manner

Previous study showed that CaWRKY58 negatively regulates the response of pepper to RSI by downregulating immunity-related genes, including CaDEF1 and CaNPR1 (Wang et al., 2013). Here, we demonstrate that CaWRKY58 positively regulates the response to LPS by upregulating CaPHR1. To address whether CaWRKY58 regulates its target genes in a context-dependent manner, the transcript levels of CaPHR1, CaNPR1, and CaDEF1 were quantified in pepper plants challenged with LPS and RSI. The expression of CaPHR1 was only upregulated by LPS, whereas that of CaDEF1 and CaNPR1 was upregulated by RSI, indicating that the expression of these genes is context dependent (Supplementary Figure S4). Furthermore, the transcript levels of CaDEF1, and CaNPR1 in CaWRKY58-silenced pepper plants were only enhanced in the presence of sufficient Pi, but not under LPS (Supplementary Figure S5). Furthermore, although the positive transcriptional regulation of CaPHR1 by CaWRKY58 was enhanced by transient co-overexpression of Ca14-3-3, the transcriptional regulation of CaDEF1 and CaNPR1 by CaWRKY58 was not affected by Ca14-3-3 (Supplementary Figure S6). These data indicate that the activation of CaPHR1 expression by CaWRKY58 occurs only in LPS but not in RSI; on the other hand, the negative regulation of CaDEF1 and CaNPR1 by CaWRKY58 does not occur under LPS.

DISCUSSION

Although WRKY and 14-3-3 proteins have been previously been shown to participate in plant immunity and the LPS response, and some members of both families physically interact, their functions in crosstalk between plant immunity and response to LPS remain uninvestigated. In this study, we provide evidence that in addition to acting as a negative regulator of immunity against RSI in pepper, CaWRKY58 positively regulates tolerance to LPS, an effect that is mediated by interaction with Ca14-3-3.

CaWRKY58 Positively Regulates the Response to LPS by Directly Regulating CaPHR1

The data from CaWRKY58 upregulation against LPS and the data from loss-of-function and gain-of-function assay indicate that CaWRKY58 positively regulates the response to LPS by regulating CaPHR1, which related to LPS tolerance (Motte and Beeckman, 2017; Wang et al., 2019), as well as by modulating RSA, a key parameter that is positively related to LPS tolerance (Jain et al., 2007; Mori et al., 2016). Primary root growth was strongly inhibited by LPS in Arabidopsis (Peret et al., 2011; Gutierrez-Alanis et al., 2018); however, we observed that the growth of primary and lateral roots in pepper was promoted by P deficiency, which was enhanced by CaWRKY58 overexpression (Figure 1B). This indicates that the regulation of RSA by LPS might differ among plant species. The data also indicate that CaWRKY58 positively regulates CaPHR1 expression directly, because the W-box within the CaPHR1 promoter was bound by CaWRKY58 according to ChIP-qPCR and EMSA (Figure 3D). PHR1 is a MYB-CC-type transcription factor that plays a key role in regulating the expression of Pi starvation-induced (PSI) genes, which leads to enhanced phosphate uptake (Nilsson et al., 2007). We conclude that CaWRKY58 positively regulates the LPS response in pepper by targeting PHR1.

Ca14-3-3 Promotes the Transcriptional Activation Activity of CaWRKY58 During LPS

In addition to regulation at the transcriptional level, the expression and function of transcription factors is frequently regulated post-translationally via interactions with other proteins (Schutze et al., 2008; Chi et al., 2013; Alves et al., 2014; Fireyre and Burow, 2015; Ohama et al., 2017). In particular, WRKY transcription factors interact with other WRKY proteins, VQ motif-containing proteins, MAPKs, chromatin remodeling proteins, calmodulin, and 14-3-3 proteins (Chi et al., 2013). 14-3-3 proteins regulate transcription by interacting with the transcription factors or activators RSG (Igarashi et al., 2001; Ishida et al., 2008), GmMYB176 (Li et al., 2012), MYBS2 (Chen et al., 2019), and CBF (Liu et al., 2017), and modifying their subcellular localization, stability, or transcription, leading to an appropriate transcriptional output. Moreover, 14-3-3 proteins have been implicated in the response to P deficiency (Wang et al., 2002; Xu et al., 2012b); however, it remains unknown whether 14-3-3 proteins function as transcription activators in these conditions. The data here indicate that the transcriptional activation of CaPHR1 by CaWRKY58 is enhanced by the interaction between CaWRKY58 and Ca14-3-3, suggesting that Ca14-3-3 might...
promote the transcriptional activation activity of CaWRKY58 during the P-deficiency response. Because the interaction between 14-3-3 proteins and their protein partners is either dependent on or related to phosphorylation (Li et al., 2012; Ito et al., 2014; Chen et al., 2019) mediated by kinases including CDPks (Ishida et al., 2008; Liu et al., 2017), we speculate that the interaction between Ca14-3-3 and CaWRKY58 might be associated with phosphorylation mediated by unidentified kinases. The further identification of these kinases might provide insight into the mechanism that underlies the LPS response in pepper.

CaWRKY58 Functions in LPS and Immune Signaling, and Is Specifically Activated in the LPS Response by Ca14-3-3
We demonstrated previously that CaWRKY58 negatively regulates the response to RSI in pepper (Wang et al., 2013); therefore, CaWRKY58 functions in the response of pepper to both RSI and LPS. These two stresses activate convergent signaling nodes, such as WRKY45 (Shimono et al., 2007; Wang et al., 2014), WRKY75 (Devaiah et al., 2007a; Encinas-Villarejo et al., 2009), and PR10a (Huang et al., 2016), suggesting that plant immunity might be closely related to phosphorus nutrition. On the one hand, application of sufficient phosphate fertilizer is generally essential to increase root ramification and strength, thereby conferring vitality and disease resistance to plants (Sharma et al., 2013). By contrast, phosphate starvation can result in the repression of plant immunity (Finkel et al., 2019), and in Arabidopsis, PHR1 also directly represses immune responses (Castrillo et al., 2017). On the other hand, pathogen infection might decrease phosphate availability (Petters et al., 2002). However, the significance of these shared regulatory proteins in plant adaptation to the environment and their contribution to the relationship between plant phosphorus nutrition and disease resistance remains to be elucidated. The data here showed that LPS tolerance and immunity-related genes were differentially activated in pepper plants upon RSI and LPS treatments, and immunity-related genes, including CaDEF1 and CaNPR1, were not negatively regulated by CaWRKY58 in plants exposed to LPS treatment, or following transient co-overexpression of CaWRKY58 and Ca14-3-3. This indicates that CaWRKY58 and Ca14-3-3 contribute to the positive regulation of CaWRKY58 in response to LPS, but do not contribute to its negative regulation in immunity against RSI. Collectively, the data in this study show that CaWRKY58 acts as positive regulator of LPS in pepper plants by directly targeting and regulating CaPHR1 and Ca14-3-3 acts as a specific transcriptional activator of CaWRKY58 during the LPS response.

DATA AVAILABILITY STATEMENT
The original contributions presented in the study are included in the article/Supplementary Material and further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS
SH and JC conceived the research and designed the experiments. JC, XH, SY, WC, JW, XX, FY, and YS performed the experiments. JC, SY, and DG analyzed the data. SH wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING
This work was supported by grants from the National Natural Science Foundation of China (31572136, 31372061, and 31902032), Development Fund Project of Fujian Agriculture and Forestry University (CXXZ2016158, CXXZ2017548).

ACKNOWLEDGMENTS
We thank Mark D. Curtis for kindly providing the Gateway destination vectors and Dr. S. P. Dinesh-Kumar of Yale University for the pTRV1 and pTRV2 vectors.

SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.607878/full#supplementary-material

REFERENCES
Alves, M. S., Dadalto, S. P., Goncalves, A. B., de Souza, G. B., Barros, V. A., and Fietto, L. G. (2014). Transcription factor functional protein-protein interactions in plant defense responses. Proteome 2, 85–106. doi: 10.3390/proteomes2010085
Aparicio-Fabre, R., Guillen, G., Loredo, M., Arrellano, J., Valdes-Lopez, O., Ramirez, M., et al. (2013). Common bean (Phaseolus vulgaris L.) PtTIFY orchestrates global changes in transcript profile response to jasmonate and phosphorus deficiency. BMC Plant Biol. 13:26. doi: 10.1186/1471-2229-13-26
Ayadi, A., David, P., Arrighi, J. F., Chiarenza, S., Thibaud, M. C., Nussaume, L., et al. (2015). Reducing the genetic redundancy of Arabidopsis PHOSPHATE TRANSPORTER1 transporters to study phosphate uptake and signaling. Plant Physiol. 167, 1511–1526. doi: 10.1104/pp.114.252338
Bakshi, M., Vahabi, K., Bhattacharya, S., Sherameti, I., Varma, A., Yeh, K. W., et al. (2015). WRKY6 restricts Piriformospora indica-stimulated and phosphate-induced root development in Arabidopsis. BMC Plant Biol. 15:305. doi: 10.1186/s12870-015-0673-4
Birkenbihl, R. P., Liu, S., and Somssich, I. E. (2017). Transcriptional events defining plant immune responses. Curr. Opin. Plant Biol. 38, 1–9. doi: 10.1016/j.pbi.2017.04.004
Briot, J. F., Rouached, H., Tissot, N., Gaymard, F., and Dubos, C. (2015). Integration of P, S, Fe, and Zn nutrition signals in Arabidopsis thaliana: potential involvement of PHOSPHATE STARVATION RESPONSE 1 (PHR1). Front. Plant Sci. 6:290. doi: 10.3389/fpls.2015.00290
Buscaill, P., and Rivas, S. (2014). Transcriptional control of plant defence responses. Curr. Opin. Plant Biol. 20, 35–46. doi: 10.1016/j.pbi.2014.04.004
Cai et al. 14-3-3/WRKY58 Interaction Modulates LPS Response

Oryza rufipogon

60, 3043–3065. doi: 10.1101/s00425-007-0569-0

Castrillo, G., Teixeira, P. J., Paredes, S. H., Law, T. F., de Lorenzo, L., Feltchen, M. E., et al. (2017). Root microbiota drive direct integration of phosphate stress and immunity. Nature 543, 513–518. doi: 10.1038/nature21417

Chen, Y. S., Ho, T. D., Liu, L., Lee, D. H., Lee, C. H., Chen, Y. R., et al. (2019). Sugar starvation-regulated MYB52 and 14-3-3 protein interactions enhance plant growth, stress tolerance, and grain weight in rice. Proc. Natl. Acad. Sci. U. S. A. 116, 21925–21935. doi: 10.1073/pnas.1904818116

Cheng, W., Xiao, Z., Cai, H., Wang, C., Hu, Y., Xiao, Y., et al. (2017). A novel leucine-rich repeat protein, CaLR51, acts as a positive regulator in the response of pepper to *Ralstonia solanacearum* infection. Mol. Plant Pathol. 18, 1089–1100. doi: 10.1111/mpp.12462

Chi, Y., Yang, Y., Zhou, Y., Zhou, J., Fan, B., Yu, J. Q., et al. (2013). Protein-protein interactions in the regulation of WRKY transcription factors. Mol. Plant 6, 287–300. doi: 10.1093/mp/sst026

Dai, X., Wang, Y., and Zhang, W. H. (2016). OsWRKY74, a WRKY transcription factor, modulates tolerance to phosphate starvation in rice. J. Exp. Bot. 67, 947–960. doi: 10.1093/jxb/erv515

Dang, F. F., Wang, Y. N., Yu, L., Eulgem, T., Lai, Y., Liu, Z. Q., et al. (2013). CaWRKY40, a WRKY protein of pepper, plays an important role in the regulation of tolerance to heat stress and resistance to *Ralstonia solanacearum* infection. Plant Cell Environ. 36, 757–774. doi: 10.1111/pce.12011

Decker, E. L., Alder, A., Hunn, S., Ferguson, J., Lehtonen, M. T., Scheler, B., et al. (2017). Strigolactone biosynthesis is evolutionarily conserved, regulated by phosphate starvation and contributes to resistance against phytopathogenic fungi in a moss, *Physcomitrella patens*. New Phytol. 216, 455–468. doi: 10.1111/nph.14506

Deng, Q. W., Luo, X. D., Chen, Y. L., Zhou, Y., Zhang, F. T., Hu, B. L., et al. (2018). Transcriptome analysis of phosphate stress responsiveness in the seedlings of Dongxiang wild rice (*Oryza sativa*). Biol. Res. 51:7. doi: 10.1186/s40659-018-0155-x

Devaiya, B. N., Karrthikeyan, A. S., and Raghothama, K. G. (2007a). WRKY75 transcription factor is a modulator of phosphate acquisition and root development in Arabidopsis. Plant Physiol. 143, 1789–1801. doi: 10.1104/pp.106.093971

Devaiya, B. N., Nagarajan, V. K., and Raghothama, K. G. (2007b). Phosphate homeostasis and root development in Arabidopsis are synchronized by the zinc finger transcription factor ZAT6. Plant Physiol. 145, 147–159. doi: 10.1104/pp.107.101615

Ding, X., Chen, H., Xu, Z., Ichinose, Y., Ishimaru, K., and Terada, M. (2012). AXR1-induced H3K4me3 is required for efficient elongation of transcription, not initiation, at AXR1-regulated genes. *PLoS Genet.* 8:e1002311. doi: 10.1371/journal.pgen.1002311

Ding, W., Wang, Y., Fang, W., Gao, S., Li, X., and Xiao, K. (2016). TaZAT8, a C2H2-ZFP type transcription factor gene in wheat, plays critical roles in disease signaling. *Curr. Opin. Plant Biol.* 29, 1–11. doi: 10.1016/j.cppb.2015.09.003

Eulgem, T. (2006). Dissecting the WRKY web of plant defense regulators. Front. Plant Sci. 2:e126. doi: 10.1371/journal.pone.000156414. doi: 10.1371/journal.pone.01356414

Hassler, S., Lemke, L., Jung, B., Mohlmann, T., Kruger, F., Schumacher, K., et al. (2012). Lack of the Golgi phosphate transporter PHT6:6 causes strong developmental defects, constitutively activated disease resistance mechanisms and altered intracellular phosphate compartmentation in Arabidopsis. *Plant J.* 72, 732–744. doi: 10.1111/j.1365-313X.2012.05106.x

Heravi, K. M., and Altenbuchner, J. (2014). Regulation of the *Bacillus subtilis* mannitol utilization genes: promoter structure and transcriptional activation by the wild-type regulator (MfrI) and its mutants. *Microbiology* 160, 91–101. doi: 10.1099/mic.0.071237.0

Hirama, K., Gerlach, N., Sacristan, S., Nakano, R. T., Haquard, S., Kracher, B., et al. (2016). Root endophyte *Colletotrichum tosfoelidum* confers plant fitness benefits that are phosphate status dependent. *Cell* 165, 464–474. doi: 10.1016/j.cell.2016.02.028

Huang, L. F., Lin, K. H., He, S. L., Chen, J. L., Jiang, J. Z., Chen, B. H., et al. (2016). Multiple patterns of regulation and overexpression of a ribonuclease-like pathogenesis-related protein gene, OsPR10a, conferring disease resistance in rice and *Arabidopsis*. *PLoS One* 11:e0156414. doi: 10.1371/journal.pone.0156414

Huang, X., Zhang, Q., Jiang, Y., Yang, C., Wang, Q., and Li, L. (2018). Shade-induced nuclear localization of PIF7 is regulated by phosphorylation and*Lucifugia* proteins in *Arabidopsis*. eLife 7:e31636. doi: 10.7554/eLife.31636

Igarashi, D., Ishida, S., Fukazawa, J., and Takahashi, Y. (2001). 14-3-3 proteins regulate intracellular localization of the bZIP transcriptional activator RSG. *Plant Cell* 13, 2483–2497. doi: 10.1105/tpc.110.00188

Ishida, S., Yuasa, T., Nakata, M., and Takahashi, Y. (2008). A tobacco calcium-dependent protein kinase, CDPK1, regulates the transcription factor repression of SHOOT GROWTH in response to gibberellins. *Plant Cell* 20, 3273–3288. doi: 10.1105/tpc.107.057489

Ishihama, N., and Yoshioka, H. (2012). Post-translational regulation of WRKY transcription factors in plant immunity. *Curr. Opin. Plant Biol.* 15, 431–437. doi: 10.1016/j.ppb.2012.02.003

Ito, T., Nakata, M., Fukazawa, J., Ishida, S., and Takahashi, Y. (2014). Scaffold function of Ca2+−DEPENDENT PROTEIN KINASE: tobacco Ca2+−DEPENDENT PROTEIN KINASE1 exchanges 14-3-3 to the substrate repression of SHOOT GROWTH after phosphorylation. *Plant Physiol.* 165, 1737–1750. doi: 10.1104/pp.114.236448

Jain, A., Poling, M. D., Karrthikeyan, A. S., Blakeslee, J. J., Peer, W. A., Tiwari, A., et al. (2016). leaf phosphate homeostasis involves pi-starvation induced responses. Curr. Opin. Plant Biol. 22, 191–197. doi: 10.1016/j.ppl.2016.04.006

Khan, G. A., Bouraine, S., Wege, S., Li, Y., de Carbonnel, M., Berthomieu, P., et al. (2014). Coordination between zinc and phosphate homeostasis involves the transcription factor PHR1, the phosphate exporter PHO1, and its...
expression in response to phosphate starvation. *Plant Physiol.* 164, 2020–2029. doi: 10.1094/pp.113.235077

Wang, Y., Zhang, F., Cui, W., Chen, K., Zhao, R., and Zhang, Z. (2019). The FvPHR1 transcription factor control phosphate homeostasis by transcriptionally regulating miR399a in woodland strawberry. *Plant Sci.* 280, 258–268. doi: 10.1016/j.plantsci.2018.12.025

Wu, Z., Ren, H., McGrath, S. P., Wu, P., and Zhao, F. J. (2011). Investigating the contribution of the phosphate transport pathway to arsenic accumulation in rice. *Plant Physiol.* 157, 498–508. doi: 10.1111/j.1744-7909.2011.03608.x

Xia, X. J., Zhou, Y. H., Shi, K., Zhou, J., Foyer, C. H., and Yu, J. Q. (2015). Interplay between reactive oxygen species and hormones in the control of plant development and stress tolerance. *J. Exp. Bot.* 66, 2839–2856. doi: 10.1093/jxb/eru089

Xu, W., Lu, J., Shi, W., Liang, J., and Zhang, J. (2012a). Smart role of plant 14-3-3 proteins in response to phosphate deficiency. *Plant Signal. Behav.* 7, 1047–1048. doi: 10.4161/psb.20997

Xu, W., Shi, W., Jia, L., Liang, J., and Zhang, J. (2012b). TTF6 and TTF7, two different members of tomato 14-3-3 gene family, play distinct roles in plant adaption to low phosphorus stress. *Plant Cell Environ.* 35, 1393–1406. doi: 10.1111/j.1365-3040.2012.02497.x

Xu, H., Zhao, X., Guo, C., Chen, L., and Li, K. (2016). Spinach 14-3-3 protein interacts with the plasma membrane H(+)–ATPase and nitrate reductase in response to excess nitrate stress. *Plant Physiol. Biochem.* 106, 187–197. doi: 10.1016/j.plaphy.2016.04.043

Xue, Y. B., Xiao, B. X., Zhu, S. N., Mo, X. H., Liang, C. Y., Tian, J., et al. (2017). GmPHR25, a GmPHR member up-regulated by phosphate starvation, controls phosphate homeostasis in soybean. *J. Exp. Bot.* 68, 4951–4967. doi: 10.1093/jxb/erx292

Xue, Y., Zhuang, Q., Zhu, S., Xiao, B., Liang, C., Liao, H., et al. (2018). Genome wide transcriptome analysis reveals complex regulatory mechanisms underlying phosphate homeostasis in soybean nodules. *Int. J. Mol. Sci.* 19:2924. doi: 10.3390/ijms19114186

Ye, Q., Wang, H., Su, T., Wu, W. H., and Chen, Y. F. (2018). The ubiquitin E3 ligase PRU1 regulates WRKY6 degradation to modulate phosphate homeostasis in response to low-pi stress in *Arabidopsis*. *Plant Cell Environ.* 30, 1062–1076. doi: 10.1111/pce.13084

Yuan, W., Zhang, D., Song, T., Xu, F., Lin, S., Xu, W., et al. (2017). *Arabidopsis* plasma membrane H(+)–ATPase genes AHA2 and AHA7 have distinct and overlapping roles in the modulation of root tip H(+) efflux in response to low-phosphorus stress. *J. Exp. Bot.* 68, 1731–1741. doi: 10.1093/jxb/erx040

Zhang, Y., Hu, L., Yu, D., Xu, X., Zhang, J., Li, X., et al. (2019). Integrative analysis of the wheat PHT1 gene family reveals a novel member involved in arbuscular mycorrhizal phosphate transport and immunity. *Cell 8:490.*

Zhang, L., Li, G., Li, Y., Min, J., Kronzucker, H. J., and Shi, W. (2018). Tomato plants ectopically expressing *Arabidopsis* GRF9 show enhanced resistance to phosphate deficiency and improved fruit production in the field. *J. Plant Physiol.* 226, 31–39. doi: 10.1016/j.jplaphy.2018.04.005

Zhang, X., Lu, X., Wang, F., Deng, M., and Yi, K. (2012). Modulating the root elongation by phosphate/nitrogen starvation in an OsGLU3 dependent way in rice. *Plant Signal. Behav.* 7, 1144–1145. doi: 10.4161/psb.21334

Zhang, J., Zhou, X., Xu, Y., Yao, M., Xie, F., Gai, J., et al. (2016). Soybean SPX1 is an important component of the response to phosphate deficiency for phosphate homeostasis. *Plant Sci.* 248, 82–91. doi: 10.1016/j.plantsci.2016.04.010

Zhong, Y., Zhang, F., Guo, J., Zhu, X., Shi, J., He, Q., et al. (2018). Rice SPX6 negatively regulates the phosphate starvation response through suppression of the transcription factor PHR2. *New Phytol.* 219, 135–148. doi: 10.1111/nph.15155

Zhou, J., Jiao, F., Wu, Z., Li, Y., Wang, X., He, X., et al. (2008). OsPHR2 is involved in phosphate-starvation signaling and excessive phosphate accumulation in shoots of plants. *Plant Physiol.* 146, 1673–1686. doi: 10.1104/pp.110.1171443

Zhou, X., Zha, M., Huang, J. L., Imran, M., and Zhang, C. (2017). SMYB44 negatively regulates phosphate transport by suppressing expression of PHOSPHATE1 in potato. *J. Exp. Bot.* 68, 1265–1281. doi: 10.1093/jxb/erx026

Zilnner, K., Jerabek-Willemsen, M., Duhr, S., Braun, D., Langst, G., and Baske, P. (2012). Microscale thermophoresis as a sensitive method to quantify protein.
nucleic acid interactions in solution. Methods Mol. Biol. 815, 241–252. doi: 10.1007/978-1-61779-424-7_18

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.