A Universal Stress Protein Involved in Oxidative Stress Is a Phosphorylation Target for Protein Kinase CIPK6[1]

Emilio Gutiérrez-Beltrán, José María Personat, Fernando de la Torre, and Olga del Pozo

Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla/Consejo Superior de Investigaciones Científicas, 41092 Sevilla, Spain

ORCID IDs: 0000-0001-7978-3164 (E.G.B.); 0000-0003-2273-5000 (J.M.P.); 0000-0002-5143-3709 (F.d.l.T.); 0000-0001-9852-0431 (O.d.P.).

Calcineurin B-like interacting protein kinases (CIPKs) decode calcium signals upon interaction with the calcium sensors calcineurin B like proteins into phosphorylation events that result into adaptation to environmental stresses. Few phosphorylation targets of CIPKs are known and therefore the molecular mechanisms underlying their downstream output responses are not fully understood. Tomato (*Solanum lycopersicum*) Cipk6 regulates immune and susceptible Programmed cell death in immunity transforming Ca^{2+} signals into reactive oxygen species (ROS) signaling. To investigate SlCipk6-induced molecular mechanisms and identify putative substrates, a yeast two-hybrid approach was carried on and a protein was identified that contained a Universal stress protein (Usp) domain present in bacteria, protozoa and plants, which we named "SlRd2". SlRd2 was an ATP-binding protein that formed homodimers in planta. SlCipk6 and SlRd2 interacted using coimmunoprecipitation and bimolecular fluorescence complementation (BiFC) assays in *Nicotiana benthamiana* leaves and the complex localized in the cytosol. SlCipk6 phosphorylated SlRd2 in vitro, thus defining, to our knowledge, a novel target for CIPKs. Heterologous SlRd2 overexpression in yeast conferred resistance to highly toxic LiCl, whereas SlRd2 expression in *Escherichia coli UspA* mutant restored bacterial viability in response to H_2O_2 treatment. Finally, transient expression of SlCipk6 in transgenic *N. benthamiana* SlRd2 overexpressors resulted in reduced ROS accumulation as compared to wild-type plants. Taken together, our results establish that SlRd2, a tomato UspA, is, to our knowledge, a novel interactor and phosphorylation target of a member of the CIPK family, SlCipk6, and functionally regulates SlCipk6-mediated ROS generation.

Environmental factors, especially those imposing stress, stimulate endogenous cellular cues, which initiate protective responses in plants. Among the concurrent events during stress are changes in the intracellular Ca^{2+} concentration, which activate an overlapping set of downstream responses. Ca^{2+} changes are perceived and decoded by an array of Ca^{2+} sensors including calmodulins or calmodulin-related proteins, Ca^{2+}-dependent protein kinases (CDPKs, CPKs), and calcineurin B-like proteins (CBLs; Dodd et al., 2010). Particularly, the CBL family has been shown to play a crucial role in different Ca^{2+}-dependent processes in plants (Sanyal et al., 2015). CBL proteins present homology to the regulatory B-subunit of calcineurin and the neuronal calcium sensor proteins from animals and yeast (Luan, 2009). The overall structure of CBLs consists of four EF-hands. Spacing of EF-hands is invariable, while the C- and N-terminal extension of CBL proteins varies in length. Posttranslational modifications of CBLs, including protein phosphorylation and lipid modifications affect their subcellular localization and their stability to interact with other proteins (Sanyal et al., 2015; Nagae et al., 2003). Thus, phosphorylation of the conserved Ser residue in the C-terminal PFPF motif of the CBL proteins enhances the interaction with CBL-interacting protein kinases (CIPKs; Du et al., 2011; Hashimoto et al., 2012).

Upon Ca^{2+} binding, CBLs physically interact with CIPKs, Ser/Thr kinases that structurally belong to Suc nonfermenting 1-related kinases, group 3, also called protein kinases related to SOS2 (PKS; Gong et al., 2004; Yu et al., 2014). CIPKs are constituted of a C-terminal or regulatory domain and a conserved kinase catalytic domain at the N terminus. Within the divergent regulatory domain, CIPKs contain an autoinhibitory NAF/FISL motif and a type 2C protein phosphatase binding site called the "PPI motif". It is well established that binding of CBLs to the NAF/FISL motif releases the C-terminal (autoinhibitory) domain from the kinase domain, thus leading the kinase into an active state

[1] This work was funded in part by the European Regional Development Fund through the Ministerio de Economía y Competitividad (grant nos. BIO2005-02136, BIO2009-08648, and BIO2013-44750R) and by the Junta de Andalucía, Spain (grant no. P07-CVI-03171, to O.d.P.); O.d.P. was also supported in part by the Junta de Andalucía, Spain (Programa de Retorno de Investigadores); F.d.l.T. was supported by Marie Curie Programme through the International Reintegration grants (MIRG-CT-2005-031174) and a Juan de la Cierva contract (Ministerio de Ciencia e Innovación, Spain); E.G.-B. was a recipient of a Formación de Personal Investigador fellowship (Ministerio de Educación, Spain); and J.M.P. was supported by grant no. BIO2013-44750R.

* Address correspondence to olga_delpozo@ibvf.csic.es.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Olga del Pozo (olga_delpozo@ibvf.csic.es).

E.G.B., O.d.P., and F.d.l.T. designed experiments and analyzed the data; O.d.P. wrote the manuscript and E.G.B. helped with the writing; E.G.B. performed most of experiments; J.M.P. generated Figure 7A.

www.plantphysiol.org/cgi/doi/10.1104/pp.16.00949

(Guo et al., 2001; Chaves-Sanjuan et al., 2014). In Arabidopsis (*Arabidopsis thaliana*), there are 10 CBL and 26 CIPK homologs (Yu et al., 2014). By yeast two-hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) assays it has been determined that CBLs show a level of specificity in targeting different CIPKs. On the other hand, a specific CIPK can also interact with different CBLs, thus allowing a single CIPK to access different cellular compartments and hence different substrates (Kim et al., 2000, 2007). It is believed that the specificity of the response to a given stimulus is achieved by decoding specific Ca^{2+} profiles by CBLs followed by the subsequent formation of different CBL/CIPK complexes in planta, and finally by phosphorylation of CIPK-specific substrates that contribute to the specific output response (Batistic et al., 2010).

At the moment, the most numerous and best characterized interactors or substrates for CBL/CIPK complexes are membrane proteins, which include salt overly sensitive 1 (SOS1; Quintero et al., 2002; Katiyar-Agarwal et al., 2006), H^+-ATPase 2 (He et al., 2004), nitrate transporter (Ho et al., 2009), K^+ transporter 1 (AKT1; Xu et al., 2006), high-affinity K^+ transporter 5 (Ragel et al., 2015), and the respiratory burst oxidase homolog F (Drerup et al., 2013). Additionally, CIPKs have also been shown to interact with nonmembrane proteins; for example, SOS2-like protein kinase 5 interacts with the chaperone DnaJ (He et al., 2004; Yang et al., 2010); AtCIPK24 interacts with GIGANTEA (Kim et al., 2013), nucleoside diphosphate kinase 2, the catalases CAT2 and CAT3 (Verslues et al., 2007) and with ABA-insensitive 2 (ABI2), a type 2C Ser/Thr phosphatase (Guo et al., 2002; Ohta et al., 2003); and CIPK26 interacts with the RING-type E3 ligase "Keep on Going" and with ABI1, ABI2 (Lyzenga et al., 2013). Although it appears that CBL/CIPK complexes could interact with several proteins, at present only few CIPK phosphorylation targets have been identified.

Previously, our group demonstrated, to our knowledge, a novel role for tomato (*Solanum lycopersicum*) Cipk6 (SlCipk6) in plant innate immunity, thus functionally implicating for the first time the participation of a CBL/CIPK module in biotic stress signaling in plants (de la Torre et al., 2013). Other studies demonstrated the participation of Cipk6 orthologs from different plant species in diverse abiotic stress responses (Chen et al., 2012, 2013; Tsou et al., 2012; Tripathi et al., 2009). As a first step to investigate SlCipk6 downstream signaling molecular mechanisms, we set to identify SlCipk6-interacting proteins using a Y2H approach. We discovered that tomato Responsive to desiccation 2 (SlRd2), which contains a Universal stress protein (Usp) domain [Pfam (http://pfam.xfam.org/) accession no. PF00582], interacted with SlCipk6 and by means of a BiFC approach, we found that the complex SlCipk6/SlRd2 is localized in the cytoplasm. In addition, we demonstrated that SlRd2 is a phosphorylation substrate of SlCipk6, thus expanding the previously described substrates for the CIPK family. Interestingly, SlRd2 is an ATP-binding protein that forms homodimers, which is required for its biological role and for interacting with SlCipk6.

The universal stress protein A (UspA) superfamily was originally discovered in *Escherichia coli*, where its expression drastically increased in response to multiple stress conditions and to starvation (Vanbogelen et al., 1990). Importantly, UspA protein accumulation was necessary for bacterial survival at the stationary phase (Nyström and Neidhardt, 1994). It was found later that *E. coli* has six *usp* genes (*uspA*, *uspC*, *uspD*, *uspF*, *uspF*, and *uspG*); however, UspA set the nomenclature for the orthologous groups of proteins. UspA family members are classified into two major groups according to their ATP binding capability. The first group is constituted by ATP-binding proteins and is represented by Mj0577 from *Methanococcus jannaschii* (Zarembinski et al., 1998). Members of the second group have no ATP-binding capability and are represented by *Haemophilus influenzae* and *E. coli* UspAs (Sousa and McKay, 2001). Both Mj0577 and HiUspA form homodimers in vivo (Zarembinski et al., 1998). At present, more than 2000 UspA (or Usp containing domain) proteins have been identified from a wide range of organisms such as bacteria, archaea, fungi, protozoa and plants, constituting an ancient and conserved group of proteins (Aravind et al., 2002). In Arabidopsis, at least 44 proteins were found to contain an Usp domain, all of which resemble ATP-binding Mj0577 protein (Kerk et al., 2003) and several plant UspAs seemed to be involved in abiotic stress. In Arabidopsis, two UspA proteins, AtPHOS32 and AtPHOS34, were phosphorylated by AtMPK3 and AtMPK6 in response to bacterial elicitors in cell suspension cultures (Merkouropoulos et al., 2008). Other reports described several UspA members as effectors of low water potential (Merkouropoulos et al., 2008). Several UspA proteins have been characterized in rice (Sauter et al., 2002), tomato (Zegzouti et al., 1999; Loukehaich et al., 2012) legumes (Becker et al., 2001; Hohnjec et al., 2000), Salicornia (Udawat et al., 2016), and cotton (Zahur et al., 2009). Still, the precise structure, regulation, biochemical function, or mechanism of function of UspA proteins in planta, are largely unknown.

RESULTS

Identification of SlRd2 as a SlCipk6-Interacting Protein

To identify SlCipk6-interacting proteins (CIPs), we carried out a Y2H approach in two separate screens, using a tomato cDNA prey library previously developed (Zhou et al., 1995) and SlCipk6 and a mutant derivative, SlCipk6(T172D), as baits (Fig. 1A). SlCipk6(T172D) displayed enhanced kinase and autophosphorylation activity compared to SlCipk6 (de la Torre et al., 2013), and we hypothesized that using either SlCipk6 or SlCipk6(T172D) as a bait, might facilitate the identification of putative regulatory proteins or phosphorylation substrates respectively, because constitutive active kinase versions stabilize the interaction with their substrates (Uno et al., 2009). Approximately 1.1×10^3 and 4.5×10^3 yeast transformants were screened for SlCipk6- and SlCipk6(T172D)-interacting proteins

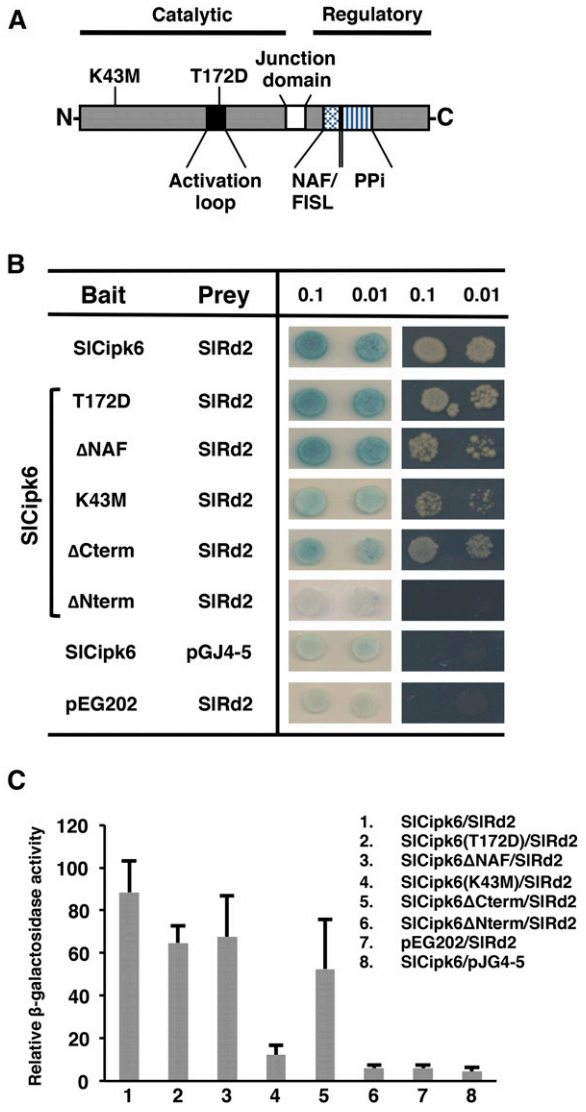

Figure 1. SlRd2 and SlCipk6 Interact in a Y2H Assay. A, Schematic structure of SlCipk6 protein representing N-terminal catalytic, junction and C-terminal regulatory domains, PPi, NAF/FISL, and activation loop domains. Residues mutagenized for SlCipk6 characterization Lys-43 to Met (K43M), and Thr-172 to Asp (T172D) are marked. B, Y2H assay performed with SlCipk6 and SlCipk6 mutant derivatives in the bait vector pEG202 and SlRd2 in the prey vector pJG4-5. Wild-type, full-length SlCipk6; K43M and T172D, single amino acid substitution of Lys-43 to Met and Thr-172 to Asp, respectively; ΔNAF, amino acids 302–321 deletion; ΔCterm amino acids 302–432 deletion; and ΔNterm, amino acids 1–302 deletion. Clones were transformed into EGY48 strain, grown in liquid culture, and spotted at OD_{600} = 0.1 and 0.01 on selective medium. Growth and blue patches indicates interaction. C, Interaction was quantitated by a β-galactosidase assay. Represented is the mean value of three independent experiments, each performed with three independent transformants. Error bars represent the SD of three different experiments.

respectively on selection plates (Supplemental Table S1). The inserts of 11 and 34 candidate prey clones from both screens were sequenced and compared with databases by BLAST in the Arabidopsis database (www.tair.org) for identification. The clone no. 29 (Cip29) was a partial open reading frame (ORF) that encoded a protein with a high similarity to Arabidopsis Response to desiccation 2 (AtRD2; At2g21620), a protein not yet characterized with no assigned function, containing a Universal stress protein domain (Usp). Clones containing different length fragments of Cip29 were identified once and two times in the screens performed with SlCipk6 and with SlCipk6(T172D), respectively, and they were among the strongest interactors. In light of these facts, its characterization was prioritized and the remaining CIPs will be published elsewhere. BLAST performed with Cip29 in the tomato database (www.solgenomics.net) identified unigene SGN-U567775 containing a full length ORF that corresponded to the tomato locus Solyc01g109710 and we will be referred to it as *SlRd2* hereafter.

We next obtained full-length *SlRd2* ORF, cloned it in the prey vector, and confirmed its interaction with SlCipk6 by Y2H (Fig. 1B). Both SlRd2 and SlCipk6 did not show autoactivation activity. To characterize SlCipk6/SlRd2 interaction, different SlCipk6 mutant derivatives were tested (de la Torre et al., 2013). SlRd2 interacted with SlCipk6, SlCipk6(T172D), SlCipk6ΔNAF (a mutant version lacking the NAF/FISL domain necessary for CBL binding) or SlCipk6ΔCterm (a deletion mutant version that lacked the C-terminal regulatory domain), but did not interact with SlCipk6ΔNterm (a deletion mutant version lacking the kinase domain) or very weakly with SlCipk6 (K43M, a reduced kinase activity version where the catalytic Lys-43 has been mutated to Met; Fig. 1, A and B). The interaction was quantitated in a β-galactosidase activity assay (Fig. 1C) and protein expression in yeast was confirmed by immunoblot (Supplemental Fig. S1). SlRd2 did not interact with SlCipk11 (Solyc06g082440) or SlCipk14 (Solyc10g085450; Supplemental Fig. S2A) and SlCipk6 did not interact with SGN-U601569, the closest tomato sequence to *SlRd2* (Supplemental Fig. S2B), indicating that SlCipk6 interaction with SlRd2 was specific. Altogether, we confirmed that SlCipk6 interacted with SlRd2 and concluded that SlCipk6 kinase domain and kinase activity seemed to be necessary and important, respectively, for SlCipk6/SlRd2 interaction.

SlCipk6 and SlRd2 Interact in Vivo

Next, we examined the in vivo interaction between SlCipk6 and SlRd2 by coimmunoprecipitation (co-IP). Although SlCipk6 interacted with SlRd2 in yeast at slightly higher levels, we used SlCipk6(T172D) for co-IP in planta because it has been described that constitutive active kinase versions stabilize the interaction with their substrates (Uno et al., 2009). For that purpose, both SlRd2 (fused to a GFP tag) and SlCipk6(T172D; fused to 2xIgG-BD and 9xMyc tags) were coexpressed via *Agrobacterium*-mediated transient transformation of leaves (agro-infiltration) in *Nicotiana benthamiana*. SlRd2 coimmunoprecipitated with SlCipk6(T172D), but did not with the GFP, used as a negative control in this experiment (Fig. 2A). Therefore, SlCipk6(T172D) and

SlRd2 interacted in planta. Because kinases and their substrates need a coordinated expression and subcellular localization, we first assessed the likelihood that both SlCipk6 and SlRd2 localized at common cellular compartments. Both proteins were fused to GFP, agroinfiltrated individually into *N. benthamiana* leaves and the localizations were determined by confocal microscopy (Supplemental Fig. S3). Both SlCipk6-GFP (Supplemental Fig. S3, a and b) and GFP-SlRd2 (Supplemental Fig. S3, c and d) localized in the cytoplasm and in the nucleus, thus showing an overlapping subcellular localization.

To gain insight where the complex localized intracellularly, we performed BiFC assays in *N. benthamiana* leaves (Walter et al., 2004). Fluorescence due to the reconstitution of yellow fluorescent protein (YFP) was observed at the confocal microscope when the combinations SlRd2-YFP^C and SlCipk6-YFP^N were agroinfiltrated in *N. benthamiana* leaves (Fig. 2B, a and d). The YFP signal showed a pattern similar to the one observed for AtFKBP12-CFP, a cytoplasmic marker fused to cyan fluorescent protein [CFP; (Faure et al., 1998)] (Fig. 2B, d–f), but did not overlap with the signal derived from the membrane-bound marker FM4-64 (Fig. 2B, a–c). No fluorescence was detected in the nucleus or when SlRd2-YFP^C or SlCipk6-YFP^N were coinfiltrated with empty vectors respectively, and neither when SlRd2-YFP^C was coexpressed with SlCbl10-YFP^C, a known interactor of SlCipk6 (Fig. 2B, g–i; de la Torre et al., 2013). These results indicate that SlCipk6 and SlRd2 interact in vivo and form a cytoplasmic complex in *N. benthamiana* leaves at the conditions assayed.

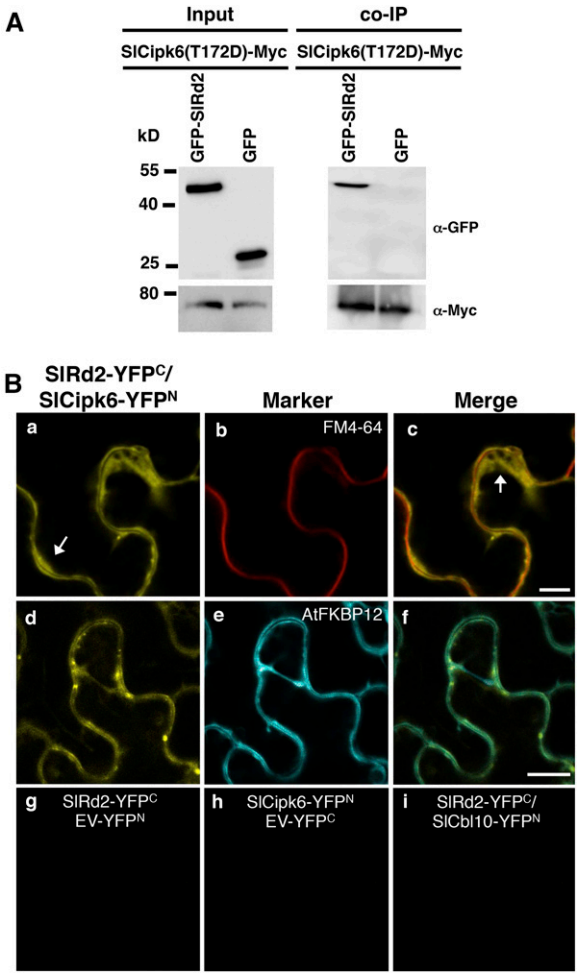

Figure 2. SlCipk6 and SlRd2 interact in planta and the complex localizes in the cytosol. A, Protein extracts from *N. benthamiana* leaves agroinfiltrated with GFP-SlRd2 and SlCipk6(T172D)-Myc, which is fused to two copies of the protein A IgG binding domains (2xIgG-BD), were incubated with IgG agarose beads, eluted and analyzed by immunoblotting using anti-Myc for SlCipk6 and anti-GFP for SlRd2. GFP protein was used as a negative control. No interaction was detected when SlCipk6(T172D) was agro-infiltrated with GFP (right upper panel). B, Fluorescence images of *N. benthamiana* leaf sections agroinfiltrated with SlRd2-YFP^C and SlCipk6-YFP^N. Colocalization analyses of SlCipk6/SlRd2 complexes (yellow, a and d) with the fluorescent membrane marker dye FM4-64 (red, b) and the cytoplasmic marker AtFKBP12-CFP (cyan, e; Faure et al., 1998). Merged images are shown in (c) and (f), respectively. White arrows show the cytoplasmic localization observed for the SlCipk6/SlRd2 complexes. Localization analysis of YFP^N (g) and YFP^C (h) empty vectors and SlCbl10/SlRd2 (i) were used as an experiment controls. Bars = 10 μm.

SlRd2 Transcript Accumulates in Response to Abiotic Stress

E. coli UspA (EcUspA) protein accumulates in response to a large and diverse number of stresses providing survival cues under adverse bacterial growth conditions (Freestone et al., 1997; Jung et al., 2015). In plants, members of the UspA family have been reported to accumulate in response to different abiotic stress conditions, including *AtRD2* (Yamaguchi-Shinozaki et al., 1992; Jung et al., 2015). Both SlCipk6 and SlRd2 displayed an overlapping subcellular localization in *N. benthamiana* epidermal cells (Supplemental Fig. S3, a–d). Next, we inquired if *SlRd2* and *SlCipk6* expression were correlated in response to abiotic stress using quantitative (Q) RT-PCR to support synchronous activity in abiotic stress response.

SlRd2 mRNA was present in all the tissues examined, however it was substantially more abundant in roots (>25-fold compared to leaves), suggesting that it might play a prominent role in this tissue (Fig. 3A). *AtRD2* accumulation increased in response to abiotic stress (Yamaguchi-Shinozaki et al., 1992). Therefore, to determine if *SlRd2* and *SlCipk6* transcript accumulation were responsive to salt and osmotic stress, we treated hydroponically grown tomato plants with 100 mM NaCl and 300 mM mannitol and analyzed *SlRd2* accumulation. NaCl treatment rapidly increased *SlRd2* transcript accumulation [approximately 12-fold, after 2 h (h)], which was maintained up to 12 h and decreased 24 h later (Fig. 3B). Osmotic pressure resulted in a similar accumulation of *SlRd2* after 2 h and 4 h, but in contrast, *SlRd2* accumulation increased up to 30-fold after 8 h to 12 h and decreased to 20-fold at 24 h (Fig. 3C). SlCipk6 orthologs from different plant species

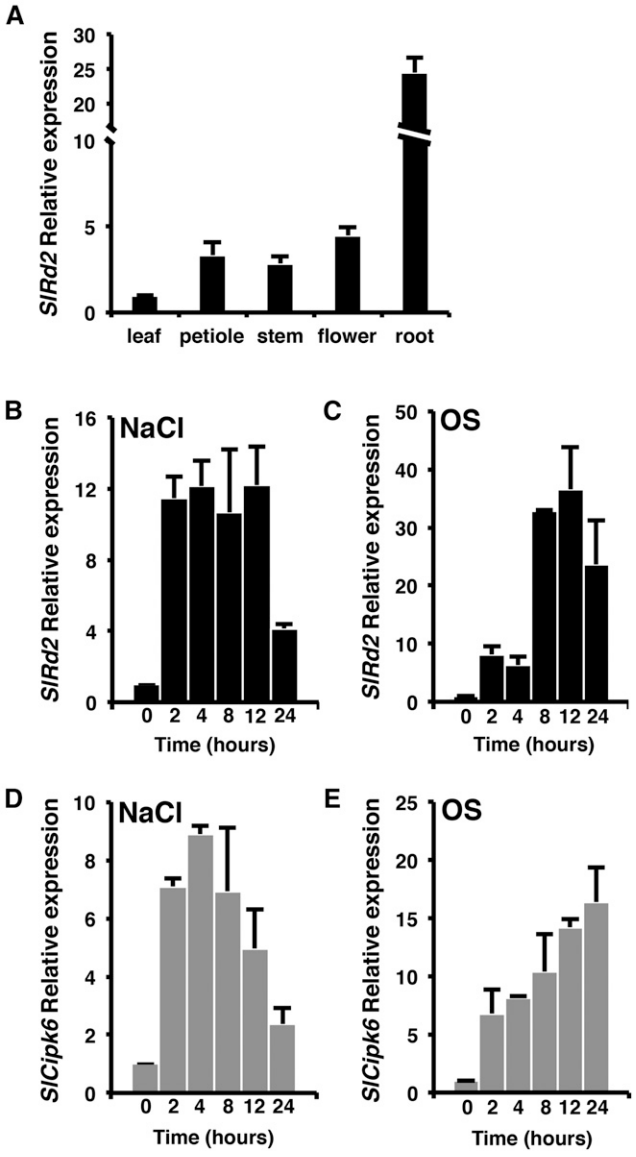

Figure 3. *SlRd2* and *SlCipk6* transcripts highly accumulate after nacl and osmotic stress in tomato. A to E, *y* axis represents mean values of Q RT-PCR of three experiments, with three biological replicates in each. Error bars represent the SE. The expression levels were normalized to *SlActin2*. Gene induction (fold increase) in infected or treated plants was compared with the expression level of control or mock inoculated plants at 0 h and is shown as relative expression, excepting in (A). A, *SlRd2* transcript accumulates in tomato leaves, petioles, stems, flowers, and roots. Relative expression on *y* axis was compared with expression level in leaves. B and C, *SlRd2*; D and E, *SlCipk6* mRNA accumulation in leaves during NaCl (B and D) and osmotic stress (C and E). Hydroponically grown tomato plants were treated with 100 mM NaCl (B and D) or 300 mM mannitol (C and E). OS, osmotic stress.

have been described to participate in different abiotic stress responses, and we tested if *SlCipk6* transcript accumulated also in response to abiotic stress. For that purpose, we treated tomato plants with NaCl and mannitol, using the same conditions as described above. After NaCl treatment, accumulation of *SlCipk6* mRNA increased 6-fold to 8-fold after 2 h and was maintained at the same levels up to 8 h, decreasing gradually thereafter, reaching a 2-fold increase at 24 h (Fig. 3D). Osmotic pressure also resulted in an increased *SlCipk6* mRNA accumulation; however, followed a different pattern compared to *SlRd2*: 2 h after *SlCipk6* transcripts increased 6-fold and steadily increased up to 15 fold 24 h after (Fig. 3E). Both *SlCipk6* and *SlRd2* transcript accumulation pattern showed similar kinetics, thus supporting the possibility of a coordinated role for SlCipk6 and SlRd2 in abiotic stress responses in tomato.

SlRd2 Belongs to the Universal Stress Protein A Superfamily

The translated sequence of SlRd2 ORF yields a 177 amino acid protein, with a M_r of 19.5 kD and a pI of 5.98. We aligned the Usp domain of SlRd2 with those of *N. benthamiana* and Arabidopsis orthologs NbRd2 and AtRD2 and other plant proteins containing the Usp domain, including tomato LeER6 (Zegzouti et al., 1999), rice (*Oryza sativa*) OsUSP1 (Sauter et al., 2002), and *Vicia fava* Enod18 (Becker et al., 2001) along with bacterial proteins belonging to the UspA family, including *E. coli* (Nyström and Neidhardt, 1992), *H. influenzae* (Sousa and McKay, 2001) and *M. jannaschii* (Zarembinski et al., 1998; Supplemental Fig. S4). Amino acid sequence alignment revealed that SlRd2 contained the residues involved in ATP binding including the Walker motif A or P-loop, (G-2X-G-9X-G(S/T)), also present in Mj0577 and UspA plant representatives but absent in *E. coli* paralogs (Sousa and McKay, 2001). This observation suggests that plant UspA proteins might also be functional ATP-binding proteins (Supplemental Figs. S4 and 5A). Overall, all UspA proteins (plant and bacterial) shared conservation within the dimerization subdomain (Supplemental Fig. S4), thus raising the possibility that plant UspAs could be present as dimers in the cell. In addition to the conserved Usp domain, SlRd2 has an N-terminal domain (amino acids 1 to 38) and a C-terminal extension (amino acids 168 to 177) with unknown function, which is shared with the Arabidopsis and *N. benthamiana* homologs (Figs. 4A and S5). Subsequently, a phylogenetic analysis was performed using *E. coli* UspG and the proteins included in the alignment shown in Supplemental Fig. S4. SlRd2 and NbRd2 were located in the same clade as AtRD2, thus supporting their orthology (Fig. 4B). Moreover, SlRd2, and all plant UspAs, are more related to Mj0577 subfamily and *E. coli* UspG than to the *E. coli* UspA (Fig. 4B).

SlRd2 Is an ATP-binding Protein and Forms Homodimers in Yeast, Bacteria, and Plants

In light of the high degree of conservation of SlRd2 amino acids putatively involved in nucleotide binding (see alignments in Figs. 5A and S4), we next inquired if SlRd2 also had the functional competence to bind ATP, as described for bacterial Mj0577. For that purpose, we

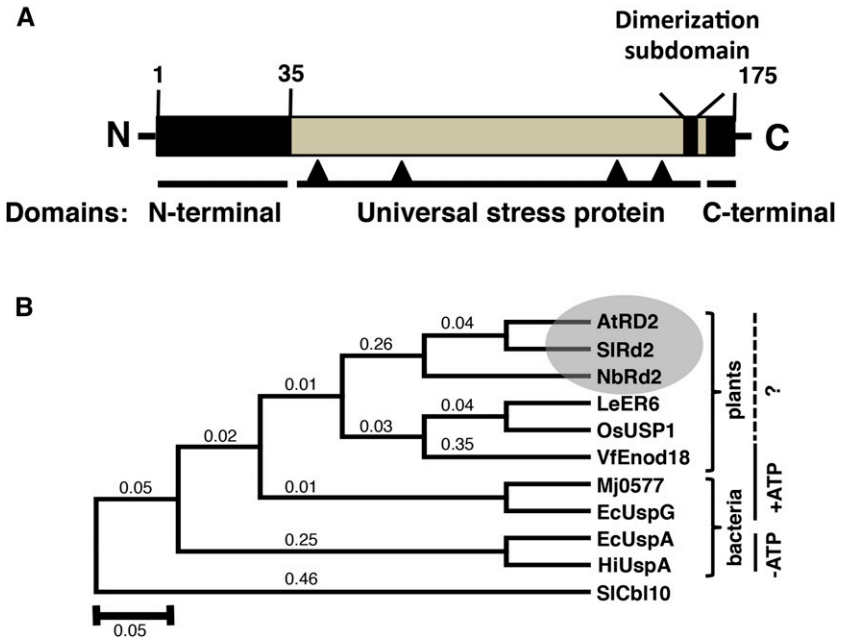

Figure 4. Tomato SlRd2 protein structure and phylogenetic analysis. A, Schematic structure of SlRd2 containing N-terminal and C-terminal domains and a conserved *Usp* domain, which encompasses a dimerization motif. Special symbols indicate the conserved residues described to be involved in ATP-binding. B, Phylogenetic relationship of proteins containing Usp domains from plants and bacteria including *A. thaliana*, AtRD2; tomato, SlRd2 and LeER6; *N. benthamiana*, NbRd2; *O. sativa*, OsUSP1; *V. faba*, VfENOD18; *E. coli*, EcUspG and EcUspA; *H. influenzae*, HiUspA; and *M. jannaschii*, Mj0577. SlCbl10 was used as the outgroup. The numbers on the tree represent bootstrap scores.

purified SlRd2 protein and performed a nucleotide binding assay in vitro by incubating SlRd2 in the presence of [α-^{32}P]ATP. Samples were then analyzed by SDS-PAGE followed by autoradiography (Fig. 5B, upper panel). Indeed, SlRd2 was able to bind [α-^{32}P]ATP, whereas no [α-^{32}P]ATP binding was observed when the GFP protein was incubated in its presence. To further confirm that SlRd2 was also able to bind ATP in vivo, a synthetic analog of ATP, (+)-biotin-hex-acyl-ATP (BHAcATP), consisting of ATP, an acyl-P linker and a biotin tag, was used (Villamor et al., 2013). Incubation of GFP-SlRd2 expressing plant extracts with BHAcATP followed by streptavidin purification revealed that SlRd2 was able to bind BHAcATP in planta (Fig. 5C, upper panel). Moreover, ATP addition suppressed BHAcATP labeling by competing with BHAcATP and saturating the nucleotide-binding site of SlRd2 (Fig. 5C, upper panel). These findings indicate that SlRd2 binds ATP and therefore functionally belongs to the ATP-binding subgroup of the UspA family represented by Mj0577.

The dimerization motif, present in UspA family proteins, is also conserved in SlRd2 and is localized close to the C terminus (Figs. 5A and S4). Mj0577 and HiUspA were shown to exist as homodimers in vivo (Sousa and McKay, 2001; Zarembinski et al., 1998). Mj0577 crystallizes as a homodimer, and each monomer binds the other through antiparallel hydrogen bonds in the fifth beta sheet within each subunit. To test whether SlRd2 was also able to form homodimers, we performed a Y2H analysis in which the yeast strain expressed SlRd2 both in the prey (pJG4-5) and in the bait (pEG202) plasmids. Indeed, SlRd2 can form homodimers because growth was observed in restrictive media and blue color developed in the presence of X-gal (Fig. 6A). No interaction was detected when Y2H analysis was performed using a SlRd2 mutant version, SlRd2Δdim (amino acids 163 to 166 deletion), in which the putative dimerization domain VIIV was deleted (Fig. 6A). SlRd2 and SlRd2Δdim, were expressed in yeast (Fig. 6B). Therefore, SlRd2 forms dimers and the conserved VIIV domain is necessary for dimerization in vivo.

It has been described that *E. coli* UspC is able to form tetramers in vivo (Nachin et al., 2008). To check if SlRd2 also formed homotetramers in vivo, an *E. coli* culture overexpressing SlRd2 tagged at the N terminus with the epitope His (His-SlRd2) was treated with a cross-linking agent, disuccinimidyl glutarate (DSG), for 30 min. Thereafter, protein extracts were obtained, analyzed by SDS-PAGE and His-SlRd2 detected by immunoblot. Two bands of approximately 42 and 24 kD were observed in the extracts treated with the crosslinker, which corresponded likely with SlRd2 dimer and monomer, whereas only the lower M_r band was observed in control conditions (Fig. 6C). Thus, we concluded that SlRd2 is able to form homodimers but not homotetramers in vivo.

To determine that SlRd2 formed homodimers in planta and their putative subcellular localization, BiFC assays were performed. *SlRd2* cDNA was cloned in the BiFC vectors, transformed into *Agrobacterium* and agroinfiltrated in *N. benthamiana* leaves. Reconstitution of YFP fluorescence was observed 2 d after under the confocal microscope when SlRd2-YFP^C and SlRd2-YFP^N

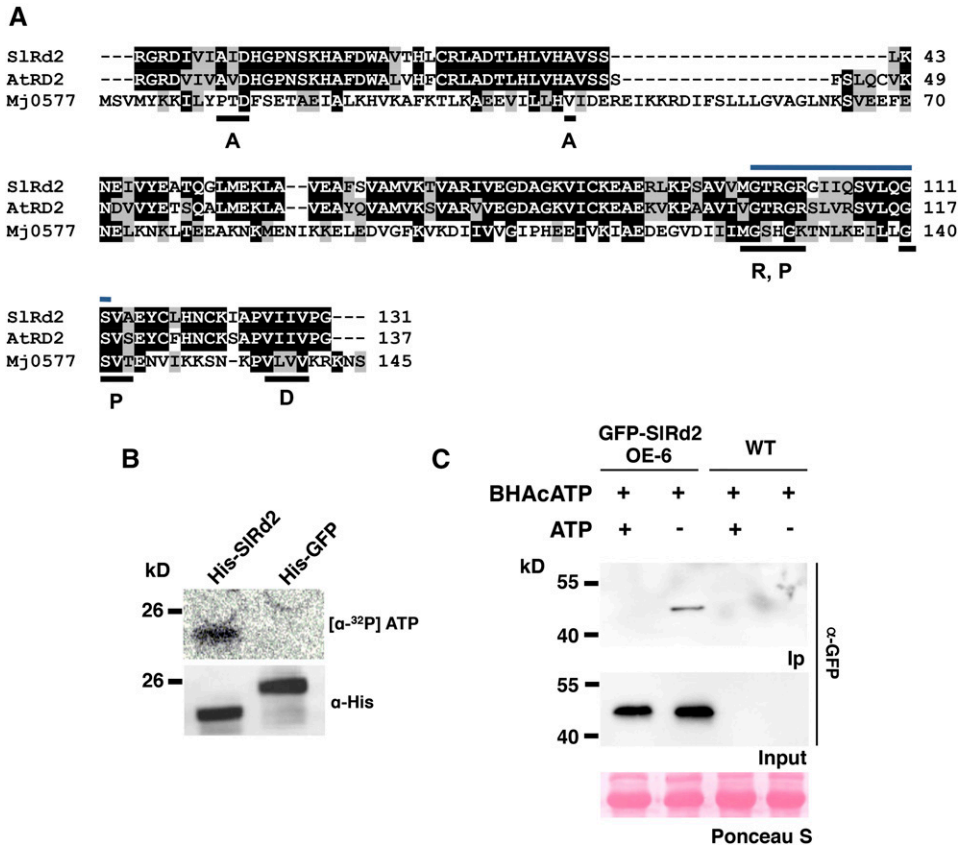

Figure 5. SlRd2 is an ATP-binding protein. A, Alignment of Usp domain of SlRd2 and AtRD2 and the full amino acid sequence of Mj0577. Conserved residues are marked in black boxes while similar residues are marked in gray boxes. Black bars underline residues putatively involved in ATP-binding. Walker motif or P loop motif G-2x-G-9x-G-(S/T) for ATP binding is marked with a blue line. Amino acid residues responsible for protein dimerization are marked as (D). B, For ATP-binding in vitro assay, 1 μg of purified His-SlRd2 and His-GFP proteins from *E. coli* were incubated with [α-^{32}P]ATP and corresponding buffer during 1 h at 30°C. Autoradiograph indicates ATP-binding (top panel); immunoblot analysis indicates protein level in the ATP binding assay (low panel). C, For ATP-binding in vivo assay, *N. benthamiana* leaf extracts from wild type and GFP-Rd2 overexpressing line 6 plants were incubated with 20 μg BHAcATP. Biotinylated proteins were purified using streptavidin beads, separated on protein gel, and detected using anti-GFP (top panel). Samples labeled with BHAcATP in presence of 10 mM of ATP were used as a control. Ponceau S staining was used as a loading control (bottom panel). Adenine, A; phosphate, P; ribose, R.

were coexpressed. The signal colocalized with that obtained by the expression of the cytoplasmic marker AtFKBP12-CFP (Fig. 6D, d–f) and with DAPI nuclear marker (Fig. 6D, g–i) but not with the cell membrane marker FM4-64 (Fig. 6D, a–c). These results demonstrate that SlRd2 can form homodimers in planta in the cytoplasm and in the nucleus.

SlRd2 Is a Phosphorylation Substrate of SlCipk6

Because SlCipk6 kinase domain and activity was necessary and important respectively for SlCipk6/SlRd2 interaction (Fig. 1), we inquired if SlRd2 was a phosphorylation target of SlCipk6. For that purpose, we set up an in vitro kinase assay in the presence of [γ-^{32}P]ATP using purified SlCipk6 (fused to the GST at the C terminus) and as substrates, SlRd2 (fused to the His epitope at the N terminus) and a mutant version, SlRd2(UspA; fused to GST at the N terminus), in which the specific putative regulatory N-terminal domain (amino acids 1 to 38) and C-terminal extension (amino acids 168 to 177) were deleted to leave only a core UspA domain. Myelin Basic Protein (MBP), a universal kinase substrate was included as a positive control for SlCipk6 kinase activity. SlCipk6 was expressed and purified from yeast, whereas SlRd2 and SlRd2(UspA) were purified from bacteria. Figure 7A shows that SlRd2 is phosphorylated by SlCipk6 but does not phosphorylate SlRd2(UspA), thus indicating that SlCipk6 phosphorylates SlRd2 specific regulatory N- or C-terminal regions. SlCipk6 also phosphorylates MBP. A faint signal can be still detected in the absence of SlCipk6, indicating that SlRd2 binds ATP (Fig. 7A, left). Protein loading levels were monitored by Coomassie staining (Fig. 7A). Quantification of SlRd2 [γ-^{32}P]ATP incorporation is presented in Figure 7B. Therefore, we conclude that SlRd2 is a phosphorylation substrate of SlCipk6 and represents, to our knowledge, a novel phosphorylation target of the CIPK family.

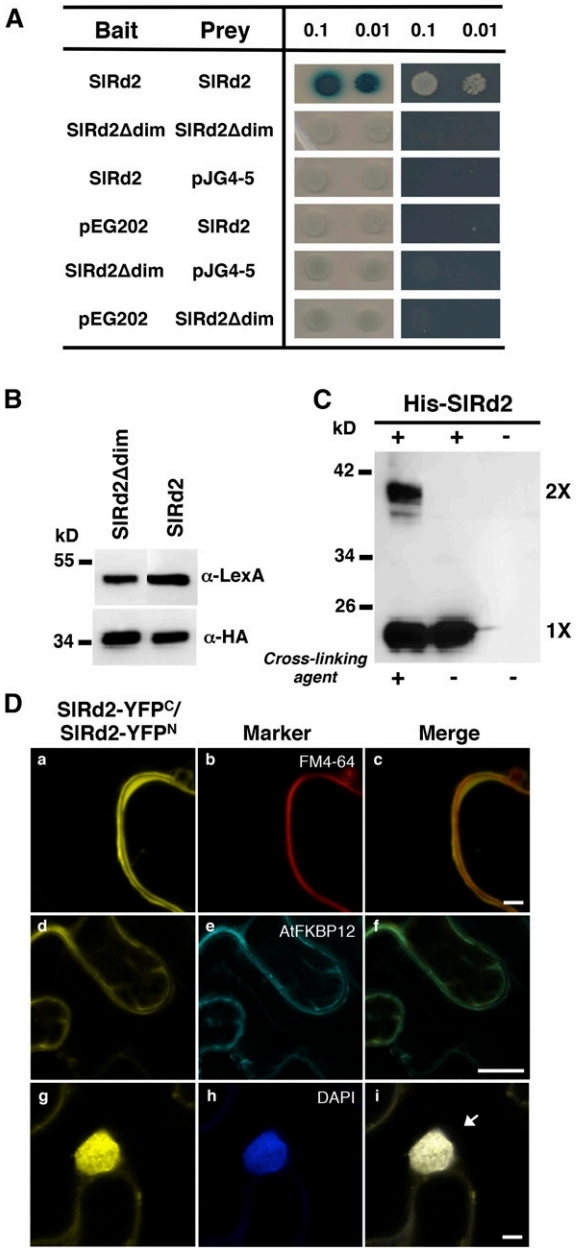

Figure 6. SlRd2 homodimerizes in the cytosol and in the nucleus. A, Y2H assay with both *SlRd2* and *SlRd2Δdim* cloned in the bait pEG202 (with a LexA fusion tag at the C terminus) and prey pJG45 (with a HA fusion tag at the C terminus) vectors in EGY48 strain, grown in liquid culture and spotted at OD_{600} = 0.1 and 0.01 on selective medium. Growth and blue patches indicates interaction. B, Expression of both SlRd2 and SlRd2Δdim proteins in yeast were confirmed by immunoblotting using anti-LexA (pEG202) and anti-HA (pJG45) antibodies, respectively. C, The cross-linking agent DSG was added to a His-SlRd2 overexpressing *E. coli* culture for 30 min. Polymerization of His-SlRd2 protein was calculated according to its molecular weight: 1×, monomer; and 2×, dimer. D, Colocalization analyses of SlRd2/SlRd2 complexes (yellow, a, d, and g) with the membrane marker dye FM4-64 (red, b), cytoplasmic marker AtFKBP12 (cyan, e), or nuclear marker DAPI (blue, h). Merged images are shown in (c), (f), and (i). Arrow denotes localization in the nucleus. Bars = 10 μm.

SlRd2 Overexpression in Yeast Confers Resistance to LiCl

In light of the strong transcriptional response of *SlRd2* to NaCl and osmotic stress, we decided to functionally test SlRd2 participation in mediating different stress responses using yeasts as a model system. Yeast has been successfully used in functional studies to characterize the role of plant proteins in stress responses (Matsumoto et al., 2001; Quintero et al., 2002; Shitan et al., 2013; Bernard et al., 2012). SlRd2 was overexpressed in the yeast strain *Saccharomyces cerevisiae* BY4741 under control of the constitutive expression vector p426GPD (p426GPD-SlRd2). As a control, BY4741 strain transformed with empty vector (EV) p426GPD was used. Both yeasts lines were subjected to the following stress-causing treatments: hygromycin B, generates changes in membrane potential (Wang et al., 2009); $MnCl_2$, causes programmed cell death at high concentrations (Liang and Zhou, 2007); $CaCl_2$ and KCl, cause osmotic stress (Liang and Zhou, 2007); DTT and H_2O_2, generate oxidative stress (Babiychuk et al., 1995); bleomycin, causes DNA damage (Aouida et al., 2004); and finally, NaCl and LiCl, cause changes in ionic homeostasis (Ye et al., 2006). SlRd2 overexpressing line grew at a similar rate than EV strain in control conditions and in all the different treatments, except in 300 mM LiCl, where it grew significantly better than EV line (Fig. 8A). Next, we tested if SlRd2 dimerization was crucial for its biological role. For that purpose, we cloned *SlRd2Δdim* into p426GPD vector, transformed it into *S. cerevisiae* BY4741 strain and performed a LiCl resistance experiment along with p426GPD-SlRd2 and EV. Figure 8B shows that a functional SlRd2 dimer was required for conferring LiCl resistance, because SlRd2Δdim line grew at the same rate as EV line and loss its protection against LiCl. Both SlRd2 and SlRd2Δdim expression in yeast was confirmed by immunoblotting (Fig. 8C). We concluded that SlRd2 dimerization was necessary for its molecular function in conferring resistance to LiCl.

Next, we asked if SlRd2 dimerization was also necessary for its interaction with SlCipk6. For that purpose, we performed a Y2H assay with SlCipk6 and SlCipk6(T172D) as baits and SlRd2Δdim as a prey (Fig. 9A). Neither SlCipk6 nor SlCipk6(T172D) interacted with SlRd2Δdim by Y2H (Fig. 9A). To test their interaction in planta, we performed a BiFC experiment, and agro-infiltration SlRd2Δdim-YFP^C and SlCipk6-YFP^N in *N. benthamiana* leaves did not reconstitute YFP fluorescence, indicating that SlCipk6 was not able to interact with SlRd2Δdim (Fig. 9B). Hence, SlRd2 homodimerization is required for interaction with SlCipk6.

SlRd2 Protects Bacteria from Oxidative Stress and Negatively Regulates Reactive Oxygen Species in Plants

E. coli mutant strain TN3151 (a knock-out of the *uspA* gene) was susceptible to H_2O_2 treatments, which did not affect the wild-type strain, W3101 (Nyström and Neidhardt, 1994). Recent results demonstrated that a

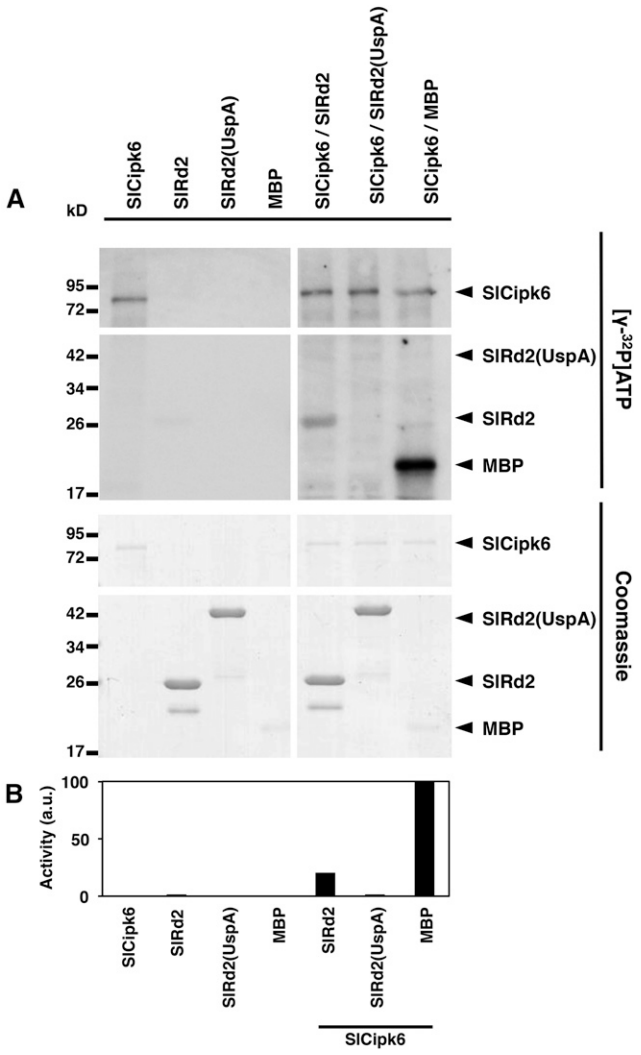

Figure 7. SlCipk6 Phosphorylates SlRd2. A, SlCipk6, SlRd2, SlRd2(UspA) and MBP alone and with SlCipk6 were incubated in the presence of [γ-^{32}P] ATP and kinase buffer, electrophoresed on SDS polyacrylamide gel and autoradiographed. They were expressed and purified as fusion proteins as follows: SlCipk6-GST, His-SlRd2, GST-SlRd2(UspA). Autoradiographs indicate SlCipk6 autophosphorylation (upper panels) and SlRd2 phosphorylation (lower panels); Coomassie protein staining indicates SlRd2, SlRd2(UspA), MBP, and SlCipk6 protein loads. Protein amounts loaded: 0.3 μg for SlCipk6, 2.5 μg for SlRd2 and SlRd2(UspA), and 0.5 μg for MBP. B, Phosphorylation was quantified using Cyclone Phosphorimager Optiquant software (Packard Bioscience, Perkin Elmer). Signal from MBP phosphorylation was not normalized to protein content to better quantify SlRd2 phosphorylation and ATP binding. Similar results were obtained in two additional experiments. a.u., arbitrary units.

UspA protein from the pathogenic bacteria *Mycobacterium tuberculosis* provided protection for the parasite against host reactive oxygen species (ROS) generated by mammalian macrophages defense (Drumm et al., 2009). SlCipk6 was demonstrated to participate in ROS generation during plant responses to bacterial pathogen attack, which was dependent on the NADPH oxidase, RbohB (de la Torre et al., 2013). Given the striking conserved structural and functional features between SlRd2 and bacterial UspA proteins, we decided to test if SlRd2 could functionally complement TN3151 in protecting bacteria in response to H_2O_2 treatment. *E. coli* wild-type W3101 (wild type), *uspa* mutant TN3151 and TN3151 complemented with either *SlRd2* (TN3151-*SlRd2*) or with the native *UspA* gene (TN3151-*UspA*) were grown in minimal media (morpholine propane sulphonic acid, MOPS) until OD_{600} reached 0.7. Then, H_2O_2 (5 mM) was added and survival was measured at different time points after (Fig. 10A). A strong growth inhibition was observed for TN3151 strain after H_2O_2 treatment, as previously described (Nyström and Neidhardt, 1994); 40 min after H_2O_2 addition, only 45% of TN3151 survived whereas 100% TN3151-*SlRd2* survived, showing a similar survival rate as either TN3151-*EcUspA* or wild-type W3101. SlRd2 and EcUspA expression in TN3151 was confirmed by immunoblotting (Fig. 10B) and lack of *UspA* gene expression in TN3151 was confirmed by PCR (Fig. 10C). In light of these results, we concluded that tomato SlRd2 functionally complements TN3151 mutant strain in protecting bacteria against oxidative stress damage and thus SlRd2 and *E. coli* UspA are functionally conserved in their ROS protection role in bacteria.

We have previously shown that kinase activity of SlCipk6 is associated with ROS production in *N. benthamiana* (de la Torre et al., 2013), so we next inquired if SlRd2 is required for SlCipk6-mediated ROS generation. To this end, c-Myc tagged versions of SlCipk6, SlCipk6(T172D) and SlCipk6(K43M; cloned into pTAPa-pYL436 vector) or EV were agro-infiltrated in *N. benthamiana* wild-type and overexpressing GFP-SlRd2 (OE-6, OE-7) leaves and production of ROS was quantified by a chemiluminescence assay in a luminometer (Fig. 10D). As expected, SlCipk6 and SlCipk6(T172D) expression in wild-type leaf discs resulted in ROS generation, which was significantly reduced in SlCipk6(K43M) expressing leaf discs (de la Torre et al., 2013). However, SlCipk6 and SlCipk6(T172D) agro-infiltration in OE-6 and OE-7 plants resulted in a significant reduction of ROS (Fig. 10D) whereas SlCipk6(K43M)-induced ROS levels remained as in wild-type discs. SlCipk6, SlCipk6(T172D), SlCipk6(K43M), and GFP-SlRd2 expression were confirmed by immunoblotting (Supplemental Fig. S6). This result clearly indicated that overexpression of SlRd2 negatively regulates SlCipk6-mediated ROS generation and that a functional link exists between both proteins.

DISCUSSION

SlRd2, a Member of the Universal Stress Protein Family, Interacts with SlCipk6

The identification and characterization of SlCipk6 targets and regulatory components is an important step for understanding how downstream SlCipk6 signaling specificity is achieved and to identify the pathways it regulates. As a first step, we set to identify

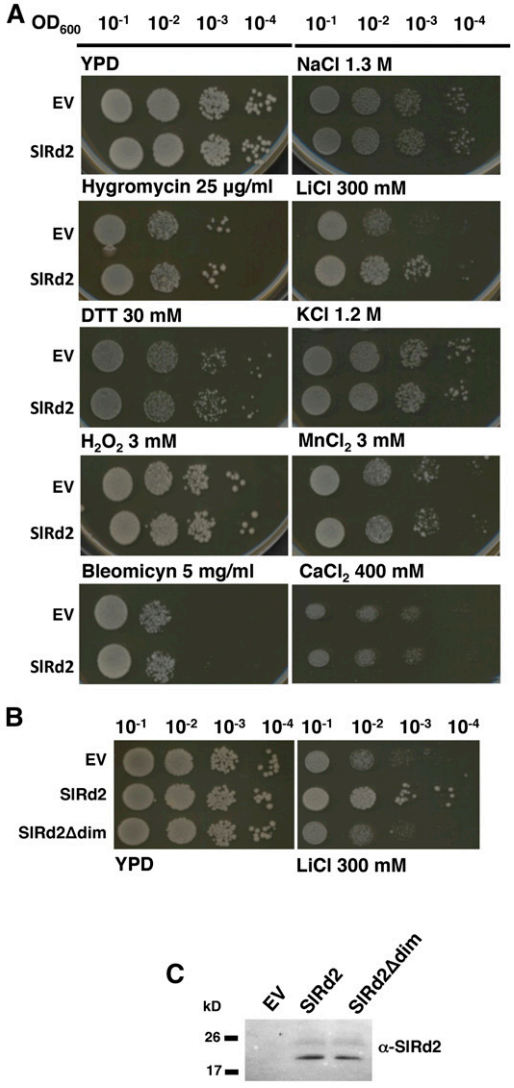

Figure 8. SlRd2 expression confers LiCl resistance in yeast and its dimerization is required for its biological function. A, BY4741 yeast strain expressing SlRd2 (cloned into p426GPD) was subjected to different stress conditions. As a control, BY4741 was transformed with the EV. Both SlRd2 and EV lines were grown in liquid culture and spotted on specific medium at different dilutions. Overexpression of SlRd2 showed a higher growth than EV in response to LiCl (300 mM). B, Overexpression of SlRd2Δdim showed the same growth rate than EV line in response to LiCl. The experiment was performed as described in (A). C, Expression of SlRd2 and SlRd2Δdim proteins in yeast were confirmed by immunoblotting using anti-SlRd2 antibodies.

SlCipk6-interacting proteins using a Y2H approach with either SlCipk6 or its enhanced kinase activity version SlCipk6(T172D) as baits, and a tomato library as a prey (Zhou et al., 1995). We expected to identify putative phosphorylation substrates or regulatory proteins. SlRd2 was identified in both screens and displayed one of the strongest interactions. SlCipk6/SlRd2 interaction was later confirmed and appeared to have some level of specificity. In addition, no assigned function was available for this protein. Hence, we prioritized its characterization. For SlCipk6/SlRd2 interaction, SlCipk6 kinase domain and kinase activity was required, thus suggesting that SlRd2 could be a phosphorylation substrate of SlCipk6. SlCipk6/SlRd2 interaction was further confirmed in planta, using coimmunoprecipitation and BiFC approaches. Thus, we compiled data using different approaches demonstrating that SlCipk6/SlRd2 interacted in *N. benthamiana*.

BiFC experiments indicated that SlCipk6/SlRd2 complex localized in the cytosol. Both GFP tagged SlCipk6 or SlRd2 localized mainly in the nucleus and in the cytoplasm of *N. benthamiana* epidermal cells, thus showing a coordinated subcellular localization pattern. However, SlCipk6/SlRd2 complex was only detected in the cytosol. We cannot rule out that the complex could localize to the nucleus or to different cellular compartments when complexed with CBLs or under stress conditions. In fact, it has been demonstrated that

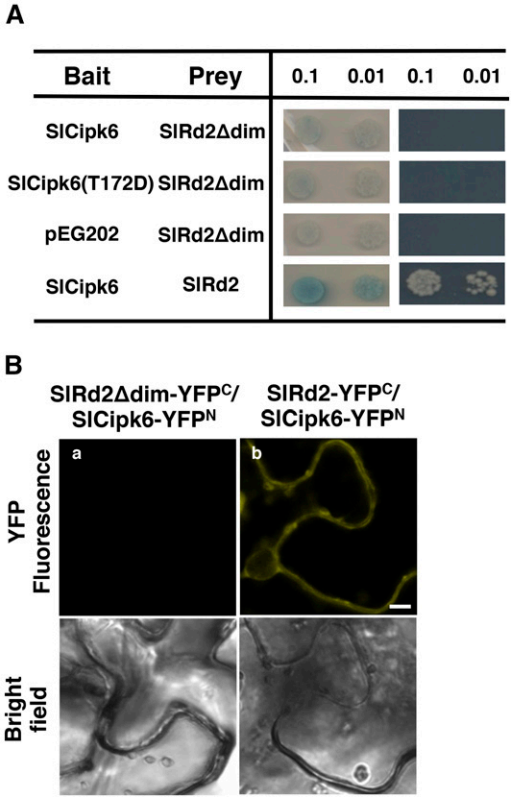

Figure 9. SlRd2 dimerization is required for its interaction with SlCipk6. A, Y2H assay performed with SlCipk6 and SlCipk6(T1172D) in the bait vector pEG202 and SlRd2Δdim in the prey vector pJG4-5. Clones were transformed into EGY48 strain, grown in liquid culture, and spotted at OD_{600} = 0.1 and 0.01 on selective medium. Growth and blue patches indicates interaction. A Y2H experiment performed with SlRd2 and SlCipk6 was used as a control. B, Fluorescence images of *N. benthamiana* epidermal cells expressing SlRd2Δdim-YFP^C and SlCipk6-YFP^N. YFP signal was not reconstituted in the BiFC experiment (a), confirming that SlRd2 dimerization is required for the interaction with SlCipk6 in planta. A BiFC experiment performed with SlRd2 and SlCipk6 was used as a control (b). Bars = 10 μm.

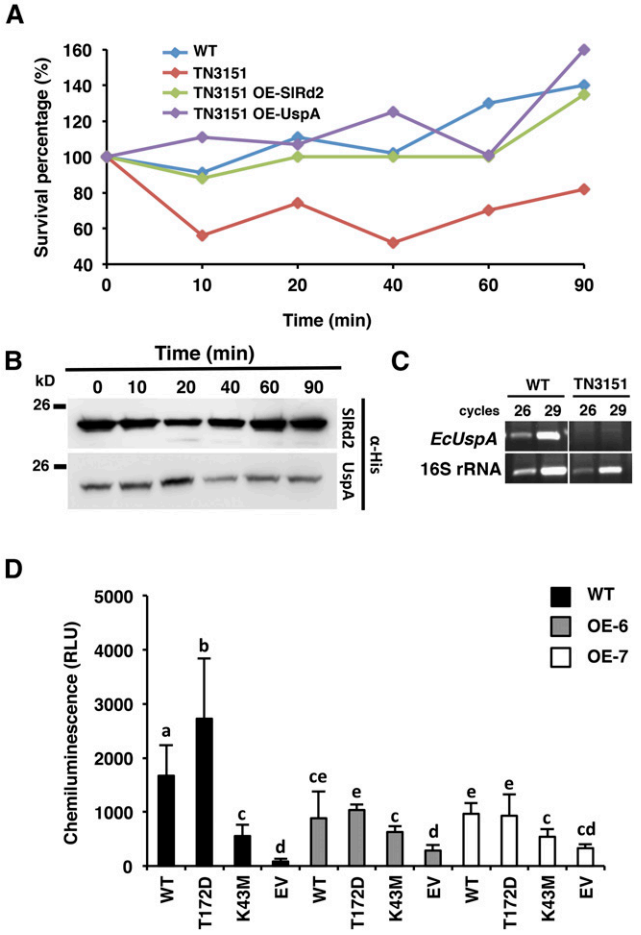

Figure 10. Tomato SlRd2 restores *E. coli* TN3151 (Δ*uspa*) survival and negatively regulates SlCipk6-mediated ROS. A, Wild type [W3101 (F-, IN (rrnD-rrnE)1, rph-1)], *Uspa* mutant TN3151 [F-, IN(rrnD-rrnE)1, UspA::kan, rph-1)], and the overexpressing TN3151 SlRd2 (OE-SlRd2) and TN3151 UspA (OE-UspA) *E. coli* lines were grown in MOPS medium and treated with H_2O_2 (5 mM) when the cultures reached OD_{600} = 0.7. B, Immunoblot experiment confirmed SlRd2 (upper panel) and UspA (lower panel) expression in TN3151 *E. coli* lines. C, Transcript abundance of *UspA* cDNA in TN3151 and wild-type strains was determined by RT-PCR. 16S rRNA transcript abundance was used as an internal standard control. D, Measurement of ROS production in *N. benthamiana* leaf disks of GFP-SlRd2 overexpressing (OE-6 and OE-7) and wild-type plants, after agro-infiltration of SlCipk6-Myc (wild type), SlCipk6(T172D)-Myc (T172D), SlCipk6(K43M)-Myc (K43M), or EV. ROS accumulation was quantified as relative light units 8 h to 12 h after. Data are presented as means ± SES of four independent experiments. Means with different letters are significantly different at $P < 0.05$, Student's *t*-test. Anti-GFP and anti-Myc were used as primary antibodies. RLU, relative light units; WT, wild type.

CIPKs can be targeted to different intracellular compartments via their interacting CBLs (Batistic et al., 2010). Accordingly, tomato Cipk6 was found to interact with SlCbl10 and with NbRbohB, a membrane based NADPH oxidase at the plasma membrane in *N. benthamiana* epidermal cells (de la Torre et al., 2013) whereas *Brassica napus* CIPK6 (also plasma membrane- and nucleus-localized), was found at the plasma membrane in complex with BnCBL1 (Chen et al., 2012). Here, we describe the localization of a CIPK in complex with its substrate, which poses additional unsolved questions, such as whether CBLs translocate CIPKs to meet their substrate at the final destination or if they could also transport the attached substrates as cargoes. CBLs and CIPKs interact with some, but not with all CIPK and CBL partners respectively, thus providing an enormous combinatorial signaling flexibility (Luan, 2009; Yu et al., 2014). At this point, we do not know which CBL(s) relay Ca^{2+} signals to SlCipk6 and SlRd2.

SlCipk6 Phosphorylates SlRd2, a Universal Stress Protein and a Novel Target of CIPK Family

We have demonstrated that SlCipk6 phosphorylates SlRd2 in vitro. In plants, Ca^{2+} intracellular increases occur after several intra- and extracellular stimuli, including biotic, abiotic, and developmental changes (Hashimoto and Kudla, 2011). It is noteworthy that tomato exposure to abiotic stress (osmotic stress and salinity) results in a rapid and strong accumulation of *SlRd2* transcripts, which resembles *SlCipk6* accumulation pattern. Similarly, in silico data indicates that under osmotic stress (mannitol 300 mM) in Arabidopsis *AtCIPK6* and *AtRD2* are transcriptionally upregulated (Arabidopsis eFP Browser). Based on our results, we propose that SlRd2 is a phosphorylation target of SlCipk6 and therefore, a Ca^{2+} signaling downstream effector or more likely, a downstream Ca^{2+} signaling component. Our results also indicate that CIPKs, in addition to membrane proteins, phosphorylate additional substrates, thus expanding their role in regulating different physiological processes. Previous work by others have shown that different UspA proteins from bacteria and plants are phosphorylated in response to stress, indicating that this posttranslational modification may be important for their role in the cell (Merkouropoulos et al., 2008; Lenman et al., 2008; Freestone et al., 1997). In addition, it was also found that the phosphosites were localized outside the Usp domain, as likely occurs in SlRd2 (Merkouropoulos et al., 2008). Likely the ancient and conserved function of the Usp domain adapts to different stress signals in different organisms acquiring specific signaling domains to modulate specific output responses. In the future, we will identify the exact SlRd2 residues phosphorylated by SlCipk6. Once identified, functional analysis in plants will be performed using transgenic plants with altered expression levels of SlRd2 or SlRd2 mutant versions in which the residues phosphorylated by SlCipk6 will be mutated to A (not a substrate) or to D (phosphomimics). These studies will help clarify SlRd2 role and SlCipk6 phosphorylation contribution in ROS generation and regulation in plants.

Using in vitro and in vivo assays, we demonstrate that SlRd2 is also an ATP-binding protein, like *E. coli* UspFG subfamily members and group representative Mj0577. Mj0577 and plant UspAs share the common motif present in Usps experimentally proven to bind

ATP (Kvint et al., 2003). Obtaining SlRd2 nucleotide binding impaired mutant versions will help to understand the physiological relevance of this feature in plants under stress responses. The ATP binding capability in some members of the UspA family has led to the speculation that nucleotide binding Usps could function as molecular switches by sensing ATP levels during stress signaling detecting cellular energy or metabolic status (O'Toole et al., 2003; Persson et al., 2007; Drumm et al., 2009). In fact, autoadenylation is observed in bacteria in late stationary phase (Weber and Jung, 2006) and it has been shown to be a key factor in microbial survival under O_2 depletion, during growth arrest and in virulence. In this line, it has been described that the ability of UspA protein Rv2623 from *M. tuberculosis* to regulate its growth and latency in the host, is dependent on its ATP-binding activity (Drumm et al., 2009). Understanding the molecular mechanisms by which Usp-proteins act has broader implications in human health, because they contribute to human pathogen's virulence and survival in the host (Seifart Gomes et al., 2011; Liu et al., 2007).

Although several reports described that UspA proteins contain a dimerization domain in its sequence, little is known about its functional implication. It has been described that EcUsp proteins have the capability to form homodimers and/or heterodimers in vivo leading to a higher adaptation to stress (Nachin et al., 2008; Heermann et al., 2009). In this work, we have found that SlRd2 forms homodimers in vivo at the cytoplasm and nucleus. However, we cannot discard that SlRd2 might form heterodimers with other UspA proteins in the plant cell. Interestingly we found that the dimerization of SlRd2 is required for both its biological stress-protection role in yeast and its interaction with SlCipk6. Similarly, Weber and Jung (2006) described that the dimerization of EcUspG was necessary for its cellular function. Unlike plants, the importance of UspA dimerization is well documented in *E. coli*. Thus, the Usp domain of KdpD (K^+ transport system) functions as a binding surface for EcUspC and it is essential for its signaling role (Heermann et al., 2009). Recently it has been found that Arabidopsis AtUSP is able to switch from low M_r species to high M_r complexes, suggesting a chaperone function in stress tolerance to heat shock and oxidative stress (Jung et al., 2015). Notably, dimerization of Hypoxia Responsive Universal Stress Protein 1 is also important for ROS regulation and apparently for subcellular localization (Gonzali et al., 2015). An important question is whether SlRd2 dimerization is also required for ATP binding, which could in turn regulate the interaction with SlCipk6. These aspects deserve further analyses in the future.

SlRd2 Protects Bacteria against ROS and Regulates SlCipk6-mediated ROS in Plants

Despite UspA proteins are widely represented in plants [with 48 members in Arabidopsis (Kerk et al., 2003; Isokpehi et al., 2011)], very little is known about their function, regulation, molecular mechanisms or their participation in physiological responses. Previously, two Arabidopsis UspA proteins (At5g54430, At4g27320), were identified as differential phosphorylation substrates in response to pathogen derived elicitors (Lenman et al., 2008; Merkouropoulos et al., 2008). In vitro kinase assays identified AtPHOS32 (At5g54430) as a mitogen-activated protein kinases AtMPK3 and AtMPK6 substrate (Merkouropoulos et al., 2008). This observation along with SlRd2 being phosphorylated by SlCipk6 (a Ca^{2+}-regulated kinase) indicates that plant UspA members might be regulated distinctly in response to a plethora of stimuli thus receiving and integrating signals from different pathways.

An interesting observation is that overexpression of SlRd2 in *S. cerevisiae* results in an increased tolerance to LiCl. In yeast, increased LiCl tolerance is promoted by a rise of activity of the K^+ transporter Trk1/2, which is in turn controlled by phosphorylation and dephosphorylation modifications (Zaidi et al., 2012; Yenush et al., 2005). In plants CIPK/CBL complexes regulate the cellular K^+ flux by interaction with the transporter AKT1 (Li et al., 2014). According to the yeast data, it is tempting to speculate that SlCipk6 and SlRd2 might act together in plants to regulate the activity of the plant K^+ transporter AKT1. In fact, in Arabidopsis, AtCIPK6 interacts with different CBLs to regulate AKT1 and AKT2 (Lee et al., 2007; Lan et al., 2011; Held et al., 2011). However, further studies should be carried out to figure out this functional implication. Significantly, we have found that SlRd2 complements an *E. coli Uspa* mutant line restoring bacterial viability to wild-type levels to otherwise lethal doses of H_2O_2 for the mutant. Because EcUspA does not contain ATP-binding regions whereas SlRd2 can bind ATP, both proteins might play a common and conserved molecular role that does not imply ATP binding, at least in bacteria (Weber and Jung, 2006). The functional complementation of *E. coli Uspa* mutant by the expression of tomato Rd2 protein indicates that the molecular function of SlRd2, a plant UspA protein in protecting cells against the toxic effects of oxidative stress in bacteria have been conserved in evolution.

A rapid ROS burst have been implicated in different physiological responses. Recently, we reported that SlCipk6 contributed to ROS generation during biotic stress response in *N. benthamiana*, which largely depends on RbohB (de la Torre et al., 2013). Also, SlCipk6 overexpression resulted in ROS generation, which was dependent on SlCipk6 kinase activity but did not occur in *RbohB* silenced *N. benthamiana* plants (de la Torre et al., 2013). The fact that overexpression of SlRd2 results in reduced SlCipk6-mediated ROS in planta clearly indicates a functional relationship between both proteins in which SlRd2 negatively regulates SlCipk6-mediated ROS output. Similarly, overexpression of *Salicornia brachiata* Usp in tobacco plants resulted in reduced accumulation of ROS during stresses (Udawat et al., 2016). Because RBOHs require posttranslational modifications for their activation (Kobayashi et al.,

2012), we propose that SlCipk6 phosphorylates the N-terminal regulatory domain of RbohB and SlRd2 directly or indirectly modulates this event, thus affecting ROS output. Because SlRd2 cancelled SlCipk6-dependent ROS, it might work in a negative feedback loop tempering the SlCipk6-RbohB signaling. If this was true, it will be important to determine whether SlRd2 inhibited SlCipk6 in a phosphorylation assay using MBP or RbohB as substrates. On the other hand, similar to UspA family members, the small GTP-binding proteins (Rac/Rop) have been postulated to act as molecular switches regulating a wide variety of important physiological functions in cells (Nibau et al., 2006; Xu et al., 2010). In this context, OsRac1 was required to activate OsRbohB in *N. benthamiana* cells (Wong et al., 2007). Interestingly, HRU1 has been found to interact with the GTPase ROP2 and RbohD, participating in the modulation of ROS levels under anoxia (Gonzali et al., 2015). Similarly, SlRd2 might act as a regulatory element of RbohB by affecting SlCipk6 activity. In the future, the molecular mechanism underlying SlRd2 regulation of SlCipk6-mediated ROS will be studied in higher detail.

MATERIALS AND METHODS

Bacteria, Yeast, and Plant Materials

Agrobacterium (*Agrobacterium tumefaciens*) strain C58C1 was grown at 30°C in Luria-Bertani (LB) medium with appropriate antibiotics. Yeast strains EGY48 (*Matα trp1 his3 ura3 leu2::6LexAop-LEU2*), BY4741 (*Matα met15Δ0 his3Δ1 ura3Δ1 leu2Δ0*) and GRF-167 (*MATα his3Δ200 ura3167*) were grown at 30°C in synthetic dropout medium with Glc as a carbon source. *Escherichia coli* strain W3101 [F-, galT22, λ-, IN (rrnD-rrnE)1, rph-1] and TN3151 [F-, λ-, IN (rrnD-rrnE)1, uspA1::kan, rph-1] were grown at 37°C in liquid morpholine propane sulphonic acid (MOPS) supplemented with Glc 0.4% (w/v). *Nicotiana benthamiana*) was grown in the greenhouse with 16 h of light and at 24°C (d) and 22°C (night). Tomatoes (*Solanum lycopersicum*) line Rio Grande-PtoR (*Pto/Pto, Prf/Prf*) was grown in hydroponic culture (Hoagland medium) in a growth chamber at the same growth conditions as *N. benthamiana*. The ORF of *SlRd2* was cloned into the binary vector pGWB6 under the control of a cauliflower mosaic virus 35S promoter. Transgenic *N. benthamiana* plants were obtained according to the procedures of Rajput et al. (2014) and Park et al. (2013), and the homozygous transgenic lines 6 and 7 from T3 progeny were used (OE-6 and OE-7). Salt and osmotic shock treatments were performed adding NaCl or mannitol to final 100 mM or 300 mM concentration respectively to the media on 4-week-old tomato plants. For ROS detection and measurement, *N. benthamiana* leaf disks (0.28 cm^2) were floated on 100 μL of distilled water in a 96-well white-bottom plate overnight at room temperature. Water was later replaced with 50 μL of distilled water and then incubated for 8 to 12 h at room temperature. For ROS detection, 50 μL of a 2× solution containing 100 μM luminol (Sigma-Aldrich) and 1 μg of horseradish peroxidase was quickly added to each well and ROS were measured in vivo as luminescence using a Varioskan Flash Multimode Reader.

SlRd2 Open Reading Frame cDNA Cloning and Deletion Mutant Generation

SlRd2 open reading frame was amplified from a tomato cDNA library prepared from leaf tissue by RT-PCR using primers OPS291 (5′-ATGGAAACGGTTATGGA-3′) / OPS292 (5′-TTAAATCACAGAGACTT-3′) and cloned into Gateway entry vector pDONR207 (Invitrogen). PCR-based site mutagenesis was performed to generate deletion of dimerization domain in *SlRd2* using the QuikChange kit (Stratagene) using OPS405 (5′-CACAACTGTAAGATAGCACCGCCTGGAAAAGAAGCTGGGG-3′) and OPS406 (5′-CCCCAGCTTCTTTTCCAGGCGGTGCTATCTTACAGTTGTG-3′) primers.

Y2H Assay

Yeast strain EGY48 (containing pS18-34 vector) was used for Y2H assays. *SlCipk6* cDNA and its mutant derivatives (de la Torre et al., 2013) were cloned into the bait vector pEG202, and *SlRd2* cDNA was cloned into the prey vector pJG4-5. To generate ΔNterm mutant derivate in *SlCipk6*, a PCR reaction was performed using OPS501 (5′-ATGTTGAATGCTTTTCATATCATTTC-3′) and OPS165 (5′-CGGAATTCATGGGGACAGAAGAAAAATGTGC-3′) primers. Yeast transformation was performed by LiAc/PEG method as described in Yeast Protocols Handbook (Clontech). Growth and blue colonies on SD (X-Gal/Gal/-Ura,-His,-Trp,-Leu) plates indicated positive interaction. Finally, β-galactosidase assay was performed as described in Yeast Protocols Handbook. Expression of bait and prey fusion proteins was verified by immunoblotting using anti-LexA mouse monoclonal antibody (Santa Cruz Biotechnology) or anti-HA rat monoclonal antibody (Roche).

Quantitative Real-Time PCR

Total RNA was isolated from tomato leaves using TRIZOL reagent (Invitrogen) and subjected to DNAse treatment using TURBO DNA-*free* (Ambion). A quantity of 2 μg of total RNA was used to synthesize cDNA using random primers and Superscript II reverse transcriptase (Invitrogen) following the manufacturer's protocol. Quantitative real-time (Q RT) PCR was performed with *SsoFast EvaGreen* Supermix (BIORAD) and a BIORAD real-time PCR system. The thermal cycle used was: 95°C for 10 min; 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Quantitative real-time PCR reactions were carried out with the following oligonucleotides: OPS389 (5′-AGCAAACACGCTTTTGATTGGGC-3′)/OPS390 (5′-CACTGTCTTCACCATAGCAACC-3′) for *SlRd2*, OPS305 (5′-ATCCATGCACTTAATATCTTCC-3′) / OPS306 (5′-GCAATGATGGGTATCTGATAGCG-3′) for *SlCipk6* and OPS281 (5′-AGCCACACAGTTCCCATCTAC-3′) / OPS282 (5′-AACTTCTCCTTCACTCCCTA-3′) for *SlActin2* as an internal standard. Relative expression levels were determined as described previously in de la Torre et al. (2013).

Co-IP

For the co-IP experiment, *SlRd2* and *SlCipk6*(*T172D*) coding sequences were cloned into pMDC43 (with a N-terminal GFP epitope) and pTAPa-pYL436 (with C-terminal 2xIgG-BD and 9xMyc tags) vectors, respectively, by gateway technology (Invitrogen; Rubio et al., 2005). *Agrobacterium* strains C58C1 carrying *GFP-SlRd2* and *SlCipk6*(*T172D*)-*Myc*, respectively, were coinfiltrated into *N. benthamiana* leaves. Coinfiltration *Agrobacterium* C58C1 cultures carrying *GFP* and *SlCipk6*(*T172D*) were used as a negative control. Two d after, leaves were collected, frozen and grounded in liquid nitrogen and resuspended in three volumes of extraction buffer [50 mM Tris-HCl pH8, 0.1% NP-40, 1× Complete protease inhibitors (Roche)] and centrifuged at 14,000 *g* for 20 min at 4°C. Supernatant was filtered through two layers of Miracloth (Calbiochem). 2 mL was incubated with 50 μL IgG beads (Amersham Biosciences) for 2 h at 4°C with gentle rotation. Beads were washed five times with 2 mL of washing buffer (50 mM Tris-HCl pH 8, 0.1% NP-40). Elution from the IgG beads was performed by boiling the samples with 1× Laemmli buffer.

ATP-Binding Assay

For in vitro ATP binding assay, 1 μg of SlRd2 and GFP proteins purified from *E. coli* were incubated in binding buffer (20 mM Tris-HCl pH 7.5, 15 mM $MgCl_2$, 1 mM DTT), 50 μM ATP and 10 μCi of [α-^{32}P]ATP [Amersham; 3000 Ci/mmol (1 Ci = 37 GBq)] during 60 min at 30°C. Then, the reactions were stopped by adding 10 μL of 4× Laemmli loading buffer. The samples were denatured by boiling 5 min and later were run on 12% SDS-PAGE gel. ATP binding was visualized by autoradiography. Protein levels were verified by immunoblot using anti-His mouse monoclonal antibody (Roche). The in vivo ATP-binding assay was performed as described in Villamor et al. (2013). *N. benthamiana* leaf samples (1 g fresh weight) expressing GFP-SlRd2 were ground in liquid nitrogen and thawed in 2 volumes of extraction buffer (50 mM Tris pH 7.5). The lysate was later cleared via centrifugation and subjected to gel filtration using DG10 columns (Bio-Rad). Labeling was performed adding 10 mm $MgCl_2$ and 20 μM of BHAcATP (Thermo Scientific) to each sample and then incubated at room temperature for 1 h. For inhibition experiments, the lysate was incubated with 10 mM ATP for 30 min before labeling with BHAcATP. Biotinylated proteins were affinity purified by incubating the samples with Streptavidin beads (Thermo Scientific) for 1 h at room temperature. The beads were washed three times with 6 M urea. Finally, the purified proteins were boiled in 4× Laemmli

buffer and analyzed by SDS-PAGE proteins gel and immunoblotted using anti-GFP mouse monoclonal antibody.

Cross-Linking Assay

The cross-linking assay was performed as described in Nachin et al. (2008). Fresh LB containing 50 μg/mL ampicillin was inoculated to a final OD_{600} of 0.3 with an overnight culture of *E. coli* harboring pDEST17-*SlRd2* construct and grown at 37°C. After 1 h, protein expression was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside. After 4 h at 37°C, cells were harvested by centrifugation and resuspended in phosphate-buffered saline 1× to a final OD_{600} of 0.7. Subsequently, 0.5 mM of cross-linking agent DSG was added to 200 μL of *E. coli* culture during 30 min at room temperature. The reaction was stopped by adding 40 μL of TS (200 mM Tris-HCl pH 8.8, 5 mM EDTA, 1 M Suc, and 0.05% (w/v) bromophenol blue)/TD (18% SDS and 0.3 M DTT; ratio 2:1). Finally, the samples were denatured by boiling 5 min and then were run on 12% SDS-PAGE gel. Protein M_r was determined by immunoblot using anti-His mouse monoclonal antibody (Roche).

In Vitro Phosphorylation Assays

For protein kinase assays, SlCipk6 full length was amplified by PCR using the primers OPS606 (5′-TCTAGACATGGGGACAGAAGAAAAATGT-3′) and OPS607 (5′-GTCGACCTCAAGCAATTGTTGGATTCTC-3′) and cloned as *Sal*I/*Xba*I fragment in the yeast expression vector pEG(KT) (Mitchell et al., 1993), and then purified from yeast (strain GRF-167) using Glutathione Sepharose 4B affinity resin (GE Healthcare). *SlRd2* cDNA was cloned into pET28a and purified from *E. coli* using a His column kit (GE Healthcare). SlRd2(UspA) was amplified by PCR using primers OPS675 (5′-AAAAAGCAGGCTCTATGGGCCGTGATATAGTGATC-3′) and OPS676 (5′-AGAAAGCTGGGTTTAAGGAACTATGATGACCGG-3′), cloned into pDEST15 vector (Invitrogen), and purified using Glutathione Sepharose 4B affinity beads. For kinase assays, 0.3 μg of SlCipk6 proteins and 2.5 μg of SlRd2, 2.5 μg of SlRd2(UspA) or 0.5 μg of MBP proteins were incubated in a final volume of 30 μL in kinase buffer (50 mM Tris-HCl pH 7.5, 2 mM $MnCl_2$, 2 mM DTT), 10 μM ATP and 10 μCi of [γ-^{32}P]ATP [Amersham; 3000 Ci/mmol (1 Ci = 37 GBq)] during 60 min at 30°C. The reactions were stopped by adding 10 μL of 4× Laemmli loading buffer. The samples were denatured by boiling 5 min and then were run on 12% SDS-PAGE gel. Kinase activity was visualized by autoradiography. Protein levels were verified by colloidal Coomassie Brilliant Blue G-250 staining.

BiFC Assay

For BiFC assays, *SlRd2* and *SlCipk6* coding sequences were cloned into $pYFP^C$ (C-terminal YFP fragment) and $pYFP^N$ (N-terminal YFP fragment) respectively by gateway technology (Invitrogen). *Agrobacterium* strains C58C1 carrying *SlRd2-YFP*^{C} and *SlCipk6-YFP*^{N} were coinfiltrated into *N. benthamiana* leaves. *Agrobacterium* cultures carrying YFP^C, YFP^N empty vectors and the mix *SlRd2-YFP*^{C}/*SlCbl10-YFP*^{N} were used as negative controls. Staining of *N. benthamiana* cells with FM4-64 was performed as described in Bolte et al. (2004). Fluorescence images were obtained 48 h after infiltration using a Leica TCS Sp2/DMRE confocal microscope with excitation wavelengths of 514 nm (YFP), 543 nm (FM4-64), and 440 nm (CFP). Transient expression of proteins in *N. benthamiana* leaves via agroinfiltration was performed as described in He et al. (2004).

Yeast Stress Tolerance Assays

For stress tolerance assays, yeast strain BY4741 harboring p426GPD EV and p426GPD-SlRd2 constructs were grown in liquid SD medium lacking Uracil (SD-Ura) containing 1% Glc (w/v) during 24 h at 30°C. Subsequently, they were diluted to the same concentrations (OD_{600} = 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) and 10 μL of each dilution was spotted onto solid YPD medium supplemented with the different stress agents. Finally, yeast were grown at 30°C during 3 d and photographed.

Preparation and Purification of SlRd2 Antibody

SlRd2 open reading frame was cloned into pET-28a in frame with an N-terminal His-tag). The pET-28a-*SlRd2* construct was transformed in BL21 (*DE3*) RIL (Stratagene) *E. coli* cells. Preparation of recombinant protein was performed as described in San-Miguel et al. (2013). Briefly, protein expression was induced at OD_{600} = 0.5 by adding of 1 mM isopropyl β-D-1-thiogalactopyranoside to LB medium supplemented with 50 μg mL ampicillin. Cells were harvested by centrifugation at 3000 *g* for 10 min at room temperature and frozen overnight at −80°C. Preparation of His-tagged recombinant protein from pET-28a-*SlRd2* was performed according to the manufacturer's instructions (Qiagen). The antiserum was raised in rabbits using the full length SlRd2 as the antigen.

Oxidative Stress Complementation Assay in *E. coli*

The complementation assay was performed as described by Nyström and Neidhardt (1994). Fresh MOPS medium containing 50 μg/mL ampicillin was inoculated with overnight cultures of W3101 [F-, galT22, λ-, IN (rrnD-rrnE)1, rph-1] and TN3151 [F-, λ-, IN (rrnD-rrnE)1, uspA1::kan, rph-1] strains harboring pDEST17-*SlRd2* and pDEST17-*UspA* constructs at an OD_{600} of 0.1 and grown at 37°C until they reached an OD_{600} = 0.7. A final concentration of 5 mM H_2O_2 was then added. 1 mL of each culture was harvested to perform the growth curve. Viability is expressed as the number of colony forming units at time divided by the number of colony forming units before the imposition of stress. Expression of SlRd2 and UspA proteins was determined by immunoblotting using anti-His mouse monoclonal antibody (Roche). mRNA *UspA* and *16S rRNA* abundance were verified by RT-PCR using gene specific primers: OPS446 (5′-ATGGCTTATAAACACATTCTC-3′) and OPS448 (5′-TTATTCTTCTTCGTCGCGCAGC-3′) for UspA and OPS600 (5′-CTCCTACGGGAGGCAGCAG-3′) and OPS601 (5′-ATTACCGCGGCKGCTG-3′) for *16S rRNA*.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: SlRd2 (KP843662), SlCipk6 (JF831200), SlCipk11 (JF831201), and SlCipk14 (JF831202).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. SlRd2, SlCipk6, and SlCipk6 mutant derivative proteins are expressed in yeast.

Supplemental Figure S2. Specificity of SlCipk6/SlRd2 interaction.

Supplemental Figure S3. Both SlCipk6 and SlRd2 are localized in the cytosol and in the nucleus.

Supplemental Figure S4. SlRd2 protein sequence alignment.

Supplemental Figure S5. SlRd2 Usp domain protein sequence alignment.

Supplemental Figure S6. SlCipk6-Myc (wild type), SlCipk6(T172D)-Myc (T172D), and SlCipk6(K43M)-Myc (K43M) are expressed in *N. benthamiana* GFP-SlRd2 overexpressing (OE-6 and OE-7) and wild-type plants.

Supplemental Table S1. Summary of the Y2H screen.

ACKNOWLEDGMENTS

We thank Jose M. Pardo (Instituto de Bioquímica Vegetal y Fotosíntesis, Consejo Superior de Investigaciones Científicas, Sevilla, Spain) for critically reading the manuscript.

Received June 15, 2016; accepted November 26, 2016; published November 29, 2016.

LITERATURE CITED

Aouida M, Tounekti O, Leduc A, Belhadj O, Mir L, Ramotar D (2004) Isolation and characterization of *Saccharomyces cerevisiae* mutants with enhanced resistance to the anticancer drug bleomycin. Curr Genet **45:** 265–272

Aravind L, Anantharaman V, Koonin EV (2002) Monophyly of class I aminoacyl tRNA synthetase, USPA, ETFP, photolyase, and PP-ATPase nucleotide-binding domains: implications for protein evolution in the RNA. Proteins **48:** 1–14

Babiychuk E, Kushnir S, Belles-Boix E, Van Montagu M, Inzé D (1995) *Arabidopsis thaliana* NADPH oxidoreductase homologs confer tolerance of yeasts toward the thiol-oxidizing drug diamide. J Biol Chem **270:** 26224–26231

Batistic O, Waadt R, Steinhorst L, Held K, Kudla J (2010) CBL-mediated targeting of CIPKs facilitates the decoding of calcium signals emanating from distinct cellular stores. Plant J **61:** 211–222

Becker JD, Moreira LM, Kapp D, Frosch SC, Pühler A, Perlic AM (2001) The nodulin vfENOD18 is an ATP-binding protein in infected cells of *Vicia faba* L. nodules. Plant Mol Biol **47:** 749–759

Bernard A, Domergue F, Pascal S, Jetter R, Renne C, Faure JD, Haslam RP, Napier JA, Lessire R, Joubès J (2012) Reconstitution of plant alkane biosynthesis in yeast demonstrates that *Arabidopsis* ECERIFERUM1 and ECERIFERUM3 are core components of a very-long-chain alkane synthesis complex. Plant Cell **24:** 3106–3118

Bolte S, Talbot C, Boutte Y, Catrice O, Read ND, Satiat-Jeunemaitre B (2004) FM-dyes as experimental probes for dissecting vesicle trafficking in living plant cells. J Microsc **214:** 159–173

Chaves-Sanjuan A, Sanchez-Barrena MJ, Gonzalez-Rubio JM, Moreno M, Ragel P, Jimenez M, Pardo JM, Martinez-Ripoll M, Quintero FJ, Albert A (2014) Structural basis of the regulatory mechanism of the plant CIPK family of protein kinases controlling ion homeostasis and abiotic stress. Proc Natl Acad Sci USA **111:** E4532–E4541

Chen L, Ren F, Zhou L, Wang QQ, Zhong H, Li XB (2012) The *Brassica napus* calcineurin B-Like 1/CBL-interacting protein kinase 6 (CBL1/CIPK6) component is involved in the plant response to abiotic stress and ABA signalling. J Exp Bot **63:** 6211–6222

Chen L, Wang QQ, Zhou L, Ren F, Li DD, Li XB (2013) *Arabidopsis* CBL-interacting protein kinase (CIPK6) is involved in plant response to salt/osmotic stress and ABA. Mol Biol Rep **40:** 4759–4767

de la Torre F, Gutiérrez-Beltrán E, Pareja-Jaime Y, Chakravarthy S, Martin GB, del Pozo O (2013) The tomato calcium sensor Cbl10 and its interacting protein kinase Cipk6 define a signaling pathway in plant immunity. Plant Cell **25:** 2748–2764

Dodd AN, Kudla J, Sanders D (2010) The language of calcium signaling. Annu Rev Plant Biol **61:** 593–620

Drerup MM, Schlücking K, Hashimoto K, Manishankar P, Steinhorst L, Kuchitsu K, Kudla J (2013) The Calcineurin B-like calcium sensors CBL1 and CBL9 together with their interacting protein kinase CIPK26 regulate the *Arabidopsis* NADPH oxidase RBOHF. Mol Plant **6:** 559–569

Drumm JE, Mi K, Bilder P, Sun M, Lim J, Bielefeldt-Ohmann H, Basaraba R, So M, Zhu G, Tufariello JM, Izzo AA, Orme IM, et al (2009) *Mycobacterium tuberculosis* universal stress protein Rv2623 regulates bacillary growth by ATP-binding: requirement for establishing chronic persistent infection. PLoS Pathog **5:** e1000460

Du W, Lin H, Chen S, Wu Y, Zhang J, Fuglsang AT, Palmgren MG, Wu W, Guo Y (2011) Phosphorylation of SOS3-like calcium-binding proteins by their interacting SOS2-like protein kinases is a common regulatory mechanism in *Arabidopsis*. Plant Physiol **156:** 2235–2243

Faure JD, Gingerich D, Howell SH (1998) An *Arabidopsis* immunophilin, AtFKBP12, binds to AtFIP37 (FKBP interacting protein) in an interaction that is disrupted by FK506. Plant J **15:** 783–789

Freestone P, Nyström T, Trinei M, Norris V (1997) The universal stress protein, UspA, of *Escherichia coli* is phosphorylated in response to stasis. J Mol Biol **274:** 318–324

Gong D, Guo Y, Schumaker KS, Zhu JK (2004) The SOS3 family of calcium sensors and SOS2 family of protein kinases in *Arabidopsis*. Plant Physiol **134:** 919–926

Gonzali S, Loreti E, Cardarelli F, Novi G, Parlanti S, Pucciariello C, Bassolino L, Banti V, Licausi F, Perata P (2015) Universal stress protein HRU1 mediates ROS homeostasis under anoxia. Nat Plants **1:** 15151

Guo Y, Halfter U, Ishitani M, Zhu JK (2001) Molecular characterization of functional domains in the protein kinase SOS2 that is required for plant salt tolerance. Plant Cell **13:** 1383–1400

Guo Y, Xiong L, Song CP, Gong D, Halfter U, Zhu JK (2002) A calcium sensor and its interacting protein kinase are global regulators of abscisic acid signaling in *Arabidopsis*. Dev Cell **3:** 233–244

Hashimoto K, Eckert C, Anschütz U, Scholz M, Held K, Waadt R, Reyer A, Hippler M, Becker D, Kudla J (2012) Phosphorylation of calcineurin B-like (CBL) calcium sensor proteins by their CBL-interacting protein kinases (CIPKs) is required for full activity of CBL-CIPK complexes toward their target proteins. J Biol Chem **287:** 7956–7968

Hashimoto K, Kudla J (2011) Calcium decoding mechanisms in plants. Biochimie **93:** 2054–2059

He X, Anderson JC, del Pozo O, Gu YQ, Tang X, Martin GB (2004) Silencing of subfamily I of protein phosphatase 2A catalytic subunits results in activation of plant defense responses and localized cell death. Plant J **38:** 563–577

Heermann R, Weber A, Mayer B, Ott M, Hauser E, Gabriel G, Pirch T, Jung K (2009) The universal stress protein UspC scaffolds the KdpD/KdpE signaling cascade of *Escherichia coli* under salt stress. J Mol Biol **386:** 134–148

Held K, Pascaud F, Eckert C, Gajdanowicz P, Hashimoto K, Corratgé-Faillie C, Offenborn JN, Lacombe B, Dreyer I, Thibaud JB, Kudla J (2011) Calcium-dependent modulation and plasma membrane targeting of the AKT2 potassium channel by the CBL4/CIPK6 calcium sensor/protein kinase complex. Cell Res **21:** 1116–1130

Ho CH, Lin SH, Hu HC, Tsay YF (2009) CHL1 functions as a nitrate sensor in plants. Cell **138:** 1184–1194

Hohnjec N, Küster H, Albus U, Frosch SC, Becker JD, Pühler A, Perlick AM, Frühling M (2000) The broad bean nodulin VfENOD18 is a member of a novel family of plant proteins with homologies to the bacterial MJ0577 superfamily. Mol Gen Genet **264:** 241–250

Isokpehi RD, Simmons SS, Cohly HH, Ekunwe SI, Begonia GB, Ayensu WK (2011) Identification of drought-responsive universal stress proteins in *viridiplantae*. Bioinform Biol Insights **5:** 41–58

Jung YJ, Melencion SM, Lee ES, Park JH, Alinapon CV, Oh HT, Yun DJ, Chi YH, Lee SY (2015) Universal stress protein exhibits a redox-dependent chaperone function in *Arabidopsis* and enhances plant tolerance to heat shock and oxidative stress. Front Plant Sci **6:** 1141

Katiyar-Agarwal S, Zhu J, Kim K, Agarwal M, Fu X, Huang A, Zhu JK (2006) The plasma membrane Na^+/H^+ antiporter SOS1 interacts with RCD1 and functions in oxidative stress tolerance in *Arabidopsis*. Proc Natl Acad Sci USA **103:** 18816–18821

Kerk D, Bulgrien J, Smith DW, Gribskov M (2003) *Arabidopsis* proteins containing similarity to the universal stress protein domain of bacteria. Plant Physiol **131:** 1209–1219

Kim BG, Waadt R, Cheong YH, Pandey GK, Dominguez-Solis JR, Schültke S, Lee SC, Kudla J, Luan S (2007) The calcium sensor CBL10 mediates salt tolerance by regulating ion homeostasis in *Arabidopsis*. Plant J **52:** 473–484

Kim KN, Cheong YH, Gupta R, Luan S (2000) Interaction specificity of *Arabidopsis* calcineurin B-like calcium sensors and their target kinases. Plant Physiol **124:** 1844–1853

Kim WY, Ali Z, Park HJ, Park SJ, Cha JY, Perez-Hormaeche J, Quintero FJ, Shin G, Kim MR, Qiang Z, Ning L, Park HC, et al (2013) Release of SOS2 kinase from sequestration with GIGANTEA determines salt tolerance in *Arabidopsis*. Nat Commun **4:** 1352

Kobayashi M, Yoshioka M, Asai S, Nomura H, Kuchimura K, Mori H, Doke N, Yoshioka H (2012) StCDPK5 confers resistance to late blight pathogen but increases susceptibility to early blight pathogen in potato via reactive oxygen species burst. New Phytol **196:** 223–237

Kvint K, Nachin L, Diez A, Nyström T (2003) The bacterial universal stress protein: function and regulation. Curr Opin Microbiol **6:** 140–145

Lan WZ, Lee SC, Che YF, Jiang YQ, Luan S (2011) Mechanistic analysis of AKT1 regulation by the CBL-CIPK-PP2CA interactions. Mol Plant **4:** 527–536

Lee SC, Lan WZ, Kim BG, Li L, Cheong YH, Pandey GK, Lu G, Buchanan BB, Luan S (2007) A protein phosphorylation/dephosphorylation network regulates a plant potassium channel. Proc Natl Acad Sci USA **104:** 15959–15964

Lenman M, Sörensson C, Andreasson E (2008) Enrichment of phosphoproteins and phosphopeptide derivatization identify universal stress proteins in elicitor-treated *Arabidopsis*. Mol Plant Microbe Interact **21:** 1275–1284

Li J, Long Y, Qi GN, Li J, Xu ZJ, Wu WH, Wang Y (2014) The Os-AKT1 channel is critical for K^+ uptake in rice roots and is modulated by the rice CBL1-CIPK23 complex. Plant Cell **26:** 3387–3402

Liang Q, Zhou B (2007) Copper and manganese induce yeast apoptosis via different pathways. Mol Biol Cell **18:** 4741–4749

Liu WT, Karavolos MH, Bulmer DM, Allaoui A, Hormaeche RD, Lee JJ, Khan CM (2007) Role of the universal stress protein UspA of *Salmonella* in growth arrest, stress and virulence. Microb Pathog **42:** 2–10

Loukehaich R, Wang T, Ouyang B, Ziaf K, Li H, Zhang J, Lu Y, Ye Z (2012) SpUSP, an annexin-interacting universal stress protein, enhances drought tolerance in tomato. J Exp Bot **63:** 5593–5606

Luan S (2009) The CBL-CIPK network in plant calcium signaling. Trends Plant Sci **14:** 37–42

Lyzenga WJ, Liu H, Schofield A, Muise-Hennessey A, Stone SL (2013) *Arabidopsis* CIPK26 interacts with KEG, components of the ABA signalling network and is degraded by the ubiquitin-proteasome system. J Exp Bot **64:** 2779–2791

Matsumoto TK, Pardo JM, Takeda S, Bressan RA, Hasegawa PM (2001) Tobacco and *Arabidopsis* SLT1 mediate salt tolerance of yeast. Plant Mol Biol **45:** 489–500

Merkouropoulos G, Andreasson E, Hess D, Boller T, Peck SC (2008) An *Arabidopsis* protein phosphorylated in response to microbial elicitation, AtPHOS32, is a substrate of MAP kinases 3 and 6. J Biol Chem **283:** 10493–10499

Mitchell DA, Marshall TK, Deschenes RJ (1993) Vectors for the inducible overexpression of glutathione S-transferase fusion proteins in yeast. Yeast **9:** 715–722

Nachin L, Brive L, Persson KC, Svensson P, Nyström T (2008) Heterodimer formation within universal stress protein classes revealed by an in silico and experimental approach. J Mol Biol **380:** 340–350

Nagae M, Nozawa A, Koizumi N, Sano H, Hashimoto H, Sato M, Shimizu T (2003) The crystal structure of the novel calcium-binding protein AtCBL2 from *Arabidopsis thaliana*. J Biol Chem **278:** 42240–42246

Nibau C, Wu HM, Cheung AY (2006) RAC/ROP GTPases: 'hubs' for signal integration and diversification in plants. Trends Plant Sci **11:** 309–315

Nyström T, Neidhardt FC (1992) Cloning, mapping and nucleotide sequencing of a gene encoding a universal stress protein in *Escherichia coli*. Mol Microbiol **6:** 3187–3198

Nyström T, Neidhardt FC (1994) Expression and role of the universal stress protein, UspA, of *Escherichia coli* during growth arrest. Mol Microbiol **11:** 537–544

O'Toole R, Smeulders MJ, Blokpoel MC, Kay EJ, Lougheed K, Williams HD (2003) A two-component regulator of universal stress protein expression and adaptation to oxygen starvation in *Mycobacterium smegmatis*. J Bacteriol **185:** 1543–1554

Ohta M, Guo Y, Halfter U, Zhu JK (2003) A novel domain in the protein kinase SOS2 mediates interaction with the protein phosphatase 2C ABI2. Proc Natl Acad Sci USA **100:** 11771–11776

Park HJ, Kim WY, Yun DJ (2013) A role for GIGANTEA: keeping the balance between flowering and salinity stress tolerance. Plant Signal Behav **8:** e24820

Persson O, Valadi A, Nyström T, Farewell A (2007) Metabolic control of the *Escherichia coli* universal stress protein response through fructose-6-phosphate. Mol Microbiol **65:** 968–978

Quintero FJ, Ohta M, Shi H, Zhu JK, Pardo JM (2002) Reconstitution in yeast of the *Arabidopsis* SOS signaling pathway for Na^+ homeostasis. Proc Natl Acad Sci USA **99:** 9061–9066

Ragel P, Ródenas R, García-Martín E, Andrés Z, Villalta I, Nieves-Cordones M, Rivero RM, Martínez V, Pardo JM, Quintero FJ, Rubio F (2015) The CBL-interacting protein kinase CIPK23 regulates HAK5-mediated high-affinity K^+ uptake in *Arabidopsis* roots. Plant Physiol **169:** 2863–2873

Rajput NA, Zhang M, Ru Y, Liu T, Xu J, Liu L, Mafurah JJ, Dou D (2014) *Phytophthora sojae* effector PsCRN70 suppresses plant defenses in *Nicotiana benthamiana*. PLoS One **9:** e98114

Rubio V, Shen Y, Saijo Y, Liu Y, Gusmaroli G, Dinesh-Kumar SP, Deng XW (2005) An alternative tandem affinity purification strategy applied to *Arabidopsis* protein complex isolation. Plant J **41:** 767–778

San-Miguel T, Pérez-Bermúdez P, Gavidia I (2013) Production of soluble eukaryotic recombinant proteins in *E. coli* is favoured in early log-phase cultures induced at low temperature. Springerplus **2:** 89

Sanyal SK, Pandey A, Pandey GK (2015) The CBL-CIPK signaling module in plants: a mechanistic perspective. Physiol Plant **155:** 89–108.

Sauter M, Rzewuski G, Marwedel T, Lorbiecke R (2002) The novel ethylene-regulated gene OsUsp1 from rice encodes a member of a plant protein family related to prokaryotic universal stress proteins. J Exp Bot **53:** 2325–2331

Seifart Gomes C, Izar B, Pazan F, Mohamed W, Mraheil MA, Mukherjee K, Billion A, Aharonowitz Y, Chakraborty T, Hain T (2011) Universal stress proteins are important for oxidative and acid stress resistance and growth of *Listeria monocytogenes* EGD-e in vitro and in vivo. PLoS One **6:** e24965

Shitan N, Dalmas F, Dan K, Kato N, Ueda K, Sato F, Forestier C, Yazaki K (2013) Characterization of *Coptis japonica* CjABCB2, an ATP-binding cassette protein involved in alkaloid transport. Phytochemistry **91:** 109–116

Sousa MC, McKay DB (2001) Structure of the universal stress protein of *Haemophilus influenzae*. Structure **9:** 1135–1141

Tripathi V, Parasuraman B, Laxmi A, Chattopadhyay D (2009) CIPK6, a CBL-interacting protein kinase is required for development and salt tolerance in plants. Plant J **58:** 778–790

Tsou PL, Lee SY, Allen NS, Winter-Sederoff H, Robertson D (2012) An ER-targeted calcium-binding peptide confers salt and drought tolerance mediated by CIPK6 in *Arabidopsis*. Planta **235:** 539–552

Udawat P, Jha RK, Sinha D, Mishra A, Jha B (2016) Overexpression of a cytosolic abiotic stress responsive universal stress protein (SbUSP) mitigates salt and osmotic stress in transgenic tobacco plants. Front Plant Sci **7:** 518

Uno Y, Rodriguez Milla MA, Maher E, Cushman JC (2009) Identification of proteins that interact with catalytically active calcium-dependent protein kinases from *Arabidopsis*. Mol Genet Genomics **281:** 375–390

Vanbogelen RA, Hutton ME, Neidhardt FC (1990) Gene-Protein database of *Escherichia coli* K-12: Edition 3. Electrophoresis **11:** 1131–1166.

Verslues PE, Batelli G, Grillo S, Agius F, Kim YS, Zhu J, Agarwal M, Katiyar-Agarwal S, Zhu JK (2007) Interaction of SOS2 with nucleoside diphosphate kinase 2 and catalases reveals a point of connection between salt stress and H2O2 signaling in *Arabidopsis thaliana*. Mol Cell Biol **27:** 7771–7780

Villamor JG, Kaschani F, Colby T, Oeljeklaus J, Zhao D, Kaiser M, Patricelli MP, van der Hoorn RA (2013) Profiling protein kinases and other ATP binding proteins in *Arabidopsis* using Acyl-ATP probes. Mol Cell Proteomics **12:** 2481–2496

Walter M, Chaban C, Schütze K, Batistic O, Weckermann K, Näke C, Blazevic D, Grefen C, Schumacher K, Oecking C, Harter K, Kudla J (2004) Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. Plant J **40:** 428–438

Wang L, Tsuda K, Sato M, Cohen JD, Katagiri F, Glazebrook J (2009) *Arabidopsis* CaM binding protein CBP60g contributes to MAMP-induced SA accumulation and is involved in disease resistance against *Pseudomonas syringae*. PLoS Pathog **5:** e1000301

Weber A, Jung K (2006) Biochemical properties of UspG, a universal stress protein of *Escherichia coli*. Biochemistry **45:** 1620–1628

Wong HL, Pinontoan R, Hayashi K, Tabata R, Yaeno T, Hasegawa K, Kojima C, Yoshioka H, Iba K, Kawasaki T, Shimamoto K (2007) Regulation of rice NADPH oxidase by binding of Rac GTPase to its N-terminal extension. Plant Cell **19:** 4022–4034

Xu J, Li HD, Chen LQ, Wang Y, Liu LL, He L, Wu WH (2006) A protein kinase, interacting with two calcineurin B-like proteins, regulates K^+ transporter AKT1 in *Arabidopsis*. Cell **125:** 1347–1360

Xu T, Wen M, Nagawa S, Fu Y, Chen JG, Wu MJ, Perrot-Rechenmann C, Friml J, Jones AM, Yang Z (2010) Cell surface- and rho GTPase-based auxin signaling controls cellular interdigitation in *Arabidopsis*. Cell **143:** 99–110

Yamaguchi-Shinozaki K, Koizumi M, Urao S, Shinozaki K (1992) Molecular cloning and characterization of 9 cDNAs for genes that are responsive to desiccation in *Arabidopsis thaliana*: sequence analysis of one cDNA clone that encode a putative transmembrane channel protein. Plant Cell Physiol **33:** 217–224

Yang Y, Qin Y, Xie C, Zhao F, Zhao J, Liu D, Chen S, Fuglsang AT, Palmgren MG, Schumaker KS, Deng XW, Guo Y (2010) The *Arabidopsis* chaperone J3 regulates the plasma membrane H^+-ATPase through interaction with the PKS5 kinase. Plant Cell **22:** 1313–1332

Ye T, García-Salcedo R, Ramos J, Hohmann S (2006) Gis4, a new component of the ion homeostasis system in the yeast *Saccharomyces cerevisiae*. Eukaryot Cell **5:** 1611–1621

Yenush L, Merchan S, Holmes J, Serrano R (2005) pH-Responsive, post-translational regulation of the Trk1 potassium transporter by the type 1-related Ppz1 phosphatase. Mol Cell Biol **25:** 8683–8692

Yu Q, An L, Li W (2014) The CBL-CIPK network mediates different signaling pathways in plants. Plant Cell Rep **33:** 203–214

Zahur M, Maqbool A, Ifran M, Barozai MY, Rashid B, Riazuddin S, Husnain T (2009) Isolation and functional analysis of cotton universal stress protein promoter in response to phytohormones and abiotic stresses. Mol Biol (Mosk) **43:** 628–635

Zaidi I, González A, Touzri M, Alvarez MC, Ramos J, Masmoudi K, Ariño J, Hanin M (2012) The wheat MAP kinase phosphatase 1 confers higher lithium tolerance in yeast. FEMS Yeast Res **12:** 774–784

Zarembinski TI, Hung LW, Mueller-Dieckmann HJ, Kim KK, Yokota H, Kim R, Kim SH (1998) Structure-based assignment of the biochemical function of a hypothetical protein: a test case of structural genomics. Proc Natl Acad Sci USA **95:** 15189–15193

Zegzouti H, Jones B, Frasse P, Marty C, Maitre B, Latch A, Pech JC, Bouzayen M (1999) Ethylene-regulated gene expression in tomato fruit: characterization of novel ethylene-responsive and ripening-related genes isolated by differential display. Plant J **18:** 589–600

Zhou J, Loh YT, Bressan RA, Martin GB (1995) The tomato gene Pti1 encodes a serine/threonine kinase that is phosphorylated by Pto and is involved in the hypersensitive response. Cell **83:** 925–935