LSU network hubs integrate abiotic and biotic stress responses via interaction with the superoxide dismutase FSD2

Antoni Garcia-Molina¹*, Melina Altmann¹, Angela Alkofer¹, Petra M. Epple²†, Jeffery L. Dangl³ and Pascal Falter-Braun⁴,⁵‡

1 Technische Universität München (TUM), School for Life Sciences Weihenstephan (WZW), Plant Systems Biology, Emil-Ramann-Straße, 4, D-85354 Freising, Germany
2 Howard Hughes Medical Institute and Department of Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA
3 BASF Plant Science LP, Research Triangle Park, NC 27709, USA
4 Institute of Network Biology (INET), Helmholtz Zentrum München (HMGU), German Research Center for Environmental Health, 85764 Neuherberg, Germany
5 Department of Microbe-Host Interactions, Ludwig-Maximilians-Universität München (LMU Munich), Planegg-Martinsried, Germany

* Present address: Ludwig-Maximilians-Universität München (LMU), Faculty of Biology, Plant Molecular Biology (Botany), Großhaderner Straße, 2–4, D-82152 Planegg-Martinsried, Germany
† Present address: BASF Plant Science LP, Research Triangle Park, NC 27709, USA
‡ Correspondence: pascal.falter-braun@helmholtz-muenchen.de

Received 16 October 2016; Editorial decision 13 December 2016; Accepted 13 December 2016

Editor: Christine Foyer, Leeds University

Abstract

In natural environments, plants often experience different stresses simultaneously, and adverse abiotic conditions can weaken the plant immune system. Interactome mapping revealed that the LOW SULPHUR UPREGULATED (LSU) proteins are hubs in an Arabidopsis protein interaction network that are targeted by virulence effectors from evolutionarily diverse pathogens. Here we show that LSU proteins are up-regulated in several abiotic and biotic stress conditions, such as nutrient depletion or salt stress, by both transcriptional and post-translational mechanisms. Interference with LSU expression prevents chloroplastic reactive oxygen species (ROS) production and proper stomatal closure during sulphur stress. We demonstrate that LSU1 interacts with the chloroplastic superoxide dismutase FSD2 and stimulates its enzymatic activity in vivo and in vitro. Pseudomonas syringae virulence effectors interfere with this interaction and preclude re-localization of LSU1 to chloroplasts. We demonstrate that reduced LSU levels cause a moderately enhanced disease susceptibility in plants exposed to abiotic stresses such as nutrient deficiency, high salinity, or heavy metal toxicity, whereas LSU1 overexpression confers significant disease resistance in several of these conditions. Our data suggest that the network hub LSU1 plays an important role in co-ordinating plant immune responses across a spectrum of abiotic stress conditions.

Key words: Abiotic stress, Arabidopsis thaliana, biotic stress, combinatorial stress, LOW SULPHUR UPREGULATED, LSU1.

Introduction

In nature and in farm fields, plants commonly encounter different forms of abiotic and biotic stress simultaneously. Due to global warming and the increasing need to farm on suboptimal soil, the incidence of combinatorial stress conditions is likely to increase. In molecular studies, stress responses are usually investigated in isolation. Although these approaches proved...
powerful for elucidating homeostatic response mechanisms for a variety of isolated stress conditions, it is becoming increasingly clear that the molecular responses to multiple simultaneous stressors differ from those to individual stressors. On a systems level, transcriptional profiling studies have demonstrated that the response to combinatorial stress qualitatively differs from the additive combination of single-stress responses (Atkinson et al., 2013; Prasch and Sonnewald, 2013; Rasmussen et al., 2013; Suzuki et al., 2014). In particular, lack of nutrients, water shortage, or high salinity can weaken the plant immune system (Triky-Dotan et al., 2005; Amtmann et al., 2008; Al-Sadi et al., 2010; You et al., 2011; Kissaoudis et al., 2015).

Bacterial pathogens such as *Pseudomonas syringae* pv. *tomato* (*Pst*) can enter the apoplast through stomata, which mediate water and gas exchange and play a central role in many abiotic stress responses. During the first steps of infection, plants recognize conserved bacterial protein patterns such as flagellin and activate a primary immune response known as pattern-triggered immunity (PTI), which includes increased reactive oxygen species (ROS) production, callose deposition, and other defence mechanisms (Jones and Dangl, 2006). Adapted pathogens can overcome initial immune responses by delivering virulence effector proteins into host cells that reach different subcellular compartments and interfere with PTI. Plants conversely possess intracellular receptors to detect the presence of virulence effectors by direct or indirect recognition, and trigger enhanced immune responses resulting in effector-triggered immunity (ETI). Beyond the idealized zig-zag model of the plant immune system (Jones and Dangl, 2006), it is clear that the perpetual arms race between pathogens and their hosts has resulted in a highly complex quantitative interplay of defence and counter-defence mechanisms.

To identify host interaction partners of virulence effectors and thus potentially novel components of the plant immune system, we recently conducted large-scale protein interaction mapping experiments between Arabidopsis proteins and virulence effectors from three evolutionarily distant pathogens (*P. syringae* and *O. congoense*; Suzuki et al., 2014; Wessling et al., 2016). Hubs in binary interaction networks were previously shown to mediate and integrate diverse cellular processes (Han et al., 2004; Yu et al., 2008). Moreover, we observed a correlation between the extent of effector convergence onto host targets and the manifestation of immune phenotypes in the corresponding genetic nulls (Mukhtar et al., 2011; Wessling et al., 2014). Incorporating population genetic data, we further discovered that protein products of Arabidopsis genes that experience the strongest balancing and positive selection preferentially interact with effector-targeted proteins (Wessling et al., 2014). Thus, despite the fact that no immune function had previously been described for most of the new effector targets, our collective data suggest that these are important in host–pathogen interactions.

Among the highly targeted host proteins are Arabidopsis *LOW SULPHUR* (S) UPREGULATED (LSU) proteins.

*Arabidopsis thaliana* has four members of the LSU protein family (LSU1–LSU4), which can be found in all higher land plants. LSU genes were named for their strong transcriptional induction in response to S deficiency (–S) (Maruyama-Nakashita et al., 2003), and a general function for the *Nicotiana benthamiana* LSU orthologue UP9C during –S stress was demonstrated (Lewandowska et al., 2010). However, the molecular and precise physiological functions of LSU proteins remain to be clarified. The observation that LSU family members are intensely targeted by evolutionarily diverse pathogens (Mukhtar et al., 2011; Wessling et al., 2014) suggests a hitherto unknown function in plant immunity.

Here we report that LSU1 and LSU2 levels are increased by transcriptional and post-translational mechanisms in a variety of abiotic stress conditions. In response to –S or salt stress, LSU gene expression is required for ROS production in guard cell chloroplasts and subsequent stomatal closure. Using genetic and biochemical approaches we demonstrate that LSU1, which is expressed in guard cells, physically interacts with the iron (Fe)-dependent superoxide dismutase (SOD) FSD2 and can activate its enzymatic activity. Virulence effectors from *P. syringae* interfere with the function and subcellular localization of LSU1. Correspondingly, in conditions of abiotic stress, we observe a moderate enhanced disease susceptibility (EDS) phenotype in seedlings with reduced LSU levels and a corresponding enhanced disease resistance (EDR) phenotype in LSU1 overexpressors. Together we uncover a physiological function for LSU proteins during combinatorial biotic and abiotic stress and propose a working mechanism of action for LSU1.

**Materials and methods**

**Plant cultivation and manipulation**

*Arabidopsis thaliana* Col-0 was cultivated on half-strength Murashige and Skoog (1/2 MS) medium or with S compounds substituted by equivalent chloride salts and supplemented with 0.5% (w/v) MES and Plant Preservative Mixture at 0.04% (v/v; Plant Cell Technology) under long-day conditions (16 h light–21 °C/8 h dark–16 °C). For stress treatments 7-day-old wild-type (WT) seedlings grown on 1/2 MS plates were transferred to liquid 1/2 MS medium without S or Fe or with 10 μM Cu, 2 mM DTT, 150 mM NaCl, or pH 8 for 1 d. For ROS detection and *P. syringae* assays, seedlings were grown on standard medium, without S, or in the presence of 25 μM Cu or 50 mM NaCl. Stable transgenic lines were generated with the *Agrobacterium tumefaciens* GV3101 (pMP90) strain by floral dipping (Konzcz and Schell, 1986; Clough and Bent, 1998). The fsd2-2 mutant corresponds to the SALK line SALK_080457C (ID NASC: N663088) (Myouga et al., 2008).

**Gene expression analysis**

RNA was prepared with the NucleoSpin RNA kit (Macherey-Nagel) following the manufacturer’s instructions. RNA quantity and quality was evaluated by spectrometry and in agarose gels prior to reverse transcription to cDNA using M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant (Promega). Quantitative PCRs (qPCRs) were conducted using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) using an initial cycle at 95 °C for 3 min and 40 cycles consisting of 95 °C for 10 s, 58 °C for 20 s, and 72 °C for 20 s. To assess expression changes of LSU genes,
the measured levels were first normalized to ACTIN2 (ACT2) and ELONGATION FACTOR1 (EF1) levels, except for salt and pH 8 treatments where these controls showed strong regulation and where ACT8 and UBQ10 (UBQ10) were used instead. The normalized expression levels are represented as fold change in expression relative to control conditions.

**Protein analysis and biochemical fractionation**

Total protein extracts were prepared in 100 mM NaCl; 50 mM Tris–HCl pH 7.5; 0.5% (v/v) Triton X-100; 1 mM DTT; 1× Complete Protease Inhibitor Cocktail Tablet (Roche), and spectrophotometrically quantified using ROTTIQUANT (Carl Roth). A 10–20 μg aliquot of protein extract was loaded onto an SDS-polyacrylamide gel, blotted on nitrocellulose membranes, probed with antibodies listed in Supplementary Table S3 at JXB online, and developed with the SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific) in a Luminescent Image Analyzer LAS4000 System (Fujifilm). Biochemical fractionation was conducted as previously described (Garcia-Molina et al., 2014) from 7-day-old CaMV35S:HA-LSU1/2 seedlings in a Sorvall MTX 150 Micro-Ultracentrifuge (Thermo Scientific) with a SS5A2 rotor. Fractions were assayed by western blots with antibodies anti-H3, anti-UGPase, anti-V-ATPase, and anti-haemagglutinin (HA) (Supplementary Table S3).

**Subcellular localization experiments**

CaMV35S:GFP-LSU12 and CaMV35S:GFP (Cutler et al., 2000) transgenic seedlings were examined in a FLUOVIEW FV1000 confocal laser microscope (Olympus) using specific green fluorescent protein (GFP) and chlorophyll filters.

**Generation of constructs and amiRNA lines**

Full-length ORFs were available as GATEWAY™ entry clones (Arabidopsis Interactome Mapping Consortium, 2011). To generate constructs for GFP and HA fusion proteins, entry clones were transferred into pMDC43 or pALLIGATOR2, respectively, using GATEWAY recombination reactions (Life Technologies) (Curtis and Grossniklaus, 2003; Benshmiren et al., 2004). For bimolecular fluorescence complementation (BiFC) assays, pYFN43 and pYFC43 were used (Belda-Palazón et al., 2012). Translational fusions to glutathione S-transferase (GST) and maltose-binding protein (MBP) were generated with pGEX6 and pMAL-DEST plasmids (Braun et al., 2002), respectively. Artificial miRNAs (amiRNAs) targeting LSU1–LSU4 were designed as in Schwab et al. (2006) with the oligonucleotides listed in Supplementary Table S2. GATEWAY-recombined into pDONR207 entry vector (Life Technologies), and subsequently into pALLIGATOR3 (Bernaudat et al., 2011).

**Stomatal aperture and water loss determination**

To determine stomatal aperture, 12-day-old Arabidopsis seedlings grown on S-sufficient or -deficient media were transferred to the indicated media supplemented with 10 μM abscisic acid (ABA; Sigma-Aldrich), or the corresponding mock treatment, for 3 h. First leaflets were abaxially observed by light microscopy, and the ratio of the width and length of ostioles (Rv) was measured. Water loss of 12-day-old seedlings was inferred from the change in dry weight over 60 min after removing seedlings from plates. For each biological replicate, at least 30 stomata were analysed.

**ROS production in guard cells**

ROS production was traced as previously described (Miao et al., 2006; Zou et al., 2015) with modifications. Briefly, detached first leaflets of 14-day-old seedlings were incubated for 10 min on 50 μM H2DCFDA (Life Technologies), washed twice with water, and observed either under laser confocal (Olympus FYV1000) or BX61 epifluorescence microscopy (Olympus) with a GFP band-pass filter. 3,3′-Diaminobenzidine (DAB) staining was used to detect H2O2 specifically. To this end, 14-day-old seedlings were incubated with 1 ml of DAB solution [1 mg ml−1 (w/v) DAB; 0.05% (v/v) Tween-20; 10 mM Na2HPO4] and vacuum-infiltrated for 5 min in the dark. Samples were left at room temperature for 5 h protected from the light, and de-stained with two washes with ethanol:acetic acid:glycerol (3:1:1) solution prior to microscopy.

**Production of recombinant proteins**

Escherichia coli Rosetta™ (Novagen) transformants were grown to log phase (OD600 0.4–0.8) in LB medium supplemented with antibiotics and with 0.2% glucose for MBP constructs; protein expression was induced with 1 mM isopropyl-p-thiogalactopyranoside (IPTG) for 4 h at 28 °C. Pelleted cells were resuspended in 1× phosphatase-buffered saline (PBS) supplemented with 1× Complete Protease Inhibitor Cocktail Tablet (Roche), sonicated, and lysates were rotated with either Amylose Resin (New England Biolabs) or Protino Glutathione Agarose 4B (Macherey-Nagel) for 2 h at 4 °C. The matrix was washed twice with 3 vols of PBS or MBP wash buffer (200 mM NaCl; 20 mM Tris–HCl pH 7.4; 1 mM EDTA; 1 mM DTT), respectively, and eluted with 2 vols of 10 mM Tris–HCl pH 8, 10 mM glutathione, or MBP wash buffer supplemented with 10 mM maltose.

**Protein–protein interaction assays**

Yeast two-hybrid (Y2H) experiments were conducted with the indicated ORFs cloned into pDEST-AD or pDEST-DB as described (Dreze et al., 2010). BiFC assays were carried out by agro-injection of pYFC43-LSU12 and pYFC43-FSD2, pYFC43-PEN1, or pYFC43-LSU12 in N. benthamiana epidermal cells at OD600 0.1 and observed after 1–2 d under epifluorescence microscopy (Olympus BX61) with a yellow fluorescent protein (YFP) band-pass filter. For in vitro pull-down assays, Protino Glutathione Agarose 4B (Macherey-Nagel) alone or coated with GST–FSD2 was incubated with equimolar combinations of purified MBP–LSU1 and/or MBP–NIMIN1, MBP–AvrXccC, MBP–AvrB2, or MBP–HopR1 in a final volume of 100 μl of PBS and incubated with gentle rotation for 3 h at 4 °C. Beads were washed four times with 3 vols of PBS each and eluted with 10 mM Tris–HCl pH 8, 10 mM glutathione. MBP–LSU1 pull-downs were analysed by western blot using anti-MBP antibodies (Supplementary Table S3).

**Determination of superoxide dismutase enzymatic activity**

SOD activity was determined as described by Kuo et al. (2013) with modifications. Briefly, samples were incubated with 1 ml of reaction buffer [5 mM Nitro Blue Tetrazolium (NBT); 50 mM PBS pH 7.8; 10 mM methionine; 0.5 mM EDTA; and 20 μM riboflavin] for 30 min in the dark, and then illuminated for 10–15 min. SOD activity was monitored by measuring inhibition of NBT–diformazan formation at A560. Nicotiana benthamiana samples were prepared in extraction buffer [50 mM PBS pH 7.8; 0.05% (v/v) NP-40; 2% (w/v) polyvinylpyrrolidone (PVPP); 1 mM EDTA]. For in vitro assays, purified recombinant GST–FSD2 (3 μg) or GST–FSD1 (1.5 μg) were incubated with the indicated recombinant proteins in 50 μl of PBS at room temperature for 30 min prior to enzymatic activity determination.

**Pseudomonas syringae infection assays**

Infection assays were conducted as previously described in Ishiga et al. (2011). Pseudomonas syringae pv. tomato DC3000, COR−, or hrcC was cultured overnight at 28 °C in NYGA medium [0.5% (w/v) bactopeptone, 0.3% (w/v) yeast extract; 2% (v/v) glycerol] supplemented with antibiotics. Infection assays were performed on
12-day-old seedlings grown in vitro by flooding with bacterial suspension at OD600 0.01 (5 × 10^6 CFU ml^-1) for 3 min. Pseudomonas syringae density was determined after 3 d. For this, shoots were harvested, disinfected with 5% (w/v) H_2O_2, and rinsed twice with water. Samples were ground and spotted in serial dilutions on selective medium (LB supplemented with 50 mg l^-1 rifampicin and 30 mg l^-1 kanamycin for all strains and also 30 mg l^-1 spectinomycin for COR) to obtain colony counts after 1 d of incubation at 28 °C. To ensure equal infection rates, WT and amiR-LSU lines were cultivated together.

**Miscellaneous methods**

Student’s t-test was used to determine significant differences from control lines or treatments as indicated. Fluorescence and band intensities were quantified with the image-processing package Fiji. Sequence analysis and multiple alignments were performed with the CLC Sequence Viewer 7 (Qiagen) software. Protein distance measure is indicated according to Jukes and Cantor (1969).

**Results**

**Transcriptional and post-translational regulation of LSU levels in abiotic stress**

Hubs in binary interaction networks were shown to co-ordinate different cellular processes (Han et al., 2004; Yu et al., 2008). Therefore, we wondered whether LSU genes might also function in abiotic stress conditions other than –S conditions (Nikiforova et al., 2003; Arabidopsis Interactome Mapping Consortium, 2011; Hawkesford et al., 2012). We investigated transcript and protein abundance in several abiotic stress conditions selecting LSU1 and LSU2 (LSU1/2) as representative members of the family (Supplementary Fig. S1A–C). LSU1/2 transcript levels were determined by qPCR in 7-day-old WT seedlings transferred to liquid media representing different abiotic stress conditions. In addition to the induction by –S, LSU transcript levels increased between 2- and 15-fold in WT seedlings exposed to increased salinity, iron (Fe) deficiency, copper (Cu) excess, or basic pH; no change was observed in response to DTT (Fig. 1A). To investigate potential post-translational regulation in the absence of transcriptional changes, we used transgenic lines expressing LSU1/2 fusion proteins from ectopic promoters. CuMV35S:GFP–LSU1/2 transgenic seedlings accumulated more GFP–LSU1 upon –S, Fe deprivation, Cu excess, and salt excess in comparison with control treatments, whereas increased GFP–LSU2 amounts were found on –S, Fe deficiency, and high Cu, but not salt excess. For the rest of the treatments, no differences or even reduced levels were observed (Fig. 1B). The LSU1 and LSU2 responsiveness towards several abiotic stress conditions suggests that LSU proteins could mediate plant responses to a variety of abiotic stress conditions.

LSU1 and LSU2 display differential subcellular and tissue localizations

Despite the high similarity of LSU proteins (Supplementary Fig. S1A–C), the interaction partners of LSU1/2 proteins previously identified in the Arabidopsis interactome only partially overlap (Arabidopsis Interactome Mapping Consortium, 2011). We wondered whether the localizations of LSU proteins further support a functional specialization and interrogated the tissue and subcellular localization of LSU1/2 in planta. A biochemical fractionation following heterologous expression in N. benthamiana showed that LSU1/2 can be found in nuclear, cytosolic, and microsomal fractions (Supplementary Fig S2A, B). We therefore investigated the tissue and subcellular localization of GFP–LSU1/2 proteins in Arabidopsis. Microscopically, GFP–LSU1 lines display a diffuse fluorescent signal in the root cytoplasm/membrane (Fig. 1C, lower panel, left). In leaflets of 5-day-old seedlings, GFP–LSU1 was prominently detected in guard cells, suggesting a function in stomatal regulation (Fig. 1C, upper panel, left). GFP–LSU2 exhibited a diffuse but ubiquitous cytosolic signal in leaves and roots, and also illuminated nuclei in root cells (Fig. 1C, right). Soluble GFP alone showed an overall similar pattern, which is expected given the small size of LSU proteins and GFP. Nonetheless, clear differences can be seen especially in the strong stomatal localization of GFP–LSU1 compared with pavement cells and more prominent nuclear localization of LSU2 in root cells (Fig. 1C; Supplementary Fig. S2C). The results support the conclusion that LSU proteins can localize to multiple cell compartments and suggest at least partially specialized and tissue-specific roles for LSU1 and LSU2. Importantly, the data point towards a likely function of LSU1 in guard cells. This conclusion is supported by markedly reduced LSU1 transcript levels in cotyledons of the stomataless mutants spch-3 and mute-3 (de Marcos et al., 2015).

**Abrogation of LSU expression constrains stomatal movements upon sulphur deficiency**

No T-DNA mutants were available for LSU1 and LSU3, and the paired duplications of the LSU1/3 and LSU2/4 paralogues further challenge generation of higher order mutants. Hence, to characterize the LSU genes phenotypically and avoid problems due to potential redundancy, three different amiRNAs targeting all LSU family members at different positions were designed and used to generate three amiR-LSU lines (amiR-LSUa–c; Supplementary Fig. S3A). Transcript levels of the four LSU genes were determined by qPCR in seedlings grown on –S. Compared with WT lines, LSU1–LSU3 transcripts were reduced by >80% and LSU4 levels by 50% in the amiR-LSUa–c (Supplementary Fig. S3B). Based on these results, the amiR-LSU lines were considered knock-down lines and used for subsequent phenotypic studies.

The amiR-LSU lines did not exhibit obvious phenotypes on soil or in vitro culture in comparison with the WT (data not shown). Since GFP–LSU1 localized to guard cells and stomatal closure is a physiological response to –S (Terry, 1976; Karmaker et al., 1991), guard cell movements in the amiR-LSU lines were characterized. The apertures of abaxial stomata of first leaflets were estimated as the ratio between the width and length of the ostiols (R_wl). Stomata were mostly open (R_wl ~0.45) in both WT and amiR-LSUa–c lines grown on S-sufficient (+S) media. S deficiency, however, led to partial stomatal closure in WT lines indicated by an ~30%
LSU proteins mediate responses to combinatorial stress

Decrease in $R_{wl}$. In contrast, stomata remained open in amiR-LSU lines in –S media (Fig. 2A; Supplementary Fig. S4A), indicating that stomatal closure during –S conditions is an LSU-dependent process. In agreement with these observations, the amiR-LSUa–c lines exhibited a more severe water loss on –S media compared with the WT (Supplementary Fig. S4B; Supplementary Table S1). Notably, stomatal closure in response to exogenous ABA was not impaired in either condition, indicating that ABA-dependent stomatal closure is not affected in the amiR-LSU lines (Fig. 2). Thus, LSU proteins, probably the prominently guard cell-localized LSU1, participate in the regulation of stomatal closure in response to –S.

LSU down-regulation compromises ROS production in guard cells upon certain abiotic stress conditions

Guard cell movements are controlled by a complex interplay of hormones and secondary signals (Daszkowska-Golec and Szarejko, 2013; Arnaud and Hwang, 2014), which culminate

![Diagram](https://example.com/diagram.png)

**Fig. 1.** Characterization of LSU1 and LSU2. (A and B) LSU1 and LSU2 expression is induced under certain abiotic stress conditions. (A) Expression of LSU1 and LSU2 transcripts in different abiotic stress conditions was determined by qPCR and expressed as the ratio between experimental and control treatments. Error bars correspond to the SD of three biologically independent measurements, and significant differences were assessed by the Student's $t$-test relative to control treatments ($^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$). (B) α-GFP western blots on total lysates of GFP–LSU1- and GFP–LSU2-expressing seedlings grown as indicated. Ponceau staining shows the large RuBisCO subunit and indicates equal loading; molecular weight marker sizes are indicated in kDa. (C) Representative confocal images of cotyledons and roots of GFP–LSU1 and GFP–LSU2 seedlings grown on –S media showing the GFP signal, chlorophyll autofluorescence, and bright field are provided (scale bar=50 µm).

**Fig. 2.** Abrogation of LSU constrains stomatal closure during abiotic stress conditions. Twelve-day-old WT and amiR-LSUa–c seedlings grown in +S or –S conditions were transferred to the same media supplemented with ABA or mock treated for 3 h. Stomatal aperture was quantified as the ratio of ostiol width to length ($R_{wl}$). Error bars correspond to the SD of ≥3 biologically independent measurements ($n$ ≥30 stomata), and significant differences were assessed by the Student’s $t$-test relative to the WT in the same conditions ($^{***}P<0.001$).
in an increase of H$_2$O$_2$ that triggers ion channel opening and a change in guard cell turgor (Pei et al., 2000; Zhang et al., 2001; Desikan et al., 2004; Wang et al., 2012). To address whether ROS production during –S stress is impaired in guard cells, we pharmacologically monitored ROS production using the ROS-mediated conversion of 2',7'-dichlorodihydro-fluorescein diacetate (H$_2$DCFDA) into fluorescent 2',7'-dichlorofluorescein (DCF) (Brandt and Keston, 1965). In +S conditions, DCF fluorescence in guard cells exhibited a diffuse pattern partly co-localizing with chloroplast autofluorescence (Fig. 3A). In contrast, in –S conditions, DCF fluorescence intensity nearly doubled (~40%) compared with +S and exclusively localized to speckles overlapping the chlorophyll autofluorescence (Fig. 3A, B). In the amiR-LSU lines, the –S-induced stimulation of ROS production was either dramatically reduced (amiR-LSUc) or completely abrogated (amiR-LSUa, b; Fig. 3B; Supplementary Fig. S4C). Among the various ROS species, H$_2$O$_2$ is of particular interest due to its varied roles as a defence and signalling molecule. To test if the observed differences in guard cell ROS production could be attributed to an H$_2$O$_2$ accumulation, DAB staining on S-deprived seedlings was conducted and the signal intensity per guard cell pair quantified. As depicted in Fig. 3C, the amiR-LSUa–c lines showed a reduced DAB staining pattern (~30%) compared with the WT. In addition, we tested whether ROS production in the amiR-LSU lines is affected in conditions other than –S. Consistent with the induction of LSU transcripts in response to high salt or Cu, the amiR-LSU lines exhibited 40–50% lower DCF signal intensity compared with the WT (Fig. 3D). These data demonstrate that LSU proteins stimulate ROS production, among them H$_2$O$_2$, in guard cell chloroplasts in response to different abiotic stress conditions.

LSU1 physically interacts with and activates the iron superoxide dismutase FSD2

H$_2$O$_2$ mainly occurs in cells as a result of the enzymatic conversion of the superoxide radical (O$_2$–) by SODs. Arabidopsis possesses three different SOD isoforms classified according to their respective metal cofactors: manganese (MnSOD), Cu/zinc (CSD1/2), and Fe (FSD1–FSD3) (Kliebenstein et al., 1998). Of these, FSD2, FSD3, and CSD2 localize to plastids and may therefore mediate the H$_2$O$_2$ production during –S stress, whereas the others function in different cell compartments. We previously identified FSD2 as an interaction partner of LSU1 and LSU2 (Arabidopsis Interactome Mapping Consortium, 2011) and aimed to verify this interaction; to ascertain its specificity, we also included all other Arabidopsis SOD proteins in this verification experiment.

Fig. 3. Abrogation of LSU impairs ROS production during abiotic stress conditions. (A) Reactive oxygen species (ROS) in chloroplast guard cells increase during sulphur starvation. Representative confocal pictures of the abaxial guard cell stomata of WT seedlings grown under +S or –S conditions showing DCF and chlorophyll fluorescence and a merged signal (scale bar=10 µm). (B) Quantification of relative DCF fluorescence intensity of stomata (n ≥30) of WT and amiR-LSUa–c seedlings grown in +S and –S conditions. (C) Hydrogen peroxide production in guard cells of amiR-LSU lines is compromised during sulphur starvation. Representative pictures of 3,3’-diaminobenzidine (DAB) staining in guard cells of seedlings grown on –S and quantification of signal intensity per guard cell pair. (D) ROS production in chloroplast guard cells is compromised during copper and salt treatments. Quantification of DCF signal intensity in guard cells of seedlings grown on 25 µM CuCl$_2$ and 50 mM NaCl, respectively. In all cases, error bars correspond to the SD of ≥3 biologically independent measurements, and significant differences were assessed by the Student’s t-test relative to the WT in the same conditions (*P<0.05; **P<0.01; ***P<0.001).
Y2H experiments using GAL4 activation domain (AD)–LSU1/2 and all Arabidopsis SODs fused to the GAL4 DNA-binding domain (DB) confirmed the specific interaction of LSU1/2 with the chloroplast-localized FSD2 (Fig. 4A). Due to self-activation of FSD3, a possible association with LSU1/2 could not be tested (Fig. 4A). BiFC in N. benthamiana demonstrates that LSU1 and LSU2 can interact with FSD2 in planta (Fig. 4B). Additional support for the interactions was obtained in biochemical pull-down experiments (see below). Moreover, upon closer inspection of confocal images, we found that in –S conditions GFP–LSU1 partly co-localized with chlorophyll autofluorescence in guard cells, suggesting plastidial localization of LSU1 in these conditions [Pearson correlation coefficient (PCC) 0.52; Fig. 4C]. Thus, LSU1 can physically interact with FSD2 in vitro and in vivo.

Given that reduced LSU levels lead to decreased ROS production in guard cells under abiotic stress conditions, we wondered if LSU proteins could directly stimulate FSD2 enzyme activity. In vivo activity measurements using N. benthamiana leaves transiently expressing GFP–LSU1 and GFP–FSD2 together or alone showed only incremental stimulation of FSD2 activity by LSU1 (Supplementary Fig. S5A, B). To eliminate confounding effects by endogenous proteins, we purified recombinant proteins and investigated the effect of LSU1 and LSU2 on FSD2 activity in vitro, using the non-interacting FSD1 to assess specificity (Fig. 5A). The functionality of GST-tagged FSD1 and FSD2 purified from Escherichia coli was confirmed by plotting SOD activity against the enzyme concentration, which yielded classic saturation curves (Supplementary Fig. S5C). Non-saturating enzyme concentrations were then incubated with increasing amounts of MBP–LSU1, and SOD activity was determined. Recombinant LSU1 specifically induced FSD2 enzyme activity, but not the activity of the non-interacting FSD1 (Fig. 4D). A similar trend was observed upon addition of GST–LSU2 but not GST alone or MBP fused to the unrelated NIM1-INTERACTING 1 (NIMIN1) (Supplementary Fig. S5D). Thus, mechanistically LSU1 and LSU2 can stimulate H$_2$O$_2$ production by direct interaction with and activation of FSD2.

Pseudomonas syringae virulence effectors interfere with LSU1 functions

Stomata are a major entry point for bacterial pathogens, and of all LSU family members the guard cell-localized LSU1 preferentially interacted with bacterial virulence effectors in our Y2H-based network mapping experiments (Melotto et al., 2006; Mukhtar et al., 2011; Wessling et al., 2014). H$_2$O$_2$ can trigger stomatal closure, but may also have signaling and defence functions (Desikan et al., 2001; Van Aken and Whelan, 2012; de Torres Zabala et al., 2015). Lastly, chloroplastic H$_2$O$_2$ production was recently shown to be an important element of PTI with which Pst virulence effectors can interfere (de Torres Zabala et al., 2015). We therefore wondered if the LSU1–FSD2 interaction may be relevant in defence responses and if LSU-interacting bacterial effectors can interfere with it. First, we confirmed by Y2H the interaction of AD–LSU1 and AD–LSU2 with DB–FSD2 by Y2H. The top panel shows diploid yeasts and successful mating; the bottom panel shows growth on selective media indicating interactions of AD–LSU1/2 with DB–FSD2 and autoactivation of DB–FSD3. (B) Bimolecular fluorescence complementation (BiFC). Nicotiana benthamiana epicotyl leaves transiently co-expressing cYFP–FSD2 and nYFP–LSU1/2 restore YFP fluorescence, whereas co-infiltration of cYFP–FSD2 and nYFP fusions with the cytosolic Arabidopsis PHOSPHATASE AND TENSIN HOMOLOG DELETED ON CHROMOSOME TEN 1 (PTEN1, AT5G39400) and with the chloroplast-localized nYFP–VARIEGATED 3 (VAR3, AT5G17790) do not (scale bar=100 $\mu$m). (C) LSU1 partly co-localizes with guard cell chloroplasts. Representative confocal images of guard cells in first leaflets of –S-grown GFP–LSU1 seedlings. Shown are GFP signal (left), chlorophyll autofluorescence (middle), and merged channels (right) (scale bar=25 $\mu$m). (D) Relative in vitro SOD activity of recombinant GST–FSD1 and GST–FSD2, respectively, in the presence of increasing concentrations of MBP–LSU1. Error bars correspond to the SD of three independent experiments, and significant differences from controls were assessed using Student’s t-test (*$P<0.05$; **$P<0.001$).
LSU proteins mediate resistance to Pseudomonas syringae infection under certain abiotic stress conditions

These results led us to investigate genetically the immune function of LSU proteins, especially during abiotic stress. First we asked if LSU down-regulation alters plant susceptibility towards *Pst*. To perform infection assays under nutrient deficiency conditions, 12-day-old WT and amiR-LSUa–c seedlings grown *in vitro* on +S or –S were flooded with *Pst* suspensions and colony density was counted 3 days post-infection (dpi). As depicted on Fig. 6A, consistent with the lack of detectable LSU expression, no significant differences in pathogen susceptibility were found among lines under standard conditions (+S). However, growth of seedlings on –S resulted in mild but significant EDS in the amiR-LSU lines, as the colony count increased ~2-fold compared with the WT (Fig. 6A; Supplementary Table S1). Consistent with this phenotype and our findings above, the amiR-LSUa–c lines showed reduced ROS production in guard cell chloroplasts following *Pst* challenge 1 dpi (Fig. 6B).

Part of the *Pst* infectivity relies on the production of coronatine (COR), a jasmonic acid analogue that mediates stomatal re-opening and promotes bacterial virulence by suppressing salicylic acid-mediated defence responses (Cui *et al.*, 2005; Melotto *et al.*, 2006). Upon infection by the COR-defective mutant COR−, the amiR-LSUa–c lines still exhibit moderate EDS on –S (on average ~2.5 times higher than in the WT) (Fig. 6C). Thus, the EDS phenotype in the amiR-LSU lines is not part of the COR-induced responses. To test if this phenotype would be a component of PTI, the same assay was repeated using the Type III Secretion System (T3SS)-defective *hrcC* mutant, which elicits PTI but...
LSU proteins mediate responses to combinatorial stress

is unable to deliver effectors into the plant cytosol (Cunnac et al., 2011) and therefore does not elicit ETI. The amiR-LSU lines did not exhibit an altered susceptibility towards hrcC in either growth condition (Fig. 6D), suggesting that the LSU-dependent immune function requires effector delivery or, formally, the T3SS itself. Consistently, infection of –S-stressed plants by hrcC, in contrast to WT Pst, did not cause an additionally increased ROS production in guard cell chloroplasts (Fig. 6B). Analysis of guard cell dynamics during initial phases of Pst infection further excluded impairments of Pst-triggered stomatal closure in amiR-LSU lines and revealed an altered pattern of stomatal dynamics in –S conditions. The WT and the amiR-LSUa–c lines effectively closed stomata 1 h post-infection (hpi) in +S and –S conditions, indicating that LSU proteins do not function during this stage of infection (Supplementary Fig. S6). However, in WT plants grown on –S media, stomata remained closed even at 4 hpi (Supplementary Fig. S6B). In amiR-LSUa and amiR-LSUc, stomata were ~20% and 30%, respectively, more open at 4 hpi compared with the WT (Supplementary Fig. S6B; Supplementary Table S3). Together, these observations reinforce the notion that in –S stress conditions, LSU proteins have a defence function that involves stimulating ROS production, but which is not mediated by COR and is not part of the hrcC-activated PTI. Consistent with this idea, the knockout line fsd2-2 also displayed a weak EDS phenotype under our S-deficient conditions (~1.5-fold more colonies than the WT; Supplementary Fig. S7).

Since LSU proteins are up-regulated in several stress conditions, we wondered if they might also have an immune function in abiotic stresses other than S deprivation. Indeed, amiR-LSU plants grown in the presence of high salt or Cu concentrations also exhibited moderate but significant EDS towards Pst, but not hrcC, demonstrating that LSU proteins support immune function in several abiotic stress conditions (Fig. 6D, E).

To test if LSU overexpression results in complementary EDR, and to gather support for our suggestion that LSU1 might be responsible for the observed amiR-LSU phenotypes, we phenotypically characterized two independent 35S:HA-LSU1 overexpression lines using Pst and hrcC. A phenotypic pattern complementary to the amiR-LSUa–c lines was observed such that LSU1 overexpression conferred significant EDR towards Pst during –S and high salt stress.

Fig. 6. LSU proteins mediate defence responses during abiotic stress. WT and amiR-LSUa–c seedlings were flooded with bacterial suspension at $5 \times 10^6$ CFU ml$^{-1}$ of the P. syringae strains: Pst DC3000 (Pst), COR$^-$, and hrcC. In (A) and (C–F), colony-forming units were determined 3 days post-infection (dpi) and normalized to fresh weight. Seedlings were grown in normal (+S) and –S conditions, or 1/2 MS conditions, or submitted to high Cu or NaCl, as indicated. (B) DCF fluorescence in guard cells of –S-grown lines at 1 dpi with the indicated bacterial strain normalized to mock treatment. Error bars correspond to the SD of at least three biologically independent experiments, and statistical differences from the WT were assessed using Student’s t-test and are indicated with asterisks (*$P<0.05$; **$P<0.01$; ***$P<0.001$).
but had no effect under normal conditions or towards hrcC (Fig. 7; Supplementary Table S1).

Discussion

In addition to understanding molecular plant response mechanisms to isolated abiotic and biotic stress, it is also important to characterize the integrated responses to combinatorial stress conditions. LSU proteins, which had only been implicated in –S stress, are highly connected hubs in a plant protein interactome network and intensely targeted by virulence effectors from evolutionarily distant pathogens (Arabidopsis Interactome Mapping Consortium, 2011; Mukhtar et al., 2011; Wessling et al., 2014). These observations motivated us to investigate the function of the encoded proteins to illuminate their normal physiological role and find out why effectors may have evolved to interfere with this function.

Our primary characterization of LSU1 and LSU2, as representatives of this protein family, suggests a general function of these genes in abiotic stress responses. Both LSU1 and LSU2 transcripts are up-regulated in several abiotic stress conditions, and apparent post-translational regulation leads to protein accumulation in seedlings and, for LSU1, to stabilization of GFP–LSU1 in guard cells. Since all LSU family members have a serine-rich domain that includes putative phosphorylation sites (Supplementary Fig. S1A) and phosphorylation of LSU3 has been detected in vivo (Phosphat; http://phosphat.uni-hohenheim.de/), the impact of this post-translational modification on protein stability warrants further investigation.

The prominent localization of GFP–LSU1 in guard cells, reinforced by the LSU1 down-regulation in cotyledons of the stomataless mutants spch-3 and mute-3 (de Marcos et al., 2015), prompted us to investigate potential functions of LSU in stomatal dynamics. Indeed, transgenic lines with reduced LSU levels display impaired stomatal closure under –S. Moreover, the amiR-LSU lines exhibited profoundly reduced ROS production in guard cells when exposed to stress conditions that induce LSU transcription, such as high salinity and –S. This suggests that LSU proteins, most probably LSU1, promote stomatal closure in response to abiotic stress by stimulating chloroplastic ROS production. In guard cells, ROS levels depend on a complex interplay between ABA and ethylene whereby ethylene negatively regulates ABA-dependent stomatal closure by alleviating ROS levels, while, conversely, ABA represses ethylene production (Tanaka et al., 2005, 2006; Watkins et al., 2014). However, upon exogenous ABA treatment, stomata of amiR-LSU lines closed normally, indicating that the ABA-triggered stomatal closure is not impaired. It is possible that, similarly to ethylene, LSUs function upstream of ABA. This appears unlikely given that S limitation negatively impacts ethylene biosynthesis due to reduced methionine availability (Bürstenbinder et al., 2007) and also leads to drastically reduced ABA levels (Cao et al., 2014). We therefore hypothesize that LSU proteins may participate in an alternative mechanism to trigger stomatal closure in some stress conditions. The exclusive presence of LSU proteins among gymno- and angiosperms provides evolutionary support for the proposed role of LSU proteins in guard cells.

A hint towards a possible mechanism by which LSUs induce ROS production came from our Arabidopsis interactome map, where LSU proteins were found to interact with FSD2 (Arabidopsis Interactome Mapping Consortium, 2011). We demonstrate here that LSU1 and LSU2 specifically interact with FSD2 in vitro and in planta, and stimulate its enzyme activity. Consistent with the idea that LSU1 activates FSD2 in planta, GFP–LSU1, but not GFP–LSU2, was found in guard cell patterns that largely overlap with chloroplast autofluorescence, and upon S deprivation amiR-LSU lines exhibit reduced H$_2$O$_2$ production in guard cells. The increased H$_2$O$_2$ levels can trigger stomatal closure and potentially other effects, such as transcriptional regulation (Vandenabeele et al., 2003; Van Aken and Whelan, 2012) (Supplementary Fig. S8). A heterodimeric complex of FSD2/FSD3 was previously shown to protect chloroplast nucleoids from ROS (Myouga et al., 2008). This study found that FSD3 localized exclusively to speckles in the nucleoids, while, consistent with our data, FSD2 exhibited a wider chloroplastic localization suggesting additional functions (Myouga et al., 2008). We propose that abiotic stress conditions lead to stabilization of LSU1, especially in guard cells, where it enters chloroplasts and stimulates ROS production by activating FSD2. The question of how LSU1, despite the lack of transit peptide, is imported to the chloroplast remains to be answered, though examples of chloroplast- and nuclear-localized proteins using non-canonical importing signals or interacting partners have been reported (Miras et al., 2002, 2007; Withers et al., 2012).

Biochemically, it is likely that FSD2 activation by LSU1 represents another example of factor-associated SOD activation, which has been previously reported for all SOD isoforms.
The capacity of LSU to stimulate FSD2, the small size, and lack of clearly defined globular domains moreover beg the question of whether LSU proteins might also have chaperone-like activity for other clients.

Besides their function for gas exchange, stomata are natural orifices that constitute entry points for bacterial pathogens into the plant apoplast (Melotto et al., 2006). Although plants reinforce stomatal immunity upon bacterial recognition during PTI, bacterial pathogens such as _Pst_ can counteract this effect by producing COR and virulence effectors to re-open stomata or prevent stomatal closure, respectively (Melotto et al., 2006; Gudesblat et al., 2009). Given that we identified LSU proteins as targets of virulence effectors, we hypothesized that LSU-mediated stomatal closure could also have a defence function with which pathogens may aim to interfere. In support of this, we found that amiR-LSU lines do exhibit an EDS phenotype in conditions of abiotic stress whereas LSU1 overexpression lines display complementary EDR phenotypes. Upon infection by _hrcC_, this phenotype was not detectable, whereas it was unaltered upon infection by a COR strain, indicating that the LSU-dependent immune function is not due to a general LSU function in these conditions and not due to PTI or COR-triggered pathways. Abrogation of _LSU_ expression did not directly impair stomatal closure in response to _Pst_ infection during S scarcity; however, it caused a moderate and faster stomatal re-opening in comparison with the WT at 4 hpi. The weak immune effects may be due to the inability of the amiR-LSU lines to maintain closed stomata during the early phase of the infection, but independent mechanisms cannot be excluded and should be further investigated.

Chloroplastic ROS were recently shown to be an important defence signal that _Pst_ can suppress by only partly known mechanisms (de Torres Zabala et al., 2015). Here, we demonstrated that _Pst_ effectors can interfere with the activating interaction of LSU1 with FSD2. In addition, _Pst_ infection prevents GFP–LSU1 from entering guard cell chloroplasts, and our genetic data confirm the immune function of LSU proteins during high salinity or –S stress. In these conditions, artificially reduced _LSU_ levels abolish the normally increased ROS production in response to _Pst_ infection and cause a modest EDS phenotype. Lack of FSD2 activity in the _fsd2-2_ line resulted in a similar degree of _Pst_ susceptibility, whereas ectopic expression of _LSU1_ results in modest EDR phenotypes.

Recent studies already implicated virulence effectors in modulating stomatal function by demonstrating that _Pst_ HopF2 and HopM1 can suppress stomatal immune responses and ROS burst _in planta_ during bacterial infection (Hurley et al., 2014; Lozano-Duran et al., 2014). Thus, we suggest that upon infection with virulent _Pst_, the antagonistic interplay of effectors triggering weak ETI (Gassmann, 2005; Fabro et al., 2011) and effectors interfering with LSU results in an overall small, but significant immune phenotype in amiR-LSU lines, demonstrating the immune function of LSU proteins in plants experiencing –S or salt stress (Supplementary Fig. S8).

Taken together, we demonstrate that LSU proteins stimulate production of _H_2O_2_ and potentially other ROS, and effect stomatal closure in response to several types of abiotic stress, including high salinity or S depletion. Mechanistically, this stimulation is at least in part achieved via LSU up-regulation and subsequent interaction with and activation of the SOD FSD2. Moreover, we show that LSU proteins are elements of the plant immune response when plants experience simultaneous abiotic stress.

**Supplementary data**

Supplementary data are available at _JXB_ online.

Fig. S1. Primary sequence analysis and additional characterization of LSU proteins.

Fig. S2. Additional characterization of amiR-LSU lines.

Fig. S3. Generation of amiR-LSU lines.

Fig. S4. Additional guard cell phenotypes of amiR-LSU lines.

Fig. S5. Determination of FSD2 activity _in vivo_ and _in vitro_.

Fig. S6. Stomatal dynamics during initial phases of _P. syringae_ infection.

Fig. S7. The knock-out line _fsd2-2_ displays a moderately enhanced disease susceptibility to _P. syringae_ during sulphur deficiency.

Fig. S8. A model for LSU1 function in guard cells during combined abiotic and biotic stress.

Table S1. Statistical analysis of experiments in the present work.

Table S2. Oligonucleotides used in the present work.

Table S3. Primary antibodies used in the present work.

**Acknowledgements**

We thank Andreas Keymer (LMU; München) for help and assistance in confocal microscopy imaging. The 35S_CuMY-GFP line was kindly provided by Kay Schneitz (TUM; München). We thank François Parcy (CEA-CNRS, Grenoble) and Alejandro Ferrando (IBMCP-CSIC, Valencia) for _pALLIGATOR2_ and _pYFN43_ and _pYFC43_ vectors, respectively. We thank Jens Steinbrenner (University Giessen) for fruitful discussions, and we thank Freddy Monteiro, Sarah R. Grant, and Nora Marin de la Rosa for discussions and critical reading of the manuscript. This work was funded by grants to PB by the Human Frontiers Science Organisation (grant RGY0080/2013) and by the DFG collaborative research grant (CRC) 924 (CRC924/18B924), and to JLD from the National Science Foundation (IOS-1257373), the National Institutes of Health (1ROI GM107444), the Gordon and Betty Moore Foundation (GBMF3030), the HHMI, and by a Distinguished Guest Professorship, Eberhard-Karls-Universität, Tübingen, Germany. JLD is an Investigator of the Howard Hughes Medical Institute. The authors declare that there are no conflicts of interest.

**Author contributions**

experiments and figures: AG-M. Assistance with Y2H experiments: MA and AA. Design of experiments: AG-M, PE, JLD, and PB. Manuscript writing: AG-M. Experiments and figures: AG-M. Assistance with Y2H experiments: MA and AA. Design of experiments: AG-M, PE, JLD, and PB. Manuscript writing: AG-M and PB. Critical manuscript reading and editing: PE and JLD. All authors discussed and commented on the article.

**References**

Ahmed M, Deadman ML. 2010. Effect of salinity on pythium damping-off
of cucumber and on the tolerance of *Pythium aphanidermatum*. Plant Pathology 59, 112–120.

Amtmann A, Troufflard S, Armengaud P. 2008. The effect of potassium nutrition on pest and disease resistance in plants. Physiologia Plantarum 133, 682–691.

Arabidopsis Interactome Mapping Consortium. 2011. Evidence for network evolution in an Arabidopsis interactome map. Science 333, 601–607.

Arnaud D, Hwang I. 2015. A sophisticated network of signaling pathways regulates stomatal defenses to bacterial pathogens. Molecular Plant 8, 566–581.

Atkinson NJ, Lilley CJ, Urwin PE. 2013. Identification of genes involved in the response of Arabidopsis to simultaneous biotic and abiotic stresses. Plant Physiology 162, 2028–2041.

Belda-Palazón B, Ruiz L, Martí E, Tarraga S, Tiburcio AF, Culliáñez F, Farràs R, Carrasco P, Ferrando A. 2012. Aminopropyltransferases involved in polyamine biosynthesis localize preferentially in the nucleus of plant cells. PLoS One 7, e46907.

Bensimhen S, To A, Lambert G, Kroj T, Giraudat J, Parcy F. 2004. Analysis of an activated AbI3 allele using a new selection method for transgenic Arabidopsis seeds. FEBS Letters 561, 127–131.

Bernaudat F, Frelet-Barrand A, Pochon N, et al. 2011. Heterologous expression of membrane proteins: choosing the appropriate host. PLoS One 6, e29191.

Brandt R, Keston AS. 1965. Synthesis of diacetyl dichlorofluorescin: a stable reagent for fluorometric analysis. Analytical Biochemistry 11, 6–9.

Braun P, Hu Y, Shen B, Halleck A, Koundinya M, Harlow E, LaBaer J. 2002. Proteome-scale purification of human proteins from bacteria. Proceedings of the National Academy of Sciences, USA 99, 2654–2659.

Bürstenbinder K, Rzewuski G, Wirtz M, Hell R, Sauter M. 2007. The role of methionine recycling for ethylene synthesis in Arabidopsis. The Plant Journal 49, 238–249.

Cao MJ, Wang Z, Zhao Q, Mao JL, Speiser A, Wirtz M, Hell R, Zhu JK, Xiang CB. 2014. Sulfate availability affects ABA levels and germination response to ABA and salt stress in Arabidopsis thaliana. The Plant Journal 77, 604–615.

Chu CC, Lee WC, Guo WY, Pan SM, Chen LJ, Li HM, Jinn TL. 2005. A copper chaperone for superoxide dismutase that confers three types of copper/zinc superoxide dismutase activity in Arabidopsis. Plant Physiology 139, 425–436.

Clough SJ, Bent AF. 1998. Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. The Plant Journal 16, 735–743.

Cui J, Bahrami AK, Pringle EG, Hernandez-Guzman G, Bender CL, Martin GB, Cunnac S, Chakravarthy S, Kvitko BH, Russell AB, Martin GB, Collmer A. 2011. Genetic disassembly and combinatorial reassembly identify a minimal functional repertoire of type III effectors in *Pseudomonas syringae* symbioses. Proceedings of the National Academy of Sciences, USA 108, 2975–2980.

Curtis MD, Grossniklaus U. 2003. A gateway cloning vector set for high-throughput functional analysis of genes in plants. Plant Physiology 133, 462–469.

Cutler SR, Ehnhardt DW, Griffits JS, Somerville CR. 2000. Random GFP::cDNA fusions enable visualization of subcellular structures in cells of Arabidopsis at a high frequency. Proceedings of the National Academy of Sciences, USA 97, 3718–3723.

Daszkowska-Golec A, Szarejko I. 2013. Open or close the gate—roles of methionine recycling for ethylene synthesis in Arabidopsis. The Plant Journal 74, 1017–1027.

Desikan R, Cheung MK, Bright J, Henson D, Hancock JT, Neill SJ. 2004. ABA, hydrogen peroxide and nitric oxide signalling in stomatal guard cells. Journal of Experimental Botany 55, 205–212.

de Torres Zabala M, Llittlejohn GJ, Jaworska S, et al. 2015. Chloroplasts play a central role in plant defence and are targeted by pathogen effectors. Nature Plants 1, 15074.

Dreze M, Monachello D, Lurin C, Cusick ME, Hill DE, Vidal M, Braun P. 2010. High-quality binary interactome mapping. Methods in Enzymology 470, 281–315.

Fabro G, Steinberg MT, Coates M, et al. 2011. Multiple candidate effectors from the oomycete pathogen *Hyaloperonospora arabidopsidis* suppress host plant immunity. PLoS Pathogens 7, e1002348.

García-Molina A, Xing S, Huijser P. 2014. Functional characterisation of Arabidopsis SPL7 conserved protein domains suggests novel regulatory mechanisms in the Cu deficiency response. BMC Plant Biology 14, 231.

Gassmann W. 2005. Natural variation in the Arabidopsis response to the avirulence gene hopPsyA uncouples the hypersensitive response from disease resistance. Molecular Plant-Microbe Interactions 18, 1054–1060.

Gudesblat GE, Torres PS, Vojnov AA. 2009. Xanthomonas campestris overcomes Arabidopsis stomatal innate immunity through a DSF cell-to-cell signal-regulated virulence factor. Plant Physiology 149, 1017–1027.

Han JD, Bertin N, Hao T, et al. 2004. Evidence for dynamically organized modularity in the yeast protein–protein interaction network. Nature 430, 88–93.

Hawkesford M, Horst W, Kichey T, Lambers H, Schjoerring J, Moller IS, White P. 2012. Functions of macronutrients. In: Marschner P, ed. Marschner’s mineral nutrition of higher plants, 3rd edn. San Diego: Academic Press, 135–189.

Hurley B, Lee D, Mott A, Wilton M, Liu J, Liu YG, Angers S, Coaker G, Gutman TS, Desveaux D. 2014. The *Pseudomonas syringae* type III effector HopF2 suppresses Arabidopsis stomatal immunity. PLoS One 9, e114921.

Ishiga Y, Ishiga T, Uppalapati SR, Mysore KS. 2011. Arabidopsis seedling flood-inoculation technique: a rapid and reliable assay for studying plant–bacterial interactions. Plant Methods 7, 32.

Jones JD, Dangl JL. 2006. The plant immune system. Nature 444, 323–329.

Jukes TH, Cantor CR. 1969. Evolution of protein molecules. New York: Academic Press, 21–132.

Karmokr JKL, Clarkson DT, Saker LR, Rooney JH, Purves JV. 1991. Sulphate deprivation depresses the transport of nitrogen to the xylem and the hydraulic conductivity of barley (Hordeum vulgare L) roots. Planta 185, 269–278.

Kissoudis C, Chowdhury R, Heusden S, Wiel C, Finkers R, Visser RGF, Bai Y, Linden G. 2015. Combined biotic and abiotic stress resistance in tomato. Euphytica 202, 317–332.

Kliebenstein DJ, Monde RA, Last RL. 1998. Superoxide dismutase in Arabidopsis: an ecletic enzyme family with disparate regulation and protein localization. Plant Physiology 118, 637–650.

Koorn MJ, Schell J. 1986. The promoter of TL- DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of Agrobacterium binary vector. Molecular and General Genetics 204, 383–396.

Kuo WY, Huang CH, Liu AC, Li SH, Chang WC, Weiss C, Azem A, Jinn TL. 2013. CHAPERONIN 20 mediates iron superoxide dismutase (FeSOD) activity independent of its co-chaperonin role in Arabidopsis chloroplasts. New Phytologist 197, 99–110.

Lewandowska M, Wawrzyńska A, Moniuszko G, et al. 2010. A contribution to identification of novel regulators of plant response to sulfur deficiency: characteristics of a tobacco gene UP9C, its protein product and the effects of UP9C silencing. Molecular Plant 3, 347–360.

Lozano-Durán R, Bourdais G, He SY, Bobatzek S. 2014. The bacterial effector HopM1 suppresses PAMP-triggered oxidative burst and stomatal immunity. New Phytologist 202, 259–269.

Luk E, Carroll M, Baker M, Cioutta VC. 2003. Manganese activation of superoxide dismutase 2 in Saccharomyces cerevisiae requires MT1, a member of the mitochondrial carrier family. Proceedings of the National Academy of Sciences, USA 100, 10353–10357.

Maruyama-Nakashita A, Inoue E, Watanabe-Takahashi A, Yamaya T, Takahashi H. 2003. Transcriptome profiling of sulfur-responsive genes in Arabidopsis reveals global effects of sulfur nutrition on multiple metabolic pathways. Plant Physiology 132, 597–605.
Melotto M, Underwood W, Koczan J, Nomura K, He SY. 2006. Plant stomata function in innate immunity against bacterial invasion. Cell 126, 969–980.

Miao Y, Lv D, Wang P, Wang XG, Chen J, Miao C, Song CP. 2006. An Arabidopsis glutathione peroxidase functions as both a redox sensor and a scavenger in abscisic acid and drought stress responses. The Plant Cell 18, 2749–2764.

Miras S, Salvi D, Ferro M, Grunwald D, Garin J, Joyard J, Rolland N. 2002. Non-canonical transit peptide for import into the chloroplast. Journal of Biological Chemistry 277, 47770–47778.

Miras S, Salvi D, Piette L, Seigneurin-Berny D, Grunwald D, Reinboth C, Joyard J, Reinboth S, Rolland N. 2007. Toc159- and Toc75-independent import of a transit sequence-less precursor into the inner envelope of chloroplasts. Journal of Biological Chemistry 282, 29482–29492.

Mukhtar MS, Carvunis AR, Dreze M, et al. 2011. Independently evolved virulence effectors converge onto hubs in a plant immune system network. Science 333, 596–601.

Myouga F, Hosoda C, Umezawa T, Izumi H, Kuromori T, Motohashi R, Shono Y, Nagata N, Ikeuchi M, Shinozaki K. 2008. A heterocomplex of iron superoxide dismutases defends chloroplast nucleoids against oxidative stress and is essential for chloroplast development in Arabidopsis. The Plant Cell 20, 3148–3162.

Nikiforova V, Freitag J, Kempa S, Adamik M, Hesse H, Hoefgen R. 2003. Transcriptome analysis of sulfur depletion in Arabidopsis thaliana: interlacing of biosynthetic pathways provides response specificity. The Plant Journal 33, 633–650.

Pei ZM, Murata Y, Benning G, Thomine S, Klüsener B, Allen GJ, Grill E, Schroeder JI. 2000. Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. Nature 406, 731–734.

Prasch CM, Sonnewald U. 2013. Simultaneous application of heat, drought, and virus to Arabidopsis plants reveals significant shifts in signalling networks. Plant Physiology 162, 1849–1866.

Rasmussen S, Barah P, Suarez-Rodriguez MC, Bressendorff S, Friis P, Costantino P, Bones AM, Nielsen HB, Mundy J. 2013. Transcriptome responses to combinations of stresses in Arabidopsis. Plant Physiology 161, 1783–1794.

Schwab R, Ossowski S, Riester M, Warthmann N, Weigel D. 2006. Highly specific gene silencing by artificial microRNAs in Arabidopsis. The Plant Cell 18, 1121–1133.

Suzuki N, Rivero RM, Shulaev V, Blumwald E, Mittler R. 2014. Abiotic and biotic stress combinations. New Phytologist 203, 32–43.

Tanaka Y, Sano T, Tamaoki M, Nakajima N, Kondo N, Hasezawa S. 2005. Ethylene inhibits abscisic acid-induced stomatal closure in Arabidopsis. Plant Physiology 138, 2337–2343.

Tanaka Y, Sano T, Tamaoki M, Nakajima N, Kondo N, Hasezawa S. 2006. Cytokinins and auxin inhibit abscisic acid-induced stomatal closure by enhancing ethylene production in Arabidopsis. Journal of Experimental Botany 57, 2259–2266.

Terry N. 1976. Effects of sulfur on the photosynthesis of intact leaves and isolated chloroplasts of sugar beets. Plant Physiology 57, 477–479.

Triky–Dotan S, Yermiyahu U, Katan J, Gamliel A. 2005. Development of crown and root rot disease of tomato under irrigation with saline water. Phytopathology 95, 1438–1444.

Van Aken O, Whelan J. 2012. Comparison of transcriptional changes to chloroplast and mitochondrial perturbations reveals common and specific responses in Arabidopsis. Frontiers in Plant Science 3, 281.

Vandenaabere S, Van Der Kelen K, Dat J, et al. 2003. A comprehensive analysis of hydrogen peroxide-induced gene expression in tobacco. Proceedings of the National Academy of Sciences, USA 100, 16113–16118.

Wang WH, Yi XQ, Han AD, Liu TW, Chen J, Wu FH, Dong XJ, He JX, Pei ZM, Zheng HL. 2012. Calcium-sensing receptor regulates stomatal closure through hydrogen peroxide and nitric oxide in response to extracellular calcium in Arabidopsis. Journal of Experimental Botany 63, 177–190.

Watkins JM, Hechler PJ, Munday GK. 2014. Ethylene-induced flavonol accumulation in guard cells suppresses reactive oxygen species and moderates stomatal aperture. Plant Physiology 164, 1707–1717.

Weßling R, Epple P, Altmann S, et al. 2014. Convergent targeting of a common host protein-network by pathogen effectors from three kingdoms of life. Cell Host and Microbe 16, 364–375.

Withers J, Yao J, Mcecy C, Howe GA, Melotto M, He SY. 2012. Transcription factor-dependent nuclear localization of a transcriptional repressor in jasmonate hormone signaling. Proceedings of the National Academy of Sciences, USA 109, 20148–20153.

You MP, Colmer TD, Barbetti MJ. 2011. Saliency drives host reaction in Phaseolus vulgaris (common bean) to Macrophomina phaseolina. Functional Plant Biology 38, 984–992.

Yu H, Braun P, Yildirim MA, et al. 2008. High-quality binary protein interaction map of the yeast interactome network. Science 322, 104–110.

Zhang X, Zhang L, Dong F, Gao J, Galbraith DW, Song CP. 2001. Hydrogen peroxide is involved in abscisic acid-induced stomatal closure in Vicia faba. Plant Physiology 126, 1438–1448.

Zou JJ, Li XD, Ratnasekera D, Wang C, Liu WX, Song LF, Zhang WZ, Wu WH. 2015. Arabidopsis CALCULUS-DEPENDENT PROTEIN KINASES and CATALASE3 function in abscisic acid-mediated signaling and H2O2 homeostasis in stomatal guard cells under drought stress. The Plant Cell 27, 1445–1460.