Sorghum Extracellular Leucine-Rich Repeat Protein SbLRR2 Mediates Lead Tolerance in Transgenic Arabidopsis

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A sorghum pathogen-inducible gene predicted to encode a simple extracellular leucine-rich repeat (LRR) protein SbLRR2 was previously isolated. LRR was the only domain identified in SbLRR2 and its homologous sequences. Phylogenetic analysis revealed that they are distinct from the simple extracellular LRR proteins reported previously. Agrobacterium-mediated transient expression in tobacco leaf cells demonstrated that the SbLRR2–EYFP (enhanced yellow fluorescent protein) fusion protein was targeted to the extracellular space. Transgenic analysis of SbLRR2 revealed its role in enhancing lead [Pb(II)] tolerance in Arabidopsis. Consequently, SbLRR2-overexpressing lines were found to show alleviated Pb(II)-induced root inhibition, lower levels of Pb(II) accumulation and enhanced transcription of AtPDR12 which encodes a plasma membrane ATP-bind cassette (ABC)-type transporter formerly shown to contribute to Pb(II) detoxification. However, all the Pb(II) tolerance responses were abolished when SbLRR2 was overexpressed in an atpdr12 T-DNA insertion line. The extracellular localization of SbLRR2 was also shown to be essential for the Pb(II) phenotypes and AtPDR12 up-regulation. Taken together, SbLRR2 appears to mediate Pb(II) tolerance through the elevation of AtPDR12 expression in transgenic Arabidopsis, thus activating a glutathione-independent mechanism for detoxification. Further investigations revealed the Pb(II)-induced transcriptional activation of SbLRR2 and several highly conserved AtPDR12 homologs in sorghum seedlings, suggesting the possibility of a common molecular mechanism for Pb(II) tolerance in diverse plant species.

Keywords: ABC transporter • Arabidopsis • Lead tolerance • Simple extracellular LRR protein • Sorghum.

Abbreviations: ABC, ATP-binding cassette; BR, brassinosteroid; BSO, buthionine sulfoximine; CaMV, Cauliflower mosaic virus; EYFP, enhanced yellow fluorescent protein; LRR, leucine-rich repeat; MS, Murashige and Skoog; OE, overexpression; PGIP, polygalacturanase-inhibiting protein; RLK, receptor-like kinase; RT–PCR, reverse transcription–PCR; SERK, somatic embryogenesis receptor-like kinase; SP, signal peptide; T-DNA, transfer DNA; WT, wild-type.

Introduction

Lead [Pb(II)] is one of the most widespread hazards threatening living organisms and the environment (Zeng et al. 2007). It occurs naturally in soil by weathering, but heavy contamination usually comes from human activities (e.g. mining) and decomposition of Pb(II)-containing organic substrates (Sharma and Dubey 2005). In general, Pb(II) exerts in vivo toxicity by inactivating enzymes and antioxidants through binding of sulfide groups and displacement of enzyme cofactors (Ercal et al. 2001). These would increase the production of reactive oxygen species in cells and in turn induce oxidation of lipids, proteins and DNA, resulting in membrane damage, protein malfunction and DNA impairment (Ercal et al. 2001). Pb(II) is particularly problematic in agriculture since it is non-essential and detrimental to plant growth (Clemens 2001). It inhibits photosynthesis, affects nutrient uptake, upsets mitosis, changes phytohormone production and disrupts cellular water balance (Sharma and Dubey 2005). Pb(II)-poisoned plants usually show stunted root and stem growth, leaf chlorosis and root darkening, thus reducing crop yield and quality substantially (Sharma and Dubey 2005). Furthermore, it is well known that humans and animals suffer from chronic and harmful illnesses following the consumption of heavy metal-contaminated food. For example, Pb(II) adversely affects the functions of most organs and the development of the brain and the nervous system in humans.

Different mechanisms coping with heavy metal stresses have been described in plants. Endogenous metal-binding molecules, such as glutathione, are widely used to compartmentalize and detoxify heavy metal ions including Pb(II) (Mehra et al. 1995, Li et al. 1997). Interestingly, the Arabidopsis (Arabidopsis thaliana) acyl-CoA binding protein ACBP1 was found to bind Pb(II) ions in vitro, and ACBP1 overexpression resulted in enhanced Pb(II) tolerance and increased Pb (II) accumulation...
in the transgenic plants (Xiao et al. 2008). These plants are potentially useful for phytoremediation (Sharma and Dubey 2005), providing an economical and environmental approach for the removal of Pb(II) from contaminated soil (Song et al. 2003). On the other hand, heavy metal contents inside plant cells could be minimized by uptake inhibition, chelation of extracellular ions or active efflux pumping (Yang et al. 2000, Sharma and Dubey 2005, Krämer et al. 2007). The Arabidopsis AtPDR12 is an ATP-binding cassette (ABC) transporter demonstrated to be associated with reduction of Pb(II) accumulation following exogenous application (Lee et al. 2005). Subsequently, a rice PiB-type heavy metal ATPase, OsHMA9, was also shown to have a similar function (Lee et al. 2007). These removal mechanisms, if optimized, would be extremely helpful in preventing Pb(II) ion from entering the food chain.

Our laboratory has been investigating defense responses in sorghum (Sorghum bicolor), and a pathogen-inducible gene predicted to encode an extracellular simple leucine-rich repeat (LRR)-containing protein (SbLRR2) was recently identified. In general, LRR domains are involved in protein–protein interactions or protein–ligand interactions (Kobe and Deisenhofer 1994, Kobe and Kajava 2001). Different classes of LRR-containing proteins have been described in plants (Zhou et al. 2009). For example, the receptor-like kinases (RLKs) contain a transmembrane α-helical structure and an intracellular kinase domain. On the other hand, the RLK-like proteins (RLPs) lack the internal kinase although they are structurally similar to the RLKs. Both RLKs and RLPs are involved in a diverse array of biological processes, such as innate immunity, phytohormone responses, cell proliferation or self-incompatibility in plants (Tor et al. 2009). Furthermore, the LRR-containing extracellular polygalacturanase-inhibiting proteins (PGLPs) could recognize and bind fungal polygalacturonases, preventing cell wall degradation and inducing defense responses in grapevine (Alexandersson et al. 2011). SbLRR2 belongs to a group of small extracellular LRR proteins (~250 amino acids) with a single LRR (4–6 repeats) domain that is highly conserved with the extracellular LRR domain of RLKs such as the somatic embryogenesis RLK (SERK) proteins (Li 2010).

Simple LRR-containing homologous proteins have been reported in other plant species, including rice OsLRR1 (Zhou et al. 2009), tomato LPR (Tornero et al. 1996), tobacco NtLPR1 (Jacques et al. 2006) and pepper CaLRR (Jung et al. 2004). Similarly, OsLRR1 and related sequences are mostly associated with plant defense responses, but, unlike SbLRR2, an additional leucine zipper domain is present upstream of their LRR regions (Zhou et al. 2009). Thus, SbLRR2 is structurally distinct from most of the simple LRR proteins previously described. To explore the potential physiological functions of SbLRR2, we overexpressed the sorghum gene in transgenic Arabidopsis for a variety of phenotypic analyses. In the present study, we provide evidence for a novel role for SbLRR2 in Pb(II) detoxification. Overexpression of SbLRR2 in Arabidopsis resulted in enhanced Pb(II) tolerance and reduced Pb(II) accumulation in the transgenic plants. Furthermore, the glutathione-independent AtPDR12 detoxification pathway was demonstrated to be essential for SbLRR2-mediated Pb(II) tolerance in Arabidopsis.

**Results**

**Structural domains, sequence analysis and phylogenetic relationships of SbLRR2**

Recently we initiated an investigation on a simple extracellular LRR protein (SbLRR2) which was identified among a collection of fungal pathogen-induced sorghum cDNA clones generated by suppression subtractive hybridization (Li 2011) (Supplementary Fig. S1). SbLRR2, which is located on chromosome 5 (Sb05g018800), is a single-copy gene possessing seven introns and eight exons (http://www.phytozome.net/sorghum). The encoded protein contains 247 amino acids with a predicted N-terminal signal peptide (SP; 0.999 probability by Signal 3.0; http://www.cbs.dtu.dk/services/SignalP/). Protein–protein BLAST searches retrieved two highly conserved homologs from maize (82% identity; ZmLRR2) and rice (74% identity; OsLRR2). The OsLRR2-encoding gene, Os11g31540, was found to have the same exon/intron structures (http://rice.plantbiology.msu.edu/) as SbLRR2. All three LRR2 proteins contain approximately 250 amino acids with six perfect/imperfect LRR motifs in the consensus sequence DLxxNx:SGxIPxxLGx LxxLxxL (Kobe and Kajava 2001) (Fig. 1). Their LRR domains share strong homology to the extracellular domains of SERK family proteins. For example, SbLRR2 is 61% identical to a 158 residue extracellular region in Arabidopsis AtSERK1.

Similar to most of the simple LRR proteins reported previously, SbLRR2 and its homologs lack the transmembrane and kinase domains (Fig. 1B). However, phylogeny analysis (Fig. 1C) placed them in a cluster distinct from OsLRR1. In fact, the leucine zipper domain upstream of the LRR region in OsLRR1 and related sequences is not present in SbLRR2 and its homologs (Fig. 1B). The sorghum gene Sb03g037580 was instead found to encode a protein (SbLRR1) highly homologous to OsLRR1 (97% identity) containing the same domain structures. Interestingly, unlike OsLRR1, there are no highly conserved homologs (>70% sequence identity) of SbLRR2 in dicot species, such as Arabidopsis or tomato. Apparently, SbLRR2 and the related cereal sequences represent a novel class of extracellular simple LRR-containing proteins in monocots.

**Subcellular localization of SbLRR2**

As described above, SbLRR2 was predicted to be an extracellular protein with a signal peptide. To determine its subcellular localization in planta, we constructed a binary vector containing the SbLRR2–enhanced yellow fluorescent protein (EYFP) fusion sequence for transient expression in tobacco leaf cells. To confirm the targeting function of the signal peptide, we also generated the SP (SbLRR2 signal peptide-encoding sequence)–EYFP and SbΔSPLRR2 (SbLRR2 without the SP sequence)–EYFP fusion sequences. The above constructs were introduced into Agrobacterium for infiltration of tobacco...
leaves. Following mannitol treatment, the plasmolyzed cells were examined by confocal microscopy (Fig. 2A). In cells expressing the SbLRR2–EYFP and SP–EYFP fusions, fluorescence was clearly visible in the extracellular space (Fig. 2A). On the other hand, in plasmolyzed cells expressing Sb/C1 SPLRR2–EYFP and EYFP, the fluorescent signals were contained in shrinking protoplasts (Fig. 2B). Thus, our data demonstrated that SbLRR2 is an extracellular protein and the SP is required for its subcellular localization. Similarly, fluorescent signals were detected in the extracellular space when the fusion construct was transiently expressed in onion epidermal cells by particle bombardment (Supplementary Fig. S2).

Arabidopsis plants overexpressing SbLRR2 exhibit enhanced Pb(II) tolerance

Transgenic Arabidopsis plants were generated to investigate the physiological functions of SbLRR2 in a heterologous system. As part of our initial abiotic stress screening experiments, the SbLRR2 overexpression (OE) lines were assayed against two non-essential heavy metals, cadmium and lead. Both of them are highly toxic when accumulated inside plants and animals. In the toxicity assays, Arabidopsis plants showed typical reduction in root growth on Murashige and Skoog (MS) medium supplemented with Pb(NO3)2 or CdCl2 (Fig. 3, 4). Root growth of the OE lines was inhibited to a similar extent to that in the wild-type (WT) plants in the presence of Cd(II) (Fig. 3). On the other hand, the OE plant roots appeared substantially longer than the WT roots by almost 1-fold on the Pb(II)-supplemented medium (Fig. 4B). The enhanced Pb(II) tolerance was also observed in the OE lines when tested at other concentrations and time points (Supplementary Fig. S3). Furthermore, the fresh weight of the OE plant roots was significantly higher compared with the WT plants in the presence of Pb(II) (Fig. 4C). When Arabidopsis plants were transformed with the OE-Sp-LRR construct in which the SP-encoding sequence was removed, the Pb(II) tolerance of the OE lines was not observed (data not shown).

Fig. 1 SbLRR2 sequence analysis. (A) Alignment of SbLRR2 with its highly conserved homologs in maize (ZmLRR2; GenBank accession No. DAA42012) and rice (OsLRR2; Os11g31540). All these sequences contain an N-terminal signal peptide and six LRR repeats as indicated. (B) Comparison of the structural domains in OsLRR1 (Zhou et al. 2009) and SbLRR2. Both proteins contain a signal peptide (SP). Note the absence of a leucine zipper (LZ) but the presence of two additional LRR repeats in SbLRR2. (C) An unrooted phylogeny tree of SbLRR2 and different extracellular simple LRR-containing proteins. The tree was constructed by MEGA4 based on the Neighbor–Joining method. Note that SbLRR2 is clustered in a group distinct from OsLRR1 and related sequences including SbLRR1 (Sb03g037580), Arabidopsis AtLRR1 (GenBank accession No. AAG40341), tobacco NtLPR1 (GenBank accession No. AAZ91738) and tomato LeLRP (GenBank accession No. CA6A664565).
The resulting transgenic lines were found to show Pb(II) inhibitory responses similar to those of the WT plants (Fig. 4). Thus, the extracellular location of SbLRR is apparently required for the expression of Pb(II) tolerance in the OE lines.

SbLRR2-mediated Pb(II) tolerance in Arabidopsis requires AtPDR12

Vacuolar sequestration and efflux pumping are two opposite mechanisms previously reported for Pb(II) tolerance in plants (Shrama et al. 2000, Gravotz et al. 2004, Lee et al. 2005). As a precursor of phytochelation which can bind Pb(II) ions, glutathione is required for detoxification via vacuolar sequestration (Mehra et al. 1995, Li et al. 1997). To investigate whether the SbLRR2-mediated Pb(II) tolerance in Arabidopsis is dependent on glutathione, buthionine sulfoximine (BSO), an inhibitor of glutathione synthesis, was included in the Pb(II)-containing MS medium. As shown in Fig. 5A, Pb(II)-induced root inhibition in both WT and OE Arabidopsis lines was elevated in the presence
of BSO, which was depleting the endogenous glutathione sources. However, the OE line still showed longer root growth than the WT under such conditions, suggesting the presence of a glutathione-independent pathway for Pb(II) tolerance. Subsequently, Pb(II) contents were measured in 3-week-old plants following 48 h of root immersion in 1 mM Pb(NO₃)₂ solution as described previously (Kim et al. 2006, Xiao et al. 2008). As shown in Fig. 5B and C, Pb(II) accumulation in roots and shoots was significantly lower in the OE lines when compared with the WT plants. Similarly, lower Pb(II) contents were detected in the OE lines when treated under the same conditions as the growth assays described above (Supplementary Fig. S3). Hence, an exclusion mechanism was likely to be involved in the SbLRR2 transgenic plants for the enhanced Pb(II) tolerance.

In Arabidopsis, AtPDR12 is a plasma membrane ABC transporter demonstrated to contribute to Pb(II) detoxification (Lee et al. 2005). Real-time PCR was then employed to examine AtPDR12 expression levels in the different transgenic lines. As shown in Fig. 6, AtPDR12 expression in the OE plants was constitutively up-regulated when compared with the WT plants. In contrast, no AtPDR12 induction was detected in the SbΔSPLRR2 OE plants in which the SP of SbLRR was removed, indicating that proper targeting of SbLRR2 is important for the observed gene activation. Similarly, Pb(II)-inducible AtPDR12 expression in the OE lines was significantly higher than in the WT and SbΔSPLRR2 OE plants.

To elucidate whether the enhanced Pb(II) tolerance in the OE lines is dependent on AtPDR12, SbLRR2 was transformed into the atpdr12 mutant (SALK_005635) for phenotypic characterization. In the presence of Pb(II), the atpdr12 mutant showed attenuated root growth (Fig. 7) as reported previously (Lee et al. 2005). However, overexpression of SbLRR2 did not improve Pb(II) tolerance in the transgenic mutant, in contrast
to the OE lines in the WT background. Thus, the extent of root inhibition was similar to that in the atpdr12 mutant (Fig. 7C). Also, the Pb(II) contents detected in the SbLRR2-OE-atpdr12 and atpdr12 plants after Pb(II) treatment were similar and were both significantly higher than those detected in the WT and OE lines (Fig. 7D). Apparently, the SbLRR2-mediated Pb(II) tolerance in transgenic Arabidopsis was abolished when AtPDR12 was not functional.

**Pb(II)-inducible expression of SbLRR2 and AtPDR12 homologs in sorghum**

To investigate whether SbLRR2 is involved in Pb(II) responses in sorghum, RNA samples were prepared from sorghum (cv. DK18) seedlings grown in the presence of Pb(NO₃)₂. As shown in Fig. 8A, SbLRR2 expression was strongly induced by Pb(II) in roots after 24 h of treatment. Furthermore, bioinformatics analysis of the sorghum genome (www.phytozome.net) retrieved nine sequences encoding proteins which are highly homologous to AtPDR12 (63–79% identity) (Supplementary Fig. S5). Interestingly, six of them showed different levels of enhanced gene expression in roots of sorghum seedlings upon Pb(II) treatment (Fig. 8B). The remaining three genes, which are less homologous to AtPDR12 (<74% identity), did not show any significant Pb(II)-inducible expression.

**Discussion**

In plants, simple extracellular LRR proteins were primarily reported to be involved in defense responses. For example, tomato LPR was proteolytically processed to a lower molecular weight form by a host-induced extracellular protease during pathogenesis, suggesting that it participated in some recognition or interaction events in the extracellular matrix (Tornero et al. 1996). In addition, tobacco NtLRP1 was shown to be a modulator of the hypersensitive response (Jacques et al. 2006). Recently, rice OsLRR1 was demonstrated to enhance resistance toward *Pseudomonas syringae* in transgenic Arabidopsis (Zhou et al. 2009). Similarly, SbLRR2 was initially identified as a
SbLRR2 mediates lead tolerance in Arabidopsis

Mechanisms for Pb(II) resistance or tolerance in plants are not well understood, and only a few molecular components have been identified and characterized. In Arabidopsis, genes/proteins implicated in either a sequestration or an exclusion strategy for coping with exogenous Pb(II) stress have been described. The acyl-CoA-binding protein ACBP1 is able to bind Pb(II) in vitro and it mediates Pb(II) tolerance with the accumulation of Pb(II) in shoots, thus representing a good candidate for applications in phytoremediation (Xiao et al. 2008). ACBP1 was suggested to be associated with Pb(II) trafficking and sequestration. In plants, glutathione-conjugated vacuolar sequestration is an important pathway of heavy metal resistance (Grill et al. 1989). Several ABC transporters in Arabidopsis, including AtATM3, AtPDR8 and AtPDR12, were reported to be associated with Pb(II) tolerance (Lee et al. 2005, Kim et al. 2006, Kim et al. 2007). AtATM3 and AtPDR8 are Pb(II) and Cd inducible, and their overexpression resulted in improved tolerance against both heavy metals. Experimental evidence suggested that AtATM3 may mediate glutathione-conjugated Cd(II) transport across the mitochondrial membrane (Kim et al. 2006), while AtPDR8 is potentially involved as an efflux pump of Cd(II) or Cd conjugates at the plasma membrane (Kim et al. 2007). However, the functional roles of AtATM3 and AtPDR8 in Pb(II) tolerance were not described (Kim et al. 2006, Kim et al. 2007). On the other hand, AtPDR12 was conclusively demonstrated to contribute to glutathione-independent Pb(II)
detoxification in Arabidopsis (Lee et al. 2005). AtPDR12 was initially suggested to serve as an efflux pump for Pb(II) exclusion. Consistently, AtPDR12-overexpressing Arabidopsis plants were more Pb(II) resistant and had lower Pb(II) contents than WT plants when grown on Pb(II)-containing medium (Lee et al. 2005).

Our investigations revealed that SbLRR2 conferred Pb(II) tolerance in transgenic Arabidopsis with reduced accumulation of Pb(II) following exogenous application (Fig. 5), suggesting that an exclusion mechanism was activated. Such a phenotype is highly desirable since heavy metal contamination of soil adversely affects plant growth and results in biological amplification along food chains. In addition, the SbLRR2-mediated Pb(II) detoxification in transgenic Arabidopsis is dependent on AtPDR12, as evidenced by the gene expression and transgenic mutant analyses (Figs. 6, 7). Therefore, our results further strengthened the contribution of AtPDR12 to Pb(II) removal in Arabidopsis. Interestingly, AtPDR12 (ABCG40) was recently demonstrated to function as a plasma membrane ABA uptake transporter (Kang et al. 2010), while direct evidence for its role as a Pb(II) efflux pump remains unavailable. It was also proposed that ABA reduced root to shoot translocation of heavy metal through stomatal closure and regulated the expression of genes involved in heavy metal tolerance (Kang et al. 2010).

Our results also demonstrated that the extracellular localization of SbLRR2 is essential for its functional expression in transgenic Arabidopsis. Thus, plants transformed with an overexpression construct devoid of the signal peptide sequence showed WT-like Pb(II) responses and a lack of AtPDR12 up-regulation (Figs. 4, 6). The corresponding EYFP fusion protein was retained intracellularly (Fig. 2). The overexpression of several extracellular LRR proteins in transgenic plants is known to activate the expression of endogenous genes which are associated with the phenotypes conferred by the transgene. For example, OsLRR1-overpressing Arabidopsis plants with enhanced bacterial resistance were found to show elevated basal expression of several defense-related genes, including PR1, PR2 and PDF1.2 (Zhou et al. 2009). When the same gene was transformed to Chinese cabbage, it resulted in enhanced resistance against bacterial soft rot along with elevated expression of PGIP2, PDF1 and a gene encoding glucanase (Park et al. 2012).

An intriguing question remains regarding the underlying mechanism for the SbLRR2-dependent up-regulation of AtPDR12 expression in our transgenic Arabidopsis plants. A large number of RLKs in plants have been demonstrated to perform the dual role on reception and activation of signals in innate immunity or developmental processes (Padmanabhan et al. 2009, Tor et al. 2009). However, SbLRR2 is structurally different by having only the LRR domain, albeit with strong homology to the extracellular LRR domains of the plant RLKs. Presumably, SbLRR2 may interact directly or indirectly with some surface receptors through its LRR domain, thereby transducing the signal intracellularly for the endogenous gene activation. Recently, OsLRR1 was found to interact with OsHIR1 which is localized on the plasma membrane (Zhou et al. 2009), and OsHIR1 was believed to enhance disease resistance by triggering a hypersensitive response (Zhou et al. 2010). It remains to be investigated whether SbLRR2-mediated Pb(II) tolerance requires interactions with any molecular components in transgenic Arabidopsis.

The newly revealed function of SbLRR2 as a transgene in Arabidopsis prompted us to examine its relationship with Pb(II) responses as an endogenous gene in sorghum. Consistently, SbLRR2 expression in roots of sorghum seedlings was demonstrated to be stimulated 24 h following Pb(II) treatment (Fig. 8). The fact that SbLRR2 from sorghum could elevate AtPDR12-dependent Pb(II) tolerance in Arabidopsis strongly suggests the presence of a common molecular mechanism for detoxification in both species. Interestingly, there are six sorghum genes encoding proteins with >75% sequence identity to AtPDR12, and their expression levels showed different extents of elevation in sorghum roots following Pb(II) exposure (Fig. 8). Thus, it is tempting to suggest that SbLRR2 also mediates Pb(II) tolerance in sorghum through transcriptional activation of the
PDR12 homologs. Particularly noteworthy is that SbLRR2 and related sequences do not have highly conserved homologs in dicots. Future investigations may reveal the presence of phylogenetically distinct extracellular proteins that are involved in the stimulation of PDR12 gene expression via similar molecular mechanisms.

**Materials and Methods**

**Plant material and growth conditions**

Tobacco (*Nicotiana tabacum* cv. SR1) plants were grown in a greenhouse 23–25°C with a light/dark cycle of 16/8 h. Arabidopsis (*Arabidopsis thaliana*) WT (Col-0) and *atpdr12* T-DNA mutant (SALK_005328, Arabidopsis Biological Resource Center), and transgenic seeds were surface-sterilized, placed in the dark at 4°C for 2 d and then germinated on MS (Sigma) agar plates containing 2% sucrose (w/v). The plates were kept in a culture room at 23°C with a light/dark cycle of 16/8 h. Sorghum seeds were planted in rolls of germination paper and kept at 28°C for 2 d before treatment.

**Transient expression of EYFP fusions in tobacco leaves**

The SbLRR2 coding region, with or without the SP sequence, was cloned in-frame with an EYFP gene under the control of the 35S promoter in a pCAMBIA-based vector (Tang et al. 2009) to generate the fusion construct 35S-SbLRR-EYFP. Other fusion constructs (35S-SbLRR2-EYFP, 35S-SbΔSPLRR2-EYFP and 35S-SbSP-EYFP) and the 35S-EYFP control were also prepared. Transformed Agrobacterium tumefaciens cells (GV3101) containing the different constructs (SbLRR2-EYFP, SbΔSPLRR2-EYFP, SbSP-EYFP and EYFP) were cultured overnight at 28°C in Luria broth (LB) with kanamycin (50 μg ml⁻¹) and gentamycin (50 μg ml⁻¹). The culture suspensions were then used to infiltrate into the lower leaf surface of 6-week-old tobacco plants. Epidermal cells were observed for fluorescent signals by confocal laser scanning microscopy (Zeiss, LSM 710). To induce plasmolysis, detached leaves were placed in 0.8 M mannitol solution and observed after 2 min.

**Generation of transgenic Arabidopsis plants**

The SbLRR2 coding region was PCR amplified and cloned into an overexpression vector (Yu et al. 2005) containing the Cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase 3’-terminator. The resulting plasmid was introduced to the binary vector pCAMBIA 1300 (CAMBIA, Australia). The SbΔSPLRR2 binary vector was constructed with the removal of the SP-encoding sequence. Agrobacterium-mediated transformation of Arabidopsis WT plants was performed by the floral dip method (Clough and Bent 1998). To generate SbLRR2 OE *atpdr12* plants, a selected OE (Col-0) line (T2 generation) was crossed to the homozygous T-DNA mutant. Transgenic mutant plants were identified by screening the F2 seeds on MS medium containing hygromycin (50 μg ml⁻¹) followed by PCR genotyping analysis.

**Pb(II)-induced root inhibition assays in Arabidopsis**

Seeds of Arabidopsis WT, OE and mutant plants were germinated on MS medium for 3 weeks. Subsequently, roots of the different plants were submerged in 0.75 mM Pb(NO3)₂ for 48 h. The plants were then washed three times with distilled water. Plant samples were harvested and digested overnight using 1 N HNO₃ at 200°C (Kim et al. 2006). The digested samples were diluted 5-fold with 0.1 N HNO₃ and the Pb(II) contents were measured using an atomic absorption spectrometer (Perkin-Elmer-AA Spectrometer 3110). For each independent plant line, six biological replicates were analyzed and each replicate contained five plants.

**Gene expression experiments**

Total RNA was extracted from different plant tissues by the RNAzol reagent (Invitrogen). RNA samples were DNase I treated (Invitrogen) and reversed transcribed by M-MLV reverse transcriptase (Promega). PCR amplifications were programmed as follows: pre-incubation (94°C for 5 min), followed by 27 cycles of 94°C for 30 s; 55°C for 45 s; 72°C for 5 s, and finalized by an extension step at 72°C for 10 min. SYBR Green Mix (Applied Biosystems) was used for quantitative PCR experiments which were conducted on the StepOne Plus real-time PCR system using the following program: pre-incubation (95°C for 10 min), followed by 40 cycles of 95°C for 15 s and 56°C for 1 min. The comparative CT value method (Schmittgen and Livak 2008) was used to determine fold changes in expression levels for the different genes under investigation.

**Statistical analysis**

The Student’s t-test (Student 1908) was used for statistical analysis of data obtained from the Pb(II) growth assays, Pb(II) content measurements and quantitative gene expression studies.

**Primer information**

Information on different primers used in cloning and gene expression experiments described above is listed in Supplementary Table S1.

**Supplementary data**

Supplementary data are available at PCP online.
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Disclosures

The authors have no conflicts of interest to declare.

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