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Isolation of Arabidopsis extracellular ATP binding proteins by affinity proteomics and identification of PHOSPHOLIPASE C-LIKE 1 as an extracellular protein essential for fumonisin B1 toxicity

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SUMMARY

ATP is secreted to the extracellular matrix, where it activates plasma membrane receptors for controlling plant growth and stress-adaptive processes. DOES NOT RESPOND TO NUCLEOTIDES 1 (DORN1), was the first plant ATP receptor to be identified but key downstream proteins remain sought after. Here, we identified 120 proteins secreted by Arabidopsis cell cultures and screened them for putative stress-responsive proteins using ATP-affinity purification. We report three Arabidopsis proteins isolated by ATP-affinity: PEROXIDASE 52, SUBTILASE-LIKE SERINE PROTEASE 1.7 and PHOSPHOLIPASE C-LIKE 1. In wild-type Arabidopsis, the expression of genes encoding all three proteins responded to fumonisin B1, a cell death-activating mycotoxin. The expression of *PEROXIDASE 52* and *PHOSPHOLIPASE C-LIKE 1* was altered in fumonisin B1-resistant *salicylic acid induction-deficient* (*sid2*) mutants. Exposure to fumonisin B1 suppressed *PHOSPHOLIPASE C-LIKE 1* expression in *sid2* mutants, suggesting that the inactivation of this gene might provide mycotoxin tolerance. Accordingly, gene knockout mutants of *PHOSPHOLIPASE C-LIKE 1* were resistant to fumonisin B1-induced death. The activation of *PHOSPHOLIPASE C-LIKE 1* gene expression by exogenous ATP was not blocked in *dorn1* loss-of-function mutants, indicating that DORN1 is not required. Furthermore, exogenous ATP rescued both the wild type and the *dorn1* mutants from fumonisin-B1 toxicity, suggesting that different ATP receptor(s) are operational in this process. Our results point to the existence of additional plant ATP receptor(s) and provide crucial downstream targets for use in designing screens to identify these receptors. Finally, PHOSPHOLIPASE C-LIKE 1 serves as a convergence point for fumonisin B1 and extracellular ATP signalling, and functions in the Arabidopsis stress response to fumonisin B1.

Keywords: Arabidopsis, extracellular ATP, fumonisin B1, extracellular matrix proteins, cell death, affinity proteomics.

INTRODUCTION

When secreted into the extracellular matrix, the high-energy molecule adenosine 5′-triphosphate (ATP) functions as an important signalling molecule. In plants this extracellular ATP activates an influx of Ca^{2+} into the cytosol (Demidchik *et al.,* 2003; Jeter *et al.,* 2004; Tanaka *et al.,* 2010) and biosynthesis of second messenger molecules, such as reactive oxygen species (Demidchik *et al.,* 2009; Song *et al.,* 2006; Wu *et al.,* 2008), nitric oxide (Foresi *et al.,* 2007; Reichler *et al.,* 2009; Tonón *et al.,* 2010; Wu and Wu, 2008) and phosphatidic acid (Sueldo *et al.,* 2010). In addition to triggering the accumulation of these signalling molecules, exogenous ATP sets off a transcriptional reprogramming of many genes (Choi *et al.,* 2014; Demidchik *et al.,* 2009; Jeter *et al.,* 2004) and remarkable changes in the growth and developmental properties of several plant organs (Demidchik *et al.,* 2009; Reichler *et al.,* 2009; Riewe *et al.,* 2008). The inhibition of Ca^{2+} influx (Jeter *et al.,* 2004; Wu and Wu, 2008) or blocking the accumulation of second messenger signal molecules (Hao *et al.,* 2012; Tonón *et al.,* 2010; Wu and Wu, 2008) prevents extracellular ATP-induced plant responses, indicating that ATP is sensed at the external cell surface to activate intracellular signalling through classical signalling intermediates.

The mechanism of extracellular ATP sensing at the plasma membrane differs between animal and plant cells.

In animal cells, extracellular ATP binds and activates purinergic 2X (P2X) and P2Y receptors, which are ATP-gated Ca^{2+} channels (Khakh and North, 2006) or heterotrimeric G-protein-coupled (Burnstock and Kennedy, 1985) plasma membrane proteins, respectively. Signals such as reactive oxygen species (Hung *et al.,* 2013), nitric oxide (Shen *et al.,* 2005) and phosphatidic acid (Pfeilschifter and Merriweather, 1993) are also mobilized in animal cells after receptor activation by extracellular ATP. DORN1, the first plant extracellular ATP receptor, was identified in Arabidopsis (Choi *et al.,* 2014) as a trans-plasma membrane receptor kinase with an extracellular ATP-binding domain and a cytosolic kinase domain. Exogenous ATP fails to activate downstream signalling in loss-of-function *dorn1* mutants (Choi *et al.,* 2014), indicating that DORN1 is an essential component of certain aspects of ATP signalling.

Part of the plant molecular machinery stimulated by extracellular ATP signalling to generate second messenger molecules is now known. For example, generation of reactive oxygen species induced by extracellular ATP in Arabidopsis occurs at the plasma membrane through the activity of the NADPH oxidase proteins; AtrbohC (Demidchik *et al.,* 2009), AtrbohD and AtrbohF (Song *et al.,* 2006). Plants devoid of these proteins become defective in their response to extracellular ATP. Thus, whereas extracellular ATP activates Ca^{2+} influx in wild-type Arabidopsis, plants in which the genes for these oxidase proteins are disrupted become insensitive to exogenous ATP and have no corresponding mobilisation of Ca^{2+} (Song *et al.,* 2006). As a consequence, the ability of extracellular ATP to activate stress-responsive genes is impaired in Arabidopsis plants with defects in these oxidase protein genes (Song *et al.,* 2006). Nitrate reductase has also been identified as a critical signal generator recruited by extracellular ATP signalling. Plant nitric oxide synthesis can occur by enzymatic conversion of nitrate to nitric oxide. Extracellular ATP-induced nitric oxide biosynthesis in Arabidopsis is carried out by the products of *NIA1* and *NIA2*, the two nitrate reductase genes of Arabidopsis. Plants with mutations in both genes fail to respond to extracellular ATP stimulation (Clark *et al.,* 2010; Reichler *et al.,* 2009), indicating that these proteins are crucial to extracellular nucleotide signalling. Finally, extracellular ATP stimulation of phosphatidic acid production is mediated via phospholipase C, phospholipase D and diacylglycerol kinase (Sueldo *et al.,* 2010). Inhibitors of these proteins block extracellular ATP-induced nitric oxide production (Sueldo *et al.,* 2010), demonstrating that they are part of the protein circuitry recruited by extracellular ATP signalling.

In plants, extracellular ATP signalling has been implicated in many processes such as root gravitropism (Tang *et al.,* 2003), pollen germination and pollen tube growth (Reichler *et al.,* 2009; Steinebrunner *et al.,* 2003), control of stomatal opening and closure (Clark *et al.,* 2011; Hao *et al.,* 2012), root nodule (McAlvin and Stacey, 2005; Tanaka *et al.,* 2011) and tuber (Riewe *et al.,* 2008) development, adaptive responses to stress (Kim *et al.,* 2009; Sun *et al.,* 2010; Thomas *et al.,* 2000) and general plant growth (Clark *et al.,* 2010; Riewe *et al.,* 2008; Tonón *et al.,* 2010; Wu *et al.,* 2007). Although still poorly understood, there is emerging evidence pointing towards a role for extracellular ATP in pathogen defence. A non-hydrolysable analogue of ATP switches on pathogen defences in tobacco (Chivasa *et al.,* 2009) and the Arabidopsis extracellular ATP receptor DOES NOT RESPOND TO NUCLEOTIDES 1 (DORN1) has been demonstrated to be an essential component of defence against fungal pathogens (Bouwmeester *et al.,* 2011; Wang *et al.,* 2016). We have previously reported that extracellular ATP also regulates plant cell death (Chivasa *et al.,* 2005). The mycotoxin fumonisin B1 (FB1) triggers programmed cell death in Arabidopsis plants and cell cultures (Chivasa *et al.,* 2005). FB1-induced cell death is preceded by depletion of extracellular ATP and can be blocked by supplying exogenous ATP (Chivasa *et al.,* 2005), indicating the critical role for extracellular ATP in this form of cell death. In tobacco cell cultures H_2O_2-induced cell death is attended by extracellular ATP depletion and can be averted by exogenous ATP (Hou *et al.,* 2020). The gene networks underpinning specific extracellular ATP physiological functions remain to be established.

Ongoing research focuses on finding additional plant extracellular ATP receptors and identifying effector proteins—the latter being extracellular matrix proteins that are regulated by directly binding ATP in the apoplast. In this study, we conducted experiments to identify putative ATP effector proteins that bind ATP in the apoplast. These proteins are potentially modulated by ATP and so could have potential functions in the physiological processes controlled by extracellular ATP. Proteomics provides an ideal platform for identifying such effector proteins of extracellular ATP functions in plants. Therefore, we employed affinity proteomics to isolate ATP binding proteins with an immobilized ATP resin and azido-ATP-biotin for photoaffinity labelling. Gene expression profiling of ATP binding protein targets in fumonisin B1-susceptible wild-type plants and fumonisin B1-resistant *sid2* mutants, followed by mycotoxin assays on gene knockout mutants, was used to screen for targets with a potential role in the Arabidopsis response to fumonisin B1. PHOSPHOLIPASE-LIKE 1 was identified as critical for the Arabidopsis cell death response to fumonisin B.

RESULTS

ATP-affinity proteomics and mass spectrometry

Proteins secreted into the growth medium of Arabidopsis cell suspension cultures were analysed using liquid chromatography and tandem mass spectrometry. High ATP

hydrolase activity in the protein fractions indicated that acetone precipitation used to recover the proteins had not denatured the proteins (Appendix S1). We identified 120 proteins belonging to a broad range of physiological processes and biochemical functional classes (Appendix S1; Tables S1 and S2). We used ATP conjugated to agarose via a covalent bond on its gamma phosphate to pull out ATP binding proteins from the extracellular matrix (ECM) fraction. The eluate from the ATP-binding resin had a different protein profile from the original ECM fraction (Figure 1a). Clearly some protein bands were more abundant in the ATP binding protein fraction, confirming that this fraction had enriched some very low abundance proteins that bind ATP. Mixing the protein with 50 mM ATP prior to incubation with the ATP-binding resin reduced the quantity of bound proteins (Figure 1b), suggesting that ATP was acting as a competitor. Lower concentrations of exogenous ATP did not block protein binding as they were degraded by endogenous ATP hydrolase activity in the protein fractions. Pixel profiles down the middle of the gel lanes in Figure 1b (cutting across the protein bands) show the pixel cross section of six major bands visible on the gel (Figure 1c). All of the peaks for the protein bands bound without competition from exogenous ATP are above the 6000-pixel intensity, whereas equivalent protein bands in the sample with competition from exogenous ATP were suppressed well below this value (Figure 1c).

Gel slices were excised from the lane with ATP binding proteins (Figure 1a), and the proteins were eluted and analysed by liquid chromatography tandem mass spectrometry. For identification, we included proteins with an identification confidence level of ≥95%. Mass spectrometry identified three proteins, which was fewer than expected from the many protein bands seen on the Sypro Ruby-stained protein gels (Figure 1a). Three independent fractions of ATP binding proteins, bulked up by pooling eluates from several rounds of affinity purification, consistently gave the same results. Although the cause for this remains unclear, a possibility could be the combination of low sensitivity of the mass spectrometer used and the extreme low abundance of most of these proteins, the visibility of which in gels was amplified by the highly sensitive Typhoon 9400 image scanner. There is precedence that some proteins may have as few as just a single tryptic cleavage site yielding peptides not readily detectable by mass spectrometry (de Godoy *et al.,* 2006).

The proteins identified in the ATP-binding eluates were PEROXIDASE 52 (PRX52), SUBTILISIN-LIKE SERINE PROTEASE 1.7, also known as ARABIDOPSIS 12 (ARA12), and PHOSPHOLIPASE C-LIKE PROTEIN, which we named PHOSPHOLIPASE C-LIKE 1 (PLCL1) (Table 1). Additional data relating to protein identification are presented in Table S3. In accordance with extracellular localization, all three proteins possess an N-terminal signal peptide (von Heijne, 1990) targeting the protein to the secretory pathway. In addition, the three proteins do not possess any known C-terminal endoplasmic reticulum retention motif (Vitale and Denecke, 1999), nor a transmembrane domain for plasma membrane localization.

We had previously identified the relatively abundant protein spots of ARA12 and PRX52 in routine two-dimensional gel electrophoresis (2DE) and mass spectrometric analyses

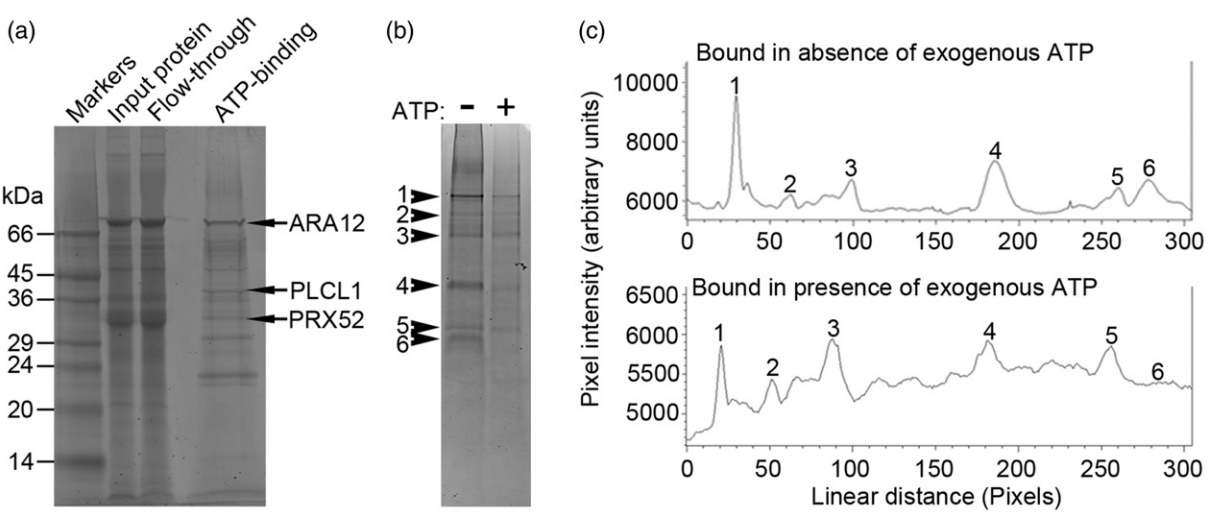

Figure 1. Affinity purification of ATP binding proteins.
(a) ATP binding proteins were affinity-purified using an immobilized ATP column. Input, the protein sample prior to affinity purification; flow-through, the protein fraction that passed through but did not bind to the ATP column. The ATP binding protein fraction eluted with buffer containing 20 mM ATP is indicated. Molecular weight markers are indicated in kDa. Positions of ARA12, PLCL1 and PRX52 are indicated by arrows on the left.
(b) Profile of proteins binding to the ATP resin in the absence (−) or presence of 50 mM ATP. Major protein bands are numbered 1–6 and indicated by arrows.
(c) Pixel intensity profile along a vertical line down the middle of the lanes in (b), indicating the peaks corresponding to protein bands 1–6. In the presence of exogenous ATP all protein bands are suppressed, indicating competition with exogenous ATP. Experiments were repeated three times with similar results.

Table 1 ATP binding proteins identified in the Arabidopsis extracellular matrix

Gene locus	Protein name	Abbreviation[a]	Signalpeptide[b]	TMD[c]	ER[d]
At5g67360	Subtilisin-like serine protease 1.7	ARA12	+	–	–
At5g05340	Peroxidase 52	PRX52	+	–	–
At1g13680	Phospholipase C-like 1	PLCL1	+	–	–

[a]Abbreviated protein name or alternative protein name.
[b]Positive sign denotes the presence of a signal peptide on the N terminus.
[c]Negative sign denotes the absence of a transmembrane domain in the protein sequence.
[d]Negative sign indicates the absence of a C-terminal endoplasmic reticulum retention motif.

of protein secreted into the growth medium of Arabidopsis cell cultures (Smith *et al.,* 2015). Therefore, we employed photo-affinity labelling as an alternative technique to confirm ATP-binding by focusing on ARA12, which is highly abundant and readily visible on protein gels and Western blots (Figure 2). Azido-ATP-biotin was added to the soluble ECM protein fraction and exposed to ultraviolet light to cross-link ATP binding proteins to the label. The azido-ATP-biotin-labelled protein sample was mixed with a sample pre-labelled with fluorescent cyanine5 NHS ester (Cy5) and the pooled sample separated by 2DE. The gel was blotted onto a membrane that was probed with a primary antibody raised against biotin and a secondary antibody conjugated to Cy3. The Western blots were scanned at two different wavelengths in order to capture the Cy3 and Cy5 profiles, with the Cy3 profile showing the image of biotinylated proteins and with the Cy5 profile showing the total protein profile. In 2D gels, ARA12 exists as a train of charge-variant spots running at the same molecular weight position (Figure 2), indicating the existence of post-translational modifications that significantly alter the charge of the protein. We labelled the ARA12 protein spots 1–6, starting from the most basic to the most acidic (Figure 2). Spots 1 and 2 were not labelled with azido-ATP-biotin, whereas spots 3–6 had incorporated the label, with spots 3 and 4 having the highest signal. In blots probed with a secondary antibody conjugated to horseradish peroxidase (HRP), the signal intensity in the ATP-binding spots could be increased without increasing the background (Figure 2). These blots confirmed the results obtained from blots probed with the Cy3-conjugated secondary antibody (Figure 2). Thus, ATP binding to ARA12 is controlled by an unidentified post-translational modification, with the more basic spots unable to bind ATP. This also demonstrates

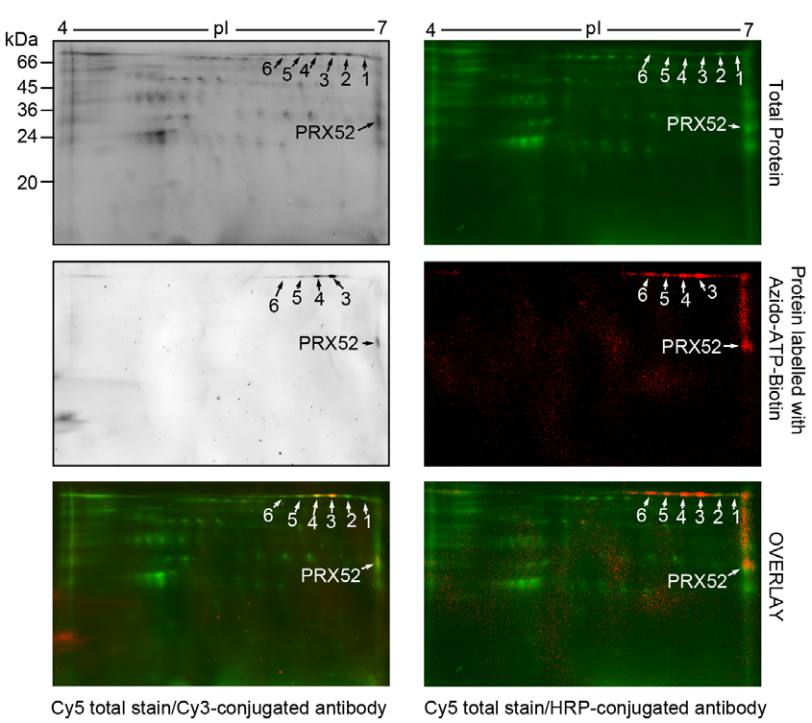

Figure 2. Western blot analysis of ATP binding proteins. Proteins secreted into the growth medium of Arabidopsis cell cultures were labelled with either Cy5 or 774 μM Azido-ATP-Biotin. The proteins were mixed, separated by two-dimensional gel electrophoresis (2DE), and analysed by Western blotting using anti-Biotin-Cy3 (left panels) or anti-Biotin-HRP serum (panels on the right). Top panels, Cy5-labelled total protein; middle panels, ATP binding proteins detected via antibodies; lower panels, overlay of total protein and ATP binding proteins. Arrows indicate six ARA12 protein spots and PRX52. Experiments were repeated at least three times with similar results.

specificity of the labelling technique as it discriminates between spots of the same protein with different post-translational modifications.

Western blots probed with either Cy3-conjugated or horseradish peroxidase (HRP)-conjugated secondary antibodies also revealed that PRX52 was cross-linked to azido-ATP-biotin (Figure 2). PRX52 has a mature protein of approximately 34.2 kDa that runs near the basic end of the pH 4–7 gels (Figure 2). However, there was a noticeable slight shift in molecular weight of the Western blot signal relative to the Cy5-labelled protein. This is accounted for by the addition of a 948.8-Da azido-ATP-biotin moiety to the protein. In protein gels, this shift in molecular weight is noticeable only in proteins of a lower molecular weight, but not in proteins with a very high molecular weight, such as ARA12 (Figure 2). These results confirmed that both ARA12 and PRX52 are ATP binding proteins. On the basis of these results, we conclude that the immobilized ATP column was indeed enriched for proteins that specifically bind ATP. Overall, these results show that only a few proteins in the soluble ECM fraction can bind ATP. These proteins are putative targets for extracellular ATP regulation of their biological functions.

Arabidopsis responses to ATP and fumonisin-B1 treatments

As ligands often activate the expression of genes encoding their target proteins (Zipfel *et al.,* 2006), we investigated whether ATP might also affect the expression of genes encoding the proteins that we identified. Infiltration of ATP into the leaf apoplast of wild-type Col-0 plants activated the expression of all three genes within an 8-h period (Figure 3a). *ARA12* expression peaked at approximately 3.5-fold, *PRX52* peaked at just over 9-fold and PLCL1 reached approximately 12-fold within 8 h (Figure 3a). Infiltration with a buffer control solution lacking ATP was followed by a smaller gene response (Figure S1), probably as a result of the wound-induced release of cytosolic ATP enabling a 60% overlap of wound-induced genes with ATP-induced genes (Choi *et al*, 2014). These results show that exogenous ATP transcriptionally activates the genes in addition to possibly regulating the proteins post-translationally via direct binding. Taken together, the gene expression profiles and ATP-binding properties suggest that all three proteins are likely to be effectors of ATP-dependent physiological processes.

Next, we investigated a possible link between the putative extracellular ATP effector genes and cell death in response to mycotoxin stress. The mycotoxin fumonisin B1 (FB1) triggers Arabidopsis cell death (Stone *et al.,* 2000), which is attenuated by exogenous ATP (Chivasa *et al.,* 2005). Infiltration of FB1 into leaves of Col-0 plants initially triggers chlorosis, which appears within 3 days and eventually develops into tissue death in leaves directly treated with the toxin (Figure 4a). By the end of 7 days, cell death symptoms spread systemically and appear in younger leaves not directly infiltrated with FB1 (Figure 4a). Although cell death in infiltrated leaves may cover the entire leaf, systemic cell death is punctate and appears as lesions. However, when exogenous ATP is simultaneously applied together with FB1, tissue death is greatly reduced in infiltrated leaves and is blocked in systemic leaves (Figure 4a). We obtained similar results from experiments using whole seedlings, initially grown on agar plates with Murashige and Skoog salts, and then transferred to solutions containing ATP ± FB1. Within 3 days of incubation with FB1, the cotyledons were bleached and dead, but the inclusion of ATP within the FB1 solution rescued the plants

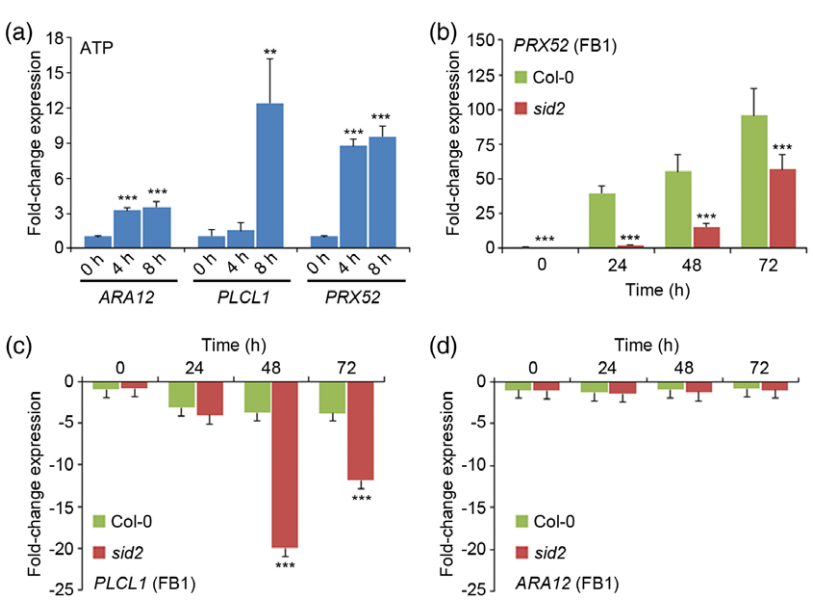

Figure 3. ATP- or fumonisin B1 (FB1)-induced gene expression.
(a) Leaves of Col-0 plants were infiltrated with 400 μM ATP and tissues were harvested at the indicated time points for RT-qPCR analysis.
(b) PRX52, (c) PLCL1 and (d) ARA12 gene expression in leaf samples of Col-0 and *sid2* plants infiltrated with 5 μM FB1 and harvested at the indicated time points. Values and error bars represent means ± SDs ($n = 3$). Two asterisks ($P \leq 0.01$) or three asterisks ($p \leq 0.001$) indicate statistically significant differences between 4 or 8 h and the 0-h control in (a), or between the wild type and *sid2* at each time point in (b)–(d). The same trend in results was observed for four similar experiments.

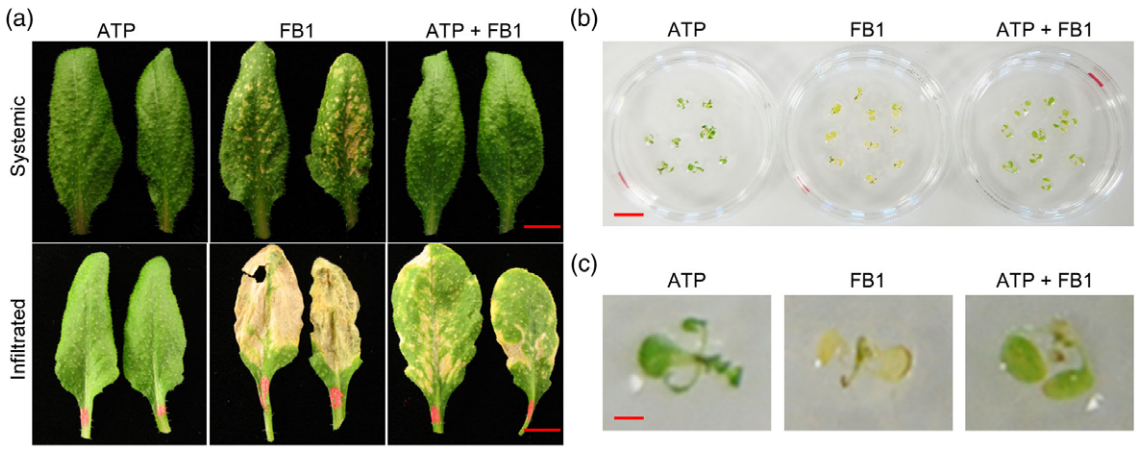

Figure 4. ATP attenuates fumonisin B1 (FB1)-induced cell death.
(a) Leaves of Col-0 plants were infiltrated with 400 μM ATP, 5 μM FB1 or a mixture of ATP + FB1 at the same concentrations. Representative directly infiltrated leaves (lower panels) and younger untreated leaves (upper panels) from the same plants were detached and photographed 7 days later. Results are from one of three independent similar experiments showing two of nine representative leaves per treatment (from three replicate plants). Scale bar: 5.5 mm.
(b) Whole Col-0 seedlings were transferred from agar plates to a dish with ATP, FB1 or ATP + FB1 solutions. Representative plants were photographed 4 days later. Scale bar: 10 mm.
(c) A close-up view of representative plants from (b). Results are from one of three similar experiments, each with three-replicate dishes per treatment, containing 25 plants. Scale bar: 1.7 mm

(Figure 4b, c). This antagonistic link between ATP and FB1 prompted us to investigate a possible role in cell death for the proteins identified in this study.

FB1-induced cell death is blocked in transgenic (expressing bacterial salicylate hydroxylase) or mutant (*phytoalexin-deficient 4-1*) plants depleted in salicylic acid (Asai *et al.,* 2000). Arabidopsis *salicylic acid induction-deficient 2* (*sid2*) mutants are similarly depleted in salicylic acid (Nawrath and Métraux, 1999) through the transfer-DNA (T-DNA)-mediated disruption of the isochorismate synthase-1 gene, the terminal enzyme in salicylic acid synthesis (Wildermuth *et al*, 2001). We monitored the expression of *PRX52*, *PLCL1* and *ARA12* in wild-type and *sid2* plants exposed to FB1. Over the 72-h period, *PRX52* was upregulated by FB1 treatment in wild-type plants, but *sid2* plants had an earlier and much stronger response, with the magnitude of *PRX52* expression close to 100-fold by 72 h (Figure 3b). *PLCL1* was moderately downregulated in wild-type plants, but the degree of suppression was significantly higher in *sid2* at 48 and 72 h (Figure 3c). However, *ARA12* had a similar expression profile between Col-0 and *sid2* plants across the time course (Figure 3d). Differential expression of *PRX52* and *PLCL1* between susceptible wild-type and FB1-resistant *sid2* plants points to a potential role in the cell death response to FB1.

PRX52 and PLCL1 belong to multiple protein gene families

We selected both *PLCL1* and *PRX52* for further analysis using gene knockout mutants because of their expression profiles in Col-0 and *sid2* plants exposed to FB1. Functional analysis using gene knockout mutants is unlikely to yield any distinct phenotype as a result of functional redundancy, which is particularly common in members of large gene families. Although the Arabidopsis peroxidase family has 73 genes (Valério *et al.,* 2004), we identified only four in the extracellular protein fractions: PRX17, PRX52, PRX53 and PRX71 (Table S1). Of these four proteins, only PRX52 was identified as ATP-binding (Table 1). Therefore, we obtained two independent T-DNA insertion mutants for *PRX52* from the SALK and JIC SM collections: *prx52-1* (SALK_081257) and *prx52-2* (SM_3_1699), respectively.

The Arabidopsis genome has three genes closely related to *PLCL1* in having a phospholipase C-like domain and a primary sequence consistent with secretion to the extracellular matrix. This suggests a protein family of four genes; we named the additional three genes *PLCL2* (At1g49740), *PLCL3* (At3g19310) and *PLCL4* (At5g67130). Comparison of gene expression in response to FB1 in wild-type plants showed a similar expression profile between *PLCL1* and *PLCL4* that was not seen in *PLCL2* and *PLCL3* (Figure S2). This raises the possibility that both PLCL1 and PLCL4 may have redundant functions in the Arabidopsis response to FB1. We found T-DNA insertion mutants of *PLCL1* in publicly available Arabidopsis mutant collections, but none were available for *PLCL4*. Therefore, we selected single gene knockout *plcl1* and *prx52* mutants for further analysis.

The response of *plcl1* and *prx52* gene knockout mutant plants to FB1

We obtained two independent T-DNA knockout lines, *plcl1-1* (SALK_048688) and *plcl1-2* (SALK_023867) (Figure 5a),

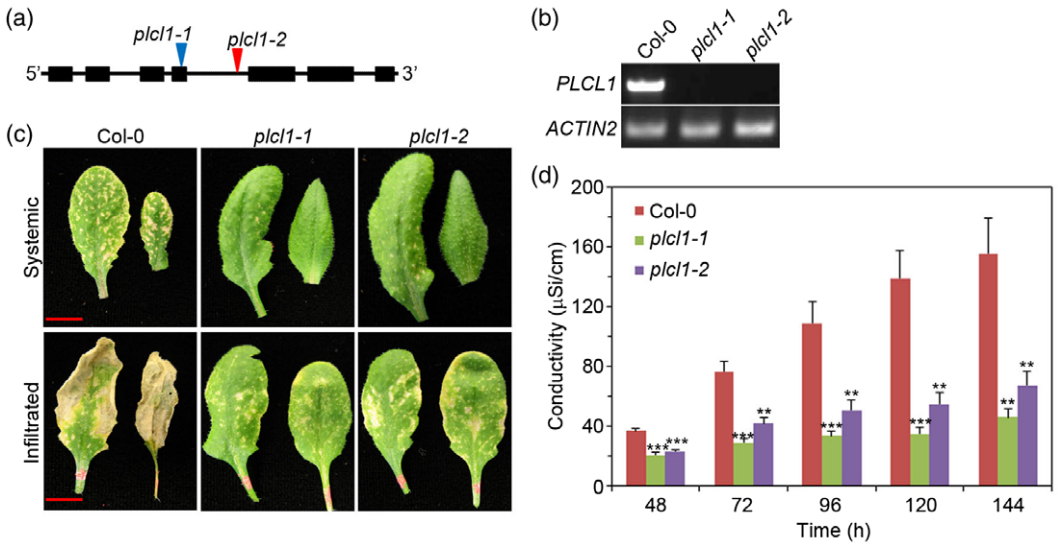

Figure 5. Response of the *plcl1* gene knockout mutants to fumonisin B1 (FB1).
(a) Schematic diagram of *PLCL1* gene structure showing seven exons (black rectangles) and the relative positions of the T-DNA insertions (inverted triangles) in the mutants.
(b) RT-PCR amplification of a *PLCL1* transcript from cDNA samples of Col-0 and T-DNA knockout lines *plcl1-1* and *plcl1-2*. *ACTIN2* was used as a constitutive reference control.
(c) Leaves of Col-0 plants were infiltrated with FB1 and representative directly infiltrated leaves (lower panels) and younger untreated leaves (upper panels) from the same plants were detached and photographed 7 days later. Results are from one of four independent similar experiments showing two of nine representative leaves per treatment (from three replicate plants). Scale bars: 6.1 mm.
(d) Cell death profiles of FB1-treated leaf discs from Col-0, *plcl1-1* and *plcl1-2* plants. Values and error bars represent means ± SDs ($n = 5$). Statistically significant differences between Col-0 and mutants at each time point are indicated by two ($P \leq 0.01$) or three ($P \leq 0.001$) asterisks. Results show the same trend obtained in four similar experiments.

from the SALK collection. No accumulation of *PLCL1* transcripts was detectable by reverse transcription polymerase chain reaction (RT-PCR) in the gene knockout plants (Figure 5b). We used these plants to investigate a possible role for PLCL1 in cell death.

Three rosette leaves of 4-week-old plants, at growth stage 5.10 (Boyes *et al.*, 2001), were infiltrated with FB1 and the development of symptoms in wild-type and knockout mutants was monitored for 7 days. Cell death lesions started to appear in all genotypes 3 days after infiltration. At this stage, the appearance of the lesions and the extent of cell death were indistinguishable between wild-type and mutant plants. However, the cell death lesions expanded, coalesced and spread in the wild-type plants until most of the tissue in directly infiltrated leaves was dead (Figure 5c). The expansion of cell death lesions in the infiltrated leaves of mutant plants was terminated, resulting in significantly reduced cell death in both *plcl1-1* and *plcl1-2* (Figure 5c). In Col-0, systemic cell death appeared in younger leaves not treated with FB1, but the equivalent systemic leaves of the mutant plants had very marginal symptoms (Figure 5c). Generally, we found that at 5 μM or lower concentrations of FB1 there were very clear differences in the cell death symptoms between Col-0 and mutant plants, but the differences were no longer apparent at higher concentrations. At higher concentrations, the initial cell death symptoms appear much earlier, within 2 days of treatment. This suggests that FB1 binding to cellular targets could be quantitative and saturable, in terms of the number of bound targets, as reflected by the speed and extent of cell death.

We also used an *in vitro* cell death assay, which relies on measuring electrolyte leakage from dying cells. In this assay, leaf discs are floated on FB1 and incubated in the dark for 48 h to enable uptake of the solution. Transferring the tissues to a 16-h light/8-h dark cycle activates cell death, with conductivity readings recorded at 24-h intervals. Conductivity of the solution on which the discs are floating is proportional to, and serves as a proxy for, FB1-induced cell death. There is a significant difference in the ion leakage profile between leaf discs floating on a control solution when compared with leaf discs floating on the FB1 solution (Figure S3). There was a significant suppression of cell death in *plcl1-1* and *plcl1-2* leaf tissues when compared with Col-0 (Figure 5d). This is in agreement with results obtained from the infiltration of leaves attached to plants (Figure 5c). However, the difference in cell-damage symptoms between mutant and wild-type tissues in the *in vitro* leaf disc assay appears smaller than that observed in infiltrated leaves attached to plants. As stated above, the difference in the cell death response between the wild type and the mutants decreases with increasing FB1 concentration.

Reduced FB1-induced cell death in *PLCL1* gene knockout plants is in agreement with a rapid suppression of *PLCL1* expression in *sid2* mutants (Figure 3c), which is concomitant with the suppression of the cell death response (Figure 5c, d). Thus, the slower and shallower suppression of *PLCL1* expression seen in Col-0 plants responding to FB1 (Figure 3c) could indicate a failed attempt at suppressing a pro-death protein. Experiments with *PRX52* T-DNA insertion mutants did not show any differences in cell-death profile between the wild type and the mutants (Figure S4), pointing either to functional redundancy or to no role for *PRX52* in FB1-induced cell death. Taken together, our results show great potential for combined ATP-affinity screening with reverse-genetic analyses in the identification of ATP targets and protein assignments to previously unknown physiological functions.

Evidence for additional extracellular ATP receptors

DORN1 is an Arabidopsis plasma membrane receptor protein that binds extracellular ATP and activates intracellular signalling events and gene expression (Choi *et al.,* 2014). To investigate whether the effects of exogenous ATP on FB1-induced cell death require DORN1, we obtained T-DNA knockout mutants, *dorn1-3* (SALK_042209) and *dorn1-4* (SALK_024581). Plants grown on agar plates for 10 days and transferred to solutions of FB1, with or without ATP, were scored for tissue damage symptoms 3–4 days later. Cotyledon damage started 3 days after FB1 application and initially appeared as chlorosis, which rapidly progressed into pigment bleaching covering the entire cotyledon within a 24-h period. ATP blocked tissue damage in both Col-0 and mutant plants (Figures 6 and S5), suggesting that ATP does not require DORN1 in the cell death signalling pathway. Moreover, the activation of *PLCL1* by ATP was unaffected in *dorn1* loss-of-function mutant plants (Figure 7). As a positive control, the ATP-induced suppression of *HSP20L* and *UMAMIT33* in wild-type plants was, respectively, blocked or reversed in *dorn1* plants. The response of *PLCL1*, *HSP20L* and *UMAMIT33* genes to infiltration with exogenous ATP was different from the low-level wounding-induced response triggered by infiltration of a control buffer solution lacking ATP (Figure S1). Overall, this confirms that unidentified alternative ATP receptors or mode of signalling exist in plants.

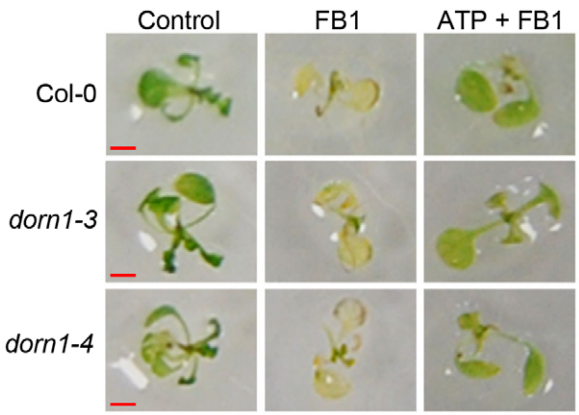

Figure 6. Exogenous ATP rescues the wild type and *dorn1* mutants from fumonisin B1 (FB1)-induced cell death. Arabidopsis seedlings were transferred from agar plates to solutions of 800 μM ATP, 5 μM FB1 or ATP + FB1, at the same concentrations. Individual representative plants were photographed 4 days after treatment. Results are from one of three independent similar experiments, each with three-replicate dishes per treatment containing 25 replicate plants. Scale bars: 1.6 mm. More images are provided in Figure S5.

DISCUSSION

ATP is an extracellular signal important for specific growth processes and stress-adaptive responses. Our long-term goal is to identify the key components of extracellular ATP signalling with a role in cell death responses. In this study, we sought to identify proteins that bind extracellular ATP in Arabidopsis cell suspension cultures. We identified ARA12, PLCL1 and PRX52 in protein fractions enriched for ATP binding proteins. We also found that application of exogenous ATP upregulated all three genes within 8 h of treatment. A similar positive feedback loop activating gene expression after a ligand binds the encoded protein has been observed before. For example, both flg22 and elf18 peptides activate the expression of genes encoding their respective cognate receptor proteins, *FLS2* and *EFR* (Zipfel *et al.,* 2006). Transcriptional and post-translational links between ATP and these proteins suggest that, in addition to reprogramming the transcriptome (Jewell *et al.,* 2019), extracellular ATP may also function by redirecting signal generation within the extracellular matrix, as explained below.

Extracellular ATP is an important regulator of cell death, with concentration-dependent effects on cell viability. The depletion of extracellular ATP activates cell death (Chivasa *et al.,* 2005), whereas too much extracellular ATP also triggers cell death (Sun *et al.,* 2012). Thus, plants maintain a delicate balance between ATP secretion and degradation for metabolic homeostasis to suit specific physiological demands. As a result of our longstanding interest in FB1-induced cell death (Chivasa *et al.,* 2005; Chivasa *et al.,* 2011; Chivasa *et al.,* 2013; Smith *et al.,* 2015), we investigated a possible link of ARA12, PRX52 and PLCL1 in this response.

We exploited the FB1 insensitivity of mutants impaired in salicylic acid accumulation and/or signalling as a filter to identify proteins with a potential role in cell death. Our hypothesis was that *sid2* mutants alter the FB1 expression profile of cell-death genes. On this basis, we selected PRX52 and PLCL1, the response of which to FB1 was changed in *sid2* plants (Figure 3). However, *prx52* gene knockout mutants had a similar kinetic profile of cell death as

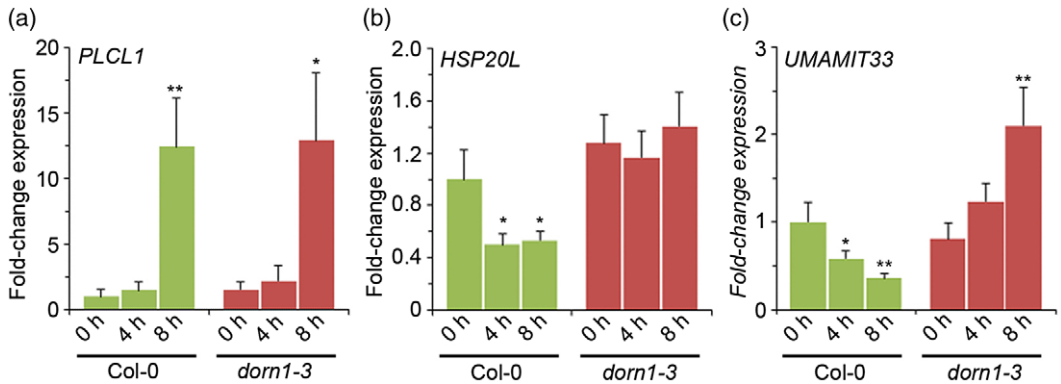

Figure 7. Effects of DORN1 loss of function on ATP-induced gene expression. Leaves of wild-type and mutant plants were infiltrated with 400 μM ATP and tissues harvested at the indicated times were analysed by RT-qPCR. Values and error bars represent means ± SDs ($n = 3$). Statistically significant differences between the 0-h and the 4- or 8-h samples within each genotype are indicated by one ($P \leq 0.05$) or two ($P \leq 0.01$) asterisks. The results show the same trend obtained in three other similar experiments.

the wild type after exposure to FB1. This probably reflects functional redundancy across the 73-gene family or that PRX52 has no direct role in cell death.

Although we did not find any role for ARA12 and PRX52 in FB1-induced death, our results link both proteins to extracellular ATP. ARA12 is an extracellular serine protease (Hamilton *et al.,* 2003) belonging to a 56-member Arabidopsis gene family (Rautengarten *et al.,* 2008). It regulates the accumulation or activation of cell wall modifying enzymes to facilitate cell wall loosening and seed mucilage swelling for rupturing the seed coat during imbibition (Rautengarten *et al.,* 2008). Whether extracellular ATP regulates the function of ARA12 in seed development or not awaits further investigation, but we found that some protein spots of ARA12 bind ATP, whereas others do not. The post-translational modifications that change the charge of ARA12 spots and alter ATP binding are not yet clear. Although the impact of ATP binding on ARA12 protease activity is yet to be determined, ATP-dependent allosteric control of certain enzymes via binding to sites other than the active site is known. For example, ATP is a substrate of phosphofructokinase, but at higher concentrations it binds to a regulatory site, distinct from the catalytic site, and allosterically inhibits the enzyme as a way to control the glycolytic pathway (Berg *et al.,* 2002).

The Arabidopsis genome has 73 members of class-III peroxidases (E.C.1.11.1.7), to which PRX52 belongs. Peroxidases are involved in diverse processes such as lignification, suberization, the cross-linking of cell wall proteins, auxin catabolism and stress-adaptive responses (Hiraga *et al.,* 2001; Penel, 2000). Whether ATP binding regulates the known enzymatic functions of ARA12 and PRX52 will require further research. However, these two ATP binding proteins may serve as signal regulatory proteins. ARA12 could potentially cleave pro-peptides to yield bioactive peptides. Potential substrates are numerous peptides that bind plasma membrane receptors to activate responses, such as defence against herbivores (Scheer and Ryan, 1999, 2002) and pathogens (Huffaker and Ryan, 2007; Yamaguchi *et al.,* 2006), cell proliferation and differentiation (Matsubayashi *et al.,* 2002; Matsubayashi and Sakagami, 1996; Matsubayashi *et al.,* 2006), the preservation of stem-cell identity in the shoot apical meristem (Fletcher *et al.,* 1999; Kondo *et al.,* 2006; Trotochaud *et al.,* 2000) and the regulation of floral organ abscission (Stenvik *et al.,* 2008). PRX52 may modulate the level of H_2O_2 during the oxidative burst triggered by the application of ATP (Demidchik *et al.,* 2009; Song *et al.,* 2006; Wu and Wu, 2008).

FB1-induced *PLCL1* suppression peaked at approximately fivefold in Col-0 but breached 20-fold in *sid2* mutants (Figure 3c). Whereas FB1 treatment led to *PLCL1* suppression, exogenous ATP activated gene expression (Figure 3a, c). The rationale accounting for the different response profiles between ATP-treated wild-type and FB1-treated *sid2* plants could be that PLCL1 plays a positive role during growth under optimal conditions, but promotes cell death in the presence of the mycotoxin in a similar fashion to mitochondrial cytochrome *c*. Under normal conditions, cytochrome *c* is involved in the positive role of electron transport in oxidative phosphorylation. However, in the presence of a death stimulus, cytochrome *c* is released from the mitochondrion and enters the cytosol to activate cell death via complex formation with Apaf-1 and caspase-9 (Li *et al.,* 1997). Thus, FB1-treated wild-type plants attempt but fail to effectively shut down *PLCL1* expression, whereas *sid2* plants mount a more robust response, blocking transcription to avert cell death. Supporting the view that PLCL1 suppression is protective against FB1 toxicity is the observation that gene knockout mutants have considerable resistance to FB1 (Figure 6). Involvement in cell death of PLCL1, a protein in the mobile phase of the extracellular matrix, is consistent with the

existence of secreted factors in growth media of Arabidopsis cell cultures known to seal the fate of cells to death when exposed to FB1 (Chivasa and Goodman, 2020).

How does PLCL1 function in FB1-induced cell death? Phospholipase C (PLC) proteins cleave membrane phospholipids to generate signalling molecules that activate downstream components, culminating in gene expression. Two types of PLCs exist in plants: phosphatidylcholine-cleaving PLCs and phosphoinositide-specific PLCs. The Arabidopsis genome has six genes encoding phosphatidylcholine-cleaving PLCs (Nakamura *et al.,* 2005) and nine genes for phosphoinositide-specific PLCs (Mueller-Roeber and Pical, 2002). Phosphoinositide-specific PLCs cleave phosphatidylinositol-4,5-biphosphate to release the second messengers inositol-1,4,5-triphosphate and 1,2-diacylglycerol. Phosphoinositide-specific PLCs have a catalytic core constituting conserved PI-PLC-X and PI-PLC-Y domains, which are flanked by regulatory domains (Pokotylo *et al.,* 2014). In animals, the catalytic domains are flanked by an N-terminal EF-hand domain for allosteric regulation by Ca^{2+} and a C-terminal C2 domain, which binds phospholipid (Kouchi *et al.,* 2005). Plant counterparts may have a truncated or completely missing EF-hand domain (Otterhag *et al.,* 2001; Pokotylo *et al.,* 2014). However, PLCL1 resembles Arabidopsis phosphoinositide-specific PLCs only in having the PI-PLC-X domain, but the PI-PLC-Y and C2 domains are missing. For catalytic activity, both the PI-PLC-X and PI-PLC-Y domains are required, casting doubt on any potential phospholipase activity in PLCL1. The other family members (PLCL2, PLCL3 and PLCL4) also have the PI-PLC-X domain. Therefore, the biochemical function of PLCL1 remains unclear and how it promotes cell death requires further work. This could be resolved by tracking its subcellular localization before and after FB1 treatment and making a recombinant protein to investigate phospholipase enzymatic activity.

Finally, we showed that DORN1 is not required for ATP to rescue plants from FB1-induced cell death, nor for the activation of *PLCL1* expression by exogenous ATP. Similarly, the ATP-dependent Ca^{2+} influx into leaf cells (Matthus *et al.,* 2019) and ATP-dependent root bending (Zhu *et al.,* 2017) in Arabidopsis still occur in *dorn1* loss-of-function mutants, albeit partially through the Ca^{2+} influx. Additionally, DORN1-independent transcriptional activation and cytosol-to-nucleus translocation of the REDOX-RESPONSIVE TRANSCRIPTION FACTOR 1 (RRTF1) was recently reported in Arabidopsis roots treated with exogenous ATP (Zhu *et al.,* 2020). RRTF1 controls root growth via the regulation of auxin distribution (Zhu *et al.,* 2020). These reports together with our results provide the basis for developing genetic screens to identify additional ATP receptors. In considering the fundamental question of why cells use a universal energy carrier as an extracellular signal (Chivasa, 2020), these studies indicate strong links between energy-demanding processes (root growth and cell viability) to the apoplastic signalling ATP pool. The reason that external ATP is preferred over intracellular ATP as a signal in these processes is related to the hypothesis of collective decision making across several cell layers (Chivasa and Goodman, 2020), with PLCL1 emerging as a key protein in these processes.

EXPERIMENTAL PROCEDURES

Materials and plant growth conditions

Arabidopsis gene knockout mutant lines *plcl1-1* (SALK_048688), *plcl1-2* (SALK_023867), *prx52-1* (SALK_081257), *dorn1-3* (SALK_042209), *dorn1-4* (SALK_024581) and *sid2* (SALK_088254) from the SALK collection (Alonso *et al.,* 2003) and *prx52-2* (SM_3_1699) from the JIC SM collection (Tissier *et al.,* 1999) were obtained from the Nottingham Arabidopsis Seed Stock Centre (NASC, http://arabidopsis.info). Gene knockout was confirmed by the PCR amplification of cDNA using the primers provided in Table S4. The mutants are in the Columbia-0 ecotype, which was then used as the wild type in all experiments. Plants were grown in soil and incubated in a growth room maintained at 23°C with a 16-h photoperiod. Lighting (approx. 200 μmol m^{-2} s^{-1}) was provided by a bank of alternating GROLUX F58W/GRO-T8 and LUMILUX L58W/865 fluorescent tubes (Sylvania, https://www.sylvania-lighting.com), an arrangement providing an optimal spectral distribution of photosynthetically active radiation. All soil-grown plants were used for experiments 4–5 weeks after sowing. For tissue culture experiments, Arabidopsis seeds were surface sterilized and grown on agar plates with Murashige and Skoog basal medium, as previously described (Chivasa *et al.,* 2005). Arabidopsis cell suspension cultures of ecotype Landsberg *erecta* (May and Leaver, 1993) were subcultured weekly by inoculating fresh growth medium (Chivasa *et al.,* 2005) with 10% (v/v) 7-day-old inoculum. Cell cultures (100 ml) were grown in 250-ml conical glass flasks on a rotating shaker incubated at 23°C with a 16-h photoperiod (approx. 150 μmol m^{-2} s^{-1}). Cell cultures were used for experiments 3–5 days after subculturing, a time window that lies within the exponential growth phase. Stock solutions of 1 mM FB1 were prepared in 70% methanol and stored at −20°C. The sodium salt of ATP was dissolved in water to make 1 mM stock solutions adjusted to pH 6.5 using KOH and stored at −80°C. Growth media components, ATP and FB1 were purchased from Sigma-Aldrich (http://www.sigmaaldrich.com).

Protein sample preparation

Arabidopsis cell cultures grown for 5 days were filtered using two layers of Mira cloth. Proteins secreted into the growth medium were recovered by acetone precipitation, as described previously (Smith *et al.,* 2015). Samples from five-replicate cell cultures were pooled prior to mass spectrometry. Methods used for liquid chromatography and tandem mass spectrometric analyses are provided in Methods S1.

Sample preparation, affinity purification and mass spectrometry

Growth medium protein samples were prepared and analysed by mass spectrometry, as described in Methods S1. To ensure that protein concentration by acetone precipitation did not denature the proteins, ATP hydrolase activity was evaluated as described in Methods S1. Affinity purification was performed using

ProteoEnrich ATP-Binders™ kit (Merck Millipore, https://www.merckmillipore.com) according to the manufacturer's instructions. Briefly, protein secreted into the growth medium of 5-day-old Arabidopsis cell cultures was precipitated with 80% acetone at −20°C, as described above. Protein was extracted from the pellets using 1 × binding buffer (60 mM $MgCl_2$, 30 mM NaCl, 25 mM HEPES, 0.05% NP-40, pH 7.2) and 500 μg total protein aliquots were used for isolating ATP binding proteins. One volume of 10X nucleotide mix (10 mM ADP, 10 mM AMP, 10 mM NADH, pH 7.0) was added to sample to give 10 volumes. This addition was made to block interactions arising from the structural similarity of ADP, AMP and NADH with ATP, so as to restrict binding to the resin to be specific for ATP. For every 1 ml of sample, 10 μl of protease inhibitor cocktail and 10 μl of freshly prepared 100 mM dithiothreitol (DTT) were added. To prepare the polyacrylamide-based resin with immobilized ATP, 15 mg was equilibrated at 4°C overnight with resin conditioning buffer (4.2 mM HEPES, 1.7% Tween 20, pH 7.2). The resin was washed twice with water and once with washing buffer (150 mM NaCl, 60 mM $MgCl_2$, 25 mM HEPES, 1 mM DTT, 0.2 mM activated Na_3VO_4, 1% protease inhibitor cocktail, 0.05% NP-40, pH 7.2). The protein sample was added to the equilibrated resin and incubated on a rotating roller at 4°C for 12–20 h. The resin + sample mixture was loaded into a Micro Bio-Spin® chromatography column (Bio-Rad, https://www.bio-rad.com) and centrifuged at 5000 ***g*** for 2 min. The flow-through was saved for a second round of affinity purification. The column was washed three times with wash buffer and eluted three times with elution buffer (25 mM HEPES, 20 mM ATP, 1 mM ADP, 1 mM AMP, 1 mM NADH, 0.05% NP-40, pH 7.2). The resin was washed and used to affinity purify a second time from the original flow-through. All eluates from the two rounds of affinity purification were pooled and analysed by gel electrophoresis and liquid chromatography tandem mass spectrometry (LC-MS/MS). ATP binding protein extraction and LC-MS/MS were conducted three times independently. For competition experiments, the sample was pre-mixed with 50 mM ATP before incubation with resin and the wash solutions contained the same concentration of ATP. These experiments were repeated three times.

The ATP binding protein fractions were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis using 12% polyacrylamide gels. The gels were stained with Sypro Ruby® (ThermoFisher Scientific, https://www.thermofisher.com) and gel slices were excised from the entire lane for protein identification. The gel slices were processed as previously described (Chivasa *et al.,* 2011) and analysed by MS and MS/MS analysis using the 4800 Proteomic Analyser mass spectrometer (Applied Biosystems, now ThermoFisher Scientific), as previously described (Chivasa *et al.,* 2013). The MS and MS/MS data were submitted to MASCOT 2.2 (Matrix Science, https://www.matrixscience.com) to search an in-house Arabidopsis database downloaded from UniProt (9 January 2015). Parameters used for database searches were as follows: digestion enzyme trypsin, single missed cleavage, variable modifications of MMTS-alkylated cysteine and oxidized methionine, and 50 pp precursor mass tolerance and 0.2 Da fragment ion tolerance. A combined protein score, incorporating the MS/MS-derived individual peptide ion scores and the peptide mass fingerprint-associated score, of more than 95% ($P \leq 0.05$) indicated a positive identification. The minimum cut-off threshold for this score in MASCOT was 76.

Labelling ATP binding proteins and Western blot analysis

Arabidopsis proteins secreted into the growth medium were recovered by acetone precipitation and resuspended in the ProteoEnrich ATP-Binders™ kit binding buffer (60 mM $MgCl_2$, 30 mM NaCl, 25 mM HEPES, 1 mM ADP, 1 mM AMP, 1 mM NADH, 1 mM DTT, 1% protease inhibitor cocktail, 0.05% NP-40, pH 7.2). To an aliquot of 150 μg protein in this buffer, 387 nmoles of 8-azidoadenosine 5′-triphosphate-[γ]-biotinyl-3,6,9-trioxaundecanediamine ($8N_3$ATP[γ]Biotin-LC-PEO-Amine; Affinity Labeling Technologies, Lexington, USA) were added in a final volume of 500 μl. The tubes were covered in aluminium foil and incubated in a rotating roller at 4°C for 12–15 h. The mixture was exposed to ultraviolet light (254 nm) three times for 4 mins each, with 1 min of vortexing between each exposure. The protein was precipitated using 80% acetone at −20°C and solubilized in an isolectric focusing solution: 9 M urea/2 M thiourea/4% (w/v) CHAPS.

Aliquots with 30 μg of protein labelled with $8N_3$ATP[γ]Biotin-LC-PEO-Amine were mixed with 10 μg of protein that had been labelled with Cy5 (GE Healthcare, https://www.gehealthcare.com), as previously described (Chivasa *et al.,* 2011). The protein mixture was resolved in two dimensions using 7-cm-long pH 4–7 isoelectric focusing gel strips (GE Healthcare) and homogeneous 8.5 × 6.5 cm^2 12% polyacrylamide gels. The gels were blotted onto nitrocellulose membrane and probed with either HRP-conjugated goat anti-biotin serum (Sigma-Aldrich) at a dilution of 1:7000 or with Cy3-conjugated mouse anti-biotin serum (Sigma-Aldrich) at 1:500 dilution using a previously described method (Chivasa *et al.,* 2002). Signals on western blots probed with HRP-conjugated antibody were developed using the ECL reagent kit (Bio-Rad). Western blots were scanned on the Typhoon 9400 (GE Healthcare) using Cy3, Cy5 and ECL channels. Cy3/Cy5 and ECL/Cy5 overlay images were examined to locate the ATP binding proteins. This experiment was repeated three times.

FB1 or ATP treatments and real-time RT-PCR analysis

Four-week-old Arabidopsis plants, at growth stage 5.10 (Boyes *et al.,* 2001), were used for these experiments. Three rosette leaves per plant of Col-0 (wild type) and *sid2* gene knockout mutant plants were infiltrated with a 5 μM FB1 solution. We avoided using the first two (oldest) rosette leaves. Triplicate plants were thus treated for harvesting at each of three time points. Treated leaves were excised at 0, 24, 48 and 72 h. At each time point, an individual sample was generated by pooling three leaves, each arising from the three independent replicate plants. Three such biological replicate samples were generated at each time point for RNA extraction and analysis of gene expression. Wild-type plants were similarly infiltrated with 400 μM ATP (adjusted to pH 6.5) and leaf tissues harvested at 0, 4 and 8 h after infiltration. The pooling of leaves in a single sample and the number of biological replicates was the same as described for the FB1 experiment.

RNA extraction and first-strand cDNA synthesis were performed as described previously (Chivasa *et al.,* 2006). Quantitative real-time polymerase chain reaction and data analysis were performed as described by Ngara *et al.,* (2018) using *ACTIN2* (At3g18780) and *EIF4* (At3g13920) as constitutive reference control genes. The primers used in the reactions are listed in Table S4.

Assay for FB1-induced cell death

Cell death assays were performed as described previously (Chivasa *et al.,* 2013), with minor modifications. Three leaves per plant from five replicate Col-0, *plcl1-1*, *plcl1-2*, *prx52-1* and *prx52-2* plants were infiltrated with 5 μM FB1. At 7 days post-treatment, representative FB1-infiltrated leaves and younger leaves not directly treated with FB1 were detached and photographed. To assess the effects of exogenous ATP on FB1-induced cell death, Col-0 plants were infiltrated with 5 μM FB1 with or without

400 μM ATP and symptom development was evaluated 7 days later as described above. Alternatively, 10-day-old seedlings grown on agar plates were pulled out and placed in Petri dishes with 10 ml of 5 μM FB1, 800 μM ATP (adjusted to pH 6.5), 5 μM FB1 + 800 μM ATP or water (serving as a control). Triplicate dishes with 20–25 plants per treatment were set up. Images of representative plants were taken 4 days after treatment. For the quantitative cell death assay, leaf discs of 1 cm in diameter were cored from 10 independent plants and floated on 10 ml of 5 μM FB1 solution in a Petri dish. For each Arabidopsis genotype, five-replicate dishes were generated, with each dish containing 20–25 plants. The dishes were incubated in the dark for 48 h and then moved into a 16-h light/8-h dark cycle thereafter. Conductivity of the FB1 solution was measured at 48 h and every 24 h thereafter.

Experimental design and statistical rationale

This study aimed to achieve three key objectives. First was to identify proteins secreted into the growth medium of cell cultures. Because the intention was to identify all proteins without any quantitative analysis of abundance, we pooled five biological replicates to ensure the composite fraction represented the full range of secreted proteins. This established the protein map of the fractions used in subsequent experiments. Second, we wanted to identify the subset of these proteins that binds ATP. Affinity proteomics using immobilized ATP was performed independently three times, and the ATP binding proteins were identified via mass spectrometry. The three experimental repeats used independent biological replicates and ensured that the observed ATP-binding events were reproducible. We also verified the results using photo-affinity labelling, which also used the same three biological replicates to ensure reproducibility. Each biological replicate was processed at different times, meaning that each consisted of a process repeat with distinct biological samples. The third objective was to perform functional analyses of selected proteins. We performed gene expression analyses after treatments with ATP and/or FB1. Three biological replicate samples were generated at each time point after treatment. Each replicate consisted of three pooled leaves, with each leaf harvested from an independently treated plant. This ensured that each sample was an average of three independent plants in order to reduce biological variation. For analysis of FB1-induced cell death using the conductivity assay, the replicate number was increased to five, with each replicate dish consisting of 10 leaf discs derived from 10 independent plants. This higher number of pooled samples was to reduce the higher biological variation seen between different leaves in the conductivity assay from previous experiments. All data analyses of gene expression and cell death conductivity assays used analysis of variance (ANOVA) and the Student's *t*-test because these data types are normally distributed. The experimental workflow described here is depicted schematically in Figure S6.

Accession numbers

Sequence information for the key genes/proteins analysed in this article is available from the Arabidopsis Information Resource (TAIR) database (https://www.arabidopsis.org) under the following gene locus identifier numbers: ACTIN2 (At3g18780), EIF4A (At3g13920), PLCL1 (At1g13680), PLCL2 (At1g49740), PLCL3 (At3g19310), PLCL4 (At5g67130), ARA12 (At5g67360), PRX52 (At5g05340), HSP20L (At4g27890), UMAMIT33 (AT4G28040) and DORN1 (At5G60300).

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AUTHOR CONTRIBUTIONS

SJS, HLG and JTMK performed the experiments. APB and WJS conducted the mass spectrometry. SC designed the research, conducted some experiments and wrote the article.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest associated with this work.

DATA AVAILABILITY STATEMENT

The protein identification raw data were submitted to the Proteomics Identifications (PRIDE) database (http://www.ebi.ac.uk/pride/archive/) and are available via ProteomeXchange with identifier PXD004729. All the other data are included within the article or supporting information.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Appendix S1. Overview of Arabidopsis growth medium proteins.

Methods S1. Proteomic analysis of Arabidopsis cell culture filtrate protein and ATP hydrolase activity.

Figure S1. Comparison of wound-induced and ATP-induced gene expression.

Figure S2. Response of the Arabidopsis *PLCL* gene family to FB1.

Figure S3. Ion leakage assay distinguishes between tissue wounding and FB1 toxicity.

Figure S4. *PRX52* gene knockout does not alter the response to FB1.

Figure S5. Exogenous ATP does not require DORN1 to block FB1-induced death.

Figure S6. Schematic representation of experimental workflow.

Table S1. List of secreted proteins identified in the growth medium of Arabidopsis cell suspension cultures.

Table S2. List of secreted proteins identified in the growth medium of Arabidopsis cell suspension cultures.

Table S3. Identification details of ATP binding proteins.

Table S4. Primer sequences used for PCR analysis.

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