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The phosphoproteome of Arabidopsis plants lacking the oxidative signal-inducible1 (OXI1) protein kinase

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Summary

- The AGC protein kinase OXI1 is a key protein in plant responses to oxidative signals, and is important for two oxidative burst-mediated processes: basal resistance to microbial pathogens and root hair growth. To identify possible components of the OXI1 signalling pathway, phosphoproteomic techniques were used to detect alterations in the abundance of phosphorylated proteins and peptides in an *oxi1* null mutant of *Arabidopsis thaliana*.
- The relative abundance of phosphorylated proteins was assessed either using two-dimensional gel electrophoresis and staining with the phosphoprotein stain Pro-Q Diamond or by the identification and quantification, by mass spectrometry, of stable-isotope labelled phosphopeptides.
- A number of proteins show altered phosphorylation in the *oxi1* mutant. Five proteins, including a putative F-box and 3-phosphoinositide-dependent kinase 1, show reduced phosphorylation in the *oxi1* mutant, and may be direct or indirect targets of OXI1. Four proteins, including ethylene insensitive 2 and phospholipase D-gamma, show increased phosphorylation in the *oxi1* mutant.
- This study has identified a range of candidate proteins from the OXI1 signalling pathway. The diverse activities of these proteins, including protein degradation and hormone signalling, may suggest crosstalk between OXI1 and other signal transduction cascades.

Introduction

Reactive oxygen species (ROS) are important signalling molecules in plants, being produced by the oxidative burst and as by-products of numerous metabolic pathways. A range of biotic and abiotic stimuli have been shown to induce ROS production, including pathogen attack, wounding and temperature stress (Apel & Hirt, 2004; Fujita *et al.*, 2006). While there is much evidence supporting a role for ROS in plant signal transduction, relatively little is known about the protein components that link ROS to downstream signalling. Previously, an AGC protein kinase, termed OXI1, has been shown to be induced directly by ROS and is also induced by stimuli that result in ROS production, such as wounding and pathogen infection (Anthony *et al.*, 2004; Rentel *et al.*, 2004). OXI1 has been shown to be important in two plant responses linked to ROS production: pathogen resistance and root hair growth. Arabidopsis plants with an *oxi1*-null mutation have enhanced susceptibility to infection by virulent strains of the oomycete pathogen *Peronospora parasitica* (Rentel *et al.*, 2004) and also the biotrophic bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (Petersen *et al.*, 2009). In addition, *oxi1*-null mutant plants exhibit reduced root hair elongation (Anthony *et al.*, 2004; Rentel *et al.*, 2004).

OXI1 may also be activated in a ROS-independent manner by 3-phosphoinositide-dependent kinase 1 (AtPDK1). Upstream of OXI1, PDK1 activity is regulated by binding to phosphatidic acid. Upon activation, PDK1 can then bind to and phosphorylate OXI1, resulting in OXI1 activation (Anthony *et al.*, 2004). Downstream, OXI1 has been shown to be required for the activation of the mitogen- activated protein kinases MPK3 and MPK6 (Rentel *et al.*, 2004) and also

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the serine/threonine protein kinase PTI1-2 (Anthony *et al.*, 2006). However, additional components of the OXI1 signalling pathway have remained elusive. The identification of such components is essential if we wish to elucidate the precise mechanisms by which OXI1 regulates plant growth and helps plants respond to biotic stress.

In the current investigation, a proteomics strategy was used for the identification of proteins that may be directly or indirectly phosphorylated by OXI1 kinase. By comparing quantitative changes in protein phosphorylation in an *oxi1*-null mutant of Arabidopsis with wild-type, it was possible to identify proteins whose phosphorylation was directly or indirectly regulated by OXI1. Altered abundance of phosphorylated proteins was assessed using either two-dimensional gel electrophoresis and staining with Pro-Q Diamond (Invitrogen, Paisley, UK) or by direct analysis using mass spectrometry of purified phosphorylated peptides labelled with heavy isotopes.

Materials and methods

Plant growth conditions

Wild-type *Arabidopsis thaliana* (L.) Heynh ecotype Ws-2 and the OXI1 T-DNA insertion mutant (Rentel *et al.*, 2004) were grown in a sterile hydroponic system. Sterilized seeds were sown on stainless steel wire mesh platforms (with a mesh diameter of 0.45 mm). Mesh platforms were placed within Magenta pots and 130 ml of Murashige and Skoog (MS) media (4.4 g l^{-1} MS, 10 g l^{-1} sucrose, 0.4 g l^{-1} MES (2-(N-morpholino)ethanesulfonic acid), pH 5.8) was added to the pots so that the final volume was just below the surface of the wire mesh. Plants were grown for 14 d under a photoperiod of 16 h and at a constant temperature of 21°C. After 14 d plant roots were treated for 30 min with 0.1% w/v cellulase (Melford Laboratories Ltd, Ipswich, UK) to induce OXI1 expression and roots were harvested, blotted on filter paper and frozen immediately in liquid nitrogen.

Protein extraction

Proteins were extracted from Arabidopsis root tissue according to the method described by Isaacson *et al.* (2006). Briefly, root tissue was homogenized in liquid nitrogen and suspended in ice-cold extraction buffer (0.7 M sucrose, 0.1 M KCl, 0.5 M Tris-HCl, pH 7.5, 50 mM EDTA, 2% v/v β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 50 mM sodium pyrophosphate, 25 mM sodium fluoride and 1 mM sodium molybdate) and an equal volume of ice-cold phenol saturated with Tris-HCl (pH 7.5). Samples were shaken for 30 min at 4°C and then centrifuged at 5000 ***g*** for 30 min at 4°C. The upper phenolic phase was collected and an equal volume of extraction buffer was added for re-extraction. Samples were shaken and centrifuged as before and the upper phenolic phase was re-extracted once more. Proteins were then precipitated from this phase with five volumes of ice-cold 0.1 M ammonium acetate in methanol at −20°C overnight. Proteins were pelleted by centrifugation at 5000 ***g*** and the protein pellet washed three times with ice-cold methanol followed by three washes with ice-cold acetone.

Two-dimensional SDS-PAGE and phosphoprotein staining

Protein samples were suspended in sample buffer (10 ml of buffer contains 3.6 g urea, 1.52 g thiourea, 0.2 g 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 0.03 g dithiothreitol (DTT)) and total protein quantified by Bradford assay. Once quantified, IPG buffer (GE Healthcare, Chalfont St Giles, UK) (200 μl per 10 ml) and trace bromophenol blue were added to the samples and 280 μg of protein was loaded on to 180 mm, pH 4–7, non-linear Immobiline DryStrips (GE Life Sciences, Buckinghamshire, UK). Isoelectric focusing (IEF) was performed on a multiphor II electrophoresis unit (GE Life Sciences) for 22 h at 20°C at the following voltage: 500 V for 5 h, gradient to 3500 V over 5 h, 3500 V for 12 h (current and power were were maintained at 1 mA and 5 W, respectively). Once IEF was complete, the Immobiline Strips were equilibrated and placed on 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels and run at 10 mA overnight. Gels were stained for protein phosphorylation using Pro-Q Diamond stain following the manufacturer's instructions. Gels were subsequently stained for total protein using colloidal Coomassie stain (10% w/v ammonium sulphate, 10% v/v phosphoric acid, 20% v/v methanol and 0.12% w/v G250). Protein spots were quantified using PD-Quest software (Bio-Rad). Data were acquired from three independent biological replicates.

Protein spots were cut and identified by digestion with trypsin followed by peptide mass fingerprinting as described by Millar *et al.* (2001) but with digestion carried out at room temperature overnight. Mass spectra were obtained using a Shimadzu Axima CFR MALDI-TOF (Shimadzu Biotech, Milton Keynes, UK) in positive ion reflectron mode, and peptide mass lists were searched against the National Centre for Biotechnology Information gene database using an in-house Mascot server (Matrix Science Ltd, London, UK). The following settings were used: peptide mass tolerance ± 50 ppm, maximum missed cleavages = 3.

Stable isotope labelling

Wild-type *A. thaliana* ecotype Ws-2 and the OXI1 T-DNA insertion mutant were grown for 14 d in a sterile hydroponic system as described earlier but with wild-type plants grown in MS media in which nitrogen compounds (potassium

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nitrate and ammonium nitrate) were replaced with ^{15}N-labelled stable isotopes (labelled to ≥ 98%; CK Gas Products Ltd, Hook, UK). Plants were treated with cellulase, roots harvested and protein extracts generated as described earlier. Wild-type and OXI1 mutant protein samples were suspended in 10 mM Hepes, 6 M urea, 2 M thiourea and quantified by Bradford assay before being combined 1 : 1 (1 mg of wild-type protein extract and 1 mg of OXI1 mutant protein extract). Samples were collected from three independent biological replicates.

Protein digestion and phosphopeptide enrichment

Protein samples were reduced for 45 min at room temperature with the addition of DTT to a final concentration of 1 mM and alkylated for 30 min with the addition of iodoacetamide to a final concentration of 5 mM. Protein samples were diluted with 10 mM Tris, pH 8.0, to achieve a final urea concentration of 2 M before digestion overnight with 20 μg trypsin. Digested protein samples were fractionated by strong-cation exchange chromatography (SCX) as described by Olsen *et al.* (2006) but using a gradient of NH_4HCO_2 of 5–200 mM. Seven 4 ml peptide fractions were collected and enriched for phosphopeptides using titanium dioxide (TiO_2) chromatography columns. One millilitre of 30 g l^{-1} 2,5-dihydroxybenzoic acid (DHB) in 80% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA) was added to each pooled peptide fraction before the addition of TiO_2 beads (5 mg of beads in 3g l^{-1} DHB with 80% ACN/0.1% TFA). Peptide fractions were incubated with TiO_2 beads, shaking for 1 h at room temperature, and the beads were harvested by centrifugation. Beads were washed with a solution of 3 g l^{-1} DHB, 80% ACN/0.1% TFA before being washed three times with 80% ACN/0.1% TFA. The beads were then loaded on to a homemade C8 column within a gel-loader tip and washed with 80% ACN. Phosphopeptides were eluted with two successive washes of 100 μl elution buffer (2% NH_4OH, 40% ACN). Eluted samples were dried in a Speed-Vac and suspended in 10 μl of 0.1% TFA before being subjected to LC-MS/MS analysis.

Mass spectrometry data were acquired on an Orbitrap mass spectrometer (Thermo Scientific, Hemel Hempstead, UK) fitted with a nanospray source (Proxeon, Thermo Scientific) coupled to a U3000 nano HPLC system (Dionex, Leeds, UK). Samples were loaded on to a 15-cm-long, 100 μm internal diameter, home-packed column made by packing a Picotip emitter (New Objective, Woburn, MA, USA) with ProntoSIL C18 phase; 120 angstrom pore, 3 μm bead C18 (ReproSil-Pur C18-AQ 3 μm; Dr Maisch GmbH, Germany). The nano HPLC was run in a direct injection configuration. Buffer A consisted of 94.5% H_2O, 5% ACN and 0.5% acetic acid, and buffer B consisted of 99.5% ACN and 0.5% acetic acid. Samples were loaded on to the column at a flow rate of 700 nl min^{-1}, and resolved using a 120 min gradient at a flow rate of 300 nl min^{-1}. The Orbitrap was run in a data-dependent acquisition mode in which the Orbitrap resolution was set at 60 000 and the top five multiply charged species were selected for MS/MS.

MS data analysis

Raw data files were searched against the NCBInr database (http://www.ncbi.nlm.nih.gov/) using MASCOT with the following settings: peptide mass tolerance ± 20 ppm, fragment mass tolerance ± 0.5 Da, maximum missed cleavages = 3, ^{15}N metabolic quantitation and the variable modifications carbamidomethylation of cysteine, oxidation of methionine and phosphorylation of serine, threonine and tyrosine. Peptides were quantified using MSQuant software (http://msquant.sourceforge.net/) with the default settings for quantification of ^{15}N-labelled peptides (Mortensen *et al.*, 2010). ^{15}N peptide ratios were corrected for inaccurate peptide ratios introduced by incomplete ^{15}N labelling using the method described by Gouw *et al.* (2008). Additional ^{15}N-labelled peptides were also detected following the method described by Gouw *et al.* (2008). The degree of ^{15}N incorporation was determined using the isotope distribution calculator ICR-2LS (Pacific Northwest National Laboratory, Richland, WA, USA; http://omics.pnl.gov/software/ICR2LS.php).

Results

Conditions for inducing *OXI1* expression

Previously OXI1 kinase has been shown to be induced in roots and leaves of *A. thaliana* in response to oxidative stress. *OXI1* expression is also up-regulated in response to cellulase and hydrogen peroxide treatment (Rentel *et al.*, 2004). Before commencing detailed phosphorylation studies we wished to confirm *OXI1* expression in our experimental system. The roots of 14-d-old Arabidopsis seedlings were treated with 0.1% (w/v) cellulase for 30 min. This time point and cellulase concentration were selected based on the results of Rentel *et al.* (2004) in which cellulase was shown to be a strong activator of *OXI1* with expression increasing over time. Using an *OXI1*:: luciferase Arabidopsis transgenic line we confirmed *OXI1* expression in the roots and leaves of Arabidopsis seedlings treated with 0.1% cellulase (data not shown).

Two-dimensional PAGE reveals differences in protein phosphorylation

A preliminary screen using two-dimensional PAGE and staining with Pro-Q Diamond, identified four candidate

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proteins that showed reduced phosphorylation in the *oxi1* null mutant background when compared with wild-type plants (Supporting Information, Fig. S1). Three of these candidate proteins were identified by mass spectrometry: citrate synthase 4 (AT2G44350), a ubiquitin-activating enzyme (AT2G30110) and COP9 signalosome 5A (AT2G30110) all showed reduced phosphorylation in the *oxi1* null mutant (Fig. S1, Table S1). All three proteins showed at least a 1.5-fold difference in phosphorylation and this difference was observed in three independent biological replicates (Fig. S2). None of these proteins were altered in overall protein abundance to a degree sufficient to account for altered Pro-Q staining (Table S2), indicating that the difference in protein phosphorylation is not the result of altered protein expression. It is important to note that a single gel spot may comprise multiple proteins, any one of which may be phosphorylated and contribute to a signal on a Pro-Q stained gel. The candidate proteins identified in this study would therefore require their phosphorylation status to be confirmed by further analysis.

Stable isotope labelling of whole plants and phosphopepetide enrichment enables a direct comparison of the abundance of wild-type and *oxi1* mutant phosphopeptides

Wild-type and *oxi1* null mutant plants were grown in a sterile hydroponic system in which the nitrogen source within the growth medium was either unlabelled (in the case of *oxi1* null mutant plants) or ^{15}N-labelled (in the case of wild-type plants). Such a strategy allows protein samples from the two lines to be combined and processed together, eliminating any variation in sample preparation and any variations in peptide identification and quantitation during mass spectrometry. An overview of the complete experimental strategy is shown in Fig. 1. The degree of ^{15}N incorporation was calculated to be 98.3%, and this value was used to adjust the quantified peptide data for improved accuracy according to the method described by Gouw *et al.* (2008). Phosphopeptide enrichment and LC-MS/MS analysis enabled the identification of over 1600 phosphopeptides (Table S3), representing a good coverage of the phosphoproteome when compared with the results of other studies (Nuhse *et al.*, 2004; Benschop *et al.*, 2007; Sugiyama *et al.*, 2008).

Quantifiable differences in phosphopeptide abundance are observed between wild-type and *oxi1* mutant plants

Stable isotope labelling of whole plants combined with phosphopeptide enrichment and peptide identification by LC-MS/MS identified a number of proteins that show altered abundance of phosphorylated peptides in the *oxi1*

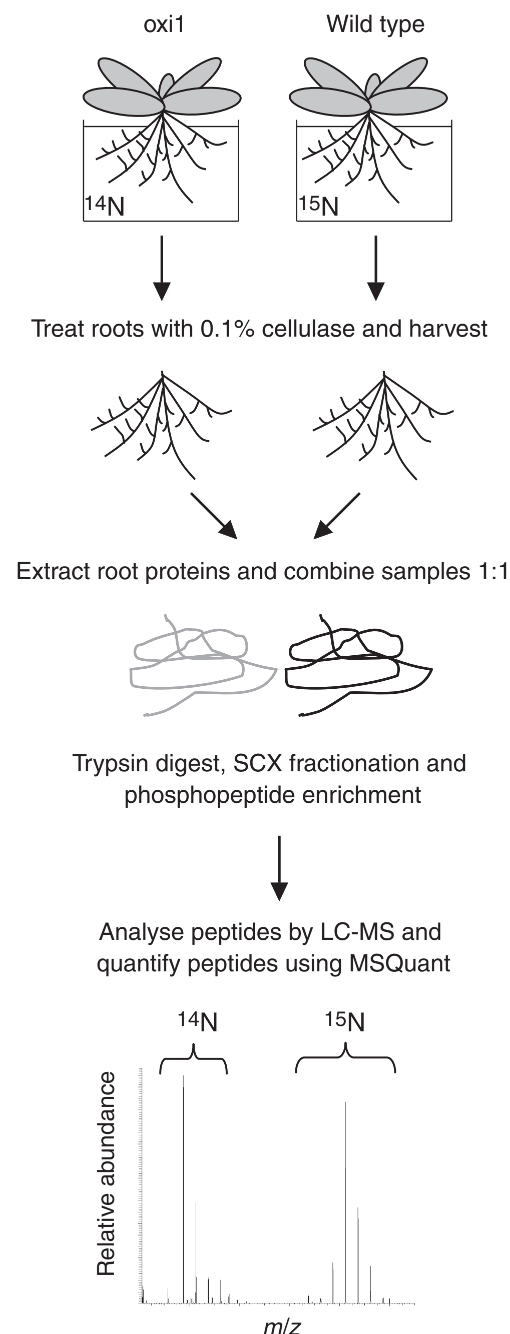

Fig. 1 Experimental strategy for isotope labelling, protein extraction, phosphopeptide enrichment and LC-MS/MS based proteomics. Wild-type and *oxi1* null mutant plants were grown in a sterile hydroponic system supplemented with light (^{14}N) or heavy (^{15}N) nitrogen. After 14 d, roots were treated with 0.1% cellulase for 30 min to induce OXI1 expression. Roots were harvested and proteins extracted before combining wild-type and OXI1 protein samples 1 : 1. The protein sample was digested with trypsin, and peptides fractionated with strong cation exchange chromatography (SCX) followed by phosphopeptide enrichment using titanium dioxide columns. Peptides were analysed by LC-MS/MS, and the ^{14}N and ^{15}N peptides, corresponding to OXI1 mutant and wild-type proteins, respectively, were quantified using MSQuant software. Data were collected from three independent biological replicates.

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null mutant. Peptides were only considered as differentially abundant if they showed at least a twofold difference in abundance in all three biological replicates. Peptides showing conflicting alterations in abundance between fractions and between the three replicates were discarded from the data set. Two categories were identified: proteins that show decreased phosphopeptide abundance in *oxi1* mutant plants compared with wild-type, and proteins that show increased phosphopeptide abundance in *oxi1* mutant plants compared with wild-type. A putative F-box protein (AT1G27540) and phosphoinositide-dependent kinase 1 (AtPDK1, AT5G04510; putative PDK1, AT3G10540) both showed decreased phosphopeptide abundance in the *oxi1* mutant compared with wild-type plants (Fig. 2, Table 1). Four proteins showed increased phosphopeptide abundance in *oxi1* mutant plants compared with wild-type: ethylene insensitive 2 (EIN2, AT5G03280), seed imbibition 1-like (SIP1, AT5G40390), phospholipase D-gamma (AT4G11850) and an unknown protein (AT1G31870) (Fig. 2, Table 1). Annotated LC-MS/MS spectra showing peptide fragmentation and predicted phosphorylation sites are shown in Fig. S3. The putative F-box protein (AT1G27540) was only sequenced by LC-MS/MS in one replicate. Additional data supporting the identity of this protein and its quantitation are provided in Fig. S4.

Discussion

This study aimed to identify components of the OXI1 signalling pathway in *A. thaliana* by examining the phosphorylation status of the proteome of wild-type and *oxi1* null mutant plants. Two-dimensional gel electrophoresis along with stable isotope labelling and phosphopeptide enrichment have enabled us to identify a number of candidates that show altered phosphoprotein/peptide abundance in a *oxi1* null mutant of Arabidopsis.

In the current investigation, six proteins were identified that showed altered phosphopeptide abundance in the *oxi1* null mutant, using isotope labelling, phosphopeptide enrichment and LC-MS/MS. All of these proteins were also

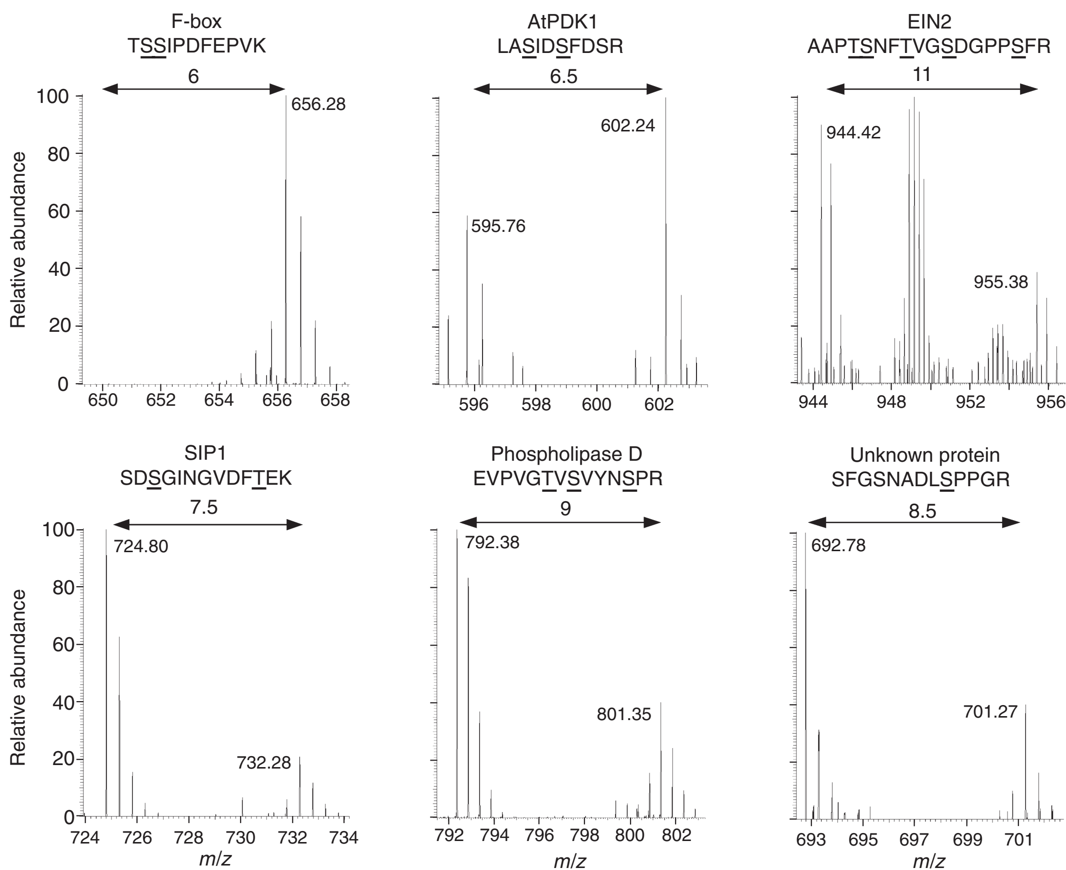

Fig. 2 Representative LC-MS spectra showing quantifiable differences in phosphopeptide abundance when comparing *oxi1* null mutant plants to wild-type. Protein identity is shown, as is the predicted position of the phosphorylated residue (underlined). The mass of the monoisotopic peak (where present) is shown for each peptide. The horizontal double-headed arrow above each spectrum indicates the pairs of ^{14}N- and ^{15}N-labelled peptides. Peptide ratios and identification data are presented in Table 1 for all peptides and for the three biological replicates. ATPDK1, 3-phosphoinositide-dependent kinase 1; EIN2, ethylene insensitive 2; SIP1, seed imbibition 1-like.

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Table 1 Identity of proteins with altered phosphorylation in the *oxi1* mutant as revealed by stable isotope labelling and phosphopeptide enrichment

	Protein	Peptide sequence	Mascot ion score	Peptide ratio (oxi1 : WT)
AT1G27540	F-box	TSSIPDFEPVK*	25	0.001
AT5G04510/AT3G10540	PDK1	LASIDSFDSR	56, 65, 53	0.42 ± 0.16
AT5G03280	EIN2	AAPTSNFTVGSDGPPSFR	46, 52, 72	2.43 ± 0.36
AT5G40390	SIP1	SDSGINGVDFTEK	67, 66, 72	4.20 ± 1.56
AT4G11850	Phospholipase D-gamma	EVPVGTVSVYNSPR	85, 52, 71	3.45 ± 1.16
AT1G31870	Unknown protein	SFGSNADLSPPGR	67, 70, 82	2.41 ± 0.54

PDK1, phosphoinositide-dependent kinase 1; EIN2, ethylene insensitive 2; SIP1, seed imbibition 1-like.
*The putative F-box protein was only sequenced by LC-MS/MS in one replicate.
Phosphopeptides with at least a twofold change in abundance in the *oxi1* mutant compared with wild-type are shown. Predicted phosphorylation sites are underlined. Where unambiguous phosphorylation site assignment cannot be achieved, multiple sites are underlined. The table shows the mascot score for each peptide and the peptide ratio (average of three biological replicates ± SD). Additional data supporting the identity of this protein and its quantitation are provided in Fig. S4.

shown to be phosphorylated in a recent study by Reiland *et al.* (2009). In fact, all but PDK1 were shown to be phosphorylated on the same peptide as those identified by Reiland *et al.* (2009), raising confidence in the identification of the phosphopeptides in the current investigation.

Within our list of phosphorylated candidates, two proteins, phosphoinositide-dependent kinase 1 (PDK1) and phopholipase D-gamma (PLD), have previously been shown to be upstream in the OXI1 signal transduction pathway. The PDK1 phosphopeptide was found in lower abundance in the *oxi1* mutant, while the PLD phosphopeptide was found in greater abundance in the *oxi1* mutant relative to wild-type. OXI1 is activated by PDK1-mediated phosphorylation and PDK1 activity is regulated by binding the lipid signalling molecule phosphatidic acid (PA). PA-induced activation of PDK1 is likely to occur through the activity of PLD (Anthony *et al.*, 2006). A range of stresses, including oxidative stress, have been shown to activate PLD and subsequent PA biosynthesis (Bargmann & Munnik, 2006). PDK1 has previously been shown to be phosphorylated in mammals and in plants, and phosphorylation is important for PDK1 activity (Casamayor *et al.*, 1999; Otterhag *et al.*, 2006). Our observation that PDK1 is phosphorylated to a reduced extent in the *oxi1* mutant may suggest feedback from OXI1 to PDK1 for the control of PDK1 activity. The phosphorylation of PDK1 may be dependent on the presence of its substrate, OXI1. The phosphorylation of PDK1 may occur directly via OXI1 kinase activity or indirectly by the activity of another kinase in the OXI1 signalling pathway. The activity of plant PLD enzymes may also be regulated by phosphorylation (Novotna *et al.*, 2003). PLD phosphorylation was increased in the *oxi1* mutant, indicating that PLD is not likely to be a direct downstream target of OXI1 but rather an indirect target during the regulation of the OXI1 signalling cascade.

A putative F-box protein was also found to have reduced phosphopeptide abundance in the *oxi1* null mutant. F-box proteins are part of the SCF (SKP1-CUL1-F-box) protein complex, which is a class of E3 ubiquitin ligases. The SCF complex catalyses the ubiquitination of target proteins, which are subsequently degraded by the 26S proteosome. The role of F-box proteins within the SCF complex is to recognize and bind a substrate protein for degradation (Lechner *et al.*, 2006). Arabidopsis is estimated to have almost 700 F-box proteins and these proteins are involved in diverse activities, including hormone signalling, root and shoot development and pathogen resistance (Xu *et al.*, 2009).

A phosphorylated peptide of the ethylene response regulator EIN2 was found to be more abundant in the *oxi1* mutant background. This increase in phosphorylation suggests that EIN2 is not a direct target of OXI1, but is affected as a secondary consequence in the *oxi1* mutant, possibly as a result of cross-talk between the OXI1 and ethylene signalling pathways. Corroborating the link between OXI1 and ethylene signalling, both *oxi1* and *ein2* mutants share a common phenotype of reduced root hair elongation (Pitts *et al.*, 1998; Rentel *et al.*, 2004). While EIN2 phosphorylation has been detected in previous large-scale phosphorylation studies (Sugiyama *et al.*, 2008, Reiland *et al.*, 2009),, the importance of this modification for the plant ethylene response is yet to be determined.

The candidate proteins identified in this investigation may be direct or indirect targets of OXI1 kinase and may be positioned upstream or downstream of OXI1 within the signal transduction pathway. Further work will be required to establish whether any of the five proteins with decreased phosphorylation in the *oxi1* mutant are direct phosphorylation targets of OXI1.

In summary, we have identified a range of alterations in the phosphoproteome of *oxi1* mutant plants and have discovered a number of new candidate proteins for the OXI1 signalling cascade. The phosphoproteomic approaches employed in this study have proved extremely sensitive for the quantification of changes in protein phosphorylation. These results may suggest that the plants' response to oxidative stress through OXI1 is closely linked to other signalling

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pathways, including plant hormone signalling and protein degradation.

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Supporting Information

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

Fig. S1 Identification of phosphorylated proteins in wild-type and *oxi1* mutant plants using two-dimensional gel electrophoresis followed by staining with Pro-Q Diamond.

Fig. S2 Full data set showing the identification of phosphorylated proteins in wild-type and *oxi1* mutant plants using two-dimensional gel electrophoresis followed by staining with Pro-Q Diamond.

Fig. S3 LC-MS/MS spectra showing peptide fragmentation and predicted phosphorylation sites for proteins of interest identified in this study.

Fig. S4 LC-MS spectra comparing the sequenced F-box peptide (replicate 1) with the unsequenced peptide that was found in replicates 2 and 3.

Table S1 Identity of candidate proteins with altered phosphorylation in the *oxi1* mutant as revealed by two-dimensional gel electrophoresis

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Table S2 The Coomassie stained spot intensity ratio for proteins isolated from wild-type and *oxi1* mutant plants

Table S3 Complete list of phosphopeptides identified in this study

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