The *Arabidopsis* protein kinase Pto-interacting 1-4 is a common target of the oxidative signal-inducible 1 and mitogen-activated protein kinases

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In *Arabidopsis thaliana*, the serine/threonine protein kinase oxidative signal-inducible 1 (OXI1), mediates oxidative stress signalling. Its activity is required for full activation of the mitogen-activated protein kinases (MAPKs), MPK3 and MPK6, in response to oxidative stress. In addition, the serine/threonine protein kinase Pto-interacting 1-2 (PTI1-2) has been positioned downstream from OXI1, but whether PTI1-2 signals through MAPK cascades is unclear. Using a yeast two-hybrid screen we show that OXI1 also interacts with PTI1-4. OXI1 and PTI1-4 are stress-responsive genes and are expressed in the same tissues. Therefore, studies were undertaken to determine whether PTI1-4 is positioned in the OXI1/MAPK signalling pathway. The interaction between OXI1 and PTI1-4 was confirmed by using *in vivo* co-immunoprecipitation experiments. OXI1 and PTI1-4 were substrates of MPK3 and MPK6 *in vitro*. Although no direct interaction was detected between OXI1 and MPK3 or MPK6, *in vitro* finding studies showed interactions between MPK3 or MPK6 with PTI1-4. In addition, PTI1-4 and MPK6 were found *in vivo* in the same protein complex. These results demonstrate that PTI1-4 signals via OXI1 and MPK6 signalling cascades.

**Structured digital abstract**

- PTI1-4 and OXI1 phosphorylate *by* protein kinase assay (View interaction)
- OXI1 physically interacts with PTI1-4 *by* two hybrid (View interaction)
- MPK6 physically interacts with PTI1-4 *by* anti tag coimmunoprecipitation (View interaction)
- MPK3 and OXI1 phosphorylate *by* protein kinase assay (View interaction)
- MPK6 *binds* to PTI1-4 *by* pull down (View interaction)
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- OXI1 physically interacts with PTI1-4 *by* anti tag coimmunoprecipitation (View interaction)
- PTI1-4 and MPK6 phosphorylate *by* protein kinase assay (View interaction)
- PTI1-4 physically interacts with AGC2-3 *by* two hybrid (View interaction)
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- PTI1-4 *interacts* to OXI1 *by* pull down (View interaction)

**Abbreviations**

3-AT, 3-Amino-1,2,4-triazole; GST, glutathione S-transferase; HA, haemagglutinin; HIS, histidine; HR, hypersensitive response; MAPK, mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase; MBP, myelin basic protein; OXI1, oxidative signal-inducible 1; PDK1, 3-phosphoinositide-dependent kinase 1; PTI1, Pto-interacting 1; ROS, reactive oxygen species.
Introduction

Reactive oxygen species (ROS) are mainly considered as toxic by-products of aerobic organisms. However, plants are also able to use ROS as signalling molecules for regulating plant development, responses to biotic, abiotic stresses and programmed cell death [1–3]. The generation of ROS, as well as their detoxification, has been well studied, but little is known as to how various cellular ROS are being perceived and which signalling network is then being activated to mediate responses in plants [4]. Recently, oxidative signal-inducible 1 (OXI1), a serine/threonine protein kinase of the AGC family (AGC2-1), was shown to be necessary for ROS-mediated responses in Arabidopsis [5]. The oxi1 mutant was compromised in ROS-dependent processes, such as root hair elongation, and displayed enhanced susceptibility to biotrophic pathogens, such as the fungal pathogen Hyaloperonospora parasitica [5] and the bacteria Pseudomonas syringae [6]. The kinase activity of OXI1 was itself induced by H₂O₂, wounding, cellulase and various elicitor treatments [5,7] mimicking pathogen attack.

The Arabidopsis genome encodes 39 AGC kinases, of which 23 are classified to the AGC VIII group [8,9]. The AGC kinases were named on the basis of their homology to the mammalian cAMP-dependent protein kinase A, cGMP-dependent protein kinase G and phospholipid-dependent protein kinase C [8]. However, the AGC VIII kinases represent a plant-specific subfamily characterized by a conserved DFD amino acid motif in subdomain VII of the catalytic domain and by the presence of an amino acid insertion of variable size between subdomains VII and VIII [8,9]. Such as OXI, other AGC kinases of the AGC VIII subfamily have been shown to be involved in various signalling pathways, including blue light signalling [10] and auxin signalling [11–13]. The majority of group VIII AGC kinases are phosphorylated and activated by another AGC kinase, 3-phosphoinositide-dependent kinase 1 (PDK1) [14–16]. Indeed, in Arabidopsis, PDK1 was shown to interact with and phosphorylate OXI1 [15]. Furthermore, Pto-interacting 1-1 (PTI1-1), PTI1-2 and PTI1-3 were identified as new downstream components of PDK1 and OXI1 [7]. These PTI1-like proteins are serine/threonine protein kinases that share strong sequence identity to the tomato PTI1 kinase. In Arabidopsis, 10 members of the PTI1 gene family have been identified and share a highly conserved kinase domain [7]. In tomato, PTI1 can physically interact with the serine/threonine kinase PTO, which confers resistance to the bacterial pathogen P. syringae pv tomato carrying the avirulence effector proteins AvrPto or AvrPtoB [17,18].

The OXI1 protein kinase was also shown to be an upstream regulator of two mitogen-activated protein kinases (MAPKs), MPK3 and MPK6, as oxi1 mutants are impaired in the activation of MPK3 and MPK6 in response to oxidative stress [5]. Different MAPK pathways respond to a variety of external stimuli and consist of three sequentially acting protein kinases: a MAPK kinase kinase, a MAPK kinase (MAPKK) and finally a MAPK [19]. However, little is known about the function and composition of the different MAPK signalling pathways. MPK3 and MPK6 were shown to be involved in regulating various developmental processes and stress responses [20,21].

Here we report that PTI1-4, another member of the PTI1-like family, interacts with OXI1. By using yeast two-hybrid assays, other members of the AGC family (AGC2-2 and AGC2-3) were shown to interact with the PTI1-4 kinase. Because various PTIs interact with different AGCs, studies were undertaken to determine whether PTI1-4 and OXI1 indeed form a complex in planta. The interaction between the two proteins was confirmed by in vivo co-immunoprecipitation experiments. We then examined how both proteins interact with MPK3 and MPK6 proteins.

Results

AGC kinases interact with PTI1 kinases in vitro

To isolate other components of the OXI1 (AGC2-1) signalling pathway, a yeast two-hybrid screen was performed. The OXI1 ORF fused to the GAL4 binding domain was used to screen a library of Arabidopsis root cDNAs fused to the GAL4 activation domain. Two serine/threonine protein kinases that share strong sequence identity to the tomato PTI1 kinase were identified. Work by Anthony et al. [7] had already positioned these kinases as new downstream OXI1 components and named the proteins PTI1-1, 1-2 and 1-3. One of the prey cDNA encoded PTI1-1 (At1g06700) and a second prey cDNA encoded another member of the family, which we named PTI1-4 (At2g47060) (Fig. 1A). To isolate additional components of this OXI1/PTI1-4 pathway, a second two-hybrid screen using PTI1-4 as bait was performed. 4.2 x 10⁵ transformed yeast colonies were screened on selective media lacking histidine and containing 1 mM 3-Amino-1,2,4-triazole (3-AT). Seven positive clones showing growth on selective media lacking adenine as well as β-galactosidase activity were further analysed (Fig. 1B). Three of the prey cDNAs encoded two other
GST-tagged proteins were pulled down with glutathione-agarose beads. The proteins were then detected by western blot analysis using an anti-HIS or an anti-GST IgG. Figure 1C shows HIS-PTI1-4 and HIS-OXI1 bound to GST-OXI1 and GST-PTI1-4, respectively, but not to GST alone. The kinase-deficient mutant, OXI1K45R, in which the lysine residue of the ATP-binding domain is mutated to arginine, still interacted with PTI1-4. These data indicate that the kinase activity of OXI1 is not required for the interaction with PTI1-4.

**OXI1 interacts with PTI1-4 in vivo**

Because various PTIs interact with AGCs VIII in vitro, the interaction between OXI1 and PTI1-4 proteins was tested in *Arabidopsis* plants. To investigate the association between OXI1 and PTI1-4 in vivo, we generated transgenic *A. thaliana* plants expressing both an *OXI1* genomic fragment tagged with haemagglutinin (HA) under the control of its own promoter (*OXI1pro:HA-OXI1*) and a *35Spro:PTI1-4-MYC* construct. The interaction between the two proteins was then tested using co-immunoprecipitation assays. When HA-OXI1 fusion proteins were immunoprecipitated from plant extracts using an anti-HA IgG, PTI1-4-MYC was detected in the HA-OXI1 immunocomplex (Fig. 2). As controls, plant extracts were also mixed with protein A-sepharose beads only and showed no PTI1-4-MYC signal. In addition, plant extracts from wild-type Col-0 plants were immunoprecipitated with an anti-HA IgG and no background signal was visible (Fig. 2). These results indicate that OXI1 and PTI1-4 interact in vivo.

**OXI1 and PTI1-4 are stress-responsive genes and show overlapping expression profiles in the root**

As Rentel *et al.* [5] showed, by northern blot analysis, that in seedlings the expression of *OXI1* was increased upon oxidative stress, we investigated whether *PTI1-4* mRNA accumulated after oxidative stress in seedlings. Real-time quantitative RT-PCR was used to show an increase in the levels of *OXI1* and *PTI1-4* transcripts in response to different stresses, such as H2O2, wound and cellulase treatment (Fig. 3A). Both genes responded to the different oxidative stress treatments in a similar pattern. The response was fast, observable within 0.5–1 h of the treatment and was transient. However, the accumulation of the *OXI1* transcript in response to oxidative stress was stronger than that of the *PTI1-4* transcript.

If *OXI1* and *PTI1-4* function together in *Arabidopsis*, the expression pattern of the two genes should be
It is known that OXI1 is expressed in the roots as well as the root hairs [5]. To examine the tissue-specific expression pattern of PTI1-4, we transformed Arabidopsis plants with a PTI1-4pro:GUS construct. Histochemical staining of transgenic Arabidopsis seedlings showed that PTI1-4 is more broadly expressed in the seedling than OXI1 (Fig. 3B). A strong expression of PTI1-4 could be detected in the roots as well as the root hairs, similar to OXI1 (Fig. 3B). Expression of both genes was observed early during plant growth and was present in the root apical meristem of the embryo. However, OXI1 expression is mainly localized to the root meristem, whereas PTI1-4 is expressed in different tissues of the embryo.

OXI1 phosphorylates PTI1-4 in vitro

Next, by using in vitro kinase assays we tested whether OXI1 could phosphorylate PTI1-4 because OXI1 is known to phosphorylate PTI1-1 and PTI1-2 in vitro and, to a lesser extent, PTI1-3 [7]. Both kinases were purified as HIS-tagged proteins and incubated with [$\gamma$-32P]-ATP. In contrast to PTI1-4, OXI1 was capable of strong autophosphorylation activity (Fig. 4A). When both proteins were incubated together, OXI1 could phosphorylate PTI1-4. As expected, the kinase inactive form of OXI1 (OXI1K45R) showed no autophosphorylation activity and showed no phosphorylation of PTI1-4. OXI1 is therefore able to use PTI1-4 as a substrate as well as the artificial substrate myelin basic protein (MBP) but not GST (Fig. 4A). Although no kinase activity could be detected for PTI1-4 in vitro, incubating OXI1 with increasing amounts of PTI1-4 enhanced the autophosphorylation activity of OXI1 (Fig. 4B) as well as the transphosphorylation of MBP. Simply by incubating the two proteins over a period of time in kinase buffer before adding the [$\gamma$-32P]-ATP was sufficient to increase the autophosphorylation activity of OXI1 as well as transphosphorylation of PTI1-4 and MBP proteins (Fig. 4B). Incubating OXI1 alone for a period of time in kinase buffer before adding the [$\gamma$-32P]-ATP did not significantly increase its autophosphorylation activity. These results suggest...
that PTI1-4 may be necessary for activation of the OXI1 kinase activity.

**MPK3 and MPK6 phosphorylate OXI1 and PTI1-4 in vitro**

Because OXI1 has been shown to play a role in the activation of MPK3 and MPK6 in response to abiotic stresses [5], we studied whether PTI1-4 was also required for the full activation of MPK3 and MPK6. However, the activity of MPK3 and MPK6 was not altered in response to wounding in \textit{pit1-4} mutant plants or to cellulase 0.1% treatment in \textit{35Spro:PTI1-4-MYC} transgenic lines compared with Col-0 (Fig. S1). We then tested whether the OXI1 protein could use MPK3 and MPK6 proteins as substrates. Because the purified GST-MPKs showed autophosphorylation activity, loss-of-function (kinase-inactive) forms of the MAPKs were produced as GST-lofMPK3 and GST-lofMPK6. However, when lofMPK3 and lofMPK6 proteins were tested for phosphorylation of OXI1, no phosphorylation was observed (Fig. 5A). On the other hand, when OXI1\textsuperscript{K45R} or PTI1-4 proteins were mixed with active MPK3 or MPK6 kinases, phosphorylation of OXI1\textsuperscript{K45R} and PTI1-4 by MPK3 as well as MPK6 proteins could be detected (Fig. 5B). As expected, no phosphorylation was seen when the kinase inactive forms lofMPK3 and lofMPK6 were tested for phosphorylation of OXI1\textsuperscript{K45R} or PTI1-4 (Fig. 5B). These results show that MPK3 and MPK6 can phosphorylate OXI1 as well as PTI1-4 in vitro.

**PTI1-4 interacts with MPK3, MPK6 in vitro and with MPK6 in vivo**

To investigate further the interaction between OXI1/PTI1-4 and MPK3/MPK6 proteins, we tested whether
HIS-OXI1 or HIS-PTI1-4 could be found to GST-MPK3 or GST-MPK6 proteins in vitro. Western blot analysis (Fig. 6A) showed that PTI1-4 could be detected in each of the MAPKs, but not to GST alone. No direct interaction between OXI1 and the MAPK proteins was detected (Fig. 6B). To confirm the interaction between PTI1-4 and MPK3/MPK6, in vivo co-immunoprecipitation experiments were undertaken. In addition, to link OXI1 to MPK3 and MPK6 proteins, we examined whether OXI1 could also be found in complexes with MPK3 or MPK6 proteins in vivo. For this purpose we used transgenic plants expressing either a 35Spro:PTI1-4-HIS or a 35Spro:OXI1-HIS construct. The different MAPK proteins were immunoprecipitated using MAPK-specific antibodies. After western blot analysis, PTI1-4 could be detected in anti-MPK6 immunoprecipitates from roots but not from anti-MPK3 immunoprecipitates (Fig. 6C). However, the MPK3 protein could also barely be detected in root extracts after immunoprecipitation with the anti-MPK3 IgG (Fig. 6D). On the other hand, the MPK6 protein was present in root extracts after immunoprecipitation with the anti-MPK6 IgG. These results indicate that PTI1-4 forms a protein complex with MPK6 in vivo. In contrast to PTI1-4, OXI1 was not detected from anti-MPK3 or anti-MPK6 immunoprecipitates. The fact that OXI1 could not be detected in a complex with the MAPK proteins might be due to the low amount of OXI1 protein in 35Spro:OXI1-MYC transgenic plants compared with 35Spro:PTI1-4-MYC overexpressors. Another possibility is that the interaction between OXI1 and MAPK proteins is triggered by stress. Thus, we then used Arabidopsis transgenic plants expressing OXI1 under the control of its promoter. When using these plant lines, we showed accumulation of the OXI1 protein in seedlings after wounding (Fig. S2). Co-immunoprecipitation experiments were then carried out using OXI1pro:HA-OXI1 seedlings wounded for either 30 min or 1 h. Even under these conditions or when using different extraction buffers, we could not find OXI1 in the same complex with MPK3 or MPK6 proteins (data not shown). However, the interaction between OXI1 and MAPK proteins could be transient and therefore difficult to detect.

**Discussion**

OXI1 was shown to interact with three different serine/threonine kinases that share strong sequence identity to the tomato PTI1 kinase and were therefore named PTI1-1, -1-2 and -1-3 [7]. In this study we showed that in vitro OXI1 can interact and phosphorylate another member of the PTI1 family, PTI1-4. Although other members of the AGC family (AGC2-2,
AGC2-3) were also identified as PTI1-4 interactors in yeast two-hybrid assays, the interaction between OXI1 and PTI1-4 was confirmed in planta. Moreover, both OXI1 and PTI1-4 expression patterns partially overlap. The two genes are strongly expressed in the root and root hairs and are induced upon oxidative stress treatments. These findings strengthen the possibility that OXI1 and PTI1-4 functionally interact in vivo.

In order to show that OXI1 and PTI1-4 function together in a signal transduction pathway, pti1-4 knockout lines were isolated and analysed to uncover phenotypic similarities to oxi1 mutants. However, pti1-4 mutants, as well as 35Spro:PTI1-4-MYC plants, showed no defects in root hair growth and pti1-4 mutants behaved like wild-type plants in response to infection with P. syringae pv tomato (data not shown). However, as Arabidopsis has 10 different members in the PTI1 family, this lack of phenotype could be explained by functional redundancy between different members of the PTI1 family. Rice has only two conserved PTI1 isoforms, OsPti1a and OsPti1b. Pathogen infection induces the hypersensitive response (HR), which is local and rapid cell death at the site of pathogen infection and limits growth of the micro-organism [22–24]. Mutants with enhanced disease resistance and exhibiting spontaneous cell death (HR-like lesions) have been identified [22, 24, 25]. The OsPti1a mutant showed spontaneous necrotic lesions on leaves and resistance to a compatible race of Magnaporthe grisea [26]. Moreover, plants overexpressing OsPti1a were more susceptible to a compatible race of the bacterial pathogen Xanthomonas oryzae pv oryzae. However, overexpression of the tomato SlPti1 in tobacco caused enhanced HR in leaves when challenged with P. syringae pv tabaci expressing AvrPto [17]. On the other hand, expression of the tomato SlPti1 cDNA in the rice OsPti1a mutant suppressed the mutant phenotype. These results indicate that PTI1 acts as a negative regulator of the HR response in rice, whereas it behaves as a positive regulator in tobacco. In Arabidopsis, the characterization of dou1 mutants between different PTI1 members may provide information on the mechanisms of PTI1 action.

The Arabidopsis MPK3 and MPK6 kinases have been extensively characterized and are known to be involved in stress responses as well as developmental processes. The two kinases are partially redundant and mpk3/mpk6 dou1 mutants are emphysema lethal [27]. The MPK3 and MPK6 kinase activity has been shown to be activated by ROS [28], as well as by bacterial and fungal elicitors [29, 30]. Because oxi1 mutant plants are impaired in the activation of MPK3 and MPK6 kinases upon oxidative stress treatments, OXI1 was positioned as an upstream regulator of the MPK3 and MPK6 cascade. Yet here we showed that OXI1 does not phosphorylate MPK3 or MPK6, but is itself phosphorylated by the MAPKs in vitro. Under these conditions, PTI1-4 is also phosphorylated by MPK3 and MPK6. These results might suggest that MPK3 and MPK6 proteins could act in a feedback loop on OXI1 and PTI1-4 (Fig. 7). On the other hand, because the kinase assays were carried out using recombinant proteins expressed in E. coli, we cannot rule out the possibility that in vitro illegitimate phosphorylations might have occurred. In addition, if these phosphorylation events occur in vivo, an interaction between the MAPK proteins and OXI1 or PTI1-4 should take place. Until now, no direct interaction between MPK3 and MPK6 has been detected with OXI1 in vitro or in vivo. However, we cannot exclude the possibility that the interaction is transient or exists under different experimental conditions. In contrast, in vitro binding studies showed an interaction of MPK3 and MPK6 with PTI1-4. In addition, PTI1-4 and MPK6 were found in the same protein complex in vivo.

Previous work by Anthony et al. [7] revealed the potential involvement of another member of the AGC kinase PDK1 in the OXI1/MAPK signalling pathway. PDK1 was shown to function upstream of OXI1 and PTI1-2 kinases and was required for the activation of

![Fig. 7. Model for PTI1-4 signal transduction.](image-url)
MPK6 upon xylanase treatment. From these results, a signalling cascade with the module PDK1/OXI1/PTI1-2 was proposed, but it was unclear how to position the MAPks in this cascade. In addition, in rice OsPdk1 was proposed to positively regulate fusal disease resistance through the OsOxi1-OsPtI1a phosphorylation cascade [31,32]. As our data show that MPK6 is found in vivo in a complex with PTI1-4, we favour a model in which MPK6 acts downstream from OXI1 and PTI1-4 (Fig. 7). However, because PTI1-4 is a common target of OXI1 and MPK6, a competition between the two proteins for binding to PTI1-4 may occur, resulting in the attenuation or amplification of a signalling pathway. Furthermore, MPK6 is known to be activated by MAPKKs, such as MKK2 [33], MKK3 [34], MKK4, MKK5 [30] and MKK9 [35]. These MAPKKs could provide an additional level of cross-talk between OXI1 and MPK6 (Fig. 7). Because MPK6 is a target of a wide set of MAPKKs, PDK1 activates many AGC kinases [15] and OXI1 interacts with PTI1-1, PTI1-2, PTI1-3 [7] and PTI1-4, future experiments would be necessary to decipher the specificity of action of each cascade and what mechanisms restrict or regulate cross-talk between distinct pathways.

**Experimental procedures**

**Yeast two-hybrid assays**

The coding sequence from *OXII* (At3g25250) or *PTII-4* (At2g47060) was cloned in the pBD-GAL4 cam (Stratagene, La Jolla, CA, USA) and were each used as bait to screen an *Arabidopsis* pACT2 cDNA library [36]. The yeast strain PJ69-4A [37] containing either pBD-OXI1 or pBD-PTI1-4 was transformed with the pACT2 cDNA library [38] and was screened for HIS auxotrophy. To confirm the interaction, the transformants were grown overnight at 30 °C in synthetic medium with dextrose (SD medium; 0.1% yeast nitrogen base without amino acids and ammonium sulfate, Difco Laboratories Ltd, West Molesey, Surrey, England; 2% dextrose, 0.5% ammonium sulfate) supplemented with the required amino acids. Ten microlitres of the suspension were then spotted on to SD agar plates lacking tryptophan, leucine and adenine and the cells were grown for 3 days at 30 °C. β-galactosidase agarose overlay assays were performed as described in the Herskowitz laboratory protocol (http://biochemistry.ucsf.edu/labs/herskowitz/xgalagar.html). Plasmids from positive yeast colonies were rescued and the cDNA inserts were identified by sequencing.

**GST pull-down assay and immunoblotting**

OXI1, PTI1-4, MPK3 and MPK6 were expressed as GST fusion proteins in the pGEX4-T1 vector (Amersham Biosciences, Little Chalfont, UK). OXI1 and PTI1-4 were expressed as HIS fusion proteins in the pET28a (+) vector (Novagen Inc., Madison, WI, USA). The OXI1K45R mutations were introduced into GST-OXI1 or HIS-OXI1 constructs using the QuickChange site-directed mutagenesis kit (Stratagene). GST- and HIS-tagged constructs were transformed into the *E. coli* strain BL-21 codon plus (Stratagene). Expression and purification of the GST-tagged proteins was carried out as described previously [39]. The HIS-tagged proteins were produced according to the manufacturer's manual (The QIAexpressionist™, Qiagen, Hilden, Germany). GST alone or GST-tagged proteins were mixed with HIS-tagged proteins in 200 μL wash buffer (50 mM Tris/HCl, pH 8, 150 mM NaCl, 1% Nonidet P-40) and were incubated for 2 h at 4 °C. Subsequently, 20 μL of glutathione-sepharose 4B beads (Amersham Biosciences) were added and the mixture was incubated for 4 h at 4 °C. Protein complexes were washed three times in wash buffer and denatured with SDS loading buffer. The proteins were separated by SDS/PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) by electroblotting. Membranes were probed with either anti-HIS monoclonal IgG (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) or with anti-GST monoclonal IgG (nanoTools Antikörpertechnik GmbH & Co. KG, Teningen, Germany). Membranes were developed by enhanced chemiluminescence, as recommended by the manufacturer (Gene Image, Amersham Biosciences).

**In vitro kinase assay**

Purified proteins were mixed together in 20 μL kinase buffer [50 mM Tris, pH 7.5, 1 mM dithiothreitol, 10 mM MgCl₂, 0.1 mM ATP and 0.1 μL mCi [γ-32P]-ATP (1 μCi)] and 1 μL MBP (10 mg/mL) when required. The reactions were incubated for 30 min at room temperature and were then stopped by adding SDS loading buffer. The reaction products were separated by SDS/PAGE and analysed by autoradiography and Coomassie Brilliant Blue R250 staining.

**Plasmids and cloning**

The *OXII* and *PTI1-4* coding sequence was amplified by PCR from total cDNA derived from Col-0 seedlings. The *OXII* coding sequence was cloned *EcoRI-SalI* into pAD, pBD (Stratagene), pGEX-4T-1 and pET-28a (*EcoRI-SalI/Xhol*). The lysine 45 (K45R) codon from OXI1 was changed to arginine by site-directed mutagenesis (Stratagene). The *PTI1-4* coding sequence was cloned *SalI-PtrI* into pAD, pBD (Stratagene) and *BamHI-SalI* into pGEX-4T-1 and pET-28a (*BamHI-SalI/Xhol*). ORFs of different MAPKs used were cloned as described previously [33].

The 35S promoter and terminator of the pRT101 vector was cloned *SalI/Xhol-NotI* into the binary vector pGreenII 0029 [40]. The MYC tag was cloned *Smal-XbaI* into this
modified pGreenII 0029 vector. OXI1 was cloned in frame to a MYC C-terminal tag EcoRI-Smal. PTII-4 was first cloned in the pRT101 vector Sac-Smal in frame to a MYC C-terminal tag. The 355geo-Pti1-4-MYC fragment was cloned HindIII in pGreenII 0029.

For OXI1pro:GUS and PTII-4pro:GUS, the intron-containing GUS gene was cloned into the binary vector pGreenII 0029. A 2.2 Kf region upstream of the OXI (At3g25250) translational start was amplified by PCR from genomic Arabidopsis Col-0 DNA and subcloned BanHI-XhoI in front of the GUS gene. For PTII-4, a 1.8 Kf region upstream of the PTII-4 (At2g47060) translational start was subcloned EcoRI-HphI.

The 2.2 Kf OXI1 promoter and the genomic sequence of OXII with the 5’UTR and 3’UTR was amplified by PCR and cloned in the pCambia 3300. The HA tag was cloned at the SalI site found at the ATG site of OXII.

**Plant material and growth conditions**

The A. thaliana (L.) Heynh. ecotype Columbia 0 was used in all the experiments. Plants were transformed using the floral dipping method [41]. OXI-MYC and PTII-4-MYC constructs were expressed in plants under the control of the 35S promoter from the binary vector pGreenII 0029. HA-OXI1 was also expressed in plants under the control of its own promoter from the binary vector pCambia 3300. In addition, plants co-expressing 355geo-Pti1-4-MYC and OXIIgeo-HA-OXII constructs were generated.

Seeds were germinated in 0.5x Murashige Skoog medium (Sigma, St Louis, MO, USA), 1% sucrose and 0.7% agar. The seeds were stratified at 4 °C for 72 h and were then transferred to 22 °C under long day conditions (16 h light, 8 h dark) for germination and growth. For stress treatments, 10-day-old seedlings of Col-0 were transferred in water overnight. They were treated in the morning with 0.1% sodium hypochlorite (Sigma, St Louis, MO, USA) and 0.7% agar. After centrifugation at 4 °C, the shoots and roots were washed three times with 0.7% sodium hypochlorite containing 1 mM 5-bromo-4-chloro-3-indolyl-d-glucuronide (Duchefa, Haarlem, The Netherlands). Tissues were cleared in ethanol and subjected to immunoblotting.

**Co-immunoprecipitation experiments**

Root extracts were prepared in extraction buffer (50 mM Tris, pH 7.8, 100 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 1 mM dithiothreitol) and proteinase inhibitor mix (Roche, Indianapolis, IN, USA). After centrifugation at 20,000 g for 30 min, the supernatant was immediately used for further experiments. Protein extracts (500 μg) were pre-cleared with 40 μL protein A-agarose beads for 2 h at 4 °C, then immunoprecipitated for 4 h at 4 °C in the presence of anti-HA IgG (Covance Carnegie Center Princeton, New Jersey, USA) and 40 μL protein A-agarose beads. Immunoprecipitation of MPK3 and MPK6 was carried out with anti-AtMPK3 and anti-AtMPK6 IgGs (Sigma). Samples were washed three times with extraction buffer and subjected to immunoblotting.

**Histochemical staining**

Plant tissues were fixed in 90% acetone for 30 min at 4 °C, washed three times with 50 mM sodium phosphate buffer (pH 7.0) and subsequently stained for up to 16 h in 50 mM sodium phosphate buffer (pH 7.0), 2 mM K3Fe(CN)6, 2 mM K4Fe(CN)6 containing 1 mM 5-bromo-4-chloro-3-indolyl-d-glucuronide (Duchefa, Haarlem, The Netherlands). Tissues were cleared in ethanol and subjected to immunoblotting with a stereomicroscope (Leica MZ16FA).

**RNA isolation and real-time quantitative RT-PCR analysis**

RNA was isolated from seedlings according to manufacturer’s instruction using the TriPure reagent (Roche). The first strand cDNA was synthesized from 1 μg RNA using the Retroscript cDNA synthesis Kit (Ambion, Austin, TX, USA). Transcript abundance was measured by real-time quantitative RT-PCR using QuantiTect SYBR Green Reagent (Qiagen) in a Rotorgene 6000 (Corbett Life Sciences, Concord, NSW). Relative expression was calculated using the 2-delta-delta CT method [42] using the ACTIN2 gene as an internal standard. PCRs were performed using the following primers: ACT2 (At3g18780): 5-ACATTGTGCTCAGTGTTGGAG-3 and 5-CTGAGGGAAGCGAAGAATGGA-3; OXI1 (At3g25250): 5-GACGAGATTATCGACGAGAAGCGAAGAATGGA-3; 35Spro:PTI1-4-MYC and 35Spro:PTI1-4:GUS. Relative expression was calculated with the 2-delta-delta CT method [42] using the ACTIN2 gene as an internal standard. PCRs were performed using the following primers: ACT2 (At3g18780): 5-ACATTGTGCTCAGTGTTGGAG-3 and 5-CTGAGGGAAGCGAAGAATGGA-3; OXI1 (At3g25250): 5-GACGAGATTATCGACGAGAAGCGAAGAATGGA-3; 35Spro:PTI1-4-MYC and 35Spro:PTI1-4:GUS. Relative expression was calculated with the 2-delta-delta CT method [42] using the ACTIN2 gene as an internal standard. PCRs were performed using the following primers: ACT2 (At3g18780): 5-ACATTGTGCTCAGTGTTGGAG-3 and 5-CTGAGGGAAGCGAAGAATGGA-3; OXI1 (At3g25250): 5-GACGAGATTATCGACGAGAAGCGAAGAATGGA-3; 35Spro:PTI1-4-MYC and 35Spro:PTI1-4:GUS.

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**References**


**Supporting information**

The following supplementary material is available:

**Fig. S1.** PTI1-4 is not required for stress-induced MPK3 or MPK6 activation.

**Fig. S2.** OXI1 protein accumulates in wounded seedlings.

This supplementary material can be found in the online version of this article.

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