

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 (OX11), 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 (PTII-2) has been positioned downstream from OX11, but whether PTII-2 signals through MAPK cascades is unclear. Using a yeast two-hybrid screen we show that OX11 also interacts with PTII-4. *OX11* and *PTII-4* are stress-responsive genes and are expressed in the same tissues. Therefore, studies were undertaken to determine whether PTII-4 is positioned in the OX11/MAPK signalling pathway. The interaction between OX11 and PTII-4 was confirmed by using *in vivo* co-immunoprecipitation experiments. OX11 and PTII-4 were substrates of MPK3 and MPK6 *in vitro*. Although no direct interaction was detected between OX11 and MPK3 or MPK6, *in vitro* binding studies showed interactions between MPK3 or MPK6 with PTII-4. In addition, PTII-4 and MPK6 were found *in vivo* in the same protein complex. These results demonstrate that PTII-4 signals via OX11 and MPK6 signalling cascades.

Structured digital abstract

- **PTII-4** and **OX11** phosphorylate by [protein kinase assay \(View interaction\)](#)
- **OX11** physically interacts with **PTII-4** by [two hybrid \(View interaction\)](#)
- **MPK6** physically interacts with **PTII-4** by [anti tag coimmunoprecipitation \(View interaction\)](#)
- **MPK3** and **OX11** phosphorylate by [protein kinase assay \(View interaction\)](#)
- **MPK6** binds to **PTII-4** by [pull down \(View interaction\)](#)
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- **OX11** phosphorylates **OX11** by [protein kinase assay \(View interaction\)](#)
- **OX11** physically interacts with **PTII-4** by [anti tag coimmunoprecipitation \(View interaction\)](#)
- **PTII-4** and **MPK6** phosphorylate by [protein kinase assay \(View interaction\)](#)
- **PTII-4** physically interacts with **AGC2-3** by [two hybrid \(View interaction\)](#)
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- **PTII-4** physically interacts with **AGC2-2** by [two hybrid \(View interaction\)](#)
- **OX11** physically interacts with **PTII-1** by [two hybrid \(View interaction\)](#)
- **PTII-4** binds to **OX11** 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; OX11, oxidative signal-inducible 1; PDK1, 3-phosphoinositide-dependent kinase 1; PTII, 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 OXI1, other AGC kinases of the AGC VIII subgroup 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 from 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 *PTII* 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 as bait 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. 4.2×10^5 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

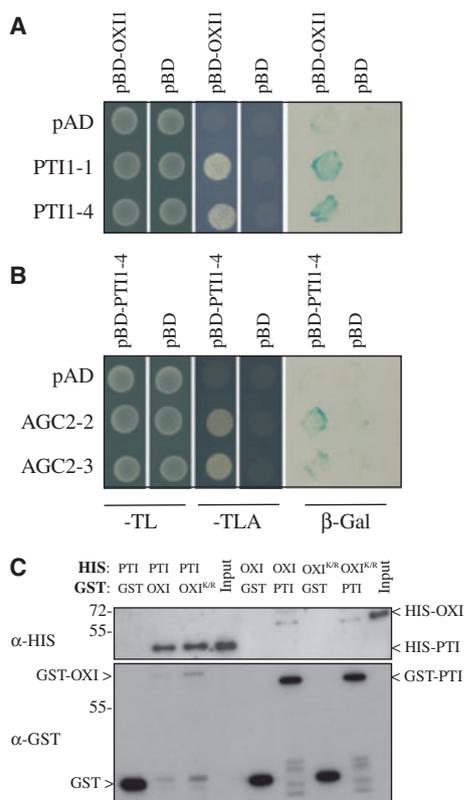


Fig. 1. *In vitro* interactions between OX11 and PTI1-4. (A) Yeast two-hybrid assays with OX11 fused to the GAL4 DNA-binding domain or the empty vector pBD, with PTI1-1 or PTI1-4 fused to the activation domain or the empty vector pAD. (B) Yeast two-hybrid assays with PTI1-4 fused to the GAL4 DNA-binding domain or the empty vector pBD, with AGC2-2 or AGC2-3 fused to the activation domain or the empty vector pAD. The left-hand side shows the growth of yeast colonies on: control plates (-TL) and plates lacking adenine (-TLA). The right-hand side shows the β-galactosidase assay. (C) *In vitro* binding assays of OX11 (OXI) and PTI1-4 (PTI). HIS- or GST-tagged proteins purified from *E. coli* were mixed together. The GST-tagged proteins were pulled down with glutathione-agarose beads. HIS-tagged proteins were then detected by western blot analysis with an anti-HIS IgG. GST alone was used as a negative control. One tenth of the input was loaded on to the gel and represents the amount of HIS-tagged proteins used for the assay. The *in vitro* binding assays were repeated twice using recombinant proteins prepared independently and showed similar results.

members of the AGC family, AGC2-2 (At4g13000) and AGC2-3 (At1g51170), which belong to group VIII [8], such as OX11/AGC2-1.

The interaction between PTI1-4 and OX11 was confirmed by *in vitro* pull-down assays. OX11 and PTI1-4 kinases were histidine (HIS)- or glutathione S-transferase (GST)-tagged and purified from *Escherichia coli*. After mixing together HIS-OX11 and GST-PTI1-4 proteins or HIS-PTI1-4 and GST-OX11 proteins, the

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-OX11 bound to GST-OX11 and GST-PTI1-4, respectively, but not to GST alone. The kinase-deficient mutant, OX11^{K45R}, 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 OX11 is not required for the interaction with PTI1-4.

OX11 interacts with PTI1-4 *in vivo*

Because various PTIs interact with AGCs VIII *in vitro*, the interaction between OX11 and PTI1-4 proteins was tested in *Arabidopsis* plants. To investigate the association between OX11 and PTI1-4 *in vivo*, we generated transgenic *A. thaliana* plants expressing both an OX11 genomic fragment tagged with haemagglutinin (HA) under the control of its own promoter (OX11_{pro}:HA-OX11) and a 35S_{pro}:PTI1-4-MYC construct. The interaction between the two proteins was then tested using co-immunoprecipitation assays. When HA-OX11 fusion proteins were immunoprecipitated from plant extracts using an anti-HA IgG, PTI1-4-MYC was detected in the HA-OX11 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 OX11 and PTI1-4 interact *in vivo*.

OX11 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 OX11 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 OX11 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 OX11 transcript in response to oxidative stress was stronger than that of the PTI1-4 transcript.

If OX11 and PTI1-4 function together in *Arabidopsis*, the expression pattern of the two genes should be

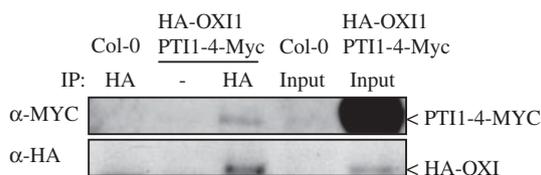


Fig. 2. *In vivo* interactions between OX11 and PTI1-4. Transgenic plants expressing both $35S_{pro}:PTI1-4-MYC$ and $OX11_{pro}:HA-OXI1$ constructs were used for *in vivo* co-immunoprecipitation. Total protein extracts from roots were immunoprecipitated with an anti-HA IgG followed by protein gel blot analysis with an anti-MYC IgG. As a negative control, total protein extracts from Col-0 wild-type roots were used. Ten micrograms of the input were used as a loading control. The bottom panel shows the level of HA-OXI1 in the anti-HA immunoprecipitates. The co-immunoprecipitation experiments were repeated three times, with similar results.

comparable. It is known that *OX11* is expressed in the roots as well as the root hairs [5]. To examine the tissue-specific expression pattern of *PTI-4*, we transformed *Arabidopsis* plants with a $PTI1-4_{pro}:GUS$ construct. Histochemical staining of transgenic *Arabidopsis* seedlings showed that *PTI1-4* is more broadly expressed in the seedling than *OX11* (Fig. 3B). A strong expression of *PTI1-4* could be detected in the roots as well as the root hairs, similar to *OX11* (Fig. 3B). Expression of both genes was observed early during plant growth and was present in the root apical meristem of the embryo. However, *OX11* expression is mainly localized to the root meristem, whereas *PTI1-4* is expressed in different tissues of the embryo.

OX11 phosphorylates PTI1-4 *in vitro*

Next, by using *in vitro* kinase assays we tested whether OX11 could phosphorylate PTI1-4 because OX11 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 $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$. In contrast to PTI1-4, OX11 was capable of strong autophosphorylation activity (Fig. 4A). When both proteins were incubated together, OX11 could phosphorylate PTI1-4. As expected, the kinase-inactive form of OX11 (OX11^{K45R}) showed no autophosphorylation activity and showed no phosphorylation of PTI1-4. OX11 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 OX11 with increasing amounts of PTI1-4 enhanced the autophosphorylation activity of OX11 (Fig. 4B) as well as the transphosphorylation of MBP. Simply by incubating the two proteins over a period of

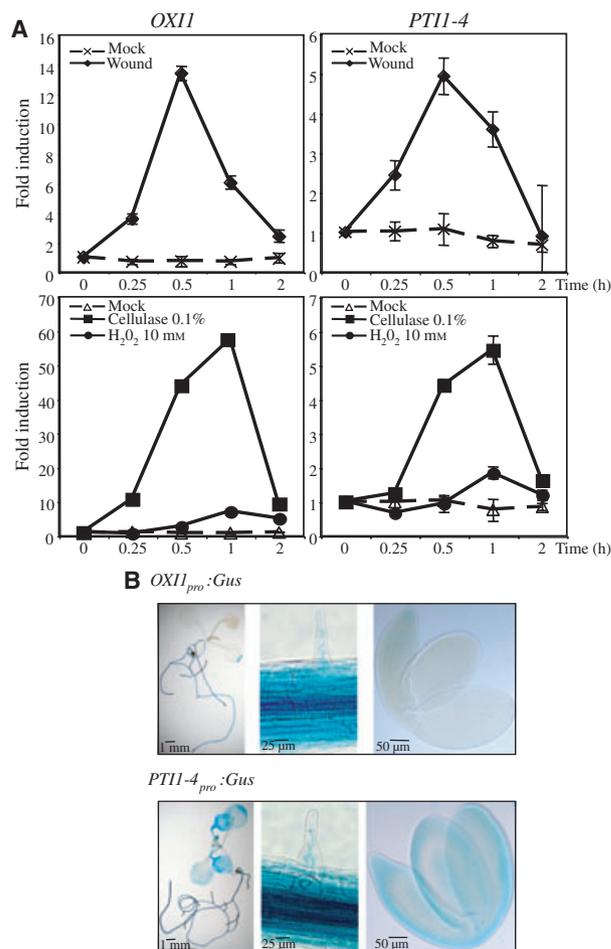


Fig. 3. *OX11* and *PTI1-4* expression in *Arabidopsis*. (A) Oxidative stress treatments increased *OX11* and *PTI1-4* transcript levels in wild-type Col-0 seedlings. RNA was extracted from 10-day-old seedlings with or without stress treatments (mock) at the time points indicated. *OX11* and *PTI1-4* transcript levels were determined by using real-time quantitative RT-PCR. The *ACTIN2* gene was used as an internal standard. The results are expressed as fold induction compared with the time point 0 of untreated plants. Each measurement is the mean and standard deviation of three replicates. Four biological repeats were analysed by RT-PCR, with similar results. One experiment was further quantified by real-time quantitative RT-PCR. (B) Expression pattern of the *GUS* reporter gene in $OX11_{pro}:GUS$ and $PTI1-4_{pro}:GUS$ transgenic *Arabidopsis* plants. *GUS* activity was examined in 10-day-old seedlings, root hairs and in embryos at torpedo stage. A similar *GUS* staining was observed in four different plant lines of $OX11_{pro}:GUS$ or $PTI1-4_{pro}:GUS$.

time in kinase buffer before adding the $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ was sufficient to increase the autophosphorylation activity of OX11 as well as transphosphorylation of PTI1-4 and MBP proteins (Fig. 4B). Incubating OX11 alone for a period of time in kinase buffer before adding the $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ did not significantly increase its autophosphorylation activity. These results suggest

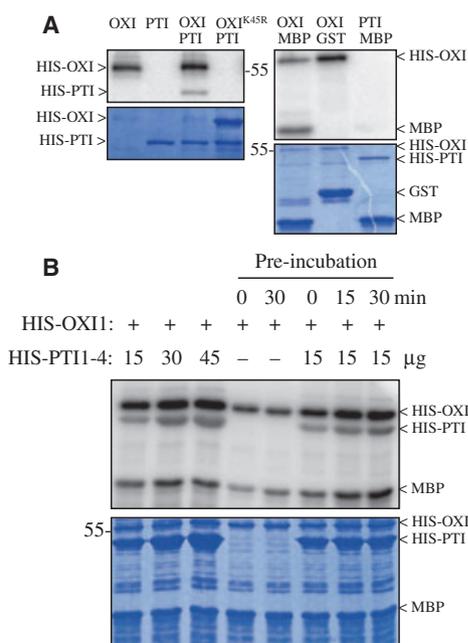


Fig. 4. OX11 phosphorylation of PTI1-4. (A) *In vitro* kinase assay using recombinant proteins: HIS-OX11 (OXI), kinase-deficient HIS-OX1^{K45R} (OXI^{K45R}) and HIS-PTI1-4 (PTI). Protein mixes were incubated in kinase buffer and [γ -³²P]-ATP. MBP was used as an artificial substrate to assess the kinase activity and GST alone was used as a negative control. The top panel shows the kinase assay visualized by autoradiography and the bottom panel shows the Coomassie Brilliant Blue-stained SDS/PAGE. The *in vitro* kinase assays were repeated three times using recombinant proteins prepared independently and showed similar results. (B) HIS-OX11 was mixed with increasing amounts of HIS-PTI1-4 or HIS-OX11 was preincubated in kinase buffer with or without HIS-PTI1-4 for the indicated time points. The mixes were then incubated with [γ -³²P]-ATP and MBP (10 μ g) for 30 min. The top panel shows the kinase assay visualized by autoradiography and the bottom panel shows the Coomassie Brilliant Blue-stained SDS/PAGE. This experiment was repeated twice with similar results.

that PTI1-4 may be necessary for activation of the OX11 kinase activity.

MPK3 and MPK6 phosphorylate OX11 and PTI1-4 *in vitro*

Because OX11 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 *pti1-4* mutant plants or to cellulase 0.1% treatment in *35S_{pro}:PTI1-4-MYC* transgenic lines compared with Col-0 (Fig. S1). We then tested whether the OX11 protein could use

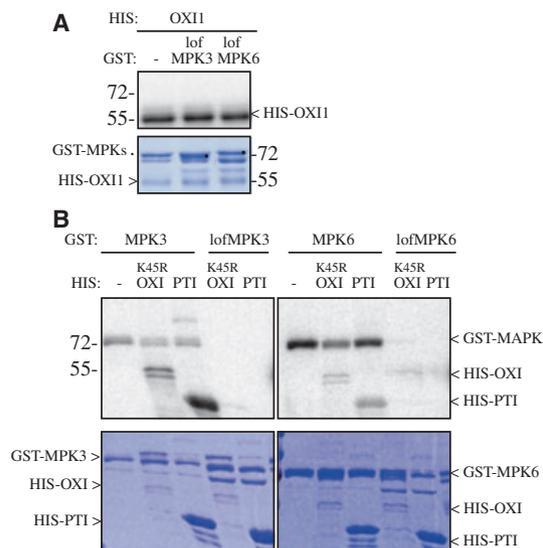


Fig. 5. MPK3 and MPK6 phosphorylate OX11 and PTI1-4. (A) Recombinant kinase-inactive GST-lofMPK3 and GST-lofMPK6 were mixed with HIS-OX11 in kinase buffer and [γ -³²P]-ATP. (B) Recombinant kinase-active GST-MPK3 and GST-MPK6 or recombinant kinase-inactive GST-lofMPK3 and GST-lofMPK6 were mixed with either HIS-OX11^{K45R} (OXI^{K45R}) or HIS-PTI1-4 (PTI) in kinase buffer and [γ -³²P]-ATP. For (A) and (B) the top panel shows the kinase assay visualized by autoradiography and the bottom panel shows the Coomassie Brilliant Blue-stained SDS/PAGE. The *in vitro* kinase assays in (A) and (B) were repeated twice using recombinant proteins prepared independently and showed similar results.

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 with OX11, no phosphorylation of lofMPK3 or lofMPK6 was observed (Fig. 5A). On the other hand, when OX11^{K45R} or PTI1-4 proteins were mixed with active MPK3 or MPK6 kinases, phosphorylation of OX11^{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 OX11^{K45R} or PTI1-4 (Fig. 5B). These results show that MPK3 and MPK6 can phosphorylate OX11 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 OX11/PTI1-4 and MPK3/MPK6 proteins, we tested whether

HIS-OX11 or HIS-PTI1-4 could bind to GST-MPK3 or GST-MPK6 proteins *in vitro*. Western blot analysis (Fig. 6A) showed that PTI1-4 could bind to each of the MAPKs, but not to GST alone. No direct interaction between OX11 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 OX11 to MPK3 and MPK6 proteins, we examined whether OX11 could also be found in complexes with MPK3 or MPK6 proteins *in vivo*. For this purpose we used transgenic plants expressing either a $35S_{pro}:PTI1-4-MYC$ or a $35S_{pro}:OX11-MYC$ 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, OX11 was not detected from anti-MPK3 or anti-MPK6 immunoprecipitates. The fact that OX11 could not be detected in a complex with the

MAPK proteins might be due to the low amount of OX11 protein in $35S_{pro}:OX11-MYC$ transgenic plants compared with $35S_{pro}:PTI1-4-MYC$ overexpressors. Another possibility is that the interaction between OX11 and MAPK proteins is triggered by stress. Thus, we then used *Arabidopsis* transgenic plants expressing OX11 under the control of its promoter. When using these plant lines, we showed accumulation of the OX11 protein in seedlings after wounding (Fig. S2). Co-immunoprecipitation experiments were then carried out using $OX11_{pro}:HA-OX11$ seedlings wounded for either 30 min or 1 h. Even under these conditions or when using different extraction buffers, we could not find OX11 in the same complex with MPK3 or MPK6 proteins (data not shown). However, the interaction between OX11 and MAPK proteins could be transient and therefore difficult to detect.

Discussion

OX11 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* OX11 can interact and phosphorylate another member of the PTI1 family, PTI1-4. Although other members of the AGC family (AGC2-2,

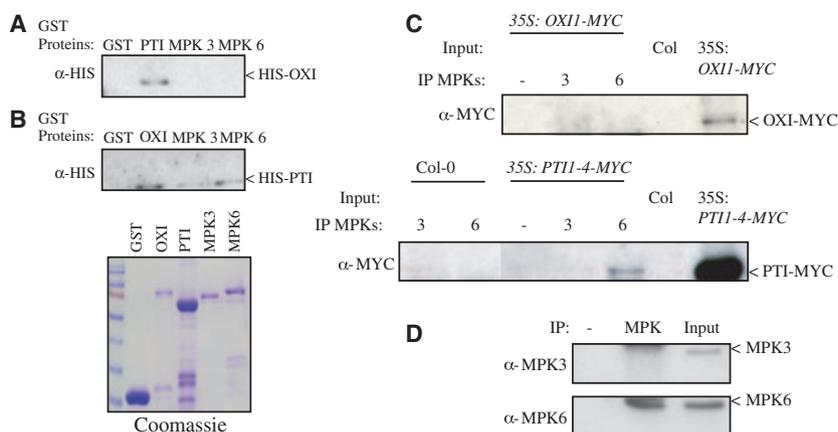


Fig. 6. *In vitro* and *in vivo* interactions between OX11, PTI1-4, MPK3 and MPK6. (A) The HIS-OX11 protein was mixed with GST alone as a control or with GST-tagged proteins. (B) The HIS-PTI1-4 protein was mixed with GST alone as a control or with GST-tagged proteins. In (A) and (B), the GST-tagged proteins were pulled down with glutathione-agarose beads. HIS-tagged proteins were then detected by western blot analysis with an anti-HIS IgG. The bottom panel shows the GST-tagged proteins separated on 10% SDS/PAGE; total proteins were stained with Coomassie Brilliant Blue. The *in vitro* binding assays were repeated twice using recombinant proteins prepared independently and showed similar results. (C) Transgenic plants expressing either $35S_{pro}:PTI1-4-MYC$ or $35S_{pro}:OX11-MYC$ were used for *in vivo* co-immunoprecipitation. Total protein extracts from roots were immunoprecipitated with anti-MPK3 or anti-MPK6 IgGs followed by protein gel blot analysis with an anti-MYC IgG. As a negative control, total protein extracts from Col-0 wild-type roots were used. Ten micrograms of the input were used as a loading control. (D) Immunoblots with anti-MPK3 and anti-MPK6 IgGs show the levels of MPKs in the anti-MPK3 and anti-MPK6 immunoprecipitates. The co-immunoprecipitation experiments were repeated three times, with similar results. Using root samples from $35S:OX11-MYC$ transgenic plants and different extraction buffers, the co-immunoprecipitation experiments were tested eight times.

AGC2-3) were also identified as PTI1-4 interactors in yeast two-hybrid assays, the interaction between OX11 and PTI1-4 was confirmed *in planta*. Moreover, both *OX11* 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 OX11 and PTI1-4 functionally interact *in vivo*.

In order to show that OX11 and PTI1-4 function together in a signal transduction pathway, *ptil-4* knockout lines were isolated and analysed to uncover phenotypic similarities to *oxil* mutants. However, *ptil-4* mutants, as well as *35S_{pro}:PTI1-4-MYC* plants, showed no defects in root hair growth and *ptil-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 *SIPti1* 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 *SIPti1* 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 double 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* double mutants are embryo 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 *oxil* mutant plants are impaired in the activation of MPK3 and MPK6 kinases upon oxidative stress treatments, OX11 was

positioned as an upstream regulator of the MPK3 and MPK6 cascade. Yet here we showed that OX11 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 OX11 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 OX11 or PTI1-4 should take place. Until now, no direct interaction between MPK3 and MPK6 has been detected with OX11 *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 OX11/MAPK signalling pathway. PDK1 was shown to function upstream of OX11 and PTI1-2 kinases and was required for the activation of

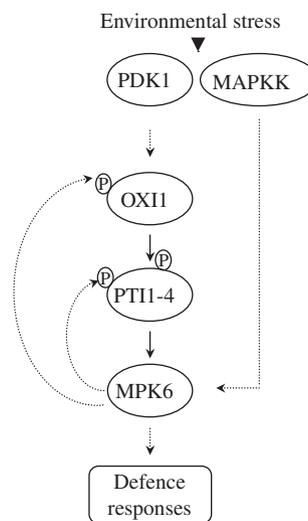


Fig. 7. Model for PTI1-4 signal transduction. (A) In response to a particular environmental stress, PDK1 interacts and activates OX11. OX11 then interacts with and phosphorylates PTI1-4, which in turn interacts with MPK6. To modulate the cascade, MPK6 phosphorylates PTI1-4 and OX11, providing a feedback loop. Because various MAPKs are known to activate MPK6, they have been positioned in a parallel pathway, probably providing a cross-talk between the pathways. Arrows with solid lines indicate an interaction between the proteins and arrows with dashed lines denote a putative link.

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 basal 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 *OXI1* (At3g25250) or *PTI1-4* (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; 0.17% 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 Biosci-

ence, Little Chalfont, UK). OXI1 and PTI1-4 were expressed as HIS fusion proteins in the pET28a (+) vector (Novagen Inc., Madison, WI, USA). The OXI^{K45R} 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 QIAexpressionistTM; Qiagen, Hilden, Germany). GST alone or GST-tagged proteins were mixed with HIS-tagged proteins in 200 μ L 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 μ L 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 (nano-Tools 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 μ L kinase buffer [50 mM Tris, pH 7.5, 1 mM dithiothreitol, 10 mM MgCl₂, 0.1 mM ATP and 0.1 μ L mCi [γ ³²P]-ATP (1 μ Ci)] and 1 μ L 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 *OXI1* and *PTI1-4* coding sequence was amplified by PCR from total cDNA derived from Col-0 seedlings. The *OXI1* coding sequence was cloned *EcoRI-SalI* into pAD, pBD (Stratagene), pGEX-4T-1 and pET-28a (*EcoRI-SalI/XhoI*). The lysine 45 (K45R) codon from OXI1 was changed to arginine by site-directed mutagenesis (Stratagene). The *PTI1-4* coding sequence was cloned *SalI-PstI* into pAD, pBD (Stratagene) and *BamHI-SalI* into pGEX-4T-1 and pET-28a (*BamHI-SalI/XhoI*). ORFs of different *MAPKs* used were cloned as described previously [33].

The 35S promoter and terminator of the pRT101 vector was cloned *SalI/XhoI-NotI* into the binary vector pGreenII 0029 [40]. The *MYC* tag was cloned *SmaI-XbaI* into this

modified pGreenII 0029 vector. *OXII* was cloned in frame to a MYC C-terminal tag *EcoRI-SmaI*. *PTII-4* was first cloned in the pRT101 vector *SacI-SmaI* in frame to a MYC C-terminal tag. The *35S_{pro}:Pti1-4-MYC* fragment was cloned *HindIII* in pGreenII 0029.

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

The 2.2 Kb *OXII* 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-OXI* was also expressed in plants under the control of its own promoter from the binary vector pCambia 3300. In addition, plants co-expressing *35S_{pro}:PTII-4-MYC* and *OXII_{pro}:HA-OXI* constructs were generated.

Seeds were germinated in 0.5× 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 H₂O₂ (10 mM), cellulase (0.1%) or were wounded with forceps and used for quantitative real-time RT-PCR analysis.

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-sepharose 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-sepharose 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 visualized 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, Concorde, NSW). 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-ACATTGTGCTCAGTGGTGGGA-3 and 5-CTGAGGGAAGCAAG AATGGA-3, *OXII* (At3g25250): 5-GACGAGATTATCAGATTTTACGC-3 and 5-AACTGGTGAAGCGGAAGAGAC-3, *PTII-4* (At2g47060): 5-CCCCAAAGAAAATGAGTTGCT-3 and 5-GCATCATTTTCTGGAGGAAAG-3.

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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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