WRKY45 phosphorylation at threonine 266 acts negatively on WRKY45-dependent blast resistance in rice

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ABSTRACT

WRKY45 is a central regulator of disease resistance mediated by salicylic acid signaling in rice and its activation involves phosphorylation by OsMPK6. OsMPK6 phosphorylates WRKY45 at Thr266, Ser294, and Ser299 in vitro. Phosphorylation of Ser294 and/or Ser299 is required for full activation of WRKY45, but the importance of Thr266 phosphorylation has remained unknown. Here, we report on the characterization of Thr266 phosphorylation of WRKY45 in rice. Transient expression of mutant WRKY45 revealed that Thr266 is phosphorylated in vivo, together with Ser294/299. Replacement of Thr266 by Asn did not affect the enhanced Magnaporthe oryzae resistance afforded by WRKY45 overexpression. By contrast, replacement by Asp negated the enhancement of M. oryzae resistance. These results suggest that Thr266 phosphorylation acts negatively on WRKY45-dependent disease resistance.

The salicylic acid (SA) signaling pathway is crucial for defense against pathogens in plants. Within this pathway, the transcriptional co-regulator NPR1 plays a central role in Arabidopsis, while the transcription factor WRKY45 and OsNPR1, the rice ortholog of NPR1, play similar roles in rice. NPR1 is dynamically modified and functionally regulated by phosphorylation, SUMOylation, and ubiquitination, and dual-mode regulation of NPR1 stability and activity is achieved by the ubiquitin-proteasome system (UPS) in Arabidopsis. Dual-mode UPS regulation also controls WRKY45 in rice, but not OsNPR1. Knock-down of WRKY45 abrogates benzo thiadiazole (BTH)-induced defense against several pathogens, including fungi such as Magnaporthe oryzae which causes blast disease, and bacteria such as Xanthomonas oryzae pv. oryzae which causes leaf blight disease. Conversely, the overexpression of WRKY45 confers BTH-independent resistance against these pathogens in rice plants.

As well as UPS regulation, WRKY45 is also regulated by phosphorylation. It is phosphorylated in vitro at Thr266, Ser294, and Ser299 by OsMPK6, a mitogen-activated protein kinase, which is activated by exogenous SA treatment. A mutant of WRKY45 with phosphomimetic missense mutations at Ser294 and Ser299, both of which are phosphorylated in vivo, exhibited increased transactivation activity compared with wild-type WRKY45. Meanwhile, the overexpression of a mutant WRKY45 in which these two sites were changed to unphosphorylatable amino acids failed to confer enhanced blast resistance to transgenic rice plants. Although the role of Ser294/299 phosphorylation in the function of WRKY45 has been defined to some extent, the phosphorylation of Thr266 in vivo and its importance in rice defense against pathogens have remained uncharacterized. Here, we validated WRKY45 phosphorylation at Thr266 in vivo and investigated its role in disease resistance using transgenic rice plants.

We first generated three missense mutants of WRKY45 in which Thr266 was replaced by unphosphorylatable Asn (NSS), Ser294 and Ser299 by Asn (TNN), or all three phospho-sites by Asn (NNN); these mutant WRKY45 proteins were fused to myc-tags as shown schematically in Fig. 1A. These proteins were transiently expressed in cultured rice cells (Os cells) and cell extracts were treated with lambda protein phosphatase (PPase). Immunoblot analysis using an anti-myc antibody revealed 2 bands each in untreated extracts from NSS- and TNN-expressing cells. However, the upper bands were missing in PPase-treated extracts from both cells (Fig. 1B), indicating that these mutant WRKY45 proteins were phosphorylated in vivo. A weak upper band was observed in the untreated extract from NNN-expressing cells, suggesting that the NNN mutant is scarcely phosphorylated in rice cells. These results indicate that Thr266, as well as Ser294 and/or Ser299, are phosphorylated in vivo as the major phosphorylation sites of WRKY45.

To investigate the role of Thr266 in defense responses, we generated stable transgenic rice plants expressing myc-tagged mutant WRKY45 proteins, NSS or DSS, in which Thr266 was replaced by Asn or Asp, respectively, under the control of a
constitutive maize ubiquitin promoter. Immunoblot analyses showed that the accumulation levels of NSS and DSS proteins were comparable with those of wild-type WRKY45 proteins in respective transgenic lines (Fig. 2A). We inoculated these plants with blast fungus, *M. oryzae*, and monitored fungal growth by the amplification of the 28S rDNA gene using quantitative PCR. Fungal growth was obviously reduced in NSS-overexpressing (ox) plants compared with non-transgenic control Nipponbare plants (NB, Fig. 2B). Fungal growth was similar between the lines overexpressing NSS- and WT WRKY45, indicating that NSS enhanced blast resistance in rice to a similar extent to WT. These results suggest that the presence of Thr266 and/or its phosphorylation is not required for WRKY45-dependent enhanced blast resistance of rice, at least when WRKY45 proteins are overexpressed. We observed no significant enhancement of blast resistance in DSS-ox plants compared with control NB plants (Fig. 2B). Thus, the replacement of Thr266 by Asp abolished the function of WRKY45 in enhancing blast resistance. Taken together, it is likely that phosphorylation at Thr266 negatively regulates WRKY45 activity.

Figure 2A shows that the bands of DSS proteins are uniform, unlike those of WT and NSS. It therefore appears that the Thr-to-Asp mutation at Thr266 canceled the activation of WRKY45 by phosphorylation at Ser294/299. To examine the phosphorylation state of DSS proteins, we treated the extracts from DSS-ox plants with PPase. Although the DSS bands were barely shifted by PPase treatment in a normal gel, unlike NSS, a shift was clearly observed in a phos-tag gel (Fig. 3). These results suggest that Ser294/299 is phosphorylated in DSS and that the dysfunction of WRKY45 caused by the Thr-to-Asp mutation at position 266 is unlikely to be because of the prevention of Ser294/299 phosphorylation.

In *Arabidopsis* and *Nicotiana benthamiana*, several group I WRKY proteins, including AtWRKY33 and NbWRKY8, are activated by phosphorylation at several Ser residues in the Pro-directed Ser residue cluster.9-11 Within this cluster, the effects of respective phosphorylations appear indistinguishable. By contrast, the effects of Thr266 or Ser294/299 phosphorylation on WRKY45 activities appear differential: phosphorylation at Ser294/299 is necessary for full activation of WRKY45 in...
rice, while that at Thr266 appears to play a negative role in the regulation of WRKY45.

The mechanism underlying the proposed negative regulation of WRKY45 by Thr266 phosphorylation remains unknown. In our previous study, the transactivation activity of the DNN mutant of WRKY45, in which Thr266 and Ser294/299 were replaced by Asp and Asn, respectively, appeared lower than that of NNN, although the difference was statistically insignificant. However, this effect appeared to be negated by phosphorylation at Ser294/299, because the transactivation activity of DDD was as high as that of NDD. Thus, it is unlikely that Thr266 phosphorylation prevents WRKY45 activation by Ser294/299 phosphorylation, consistent with the results in Fig. 3 in which the Thr-to-Asp mutation at Thr266 does not prevent Ser294/299 phosphorylation. Another possibility is that Thr266 phosphorylation affects protein stability. It has been reported that Ser phosphorylation in AtWRKY46 negatively regulates the stability of this protein. However, the Thr-to-Asp mutation does not appear to affect the stability of WRKY45 (Fig. 2A).

The WRKY45 proteins overexpressed in transgenic rice were phosphorylated at both Ser294/299 and Thr266. It is of interest that the different phospho-sites possibly involved in positive and negative regulation of WRKY45 are phosphorylated simultaneously under our conditions, in which the transgenic plants overexpressing WRKY45 were not infected or treated with any chemical. OsMPK6 phosphorylates all 3 sites in vitro, while OsMPK4 can also phosphorylate WRKY45 at the same sites in vitro (data not shown). It is possible that WRKY45 is phosphorylated differentially in response to different signals mediated by these protein kinases in planta under non-overexpression situation. The elucidation of such differential phosphorylation could lead to the unraveling of new aspects in the regulation of WRKY45.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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**Abbreviations**

| Acronym | Definition |
|---------|------------|
| NPR1    | nonexressor of pathogenesis-related gene 1 (Oryza sativa) |
| OsNPR1  | salicylic acid |
| Ser294 and Ser299 | serines at the positions of 294 and 299 respectively |
| Thr266 | threonine at the position of 266 |
| UPS | ubiquitin-proteasome system |
| WT | wild-type |

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