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OsLYP4 and OsLYP6 play critical roles in rice defense signal transduction

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Plant innate immunity relies on successful detection of trespassing pathogens through recognizing their microbe-associated molecular patterns (MAMPs) by pattern recognition receptors (PRRs) at the cell surface. We recently reported two rice lysin motif (LysM)-containing proteins, OsLYP4 and OsLYP6, as dual functional PRRs sensing bacterial peptidoglycan (PGN) and fungal chitin. Here we further demonstrated the important roles of OsLYP4 and OsLYP6 in rice defense signaling, as silencing of either *LYP* impaired the defense marker gene activation induced by either bacterial pathogen *Xanthomonas oryzaecola* or fungal pathogen *Magnaporthe oryzae*. Moreover, we found that OsLYP4 and OsLYP6 could form homo- and hetero-dimers, and could interact with CEBiP, suggesting an unexpected complexity of chitin perception in rice.

OsLYP4 and OsLYP6 Affected Pathogen-Induced Defense-Related Gene Activation in Rice

LysM is a ubiquitous protein motif found in virtually every living organism except for Archaea.[1] The characterized LysM-containing proteins in high plants were mostly involved in rhizobial symbiosis or perception of chitin signals,[2] and could be roughly categorized into LysM-containing receptor-like kinases (LysM-RLKs or LYKs) and non-receptor kinase LysM proteins (LYPs).[3,4] The LysM-RLKs such as NFR1 and NFR5 are genetically defined as receptors for the chitin-derived rhizobial nodulation factor and are coupled to the symbiosis progress.[5,6] On the other hand, rice chitin receptor complex components CEBiP and co-receptor OsCERK1 both contain LysMs, and intact LysM domain is necessary for chitin binding in Arabidopsis CERK1.[7,8] Interestingly, Arabidopsis LYM1/LYM3 was found to contribute to PGN sensing but not chitin.[9] Recently, we identified two rice LysM proteins, OsLYP4 and OsLYP6, play dual function in chitin and PGN perception.[10] In this research, to investigate the influence of OsLYP4 and OsLYP6 in pathogen-induced defense-related gene activation in rice, we tested the selected marker genes, such as *Beta-Glu* (Os05 g0495900) and *PAL* (Os02 g0627100) in *OsLYP4* and *OsLYP6* RNAi rice. The induction of the selected marker genes was monitored by qPCR at different time points after the fungal blast pathogen *Magnaporthe oryzae*, or the bacterial streak pathogen *X. oryzaecola* treatment. As expected, the upregulation of selected defense marker genes were suppressed to different extents in *OsLYP4*-RNAi and *OsLYP6*-RNAi transgenic lines compared with that in control (CK) rice after *M. oryzae* inoculation (**Fig. 1A**) or *X. oryzaecola* inoculation (**Fig. 1B**). These results are consistent with our former data, in which knockdown of either *OsLYP* genes could increase rice susceptibility to both fungal and bacterial pathogens.[10] Considering that these microbial pathogens contain a cocktail of MAMPs,[11] these data suggested that PGN or chitin signaling mediated by OsLYP4 and OsLYP6 contributes significantly to rice defense responses against bacteria and fungi.

OsLYP4 and OsLYP6 May Cooperate With Other LysM-Containing Receptors to Transduce PGN or Chitin Signal

Our previous study has suggested that OsLYP4 and OsLYP6 are not functionally redundant in chitin and PGN signaling pathways.[10] Moreover, another lysin motif-containing protein, CEBiP has been reported to play important role in chitin sensing and signal transduction.[12,13] Therefore, we investigated the protein-protein interactions between these LYP proteins with yeast two-hybrid (Y2H).

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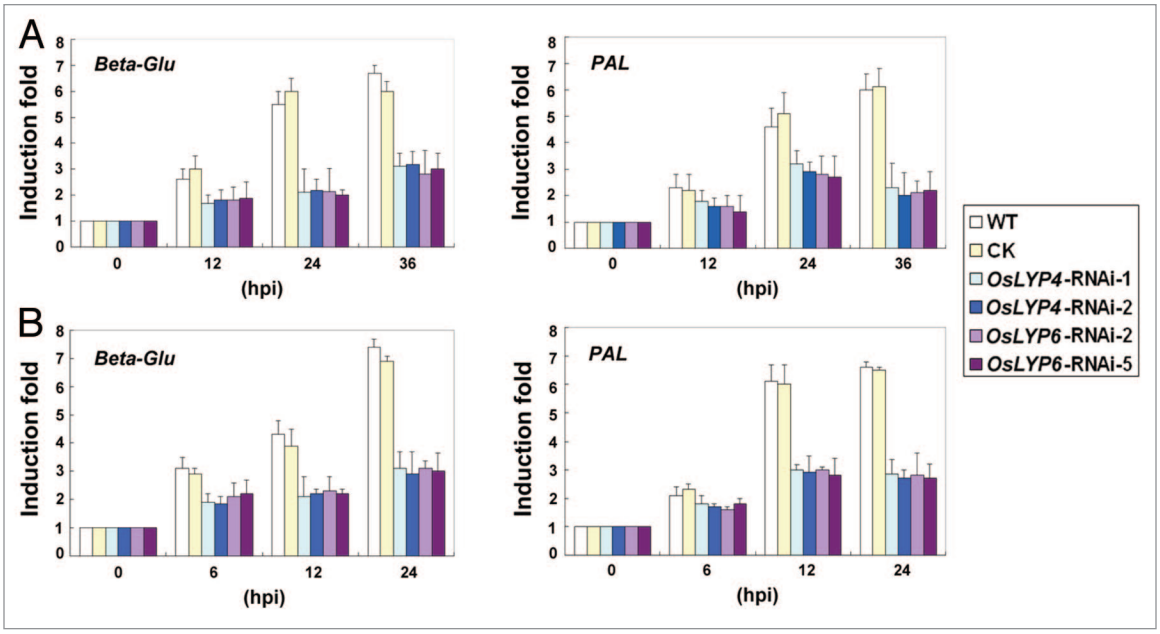

Figure 1. Suppression of pathogen-induced gene expression in *OsLYP4*- and *OsLYP6*-RNAi rice plants. (**A**) The fourth leafs at the five-leaf stage from each rice line were inoculated with *M. oryzae* (10^6 colony-forming units/mL) and the induction fold of *Beta-Glu* or *PAL* gene was examined by qPCR at 0, 12, 24 and 36 h after inoculation. (**B**) The induction fold of *Beta-Glu* or *PAL* gene was examined by qPCR at 0, 6, 12 and 24 h after *X. oryzaecola* (10^6 colony-forming units/mL) inoculation. The induction fold of each gene was calculated by the gene expression level in pathogen–treated seedlings relative to that in mock-treated seedlings at the same time point. hpi, hour post inoculation. Three biological repeats were conducted, and the data represent means ± SD (10 leaves in each transgenic line, three independent experiments). WT: wild type, CK: control transgenic rice transformed with empty vector, *OsLYP4*-RNAi-line -1, *OsLYP4*-RNAi -2, *OsLYP6*-RNAi line -2 and *OsLYP6*-RNAi line -5 were compared.

In Y2H assay, we indeed detected positive interactions between OsLYP4 and OsLYP6 (**Fig. 2A**), and between CEBiP and OsLYP4 (**Fig. 2B**). The false positives were excluded by the negative control (data not shown). Furthermore, the CEBiP and OsLYP4 interaction was further verified in rice protoplasts by bimolecular fluorescence complementation (BiFC) (**Fig. 2B**). Interestingly, we also found that OsLYP4 and OsLYP6 could form homodimers in Y2H (**Fig. 2A**).

These data consistently support a cooperative relationship between OsLYP4 and OsLYP6 as our previous genetic analyze. Therefore, we propose that OsLYP4 and OsLYP6 work in the same complex for PGN or chitin perception and subsequent signal transduction. The identification of OsLYP4 and OsLYP6 as additional chitin receptors in rice raises the question regarding the relationship between the OsLYP4-OsLYP6 chitin receptor complex and the reported CEBiP-OsCERK1 chitin receptor complex. Shimizu et al. has found that a major portion of CEBiP proteins were visualized as homodimers by the blue native PAGE analysis.[13] However, a small fraction of CEBiP proteins did exist as larger-size oligomers,[13] which may suggest that some CEBiP proteins can be in the same complexes with OsLYP4 and OsLYP6. Our data that CEBiP interacted with OsLYP4 in yeast and in plant cells (**Fig. 2B**) supported such a possibility. As CEBiP, OsLYP4, and OsLYP6 could all form homodimers (**Fig. 2A**, and reviewed in ref. 13), and these OsLYPs apparently need a signaling partner (e.g., OsCERK1)[13] with an intracellular kinase domain for defense signal transduction, the composition and the stoichiometry of different chitin or PGN receptor complexes in rice await further investigation, which will shed new light on the rice innate immunity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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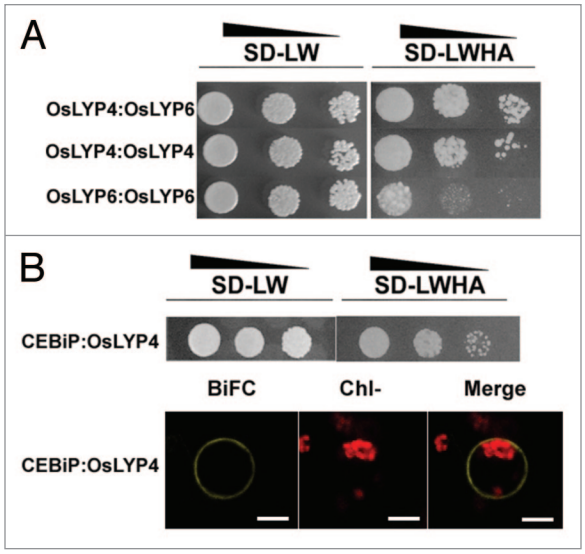

Figure 2. OsLYP4 and OsLYP6 Can Interact with other LYPs. (**A**) Yeast two-hybrid analysis of the protein-protein interaction between OsLYP4, and OsLYP6. Yeast cells harboring the indicated bait (cloned into the pGADT7) and prey plasmids (cloned into the pGBKT7) were diluted by 1:1, 1:10 or 1:100 and spotted on the selective medium SD-LW (medium without leucine and tryptophan) and SD-LWHA (medium without leucine, tryptophan, histidine and adenine). Growth on the SD-LWHA medium indicates a positive interaction. (**B**) Interactions between OsLYP4 and CEBiP proteins in yeast and rice protoplasts. BiFC (bimolecular fluorescence complementation) indicated the complementary yellow fluorescence, Chl- indicated the chloroplast autofluorescence in the protoplast. Bar = 10 μm.

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