Crystal structures of the RLPH2 protein phosphatase from *Arabidopsis thaliana* reveal a novel mechanism for recognizing dually phosphorylated substrates

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**Abstract:** Despite belonging to the phosphoserine- and phosphothreonine-specific phosphoprotein phosphatase (PPP) family, *Arabidopsis thaliana* *Rhizobiales*-like phosphatase 2 (RLPH2) strongly prefers substrates bearing phosphorylated tyrosine residues. We used the anomalous scattering signal from sulfur atoms in the native protein to calculate phases for modest-quality diffraction patterns measured from needle-like crystals (1.0 x 0.01 x 0.01 mm) of RLPH2 (P6₁; dmin = 2.2 Å; Rsym = 0.092; redundancy = 30.9; \( \lambda = 1.853 \) Å). As expected for a PPP-family enzyme, the structure of RLPH2 contains a central domain that forms a binding site for two divalent metal ions. Distinctive structural elements from two flanking domains contribute structural elements that suggest a novel mechanism for the selective dephosphorylation of phosphotyrosine residues. Co-crystallization with the phosphate mimetic tungstate also suggests how positively charged residues that are highly conserved in the RLPH2 class form an additional pocket that is specific for a phosphothreonine residue located near the phosphotyrosine residue that is bound to the active site. Site-directed mutagenesis confirmed that this auxiliary recognition element facilitates the recruitment of dual-phosphorylated substrates containing a pTxpY motif. The combination of an auxiliary site specific for phosphothreonine and the active-site specific for phosphotyrosine reveals a novel mechanism for the recognition of dually phosphorylated substrates that appears to be conserved in the RLPH family of plant protein phosphatases.