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Interactions in the pollen-specific receptor-like kinases-containing signaling network

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ABSTRACT

The pollen-specific receptor-like kinases (PRKs) from *Solanum lycopersicum*, *Le*PRK1 and *Le*PRK2, are believed to be involved in the regulation of pollen germination and pollen tube growth. They appear to be part of a multimeric complex in which the transmembranic *Le*PRKs presumably have a key position in transducing exogenous signals through the plasma membrane. Here, we focused on extra- and intracellular interactions involving the *Le*PRKs. We show in yeast two-hybrid experiments a cross-interaction of putative PRK-ligands, the oligomerization of *Le*PRK2 and a direct contact of *Le*PRKs to activated Rho proteins of plants (ROPs). Moreover, we observed that pollen-specific RopGEFs, which catalyze ROP activation and may be regulated by PRK interaction, are active *in vitro* while autoinhibition seems to occur *in vivo*. We suggest that activation of RopGEFs as a checkpoint in PRK signal transduction is a more complex event including further components *in planta*. Our findings point to some new aspects in PRK-mediated signal transduction implying a *Le*PRK2 complex with different signaling activity and a further direct control of *Le*PRKs by activated ROP.

Introduction

Plant receptor-like kinases (RLKs) belong to a large family of proteins that are involved in various signal transduction cascades. They resemble in their domain organization animal receptor tyrosine kinases, exhibiting an extracellular amino-terminal domain, a transmembrane region and a carboxy-terminal kinase domain in the cytosol. In most plant RLKs this intracellular domain displays serine/threonine kinase specificity, while the majority of the animal receptors are receptor tyrosine kinases (RTKs) (De Smet et al., 2009; Shiu and Bleecker, 2001; Shiu et al., 2004). In the model plant *Arabidopsis thaliana* over 600 RLK homologues have been identified and more than 1000 putatively secreted peptides make up the group of their potential protein ligands (Butenko et al., 2009; Lease and Walker, 2006). However, for most RLKs and the potential ligands just little is reported about their function, signaling capacities and interaction partners (Butenko et al., 2009; Shiu and Bleecker, 2001). On the other hand, much more is known about some processes, that are regulated and mediated by RLKs, including disease resistance, symbiosis, self-incompatibility, cell growth and reproduction (De Smet et al., 2009; Shiu and Bleecker, 2001). In tomato (*Solanum lycopersicum*), two pollen-specific RLKs (PRKs), *Le*PRK1 and *Le*PRK2, have been implicated to be involved in the regulation of pollen germination and pollen tube growth. Both are specifically expressed in mature and germinated pollen (Kim et al., 2002; Muschietti et al., 1998). They are localized to the plasma membrane of growing pollen tubes and are believed to form heterodimers (Wengier et al., 2003; Zhang et al., 2008). It was shown that *Le*PRK2 is phosphorylated in pollen membranes and dephosphorylation occurs after incubation with style extract when the heterodimer dissociates (Muschietti et al., 1998; Wengier et al., 2003). *Le*PRK1 and *Le*PRK2 belong to the group of LRR-RLKs containing a variable number of leucine-rich repeats (LRRs) in the extracellular domain (Muschietti et al., 1998). LRRs are known as binding motifs that are often involved in protein-protein interaction and thus they may mediate the binding of proteinaceous signaling molecules (Shiu and Bleecker, 2001). Three extracellular proteins are discussed as potential ligands for *Le*PRK1 and *Le*PRK2. LAT52 (late anther tomato 52) and *Le*SHY are pollen-specific proteins while *Le*STIG1 (stigma-specific protein 1) is exclusively expressed in the stigma-part of the flower pistil (Guyon et al., 2004; Tang et al., 2002, 2004). LAT52 is a small cysteine-rich protein which interacts with the extracellular domain of *Le*PRK2 before, but not after pollen germination (Tang et al., 2002). *Le*SHY is a LRR-protein binding also to the extracellular domain of *Le*PRK2. It is highly expressed in mature pollen and after pollen germination (Tang et al., 2004). *Le*STIG1 is also a small cysteine-rich protein which is unrelated to LAT52. It is specifically expressed in the stigmatic secretory zone and was shown to bind to the extracellular domain

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of both *Le*PRK1 and *Le*PRK2 in yeast two-hybrid analyses (Tang et al., 2004). Although the potential ligands and PRKs appear to contribute to germination and pollen tube growth, the underlying molecular mechanisms of signal transduction are still largely unknown.

Rho proteins of plants (ROPs) also participate in signal transduction during pollen tube growth (Berken, 2006; Yang, 2008). They switch between an inactive GDP-bound conformation and an active GTP-bound state (Berken and Wittinghofer, 2008) and thereby play a major role in regulating the polarity of growing pollen tubes. ROP was identified in pollen extracts as a component of a high molecular weight protein complex, which also included *Le*PRK1 and *Le*PRK2 (Wengier et al., 2003). A connection between *Le*PRKs and intracellular ROP signaling was brought up with the identification of RopGEFs (GEF: guanine nucleotide exchange factor) (Berken et al., 2005), and when the tomato pollen-specific RopGEF KPP (kinase partner protein) was shown to interact with the cytosolic domains of the *Le*PRKs (Kaothien et al., 2005). RopGEFs activate ROPs by promoting the exchange of GDP to GTP via their catalytic PRONE (plant-specific ROP nucleotide exchanger) domain (Berken et al., 2005; Thomas et al., 2007, 2009) and in this function the GEFs also play a crucial role for pollen tube growth (Gu et al., 2006). In this scenario, the interaction between PRKs and RopGEF may be crucial for RopGEF regulation (Zhang and McCormick, 2007). Taken together, several hints point towards a signal transduction cascade from *Le*PRKs via RopGEF to ROPs and this may depend on potential PRK-ligands. Yet, the interactions of the involved components have not been exhaustively investigated. Here, we further analyzed the extracellular and intracellular interactions involving *Le*PRKs. We report novel interactions between potential *Le*PRK-ligands, homodimerization of *Le*PRK2 as well as direct interaction between activated ROP and the *Le*PRKs. While PRKs are believed to regulate RopGEF function by possibly releasing their autoinhibition *in vivo* (Zhang et al., 2008; Zhang and McCormick, 2007), we show here that pollen-specific RopGEFs from *Arabidopsis thaliana* and *Solanum lycopersicum* are active *in vitro*, arguing for a more complex regulatory mechanism *in planta*.

Material and methods

Molecular cloning

The signal peptides (SP) of putative PRK-ligands were excluded by PCR-cloning. LAT52ΔSP containing amino acids (aa) 18-160 of LAT52 (X15855) was cloned from the plasmid LAT52-pGEX-4T3 (Tang et al., 2004). *Le*STIG1ΔSP encompassing aa 12-143 of *Le*STIG1 (AY376851) and *Le*SHYΔSP consisting of aa 19-350 of *Le*SHY (AY376852) were cloned from reverse transcribed (RT: Superscript II, Invitrogen) mRNA isolated from pollen of *S. lycopersicum* (VF36) with the RNeasy Plant Mini Kit (Qiagen). PCR-fragments were cloned by Gateway® Technology (Invitrogen) in modified pAS2-1 or pACT2 vectors (Clontech) expressing fusions with the Gal4 DNA-binding domain (BD) or the Gal4 activation domain (AD).

The cDNA constructs for the extracellular domain (EX) of *Le*PRK1 (U58474, aa 37-256) and the EX of *Le*PRK2 (U58473, aa 30-238) were also cloned by PCR and Gateway®-Technology from reverse transcribed mRNA isolated from pollen and inserted into modified pAS2-1 or pACT2 vectors.

Plasmids for split-ubiquitin assays encoding full length *Le*PRK1 and *Le*PRK2 as fusions with NubG and Cub-PLV, respectively, were generated from reverse transcribed mRNA isolated from VF36 pollen. PCR-fragments were cloned by Gateway®-Technology using pMetYC-Dest and pNx22-Dest vectors obtained from Klaus Harter (University of Tübingen).

The following constructs cloned from different sources by PCR were inserted into pGEX-6P-1 (GE Healthcare) for heterologous protein expression in *Escherichia coli*: KPP (AY730762) was cloned from reverse transcribed mRNA isolated from tomato pollen. RopGEF9 (At4g13240) cDNA was isolated from a two-hybrid cDNA library of floral buds of *A. thaliana* (Landsberg erecta) (Fan et al., 1997). RopGEF1 (At4g38430) and ROP1 (At3g51300) were cloned by RT-PCR from flowers of *A. thaliana* (Col-0). Our sequence for *Le*ROP was isolated from cDNA generated by RT of mRNA from tomato pollen with degenerative primers based on *in silico* data of expressed sequence tags for the unigene SGN-U314374. Our cDNA matches the open reading frame of AK324083 with the exception of three silent exchanges at position 6(c/t), 9(a/t) and 588(t/c). Site-specific mutagenesis of *Le*ROP was carried out using the QuickChange Kit (Stratagene) according to the manufacturer's protocol.

Yeast two-hybrid and split-ubiquitin assays

Yeast techniques were performed according to the Matchmaker GAL4 Two-Hybrid System 3 manual (Clontech).

Two-hybrid tests were carried out with *Saccharomyces cerevisiae* AH109 while DYS-1 was used in split-ubiquitin assays. Yeasts were co-transformed with different plasmid combinations and successful co-transformation was confirmed on minimal medium lacking tryptophan (-W) and leucine (-L). In two-hybrid assays, positive interactions were tested by spotting cells (OD_{600} 0,2) on plates lacking tryptophan, leucine, adenine (-W-L-A). In split-ubiquitin assays the interaction of NubG and Cub-PLV was monitored on medium lacking tryptophan, leucine, histidine (-W-L-H) with 2.5 mM 3-amino-1,2,4-triazol. Empty vectors were used as negative and autoactivation controls.

In interaction assays with *Le*ROP and *Le*PRK1 and *Le*PRK2, respectively, we also tested expression of the β-galactosidase reporter gene by staining based on the conversion of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal: 0.1 mg/ml medium).

Recombinant protein production

RopGEF and ROP proteins were produced as described before (Thomas et al., 2006) from the cloned cDNAs encoding full length or the PRONE domain of RopGEFs and full length *Le*ROP or C-terminally truncated ROPs. Deletion constructs included the following amino acids (aa): KPP-PRONE (KPP aa 55-400), PRONE9 (RopGEF9 aa 67-429), PRONE1 (RopGEF1 aa 76-460) (Berken et al., 2005), ROP1 (ROP1 aa 1-180), ROP4 (ROP4 aa 1-180) (Thomas et al., 2007).

Guanine nucleotide exchange assay

GEF activity was measured in guanine nucleotide exchange assays as published earlier (Thomas et al., 2007). Nucleotide release of N-methylanthraniloyl[mant]-GDP labeled ROP (200 nM) was examined by spectrofluorometry in the absence or presence of 10-fold molar excess of RopGEF protein after addition of 500-fold molar excess of unlabeled GDP. Reactions were carried out at 20 °C in 50 mM Tris (pH 7.5), 100 mM NaCl, 3 mM β-mercaptoethanol, and 10 mM $MgCl_2$. Proteins were used without GST-tags except for PRONE1. Tag removal did not alter GEF activity of PRONE1. Single exponential decay functions were fitted to data with Origin7 (OriginLab) to determine apparent rate constants k_{obs} for the intrinsic and GEF catalyzed reaction.