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Short communication

# Oligomerization studies show that the kinase domain of the tomato pollen receptor kinase LePRK2 is necessary for interaction with LePRK1

Tamara Marcela Salem [a,1], María Laura Barberini [a], Diego Leonardo Wengier [a,2], María Laura Cabanas [a,3], Pablo de Paz [a], Jorge Muschietti [a,b,*]

[a] Instituto de Ingeniería Genética y Biología Molecular INGEBI - Consejo Nacional de Investigaciones Científicas y Técnicas, 1428 Buenos Aires, Argentina
[b] Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

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ABSTRACT

LePRK1 and LePRK2 are two pollen-specific receptor-like kinases from *Solanum lycopersicum* that are involved in signaling during pollen-pistil communication. Previously, we showed that both proteins interact in pollen and when expressed in yeast. We also showed that pollen tube length was regulated by phosphorylation of specific residues in the juxtamembrane domain of LePRK2. To determine the domains responsible for the interaction between LePRK1 and LePRK2, we constructed a series of deletions, expressed them in yeast and determined their association by co-immunoprecipitation assays. We show that deletions containing extracellular domains of LePRK1 and LePRK2 were glycosylated in yeast and were sufficient for interaction with the corresponding full-length receptor. The juxtamembrane domain of LePRK1 was sufficient for its interaction with LePRK2, whereas LePRK2 required its kinase domain for interaction with LePRK1. These findings suggest a role for the juxtamembrane domain of LePRK2 in mediating intracellular dimerization and thus receptor kinase phosphorylation.



## 1. Introduction

Plants have different types of surface receptors, which sense various environmental stimuli and transduce them to the cytoplasm of the cell. Protein phosphorylation is one of the most studied mechanisms for mediating signal transduction. In Arabidopsis there are over 600 receptor kinases that participate in different plant processes such as development, perception of steroid hormones, and disease resistance [1]. Typically, kinase receptors have an amino-terminal extracellular domain involved in ligand perception, a transmembrane region, a juxtamembrane domain, a serine/threonine kinase domain and a carboxy-terminal domain [1]. A major group of plant receptor kinases have in their extracellular domain a variable number of leucine-rich repeats (LRRs), which are presumably involved in protein–protein interactions [2]. For example, CLAVATA1 (CLV1) is a 20 LRR-containing receptor that together with CLV2, binds their ligand CLV3 [3]. Perception of brassinosteroids is mediated by a 25 LRR-receptor kinase called BRI1 (Brassinosteroid-Insensitive1) and a 5 LRR-receptor kinase called BAK1 (BR1I-Associated Kinase1) [4]. Both proteins interact *in vitro* when expressed in yeast cells, and can phosphorylate each other *in vitro*.

LePRK1 and LePRK2 are two pollen-specific receptor-like kinases from *Solanum lycopersicum*, localized in the plasma membrane of pollen tubes and involved in pollen-pistil interactions [5]. The extracellular domains of LePRK1 and LePRK2 interact with different pistil or pollen proteins along its path to the ovary [6–9]. In the pollen cytoplasm, the kinase domain of LePRK2 interacts with a Rop-specific guanine exchange factor called KPP [10]. Therefore, transduction of pollen and pistil signals by their kinase activity might initiate a signaling cascade that would activate ROPs that in turn regulate pollen tube growth [11]. It was shown that in tomato pollen membranes, LePRK2 can be phosphorylated *in vitro* (Muschietti et al., 1998) and *in vivo*, exhibiting multiple phosphorylated isoforms [12]. The kinase activity of LePRK2 is necessary for interaction with LePRK1 [13].

In order to delineate the structural basis of LePRK1 and LePRK2 interaction, we generated five deletion mutants for each of the two

* Corresponding author. INGEBI, 2490 Vuelta de Obligado, Buenos Aires, Argentina. Tel.: +54 11 4783 2871; fax: +54 11 4786 8578.
*E-mail address:* prometeo@dna.uba.ar (J. Muschietti).
[1] Institut de Génétique Moléculaire de Montpellier, UMR5535, CNRS, Montpellier Cedex 5, France.
[2] Department of Biology, Stanford University, Stanford, CA, USA.
[3] Gema Biotech, Buenos Aires, Argentina.

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receptor-like kinases, LePRK1 and LePRK2. We expressed in yeast a pair of constructs consisting of one full-length receptor kinase together with one of each of the five deletions of the other receptor kinase and assayed their interaction by co-inmunoprecipitation assays. Our results showed that the LePRK2 kinase domain is necessary for interaction with full-length LePRK1, while the LePRK1 juxtamembrane domain is sufficient for interaction with full-length LePRK2.

## 2. Experimental procedures/materials and methods

### 2.1. Plasmids

The different domains of LePRK1 (deletions A–E) or LePRK2 (deletions 1–5) were first amplified by PCR with synthetic nucleotides. The primers used to amplify LePRK1 and LePRK2 deletions are listed in Table 1.

PCR products were digested with restriction enzymes SacII and SacI and ligated into a shuttle vector YcpIF7-MF1-3HA, a modified version of YcpIF7 [14]. To create the YcpIF7-MF1-3HA vector, the yeast MF1 signal peptide was used [15] and cloned into the SalI - SpeI sites of YcpIF7 using the MF1 primers listed in Table 1. Three tandem repeats of the influenza virus hemagglutinin epitope (3HA) [16] were inserted into the SpeI - NotI sites of YcpIF7-MF1 in frame with MF1 and then the deletion products were ligated. The final vectors were obtained by digesting the MF1-3HA-deletion fragments from YcpIF7-MF1-3HA with SalI and SacI and ligating them either into SalI/SacI-digested YcpIF1 (for deletions A–E of LePRK1) or into SalI - SacI-digested YcpIF4 (for deletions 1–5 of LePRK2). DNA manipulations, including PCR, restriction enzyme digestions, ligations, plasmid isolation, and *E. coli* transformation, were carried out by standard methods.

### 2.2. Yeast expression and immunoprecipitation

BJ2168 yeast strain (strain 208277 from ATCC) was transformed in pairs with LePRK1 (cloned into YCpIF3 vector) or with LePRK2 (cloned into YCpIF6 vector), together with a deletion construct of LePRK2 or LePRK1 respectively. All cloned genes were under the control of the *GAL1* promoter and, therefore, were induced in cells grown in the presence of galactose. Yeast transformation was performed as described in Gietz et al. [17]. Yeast microsomal ($P_{100}$) preparations and co-immunoprecipitation experiments were as previously described [13]. Co-immunoprecipitations of 750 μg of microsomal proteins were carried out with low detergent buffer (150 mM NaCl, 0.5% NP40, 50 mM Tris pH 8) except when noted.

### 2.3. SDS-PAGE and immunoblotting

Eighty micrograms of tomato pollen $P_{100}$ membrane or yeast membrane $P_{100}$ proteins or the immunopellet of each co-immunoprecipitation were separated by SDS-PAGE and blotted to Hybond ECL nitrocellulose membrane (GE Healthcare). The membranes were blocked with 0.2% Triton X-100, 4% non-fat dry milk, 2% glycine in TBS for 30 min. The blocked membranes were incubated with antibodies raised against the extracellular domain of LePRK1 (ECD1), LePRK2 (ECD2), or anti-HA (Covance), diluted to 1:1000 in 0.2% Triton X-100, 2% non-fat dry milk, and 2% glycine in TBS for 1 h at room temperature. After three washes of 10 min each with 0.2% Triton X-100 in TBS, the membranes were incubated with a 1:5000 dilution of sheep anti-mouse polyclonal secondary antibodies conjugated with horseradish peroxidase (GE Healthcare) in 0.2% Triton X-100, 2% non-fat dry milk, and 2% glycine in TBS for 1 h at room temperature, washed as described above, and developed using an enhanced chemiluminescence kit (GE Healthcare).

### 2.4. Deglycosylation assays

PNGase F (New England Biolabs) reactions were performed as indicated by the manufacturer. Briefly, 100 μg of tomato pollen $P_{100}$ membrane or 25 μg of yeast $P_{100}$ proteins were denatured in 1X denaturation buffer (10X: 5% SDS and 10% β-mercaptoethanol) for 10 min at 100 °C. Then, 1/10 of buffer G7 1X (10X: 0.5 M $Na_3PO_4$ pH 7.5 at 25 °C), 1/10 of NP-40 10% and 1/10 of PNGase F were added. The reaction was incubated ON at 37 °C. Finally, 45 μl of 5X Laemmli-SDS-PAGE sample buffer was added to the reaction and boiled for 5 min at 100 °C. After centrifugation for 1 min, the supernatant was loaded into an SDS-PAGE and immunoblotted.

**Table 1**
Primers used in this study.

| | Deletion | Sequence (5′ to 3′) | Direction[a] |
|---|---|---|---|
| LePRK1 deletions | ΔA | 5′-AAACCGCGGAGATAATAACGAGGCAGAAATTCTC-3′ | S |
| | | 5′-ATTGAGCTCTTAAAGAATGATGATAACAACAACAAT-3′ | AS |
| | ΔB | 5′-AAACCGCGGAGATAATAACGAGGCAGAAATTCTC-3′ | S |
| | | 5′-AGTGAGCTCTTAATCTGGCAAATCAAATTTTTC-3′ | AS |
| | ΔC | 5′-TAACCGCGGAATTATATTGGTGGTTATCGCAGTTGC-3′ | S |
| | | 5′-CTTTTTAAACTAAACGGTCTAATTCTCGAGTGA-3′ | AS |
| | ΔD | 5′-TAACCGCGGAATTATATTGGTGGTTATCGCAGTTGC-3′ | S |
| | | 5′-CGTTACCTTTCCTAACTCATTCTCGAGCCA-3′ | AS |
| | ΔE | 5′-TAACCGCGGAATTATATTGGTGGTTATCGCAGTTGC-3′ | S |
| | | 5′-GTCCAAAGCTACTTGTAAATCCTCGAGCCA-3′ | AS |
| LePRK2 deletions | Δ1 | 5′-AAACCGCGGAAACTTATCAGAGCCTGA-3′ | S |
| | | 5′-AGTGAGCTCTTAATCAGGCAAGTCAAATTTCTC-3′ | AS |
| | Δ2 | 5′-AAACCGCGGAGATAATAACGAGGCAGAAATTCTC-3′ | S |
| | | 5′-AGTGAGCTCTTAATCAGGCAAGTCAAATTTCTC-3′ | AS |
| | Δ3 | 5′-ATTCCGCGGTATTGCCCTTATTGTGGTTATAG-3′ | S |
| | | 5′-CTCTTTAAACTGAACGGACTAATTCTCGAGTGA-3′ | AS |
| | Δ4 | 5′-ATTCCGCGGTATTGCCCTTATTGTGGTTATAG-3′ | S |
| | | 5′-CTCCGTCAACTTTCCTAACTAATTCTCGAGCCA-3′ | AS |
| | Δ5 | 5′-ATTCCGCGGTATTGCCCTTATTGTGGTTATAG-3′ | S |
| | | 5′-CAACTGTTATACGTATGAAGTTCTATTCTCGAGTTA-3′ | AS |
| MF1 | | 5′-ATTTGTCGACTTAACCATGGCATTTCCTTC-3′ | S |
| | | 5′-GGACTAGTGCGACCGGTAGCTTCAGCCTCTCTTTTATCC-3′ | AS |

[a] S, sense; AS, antisense.

## 3. Results

In order to address the relevance for interaction of the different domains of LePRK1 and LePRK2, we made five deletion constructs (Fig. 1) for each receptor kinase and expressed them in yeast. Both wild type LePRK1 and LePRK2 bear an extracellular domain (ECD) with 6 and 5 LRRs respectively, a transmembrane domain (TM), a juxtamembrane domain (JM), a kinase domain (KD) and a COOH-terminal domain (CtD) [5]. Constructs LePRK1-ΔA and LePRK2-Δ1 contained the ECD and the TM domains of LePRK1 and LePRK2 respectively, while constructs LePRK1-ΔB and LePRK2-Δ2 contained the ECD-TM-JM of LePRK1 and LePRK2, respectively. To study the roles of the different cytoplasmic domains for interaction, three pairs of deletion constructs were made: LePRK1-ΔC and LePRK2-Δ3 contained the TM-JM, LePRK1-ΔD and LePRK2-Δ4 included the TM-JM-KD and LePRK1-ΔE and LePRK2-Δ5 contained the TM-JM-KD-CtD. All deletion constructs had a signal sequence from the yeast α-mating factor (MF1) to assure entry into the secretory pathway [15]. They also had an N-terminus 3HA epitope tag so that they could be precipitated by a commercial anti-HA antibody [16]. Predicted molecular weights for each construct are shown in Fig. 1.

Each deletion construct was co-expressed in yeast with a full-length clone of the other corresponding receptor. Expression in yeast was confirmed by western blot (Fig. 2) using an anti-HA antibody that recognizes the deletion constructs and the anti-ECD1 or anti-ECD2 antibodies that specifically recognize the extracellular domains of LePRK1 and LePRK2, respectively [13]. Yeast expressing deletion proteins that contain the extracellular domains of LePRK1 (LePRK1-ΔA and LePRK1-ΔB) and LePRK2 (LePRK2-Δ1 and LePRK2-Δ2) showed bands with higher molecular weight than expected (Fig. 2A). To determine whether these bands corresponded to glycosylated versions of the deletion proteins, $P_{100}$ fractions from yeast were treated with PNGase F, a deglycosylating enzyme. Fig. 2A showed that upon PNGase F treatment most of the high molecular weight yeast bands disappeared and a band that corresponded to the predicted molecular weight of the deletion proteins became more prominent. Full-length LePRK1 treated with PNGase F also showed slightly increased mobility when compared to non-treated LePRK1 (Fig. 2A), although this was not true for LePRK2. However, no differences were seen when pollen membrane $P_{100}$ fractions were incubated, or not, with PNGase F (Fig. 2B). The deletion proteins lacking the ECDs showed the predicted molecular weight (Fig. 2C).

We used co-immunoprecipitation to determine which domains of LePRK1 and LePRK2 were important for their interaction. $P_{100}$ proteins from yeast were co-immunoprecipitated with anti-HA that recognizes the deletion mutant and immunoblots were developed with anti-ECD1 or anti-ECD2 antibodies.

We first tested whether the cytoplasmic domains were required for the interaction between LePRK1 and LePRK2 (Fig. 3A and B). Membrane proteins from yeast expressing the pairs LePRK1/LePRK2-Δ3, LePRK1/LePRK2-Δ4 or LePRK1/LePRK2-Δ5 were co-immunoprecipitated with anti-HA antibody and the presence of LePRK1 was detected with anti-ECD1 (Fig. 3A). Because LePRK1 was in the immunopellet when constructs LePRK2-Δ4 and LePRK2-Δ5, but not LePRK2-Δ3, were used, we concluded that the kinase domain of LePRK2 was necessary for the interaction with LePRK1, whereas the presence of the JM of LePRK2 was not sufficient. This result correlates with our previous finding that the LePRK2 kinase activity was required for LePRK1 binding in yeast [13].

We then analyzed the interactions between LePRK2 and the cytoplasmic deletions of LePRK1 (Fig. 3B). $P_{100}$ proteins from yeast expressing the pairs LePRK2/LePRK1-ΔC, LePRK2/LePRK1-ΔD or LePRK2/LePRK1-ΔE were co-immunoprecipitated with anti-HA antibody and the presence of LePRK2 was detected with anti-ECD2. Fig. 3B showed that LePRK2 was co-immunoprecipitated with all three cytoplasmic deletions of LePRK1. In all cases yeast expressing both full-length LePRK1 and LePRK2 immunoprecipitated using either anti-ECD1 or anti-ECD2 were used as controls (data not shown) [13]. These results suggest that the presence of the JM of LePRK1 was sufficient for the interaction with LePRK2.

We next analyzed whether the extracellular domains of LePRK1 and LePRK2 were required for their interaction (Fig. 4). Membrane proteins from yeast expressing LePRK1/LePRK2-Δ1 or LePRK1/LePRK2-Δ2 were immunoprecipitated using anti-HA antibody and the presence of LePRK1 was detected with anti-ECD1 (Fig. 4, left panel). Similarly, LePRK2/LePRK1-ΔA or LePRK2/LePRK1-ΔB was immunoprecipitated using anti-HA antibody and immunoblots were developed with anti-ECD2 (Fig. 4, right panel). Fig. 4 showed that LePRK1 was co-immunoprecipitated from yeast expressing either extracellular domain deletion constructs, i.e. LePRK2-Δ1 and LePRK2-Δ2 (Fig. 4, left panel). The same result was obtained when LePRK2 was co-immunoprecipitated with the LePRK1 extracellular

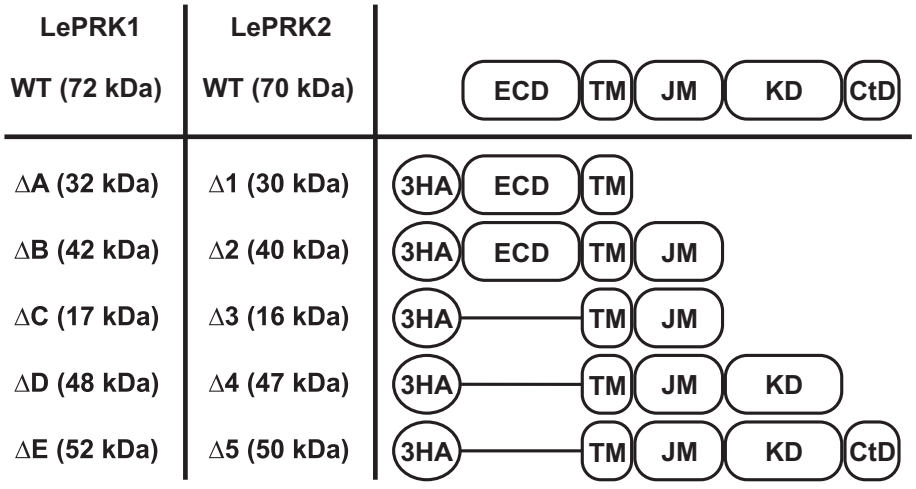


**Fig. 1.** Schematic representation of LePRK1 and LePRK2 deletions. Diagram of LePRK protein domains. ECD, Extracellular domain; TM, Transmembrane domain; JM, Juxtamembrane domain; KD Kinase domain; CtD: C-terminal domain. WT, wild type protein. 3HA, three tandem repeats of the influenza virus hemagglutinin epitope. Δ"*letter*" (A, B, C, D, E), corresponds to deletions of LePRK1 domains while Δ"*number*" (1, 2, 3, 4, 5) corresponds to deletions of LePRK2 domains. The molecular weight of each deletion is indicated in parentheses.

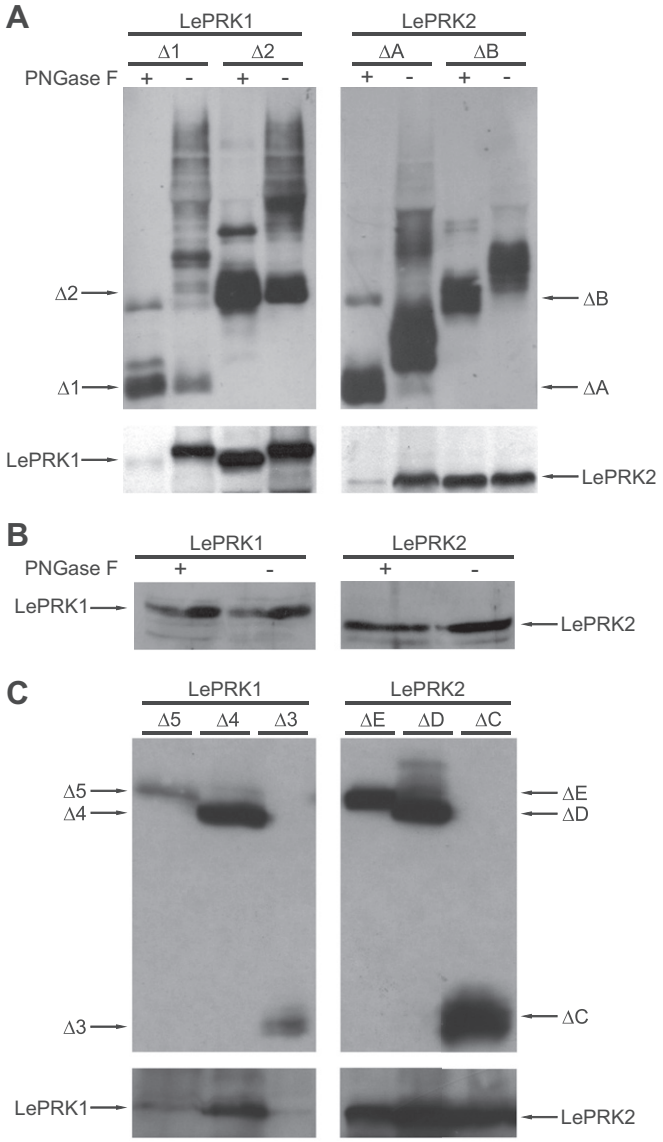


**Fig. 2.** Extracellular domain deletions of LePRK1 and LePRK2 and full-length LePRK1 are glycosylated in yeast. Tomato mature pollen microsomal ($P_{100}$) proteins or microsomal ($P_{100}$) proteins from yeast expressing the deletion proteins with their corresponding full-length receptor proteins were subjected to SDS-PAGE. Deletion proteins were detected with anti-HA antibody (Fig. 2A and C, upper panels) and full-length LePRK1 and LePRK2 kinases (Fig. 2B and lower panels of Fig. 2A and C) were detected with anti-ECD1 (left panels) or anti-ECD2 (right panels) antibodies. Arrows indicate the expected position of the proteins. (A) $P_{100}$ proteins from yeast expressing extracellular deletions of LePRK1 (LePRK1-ΔA and LePRK1-ΔB) and LePRK2 (LePRK2-Δ1 and LePRK2-Δ2) with their corresponding full-length receptor, were treated (+) or not treated (−) with PNGase F. After treatment, samples were subjected to SDS-PAGE. (B) Tomato mature pollen microsomal ($P_{100}$) proteins were treated (+) or not treated (−) with PNGase F and then subjected to SDS-PAGE. (C) $P_{100}$ proteins from yeast expressing cytoplasmic deletions of LePRK1 (LePRK1-ΔC, LePRK1-ΔD and LePRK1-ΔE) and LePRK2 (LePRK2-Δ3, LePRK2-Δ4 and LePRK2-Δ5) with their corresponding full-length receptor were subjected to SDS-PAGE. Arrows indicate the expected position of the expressed yeast proteins.

domain deletion constructs, i.e. LePRK1-ΔA and LePRK1-ΔB (Fig. 4, right panel). The absence of a band in the co-immunoprecipitation control LePRK1/LePRK2-Δ3 (Fig. 4, left panel) further confirms that the co-immunoprecipitation of LePRK2-Δ1 and LePRK2-Δ2 (Fig. 4 lanes 1 and 2, left panel) were specific. All these results suggest that the extracellular domains of LePRK1 and LePRK2 are sufficient for their interaction with the corresponding full-length receptor kinase. When co-immunoprecipitations were performed using a buffer with a higher detergent concentration (1% NP-40 and 0.1% SDS instead of 0.5% NP-40) different results were obtained. Then only the cytoplasmic deletion proteins (LePRK1-ΔC, LePRK1-ΔD, LePRK1-ΔE, LePRK2-Δ4 and LePRK2-Δ5) were capable of interacting with their corresponding full-length receptors, while all four extracellular domain deletion proteins (LePRK1-ΔA, LePRK1-ΔB, LePRK2-Δ1 and LePRK2-Δ2) showed no interaction (data not shown). All these results suggest that the interaction through their cytoplasmic domains is stronger than through their extracellular domains.

## 4. Discussion

In this work, we studied the interaction between the tomato pollen receptor kinases LePRK1 and LePRK2 by heterologous expression and co-immunoprecipitation in yeast. First we showed that full-length LePRK1 and all deletions containing the extracellular LePRK1 (LePRK1-ΔA and LePRK2-ΔB) and LePRK2 domains (LePRK2-Δ1 and LePRK2-Δ2) were N-glycosylated in yeast (Fig. 2A). However, the glycosylation was observed when full-length LePRK1 was expressed in yeast but not *in planta* (Fig. 2B). In contrast, full-length LePRK2 appears not to be glycosylated neither *in planta* nor in yeast, since no apparent shift in SDS-PAGE mobility was observed (Fig. 2A and B). The results observed in yeast are consistent with the presence of two potential N glycosylation sites in the extracellular domain of LePRK1, but none in LePRK2 [18]. However, it is not clear why the deletions containing the extracellular domains of LePRK2 (LePRK2-Δ1 and LePRK2-Δ2) were glycosylated in yeast. It has been previously described that glycosylation of heterologous proteins expressed in yeast is disproportionate [19]. The glycosylation pattern was not caused by the presence of the epitope 3HA, because there was no shift in the SDS-PAGE mobility of any of the cytoplasmic deletion proteins of LePRK1 and LePRK2.

We showed that the LePRK2 deletion that only has the transmembrane and juxtamembrane domain (LePRK2-Δ3) did not interact with LePRK1. On the other hand, the juxtamembrane domain of LePRK1 was sufficient for interaction with LePRK2, as shown with the LePRK1-ΔC deletion, suggesting that the kinase activity of LePRK1 is not relevant for the interaction. This correlates well with our previous finding that the full kinase domain of LePRK2, but not LePRK1, is necessary for the interaction with LePRK1 [13] and for proper pollen tube growth [12]. This is comparable to Arabidopsis brassinosteroid signaling, where the kinase activity of BRI1 is required for the association between BRI1 and BAK1. However, the kinase dead mutant BAK1 associates with wild-type BRI1, suggesting that the kinase activity of BAK1 is not essential for this interaction [20].

We have previously shown that full-length LePRK1 and LePRK2 interacted in yeast microsomal fractions [13]. This result suggested that no other cytoplasmic pollen proteins or extracellular ligands were required for the LePRK1 and LePRK2 interaction in yeast membranes. Here, we showed that deletions containing only the extracellular domains of LePRK1 or LePRK2 were sufficient for interacting with the corresponding full-length receptor. These results agree with a previous report that showed, using yeast two hybrid, that full length or extracellular domain of LePRK1 interacted with full length or extracellular domain of LePRK2, respectively [21]. They also showed that full length or extracellular domain of LePRK2, but not LePRK1, interacted with itself [21], suggesting that LePRK2, but not LePRK1, was able to homodimerize.

Interactions between the extracellular domains of plant receptor kinases were reported for BRI1 receptor [22], AtSERK1 (*Arabidopsis thaliana* Somatic Embryogenesis Receptor Kinase1) [23] and CLV1 (CLAVATA1 from *A. thaliana* [24]). Our results showed

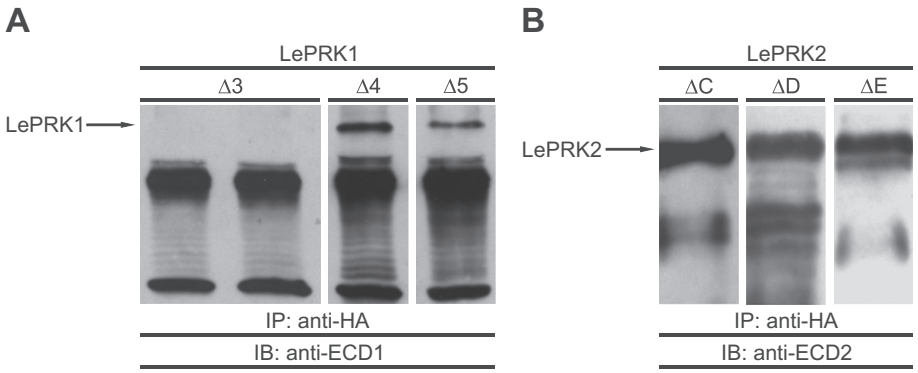


**Fig. 3.** The kinase domain of LePRK2 is necessary for the interaction with LePRK1. $P_{100}$ proteins from yeast expressing the deletion proteins with their corresponding full-length receptor proteins were immunoprecipitated (IP) with anti-HA antibody and then subjected to SDS-PAGE. Immunoprecipitated LePRK1 and LePRK2 were immunoblotted (IB) using anti-ECD1 (Fig. 3A) or anti-ECD2 (Fig. 3B) antibodies respectively. (A) $P_{100}$ proteins from yeast expressing LePRK1 together with the LePRK2 cytoplasmic deletions (LePRK2-Δ3, LePRK2-Δ4 or LePRK2-Δ5) were immunoprecipitated with anti-HA antibody and subjected to SDS-PAGE. (B) $P_{100}$ proteins from yeast expressing LePRK2 together with the LePRK1 cytoplasmic deletions (LePRK1-ΔC, LePRK1-ΔD or LePRK1-ΔE) were immunoprecipitated with anti-HA antibody and subjected to SDS-PAGE. Arrows indicate the position of LePRK1 and LePRK2 co-immunoprecipitated proteins.

that the interaction through the extracellular domains of LePRK1 and LePRK2 is weaker than through their cytoplasmic domains. A similar situation seems to occur with the brassinosteroids signaling where the kinase domains of BRI1 and BAK1 are necessary for their association induced by brassinosteroids. This was illustrated by the fact that the kinase dead mutant BRI1 did not interact with BAK1 in the presence of brassinosteroids suggesting that the only presence of the extracellular domains is not enough for BRI1–BAK1 association [20].

In other case, the LRR receptor-like protein CLV2 (CLAVATA 2), which bears an extracellular domain, a single transmembrane domain and a short cytoplasmic tail, and CRN (CORYNE) a kinase-related protein that has a short extracellular domain, a transmembrane domain and a protein kinase domain, interact through their transmembrane domains [25,26]. In our case, we can not rule out the possibility that the transmembrane domain of the deletion protein LePRK1-ΔC was important for association with LePRK2.

The role of LePRK1 kinase activity is not clear. Even though we showed *in vitro* kinase activity of the kinase domain of LePRK1, we never found labeled LePRK1 when pollen membranes were phosphorylated *in vitro* [5]. We previously showed that the kinase activity of LePRK1 was dispensable for the interaction with LePRK2, because a mutant dead kinase LePRK1 co-immunoprecipitated LePRK2 in yeast membranes [13]. There is a possibility that LePRK1 belongs to the group of atypical receptor kinases that bear a kinase dead domain [27] but still signal because they are phosphorylated by other kinases. This possibility is supported because 2D gel followed by western blot analysis of tomato pollen membranes showed the presence of few putatively phosphorylated spots that corresponded to LePRK1 (data not shown).

All these results suggest that the LePRK2 kinase domain is relevant for the interaction with LePRK1 and supports the idea that the kinase domain of LePRK2 is necessary for transducing the style signals by phosphorylation of diverse pollen proteins.

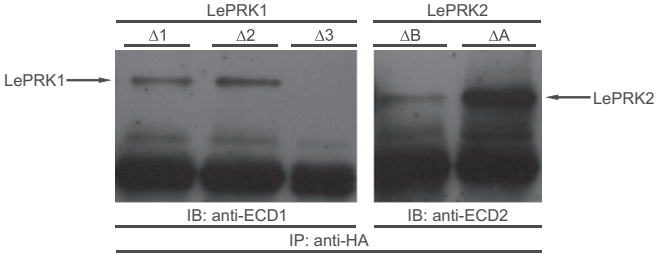


**Fig. 4.** The extracellular domains of LePRK1 and LePRK2 are sufficient for the interaction with full-length LePRK2 and LePRK1 respectively. $P_{100}$ proteins from yeast expressing the deletion proteins with their corresponding full-length receptor proteins were immunoprecipitated (IP) with anti-HA antibody and then subjected to SDS-PAGE. Immunoprecipitated LePRK1 and LePRK2 were immunoblotted (IB) using anti-ECD1 (Fig. 4A) or anti-ECD2 (Fig. 4B) antibodies respectively. (A) $P_{100}$ proteins from yeast expressing LePRK1 together with the LePRK2 extracellular deletions (LePRK2-Δ1 or LePRK2-Δ2) or one of the cytoplasmic deletions (LePRK2-Δ3) were immunoprecipitated with anti-HA antibody and subjected to SDS-PAGE. (B) $P_{100}$ proteins from yeast expressing LePRK2 together with the LePRK1 extracellular deletions (LePRK1-ΔA or LePRK1-ΔB) were immunoprecipitated with anti-HA antibody and subjected to SDS-PAGE. Arrows indicate the position of LePRK1 and LePRK2 co-immunoprecipitated proteins.

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## References

[1] E.R. Morris, J.C. Walker, Receptor-like protein kinases: the keys to response, Curr. Opin. Plant Biol. 6 (2003) 339–342.
[2] K.U. Torii, Leucine-rich repeat receptor kinases in plants: structure, function, and signal transduction pathways, Int. Rev. Cytol. 234 (2004) 1–46.
[3] A.E. Trotochaud, S. Jeong, S.E. Clark, CLAVATA3, a multimeric ligand for the CLAVATA1 receptor-kinase, Science 289 (2000) 613–617.
[4] J.M. Gendron, Z.Y. Wang, Multiple mechanisms modulate brassinosteroid signaling, Curr. Opin. Plant Biol. 10 (2007) 436–441.
[5] J. Muschietti, Y. Eyal, S. McCormick, Pollen tube localization implies a role in pollen-pistil interactions for the tomato receptor-like protein kinases LePRK1 and LePRK2, Plant Cell 10 (1998) 319–330.
[6] V. Guyon, W.H. Tang, M.M. Monti, A. Raiola, G.D. Lorenzo, S. McCormick, L.P. Taylor, Antisense phenotypes reveal a role for SHY, a pollen-specific leucine-rich repeat protein, in pollen tube growth, Plant J. 39 (2004) 643–654.
[7] W. Tang, I. Ezcurra, J. Muschietti, S. McCormick, A cysteine-rich extracellular protein, LAT52, interacts with the extracellular domain of the pollen receptor kinase LePRK2, Plant Cell 14 (2002) 2277–2287.
[8] W. Tang, D. Kelley, I. Ezcurra, R. Cotter, S. McCormick, LeSTIG1, an extracellular binding partner for the pollen receptor kinases LePRK1 and LePRK2, promotes pollen tube growth in vitro, Plant J. 39 (2004) 343–353.
[9] D.L. Wengier, M.A. Mazzella, T.M. Salem, S. McCormick, J.P. Muschietti, STIL, a peculiar molecule from styles, specifically dephosphorylates the pollen receptor kinase LePRK2 and stimulates pollen tube growth in vitro, BMC Plant Biol. 10 (2010) 33.
[10] P. Kaothien, S.H. Ok, B. Shuai, D. Wengier, R. Cotter, D. Kelley, S. Kiriakopolos, J. Muschietti, S. McCormick, Kinase partner protein interacts with the LePRK1 and LePRK2 receptor kinases and plays a role in polarized pollen tube growth, Plant J. 42 (2005) 492–503.

[11] D. Zhang, D. Wengier, B. Shuai, C.P. Gui, J. Muschietti, S. McCormick, W.H. Tang, The pollen receptor kinase LePRK2 mediates growth-promoting signals and positively regulates pollen germination and tube growth, Plant Physiol. 148 (2008) 1368–1379.
[12] T. Salem, A. Mazzella, M.L. Barberini, D. Wengier, V. Motillo, G. Parisi, J. Muschietti, Mutations in two putative phosphorylation motifs in the tomato pollen receptor kinase LePRK2 show antagonistic effects on pollen tube length, J. Biol. Chem. 286 (2011) 4882–4891.
[13] D. Wengier, I. Valsecchi, M.L. Cabanas, W.H. Tang, S. McCormick, J. Muschietti, The receptor kinases LePRK1 and LePRK2 associate in pollen and when expressed in yeast, but dissociate in the presence of style extract, Proc. Natl. Acad. Sci. U S A 100 (2003) 6860–6865.
[14] P.K. Foreman, R.W. Davis, Cloning vectors for the synthesis of epitope-tagged, truncated and chimeric proteins in Saccharomyces cerevisiae, Gene 144 (1994) 63–68.
[15] J.M. White, M.D. Rose, Yeast mating: getting close to membrane merger, Curr. Biol. 11 (2001) R16–R20.
[16] M.S. Longtine, A. McKenzie 3rd, D.J. Demarini, N.G. Shah, A. Wach, A. Brachat, P. Philippsen, J.R. Pringle, Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae, Yeast 14 (1998) 953–961.
[17] R.D. Gietz, R.A. Woods, Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method, Methods Enzymol. 350 (2002) 87–96.
[18] H.U. Kim, R. Cotter, S. Johnson, M. Senda, P. Dodds, R. Kulikauska, W. Tang, I. Ezcura, P. Herzmark, S. McCormick, New pollen-specific receptor kinases identified in tomato, maize and Arabidopsis: the tomato kinases show overlapping but distinct localization patterns on pollen tubes, Plant Mol. Biol. 50 (2002) 1–16.
[19] M. Wyss, L. Pasamontes, A. Friedlein, R. Remy, M. Tessier, A. Kronenberger, A. Middendorf, M. Lehmann, L. Schnoebelen, U. Rothlisberger, E. Kusznir, G. Wahl, F. Muller, H.W. Lahm, K. Vogel, A.P. van Loon, Biophysical characterization of fungal phytases (myo-inositol hexakisphosphate phosphohydrolases): molecular size, glycosylation pattern, and engineering of proteolytic resistance, Appl. Environ. Microbiol. 65 (1999) 359–366.
[20] X. Wang, U. Kota, K. He, K. Blackburn, J. Li, M.B. Goshe, S.C. Huber, S.D. Clouse, Sequential transphosphorylation of the BRI1/BAK1 receptor kinase complex impacts early events in brassinosteroid signaling, Dev. Cell 15 (2008) 220–235.
[21] S. Locke, I. Fricke, E. Mucha, M.L. Humpert, A. Berken, Interactions in the pollen-specific receptor-like kinases-containing signaling network, Eur. J. Cell Biol. 89 (2010) 917–923.
[22] A. Dievart, S.E. Clark, Using mutant alleles to determine the structure and function of leucine-rich repeat receptor-like kinases, Curr. Opin. Plant Biol. 6 (2003) 507–516.
[23] K. Shah, T.W. Gadella Jr., H. van Erp, V. Hecht, S.C. de Vries, Subcellular localization and oligomerization of the Arabidopsis thaliana somatic embryogenesis receptor kinase 1 protein, J. Mol. Biol. 309 (2001) 641–655.
[24] S.E. Clark, Meristems: start your signaling, Curr. Opin. Plant Biol. 4 (2001) 28–32.
[25] A. Bleckmann, S. Weidtkamp-Peters, C.A. Seidel, R. Simon, Stem cell signaling in Arabidopsis requires CRN to localize CLV2 to the plasma membrane, Plant Physiology 152 (2010) 166–176.
[26] Y. Guo, L. Han, M. Hymes, R. Denver, S.E. Clark, CLAVATA2 forms a distinct CLE-binding receptor complex regulating Arabidopsis stem cell specification, Plant journal: for cell Molecular Biology 63 (2010) 889–900.
[27] E. Castells, J.M. Casacuberta, Signalling through kinase-defective domains: the prevalence of atypical receptor-like kinases in plants, J. Exp. Bot. 58 (2007) 3503–3511.