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Characterization of ScORK28, a transmembrane functional protein receptor kinase predominantly expressed in ovaries from the wild potato species *Solanum chacoense*☆

Hugo Germain[a], Josée Houde[a], Madoka Gray-Mitsumune[a], Tatsuya Sawasaki[b], Yaeta Endo[b], Jean Rivoal[a], Daniel P. Matton[a,*]

[a] *Institut de Recherche en Biologie Végétale (IRBV), Département de Sciences Biologiques, Université de Montréal, 4101 Sherbrooke Est, Montréal, QC, Canada H1X 2B2*

[b] *Department of Applied Chemistry, Faculty of Engineering, and Venture Business Laboratory, Ehime University, Matsuyama 790-8577, Japan*

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Abstract *Solanum chacoense* ovule receptor kinase 28 (*ScORK28*) was found among 30 receptor kinases from an ovule cDNA library enriched for weakly expressed mRNAs. This LRR-RLK displayed high level of tissue specificity at the RNA and protein levels and was predominantly expressed in female reproductive tissues. Protein expression analyses in planta revealed that ScORK28 was *N*-glycosylated and ScORK28::GFP fusion analyses showed that it was localized at the plasma membrane. Bacterial expression of ScORK28 catalytic domain followed by kinase activity assays revealed that ScORK28 is an active Mg^{2+}-dependent protein kinase and that the juxtamembrane domain is necessary for kinase activity.

Keywords: Receptor-like kinase; Ovule; Fertilization; *Solanum chacoense*

1. Introduction

One key aspect of all developing organism is their ability to sense their extracellular environment. This function is mediated, in part, by receptor kinases. These proteins are composed of three different subdomains: an extracellular domain that perceives the signal or ligand, a hydrophobic transmembrane domain that anchors the protein to the phospholipids bi-layer, and a cytoplasmic kinase domain that transduces the signal to intracellular target(s). Although most of the knowledge regarding the *modus operandi* of these signal transducer was gained in animal cell, numerous studies indicate that it will be similar in plants [1]. Upon ligand binding the receptor kinase dimerizes thus bringing the kinase domains in close proximity to allow transphosphorylation (activation), enabling the recruitment and/or phosphorylation of downstream effectors [2]. The receptor-like kinase (RLK) gene family represents one of the largest gene family in *Arabidopsis* with 417 members [3]. Mutant analysis have revealed that RLKs are involved in diverse biological processes including development, defense, hormone sensing, and reproduction [4]. In contrast to this, biochemical characterization of the intracellular domain of plant receptor kinase has only been performed for a few receptor kinase such as in [5–11].

Using a negative selection screen we have previously identified 30 receptor kinases expressed in ovules and ovaries from *Solanum chacoense* [12]. One of these, ovule receptor kinase 28 (*ScORK28*) displayed an expression pattern highly specific to ovaries and was therefore chosen for further biochemical, cellular, and molecular characterization to determine its involvement in fertilization and early fruit development.

2. Materials and methods

2.1. Sequence analysis

DNA sequence was in silico translated using MacVector 8.1.2 (Accelrys). The presence of a signal peptide was assessed using SignalP 3.0 and the location of the transmembrane domain was positioned using TMHMM 2.0. Putative *N*-glycosylation sites were analysed using NetGly 1.0 and the structure of the leucine-rich repeat domain was analyzed using ScanSite 2.0. The phylogenic tree was inferred using a neighbor-joining algorithm with 1000 bootstraps either on the full length sequences or on the trimmed catalytic domains.

2.2. Transgenic plants

Arabidopsis plants consisted of T-DNA lines obtained from the SALK institute (*SRF6*/At1g53730 SALK line number: 116320, 116318, 062310, 054337, 077702 and *SRF7*/At3g14350 SALK line number: 039120, 068033, 110007). The *S. chacoense* transgenic was an overexpression line (OX) with the full length *ScORK28* cDNA cloned downstream of the CaMV35S promoter while the truncated construct consisted of the *ScORK28* ectodomain with its transmembrane domain.

2.3. Protein extraction, Western blot analysis, and deglycosylation assay

Protein were extracted in a buffer containing 0.1 M Tris–HCl, pH 8, 0.1% SDS, 2% β-mercaptoethanol, and 1× of protease cocktail inhibitor (Roche). Equal amount of protein was loaded in each well as determined by the Bio-Rad Protein Assay. The 8% polyacrylamide gel was run in Tris-glycine buffer and then transferred to a PVDF membrane [13]. To generate specific polyclonal antibodies the extracellular domain of ScORK28 (to minimize the possibility of cross-reaction with other RLKs) was expressed in a large-scale in vitro wheat germ extract system as previously described [14]. The protein was pre-purified by precipitation at 15000 × *g*/15 min/4 °C, solubilized in 2-D buffer, separated on several 2-D gels, electroeluted, and injected into NZ white

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*Corresponding author. Fax: +1 514 8729406.
E-mail address: dp.matton@umontreal.ca (D.P. Matton).

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rabbits. Pre-bleed, a test bleed at 28 days, and a final bleed at 35 days were collected. Appropriate control experiments were performed to ascertain that the pre-bleed serum did not react against *S. chacoense* proteins.

Immunoblotting was done as previously described [15]. For *N*-deglycosylation assay, the ScORK28 complete protein sequence was cloned into the tandem affinity purification vector pC-TAPa [16] and expressed in planta through agroinfiltration of *Nicotiana benthamiana* leaves [17]. Crude extracts from infected leaves were treated with *N*-glycosidase F (PNGase F) following the manufacturer instructions (New England Bio-Lab). The extract was separated on gel, transferred on membrane and detected using an anti-HIS antibody (Sigma).

2.4. Transient expression for protein localization

ScORK28 was PCR amplified from the cDNA clone and introduced by recombination in the Gateway pZeo donor vector (Invitrogen), and then transferred in the pMDC83 vector [18]. Onion cells were transiently transformed by particle bombardment using this *ScORK28-GFP* construct.

2.5. Protein kinase activity assay

The kinase domain of *ScORK28* was cloned in fusion with a His-tag in the pQE30 vector (Qiagen), expressed in the Rosetta-gami bacterial strain (Novagen) and proteins were purified on nickel Sepharose beads (GE Healthcare). Two versions of the kinase domain of ScORK28-6×His were expressed. The first one with the full length intracellular domain and named ScORK28$_{308–711}$. The second construct was made without the internal juxtamembrane domain and named ScORK28$_{409–711}$. Kinase activity was assayed as in [19] and the product was separated on acrylamide gel, transferred to a membrane, exposed on a europium screen and scanned on a Phosphorimager (GE healthcare). Three replicates were performed for all assays.

3. Results

3.1. ScORK28 sequence analysis and phylogeny

We have previously isolated 30 receptor-like kinases from a negative screen that focused on weakly expressed transcripts in ovule and ovary tissues following fertilization [12]. A few RLKs were chosen for further analysis, based on their high ovary-specific expression and low expression in other tissues. One of these RLK is ScORK28 and is described hereafter.

The EST corresponding to *ScORK28* (DN98256) was fully sequenced and most probably contained the full-length or near full-length *ScORK28* cDNA (2329 bp) since its 5′-UTR sequence contained three in frame stop codons upstream of the first methionine. A signal peptide sequence targeting the protein to the secretory pathway is also present in the first 23 amino acids (Fig. 1A). The signal peptide is followed by seven imperfect leucine-rich repeat (Fig. 1B) from amino acid position 71 to 232. A small island of prolines (8 out of 13 residues) is also found after the LRRs between amino acids 246 and 258. A total of eight phosphorylation sites were predicted. A putative *N*-glycosylation site is also predicted on the asparagine located at position 145 of the deduced protein.

A global phylogenetic analysis against all *Arabidopsis* RLKs, and using only the catalytic kinase domain, confirmed that ScORK28 belonged to the small RLK subfamily LRR-V (data not shown). This family has nine members in *Arabidopsis* and the best known member of the family is *STRUBBELIG/SCRAMBLED* [20,21]. Fig. 1C shows a phylogenetic analysis using the complete amino acid sequence of the nine RLK-LRR-V subfamily members (also known as the SRF family) from *Arabidopsis* (SUB and SRF1 to 8) and a RLK of the LRR-VI subfamily (At5g07150) as the outgroup. This analysis shows that ScORK28 is more closely related to the SRF6 (At1g53730) and SRF7 (At3g14350) members of the SRF family.

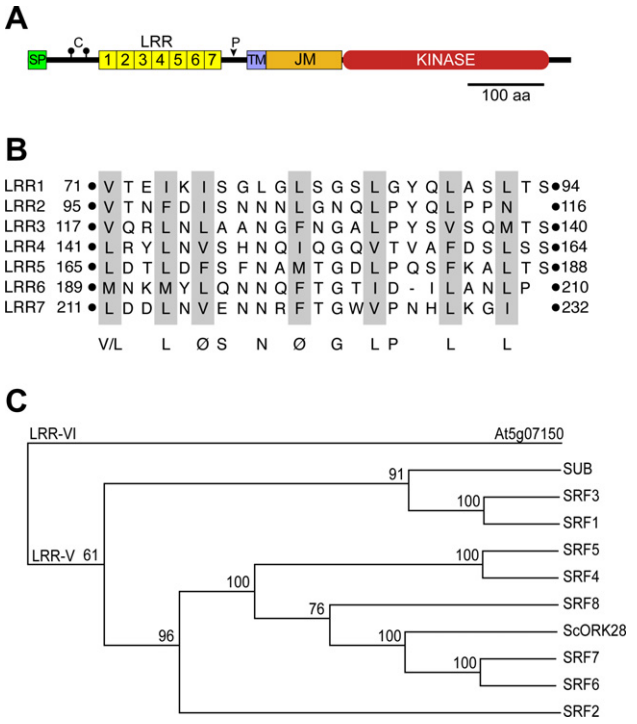

Fig. 1. (A) ScORK28 schematic structure. The dots represents the cystein pair (C). SP, signal peptide; LRR, leucine-rich repeat; P, proline-rich island; TM, transmembrane domain; JM, intracellular juxtamembrane domain; KINASE, kinase catalytic domain. (B) The seven LRR are aligned to emphasize most frequent amino acids found at regularly spaced positions. Grey boxes represent conserved hydrophobic residues. A consensus sequence is presented underneath the alignment and represents amino acids conserved in >50% of the LRR. ∅, aliphatic amino acid. (C) A neighbour-joining clustering from the complete amino acid sequences of the ScORK28 receptor kinase and the most closely related RLKs from *A. thaliana* (LRR-V subfamily). A LRR-VI RLK was chosen as the outgroup. Bootstrap values were calculated from 1000 replicas. Identical clustering was also obtained using only the kinase catalytic domain.

3.2. ScORK28 is predominantly expressed in young fruits and is glycosylated

To determine the protein expression pattern of *ScORK28*, a specific antibody directed against the ectodomain of ScORK28 was produced. Immunoblotting using an anti-ScORK28 antibody (Fig. 2B) clearly shows a predominant ScORK28 accumulation in ovaries, as determined previously for *ScORK28* mRNA levels [12]. Peak accumulation was observed in unfertilized ovules and declined slightly following fertilization that occurs ~42 h after pollination (HAP). By 96 HAP, ovules would still harbor embryos at the zygotic or at the two-celled proembryo stages (Lafleur, E. and Matton, D. P., unpublished observations). Furthermore, although low levels of *ScORK28* mRNAs were detected in root, stem, leaf, petal, anther, and pollen [12], no translation product of *ScORK28* could be detected in these tissues suggesting that there is a strict translational control of the *ScORK28* mRNAs.

The ScORK28 molecular weight was estimated at around 79 kDa on SDS–PAGE gel, compared to a predicted 74 kDa MW as deduced from the primary amino acid sequence (with-

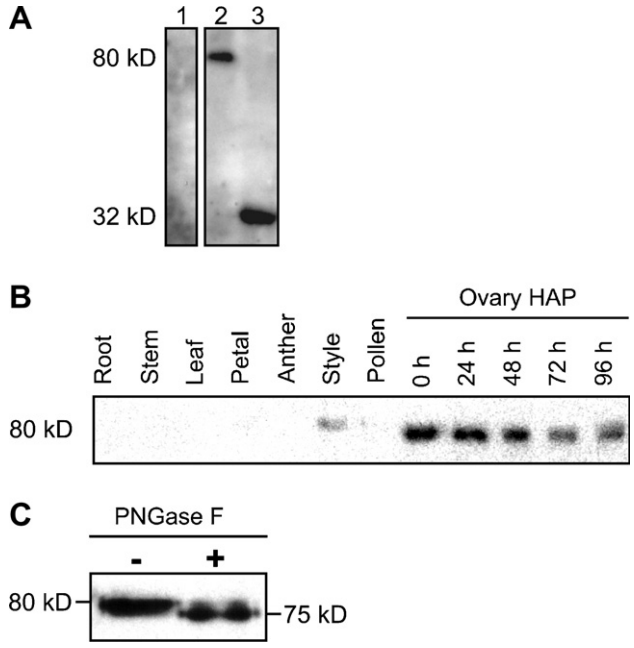

Fig. 2. Protein expression profile of ScORK28 and deglycosylation assay. (A) ScORK28 antibody specificity. Lane 1, pre-immune sera. Lane 2, sera from the final bleed was used to detect ScORK28 from whole flowers or, lane 3, recombinant ScORK28 ectodomain. (B) Western blot analysis on various vegetative and reproductive tissues. (C) Deglycosylation assay from transiently expressed ScORK28 protein in *Nicotiana benthamiana* leaves.

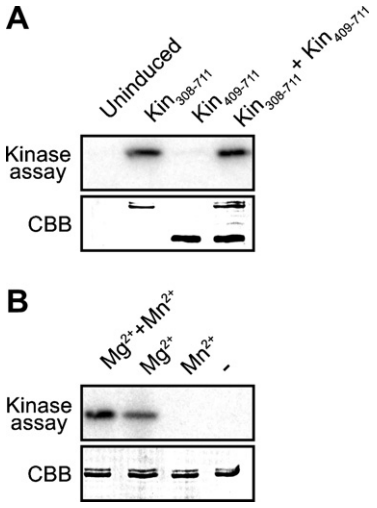

Fig. 3. Kinase activity assay. (A) ScORK28 kinase activity from bacterially expressed proteins. Two histidine-tagged kinase constructs were used for the phosphosphylation assay. Lane 1, non-induced control. Lane 2 $Kin_{308–711}$. Lane 3 $Kin_{409–711}$. Lane 4 $Kin_{308–711}$ and $Kin_{409–711}$. (B) Bivalent cation requirement for ScORK28 activity ($Kin_{308–711}$ construct). Lower panels, Coomassie Brilliant Blue (CBB) staining.

out the signal peptide). Since ScORK28 possesses one predicted *N*-glycosylation site in the ectodomain (position 145), a *N*-deglycosylation assay was performed to determine if ScORK28 is glycosylated. The ScORK28 open reading frame (ORF) fused to a hexahistidine (6×His) tag was transiently expressed in *Nicotiana benthamiana* leaves and a crude protein extract was submitted to deglycosylation with *N*-glycosidase F (PNGase F). A small mobility shift of ~5 kDa was detected with an anti-His antibody (Fig. 2C), confirming that the protein is *N*-glycosylated.

3.3. ScORK28 is a functional protein kinase

In order to determine if ScORK28 was a functional protein kinase, hexahistidine-tagged truncated constructs were expressed in *E. coli*. Fig. 3A shows that $ScORK28_{308–711}$ can autophosphorylate (or transphosphorylate) while $ScORK28_{409–711}$ cannot (equal loading is shown on Fig. 3A, bottom panel). When both bacterially expressed proteins are mixed together, phosphorylation is only detected on the higher molecular weight $ScORK28_{308–711}$ protein construct (Fig. 3A, $KIN_{308–711}$ + $KIN_{409–711}$). This suggests that $ScORK28_{308–711}$ cannot phosphorylate the shorter $ScORK28_{409–711}$, indicating that the juxtamembrane domain is either necessary for the association of the two proteins or it encompasses the targeted phosphorylation site(s). Bivalent metal ion requirements for ScORK28 kinase activity were also assessed using the bacterially expressed $ScORK28_{308–711}$ construct (Fig. 3B). The enzymatic assay was performed with or without either Mg^{2+} or Mn^{2+}, or with both ions. Bivalent metal ion are clearly necessary for ScORK28 phosphorylation activity since no activity can be detected without Mg^{2+} while Mn^{2+} alone cannot stimulate its phosphorylation activity. Presence of both bivalent cations slightly increased the detected activity when compared to Mg^{2+} alone.

3.4. ScORK28 is targeted to the plasma membrane

Although LRR-RLKs possess a transmembrane domain and a putative signal peptide, the subcellular localization of only a limited number of RLKs has been investigated, such as in [22–27]. To determine the subcellular localization of ScORK28, a fusion protein harboring a green fluorescent protein (GFP) downstream of the ScORK28 ORF was constructed. Transient expression through *Agrobacterium* infiltration of *N. benthamiana* leaves showed that the tagged protein is located at the cell periphery but does not allow a clear discrimination between a cell wall or plasma membrane localization (Fig. 4A). To better localize ScORK28, transient expression through particle bombardment of onion epidermal cells was performed. Again, ScORK28 could be unambiguously localized to the cell periphery in non-plasmolyzed cells (Fig. 4B) and was clearly associated with the cell plasma membrane after plasmolysis (Fig. 4D). A DIC image of the same cell layer shows the cell structures after plasmolysis, clearly showing the cell wall and the plasma membrane of the shrunken protoplasm (Fig. 4C).

3.5. Transgenic plants analysis

In order to uncover the function of ScORK28 in planta, transgenic plants overexpressing the full length *ScORK28* cDNA were made. *S. chacoense* plants strongly overexpressing the mRNA corresponding to the full length transgene were obtained (Fig. 5, upper panel) but a Western blot analysis of these plants revealed that the ScORK28 protein level was not significantly affected and remained steady despite the fact that the expression of the transgene is under the control of the strong CaMV 35S promoter (Fig. 5, lower panel). This result again suggests that the protein level is tightly regulated

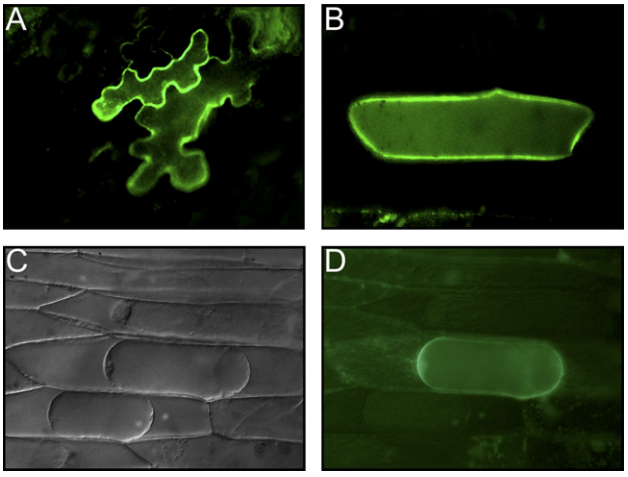

Fig. 4. Plasma membrane localization of ScORK28. (A) The ScORK28 construct fused to the GFP was expressed in *N. benthamiana* epidermal cells along with the p19 silencing suppression construct and observed under fluorescent illumination. (B) Epidermal onion cells expressing the ScORK28-GFP construct after microparticles bombardment and observed under fluorescent illumination. (C) Differential interference contrast (DIC) microscopy of the same cells shown in B after plasmolysis showing the shrinkage of the protoplast (white arrow). (D) Same cell as in B but after plasmolysis showing the retention of the fluorescent signal around the plasma membrane.

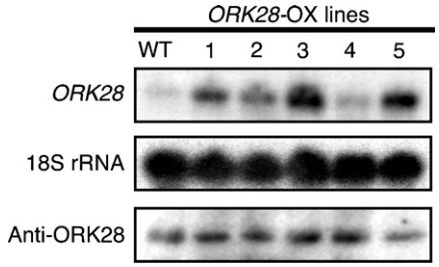

Fig. 5. Analysis of transgenic lines expressing full length *ScORK28* constructs. A. Selection of transgenic lines overexpressing *ScORK28* under the control of the CaMV 35S promoter. Upper panel: RNA gel blot analysis of *ScORK28*-OX lines probed with the *ScORK28* full length ^{32}P labeled insert. Middle panel: RNA gel blot analysis with *ScORK28*-OX lines with a 18S ribosomal probe. Lower panel: Western blot analysis of the same *ScORK28*-OX lines with an anti-ScORK28 antibody.

post-transcriptionally. Phenotypical analysis of these *S. chacoense* transgenic plants overexpressing *ScORK28* mRNA did not reveal any abnormalities, both at the vegetative and reproductive levels.

Arabidopsis thaliana T-DNA mutants corresponding to the two closest ScORK28 putative orthologs (At1g53730 and At3g14350, see Section 2 for mutant line accessions) were analyzed for altered phenotypes, including overall plant size, silique length, seed number per pod, seed germination ratio, and root growth assay on MS media. Among these lines, homozygous lines were obtained from two lines that have been shown to bear insertions in exons through sequencing of the T-DNA left border/gene junction sequence (SRF6 Salk_07772 and SRF7 Salk_03910, D.P. Matton, unpublished results). Even in these lines, only wild-type looking plants were observed in single mutants indicating a possible redundancy effect in this family of eight genes.

4. Discussion

The importance of receptor kinases in plants has emerged in the early 1990s with the discovery of the first plant receptor-like kinase [28]. Since then hundreds of plant receptor kinases have been found, mainly through the analysis of completely sequenced genomes [29]. Protein kinases and receptor kinases can also be further divided into RD or non-RD kinase [30]. RD kinases are regulated by activation loop phosphorylation and typically carry a conserved arginine (R) residue immediately preceding the invariant aspartate in the kinase subdomain VI required for catalytic activity. Most RD kinases are much more active than non-RD kinases, although there are exceptions [30]. Recently, Dardick and Ronald [31] made a thorough classification of all protein kinases in rice and *Arabidopsis*. RD kinases comprises 70% of *Arabidopsis* kinome and include well known RLKs like ERECTA, BRI1, CLAVATA1, and HAESA. Non-RD kinases comprises 10% of the *Arabidopsis* kinases, show very little kinase activity and have been associated in plants and animals with innate immunity and pathogen perception. A third class, termed ACF for alternative catalytic function comprises 20% of the *Arabidopsis* kinome and these protein kinases lack one or more of three highly conserved residues.

Amino acid sequence analysis and phylogenetic analysis revealed that the ScORK28 kinase belonged to the LRR-V subfamily. In *Arabidopsis*, this family comprises nine members classified as ACF kinases, including *STRUBBELIG/SCRAMBLED*, a RLK originally isolated on the basis of its defect in ovule development and the fact that it produced twisted siliques, but that was also showed to affect the formation and shape of several organs, as well as cell proliferation, orientation of the division plane, and position-dependant specification of root epidermal cells [20,21]. The two SRF members that show closest homology with *ScORK28* are *SRF6* and *SRF7*. It was hypothesized, based on expression analysis that *SRF6* could be involved in defense response against pathogenic fungi or in other stress-related process. The authors observed that neither the *SRF6* or *SRF7* single-mutant nor did the double mutant showed difference with wild-type plants, although this could be explained by the fact that the *SRF6* and *SRF7* mRNA was still produced in the T-DNA lines used. It should be noted however that ectopic expression of *SRF7* lead to male sterility [32].

Although ScORK28 is an ACF kinase, in vitro autophosphorylation activity could be detected in the presence of magnesium ions but not in the presence of manganese ions (Fig. 3). The only other RLK in the LRR-V family tested for kinase activity, the SUB RLK, was shown not to have any kinase activity and therefore this is the first result showing in vitro kinase activity for a member of the LRR-V RLK family. SUB is also an ACF kinase, like all LRR-V kinases, but also has other mutated residues in highly conserved regions [20]. The phosphorylation sites of more than 50 plant receptor kinases were thoroughly studied by the Peck's group using mass spectrometry [33]. They showed that most phosphorylation events take place either within the juxtamembrane domain or in the C-terminal domain and only a minor fraction takes place in the kinase domain. These results are supported by the fact that BRI1 has four confirmed phosphorylation sites (out of five) in its juxtamembrane domain [34]. For ScORK28, four predicted phosphorylation sites (out of eight) are located in the juxta-

membrane domain. A bacterially expressed construct encompassing only the kinase domain ($Kin_{409–711}$) was inactive (Fig. 3), suggesting that phosphorylated residues are located in the JM domain, in agreement with the results of Nuhse et al. [33]. Furthermore, it was recently shown that upon flagellin treatment of *Arabidopsis* cell cultures, a member of the LRR-V family of RLKs, SRF3, was phosphorylated in the JM domain, supporting our kinase activity data. Kinase activity for other RLK has also been obtained in constructs that included the JM domain [33,34]. These results taken together strongly suggest that ScORK28 phosphorylation occurs in the JM domain although we cannot exclude the possibility that the juxtamembrane domain is necessary for protein dimerization and transphosphorylation. In the latter case, absence of dimerization with the $ScORK28_{409–711}$ construct would preclude phosphorylation. Requirements for bivalent cation have been tested in only a few cases and suggest a high degree of variability in plant receptor kinases. For example, analyses of bivalent cation preferences performed by several groups suggested a preference of Mn^{2+} over Mg^{2+} [5,7,10,11], while Yoshida et al. observed no such difference between Mg^{2+} or Mn^{2+} [9]. Contrary to this, Shah et al. showed SERK1 to be Mg^{2+} dependant while being inhibited by Mn^{2+} [8]. Here, we show for ScORK28, another variation on the theme of ionic requirements. Kinase activity of ScORK28 required Mg^{2+}, while Mn^{2+} alone was totally ineffective. Presence of both ions has a slight synergistic effect, showing the great variety of biochemical requirements for the activity of protein receptor kinases.

It is generally assumed that receptor kinase proteins localize to the plasma membrane. This assumption may prove wrong as for the ethylene histidine-kinase receptor (ETR1) in *Arabidopsis* that localizes mainly to the ER [35], although localization to other membranes cannot be ruled out as observed for the tobacco NTHK1putative histidine kinase-like ethylene receptor that localizes at the plasma membrane [36]. Evidence also exist showing that some receptor kinases may be localized to both plasma and intracellular membranes as well as in the cytosol [5]. Our results in transformed onion cells or through leaf agroinfiltration clearly demonstrated that ScORK28 localizes to the plasma membrane (Fig. 4), as would be expected for a receptor-kinase involved in intercellular communication.

Although we have obtained valuable and novel information regarding the *modus operandi* of ScORK28 as a receptor kinase, including the requirement for the juxtamembrane domain and of Mg^{2+} for its phosphorylation activity, its plasma membrane localization, and its glycosylation status, functional analyses in transgenic plants has not yet revealed a precise role for this RLK. Analysis of eight T-DNA lines in *Arabidopsis* (see Section 2 for the line accessions) corresponding to putative *S. chacoense* orthologs (Fig. 1) and *S. chacoense* transgenic plants overexpressing the full length *ScORK28* clone did not yield plants showing obvious developmental defects. Although overexpression could be found at the mRNA level, tight control at the post-transcriptional level might have precluded accumulation of ScORK28 protein at levels significantly higher than WT. Such a mechanism is not uncommon and has been postulated to be caused by the host silencing mechanism [37].

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