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Lily Cdc42/Rac-interactive binding motif-containing protein, a Rop target, involves calcium influx and phosphoproteins during pollen germination and tube growth

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We report unique desiccation-associated ABA signaling transduction through which the Rop (Rho GTPase of plants) and its target LLP12-2 are regulated during the stage of pollen maturation and tube growth. Overexpression of LLP12-2 drastically inhibited pollen germination and tube growth. Studies on the germination inhibitors, Ca^{2+} influx blocking agents $LaCl_3$ and EGTA and an actin-depolymerizing drug, latrunculin B (LatB), revealed that the LLP12-2-induced inhibition of germination and tube growth is significantly suppressed by $LaCl_3$ and EGTA in the LLP12-2-overexpressing pollen but not by LatB. These results suggested that LLP12-2 is associated with Ca^{2+} influx in the cytoplasm and may be not with actin assembly. With the addition of $LaCl_3$ and EGTA, LLP12-2-overexpressing pollen increased germination and tube growth compared with the one without addition, whereas pollen expressing GFP decreased germination and tube growth. Thus, an optimum level of $[Ca^{2+}]_{cyt}$ influx is crucial for normal germination and tube growth. Studies on the inhibitors, staurosporine and okadaic acid in the LLP12-2-overexpressing pollen, showed no appreciable increase in germination when compared with the one without addition, suggesting that staurosporine-sensitive protein kinases and dephosphorylation of phosphoproteins may be not involved in the LLP12-2 mediated germination. However, the LLP12-2-induced inhibition of tube length was slightly but significantly suppressed by staurosporine, suggesting that staurosporine-sensitive protein kinases involve in the LLP12-2-induced inhibition of tube growth.

Key words: calcium influx, phosphoprotein, pollen tube growth, RIC protein, rop GTPase

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Rop (Rho GTPase of plants) was newly reported as a master regulator for plant signaling.[1,2] It participates in concerted actions of many signaling pathways that influence growth and development, and the adaptation of plants to various environmental situations.[3-5] In contrast to be negative regulators in ABA signaling,[6] Rops might work as positive regulators in auxin signaling pathways.[7,8] Recently, we have reported unique desiccation-associated ABA signaling in which the *LLP-Rop1* gene is not only negatively regulated by desiccation but also positively regulated by developmental cues independent of ABA during pollen maturation.[9] Although LLP-Rop1 and its target, LLP12-2, accumulate in abundance in the matured and dried pollen upon dehydration, the activity of LLP-Rop1 and LLP12-2 is likely restricted at the stage of pollen maturation.[9] As pollen germinates, ABA content decreases its level in the growing tube and thus, the activity of Rop is less restricted than that in the dried pollen and subsequently Rops become powerful regulators playing crucial roles during pollen tube growth.

Pollen germination and tube growth are a continuous and highly polarized process characteristic of tip growth. As soon as pollen hydrates and germinates, a tip-focused cytoplasmic Ca^{2+} gradient is established and sustained while a pollen tube grows forward.[10,11] The Ca^{2+}-permeable channels that modulate $[Ca^{2+}]_{cyt}$ influx in germinating pollen grains have been identified in

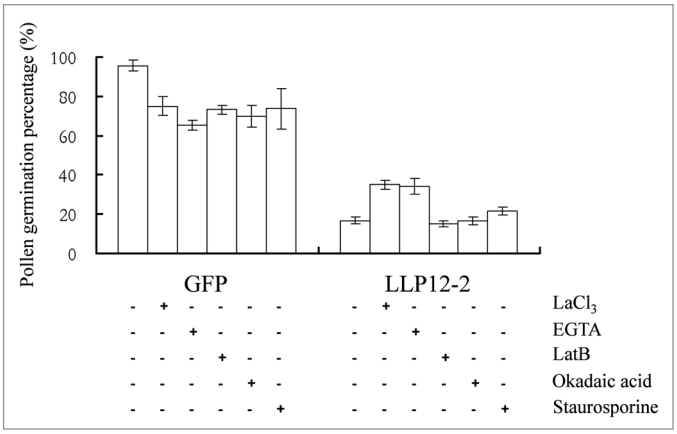

Figure 1. LLP12-2 inhibits pollen germination by regulating the calcium influx channels. Germination percentages were determined 9 h after particle bombardment and subsequent in vitro germination. Equal amounts of *GFP* and *LLP12-2* DNA (7.5 μg) were transiently expressed in lily pollen after which pollen was treated without (control) or with either $LaCl_3$ (1 μM), EGTA (0.5 mM), LatB (0.05 nM), okadaic acid (5 nM) or staurosporine (1 μM) during germination.

Arabidopsis,[12,13] lily[14] and pear.[15] When a pollen tube grows, Rop-interactive Cdc42/Rac-interactive binding (CRIB) motif-containing proteins (RICs) play an important role as Rop GTPase targets and control a variety of Rop-dependent signaling pathways.[16] RICs contain a CRIB motif required for their specific interaction with GTP-bound Rop. They are grouped into five classes that share little sequence similarity outside of the Rop-interactive domain.[16] Different RICs expressed in various reproductive and vegetative parts of the Arabidopsis plant may act as Rop targets to control different Rop-dependent pathways in pollen tubes and in other organ development. For instances, RIC4 has been demonstrated to promote F-actin assembly, whereas RIC3 activates Ca^{2+} signaling by affecting $[Ca^{2+}]_{cyt}$ influx that subsequently results in F-actin disassembly in pollen tube growth.[17] The two RICs, both activated by AtRop1, counteract each other to control the actin dynamics and polar pollen tube growth.[17]

We have demonstrated that LLP12-2, a RIC protein, interacts with active LLP-Rop1 in vivo.[9] To examine the function of LLP12-2 in the growing tubes, the purified *LLP12-2* PCR product digested with *Xba*I and *Sac*I was cloned into the corresponding sites of *Zm13::GFP* construct to generate the *Zm13::GFP-LLP12-2* construct. The transient expression of GFP-LLP12-2 in pollen using particle bombardment was investigated. Pollen germination and tube length were measured after particle bombardment and subsequent in vitro germination. With the treatment of Ca^{2+} influx blocking agents $LaCl_3$ and EGTA, pollen expressing GFP alone significantly decreased germination and tube elongation, suggesting that a decrease in $[Ca^{2+}]_{cyt}$ influx may cause the inhibition of pollen germination and tube growth (**Fig. 1 and Table 1**). The chemical $LaCl_3$ blocks plasma membrane (PM)-localized inward Ca^{2+} channels whereas EGTA is a Ca^{2+} chelating agent.[17]

The inhibition of germination and tube growth was further enhanced in the pollen overexpressing GFP-LLP12-2 when compared with the pollen expressing GFP only (**Fig. 1 and Table 1**). The LLP12-2-induced inhibition of germination and tube growth in the LLP12-2-overexpressing pollen strongly indicated that LLP12-2 plays a crucial role during pollen germination and growth. The function of LLP12-2 in pollen was further investigated not only by the addition of $LaCl_3$ and EGTA but also by the treatment of an actin-depolymerizing drug, LatB. When $LaCl_3$ or EGTA was applied, pollen germination increased two- to three-fold, and tube length increased by approximately 27% compared with those without drug addition (**Fig. 1 and Table 1**). On the contrary, no increase in germination percentage and tube length was observed with the application of LatB (**Fig. 1 and Table 1**). These results suggested that LLP12-2 is indeed involved in $[Ca^{2+}]_{cyt}$ influx but may be not with actin assembly. It is rational to hypothesize that pollen overexpressing LLP12-2 may regulate the activity of PM-localized inward Ca^{2+} channel proteins, resulting in an increase in Ca^{2+} influx in the cytoplasm and lead to inhibit germination and tube growth. The hypothesis is supported by the fact that, with the addition of $LaCl_3$, an increase in the level of $[Ca^{2+}]_{cyt}$ influx is adversely affected in the LLP12-2-overexpressing pollen. As a result, both germination and tube growth increase (**Fig. 1 and Table 1**). A similar result was also obtained with the application of EGTA, a Ca^{2+} chelating agent where a decrease in the level of $[Ca^{2+}]_{cyt}$ influx alleviates the LLP12-2-induced inhibition of germination and tube growth (**Fig. 1 and Table 1**). Based on these analyses, it is likely that an optimal level of Ca^{2+} influx in the cytoplasm of pollen is crucial for normal germination and tube growth as illustrated in **Figure 2**. Any perturbation of $[Ca^{2+}]_{cyt}$ influx in the pollen would decrease germination and tube growth. The function of LLP12-2 mimics that of RIC3, Group III of Arabidopsis RICs family. It has been reported that RIC3 activates Ca^{2+} signaling, which leads to F-actin disassembly, whereas RIC4 promotes F-actin assembly.[17] Alike RIC3, overexpression of LLP12-2 causes an excess amount of tip-localized calcium in the cytoplasm of the tube and subsequently results in the inhibition of germination and tube growth. It should be noted that the LLP12-2-induced inhibition of germination and tube growth only partially rescued with the treatment of $LaCl_3$ or EGTA, implying that factors other than calcium involves in the modulation of pollen germination and tube growth.[18-20]

Protein kinases such as calcium-dependent protein kinase (CDPK) have been reported to involve in the regulation of pollen germination and tube growth.[21,22] Studies have shown that CDPK comprises a kinase domain and a calmodulin-like domain in a single protein. Thus, it acts not only as a Ca^{2+} sensor but also as an effector affecting growth polarity, elevated cytosolic Ca^{2+}, and plant cytoskeleton during pollen germination and tube growth.[21,23] Aside from CDPKs, calcineurin B-like proteins (CBLs), a new family

Table 1. Effects of $LaCl_3$, EGTA, LatB, okadaic acid or staurosporine on the length of pollen tube expressing *LLP12-2*

	Pollen tube length (μm)					
	Control	**$LaCl_3$**	**EGTA**	**LatB**	**Okadaic acid**	**Staurosporine**
GFP	>1,500	1,286 ± 26	1,262 ± 23	1,248 ± 32	1,265 ± 36	1,254 ± 31
LLP12-2	1,028 ± 22	1,305 ± 34	1,309 ± 31	1,021 ± 24	1,089 ± 35	1,186 ± 23

Pollen tube length was measured 9 h after particle bombardment. Data are mean ± SD (μm) of three individual experiments (n = 10, per experiment).

of Ca^{2+} sensor, interact specifically with CBL-interacting protein kinases.[24] These putative Ca^{2+} sensors are responsible for the regulation of calcium-dependent tip growth and growth oscillation in pollen tubes.

To examine the signaling of protein kinases associated with LLP12-2 during germination and tube elongation, bombarded pollen was incubated in the absence or presence of okadaic acid or staurosporine. Okadaic acid is a membrane-permeable inhibitor of serine/threonine protein phosphatases types 1 and 2A,[25] whereas staurosporine is a potent broad-spectrum inhibitor of serine/threonine kinases.[26] The LLP12-2-overexpressing pollen did not exhibit appreciable increase in germination with the treatment of either staurosporine or okadaic acid when compared with that without treatment (**Fig. 1**). This implies that staurosporine-sensitive protein kinases and dephosphorylation of phosphoproteins may be not involved in the LLP12-2-regulated germination. Nevertheless, it is intriguing that the LLP12-2-induced inhibition of tube growth was slightly but significantly suppressed by staurosporine, suggesting that staurosporine-sensitive protein kinases involve in the LLP12-2-induced inhibition of tube elongation (**Table 1**). Thus, staurosporine-sensitive protein kinases play a role governing pollen tube growth as illustrated in **Figure 2**. It is consistent with the observation that a double mutation of two CDPKs severely reduces tube length but does not reduce germination.[27]

In conclusion, we report unique desiccation-associated ABA signaling transduction through which the Rop and its target LLP12-2 are regulated during pollen maturation and tube growth. Overexpression of LLP12-2 drastically inhibits pollen germination and tube growth. An optimum level of $[Ca^{2+}]_{cyt}$ influx is crucial for normal germination and tube growth.

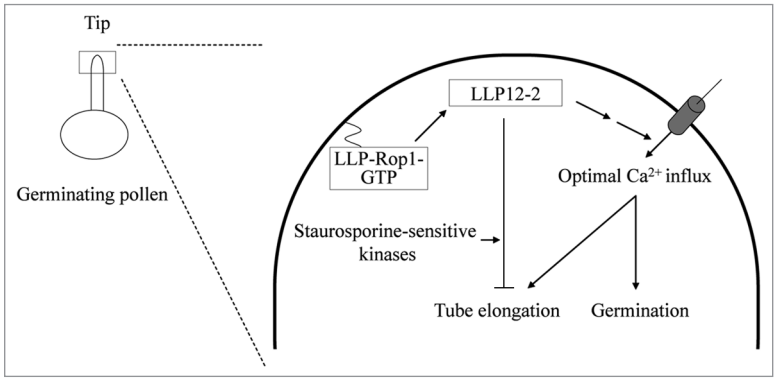

Figure 2. Schematic diagram of the LLP-Rop1 signaling during pollen germination and tube growth. During germination and tube growth, LLP-Rop1 is activated at the tip and activates LLP12-2, which affects calcium influx in the cytoplasm that in turn promotes germination and tube elongation. In addition, staurosporine-sensitive protein kinases are involved in the LLP12-2-induced inhibition of pollen tube elongation.

In addition, staurosporine-sensitive protein kinases also involve in the LLP12-2-induced inhibition of tube growth, but may be not involved in germination.

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