was 3.4 ± 0.3 (Fig. 3B and Table1). The amplitude range of the ratio was 0.4 ± 0.3, which was significantly different ($P<0.01$) from that of the regular $[Ca^{2+}]_{cyt}$ oscillations observed *in vitro* (Fig. 1C). In addition, compared with the $[Ca^{2+}]_{cyt}$ oscillations shown in Figure 1C, the periodicity of the $[Ca^{2+}]_{cyt}$ oscillations was more irregular. The data obtained in this experiment were not affected by the monitoring interval (data not shown). Irregular $[Ca^{2+}]_{cyt}$ oscillations were observed in all experiments under the *in vivo* condition (10/10 samples). In summary, regular $[Ca^{2+}]_{cyt}$ oscillations were not observed in pollen tubes as they grew through the papilla cell wall.

Ca^{2+} imaging in *Arabidopsis* pollen tubes growing under semi-*in vivo* conditions

Arabidopsis pollen tubes were examined under the semi-*in vivo* condition after they grew through the pistil; this method facilitated the monitoring process because we were able to monitor a number of elongating pollen tubes simultaneously and the growth conditions better represented the *in vivo* condition (Iwano et al., 2004). We monitored the $[Ca^{2+}]_{cyt}$ in pollen tubes growing in the semi-*in vivo* condition at 50-msec to 2-sec intervals. The mean growth rate was 3.1 ± 0.8 μm/min, and irregular $[Ca^{2+}]_{cyt}$ oscillations were in the pollen tube tips (Fig. 3C, 3D and Supplementary Video 5). The mean values of the maximum and minimum ratios were 5.1 ± 0.5 and 4.5 ± 0.5, respectively, with an amplitude range of 0.3 ± 0.2 (Fig. 3D and Table1). Similar irregular $[Ca^{2+}]_{cyt}$ oscillations were observed in all experiments under the semi-*in vivo* condition (48/48 samples).

In order to confirm that the cameleon can technically detect $[Ca^{2+}]_{cyt}$ oscillations in semi-*in vivo* condition, we obtained fluorescence spectra from the pollen tubes growing in the semi-*in vivo* condition in the presence of ionophore or in the presence of ionophore and EGTA using a spectral-imaging microscope system with excitation at 458 nm. Compared with that in the presence of ionophore alone (Fig. 4A), fluorescence of Venus component of YC3.60 was decreased in the presence of ionophore

and EGTA, which is almost free from Ca^{2+} (Fig. 4B) Therefore, these results showed that Ca^{2+} dependent energy transfer from ECFP to Venus occurs also in the semi-*in vivo* condition.

Effects of pollen tube growth inhibitors on Ca^{2+} dynamics in *Arabidopsis* pollen tubes

We only observed regular $[Ca^{2+}]_{cyt}$ oscillations when the pollen tubes had stopped growing or when they grew slowly. We therefore speculated that regular $[Ca^{2+}]_{cyt}$ oscillations were directly related to arrest of pollen tube growth. It was previously shown that the growth rate and changes in the $[Ca^{2+}]_{cyt}$ were affected by Ca^{2+} channel blockers and chelators, such as Gd^{3+} and EGTA (Malho et al., 1994; Geitmann & Cresti, 1998; Franklin-Tong et al., 2002). In addition, cyclopiazonic acid (CPA), a specific inhibitor of animal SERCA-type Ca^{2+}-ATPases (Inesi & Sagara, 1994) and plant P-type IIA Ca^{2+}-ATPases (Geisler et al., 2000), affects the growth rate and $[Ca^{2+}]_{cyt}$ dynamics of pollen tubes.

First, we examined the effects of these inhibitors on pollen germination and the growth rate. Gd^{3+}, EGTA, and CPA arrested pollen tube growth in the semi-*in vivo* condition (Fig. 5). Next, we examined the effects of these inhibitors on $[Ca^{2+}]_{cyt}$ oscillations in pollen tubes growing in the semi-*in vivo* condition. The maximum $[Ca^{2+}]_{cyt}$ did not change, whereas the area with a high $[Ca^{2+}]_{cyt}$ increased in size just after the addition of CPA to the culture medium (final concentration: approximately 5 μM; Fig. 6). Regular $[Ca^{2+}]_{cyt}$ oscillations then were induced. These regular $[Ca^{2+}]_{cyt}$ oscillations were induced in 5 of 10 pollen tubes treated with CPA. 2 pollen tubes did not induce and the other 3 bursted during monitoring. After the addition of Gd^{3+} to these samples, the amplitude of the oscillations decreased and irregular oscillations were observed (Supplementary Video 6). On the other hand, application of Gd^{3+} alone arrested pollen

tube growth but did not induce $[Ca^{2+}]_{cyt}$ oscillation at the tip (Fig. 7 and Supplementary Video 7) yet caused a decrease of $[Ca^{2+}]_{cyt}$ at the tip, namely the disappearance of a tip-focused $[Ca^{2+}]_{cyt}$ gradient (2 in Fig. 7a). Finally, many of the pollen tips burst and the elevation of $[Ca^{2+}]_{cyt}$ over the whole area of pollen tube was caused by the influx of extracellular Ca^{2+} (3 in Fig. 7a). These results were repeatedly observed in 10 of 15 pollen tubes treated with Gd^{3+}. The other 5 bursted before monitoring. These results showed that inhibitors of pollen tube growth induced abnormal $[Ca^{2+}]_{cyt}$ dynamics, including regular $[Ca^{2+}]_{cyt}$ oscillations and a disappearance of the tip focused $[Ca^{2+}]_{cyt}$ gradient. Furthermore, these results suggested that the action site of Gd^{3+} is different from that of CPA and is related to the formation of the tip-focused $[Ca^{2+}]_{cyt}$ gradient.

Ca^{2+} dynamics in the ER of *Arabidopsis* pollen tubes growing in the semi-*in vivo* condition

To confirm that ER functions as a Ca^{2+} store in the pollen tube, we monitored the $[Ca^{2+}]$ in the ER ($[Ca^{2+}]_{ER}$) of pollen tubes growing in the semi-*in vivo* condition and examine the effects of CPA treatment. We also generated transgenic *Arabidopsis* plants expressing a chimeric protein consisting of a signal peptide, YC4.60, and an ER retention signal. We monitored ECFP and Venus (FRET imaging) in a transgenic pollen tube passing through a pistil at 1- to 3-sec intervals with excitation at 442 nm; the Venus/CFP fluorescence ratio was then calculated. Venus-labeled ER was localized longitudinally in the pollen tube (Fig. 8). Areas with a high $[Ca^{2+}]_{ER}$ were distributed sporadically throughout the pollen tube (Fig. 8 and Supplementary Video 8) and no ER with a high $[Ca^{2+}]$ was observed in the tip of the growing pollen tube. The maximum ratio of the high $[Ca^{2+}]_{ER}$ areas was 9.5, which was estimated to correspond to between 100 and 500 μM (Fig. 2B).

When CPA was added to pollen tubes in the semi-*in vivo* condition (final concentration: approximately 5 μM), the ratio decreased in 2 min from approximately 9 to

5.5, which corresponds to between 0.1 and 1 μM, although pollen tube growth was not completely inhibited (Fig. 9). When additional CPA was added to the culture medium (final concentration: approximately 10 μM), the ratio decreased further and pollen tube growth was completely arrested. Similar decreases of the ratio by CPA were observed repeatedly 8 of 10 pollen tubes treated with CPA (8/10 samples). The other 2 bursted during monitoring. On the other hand, thapsigargin, another specific inhibitor of animal SERCA-type Ca^{2+}-ATPases, did not change the $[Ca^{2+}]_{ER}$ (data not shown). In these experiments, we assumed the specificity of CPA to be ER-type Ca^{2+}-ATPases. These results suggested that the pollen tube ER contains CPA-sensitive Ca^{2+}-ATPases, and that the $[Ca^{2+}]_{ER}$ is maintained at high level in the growing pollen tube. Moreover, it is likely that the ER is a Ca^{2+} store in pollen tubes and that CPA-sensitive Ca^{2+}-ATPases are required for pollen tube growth.

Ca^{2+} imaging in *N. tabacum* pollen tubes

Regular $[Ca^{2+}]_{cyt}$ oscillations in the tip region were rarely observed in the growing pollen tubes of YC3.60-expressing *Arabidopsis*, even though we monitored $[Ca^{2+}]_{cyt}$ dynamics under three conditions: *in vivo*, semi-*in vivo* and *in vitro*. Previous study, however, have reported regular *in vitro* $[Ca^{2+}]_{cyt}$ oscillations in growing pollen tubes from *L. longiflorum* and *N. tabacum* (Watahiki et al., 2004). To determine whether this discrepancy was caused by differences between the species, we monitored $[Ca^{2+}]_{cyt}$ dynamics under various conditions using transgenic YC3.60-expressing *N. tabacum*. Under the *in vitro* condition, more than 90% of the pollen grains germinated with elongated pollen tubes 5 h after dissemination. We monitored growing pollen tubes that were more than 200 μm in length at 5-sec intervals (growth rate: 4.5 ± 1.8 μm/min). $[Ca^{2+}]_{cyt}$ gradients and regular $[Ca^{2+}]_{cyt}$ oscillations were evident in 3 of 10 pollen tubes (3/10 samples) (Fig. 10A, 10B and Supplementary Video 9). In these cases, the mean value of the maximum ratio was

4.0 ± 0.2, whereas the mean value of the minimum ratio was 3.6 ± 0.2 (Fig. 10B). The mean amplitude range was 0.2 ± 0.1 and the periodicity of the $[Ca^{2+}]_{cyt}$ oscillation was long, ranging from 40 sec to 60 sec (Fig. 10B). These observations were similar to previously reported results. However, irregular oscillations were also observed in 17 of 20 pollen tubes in monitoring at 1-2 sec intervals (17/20 samples) (Fig. 10C, Table1 and Supplementary Video 10). Moreover, distinct regular oscillations were observed in 3 of 20 pollen tubes when growth ceased or was very slow (3/20 samples) (Fig. 10D, Table1 and Supplementary Video 11). In these cases, the mean value of the maximum ratio was 4.8 ± 0.4, whereas the mean value of the minimum ratio was 2.8 ± 0.2 (Fig. 10D). The mean amplitude range was large, 1.0 ± 0.1, which was significantly different from the results shown in Figure 10B, although the periodicities of the $[Ca^{2+}]_{cyt}$ oscillations were similar. These data suggested that regular oscillations were not directly related to the growth rate in *N. tabacum* or *Arabidopsis*.

To examine whether regular $[Ca^{2+}]_{cyt}$ oscillations occurred under the *in vivo* condition, we observed the stigma surface 3 h after pollination and monitored the germinated pollen tubes. Although growth was very slow in the lipophilic environment of the stigma surface, regular $[Ca^{2+}]_{cyt}$ oscillations were not observed in all experiments (15/15 samples) (Fig. 11A, Table1 and Supplementary Video 12). Furthermore, we monitored pollen tubes after they grew through the pistil (the semi-*in vivo* condition). When a pollen tube was growing well, a $[Ca^{2+}]_{cyt}$ gradient was evident but regular $[Ca^{2+}]_{cyt}$ oscillations were not observed in all experiments (30/30 samples) (Fig. 11B, Table1 and Supplementary Video 13). These results were not affected by the monitoring interval. Thus, regular $[Ca^{2+}]_{cyt}$ oscillations with large amplitudes were not observed in normally growing pollen tubes under three conditions: *in vivo*, semi-*in vivo* and *in vitro*. These results suggested that regular $[Ca^{2+}]_{cyt}$ oscillations are not essential for pollen tube growth in *N. tabacum* or *Arabidopsis*.

Discussion

Previously, the relationship between pollen tube growth and $[Ca^{2+}]_{cyt}$ dynamics has been examined *in vitro*. In this study, we monitored pollen tube growth and $[Ca^{2+}]_{cyt}$ dynamics in the pollen tubes of transgenic *Arabidopsis* and *N. tabacum* expressing YC3.60 using three different systems: *in vitro*, *in vivo* and semi-*in vivo*. In addition, we examined the effects of inhibitors of pollen tube growth on $[Ca^{2+}]_{cyt}$ dynamics in the semi-*in vivo* system.

In *Arabidopsis*, regular oscillations were not observed under the *in vivo* condition, in which the pollen tube grew in the papilla cell wall. Further, under the semi-*in vivo* condition, which is thought to mimic the *in vivo* condition, only irregular oscillations were observed. Finally, under the *in vitro* condition, irregular oscillations were observed in growing pollen tubes, whereas regular oscillations were observed only when growth stopped or was very slow. These results suggested that regular oscillations are not essential for pollen tube growth in *Arabidopsis*.

In *N. tabacum*, both regular and irregular $[Ca^{2+}]_{cyt}$ oscillations were observed in the tip region of normally growing pollen tubes under the *in vitro* condition. In addition, regular oscillations also were observed when the growth stopped or was relatively slow. Comparing the amplitudes of the regular oscillations revealed that the amplitude in slow-growing pollen tubes was 5-fold greater than that observed in normally growing pollen tubes. In the previous studies of *N. tabacum* and *L. longiflorum* transiently expressing YC2.1, regular $[Ca^{2+}]_{cyt}$ oscillations were observed in normally growing pollen tubes. The amplitude was small, however, ranging from 0.1 to 0.15 in *N. tabacum*, and the maximum ratio of the oscillation was 1.6, considerably lower than the maximum ratio of 2.7 observed after addition of the ionophore (Watahiki et al., 2004). Moreover, the amplitude was lower in *L. longiflorum* than in *N. tabacum* (Watahiki et al., 2004).

Interestingly, in regular oscillations from normally growing pollen tubes, the amplitude was small in both this study and the previous study and was different from the value observed in pollen tubes that had stopped growing. Furthermore, regular $[Ca^{2+}]_{cyt}$ oscillations were rarely observed in either the *in vivo* or semi-*in vivo* condition. These results suggested that regular oscillations are not essential for the pollen tube growth in *N. tabacum* as well as in *Arabidopsis*. $[Ca^{2+}]_{cyt}$ oscillations, which have a large amplitude range, also are not likely to be improper for pollen tube growth. In the previous experiments in *L. longiflorum*, $[Ca^{2+}]_{cyt}$ do not regularly oscillate in the short pollen tubes about 30 min after germination, while $[Ca^{2+}]_{cyt}$ regularly oscillates in the elongated pollen tubes 3 hours after germination (Pierson et al., 1996; Messerli and Robinson, 1997; Feijo et al., 2001). These reports reinforced our conculusion that regular oscillations are not essential for the pollen tube growth. On the other hand, based on the data that the tip-focused $[Ca^{2+}]_{cyt}$ gradient oscillates with the same period as the growth rate (Pierson et al., 1996; Messerli et al., 2000; Holdaway-Clarke & Hepler, 2003; Holdaway-Clarke & Hepler, 2003; Messerli & Robinson, 2003), models that the growth rate and $[Ca^{2+}]_{cyt}$ were correlated have been presented (Holdaway-Clarke et al., 1997). In addition, it has been reported that the Rop GTPase activity regulating $[Ca^{2+}]_{cyt}$ influx oscillated with the same period as the growth (Li et al., 1999; Hwang et al., 2005). Although we could not examine whether regular oscillation occurs in the non-growing pollen tube of lily nor whether Rop GTPase activity oscillates in *Arabidopsis*, our results showed cases where $[Ca^{2+}]_{cyt}$ oscillation was not correlated with the growth rate. For pollen tube growth, it is both important and essential to fine tune the components that regulate $[Ca^{2+}]_{cyt}$. In living cells, the $[Ca^{2+}]_{cyt}$ is critical for many cellular responses and must be maintained at a level approximately 2×10^4 lower than the extracellular $[Ca^{2+}]$ (Petersen et al., 2005; Clapham 2007). In addition, $[Ca^{2+}]_{cyt}$ oscillations are thought to control a diverse range of intracellular processes, which may be regulated by the amplitude, frequency, shape, or

any combination of characteristics related to the oscillations (Petersen et al., 2005). We speculate that maintaining the $[Ca^{2+}]_{cyt}$ in a narrow range is important for Ca^{2+} homeostasis in the pollen tube.

In *L. longiflorum*, $[Ca^{2+}]_{cyt}$ dynamics in pollen tubes growing *in vitro* has been examined, revealing that regular $[Ca^{2+}]_{cyt}$ oscillations correlated with the growth rate (Pierson et al., 1996; Messerli et al., 2000; Holdaway-Clarke & Hepler, 2003; Holdaway-Clarke & Hepler, 2003; Messerli & Robinson, 2003). Although we could not compare the *in vitro* $[Ca^{2+}]_{cyt}$ dynamics with those observed under the *in vivo* and semi-*in vivo* conditions, irregular oscillations may occur under the *in vivo* or semi-*in vivo* condition in lily species and *N. tabacum*. In fact, TTS (transmitting tissue specific protein), which is secreted by the pistil, has been shown to attract pollen tubes and stimulate their growth in tobacco plants (Cheung *et al.* 1995). Thus, molecules released from the pistil may affect $[Ca^{2+}]_{cyt}$ dynamics in the pollen tube under *in vivo* or semi-*in vivo* conditions.

Changes in the $[Ca^{2+}]_{cyt}$ are thought to be regulated by transporters localized in Ca^{2+} stores and the plasma membrane (Sze et al., 2000). The identities of the compartments that function as Ca^{2+} stores in the pollen tube, however, were previously unknown. In this study, we visualized $[Ca^{2+}]_{ER}$ dynamics using transgenic *Arabidopsis* pollen expressing YC4.60, a modified fluorescent Ca^{2+} indicator with a relatively low affinity for Ca^{2+}, making it suitable for the analysis of $[Ca^{2+}]_{ER}$ dynamics against a high background $[Ca^{2+}]_{ER}$. Although the ERs were localized throughout the pollen tube, the $[Ca^{2+}]_{ER}$ was not homogeneous and areas containing a high local $[Ca^{2+}]_{ER}$ (e.g., 100-500 μM) were scattered throughout the pollen tubes. This result supports the idea that the $[Ca^{2+}]_{ER}$ is heterogeneous due to an uneven distribution of ER Ca^{2+}-binding proteins (Clapham 2007). Thus, our results indicated that the ER is a Ca^{2+} store in growing pollen tubes.

Ca^{2+}-sensitive vibrating electrodes and patch-clamp electrophysiology have

been used to examine Ca^{2+} influx in the tip region (Kuhtreiber & Jaffe, 1990; Pierson et al., 1994; Holdaway-Clarke et al., 1997; Franklin-Tong et al., 2002; Dutta & Robinson, 2004). In this study, we always observed a tip-focused $[Ca^{2+}]_{cyt}$ gradient in growing pollen tubes, with a submicromolar $[Ca^{2+}]_{cyt}$ in the tip region. In addition, the regions showing a high $[Ca^{2+}]_{ER}$ were scattered throughout the pollen tubes. These data suggested that Ca^{2+} imported through the plasma membrane in the tip region immediately moved into Ca^{2+} stores.

When Gd^{3+} was added to the samples, $[Ca^{2+}]_{cyt}$ at the tip region decreased and the $[Ca^{2+}]_{cyt}$ gradient disappeared from the tip region. On the other hand, just after the addition of CPA to the culture medium, the area with a high $[Ca^{2+}]_{cyt}$ increased in size. The $[Ca^{2+}]_{cyt}$ increase is thought to be the result of CPA impairing the ability to restore the cytoplasmic Ca^{2+}. After the $[Ca^{2+}]_{cyt}$ increase, $[Ca^{2+}]_{cyt}$ was decreased and then regular $[Ca^{2+}]_{cyt}$ oscillations were induced. The result that Gd^{3+} inhibited $[Ca^{2+}]_{cyt}$ oscillations suggested that an imbalance of extracellular Ca^{2+} influx and Ca^{2+} efflux to outside would induce the regular $[Ca^{2+}]_{cyt}$ oscillations.

In this study, the results that CPA decreased $[Ca^{2+}]_{ER}$ and induced $[Ca^{2+}]$ oscillations suggested that the ER contains CPA-sensitive Ca^{2+}-ATPases and that CPA affected Ca^{2+} efflux from the cytoplasm to the ER. Thapsigargin, another inhibitor of type IIA Ca^{2+}-ATPases, did not induce a decrease in the $[Ca^{2+}]_{ER}$, however. Four different IIA-type Ca^{2+}-ATPases and ten different IIB-type Ca^{2+}-ATPases have been identified in *Arabidopsis*. One of the IIA-type Ca^{2+}-ATPases, ECA1, is inhibited by CPA but not thapsigargin (Liang et al., 1997; Liang & Sze, 1998; Sze et al., 2000), making it a good candidate for the CPA-sensitive Ca^{2+}-ATPase in the ER of the pollen tube. On the other hand, one of the IIB-type Ca^{2+}-ATPases, ACA2, is not inhibited by CPA, is present in ER, and is expressed in pollen grains (Harper et al., 1998; Hwang et al., 2000). As CPA at a concentration that induced $[Ca^{2+}]_{cyt}$ oscillations did not completely deplete the $[Ca^{2+}]_{ER}$ in

this study, a CPA-insensitive Ca^{2+}-ATPase in the ER might be related with the $[Ca^{2+}]_{cyt}$ oscillations. In addition, ECA3, another IIA-type Ca^{2+}-ATPase from *Arabidopsis*, has been detected in the Golgi apparatus (Mills et al., 2008), and a CPA-sensitive Ca^{2+} pump has been identified in both ER vesicles and the Golgi apparatus of *Pisum sativum* (Ordenes et al., 2002). Furthermore, vesicle accumulation independent of the ER was observed in the pollen tip of *L. longiflorum* (Parton et al., 2003; Bove et al., 2008). In addition, there is a possibility that a CPA-sensitive Ca^{2+} pump exists in vacuolar membrane. Therefore, the induction of regular $[Ca^{2+}]_{cyt}$ oscillations by CPA may be related to Ca^{2+}-ATPases not only in the ER but also in other intracellular compartments such as Golgi vesicles and vaculoles.

In this study, we visualized cytoplasmic Ca^{2+} dynamics in pollen tubes growing under *in vitro*, *in vivo*, and semi-*in vivo* conditions in *Arabidopsis* and *N. tabacum* with a higher resolution, higher speed, and higher FRET efficiency compared with previously reported results from YC3.1-expressing *Arabidopsis* (Iwano et al., 2004). The tip-focused $[Ca^{2+}]_{cyt}$ gradient was always observed in growing pollen tubes. Regular oscillations of the $[Ca^{2+}]_{cyt}$, however, were rarely identified in *Arabidopsis* or *N. tabacum* pollen tubes grown under the *in vivo* condition, or in the semi-*in vivo* condition. On the other hand, regular oscillations were observed *in vitro* in both growing and nongrowing pollen tubes, although the oscillation amplitude was 5-fold greater in the nongrowing pollen tubes compared with growing pollen tubes. Although models that the growth rate and $[Ca^{2+}]_{cyt}$ were correlated have been presented previously (Holdaway-Clarke et al., 1997), it was shown that $[Ca^{2+}]_{cyt}$ oscillation was not always correlated with the growth rate. Our results suggested that a submicromolar $[Ca^{2+}]_{cyt}$ in the tip region is essential for pollen tube growth, whereas a regular $[Ca^{2+}]$ oscillation is not. In addition, our results revealed that the ER acts as a Ca^{2+} store in pollen tubes, and that CPA-sensitive Ca^{2+}-ATPases in the ER function to maintain a high $[Ca^{2+}]_{ER}$. Furthermore, CPA-sensitive Ca^{2+}-ATPases

are likely required to maintain a narrow range of the $[Ca^{2+}]_{cyt}$ in growing pollen tubes.

Materials and Methods

Plant materials and growth conditions

Arabidopsis ecotype Columbia was used in all of the experiments. *Arabidopsis* plants were grown in mixed soil in a growth chamber. The light intensity was 120-150 μmol m^{-2} s^{-1} during the daily 12-h light period. The temperature was maintained at 22 ± 2°C. *N. tabacum* cv. SR1 was grown in mixed soil in a greenhouse, in which the temperature was maintained at 25 ± 2°C.

Transgenic constructs

A cassette containing the YC3.60-coding region followed by the nopaline synthase polyadenylation signal from pBIYC3.60 was constructed by replacing the GUS-coding sequence of pBI221 (Clontech, Palo Alto, CA) with the YC3.60-coding region. The 1.5-kb fragment upstream of the *Act1* gene, which is highly expressed in reproductive tissues (An et al., 1996), was PCR amplified using the specific primers 5'-GAAGCTTTCTCTTTAAAAGTTAAGTTTTCTTTGTACATGTCTCTAAGC-3' and 5'-GTCTAGATTTCTTCTACCTTTATGCAAATCCAAACATTGTTTAAAGATC-3' (the underlined sequences show the incorporated *Hin*dIII and *Xba*I sites, respectively). The amplified fragments were subcloned into pBIYC3.60 to yield Act1 promoter-YC3.60. The chimeric gene comprising the 1.5-kb promoter region of the *Act1* gene, the YC3.60 coding sequence, and the nopaline synthase transcription terminator was inserted into the binary vector pSLJ1006 (Jones et al., 1992) to create pAct1::YC3.60.

To express YC4.60 in the pollen tube ER, the DNA fragment encoding YC3.60 in pSLG9-YC3.60 was replaced with that encoding YC4.60 in pcDNA-YC4.60, which yielded pSLG9-YC4.60. To construct a vector encoding ER-targeted YC4.60, a signal

peptide and ER retention signal sequence were designed according to a previous report (Mitsuhashi, Shimada, Mano, Nishimura, & Hara-Nishimura, 2000). A double-strand oligonucleotide encoding the signal peptide (5'-GGCCGGATCCATGGCTAGATTGACTTCTATTATTGCTTTGTTTGCTGTTGC TTTGTTGGTTGCTGATGCTTATGCTTATCGTAC-3' as the sense strand and 5'-CATGGTACGATAAGCATAAGCATCAGCAACCAACAAAGCAACAGCAAAC AAAGCAATAATAGAAGTCAATCTAGCCATGGATCC-3' as the antisense strand) was synthesized and ligated into the *Eco*52I and *Nco*I sites of pSLG9::YC4.60, yielding pSLG9::SP-YC4.60. To synthesize a DNA fragment encoding the C-terminal portion of YC4.60 with an ER retention signal, a PCR was performed using specific primers (5'-AAGAAGATCTCCAGCTCCGGGGCACTGGAGCTTAT-3' and 5'-AATAGAGCTCACAATTCATCATGATGATGATGATGATGTCCACCTCCCTCG ATGTTGTGGCGGATCT-3') and pSLG9::YC4.60 as the template. The obtained fragment was digested with *Bgl*II and *Sac*I, and ligated to the *Bgl*II and *Sac*I sites of pSLG9::SP-YC4.60, yielding pSLG9::SP-YC4.60-ER.

A DNA fragment from pSLG9::SP-YC4.60-ER encoding SP-YC4.60-ER was PCR-amplified using specific primers (5'-ACGGTCTAGATGGCTAGATTGACTTCTATTATTG-3' and 5'-AATAGAGCTCACAATTCATCATGATGATGATGATGATGTCCACCTCCCTCG ATGTTGTGGCGGATCT-3'). The obtained DNA fragment was digested with *Xba*I and *Sac*I and ligated into the *Xba*I and *Sac*I sites of pBI121-pAct1::YC3.60, resulting in pBI121-pAct1::YC4.60(ER).

To express YC3.60 in the tobacco pollen tube cytoplasm, the 35S promoter and GUS reporter gene in pBI121 were replaced with the microspore-specific Lat52 promoter from pBI121-pLat52::GUS (Twell et al., 1991) and the YC3.60-coding fragment from pSLG9-YC3.60, which yielded pLat52::YC3.60.

Transformation

The pSLJAct1-YC3.60 and pSLJAct1-YC4.60 plasmids were electroporated into *Agrobacterium tumefaciens* strain EHA105 (Hood et al., 1993). For Arabidopsis, the *Agrobacterium* infiltration procedure was performed with unopened flower buds of *Arabidopsis* ecotype Columbia as previously described (Bechtold et al., 1993). The transformed seeds were selected on 1/2 MS plates containing kanamycin (50 μg/ml) and were analyzed using PCRs to test for the presence of the YC3.60 gene. For *N. tabacum*, the *Agrobacterium* infiltration procedure was performed with leaf disks from *N. tabacum* cv. SR1 as previously described (Horsch et al., 1985).

Pollen tube growth, ratiometric imaging, and image analysis

For *in vitro* imaging, pollen grains from freshly dehisced anthers of YC3.60-expressing plants were mounted on modified germination medium containing 2 mM $CaCl_2$, 0.01% boric acid, 1 mM $MgSO_4$, 1% (w/v) agar (ultra-low gelling temperature type IX-A, Sigma), and 17% (w/v) sucrose (pH adjusted to 7.0 using KOH) in moistened glass-bottomed dishes (Palanivelu et al., 2003) for *Arabidopsis* or germination medium (Read et al., 1993) containing 0.5% (w/v) agar for *N. tabacum*. After 5 h at 20°C for *Arabidopsis* and at 25°C for *N. tabacum*, pollen tubes that were at least 200 μm in length were imaged with an Olympus IX81 inverted microscope equipped with a CSU-22 spinning Nipkow disk confocal unit (Yokogawa, Tokyo, Japan), an EM-CCD C9100 camera (Hamamatsu, Hamamatsu, Japan), an image splitter, Dual-View (Optical Insights, Santa Fe, NM), and a diode-pumped solid-state 445-nm laser (iFLEX2000, Point Source, Hamble, UK). Imaging of the cameleon emission ratio was accomplished using two emission filters (480/30 for ECFP and 535/40 for Venus). After a background subtraction, the Venus/ECFP ratio was determined using MetaMorph software. An Olympus

UPlanSApo 60×/1.35w immersion objective lens was used for imaging. In each experiment, the ratio in a region with a diameter of 6 μm was measured using the MetaMorph software and is shown as sequential line graphs. For analysis of the changes in the [Ca^{2+}], the maximum and the minimum values in each periodicity were measured, allowing calculation of the amplitude in each periodicity. In each experiment, the mean maximum, minimum, and amplitude values were calculated using EXCEL software. Exposure times were typically 50-500 msec, and images were collected every 0.5-5 sec.

To confirm that full-length cameleon was expressed in the pollen tube, elongated pollen tubes were monitored with excitation at 458 nm using a spectral imaging fluorescence microscope system (LSM510 META, Carl Zeiss, Jena, Germany). This system is capable of resolving the spectra of various fluorescence images; therefore, we were able to obtain images with no interference from the overlapping fluorescence emissions (Haraguchi et al., 2002; Iwano et al., 2004). A Zeiss 63× W Korr objective lens (N.A.: 1.2) was used to image the pollen tubes.

To examine the effects of a Ca^{2+} channel blocker, Gd^{3+} (Sigma, Poole, UK) was dissolved in the germination medium. To examine the effects of a P-type IIA Ca^{2+}-ATPase inhibitor, CPA was dissolved in DMSO and added to the germination medium to a final concentration of 10 μM to 100 μM. DMSO alone had no discernible effects on germination or tube growth.

For *in vivo* imaging in *Arabidopsis*, a pistil was mounted on a cover slip prior to pollination, fixed with double-sided tape, and covered with 1% agar except for the stigma. After a pollen grain from a transgenic *Arabidopsis* was mounted on a wild-type *A. thaliana* papilla cell using a micromanipulator, the $[Ca^{2+}]_{cyt}$ in the pollen tube growing through the papilla cell wall was monitored under dry conditions using the microscope system described above. For *N. tabacum*, the pistil was mounted on a cover slip 3 h after pollination, fixed with double-sided tape, covered with 1% agar except for the stigma, and

monitored. These experiments were carried out more than 10 times.

For imaging under the semi-*in vivo* condition, wild-type *A. thaliana* flowers excised before they dehisced were attached to an agar plate after the anthers of a transgenic *Arabidopsis* were removed from the flowers. Pollen grains from freshly dehisced anthers of YC3.60- or YC4.60-expressing plants were attached to the wild-type stigma. Thirty minutes after pollination, the upper half of the pollinated pistil was excised and mounted in germination medium in a moistened glass-bottomed dish. After 2 h at 20°C, the $[Ca^{2+}]_{cyt}$ in pollen tubes growing through the style was monitored using the microscope system described above. For *N. tabacum*, styles, excised before the flowers were dehisced, were placed into tobacco germination medium and pollinated with pollen grains from freshly dehisced anthers of YC3.60-expressing plants. After 18 h at 25°C, the $[Ca^{2+}]_{cyt}$ in pollen tubes growing through the style was monitored using the microscope system described above. These experiments were carried out more than 30 times.

Calibration of YC3.60 and YC4.60 ratiometric changes

Calibration of the $[Ca^{2+}]_{cyt}$ was carried out as described previously (Allen et al., 1999). Serial dilutions of purified YC3.60 and YC4.60 were made in Ca^{2+} calibration buffer (Molecular Probes), in which the free $[Ca^{2+}]$ ranged from 0 μM to 1 mM. Dilutions of YC3.60 and YC4.60 that resulted in similar signal intensities to those seen in YC3.60- and YC4.60-expressing pollen tubes were used to determine R_{min} and R_{max}. R_{min} and R_{max} values were 2.95 and 8.33 for YC3.60, and 3.53 and 9.87 for YC4.60, respectively. These values were used to convert the YC3.60 and YC4.60 fluorescence ratios into a $[Ca^{2+}]_{cyt}$ by fitting them to YC3.60 and YC4.60 calibration curves obtained *in vitro*.

Pollen tube growth rate

The growth rate, calculated from the elongation length during a 3-min period, was

obtained using Venus fluorescence in the growing pollen tube and MetaMorph software.

Statistical analysis

Statistical analyses were performed using Student's *t*-tests, when necessary.

Acknowledgments

We thank and Mrs. Onishi, Mrs. Yamamoto, Mrs. Matsumura Mrs. Okamura and Mrs. Ichikawa for their technical assistance and Dr. Pulla Nakayama for her critical reading. This work was supported in part by Grants-in-Aid for Special Research on Priority Areas (No. 16GS0316 and 18075008), Grants-in-Aid for Special Research (C) (No. 19570040) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

Allen GJ, Chu SP, Harrington CL, Schumacher K, Hoffman T, Tang YY, Grill E, Schroeder JI (2001) A defined range of guard cell calcium oscillation parameters encodes stomatal movements, Nature **411**: 1053-1057 PMID: 11429606

Allen GJ, Chu SP, Schumacher K, Shimazaki CT, Vafeados D, Kemper A, Hawke SD, Tallman G Tsien RY, Harper JF, Chory J, Schroeder JI (2000) Alteration of stimulus-specific guard cell calcium oscillations and stomatal closing in Arabidopsis det3 mutant, Science **289**: 2338-2342 PMID: 11009417

Allen GJ, Kwak JM, Chu SP, Llopis J, Tsien RY, Harper JF, Schroeder JI (1999) Cameleon calcium indicator reports cytoplasmic calcium dynamics in Arabidopsis guard cells, Plant J **19**: 735-747 PMID: 10571859

An YQ, Huang S, McDowell JM, McKinney EC, Meagher RB (1996) Conserved expression of the Arabidopsis ACT1 and ACT3 actin subclass in organ primordia and mature pollen, Plant Cell **8**: 15-30 PMID: 8597657

Bechtold N, Ellis J, Pelletier, G (1993) In planta agrobacterium mediated gene transfer by infiltration of adult arabidopsis thaliana plants. C. R. Acad. Sci. Paris, Life Sciences, **316**: 1194-1199

Bove J, Vaillancourt B, Kroeger J, Hepler PK, Wiseman PW, Geitmann A (2008) Magnitude and direction of vesicle dynamics in growing pollen tubes using spatiotemporal image correlation spectroscopy and fluorescence recovery after photobleaching. Plant Physiol **147**: 1646-1658 PMID: 18508956

Brewbaker JL, Kwack BH (1963) The essential role of calcium ion in pollen germination and pollen tube growth, Amer J Bot **50**: 859-865

Cheung AY, Wang H, Wu HM (1995) A floral transmitting tissue-specific glycoprotein attracts pollen tubes and stimulates their growth. Cell **82**: 383-93 PMID: 7634328

Cheung AY, Wu HM (2008) Structural and signaling networks for the polar cell growth

machinery in pollen tubes. Annu Rev Plant Biol **59**: 547-572 PMID: 18444907

Clapham DE (2007) Calcium signaling, Cell **131**: 1047-1058 PMID: 18083096

Dutta R, Robinson KR (2004) Identification and characterization of stretch-activated ion channels in pollen protoplasts, Plant Physiol **135**: 1398-1406 PMID: 15247410

Feijo JA, Sainhas J, Holdaway-Clarke, Cordeiro MS, Kunkel JG, Hepler PK (2001) Cellular oscillations and the regulation of growth: the pollen tube paradigm. BioEssays **23**: 86-94 PMID: 11135313

Franklin-Tong VE, Holdaway-Clarke TL, Straatman KR, Kunkel JG, Hepler PK (2002) Involvement of extracellular calcium influx in the self-incompatibility response of *Papaver rhoeas*. Plant J **29**: 333-345 PMID: 11844110

Frietsch S, Wang YF, Sladek C, Poulsen LR, Romanowsky SM, Schroeder JI (2007) A cyclic nucleotide-gated channel is essential for polarized tip growth of pollen. Proc Natl Acad Sci USA **104**: 14531-14536 PMID: 17726111

Geisler M, Axelsen KB, Harper JF, Palmgren MG (2000) Molecular aspects of higher plant P-type Ca^{2+}-ATPases. Biochem Biophys Acta **1465**: 52-78 PMID: 10748247

Geitmann A, Cresti M (1998) Ca^{2+} channels control the rapid expansions in pulsating growth of Petunia hybrida pollen tubes, J Plant Physiol **152**: 439-447

Haraguchi T, Shimi T, Koujin T, Hashiguchi N, Hiraoka Y (2002) Spectral imaging fluorescence microscopy. Gene Cell **7**: 881-887 PMID: 12296819

Harper JF, Hong B, Hwang I, Guo HQ, Stoddard R, Huang JF, Palmgren MG, Sze H (1998) A novel calmodulin-regulated Ca^{2+}-ATPase (ACA2) from Arabidopsis with an N-terminal autoinhibitory domain. J Biol Chem **273**: 1099-106 PMID: 9422775

Holdaway-Clarke TL, Feijo JA, Hackett GR, Kunkel JG, Hepler PK (1997) Pollen tube growth and the intracellular cytosolic calcium gradient oscillate in phase while extracellular calcium influx is delayed. Plant Cell **9**: 1999-2010 PMID:

12237353

Holdaway-Clarke T, Hepler P (2003) Control of pollen tube growth: Role of ion gradients and fluxes. New Phytol **159**: 539-563

Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993) New agrobacterium helper plasmids for gene transfer to plants. Transgenic Res **2**: 208-218

Horsch RB, Fry JE, Hoffmann NL, Eichholtz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. Science **227**: 1229-1231 PMID: 17757866

Hwang JU, Gu Y, Lee YJ, Yang Z (2005) Oscillatory ROP GTPase activation leads the oscillatory polarized growth of pollen tubes. Mol Biol Cell **16**: 5385-5399 PMID: 16148045

Hwang I, Sze H, Harper JF (2000) A calcium-dependent protein kinase can inhibit a calmodulin-stimulated Ca^{2+} pump (ACA2) located in the endoplasmic reticulum of *Arabidopsis*. Proc Natl Acad Sci USA **97**: 6224-6229 PMID: 10823962

Inesi G, Sagara Y (1994) Specific inhibitors of intracellular Ca^{2+} transport ATPases. J Memb Biol **141**: 1-6 PMID: 7966241

Iwano M, Shiba H, Miwa T, Che FS, Takayama S, Nagai T, Miyawaki A, Isogai A (2004) Ca^{2+} dynamics in a pollen grain and papilla cell during pollination of arabidopsis. Plant Physiol **136**: 3562-3571 PMID: 15489279

Jones JDG, Shlumukov L, Carland F, English J, Scofield SR, Bishop GJ, Harrison K (1992) Effective vectors for transformation, expression of heterologous genes, and assaying transposon excision in transgenic plants. Transgenic Res **1**: 285-297 PMID: 1338696

Kuhtreiber WM, Jaffe LF (1990) Detection of extracellular calcium gradients with a calcium-specific vibrating electrode. J Cell Biol **110**: 1565-1573 PMID: 2335563

Li H, Lin Y, Heath RM, Zhu MX, Yang Z (1999) Control of pollen tube tip growth by a

Rop GTPase-dependent pathway that leads to tip-localized calcium influx. Plant Cell **11**: 1731-42 PMID: 10488239

Liang F, Sze H (1998) A high-affinity Ca^{2+} pump, ECA1, from the endoplasmic reticulum is inhibited by cyclopiazonic acid but not by thapsigargin. Plant Physiol **118**: 817-825 PMID: 9808725

Liang F, Cunningham KW, Harper JF, Sze H (1997) ECA1 complements yeast mutants defective in Ca^{2+} pumps and encodes an endoplasmic reticulum-type Ca^{2+}-ATPase in arabidopsis thaliana. Proc Natl Acad Sci USA **94**: 8579-8584 PMID: 9238019

Malhó R, Read ND, Pais MS, Trewavas AJ (1994) Role of cytosolic free calcium in the reorientation of pollen tube growth. Plant J. **5**: 331– 341

Messerli MA, Robinson KR (2003) Ionic and osmotic disruptions of the lily pollen tube oscillator: Testing proposed models. Planta **217**: 147-157 PMID: 12721859

Messerli MA, Creton R, Jaffe LF, Robinson KR (2000) Periodic increases in elongation rate precede increases in cytosolic Ca^{2+} during pollen tube growth. Develop Cell **222**: 84-98 PMID: 10885748

Messerli M, Robinson KR (1997) Tip localized Ca^{2+} pulses are coincident with peak pulsatile growth rates in pollen tubes of lilium longiflorum. J Cell Sci **110**: 1269-1278 PMID: 9202387

Mills RF, Doherty ML, Lopez-Marques RL, Weimar T, Dupree P, Palmgren MG, Pittman JK, Williams LE (2008) ECA3, a golgi-localized P2A-type ATPase, plays a crucial role in manganese nutrition in arabidopsis. Plant Physiol **146**: 116-128 PMID: 18024560

Mitsuhashi N, Shimada T, Mano S, Nishimura M, Hara-Nishimura I (2000) Characterization of organelles in the vacuolar-sorting pathway by visualization with GFP in tobacco BY-2 cells. Plant Cell Physiol **41**: 993-1001 PMID: 11100771