A Pollen-Specific RALF from Tomato That Regulates Pollen Tube Elongation¹,²[W][OA]

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Rapid Alkalinization Factors (RALFs) are plant peptides that rapidly increase the pH of plant suspension cell culture medium and inhibit root growth. A pollen-specific tomato (Solanum lycopersicum) RALF (SIPRALF) has been identified. The SIPRALF gene encodes a preproprotein that appears to be processed and released from the pollen tube as an active peptide. A synthetic SIPRALF peptide based on the putative active peptide did not affect pollen hydration or viability but inhibited the elongation of normal pollen tubes in an in vitro growth system. Inhibitory effects of SIPRALF were detectable at concentrations as low as 10 nM, and complete inhibition was observed at 1 μM peptide. At least 10-fold higher levels of alkSIPRALF, which lacks disulfide bonds, were required to see similar effects. A greater effect of peptide was observed in low-pH-buffered medium. Inhibition of pollen tube elongation was reversible if peptide was removed within 15 min of exposure. Addition of 100 nM disulfide bonds, were required to see similar effects. A greater effect of peptide was observed in low-pH-buffered medium.

Peptide signaling regulates a variety of developmental processes and environmental responses in plants (Olsen et al., 2002; Ryan et al., 2002; Boller, 2005; Germain et al., 2006; Matsubayashi and Sakagami, 2006; Farrokhi et al., 2008). For example, the CLAVATA3 peptide regulates meristem size (Fletcher et al., 1999), and the SCR peptide is the pollen self-incompatibility recognition factor in the Brassicaceae (Schopfer et al., 1999; Takayama et al., 2000). The peptide systemin induces the systemic defense response (Ryan and Pearce, 2003), and defensins are small Cys-rich proteins that are involved in the innate immune system of plants (Lay and Anderson, 2005; Okuda et al., 2009).

The Rapid Alkalinization Factor (RALF) family of peptides was discovered a factor that could rapidly cause alkalinization of suspension culture medium in a search for tobacco (Nicotiana tabacum) systemins (Pearce et al., 2001). Tomato (Solanum lycopersicum) RALF (now called SIRALF, previously LeRALF [Scheer et al., 2005]) is a 49-amino acid active peptide produced from a 115-amino acid propeptide. When Arabidopsis (Arabidopsis thaliana) or tomato seeds were germinated in the presence of 10 μM synthetic RALF, root growth and root hair initiation were inhibited, suggesting a negative growth regulatory function for this peptide. Subsequently, RALF-encoding genes have been identified in a wide variety of plant species, including dicots, monocots, and gymnosperms (Pearce et al., 2001). The 40 RALF or RALF-like (RALFL) genes encoded in Arabidopsis have variable expression patterns, and ovule-specific expression of a subset of RALF genes has been observed in Solanum chacoense (Olsen et al., 2002; Germain et al., 2005; Punwani et al., 2007). A study of NaRALF down-regulation in Nicotiana attenuata has demonstrated that this root-expressed RALF is required for normal root and root hair growth (Wu et al., 2007). Overexpression of either of two Arabidopsis RALF genes, AtRALF1 or AtRALF23, results in a dwarf phenotype (Matos et al., 2008; Srivastava et al., 2009). AtRALF1 was identified in a search for bioactive peptides using Arabidopsis seedlings expressing aequorin-a bioluminescent Ca²⁺ reporter as a factor that can rapidly (within 20 s) induce a transient internal Ca²⁺ spike (Haruta et al., 2008), suggesting that RALF peptides may act through a calcium signaling pathway. Recent experiments using exogenous RALF peptide purified from sugarcane seeds have demonstrated that RALF peptides can induce a rapid alkalinization of suspension culture medium.

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² This article is dedicated to the memory of Clarence “Bud” Ryan.

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Saccharum officinarum) leaves (SacRALF) demonstrated that the peptide inhibits sugarcane microcalli development and that SacRALF transcripts were found in actively expanding zones of both roots and leaves. These studies also showed that exogenous recombinant AtRALF1 reduced Arabidopsis hypocotyl growth (Mingossi et al., 2010). RALF gene expression in turn appears to be regulated by other plant hormones: PtdRALF2 transcripts are down-regulated by methyl jasmonate in poplar (Populus spp.) cell suspension cultures (Haruta and Constabel, 2003), and AtRALF23 is significantly down-regulated by brassinolide (Srivastava et al., 2009).

Prohormone proteins in animals and yeast are typically processed at dibasic sites by Golgi-localized subtilisin-related proteinases called proprotein convertases, and the processed, active peptides are released into the extracellular matrix (Nakayama, 1997; Seidah and Chretien, 1999). RALF precursors also possess a conserved dibasic site upstream to the active peptide, suggesting that the processing mechanism may be similar. Recent studies show that both AtRALF23 and AtRALF1 are processed at the dibasic site by Golgi-located plant subtilisin-like Ser proteases and that this processing step is required for the activation of the peptide (Matos et al., 2008; Srivastava et al., 2009).

Pollen tube germination and growth are auto-regulated by several pollen-produced peptides. For example, phytosulfokines are responsible for the stimulatory “pollen population effect” for in vitro pollen tube germination (Chen et al., 2000). The small Cys-rich LAT52 protein is required for normal pollen hydration and germination (Muschietti et al., 1994). In both of these cases, the peptides can act in an “autocrine”-like manner to regulate pollen tube germination (Tang et al., 2002; Johnson and Preuss, 2003).

In this article, we describe the discovery of SIPRALF, a pollen-specific RALF peptide from tomato that does not affect pollen viability, hydration, or early germination events but does inhibit the elongation of pollen tubes within a specific developmental window.

RESULTS

Discovery of a Pollen-Expressed RALF Gene

A yeast two-hybrid screen was conducted to identify potential proteins that interact with the recognition domain of the tomato pollen-specific cell wall-localized Leu-rich repeat extensin chimera (LRX) protein (Rubinstein et al., 1995a, 1995b; Stratford et al., 2001; Baumberger et al., 2003). One pollen cDNA identified by this screen (Pol2) encodes a peptide similar to SIRALF, a factor shown to rapidly increase the pH of plant tissue culture medium and to inhibit root growth (Pearce et al., 2001). While additional experiments to confirm and characterize pollen LRX-RALF interactions are in progress, the effects of the pollen-expressed RALF on pollen tube germination and growth became the focus of this study. The Pol2 cDNA sequence was used to query the Sol Genomics Network (SGN) unigene collection (http://www.sgn.cornell.edu/tools/blast/), and a single tomato unigene constructed from S. lycopersicum and Solanum pennellii ESTs was identified (SGN-U324197; SIRALF in Fig. 1). Like the previously identified vegetative tissue-expressed SIRALF (Pearce et al., 2001), the Pol2 gene encodes a propreptide predicted to be targeted to the endomembrane system and then proteolytically processed near a conserved dibasic site that, in Arabidopsis, is required for propeptide processing (and activity) of both AtRALF1 and AtRALF23 (Matos et al., 2008; Srivastava et al., 2009). The predicted active Pol2 peptide includes four conserved Cys residues likely to be involved in disulfide bridges that have been shown to be required for both alkalinization of somatic suspension cell culture medium and root growth inhibi-
tion (Pearce et al., 2001). The predicted mature Pol2 peptide also possesses additional well-conserved sequences found in the original SlRALF and RALFL peptides, including an ISY motif near the mature N terminus, a GASYY motif between the first and second conserved Cys residues, and an YXRGCS motif that contains the third conserved Cys residue.

Pol2 Expression Is pollen Specific

Pollen expression of the Pol2 gene could be inferred, since the Pol2 cDNA was identified using a pollen cDNA library and the corresponding SGN unigene was compiled mainly from S. pennellii pollen cDNA sequences. In order to assess expression in other tissues, an RNA blot with RNA from tomato root, etiolated seedlings, leaf, stem, sepal, petal, stamen, pollen, unpollinated carpels from closed flowers, and 16 d-after-pollination fruit was probed with Pol2 sequences. As seen in Figure 2, a strong signal was only detected in pollen and stamens, which contain pollen. Thus, the Pol2 sequence is most highly expressed in pollen. Therefore, we have named this tomato gene SIPRALF for S. lycopersicum pollen RALF. The Arabidopsis genome contains 40 genes encoding RALFL proteins (Olsen et al., 2002; www.arabidopsis.org), and at least six Arabidopsis RALFL genes are expressed in pollen (Honys and Twell, 2004; Pina et al., 2005; Schmid et al., 2005); At1g28270_RALFL4 is the Arabidopsis pollen-expressed gene most closely related to SIPRALF. The encoded amino acid sequence of this gene as well as a pollen/anther-expressed RALF from Petunia hybrida (PhanthRALF) are shown in the amino acid alignment in Figure 1. No clear pollen-specific motif is evident when pollen-expressed RALFs are compared with vegetative SIRALF, other than an enrichment of positively charged amino acids between the third and fourth Cys residues in the active peptide region.

Detection of SIPRALF in Growing Pollen Tubes and Germination Medium

The SIPRALF gene encodes a predicted preproprotein of 129 amino acids with a secretion prediction score of 0.996 using TargetP (http://www.cbs.dtu.dk/services/TargetP/; Emanuelsson et al., 2000). Immunolocalization studies indicated that a SIPRALF signal is punctate, with an increasing gradient of the protein toward the tip of the tube (Fig. 3A). Protein-blotting
experiments showed that the pollen tube-localized protein may correspond to the unprocessed form of SlPRALF, based on the size of immune-reactive bands ($17 \text{ kD}$; Fig. 3B). The diffuse bands observed may represent either posttranslationally modified versions or oligomers that are insensitive to SDS-reducing agent treatment (Leite et al., 2000). A similar diffuse banding pattern was observed when AtRALF23 was overexpressed in Arabidopsis and resolved on denaturing gels (Srivastava et al., 2009). The processed form of SlPRALF (approximately 7 kD) was not detected (even after overloading the gel with more than 100 $\mu$g of protein per lane) either in total pollen tube extracts or in subcellular fractions of pollen extracts, indicating that it is either degraded rapidly or immediately secreted to outside the pollen tube after processing. The processed form of SlPRALF was detected only in the medium, and no protein larger than the processed form was found in the medium, suggesting that only the mature peptide is secreted to outside the pollen tube.

### SlPRALF Alkalination Activity

A synthetic form of the predicted mature SlPRALF peptide sequence derived from the SGN unigene U324197 was tested for activity in somatic cell culture assays using *Solanum peruvianum* and Arabidopsis suspension cultures. Interestingly, *S. peruvianum* cells, which exhibited a strong alkalinizing response with tomato and tobacco RALFs purified from leaves, responded very weakly to SlPRALF. However, both the SIRALF and the SlPRALF peptides produced a very strong alkalinizing response with Arabidopsis suspension cells, and this assay was utilized for the purification of oxidized SlPRALF (Fig. 4).

To test whether a similar alkalinization response could be detected in an in vitro pollen germination system, synthetic SlPRALF was added to a final concentration of 0.1 $\mu$M to approximately $3.65 \times 10^5$ tomato (cv VF36) pollen grains in 0.5 mL of buffered or unbuffered pollen germination medium (PGM). No significant change in pH was detected (Supplemental Table S1). However, it is important to note that localized changes in pH at the pollen tube plasma membrane would not be detected using this assay.

### SlPRALF Does Not Affect Pollen Hydration or Viability

Synthetic SlPRALF was tested for effects on tomato pollen hydration and viability. The peptide did not affect pollen hydration in the range of 0.005 to 1 $\mu$M (Table I). Viability of treated pollen was tested using the fluorochromatic response with the stain fluorescein diacetate (Rotman and Papermaster, 1966; Heslop-Harrison and Heslop-Harrison, 1970). As shown in Figure 5, the presence of the SlPRALF peptide at 1 $\mu$M, the highest concentration tested, did not significantly affect pollen viability compared with untreated controls (Fig. 5A). The slight increase in viability seen in the presence of SlPRALF is likely due to the lack of elongating pollen tubes, which are vulnerable to breakage (Fig. 5, B and C).

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### Table I. SlPRALF effects on pollen hydration

Values are average percentages of NHP from five flowers ± s.e at each indicated peptide concentration after 1 h in pollen germination medium.

<table>
<thead>
<tr>
<th>SIPRALF $\mu$M</th>
<th>NHP</th>
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<tbody>
<tr>
<td>0.0</td>
<td>21.9 ± 1.82</td>
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<tr>
<td>0.005</td>
<td>20.8 ± 3.15</td>
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<tr>
<td>0.01</td>
<td>23.1 ± 4.54</td>
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<tr>
<td>0.025</td>
<td>24.03 ± 2.67</td>
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<tr>
<td>0.05</td>
<td>21.9 ± 4.26</td>
</tr>
<tr>
<td>0.1</td>
<td>20.2 ± 4.89</td>
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<tr>
<td>0.25</td>
<td>22.12 ± 4.78</td>
</tr>
<tr>
<td>0.5</td>
<td>20.7 ± 3.09</td>
</tr>
<tr>
<td>1.0</td>
<td>18.64 ± 0.85</td>
</tr>
</tbody>
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Figure 4. Suspension cell culture alkalinization assay. Change in pH was measured in cell suspension culture 15 min after exposure to unaltered or alkalinized vegetative (SIRALF and alkSIRALF) and pollen (SIPRALF and alkSIPRALF) RALF peptides at 0.25 nM (white bars), 2.5 nM (light gray bars), 25 nM (dark gray bars), and 250 nM (black bars). A, *S. peruvianum* suspension cell culture assay. B, Arabidopsis suspension cell culture assay.
SIPRALF Affects Pollen Tube Elongation

Although SIPRALF had no effect on pollen hydration or viability, this peptide clearly had a dramatic effect on pollen tube elongation (Fig. 5C). To test the effect of the peptide, pollen was placed in PGM with varying concentrations of SIPRALF (Fig. 6). After 1 and 3 h, hydrated pollen was classified as having tubes greater than the radius (TGR; Fig. 6A), tubes less than the radius (TLR; Fig. 6B), no detectable tubes (nongerminated pollen [NGP]; Fig. 6C), or burst pollen (BP; Fig. 6D). In the absence of peptide, approximately 85% of pollen grains had TGR after 1 h. As shown in Figure 6E, at SIPRALF concentrations as low as 0.01 μM, a decrease in the TGR class of pollen was detected.

Figure 5. Pollen viability assessed by FDA staining. A, Histogram of pollen fluorescence, with white bars representing positive FDA-stained fluorescent (live) pollen and gray bars representing nonfluorescent (dead) pollen from nontreated (no SIPRALF), treated (1 μM SIPRALF), and heat-killed pollen. FDA staining was done 1 h after the start of pollen germination, ±SD, n ≥ 100 pollen grains. B, Micrograph of stained untreated pollen. C, Micrograph of stained SIPRALF-treated pollen.

Figure 6. Effects of SIPRALF and alkSIPRALF on pollen tube germination and growth. Pollen was exposed to exogenous concentrations of SIPRALF peptide ranging from 0.005 to 1 μM. Peptide effects were classified according to pollen morphology. A, Micrograph of TGR class. B, Micrograph of TLR class. The arrow indicates the glebula. C, Micrograph of NGP class. D, Micrograph of BP class. Bars = 25 μm. E, The average percentage of hydrated pollen in each classification group, ±se, for pollen treated with each concentration of SIPRALF peptide for 1 h. F, The average percentage of hydrated pollen in each classification group, ±se, for pollen treated with SIPRALF peptide for 3 h. G, The average percentage of hydrated pollen in each classification group, ±se, for pollen treated with alkSIPRALF peptide for 1 h. H, The average percentage of hydrated pollen in each classification group, ±se, for pollen treated with alkSIPRALF peptide for 3 h. n ≥ 100 for each treatment.
with an almost complete absence at 0.1 μM SIRALF. Instead, at concentrations higher than 0.05 μM SIRALF, the predominant class of pollen was TLR, with small incipient pollen tubes (4.58 ± 1.9 μm), which we call “glebula” (“small mound” in Latin; arrow in Fig. 6B). For example, at 0.1 μM SIRALF, about 57% of the pollen had glebula (TLR). Since early germination events including glebula formation do not appear to be affected by SIRALF at low concentrations, the primary process affected by SIRALF seems to be the elongation of pollen tubes.

After prolonged treatment (3 h; Fig. 6F), increases in both the normal pollen (TGR) and in the BP are observed relative to the shorter, 1-h treatment. For example, at 3 h in 0.1 μM SIRALF, 43.1% of pollen falls into the TGR class and 41.3% into the BP class. This increase in burst or normal pollen with a concomitant decrease in the TLR class suggests that, over time, pollen with arrested tubes can either recover or will tend to burst, possibly due to a buildup of turgor pressure as tube elongation is inhibited.

SIRALF that has been reduced and alkylated by treatment with iodoacetamide (alkSIRALF) cannot form disulfide bridges. alkSIRALF was much less effective in the inhibition of pollen tube elongation (10–20 times higher concentrations are needed to see any effect), as shown in Figure 6, G and H. This result is consistent with the results of Pearce et al. (2001) demonstrating that disulfide bonds are important for SIRALF peptide activity in the cell culture medium alkalization and root growth inhibition bioassays. At least five times higher concentrations of exogenous vegetatively expressed SIRALF peptide were required to see any affect on pollen tube elongation (Supplemental Fig. S1).

**pH Effects on Pollen Germination and Growth**

In *NaRALF* down-regulated plants, a reduced pH of the growth medium partially ameliorated an abnormal root hair growth phenotype (Wu et al., 2007). To test whether the pH of the medium alters SIRALF effects on pollen tube growth, 0.05 μM peptide was added to PGM at pH 5.5, 6, 6.5, and 7, and the percentage of normal pollen with TGR was calculated (Fig. 7). The highest percentage of normal pollen tubes in the absence of peptide was observed at pH 6 and 6.5, indicating that this range of pH is optimal for pollen germination and tube growth. The greatest effect of the peptide was seen at the lowest pH tested (pH 5.5), and

![Graph showing effects of peptide on pollen tube growth at different pH environments](image)

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**Figure 7.** Effects of peptide on pollen tube growth at different pH environments. Tomato pollen was germinated for 1 h in PGM-MES medium buffered to pH 5.5, 6, 6.5, or 7 containing either no peptide (white bars) or 0.05 μM SIRALF (gray bars). Average number of normal pollen grains with tubes greater than the radius of the grain is shown as the percentage of normal pollen tubes ± se. n ≥ 100 for each pH.

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**Figure 8.** Reversibility of SIRALF inhibition. A, Histogram of percentage of hydrated pollen with TGR ± se 1 h after time-limited treatment with 0.1 μM SIRALF. Pollen was treated with peptide for designated exposure times, pelleted by centrifugation, and resuspended in medium containing 0.1 μM SIRALF (constant SIRALF, spin = black bars) or no peptide (SIRALF removed, spin = dark gray bars). Samples not treated with peptide were analyzed without centrifugation (no SIRALF, no spin = white bars) or with centrifugation (no SIRALF, spin = light gray bars). n ≥ 100. B, Micrograph of pollen in peptide-removed PGM 1 h after 15 min of SIRALF treatment. C, Micrograph of pollen in peptide-removed PGM 1 h after 20 min of SIRALF treatment.
the least effect was seen at the highest pH tested (pH 7). This is not unexpected if exogenous SIPRALF directly or indirectly acts to decrease cytoplasmic pH; a higher external proton concentration would tend to exacerbate an increase of protons inside the pollen tube.

**Reversibility of SIPRALF Effects**

Although seedling root growth is inhibited by SIRALF, seedlings will recover normal root growth when transferred from SIRALF-containing medium to peptide-free medium (Pearce et al., 2001). In order to test whether the inhibitory effects of SIPRALF on pollen tube elongation are reversible, ungerminated pollen was exposed to 0.1 μM SIPRALF for varying time periods and then pelleted and resuspended in peptide-free medium (Fig. 8; Supplemental Fig. S2). Centrifugation had no significant detrimental effect on pollen tube growth. If peptide was removed prior to or at 15 min of exposure, pollen was able to recover and generate normal pollen tubes in peptide-free medium (Fig. 8, A and B). However, pollen exposed to peptide for 20 min or longer was unable to generate normal pollen tubes after peptide was removed (Fig. 8, A and C). Thus, after 20 min of continuous exposure to SIPRALF, pollen tube elongation is irreversibly inhibited.

**SIPRALF Effects on Growth of Elongating Pollen Tubes**

To assess the effect of peptide on actively growing pollen tubes, pollen tubes were allowed to grow prior to the addition of 0.1 μM SIPRALF. As shown in Figure 9A, SIPRALF completely inhibited further pollen tube elongation when added up to 40 min after pollen germination. However, when SIPRALF was added at 50 min, a small population of tubes appeared to be resistant to the peptide (Fig. 9B). When peptide was added at 60 min, two distinct populations of tubes were observed (Fig. 9C, P1 and P2). At this time, about half of the tubes (46.8%) were sensitive to SIPRALF and did not elongate further after peptide addition. However, about half of the tubes (53.2%) were resistant to inhibition by the peptide and grew at the same rate as untreated pollen tubes. The lack of tubes of intermediate lengths suggests that tube growth is either completely inhibited or completely insensitive to inhibition. The bimodal distribution of sensitive and resistant pollen tubes at this time point is most likely due to variation in pollen tube length within the normal distribution of the pollen population, rather than to a true dichotomy in sensitivity to SIPRALF. Figure 9. SIPRALF effects on elongating pollen tubes. Histograms show pollen tube lengths of pollen tubes exposed to 0.1 μM SIPRALF at 40 (A), 50 (B), 60 (C), or 90 (D) min after addition of pollen to PGM. Pollen tube lengths were measured at the time of SIPRALF addition (green bars) and again 2 h later for nontreated (magenta bars) and 0.1 μM SIPRALF-treated (black bars) samples. Pollen tube lengths are grouped into 10-μm bin increments and represented as frequency of pollen tube lengths. The average pollen tube length for each treatment ± se is shown above the corresponding peak. n = 100. The average pollen tube length for populations showing bimodal distributions (B and C) were calculated separately (P1 and P2).

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than to genetic variation in pollen from the highly inbred cv VF36 (Williams and St. Clair, 1993). When pollen tube lengths average 58 μm at 90 min (Fig. 9D), elongating tubes were no longer sensitive to inhibition by peptide. From these results, we hypothesize that when tubes that have reached a critical threshold length between 38.4 and 58 μm, they become resistant to SfPRRALF peptide.

Migration of the Male Germ Unit as a Reference Point for Sensitivity to SfPRRALF

We have demonstrated that pollen tubes are inhibited by exogenous SfPRRALF within a defined period; pollen tubes become resistant to SfPRRALF once they have reached a length of about 50 μm. Generative cell mitosis and callose plug formation are distinctive cellular events in pollen tube growth, but both of these occur much later than this time frame. The migration of the male germ unit (MGU; comprising the vegetative nucleus and the generative cell) from the pollen grain into the pollen tube is an earlier event that may occur during this period. Migration of the MGU was assessed with regard to tube length by fixing and staining of pollen tubes with 4',6-diamidino-2-phenylindole (DAPI) after 0.5 to 2 h of growth. As shown in Figure 10, the first migration events are detected when tubes are 10 to 20 μm. The majority of germinated pollen has at least one nucleus/generative cell in the tube by 30 to 40 μm, and the majority have both nuclei in the tube by 50 to 60 μm. MGU migration is essentially complete when tubes are greater than 70 μm in length. Thus, the movement of the MGU into the pollen tube correlates developmentally with the end of the window of pollen tube sensitivity to exogenous SfPRRALF. While the mechanism of SfPRRALF action is probably not directly related to MGU movement, this event provides a convenient reference point for further investigation into the developmental timing of sensitivity to SfPRRALF.

DISCUSSION

RALFs are recently discovered plant peptides that can increase the pH of suspension cell culture medium more rapidly than systemin (Pearce et al., 2001). However, RALF genes are not induced by wounding, suggesting that RALF peptides do not play a role in defense like the systemin family of peptides. Rather, a role for RALF peptides in development is suggested by the inhibition of root growth by exogenously added peptide (Pearce et al., 2001; Mingossi et al., 2010) and by the tissue-specific regulation of RALF gene expression (Germain et al., 2005; Punwani et al., 2007; Mingossi et al., 2010). Down-regulation of a root-expressed NaRALF gene results in increased root growth and abnormal root hair growth, further supporting this notion (Wu et al., 2007). A growth inhibitory role for RALF is also consistent with the dwarf phenotypes that are observed in transgenic overexpression lines of AtRALF1 and AtRALF23 (Matos et al., 2008; Srivastava et al., 2009). Analogous to its vegetative orthologs, the pollen-specific tomato SfPRRALF gene described in this article seems to be a negative regulator of growth, as suggested by the effects of a synthetic SfPRRALF peptide on pollen tube growth in vitro.

The finding that SfPRRALF inhibits pollen tube growth may at first seem puzzling, since pollen itself produces this peptide. However, pollen tube germination and growth is known to be self-regulating. For example, the peptide phytosulfokine-α is produced by pollen and stimulates germination and pollen tube growth (Chen et al., 2000). The proposed role of the small Cys-rich pollen protein LAT52 is more complex. From antisense studies, LAT52 protein appears to be required for pollen hydration and germination (Muschietti et al., 1994). This positive regulation may
be mediated by LAT52 binding to the pollen LePRK2 kinase receptors (Tang et al., 2002). After these early events, the LAT52 protein may be displaced by a stigma factor, possibly LeSTIG1, which binds to the LePRK2 receptor and promotes pollen tube growth (Tang et al., 2004). In this way, LAT52 has an autocrine-like effect on several different aspects of pollen tube growth. SIPRALF-mediated inhibition of growth could have a role in modulating pollen tube growth at its earliest phase in response to an unfavorable environment, for example a nonreceptive immature stigma. Transient inhibition of pollen tube growth by SIPRALF could be abrogated by female factors once pollen tube growth is established within a receptive pistil.

Although the dynamics of endogenous SIPRALF protein production and processing are yet to be determined, our results indicate that the preproprotein appears to enter the endomembrane system, where it may be processed at a dibasic site similarly to other RALFs (Matos et al., 2008; Srivastava et al., 2009) and released into the medium as an active peptide (Fig. 2), indicating that the peptide hormone acts extracellu-

larly. The localization of the SIPRALF preprotein within pollen tubes and the active peptide in the medium is in accordance with the distribution of unprocessed and processed forms of several well-characterized peptide growth regulators (Iida and Shibata, 1994; Shankaran et al., 2008). These results are also consistent with the localization of a Nicotiana benthamiana RALF fused to GFP, which is located first to the endoplasmic reticulum and later to the cell wall in N. benthamiana leaf cells (Escobar et al., 2003). The extracellular location of SIPRALF also provides the opportunity for this peptide to interact with the cell wall-localized pollen LRX protein.

Exogenously added synthetic SIPRALF has a negative effect on pollen tube growth, and the peptide is effective only during a specific window in tube development (Fig. 11). SIPRALF effects are reversible within the first 20 min after pollen has been exposed to the peptide, and actively growing tubes become resistant to exogenously added SIPRALF once they are 40 to 60 μm long. The exit of the MGU from the pollen grain into the pollen tube provides a developmental reference point for the onset of resistance, in that both MGU movement and pollen sensitivity to exogenous SIPRALF characterize an early phase of pollen tube growth. How this window of sensitivity is delimited is not currently understood and will require further experimentation. A recent spatiotemporal study of membrane-related proteins during lily (Lilium longiflorum) pollen tube hydration, early germination, and later tube growth revealed that ion transport-associated proteins were particularly dynamic during early germination (Pertl et al., 2009). The determination of the as yet unknown identity of a putative membrane-localized SIPRALF-binding protein could be helpful in elucidating the dynamics of this process (Scheer et al., 2005). In any case, the results presented here are consistent with the idea that SIPRALF can regulate the elongation of pollen tubes within a specific developmental window.

A consistent picture of RALF function as a growth inhibitor is emerging from experiments with exogenously added peptide and gene regulation studies. Exogenous RALF peptide inhibits root and root hair growth, sugarcane microcalli development, and Arabidopsis hypocotyl elongation (Pearce et al., 2001; Mingossi et al., 2010). We have shown that exogenous SIPRALF peptide inhibits pollen tube growth. Overexpressed AtRLF1 and AtRLF23 causes stunted vegetative growth (Matos et al., 2008; Srivastava et al., 2009). In contrast, down-regulation of NaRALF promotes root growth and causes abnormal bulbous root hair growth, followed by cell bursting (Wu et al., 2007). Given these observations and our own results, it is possible that SIPRALF plays a role in regulating localized cell expansion in emerging pollen tubes to produce a cylindrical allometrically expanding structure with growth limited to the tip (Lancelle and Hepler, 1992; Geitmann and Dumais, 2009).

The mechanism(s) by which RALFs regulate cell growth will require further study. RALFs affect a number of processes, including proton flux, mitogen-activated protein kinase activity, and cytoplasmic Ca2+ levels (Pearce et al., 2001; Wu et al., 2007; Haruta et al.,

Figure 11. Summary of exogenous SIPRALF effects on pollen tube growth. Pollen tubes are sensitive to inhibition by SIPRALF peptide after hydration and early germination events, including glebula formation. This inhibition is reversible up to 15 min of exposure to peptide. Growing pollen tubes become resistant to exogenously added SIPRALF once they have reached lengths of 40 to 60 μm, when MGU migration is nearing completion.
A dramatic increase in extracellular pH was the basis for the initial discovery of RALF peptides. Our observations of an increased peptide effect with increased extracellular proton concentration (Fig. 7) are consistent with observations in roots of transgenic down-regulated NaRALF plants (Wu et al., 2007), wherein a decreased pH of the medium partially rescued an abnormal growth phenotype. If proton flux regulation is the primary action of RALFs, these peptides may function to modulate the rigidity of the extracellular landscape of the cells by influencing the activity of pH-sensitive cell wall modification proteins, including pectin methyltransferases, exo-α-glucanases, and/or expansins (Kotake et al., 2000; Cosgrove et al., 2002; Holdaway-Clarke and Hepler, 2003; Bosch and Hepler, 2005; Jiang et al., 2005; Sampredo and Cosgrove, 2005; Choi et al., 2006). Alternatively, RALFs could exert their effects intracellularly by modifying the pH of the cytoplasm. In pollen tubes, for example, an internal alkaline band that may be required for normal growth has been observed behind the growing tip (Feijo et al., 1999). It is possible that SIPRALF only acts in a localized fashion in vivo, perhaps increasing pollen tube elongation rates (e.g. by fine-tuning the localization of necessary proton fluxes). However, changes in proton fluxes may be downstream from initial events triggered by RALFs, as suggested by the observation that both down-regulation (Wu et al., 2007) and overexpression (Srivastava et al., 2009) of root-expressed RALFs result in transgenic plants being unable to acidify growth medium. These observations make a simple direct proton-pump activation model for RALFs improbable. Activation of mitogen-activated protein kinases occurs just as rapidly (within 5 min) as alkalization of cell medium (Pearce et al., 2001). RALFs can cause even more rapid changes in cytoplasmic Ca²⁺ concentration (Haruta et al., 2008). AtRALF1 was identified as the predominant peptide factor in seedling lysates that could elicit cytoplasmic Ca²⁺ elevation in Arabidopsis seedlings and roots within 40 s (at nanomolar concentrations equivalent to those used in these studies), suggesting that a Ca²⁺ signaling cascade may be the primary RALF response. It is well known that highly regulated ion flow is required for normal tube growth (Feijo et al., 1995, 1999; Messerli and Robinson, 1998; Messerli et al., 1999; Robinson and Messerli, 2002; Holdaway-Clarke and Hepler, 2003; Bosch and Hepler, 2005; Lovvy-Wheeler et al., 2006). In particular, a steep Ca²⁺ gradient at the pollen tube tip has been shown to be a critical regulator of pollen tube growth (Pierson et al., 1996; Hepler, 1997; Holdaway-Clarke et al., 2003). Given the well-characterized physiology of growing pollen tubes and their relatively simple cellular structure, pollen may provide the ideal system for probing the molecular mechanisms of RALF action. In any case, RALF peptides are likely to be key regulators of plant growth and development and are richly deserving of intensive investigation.

### MATERIALS AND METHODS

#### Plants

Tomato (Solanum lycopersicum ‘VF36’) plants were grown in a greenhouse in Pro Mix BX soil (Premier Horticulture) and supplemented weekly with 10:20:10 nitrogen:phosphorus:potassium fertilizer. Plants were grown throughout the year, and natural photoperiod was supplemented to a 12-h light (24°C)/12-h-dark (21°C) photoperiod. Mature flower-producing plants were used for pollen collection.

#### Pollen Collection

Tomato pollen was collected from stage 15 flowers (Brukhin et al., 2003) using a vibrating hand-held tooth polisher (Dental Concepts) into tubes with PGM. PGM solutions contained 24% (w/v) polyethylene glycol (PEG) 4000, 0.01% (w/v) boric acid, 2% (w/v) Suc, 20 mM MES buffer, pH 6.0, 3 mM Ca (NO₃)₂·4H₂O, 0.02% (w/v) MgSO₄·7H₂O, and 1 mM KNO₃. To prevent pollen from clumping in PGM, the collection tubes were vortexed for 15 s using the tooth polisher. Aliquots of the pollen/PGM mixture were then immediately further diluted for each experiment as described below.

#### Yeast Two-Hybrid Screen

A yeast two-hybrid library constructed using mature tomato cv VF36 pollen cDNA cloned into pAD-GAL4-2.1 (Stratagene) was a generous gift from Dr. Sheila McCormick (Tang et al., 2002). Sequences encoding the recognition domain (amino acids 22–392, from the mature N terminus to the end of the Cys-rich region adjacent to the extensin domain) of the tomato pollen LXR gene (Stratford et al., 2001) were cloned in-frame with the DNA-binding domain of pBD-GAL4 Cam (Stratagene) and used as bait. A modified lithium acetate method (Gietz et al., 1992) was used to transform Pfeno-4A yeast cells (James et al., 1996) with bait plasmid, and positive transformants were transformed with DNA from the pAD-GAL4 pollen library. Selection was on synthetic complete medium lacking His, Leu, and Trp and containing 3 μg 3-amino-1,2,4-triazole.

#### Sequence Alignment

Sequences were aligned using the MUSCLE algorithm (Edgar, 2004) within Jalview (Clamp et al., 2004). The alignments were then edited by eye to produce the final alignment and colored using Boxshade (http://bioweb.pasteur.fr/seqanal/interfaces/boxshade.html).

#### RNA Gel-Blot Analysis

Tissue was harvested from tomato plants, frozen in liquid nitrogen, and stored at −80°C until processed. Tissue was either powdered using a mortar and pestle in liquid nitrogen and added to Trizol (Invitrogen) or Trizol was added to the frozen tissue, which was then disrupted using a micropestle attached to an electric motor. All subsequent steps followed the manufacturer’s protocol. Five micrograms of total RNA from each tissue was electrophoresed on a 1.2% (w/v) agarose gel containing 2.2M formaldehyde, transferred to a nylon membrane, and probed for the presence of SIPRALF RNA using standard protocols (Sambrook et al., 1989). The probe, prepared using the RediPrime II kit (Amerham Biosciences) according to the manufacturer’s protocol, consisted of the Pol2 cDNA clone (encoding 48 of the 50 amino acid residues in the putative active peptide). The washed blot was exposed to a phosphorimaging screen and read using a Storm model 840 (Molecular Dynamics).

#### Antibodies

Polyclonal antibodies were raised in a rabbit using a recombinant fusion protein composed of mature SIPRALF sequences fused to glutathione S-transferase (GST) as an antigen at La Trobe University. IgG fractions from immune and preimmune sera were purified using protein A-Sepharose. Anti-SIPRALF antibodies were further purified from anti-GST antibodies before use using recombinant GST protein bound to glutathione-Sepharose.

#### Immunolocalization

Tomato pollen was germinated in PGM for 1 h and pelleted at 3,000 rpm for 1 min. Pollen tubes were fixed in HistoChoice (Amresco) for 20 min and

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washed three times in wash buffer (50 mM Tris-0.15 mM NaCl [pH 7.5] containing 0.05% Tween 20, 0.02% sodium azide, and 1% bovine serum albumin). The sample was divided into two aliquots and incubated with either immune serum or preimmune serum diluted 1:500 in wash buffer for 2 to 3 h. Pollen tubes were washed four times in wash buffer and incubated in goat anti-rabbit antibodies conjugated with either tetramethyl rhodamine isothiocyanate (TRITC) or fluorescein isothiocyanate (FITC; Sigma-Aldrich) at 1:1,000 dilution in wash buffer. Samples were washed three times in wash buffer and imaged using an Olympus inverted microscope (model IX18) equipped with a CSU22 spinning-disc confocal system. The samples were excited with 473-nm (for FITC) or 561-nm (for TRITC) lasers, and fluorescent and differential interference contrast images were collected using Slide Book version 5 software.

**PGM and Pollen Protein Analysis**

Since PEG interfered with the analysis of proteins in the medium, pollen was germinated for 1 h in a PEG-free medium. In the modified PGM, PEG was substituted with 12% Suc and 2% raffinose. Pollen proteins were extracted by maceration and heated to 70°C for 10 min in SDS sample buffer containing sample reducing agent as recommended by the manufacturer (Invitrogen). Proteins of all sizes were efficiently retrieved from PGM using a chloroform extraction method (Obayama et al., 2008). Protein was quantified using a Bradford protein assay. A mixture of microsomal and Bio-Rad ProtoBlue reagents was separated on a HPLC gel (4-12% acrylamide gradient; Invitrogen) and blotted onto Immobilon-PSV membranes (Millipore). Blots were blocked in 2% gelatin and probed with SIRPRLF antiserum (1:5,000 dilution). Signals were detected by chemiluminescence using horseradish peroxidase-conjugated goat anti-rabbit secondary antiserum.

**SIRPRLF and alkSIRPRLF Synthesis**

The synthetic peptide sequence was based upon the Solanum pennellii unigene SCN-US24197 from the SGN Web site (http://www.sgn.cornell.edu/). SIRPRLF was synthesized by 9-fluorenylmethyl chloroformate solid-phase synthesis with p-methyl benzhydrylamine resin according to the manufacturer’s protocol (model 431A; Applied Biosystems). The synthesized peptide was oxidized as described previously (Pearce et al., 2001). A mixture of oxidized products was obtained, and the active form was identified by fractionation on strong cation-exchange HPLC and utilization of the alkalization assay (Pearce et al., 2001). Strong cation-exchange HPLC was performed on a poly-SULPHOETHYL aspartamide column (5 μm, 4.6 × 200 mm; Nest Group). The column was equilibrated in 5 mM potassium chloride, pH 3, in 25% (v/v) acetonitrile, and the oxidized SIRPRLF (20 mg) was loaded onto the column. After 2 min, a 90-min gradient was applied to 100% (v/v) elution buffer, consisting of 5 mM potassium phosphate and 1 M potassium chloride, pH 3, in 25% (v/v) acetonitrile. A flow rate of 1 mL min⁻¹ was employed, and 1-min fractions were collected and assayed in the suspension cell alkalization assay as described below. The active fraction (72-74 min) was desalted by C18 HPLC and lyophilized.

Active synthetic SIRPRLF was reduced and alkylated using diithothreitol and iodoacetamide as described previously (Pearce et al., 2001). The resulting alkSIRPRLF peptide displayed no activity in the Arabidopsis (Arabidopsis thaliana) suspension cell alkalization assay.

Oxidized and alkylated SIRPRLF were analyzed for correctness and purity by matrix-assisted laser desorption-ionization mass spectrometry using a PerSeptive Biosystems Voyager time of flight mass spectrometer with α-cyano-4-hydroxycinnamic acid as the matrix.

The disulfide linkages of the purified, oxidized SIRPRLF were determined by fragmenting the peptide by cyanogen bromide treatment to cleave the peptide at Met⁹ and Met⁵⁹ and analyzing the products by matrix-assisted laser desorption-ionization mass spectrometry. Major mass peaks found after cyanogen bromide treatment included 3,404 D (amino acids 1–29), 2,274 D (amino acids 30–48), and 2,405 D (amino acids 10–29). These masses indicate cyanogen bromide treatment included 3,404 D (amino acids 1–29), 2,274 D (amino acids 30–48), and 2,405 D (amino acids 10–29), and 2,405 D (amino acids 10–29). These masses indicate cyanogen bromide treatment included 3,404 D (amino acids 1–29), 2,274 D (amino acids 30–48), and 2,405 D (amino acids 10–29).

**SlPRALF Regulation of Pollen Tube Elongation**

**Pollen Viability Staining**

Pollen exposed to SIRPRLF peptide was assessed for viability using fluorescein diacetate (FDA). Pollen was collected as described above and immediately diluted 1:10 into PGM plus or minus 1 μM SIRPRLF, and a negative “heat-killed” control aliquot was exposed to 50°C for 10 min. Aliquots were transferred into individual wells of a flat-bottom 96-well microtiter plate and allowed to germinate and grow for 1 h. At 1 h, FDA was added to a final concentration of 0.2 mg mL⁻¹. After a 10-min incubation with FDA at room temperature, images were captured using a Nikon DIAPHOT-TMD inverted microscope, a Photometrics Cool Snap CF camera, and Image Pro Software at 100× magnification with UV excitation and a long-pass GFP emission filter (FDA has a broad range emission, with a peak at 518 nm). A minimum of 100 pollen grains were counted for each treatment.

**Assay for Effects of SIRPRLF Peptides on Pollen Hydration and Germination**

Pollen from five flowers was collected separately into tubes containing PGM, vortexed, and further diluted 1:10 into individual treatment reaction tubes containing peptide at final concentrations of 0, 0.005, 0.01, 0.025, 0.05, 0.01, 0.25, 0.5, and 1 μM. Each treatment tube was vortexed for 10 s after addition of pollen. Aliquots of 100 μL each from the reaction were transferred into wells of a flat-bottomed 96-well microtiter plate, which was then enclosed in a humidified container. Pollen in wells was examined using an inverted microscope as described previously. Bright-field images encompassing at least 100 pollen grains were photographed at 100× magnification for each treatment reaction after 1 and 3 h.

Pollen was classified based upon specific morphological characteristics. Nonhydrated pollen grains (NHP) were classified by their oblong morphology, which resembles a deflated football, as opposed to round hydrated pollen. Quantification of NHP was scored as the percentage of the total pollen hydrated for each peptide concentration after 1 h. NHP was calculated using data with pollen from five flowers for each concentration of peptide after 1 h of germination. Similar results were obtained with SIRPRLF treatment for 3 h and for alkSIRPRLF treatment for 3 and 1 h (data not shown).

After 1 and 3 h of peptide treatment, hydrated pollen grains were categorized into four classifications. The first class consisted of hydrated pollen with TGR. The average hydrated pollen radius is 15 μm. The second class consists of grains with no apparent tube or protrusion, designated as NGP (but hydrated) grains. The third class is pollen with TLP. The average hydrated pollen radius is 20 μm. The fourth class is pollen with TLR. The average hydrated pollen radius is 20 μm. The average hydrated pollen radius is 20 μm. The average hydrated pollen radius is 20 μm. The average hydrated pollen radius is 20 μm. The average hydrated pollen radius is 20 μm. The average hydrated pollen radius is 20 μm. The average hydrated pollen radius is 20 μm.

**pH Effects on Pollen Germination and Growth**

Pollen was collected separately from three flowers into nonbuffered PGM (pH 6.07) and diluted 1:10 into PGM with 20 mM MES buffered to pH 5.5, 6.5, or 7. Each treatment tube was vortexed for 10 s after addition of pollen. After 1 h, samples encompassing at least 100 pollen grains were photographed at 100× magnification for each pH treatment and categorized into four morphological classifications as described previously. The TGR class of hydrated pollen grain was scored and represented as a percentage of total hydrated pollen ± se for each peptide concentration.

**Reversibility Tests**

Pollen was collected separately from three flowers and further diluted 1:10 in PGM. One sample was incubated at room temperature in plain PGM (“no-SIRPRLF” control), while the other samples were incubated in PGM plus 0.1 μM SIRPRLF. Exposure times were 5, 10, 15, 20, 40, and 60 min. After the designated exposure time, tubes were spun at 13,000 rpm for 30 s and an aspirator was used to carefully remove the supernatant from the pollen pellet. Pollen in the no-SIRPRLF control and the “SIRPRLF-removed” SIRPRLF-treated pellets were resuspended in plain PGM. The “constant-SIRPRLF”
pellet was resuspended in PGM containing 0.1 μM SIPRALF. Each treatment aliquot was gently vortexed for 10 s at a low setting to resuspend the pollen pellet. Images were collected as described previously 1 h after resuspension. The average percentages of TGR ≤ SE, TLR ≥ SE, NHP ≥ SE, and BP ≤ SE were calculated for three replicates of each treatment at each exposure time. To confirm that centrifugation does not alter pollen tube growth, additional noncentrifuged pollen samples with either 0 or 0.1 μM SIPRALF were compared with centrifuged samples. At least 100 hydrated pollen grains were analyzed for each treatment time.

Assay for Effects of SIPRALF on Pollen Tube Elongation

Pollen from three flowers was collected separately in PGM, further diluted 1:10 into PGM, and allowed to germinate and grow for 40, 50, 60, or 90, min. Two 50-μL aliquots were transferred into separate wells for each time treatment interval. One aliquot received an equal volume of PGM as an untreated control, and the other received an equal volume of 0.2 μM SIPRALF in PGM for a final concentration of 0.1 μM SIPRALF. Images were collected as described previously. Pollen tubes were imaged after 0.5, 1, and 2 h from the time of peptide addition. For simplicity, only the data for 2 h after peptide addition are shown. Pollen tube lengths were measured using ImageJ 1.38u (http://rsb.info.nih.gov/ij/) immediately before peptide addition and 2 h after peptide addition (with or without 0.1 μM SIPRALF treatments). Tube lengths for each time treatment were grouped in bins of 10 μm as frequencies of lengths. The mean pollen tube length ± SE was calculated for the bell curve of each treatment. Calculation of the mean for data demonstrating a bimodal distribution was determined by manually defining a cutoff value of the two peaks. The peak at shorter pollen tube lengths (P1) was then calculated separately from the longer pollen tube length peak (P2). At least 100 pollen grains were analyzed for each treatment.

Pollen DNA Staining

Pollen was collected separately from three flowers and then diluted 1:5 into PGM. Pollen was germinated for 1 to 3 h. Pollen was then fixed by carefully removing the pollen from the pollen and replacing it with a fixative solution of ethanol/glacial acetic acid (3:1, v/v). To stain, fixative was removed from the pollen, gently mixed with 10 μL of 0.4 mg mL⁻¹ DAPI in buffer (0.1 M NaH₂PO₄, pH 7, 1 mM EDTA, and 0.1% [v/v] Triton X-100), and stained for 15 min. Stained pollen was imaged using oil immersion at 400× with excitation filters (461 nm) and emission filter (471 nm). Tube length and the distance of the leading edge of the pollen tube to the tip of the pollen grain were measured as described previously. Tube lengths were grouped in bins of 10 μm. Any tube length greater than 80 μm was placed in one bin, 80+. For each bin length, the frequency of nuclear migration was scored. Tubes that did not show any migration of the leading edge of the VN or GC into the pollen tube were scored as “neither out,” and those that showed at least one, or both, leading edge of the VN or GC migrating into the pollen tube were scored as “one out” or “both out,” respectively.

Sequence data from this article can be found in the SGN (www.sgn.cornell.edu) data library under accession numbers SGN_U316452 (SIRALF), SGN_U324197 (SIPRALF), and SGN_U207478 (PhanRHALF) and in the GenBank/EMBL data library under the Arabidopsis Genome Initiative accession number A1g28270 (AIRALFL4).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Vegetative SIRALF has a reduced effect on pollen.

Supplemental Figure S2. SIPRALF inhibition reversibility for abnormal pollen morphology classes.

Supplemental Table S1. Effect of SIPRALF on media pH.

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