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Research article

Biological activity of nine recombinant AtRALF peptides: Implications for their perception and function in *Arabidopsis*

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ABSTRACT

RALF is a small (5 kDa) and ubiquitous plant peptide signal. It was first isolated from tobacco leaf protein extracts owing to its capacity to alkalinize the extracellular media of cell suspensions. RALFs inhibit root growth and hypocotyl elongation, and a role for RALFs in cell expansion has also been proposed. Arabidopsis has 37 RALF isoforms (AtRALF), but only a small group of nine has high primary structure identity to the original RALF peptide isolated from tobacco. Herein, we report the heterologous production of these nine peptides in *Escherichia coli* and the evaluation of their activity in five biological assays. All AtRALF peptides produced showed strong alkalinizing activities, with the exception of the pollen-specific isoform AtRALF4. Although it exhibited no inhibitory activity in the root growth and hypocotyl elongation assays, AtRALF4 is a strong inhibitor of pollen germination. Our data demonstrate that the divergence in the tissue specificity and gene expression patterns of the different AtRALFs does not change the fact that their main role seems to be the regulation of cell expansion. Furthermore, different activities in the alkalinization assays upon the addition of two consecutive and saturating doses of the peptides suggest that the peptides are likely being sensed by specific receptors.

1. Introduction

Peptide signals are small proteins involved in intercellular communication and the coordination of different processes, such as growth, development, defense and reproduction (Ryan et al., 2002; Matsubayashi and Sakagami, 2006; Sugano et al., 2010; Kondo et al., 2011; Whitford et al., 2012). RALF is a secreted, 5 kDa peptide signal that was first isolated from tobacco leaves (Pearce et al., 2001). Since its discovery, homologs of the RALF peptide have been biochemically isolated from several species (Pearce et al., 2001; Haruta and Constabel, 2003; Neumann, 2007; Haruta et al., 2008; Mingossi et al., 2010), and their genes have been either cloned or simply identified from almost all genomes that have been searched (Pearce et al., 2001; Germain et al., 2005; Zhang et al., 2011; Li et al., 2011; Combier et al., 2008). RALF genes are found either as single copy genes, as in the native tobacco plant *Nicotiana attenuata*, or as multigenic families, as in the model plants *Arabidopsis*, rice, poplar and maize (Wu et al., 2007; Cao and Shi, 2012). The biological activities of RALF peptides have been associated with alkalinization of the extracellular medium of suspension cells, activation of a MAP kinase, mobilization of calcium, and inhibition of root growth and cell expansion (Pearce et al., 2001; Haruta et al., 2008; Mingossi et al., 2010; Covey et al., 2010). RALF peptides are processed from larger precursors of up to 134 amino acids. The mature active peptide is located at the C-terminus of the precursor and is processed by a subtilase family of proteases (Matos et al., 2008; Srivastava et al., 2009). A truncated tobacco RALF that was fused to the green fluorescent protein (GFP) and that was transiently

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expressed in tobacco plants showed fluorescence in the endoplasmic reticulum and in the apoplast (Escobar et al., 2003).

The mature peptide has two disulfide bridges that are essential for its activity (Pearce et al., 2001). Although the size of RALF peptides limits more common techniques such as alanine scanning to perform structure-activity studies, Pearce et al. (2010) found that the tomato peptide SlRALF loses activity when the first eight amino acids including a conserved motif – YISY – are deleted. A single substitution of the Ile with an Ala in the – YISY – motif renders SlRALF inactive. Synthesis of two SlRALF fragments that incorporate the entire RALF sequence, when added simultaneously in the alkalinization assay, were totally inactive (Pearce et al., 2010).

In Arabidopsis, RALF peptides (AtRALFs) comprise a family of 37 members with highly diverse expression patterns (Cao and Shi, 2012; Olsen et al., 2002; Lamesch et al., 2011). A recombinant version of the AtRALF1 peptide, an Arabidopsis RALF isoform expressed mainly in roots, was as active as the native tomato RALF in the alkalinization assay and inhibited the hypocotyl elongation of Arabidopsis plants (Mingossi et al., 2010).

We show here that His-tagged recombinant AtRALF peptides have the same activity as a synthetic RALF peptide, making their use instrumental in analyses of RALF perception and function. Of the nine AtRALFs chosen for the study, only the pollen-specific isoform AtRALF4 showed no activity in the alkalinization assay; the calculated half-maximal activities of all the other peptides were in the low nanomolar range. Neither root nor hypocotyl growth were inhibited by AtRALF4, whereas all other AtRALF peptides tested showed inhibitory activities characteristic of RALF. Despite the poor performance of the AtRALF4 isoform in several bioassays, the peptide was able to inhibit the germination of over 80% of the pollen grains in the pollen germination assay. Our results demonstrate that although the AtRALF peptides diverge in their tissue-specific gene expression patterns, their role in the regulation of cell expansion seems to be preserved. These results prompted us to investigate how specific the perception of AtRALF peptides would be. We have evaluated the specificity of perception by measuring the alkalinization caused by each peptide when applied immediately following a saturating dose of one of the other peptides. Although some promiscuous binding of the peptides may occur, our results suggest the existence of a family of receptors specific for each AtRALF peptide.

2. Results

2.1. Production, purification and alkalinizing activity of nine $_{His}$AtRALFs

RALF peptides in Arabidopsis form a gene family of 37 homologous members that may play similar roles. To evaluate how they diverge functionally, we selected a subgroup of nine isoforms and generated plasmids to heterologously express the recombinant peptides in *Escherichia coli*. The specific isoforms were selected based on the similarity between their primary structures and that of the original RALF peptide isolated from tobacco leaves (Pearce et al., 2001) (Supplementary data, dotted box in Fig. S1). The selected isoforms have heterogeneous expression patterns: two isoforms, AtRALF1 and 4, are specific to roots and inflorescences, respectively; another five, AtRALF22, 24, 31, 33 and 34, are mainly expressed at different levels in leaves and inflorescences; and two, AtRALF19 and 23, are ubiquitously expressed (Supplementary data, Fig. S2). Because our data and conclusions are dependent on how accurately the recombinant peptides recapitulate the biological activity of the native peptides, we first evaluated if the addition of a His-tag at the N- or C-terminus would modify the biological activity of the peptides. Considering the high primary structure conservation among AtRALFs, we chose the AtRALF1 isoform and compared the performance of the recombinant peptides with a synthetic version of the native AtRALF1. In the alkalinization assay, the recombinant AtRALF1 peptides, with a His-tag at either the N-terminus ($_{His}$AtRALF1) or the C-terminus (AtRALF1$_{His}$), increased the pH of the cell suspension media to a similar level as the synthetic AtRALF1 peptide ($_{syn}$AtRALF1) (Fig. 1A). The half-maximal activities, estimated using double-reciprocal plots, were 4.1, 4.6 and 4.4 nM for the $_{syn}$AtRALF1, $_{His}$AtRALF1 and AtRALF1$_{His}$ peptides, respectively (Fig. 1B).

Using a standard Ni^{2+}-resin affinity-purification protocol, we produced up to 2 mg of purified recombinant AtRALF peptide per liter of bacterial culture. After affinity purification, the peptides were extensively dialyzed against 1% (v/v) formic acid and later freeze-dried. Peptides resuspended in 0.1% formic acid were run on HPLC for quantification and evaluation of purity. When the purified $_{His}$AtRALFs were separated on an SDS-PAGE gel, bands with the expected molecular weight were detected for each peptide (Fig. 2A). Antibodies raised against $_{His}$AtRALF1 recognized all the purified peptides, but with different strengths. As little as 0.1 μg was enough for the detection of $_{His}$AtRALF1, 22, 23 and 33, whereas ten-fold higher levels of the $_{His}$AtRALF4, 19, 24, 31 and 34 peptides

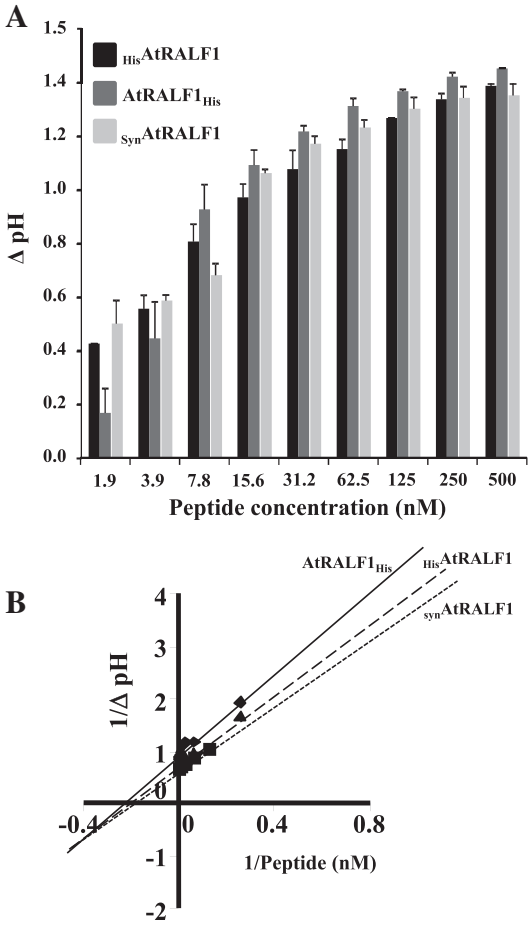

Fig. 1. The effect of adding a Histidine-tag at either the N-terminus ($_{His}$AtRALF1) or the C-terminus (AtRALF1$_{His}$) on the activity of the AtRALF1 peptide in the alkalinization assay. Comparison of the alkalinization activity of the recombinant AtRALF1 peptides with the synthetic AtRALF1 (synAtRALF1) at 1.9, 3.9, 7.8, 15.6, 31.2, 62.5, 125, 250, and 500 nM concentrations (A). Lineweaver-Burk plot used to estimate the half-maximal activity values of the peptides (B). Values are the means ± SD of two independent experiments with three replicates each. Experiments were repeated at least three times.

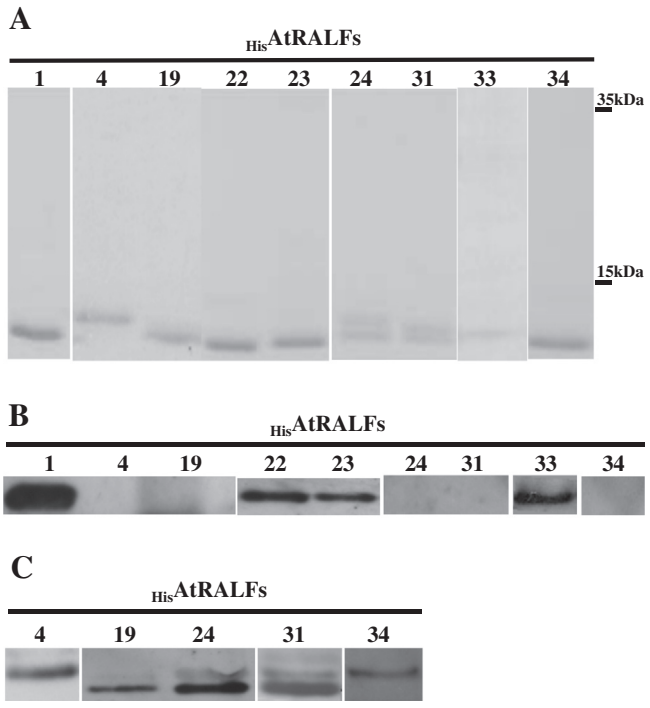

Fig. 2. Purified recombinant $_{His}$AtRALF peptides. For each $_{His}$AtRALF peptide, 1 μg was separated on a 12.5% SDS-PAGE gel followed by Coomassie staining (A). Numbers at the top identify each $_{His}$AtRALF peptide and marks on the right indicate the position of molecular weight markers. Protein blots probed with anti-AtRALF1 antibody. Lanes were loaded with 0.1 μg (B) or 1 μg (C) of each $_{His}$AtRALF peptide.

Table 1
Half-maximal activities of the recombinant AtRALFs in the alkalinization assay.

Peptide	Half-maximal activity[a]
$_{His}$AtRALF1	4.1 nM
$_{His}$AtRALF4	No
$_{His}$AtRALF19	4.6 nM
$_{His}$AtRALF22	8.1 nM
$_{His}$AtRALF23	33.2 nM
$_{His}$AtRALF24	32.0 nM
$_{His}$AtRALF31	10.0 nM
$_{His}$AtRALF33	6.1 nM
$_{His}$AtRALF34	5.8 nM

[a] values estimated using up to 500 nM and measuring the pH changes 5 min after the addition of the peptide. No, non-observed.

were required for detection (Fig. 2B and C). HPLC profiles and mass spectrometer data confirmed the purity and identity, respectively, of the AtRALF peptides (Supplementary data, Fig. S3 and S4A-S4I). AtRALF19, 24 and 31 ran as double bands on SDS-PAGE, and both bands were recognized by the anti-$_{His}$AtRALF1 antibody. Based on the protein blot, HPLC and mass data, we conclude that the double band pattern resulted from abnormal migration rather than contamination.

RALF peptides were originally isolated based on their capacity to rapidly increase the pH of the extracellular medium of cell suspensions (Pearce et al., 2001). With the exception of the $_{His}$AtRALF4 peptide, all the other recombinant $_{His}$AtRALF peptides tested were able to rapidly induce the alkalinization of the cell suspension medium at concentrations in the low nanomolar range (Fig. 3). $_{His}$AtRALF23 caused a pH change of 1.2 units in 5 min, which was the highest pH difference; all the others varied between 0.8 and 1.0 pH units. The estimated half-maximal activities for the peptides ranged from 4.1 to 33.2 nM (Table 1). The $_{His}$AtRALF4 peptide showed no alkalinizing activity in the assay, and significant increases in the extracellular pH (>0.2 units) were detected only when measurements were taken after 30 min and at peptide concentrations above 200 nM (Supplementary data, Fig. S5). Although an activity plateau could not be reached for $_{His}$AtRALF4, even after adding micromolar concentrations of the peptide, the estimated activity based on the data collected at 30 min shows that its half-maximal activity is in the micromolar range.

2.2. Effect of nine $_{His}$AtRALFs on root growth and hypocotyl elongation

An inhibitory effect on root growth and development is observed when tomato or Arabidopsis seedlings are incubated with 10 μM tomato RALF (Pearce et al., 2001). We observed a similar effect on Arabidopsis roots when they were incubated with 10 μM $_{His}$AtRALF1, 19, 22, 23, 24, 31, 33 and 34 (Fig. 4A). The greatest inhibition was observed with AtRALF22, 23 and 33. Arabidopsis seedlings incubated with 10 μM $_{His}$AtRALF4 were indistinguishable from seedlings treated with water only (control treatment). A more sensitive biological assay for the effect of RALF peptides is the

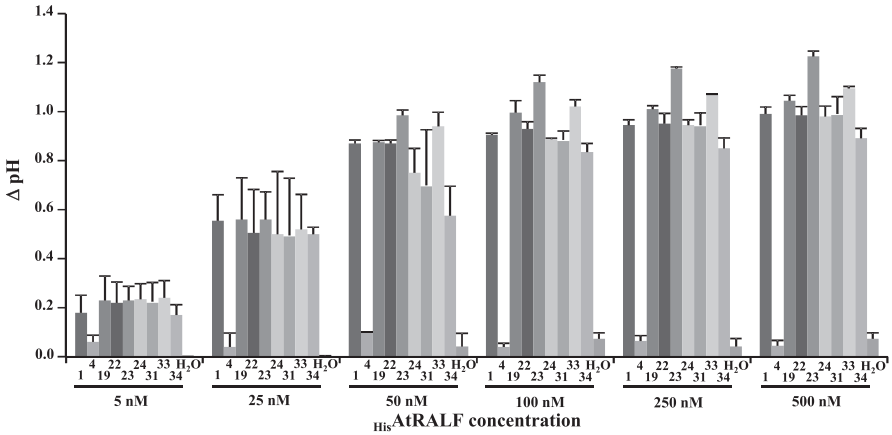

Fig. 3. Alkalinization activity of the nine AtRALF peptides. Six different concentrations (5, 25, 50, 100, 250 and 500 nM) were evaluated and the change in pH was measured after 5 min. $_{His}$AtRALF1 and water were included for comparison and as a control. Values are the means ± SD of two independent experiments with three replicates each. Experiments were repeated at least twice.

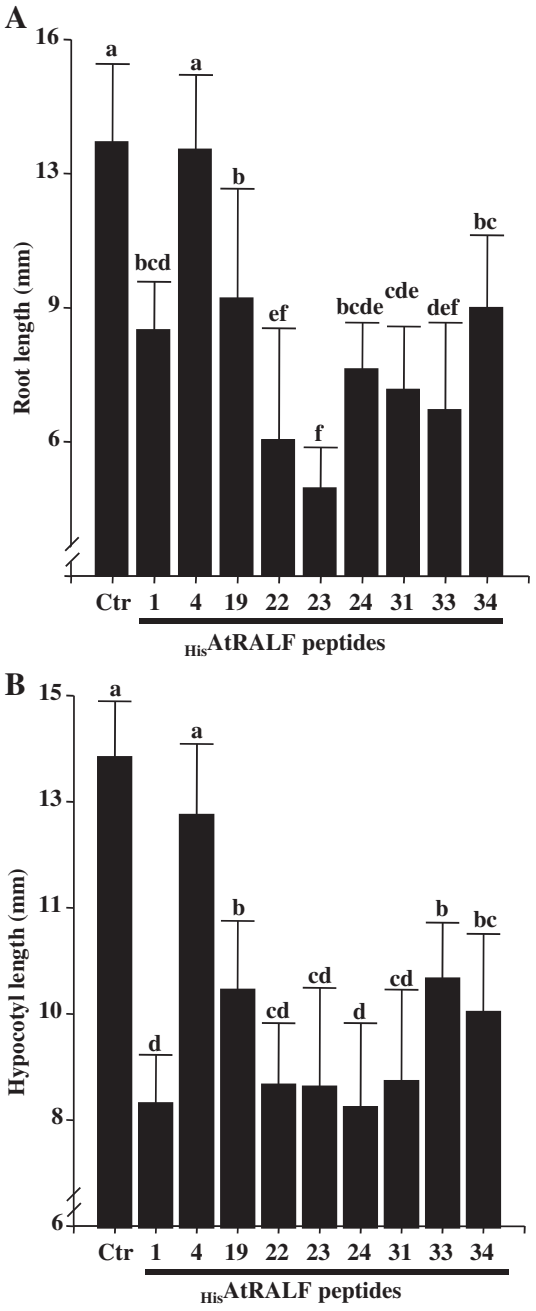

Fig. 4. Effect of $_{His}$AtRALFs on root growth and hypocotyl elongation. $_{His}$AtRALF inhibition of root growth (A). Arabidopsis seeds were germinated in half-strength MS liquid medium; after two days, 10 μM of each $_{His}$AtRALF peptide, or an equal volume of water (Ctr), was added to the medium. Root growth was measured after three days of exposure to the peptides. $_{His}$AtRALF inhibition of hypocotyl elongation (B). Arabidopsis seeds were germinated in the dark in half-strength MS liquid medium; after two days, 1 μM of each $_{His}$AtRALF peptide, or an equal volume of water (Ctr), was added to the medium. Hypocotyl length was measured after four days of exposure to the peptides. Values are the means ± SD of at least 30 seedlings. The means with the same letter are not significantly different from each other (Tukey test, $P \leq 0.01$). The experiment was repeated at least three times with similar results.

evaluation of dark-grown hypocotyl elongation in the presence or absence of 1 μM RALF (Mingossi et al., 2010). $_{His}$AtRALF1, 19, 22, 23, 24, 31, 33 and 34 inhibited hypocotyl elongation, and again the only exception was $_{His}$AtRALF4 (Fig. 4B). Treatment with AtRALF1, 22, 23, 24, and 31 produced the shortest hypocotyls.

2.3. Effect of nine $_{His}$AtRALFs on pollen germination

Covey et al. (2010) showed that a pollen-specific RALF could inhibit pollen germination at nanomolar concentrations. We tested all the recombinant peptides to evaluate their capacity to inhibit pollen germination: $_{His}$AtRALF1, 23 and 34 failed to inhibit at 10 nM concentrations (Fig. 5), $_{His}$AtRALF31 was weakly inhibitory (>50%) and $_{His}$AtRALF4, 19 and 33 were the strongest inhibitors (<20%). An intermediate germination rate was observed when either $_{His}$AtRALF22 or 24 was added to the media (Fig. 5). We did not observe a specific effect on pollen hydration for any of the AtRALFs tested (Supplementary data, Table S2).

2.4. Ca^{2+} mobilization by $_{His}$AtRALFs measured by the Ca^{2+} sensor protein aequorin

RALF peptides mobilize Ca^{2+} from extracellular and intracellular stores, and the luminescent Ca^{2+} sensor protein aequorin is an established tool for monitoring Ca^{2+} mobilization *in vivo* (Haruta et al., 2008). Recombinant $_{His}$AtRALF1 induced an increase in the cytosolic Ca^{2+} concentration within 40 s of the peptide being applied, just as previously reported by Haruta et al. (2008) (Fig. 6A). None of the other $_{His}$AtRALFs tested showed similar Ca^{2+} induction. $_{His}$AtRALF22-induced Ca^{2+} elevation also peaked at 40 s but with approximately half the intensity observed for $_{His}$AtRALF1. Total luminescence counts over a period of 160 s clearly show the strong Ca^{2+} elevation induced by $_{His}$AtRALF1 treatment and the minor Ca^{2+} changes produced by the other peptides (Fig. 6B). Increased responses were observed when 10-fold higher concentrations of the $_{His}$AtRALF peptides 19, 22, 23, 24, 31, 33 and 34 were used. $_{His}$AtRALF1 showed no change in the Ca^{2+} mobilization response when used at 100 nM $_{His}$AtRALF4 caused no Ca^{2+} induction, even at a concentration of 100 nM (Fig. 6B).

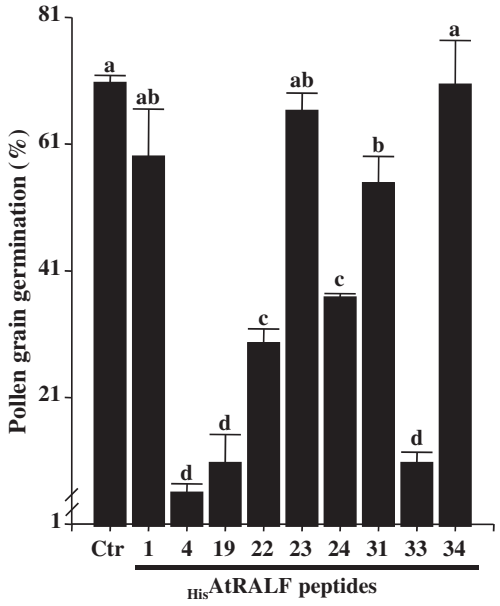

Fig. 5. Effect of $_{His}$AtRALFs on pollen germination. Pollen grains were distributed on top of the pollen germination medium (PGM) with or without (control, Ctr) 10 nM of each $_{His}$AtRALF peptide. Pollen germination was evaluated after the glass slides containing the PGM and pollen grains remained for 18 h in a humidified chamber. Values are means ± SD of three experiments ($N = 60$ pollen grains for each experiment). The means with the same letter are not significantly different from each other (Tukey test, $P \leq 0.01$).

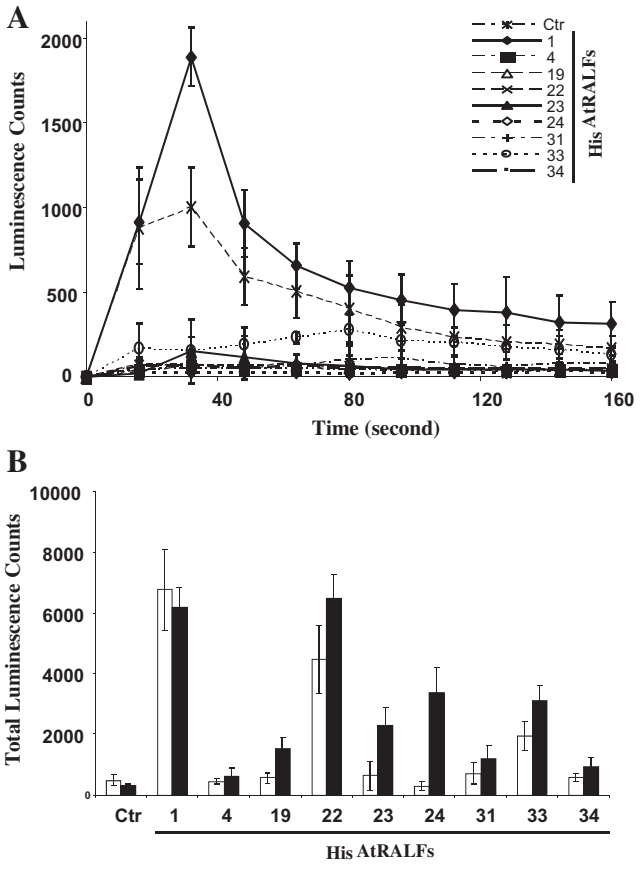

Fig. 6. Effect of recombinant $_{His}$AtRALFs on Ca^{2+} mobilization. Kinetics of Ca^{2+} mobilization triggered by 10 nM of each $_{His}$AtRALF (A). The results are presented as the means ± SD of three measurements. Total activity of each AtRALF peptide measured over 160 s (B). A total of 10 time-points was measured and summed at 10 nM (empty bars) and 100 nM (solid bars) concentrations. The results are the means ± SD of three measurements. The experiment was repeated twice with similar results.

2.5. Alkalinizing activity of $_{His}$AtRALFs when applied following saturating doses of other $_{His}$AtRALFs

Our assays to determine the activities of the recombinant AtRALF peptides have different sensitivities, but they all suggest specificity in the perception of each peptide. Based on the alkalinization, pollen germination and Ca^{2+} mobilization assays, it is clear that at least two or three different pathways for sensing the peptides are functioning in the same tissue. These results prompted us to investigate the diversity of AtRALF perception. We used the alkalinization of the extracellular medium of suspension cells to evaluate whether two different peptides applied sequentially as a first and second dose would be able to induce a higher alkalinization response than that produced when the same peptide was applied as both the first and second doses. We used saturating concentrations (1 μM) for both doses and interpreted further alkalinization after a second dose as evidence that a different set of receptors was being turned on. Our rationale is that if the same peptide does not elicit an alkalinization response when applied as second dose but a different peptide does, then the peptide that elicits the alkalinization response as a second dose is likely using a different receptor(s). Two controls were essential for this experiment: the demonstration that a second dose of the same peptide does not cause an extra increase in the pH, which would provide evidence that saturation has been reached; and the demonstration that the cells are still able to respond after a saturating dose of the peptide. This second control was performed using AtPep1, a peptide of a different nature that also induces an alkalinization response and for which the receptors are known (Huffaker et al., 2006; Yamaguchi et al., 2006). With that hypothesis in mind, we tested all the peptides as the first dose with all the peptides as the second dose (Fig. 7). When the same peptide was applied sequentially as both the first and second doses, the alkalinization response was identical to the response produced by a single dose of that same peptide (Fig. 7, intercept of rows and columns: $_{His}$AtRALF1–$_{His}$AtRALF1, $_{His}$AtRALF4–$_{His}$AtRALF4, …, $_{His}$AtRALF34-$_{His}$AtRALF34). Note that $_{His}$AtRALF4 caused detectable alkalinization because a saturating concentration, 1 μM, was used in the assays. Arabidopsis suspension cells induced an additional pH increase when AtPep1 was applied as a second dose after each of the 9 recombinant $_{His}$AtRALFs was used as a first dose, demonstrating that the cells are able to respond to a second stimulus (Fig. 7, first row). All the peptides induced alkalinization when used as a second dose after using $_{His}$AtRALF4 as a first dose (Fig. 7, second column). None of the $_{His}$AtRALFs produced any additional alkalinization when applied as the second dose following $_{His}$AtRALF23 as the first dose; however, when used as the second dose, $_{His}$AtRALF23 induced alkalinization regardless of which peptide was used first. All activities showed dependence on the peptide used as the second dose and also on the order in which they were applied, namely, as the first or second dose. To untangle the data produced by the alkalinization assays, we assigned plus and minus values to the presence and absence of further alkalinization after a second dose, respectively, and performed a cluster analysis in an attempt to group peptides with similar response patterns (Fig. 8, see M&M for details). The analysis produced a cluster containing the peptides $_{His}$AtRALF1, 22, 24, 31 and 33 and also indicated a separate group formed by $_{His}$AtRALF19 and 34. Both $_{His}$AtRALF4 and 23 were ungrouped.

2.6. Effect of suramin on the alkalinizing activities of $_{His}$AtRALFs

Another way to dissect the different ways that cell suspensions perceive the AtRALF peptides is by using the drug suramin. Suramin is a polysulfonated naphthylurea molecule known to inhibit ligand–receptor interactions such as cytokine and growth factor receptor in animals and the systemin receptor interaction in plants (Stratmann et al., 2000). Different receptors would interact differently with suramin and consequently produce different effects on the estimated half-maximal activity of each AtRALF. Peptides were again separated into different groups: $_{His}$AtRALF1, 19, 22, 24, 31 and 33 shifted their half maximal activity to over 100 nM, whereas $_{His}$AtRALF23 and 34 were less affected, and the shift in their half maximal activity was under 100 nM (Table 2). Surprisingly, $_{His}$AtRALF4 reduced its half maximal activity to 106 nM, meaning that suramin, rather than negatively affecting the ligand–receptor interaction, somehow facilitated the interaction (Table 2).

3. Discussion

The large diversity of Arabidopsis RALF isoforms led us to investigate how such well-conserved peptides would perform in the available biological assays for RALF activity. RALF peptides elicit an increase in the extracellular pH of cell suspension cultures, inhibit root growth, induce Ca^{2+} mobilization, and inhibit hypocotyl and pollen tube elongation (Pearce et al., 2001; Haruta et al., 2008; Covey et al., 2010). The AtRALF peptides we selected were recognized by the $_{His}$AtRALF1 antibody in a concentration-dependent manner that recapitulates the sequence identity among the peptides (Fig. 2B–C and Supplementary data, Fig. S1). However, the same did not hold for their biological activities. The results for all biological assays are summarized in Table 3. Although

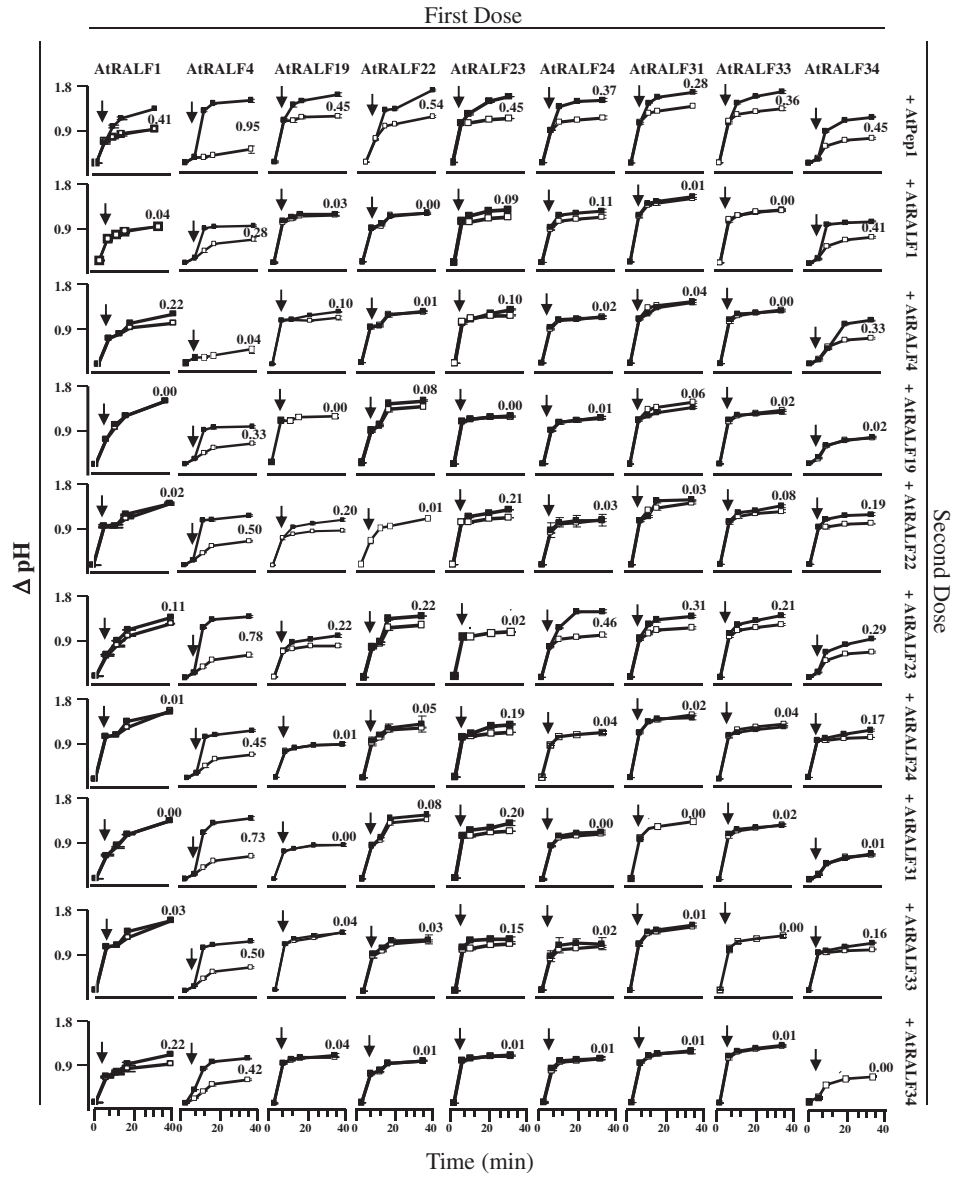

Fig. 7. Alkalinizing activity of two consecutive saturating doses (1 μM) of $_{His}$AtRALFs. The second dose was added 5 min after the first dose (arrows). AtPep1 was used to demonstrate that the cells were able to respond after the first dose of the $_{His}$AtRALFs. Open squares, alkalinization response caused when the same peptide is used as both the first and second dose. Filled squares, alkalinization response caused when different peptides are used as the first and second doses. Numbers inside each graph represent the maximum pH change observed when the two peptides were added consecutively. Values are the means ± SD of three measurements. Experiments were repeated at least twice.

the estimated half-maximal activities of $_{His}$AtRALF1, 19, 22, 23, 24, 31, 33 and 34 are in the same range as those that have been reported so far (Pearce et al., 2001; Haruta and Constabel, 2003; Covey et al., 2010), $_{His}$AtRALF4 showed no detectable activity in the alkalinization assay at nanomolar concentrations (Fig. 3, Table 1). The closest isoform to AtRALF4 is AtRALF19. These peptides share 85% sequence identity with only 7 amino acid differences between them (40/47 from R61 to R107 of preproAtRALF4 locus AT1G28270). $_{His}$AtRALF19 has a half-maximal activity of 4.6 nM, whereas $_{His}$AtRALF4 only showed activity in the alkalinization assay when higher concentrations were used and when responses were evaluated at later times (Supplementary data, Fig. S4). This discrepancy between the high primary structure identity and the difference in activity suggests that a few amino acids may determine the alkalinization activity. Pearce et al. (2010) showed that the activities of RALFs might be sensitive to amino acid substitutions, when a single substitution of an isoleucine at position six for an alanine rendered the tomato RALF inactive in the alkalinization and root growth inhibition assays. Our data suggest that the seven-amino-acid difference between AtRALF4 and 19 may contain the determinants not only for alkalinization but perhaps also for receptor-binding and for all the different RALF-induced biological responses. Covey et al. (2010) reported that SlPRALF, a pollen-specific tomato RALF that inhibits pollen tube germination, showed low activity in the alkalinization assay using tomato and tobacco suspension cells, as opposed to the leaf isoforms of both tomato and tobacco RALF. Interestingly enough, AtRALF4 is the closest Arabidopsis homolog of SlPRALF.

Apart from AtRALF4, all AtRALFs tested showed inhibitory effects in the root and hypocotyl elongation experiments (Fig. 4A–B). The inhibitory activity towards root growth does not seem to be related to the level of expression that each isoform has in each

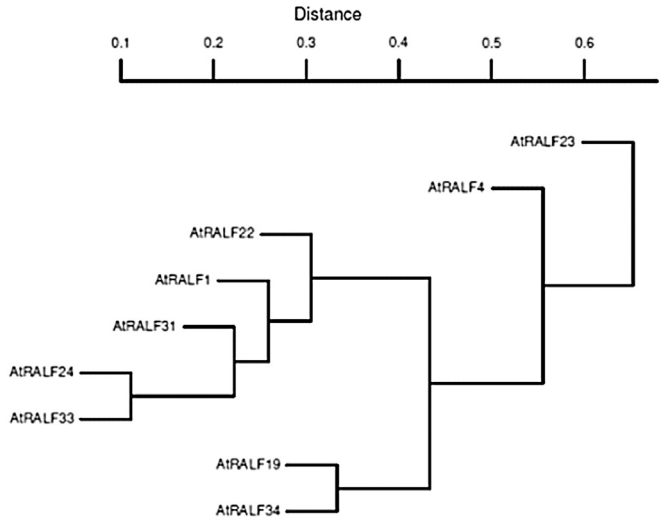

Fig. 8. Cluster analysis of the alkalinizing activity of two consecutive saturating doses of $_{His}$AtRALFs.

tissue (compare Supplementary data, Fig. S2 and Fig. 4A–B). It has been reported, and our expression data confirms, that AtRALF1 is mainly expressed in roots (Haruta et al., 2008) (Supplementary data, Fig. S2); however, it does not show the highest inhibitory activity. Rather, the highest inhibitory activity is shown by AtRALF23, and as observed in our RT-PCR data and in the e-FP Browser, this isoform is only weakly expressed in root tissues (Winter et al., 2007) (Supplementary data, Fig. S2). The inhibition of pollen germination shows no correlation to gene expression, with exception of AtRALF4, which has a high level of expression in inflorescences and does inhibit pollen germination (Fig. 5). One hypothesis to explain this apparent discrepancy could be cell-specific gene expression within the root cell layers. Thus, exogenously applied AtRALFs would have different responses (magnified or minimized) according to the number of receptors that would bind, and gene expression levels based on total RNA from roots would not reflect the actual level of expression in a specific cell layer. In this case, the level of permissiveness of each receptor for different ligands would determine the inhibitory capacity of each AtRALF when applied exogenously or ectopically. This likely accounts for the minor differences in the inhibitory efficiency that arise when the responses for each AtRALF in the root and hypocotyl elongation assays are compared (Fig. 4A–B). Receptor permissiveness towards different AtRALFs can also be deduced from the semi-dwarf phenotype of plants overexpressing the root-specific isoform AtRALF1, which is identical to the phenotype shown by the overexpression of the ubiquitous isoform AtRALF23 (Matos et al., 2008; Srivastava et al., 2009). In this sense, AtRALFs and their still-unidentified receptors behave similarly to AtPep1 peptides and their receptors AtPEPR1 and R2. The AtPep1 gene family is made of 7 members that all seem to function as ligands for AtPEPR1, with only AtPep1 and 2 binding AtPEPR2 (Krol et al., 2010; Yamaguchi et al., 2010). For the CLV3 and CLE peptides, scenarios have been reported for both high specificity between ligands and receptors and a lack of specificity (Strabala et al., 2006; Kinoshita et al., 2010; Meng and Feldman, 2010).

Table 2
AtRALFs alkalinizing activity after 5 min pretreatment of the cells with suramin (10 μM).

Peptide	Half-maximal activity[a]
AtRALF1	389 nM
AtRALF4	106 nM
AtRALF19	223 nM
AtRALF22	230 nM
AtRALF23	120 nM
AtRALF24	203 nM
AtRALF31	476 nM
AtRALF33	463 nM
AtRALF34	51 nM

[a] values estimated measuring the pH, changes 5 min after the addition of the, peptides.

The calcium mobilization assay provided the most specific response (Fig. 6A). The aequorin luminescence in this assay has been shown to be derived mainly from the roots (Haruta et al., 2008). Therefore, it is not surprisingly that the only isoform with a strong signal in this assay is AtRALF1, which is mainly expressed in roots. At higher concentrations, other isoforms such as AtRALF22, 24 and 33 showed an increase in the total luminescence counts over the duration of the experiment (Fig. 6B). The AtRALF1-specific response associated with this conditional mobilization of calcium could be interpreted as evidence that, at least in roots, a single AtRALF receptor is in operation.

To biochemically shed some light onto the number of possible receptors that may be involved in AtRALF binding, we used the alkalinization assay to test the sequential addition of saturating amounts of different peptides (Fig. 7). Although the data present some clear interpretations, such as for the uniqueness of the AtRALF4 response, interpretation of the data from other peptides is challenging. We took advantage of cluster analyses to separate the peptides into groups of similar responses, and at least four groups that most likely share receptors could be distinguished (Fig. 8). To further explore the alkalinization assay as a way to probe the number of AtRALF receptors, we pre-treated the cells with suramin, an inhibitor of ligand–receptor interactions. Three groups could be distinguished according to their response to the addition of suramin, and AtRALF4 was confirmed to be in a separate group because of its specific and unusual response to suramin (Table 2). Although suramin usually works as an inhibitor of ligand–receptor interactions, it has also been reported as an activator for a specific class of Ryanodine receptors in animals (Sitsapesan and Williams, 1996).

4. Materials and methods

4.1. Production and purification of recombinant $_{His}$AtRALF peptides

The pET (Novagen) and Gateway (Life Technologies) systems were used for the production of recombinant $_{His}$AtRALF peptides. The coding region corresponding to the mature sequence of each AtRALF peptide was amplified from genomic DNA by conventional PCR using specific primers (Supplementary data, Table S1). The amplified fragments of isoforms AtRALF1, 33 and 34 were cloned into the pET-28b expression vector, and isoforms AtRALF4, 19, 22, 23, 24 and 31 were cloned into the pDEST17 vector. For the Gateway system, fragments were first cloned into the pENTR D-TOPO vector and later transferred by recombination to the pDEST17 vector. All peptides were fused to a histidine-tag at their N-termini with exception of AtRALF1, which was also fused to a histidine-tag at its C-terminus. The vectors were introduced into the BL21 or Rosetta *E. coli* strains. Bacterial cultures were grown in LB medium (tryptone 10 g l^{-1}, yeast extract 5 g l^{-1}, NaCl 10 g l^{-1} and pH 7.5), at 37 °C and at 200 rpm. After reaching an O.D. of 0.6, the cells were induced for 4 h using 1 mM IPTG. Extraction and purification were performed using standard Ni^{2+}-resin affinity chromatography (Ni-NTA, Qiagen). After collecting the cells by centrifugation (8 min, 18 °C, 5000× *g*), they were frozen and then incubated in denaturant

Table 3
Summary of $_{His}$AtRALFs activity in the biological assays. Activities were ranked within each assay according to the statistical analysis.

$_{His}$AtRALFs	Alkalinization[a] (up to 500 nM in 5 min)	Inhibition of root growth (10 μM)	Inhibition of hyp. elongation (1 μM)	Inhibition of pollen germin (10 nM)	Ca^{2+}[b] mobilization (10 nM)
1	+	++	+++	No	+++
4	No	No	No	+++	No
19	+	+	+	+++	No
22	+	++	++	++	++
23	+	+++	++	No	No
24	+	++	+++	++	No
31	+	++	++	+	No
33	+	++	+	+++	+
34	+	+	+	No	No

[a] No, non-observed.
[b] Rank based on total counts.

buffer (100 mM NaH_2PO_4, 20 mM Tris–HCl, 8 M urea, pH 8.0) under agitation for 40 min, lysed using cell disruption by nitrogen decompression in a Parr bomb and centrifuged (40 min, 4 °C, 12,000× *g*). The supernatant was directly applied to a column filled with Ni-NTA resin equilibrated with 10 volumes of binding buffer (100 mM NaH_2PO4, 20 mM Tris–HCl, 8 M urea, pH 8.0). The column was washed with 5 volumes of washing buffer (100 mM NaH_2PO_4, 20 mM Tris–HCl, 8 M urea, pH 6.3) and the proteins of interest were eluted with 5 ml of eluting buffer (100 mM NaH_2PO_4, 10 mM Tris–HCl, 8 M urea, pH 3.3). The purified recombinant proteins were dialyzed against 4 l of 0.1% (v/v) formic acid, in 3 steps of approximately 24 h each (4 °C under gentle agitation). After dialysis, the samples were lyophilized and quantified by HPLC. A gradient from 0 to 40% acetonitrile/0.1% formic acid and a flow rate of 1 ml/min were used. The lyophilized powder was dissolved in 1 ml of 0.1% formic acid and injected into a semipreparative reversed-phase C18-HPLC column (Kromasil®).

4.2. Mass spectrometry

Trypsinization of the recombinant AtRALFs was performed after separation on SDS-PAGE gels. Bands of interest were cut into smaller fragments and incubated in 75 mM NH_4HCO_3 in 40% (v/v) ethanol for 60 min at room temperature. The process was repeated until the gel was completely discolored. Proteins were reduced with the addition of 5 mM dithiothreitol (DTT) in 25 mM NH_4HCO_3 for 30 min at 60 °C, followed by the alkylation reaction with 55 mM of iodoacetamide in 25 mM NH_4HCO_3 for 30 min at room temperature and in the dark. Gel fragments were washed once with 25 mM NH_4HCO_3 and once with acetonitrile. The fragments were then dehydrated with acetonitrile (3 incubations of 10 min each) and dried. Rehydration was performed in a solution containing 40 mg ml^{-1} trypsin in 50 mM NH_4HCO_3 for 45 min on ice, followed by incubation at 30 °C for 16 h. The fragments were extracted with the addition of 50 ml of 50 mM NH_4HCO_3 (incubation for 10 min in an ultrasound bath) and addition of 50 ml of acetonitrile. This procedure was repeated 3 times. The extracted peptides were dried under vacuum and sent to the Central Analitica, University of São Paulo (USP) for analysis using ESI-TOF (Electrospray Ionization-Time of Flight).

4.3. Protein blot

Purified peptides were separated by SDS-PAGE and electro-transferred onto a nitrocellulose membrane (1 h, 100 V). After transfer, the membrane was blocked for at least 30 min with TTBS [50 mM TRIS, 150 mM NaCl and 0.1% (v/v) Tween 20] containing 5% skimmed milk powder. The anti-AtRALF1 antibody was added (1:2000) in TTBS solution containing 2% (w/v) skimmed milk powder and the membrane was incubated in this solution for 4 h under gentle agitation. After 4 washes of 5 min with TTBS, the secondary antibody (1:500 alkaline phosphatase-conjugated anti rabbit IgG, Bio-RAD) was added in the same solution and the membrane was incubated under agitation for 2 h. Final washes were made with TBS buffer and the bands were visualized using Lumi-Phos WB Chemiluminescent Substrate for AP (Pierce) according to the manufacturer's instructions.

4.4. Root growth and hypocotyl elongation inhibition assays

Arabidopsis seeds (Columbia) were surface sterilized in a 50% (v/v) bleach solution for 15 min followed by several washes using sterile Milli-Q water. The water was replaced by half-strength MS media without sucrose (Murashige and Skoog, 1962), and aliquots of 1 ml were distributed in 24-well plates. After incubation at 4 °C for 72 h, the plates were transferred to the growth room where they stayed in constant light conditions at 24 °C [150 μE m^{-2} s^{-1} (E, Einstein; 1 E = 1 mol of photons)]. After 48 h, recombinant peptides (final concentration of 10 μM) were added and root growth was evaluated after 72 h of incubation under the same conditions. For hypocotyl elongation, the plates were also transferred to the growth room but remained in the dark. On the second day, recombinant peptides (final concentration of 1 μM) were added and growth was evaluated 4 days after germination. Quantitative data were obtained using the methodology described by Weigel and Glazebrook (2002) using Image J software (Abramoff et al., 2004). Control plants were kept under identical conditions in a peptide-free medium.

4.5. Alkalinization assay

Arabidopsis suspension-cultured cells (MM1) were maintained as described (Menges and Murray, 2002). Suspension cells were transferred on a weekly basis (8 ml per 80 ml media) and utilized in the assays 3–5 days after transfer. Aliquots of 1 ml were distributed into 24-well non-tissue culture-treated plates (Corning) and allowed to equilibrate for 1 h on an orbital shaker at 130 rpm at 25 °C. Peptides were added to suspension cells and the pH was measured after 5 min unless stated otherwise. The pH was monitored using an Orion Model EA940 pH meter with an Orion semi-micro pH electrode. In the assays to evaluate the activity of $_{His}$AtRALFs when applied after other $_{His}$AtRALFs at saturating concentrations, the first dose (1 μM) was applied 5 min before the first measurement and the second dose (1 μM). After the second dose, the pH was recorded at 5, 15 and 30 min.

For the suramin assays, suramin (Sigma–Aldrich) was added to the suspension cells at a concentration of 10 μM, and the peptides were added to these cells after 5 min. The pH was measured 5 min

after the addition of the peptides. Data to estimate the half-maximal activity after suramin treatment were collected using 0, 100, 250, 500 and 1000 nM of each peptide.

For cluster analyses, the alkalinization activity assay results for the nine peptides were considered as a non-symmetric matrix because the order in which they were added in the assay could influence the results. The alkalinizing activities were converted into plus and minus signs for each pair of peptides according to the presence (+) or absence (−) of activity after the first dose of the peptides. Simple pairwise matching coefficients (Meyer et al., 2004) for the plus and minus signs were calculated considering rows and columns as the subjects in two separate analyses. The results were then averaged for the two simple matching matrices, resulting in a similarity matrix with all peptides. A dendrogram was then obtained using the UPGMA algorithm.

4.6. Pollen germination assay

Arabidopsis pollen was collected from plants that were grown in mixed soil in a growth chamber. Unopened flowers were collected and dipped in a 10% (w/v) sucrose solution overnight in the dark at 22 °C. The sucrose solution containing pollen grains was placed onto glass slides covered with pollen germination medium (PGM) with or without the peptides. The PGM was made with 18% (w/v) sucrose, 0.01% (v/v) boric acid, 1 mM $CaCl_2$, 1 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$ and 1.5% (w/v) low melting point agarose, with the pH adjusted to 7. Glass slides were prepared using 200 μl of PGM and peptides at 10 nM concentration. The pollen grains were allowed to germinate for 18 h at 22 °C in a humidified chamber and were visualized using a light microscope (Nikon 145390, Model SC, Japan) at 40× magnification. Pollen grains were counted as germinated only if the tubes were greater than the radius.

4.7. Ca^{2+} mobilization assay

The cytoplasmic Ca^{2+} assay in Arabidopsis seedlings was previously described by Haruta et al. (2008). To assay for changes in Ca^{2+} levels, an Arabidopsis line homozygous for a single insertion of a transgene encoding a cytoplasmically expressed apoaequorin driven by a cauliflower mosaic virus 35S promoter was used (Lewis et al., 1997). Seeds were sterilized as described before and plated on medium containing Murashige–Skoog salts, 3% (w/v) sucrose, and 0.4% (w/v) agar. The plates were kept at 4 °C for 72 h and incubated at 24 °C under continuous light [150 μE m^{-2} s^{-1} (E, Einstein; 1 E = 1 mol of photons)] for 4 days. A single seedling was transferred into each well of a 96-well white microplate (Thermo Labsystems) containing 200 μl of liquid Murashige–Skoog medium supplemented with 2.5 μM coelenterazine cp (Sigma) and incubated in the dark at 24 °C for 16 h. Each well of the plate received 20 μl of the recombinant peptides for a final concentration of 10 or 100 nM. The resulting luminescence emission was monitored using a microplate reader (Biotec ELx 800) for 10 time-points over approximately 160 s.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2013.12.005.

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