Review

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Understanding the RALF family: a tale of many species

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Small secreted peptides are gaining importance as signalling molecules in plants. Among the 1000 open reading frames (ORFs) in the *Arabidopsis* (*Arabidopsis thaliana*) genome potentially encoding small secreted peptides, the members of the RAPID ALKALINIZATION FACTOR (RALF) family of peptides have been linked to several physiological and developmental processes. Here, we provide a comprehensive overview of current knowledge on the RALF family. Discovered in tobacco (*Nicotiana tabacum*), the role of RALF peptides has been investigated in numerous plant species. Together, these observations suggest that RALF peptides impact on acidification and cell expansion during growth and development. Although few components of the signalling pathway have been revealed, the recent identification of FERONIA (FER) as a RALF receptor and plasma membrane H^+-ATPase 2 as a downstream target provide a major step forward.

Small signalling peptides: a brief introduction

Cell-to-cell communication in plants has canonically revolved around phytohormones, such as auxin [1]. However, over the past few decades, a focus on short distance communication has highlighted the role of small signalling peptides during tissue patterning and development. These peptides have been implicated in almost all developmental processes [2,3]. Among the many signalling peptides discovered to date, these small proteins can be grouped into two broad categories: small post-translationally modified peptides and cysteine-rich peptides. The first class, small post-translationally modified peptides, currently outnumber the known cysteine-rich peptides in *Arabidopsis*, and are involved in many developmental processes [2,4–8]. Although smaller than cysteine-rich peptides (less than 20 amino acids), they are subject to numerous post-translational modifications, such as tyrosine sulfation, proline hydroxylation, and hydroxyproline arabinosylation, which may conformationally alter the peptides into an active or inactive state [2,6,7,9–12]. Small post-translationally modified peptides are often grouped in highly conserved families, including C-TERMINALLY ENCODED PEPTIDE (CEP), CLAVATA3/EMBRYO SURROUNDING REGION-RELATED (CLE), and ROOT GROWTH FACTOR (RGF)/GOLVEN (GLV)/CLE-LIKE (CLEL) peptides, with great developmental redundancy within the families [2,7,13–16]. The second class of peptides, cysteine-rich peptides, have many structural features in common despite their greater diversity in amino acid sequences. Cysteine-rich peptides are usually much larger than small post-translationally modified peptides (less than 160 amino acids), are positively charged, and generally contain 4–16 cysteine residues. These residues appear to be integral to the correct conformational folding of the mature peptide, but, unlike small post-translationally modified peptides, have not yet been shown to be post-translationally modified [17]. Similar to small post-translationally modified peptides, they contain a conserved N-terminal signal peptide, which directs the peptide to the correct cellular location via the secretory pathway [18]. To date, the known cysteine-rich peptides family of peptides includes RALF, EPIDERMAL PATTERNING FACTOR1 (EPF1), EPF2, TAPETUM DETERMINANT1 (TPD1), STOMAGEN/EPF-LIKE9 (EPFL9), and EARLY NODULIN [2,17,19–22].

Here, we provide a comprehensive overview of our current knowledge on the RALF family. Based on observations in numerous plant species, it appears that RALF peptides impact on acidification and cell expansion during growth and development. Although few components of the signalling pathway have been revealed, the recent identification of FERONIA as a RALF receptor and plasma membrane H^+-ATPase 2 as a downstream target provides a major step forward.

RALF function: a many species saga

The RALF peptide family was discovered just over a decade ago and current knowledge on their function is limited. Here, we summarise the RALF-related research from different species, which exposes a general trend in the function of RALF peptides (Figure 1 and Table 1).

Nicotiana tabacum

RALFs were discovered during a peptide screen for systemin proteins (defence-related, alkalinisation-inducing peptides) in *Nicotiana tabacum* leaf cells, utilising an assay developed to identify peptides that cause an increase in pH of the medium [17]. Having caused a stronger and more rapid alkalinisation of the medium compared with

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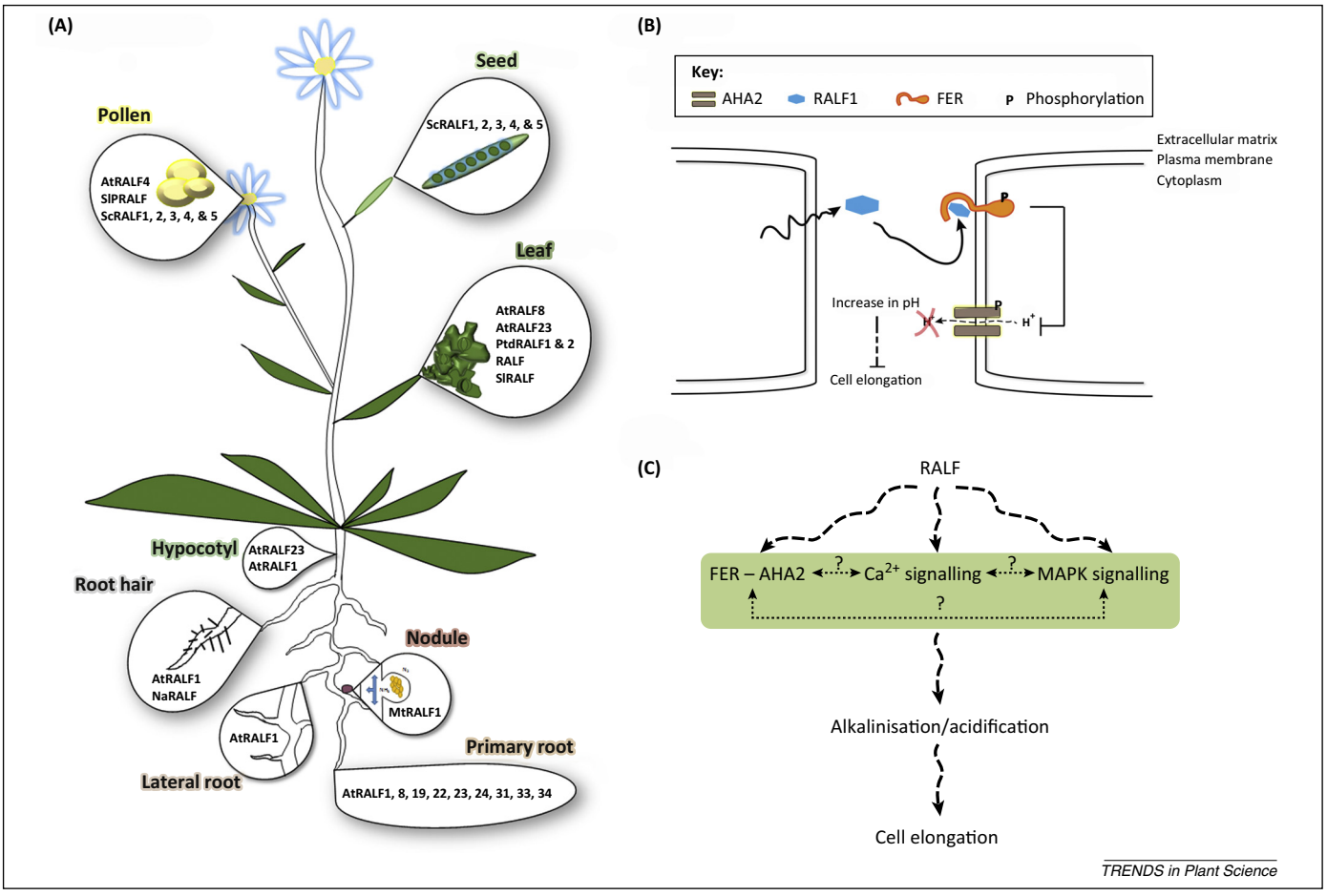

Figure 1. RAPID ALKALINIZATION FACTOR (RALF) peptides in plant growth and development. **(A)** Schematic of a plant highlighting where RALFs have a role (combined observations from various species). **(B)** RALF binding to the FERONIA (FER) receptor and regulating H^+-ATPase 2 (AHA2) activity. **(C)** Summary of events downstream of RALF, without any specific order of events or interactions and indicating possible connections (broken line and '?').

two systemin peptides, the then unknown peptide was investigated further. Through MALDI-MS on HPLC purified leaf tissue samples, RALF was identified to be a 5-kDa polypeptide, with a 115 amino acid preproprotein. The new RALF peptide was dismissed as being a systemin because it was shown to induce MITOGEN-ACTIVATED PROTEIN (MAP) KINASE activity peaking earlier than previously identified systemin proteins. Furthermore, the systemin family of peptides induces the synthesis of tobacco trypsin inhibitors as a defensive wound signal; however, when exogenously applied to tobacco leaves, RALF did not induce this wound response, thus firmly dissociating this novel peptide from the systemin peptide family and beginning the journey to discovering the function of these novel peptides [17].

Hybrid poplar (Populus trichocarpa x Populus deltoides)

The difference in RALF compared with the systemin peptide family was reinforced when novel poplar-derived RALF peptides also did not induce a defence response. Using similar methodology to [23], poplar RALFs (specifically *Ptd*RALF1 and *Ptd*RALF2) were isolated from hybrid poplar leaf cells, and were shown to alkalinise strongly the culture medium, be nonresponsive to fungal treatment (no change in expression), and to not elicit a defence response (in this case, no production of PHENYLALANINE AMMONIA LYASE, which is often associated with a defence response [24]), suggesting that indeed a novel, nondefence-related family of peptides had been discovered [25].

Nicotiana attenuata

To explore RALF functionality in a *N. tabacum*-related species, *NaRALF* was silenced in *Nicotiana attenuata* with RNA interference (RNAi), which showed increased primary root length and produced trichoblasts that formed abnormal root hairs [26]. In these *NaRALF* RNAi lines, slow apoplastic pH oscillations were observed with increased pH at the trichoblast tip. This phenotype was partially restored when the inverted-repeat RALF construct (*irRALF*) plants were grown in low pH-buffered medium, indicating the first regulatory role for RALF peptides in modulating root hair pH and pH-related growth [26].

Solanum lycopersicum (teaming up with A. thaliana)

The physiological role of RALFs was investigated further by developing a synthetic RALF peptide based on tomato-derived endogenous RALF [17]. Exogenously applying synthetic tomato RALF peptide to germinating tomato seeds caused a significant arrest in root elongation of the newly emerged primary roots. This synthetic peptide was then applied to germinating *Arabidopsis* seeds and a