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ORIGINAL ARTICLE

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Characterization of five RALF-like genes from *Solanum chacoense* provides support for a developmental role in plants

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Abstract Five RALF (rapid alkalinization factor)-like genes, named ScRALF1 to 5, were isolated from fertilized ovule and ovary cDNA libraries of *Solanum chacoense*. They showed high sequence similarities with the RALF protein sequence from *Nicotiana tabacum*, and exhibited the characteristic architecture of RALF polypeptides. All ScRALFs were moderately to highly expressed at some stage of fruit maturation. ScRALF1 and ScRALF3 were predominantly expressed in ovaries and larger fruits, while ScRALF2, ScRALF4, and ScRALF5 were also expressed in other tissues, indicating that while some RALFs may be involved in fruit maturation, others could be involved in other developmental processes. Wounding or treatment of plants with growth regulators involved in plant defense responses had no significant impact on the mRNA level of any of these genes. These results suggest and support previous data showing that RALF peptides are more likely to act as a small peptide involved in plant development than in defense responses.

Keywords Ligand · Peptide signaling · RALF

Abbreviations EST: Expressed sequence tag · RALF: Rapid alkalinization factor

GenBank accession numbers: ScRALF1, AY422824; ScRALF2, AY422825; ScRALF3, AY422826; ScRALF4, AY422827; ScRALF5, AY655141

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Introduction

The extracellular domains of plant receptor kinases confer extreme specificity to their binding partners (Kajava 1998; Kobe and Deisenhofer 1994). Only a few binding partners, most commonly referred to as ligands, have been isolated in plants, but they already appear to be very diverse in nature: ethylene is a very small gaseous molecule; brassinolide is a steroid molecule; cytokinin, one of the classical plant hormones, is a small molecule derived from a nucleic acid precursor, and others, which appear to account for a large number of these signaling molecules, are small polypeptide hormones that can originate from the plant itself (Takayama and Sakagami 2002) or are of pathogenic (Asai et al. 2002) or parasitic (Olsen and Skriver 2003) origin. Some ligands, although very different in nature, like brassinolide and systemin, might even bind to similar receptors to trigger different responses (Szekeres 2003).

Some of these predicted peptides seem to be part of large putative ligand families. The CLE (CLAVATA3-ESR related) polypeptides appear to be such a family. All CLE polypeptides have a signal peptide, most are intronless, and all share a highly conserved carboxy-terminal domain composed of 14 amino acids (Cock and McCormick 2001). Other families involved in various processes, such as abscission [IDA (inflorescence deficient in abscission); Butenko et al. 2003], sporophytic self-incompatibility [SCR (S-locus cysteine-rich protein); Schopfer et al. 1999; Shiba et al. 2001], or gametophytic self-incompatibility (the small asparagine-rich HT protein; O'Brien et al. 2002) have also been identified. Families of small peptides having either pleiotropic developmental roles [DVL (Devil); Wen et al. 2004] or a nodule-specific expression pattern [NCR (nodule cysteine-rich); Mergaert et al. 2003] were also recently uncovered. The rapid alkalinization factor (RALF)-like peptides also form such a large family (Olsen et al. 2002). RALF is a small 5 kDa polypeptide that causes alkalinization of the medium, can stop root growth, and

activates intracellular MAPKs (Pearce et al. 2001b). It was found as a 49 amino acid peptide, but is processed from a larger precursor peptide of 115 amino acids. Proteolytic processing from a longer precursor was also found to occur in other plant polypeptide hormones such as phytosulfokine (Yang et al. 1999) and systemin (Pearce et al. 2001a). Following N-terminal cleavage of the signal peptide, further N-terminal processing would occur at well conserved dibasic residues just upstream of the mature polypeptide. According to a recent review, tobacco RALF binds a 120 kDa protein concomitantly with a 25 kDa protein (Ryan et al. 2002). An apoplastic localization has also been recently determined using a RALF-GFP fusion expressed in *Nicotiana benthamiana* cells (Escobar et al. 2003). In recent database searches, 34 RALF-like genes were found in the *Arabidopsis thaliana* genome (Olsen et al. 2002; Ryan and Pearce 2001).

Because it has the property of inducing rapid alkalinization of the cell culture medium, as well as the ability to induce MAPK activation (like tobacco systemin, with which it was concomitantly isolated), RALF was originally associated with plant wound or defense responses. This was counterbalanced by the fact that, unlike systemin, it could not induce the synthesis of tobacco trypsin inhibitor in leaves treated with the purified peptide. The only biological activity associated with RALF was its ability to arrest root growth (Pearce et al. 2001b). Furthermore, recent experiments have shown that a poplar RALF could not be induced by chitosan or *Phytophtora megasperma* elicitors, and the expression of one RALF was decreased following addition of methyl jasmonate (MeJA) to the culture medium (Haruta and Constabel 2003). In addition, results obtained by Olsen et al. (2002) showed no alteration in the expression level of seven RALFs in a *mpk4* background (constitutive systemic acquired resistance), nor in the *ctr1* (constitutive ethylene response) mutant. Although alkalinization of the medium is often related to defense responses (Bolwell 1995), the inability to induce RALF by defense response elicitors suggested that RALF could play other roles in planta, such as a developmental role, as pointed out by Pearce et al. (2001b).

A Blast search for RALF-like peptides against our internal EST database generated from ovary tissues from *S. chacoense* (a close relative of tomato and potato) and enriched for rare mRNAs, allowed us to find five new members of the RALF peptide family. RNA gel blots were used to gain further insight into the expression profiles of these putative peptides in various tissues, and to established if their expression profiles were altered when the plant was submitted to various plant growth regulators, stress treatments, or following developmental cues. We provide evidence that expression of the RALF peptide cDNAs is not modulated by wounding or stress hormones, and that expression is tissue-specific, thus suggesting that these peptides most probably play a developmental role in plants.

Materials and methods

cDNA library construction and sequence analysis

Solanum chacoense RALF sequences (ScRALF) were found using both the complete tobacco original RALF peptide (NtRALF) sequence, and highly conserved short amino acid sequence motifs found in published RALF sequences in a Blast search (Altschul et al. 1990) against our internal *S. chacoense* EST database. This EST database was generated by a modified selection screen based on a virtual subtraction procedure (Li and Thomas 1998), using fertilized ovary tissues as the starting material. Briefly, the cDNA libraries were prepared from 2- to 6-day post-pollination depericarped ovaries and hand-dissected ovules from 7 to 17 days-after-pollination ovaries in the Uni-ZAP vector following the manufacturer's instruction (Stratagene, La Jolla, Calif.). The phage libraries were converted to plasmid cDNA library by mass excision. Colonies (50,000) were transferred to positively charged nylon membranes and denatured in 0.5 *N* NaOH and 1.5 M NaCl. The resulting membranes were hybridized with a probe derived from α-^{32}P dATP random-labeled cDNA leftovers (library target tissues) obtained during the library construction procedure, and were exposed at −85°C with one intensifying screen on Kodak Biomax MR film (Interscience, Markham, ONT, Canada). The cDNA probes produced correspond exactly to the abundance of each mRNA species found in the tissue tested. Rare mRNA species are underrepresented and thus, make poor probes. This enabled selection of weakly expressed genes. Of the 50,000 colonies plated, only the lowest fifth in expression level (as determined by densitometric scans and visual inspection) were selected for sequencing on an automated sequencer (ABI DNA analyzer 3700). RALF sequences were aligned using ClustalX and phylogenetic trees were inferred using neighbor-joining (and parsimony) in PAUP 4.0b10 (Swofford 2002). RALF homologues, according to Olsen's classification (Olsen et al. 2002), were assigned to the neighbor(s) on the closest branch.

Phytohormone and wounding treatments

All plant material was collected from *S. chacoense* Bitt. genotype G4 (self-incompatibility alleles $S_{12}S_{14}$). For fertilization-related events, *S. chacoense* genotype V22 (self-incompatibility alleles $S_{11}S_{13}$) was used as the pollen donor. These genotypes were obtained from crosses between line PI 458314 (self-incompatibility alleles $S_{11}S_{12}$) and line PI 230582 (self-incompatibility alleles $S_{13}S_{14}$) originally obtained from the Potato Introduction Station (Sturgeon Bay, Wis.) (van Sint Jan et al. 1996). Phytohormone treatments were performed as described previously (Lantin et al. 1999) with the following compounds: Tween (control), abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA), methanol (control), MeJA

and trans-2-hexenal (HEX). Briefly, flowers and leaves were sprayed with an aqueous solution (in 0.05% Tween) of either 50 mM ABA, 50 mM JA (mixed isomers), 5 mM SA, or 0.05% Tween. Tissues were collected 48 h post-treatment. For wounding, forceps were used to slightly tweeze leaves. For volatile treament, MeJA or HEX were first diluted to 0.1 M in ice-cold methanol and 250 µl of the compound (equivalent to 10ml/l air space) was added to a piece of Whatman paper that was placed in an air tight container along with the plant. As a control, 250 µl methanol was applied in the same way. Treatment with each volatile compound was applied to distinct plants and tissues were collected after 6 h and 48 h.

Isolation and gel blot analysis of RNA and DNA

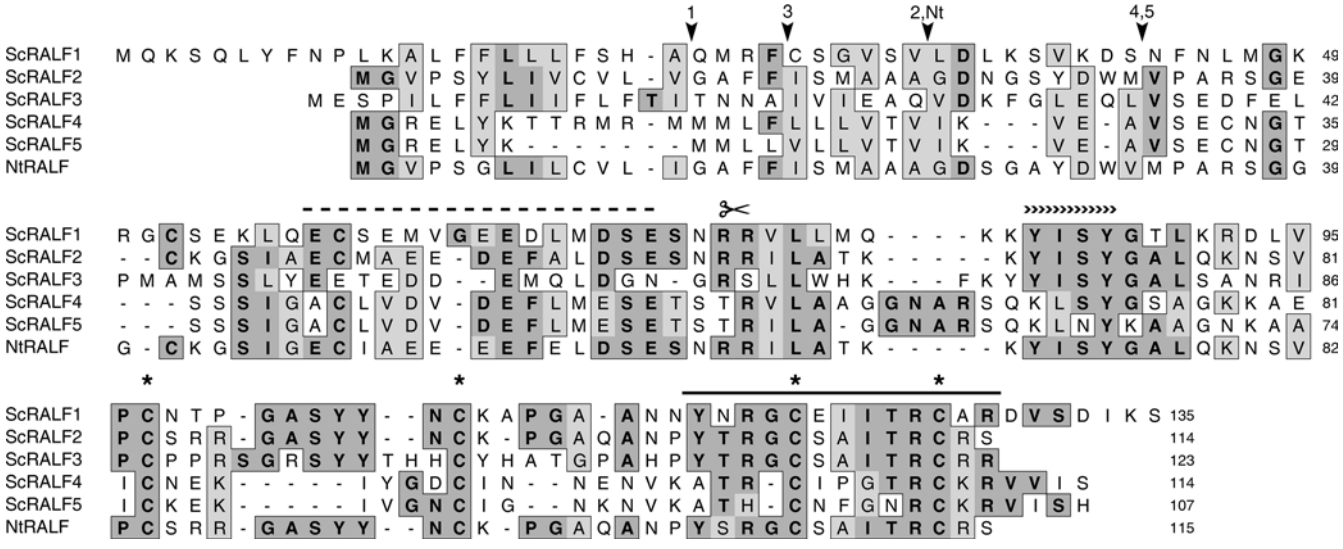
Total RNA was isolated as described previously (Jones et al. 1985). RNA from tissues that were wounded or treated with phytohormones was extracted using the RNeasy Plant Mini Kit (Qiagen; Mississauga, ONT, Canada). The RNA concentration was determined by measuring its absorbance at 260 nm and verified by agarose gel electrophoresis and ethidium bromide staining. Equal loading of total RNA on RNA gel blots was verified with a *S. chacoense* 18S RNA probe. Genomic DNA was extracted using a modified version of the protocol of Murray and Thompson (1980). RNA and DNA gel blot analyses were performed as described previously (O'Brien et al. 2002). Probes for RNA and DNA blots were synthesized using a random-labeled PCR kit (Ambion, Austin, Tex.). Membranes were exposed at room temperature on an europium screen and scanned on a Typhoon 9200 Phosphorimager (Amersham, Baie d'Urfé, QC, Canada).

Results

ScRALF isolation and sequence analysis

Using a negative selection procedure, we produced a small EST database of weakly expressed genes in *S. chacoense* ovules or ovaries after fertilization; 7,741 ESTs were sequenced and annotated, and statistical analysis of our data set showed that our subtraction procedure highly enriched our EST pool for unique sequences (82% unigenes). We used the RALF peptide sequence from *N. tabacum* for a Blast search against our EST database and retrieved five distinct homologues from *S. chacoense*, hereafter named ScRALF1, ScRALF2, ScRALF3, ScRALF4 and ScRALF5 (Fig. 1). According to phylogenetic analysis performed using both neighbor-joining and parsimony methods with the full-length protein or the putative mature polypeptide, ScRALF proteins 1–3 are related to *A. thaliana* AtRALF 24/31, AtRALF 22/23/33, and AtRALF 34 respectively, while ScRALF4 and 5 are related to AtRALF 27 (Fig. 2).

The ScRALF1 clone (731 bp) codes for a predicted open reading frame of 174 amino acids, with two putative in-frame translation start sites found at position 23 and 40, with no stop codon found upstream. A similar situation is found in the tobacco RALF cDNA, where 44 in-frame amino acids are found before the first methionine identified. Although both putative ScRALF1 starting methionines are in non-optimal translation initiation context (Joshi et al. 1997; Kozak 1999), an analysis for the presence of a signal peptide



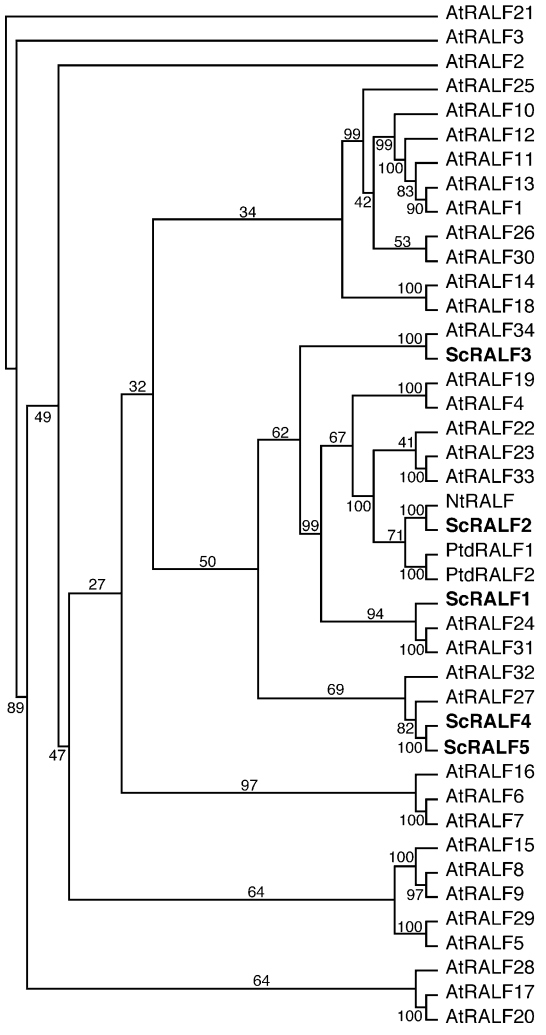


Fig. 2 Phylogenetic distance analysis (neighbor-joining) of RALFs. Amino acid sequences from the predicted mature RALF peptide were aligned using ClustalX and the tree was inferred using PAUP 4.0b10; 1,000-bootstraps were used to calculate branch support. AtRALF21 was used as an outgroup as it was the most distantly related to all our ScRALFs. The bootstrap support values are indicated on the left

(predicted in all RALF-like proteins described so far) strongly suggests the presence of such a signal immediately downstream of the second methionine. If this methionine is taken as the translation start site, the deduced ScRALF1 protein has the characteristic length of most other RALF sequences (this shorter RALF sequence was thus used for the sequence alignment shown in Fig. 1). ScRALF1 has all the features typical of RALF peptides, including an acidic region preceding the dibasic residues, an YIXY motif, and four conserved cysteines involved in disulfide bridges in the mature protein.

ScRALF2, our closest homologue to NtRALF (Fig. 2), encompasses 789 bp and showed 56% identity (69% similarity) with PtRALF1 and 52% identity (65% similarity) with PtRALF2 from hybrid poplar (Haruta and Constabel 2003). Its signal peptide is 87% identical to that found in NtRALF. The overall identity between ScRALF2 and NtRALF is 87% (93% similarity) and they share 94% identity (99% similarity) in the highly charged C-terminal domain. All domains found in RALFs are present, including the YIXY motif, which for three of the five ScRALFs analyzed was YISY. Based on this very high amino acid sequence similarity, ScRALF2 is most probably the true orthologue of NtRALF. Furthermore, the phylogenetic analysis (Fig. 2) also showed that ScRALF2 is more closely related to NtRALF and PtdRALF1 and 2 than to any AtRALFs.

ScRALF3 encodes a cDNA of 688 bp. This ScRALF is different from the preceding ScRALFs since it does not have the dibasic residues suspected to be involved in polypeptide maturation. This motif is shared by 59% (20/34) of AtRALFs (Olsen et al. 2002). ScRALF3 has GR residues instead of RR, which could be the result of an A → G point mutation. The spacing between the conserved cysteines also differs from the conserved consensus sequence. Identity with NtRALF C-terminal domain is 76% (87% similarity) and other features such as the signal peptide, the acidic sequence and the YIXY motif are present.

ScRALF4 and 5 are by far our least similar ScRALFs when compared to the original NtRALF sequence (Fig. 1), and clearly group together in the phylogenetic analysis (Fig. 2). ScRALF4 codes for a small cDNA (532 bp) and has a signal peptide, the acidic region, and a single basic residue. The YIXY motif (present in 56% of AtRALFs) is replaced by KLSY and it has the four conserved cysteines. Finally, it shares only 23% overall amino acid sequence identity (42% similarity) with NtRALF. ScRALF5 codes for an EST of 517 bp and shares 75% identity with ScRALF4 (20% overall amino acid sequence identity and 35% similarity with NtRALF). ScRALF5 shares the same features found in ScRALF4, except that the YIXY motif is replaced by KLNY.

All of the ScRALFs found share four universal motifs found in RALFs: an N-terminal signal peptide sequence; an acidic region; a highly charged C-terminal tail; and the presence of four conserved cysteines in the predicted mature peptide. Other domains, such as the dibasic residues located immediately upstream of the predicted mature peptide, and the YIXY motif are present in our ScRALF sequences in proportions similar to the situation found in the AtRALFs (Olsen et al. 2002).

ScRALF1–5 are single-copy genes in *S. chacoense*

The full-length clone of each ScRALF was used as a probe to hybridize digested genomic DNA in order to obtain information about their gene copy number in the *S. chacoense* genome (Fig. 3a). For ScRALF1, one hybridizing fragment was detected with *Eco*RI, and two fragments were detected with the restriction enzymes

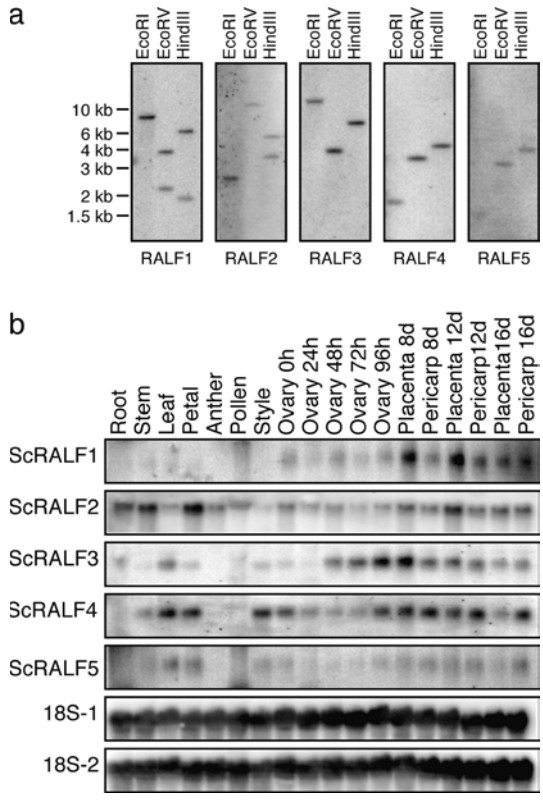


Fig. 3 **a** DNA gel blot analysis of the five ScRALF genes. Genomic DNA (10 µg) was digested with *Eco*RI, *Eco*RV or *Hin*dIII. Membranes were exposed for 2 days as described in Materials and methods and stripped with Ambion StripEZ. **b** RNA gel blot analysis of the five *S. chacoense* RALF-like genes. Total RNA (10 µg) was used for each tissue. Full-length cDNA clones were used as probes. 18S-1 was used as a control for loading of ScRALF1, 3, and 4, and 18S-2 for ScRALF2 and 5. ScRALF1, 2, 4 and 5 were exposed for 2 days, ScRALF3 was exposed 4 days on a europium phosphorscreen. Calculated sizes for ScRALF1, ScRALF2, ScRALF3, ScRALF4, and ScRALF5 mRNAs were 801, 766, 680, 541, and 710 nucleotides, respectively

*Eco*RV and *Hin*dIII. Since the ScRALF1 cDNA contained no *Eco*RI site and one *Hin*dIII site, one and two fragments, respectively, were expected for these enzymes. Since no *Eco*RV site was found in the ScRALF1 cDNA, the two fragments observed with the *Eco*RV digestion could result from the presence of a restriction site in a putative intron. For ScRALF2, no restriction sites for the three enzymes tested are present in the cDNA sequence. As expected, only one hybridizing fragment was detected with *Eco*RI and *Eco*RV. For *Hin*dIII, two bands were observed. As in the case of ScRALF1, this second unexpected fragment could be explained by the presence of a restriction site in a putative intron. Cross-hybridization with a highly related member of the family could also explain this hybridization pattern, although this is quite unlikely since a similar pattern would have been obtained for the other two restriction enzymes. For ScRALF 3, 4 and 5, only one hybridizing fragment was detected in *Eco*RI-, *Eco*RV- and *Hin*dIII-digested genomic DNA, as expected from the absence of these restriction sites in the corresponding cDNA sequences. Although ScRALF 4 and 5 showed an identical banding pattern on DNA gel blot analysis, the size of the bands were consistently different, being slightly shorter for ScRALF5. Furthermore, in RNA gel blot analyses (Fig. 3b), no cross-hybridization was detected between ScRALF4 and ScRALF5 and they migrated at different sizes. This, combined with the phylogenetic analysis, suggests that these genes could be the result of a recent duplication event. These results strongly suggest that all five ScRALFs are single copy genes in *S. chacoense*. Furthermore, the lack of cross-hybridization between our five ScRALFs under the hybridization and washing conditions used, indicates that the mRNA expression patterns obtained (see below) are also specific to each ScRALF tested.

ScRALF1–5 mRNA expression in mature tissues

ScRALF1 is expressed exclusively in ovary tissues, particularly in the placenta during later stages of fruit development (Fig. 3b). No expression profile comparison could be established for this clone, since its closest *Arabidopsis* homologues, AtRALF24 and AtRALF31, have not yet been analyzed at this level (Olsen et al. 2002). ScRALF2 showed expression in all tissues tested (Fig. 3b). It was most strongly expressed in roots, stems and petals. Strong expression could also be detected during fruit development. AtRALF33 (one of the ScRALF2 homologs) also showed strongest expression in stems and roots. ScRALF3 was expressed mainly in ovaries following fertilization (fertilization takes place from 36 to 48 h post-pollination in *S. chacoense*). Expression in young fruits gradually declined after 8 days after pollination. Weaker expression levels could also be detected in leaf, petal, style and pollen. Since *S. chacoense* is a self-incompatible species, we took advantage of this reproductive barrier to determine if ScRALF3 was fertilization-induced or if its expression level was modulated by pollination. After a self-incompatible pollination, no strong increase in ScRALF3 mRNA levels could be observed in ovaries, 24, 48 or 96 h after pollination (data not shown), confirming that the strong increase observed was fertilization-induced. The *Arabidopsis* ScRALF3 putative homologue (AtRALF 34) was shown by RT-PCR to be expressed in various tissues including stem, leaf, and root, but no expression was detected in flowers or siliques containing mature seeds (Olsen et al. 2002). These differences might be explained by the low expression observed in non-fertilized flowers in *S. chacoense* and the gradual decrease in ScRALF3 expression observed in maturing fruit. ScRALF4 showed strong expression in floral organs, including style and petals, with a peak accumulation in ovaries and young fruits (Fig. 3b). ScRALF5 showed a similar expression pattern, albeit weaker (Fig. 3b). Strong expression in leaf was also detected for these two ScRALFs. ScRALF4 mRNA has an apparent

size of 541 nt whereas that of ScRALF5 is 710 nt. Therefore, the expression profile determined for these two highly similar ScRALFs was specific. Their almost identical expression pattern also suggests that these genes are the result of a recent duplication event and that their respective promoter regions have not significantly diverged.

Is ScRALF expression modulated by wounding or growth regulator treatments?

In order to establish if ScRALFs could be induced by stress-related hormones, mRNA was extracted from tissues that had been submitted to wounding or various phytohormone and stress hormone treatments. The different treatments tested were: wounding, ABA, JA, SA, and at 6 h or 48 h post-treatment, the two volatile compounds used were HEX and MeJA (Tween, methanol 6 h and methanol 48 h were used as controls). ScRALF1 showed induction following treatment with MeJA in all tissues tested as well as following HEX treatment in leaves (Fig. 4a–c). ScRALF2 is induced in styles by wounding (Fig. 4b). Although Haruta and Constabel (2003) tested MeJA with PtRALF1 and PtRALF2, we cannot compare their results with ours due to differences in the experimental set-up (treated cell cultures compared to whole plants in this study). ScRALF3, already very weak in RNA gel blot analyses, could not be detected in any of the blots with RNA from treated plants. ScRALF4 and ScRALF5 expression was mostly unaffected by these treatments in leaves or styles, but was slightly induced in ovaries following HEX or MeJA, as early as 6 h after treatment (Fig. 4c).

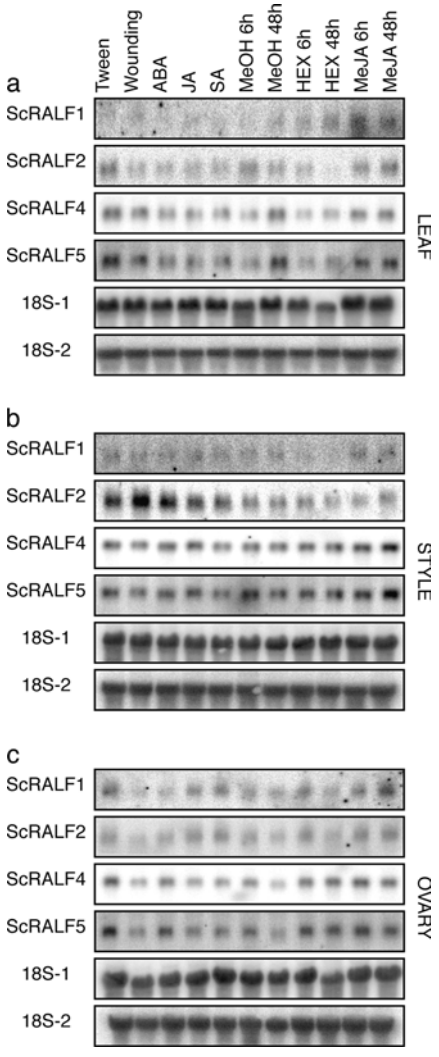


Fig. 4 Effect of wounding and various stress-related hormones on mRNA levels of four ScRALF in **a** leaf, **b** style, and **c** ovary. Total RNA (10 μg) was probed with full-length ScRALF clones. 18S-1 was used as a control for RNA loading of ScRALF1 and 2, and 18S-2 for ScRALF4 and 5. ScRALF3 is not shown, since it was barely detectable on RNA gel blots

Discussion

RALF polypeptides were initially isolated along with systemin in a search for media-alkalinization inducing peptides (Pearce et al. 2001a). Because it was found to cause very rapid alkalinization of the medium (faster than systemin) and since, like systemin, it could trigger a MAPK response, RALF was first associated with the plant defense response. Later, it was shown that RALF could not induce the synthesis of the tobacco trypsin inhibitors when supplied to tobacco plants (Ryan et al. 2002). In poplar, a RALF homologue was also able to induce medium alkalinization faster than other elicitors, but could not induce the defense response gene phenylalanine ammonia lyase (PAL) (Haruta and Constabel 2003). For these reasons it was suggested that RALF is most probably not involved in plant defense responses. We have identified five RALF-like genes that share a similar architecture with previously described RALFs from *Nicotiana* and *Arabidopsis* (Olsen et al. 2002): an amino terminal signal peptide followed by an acidic region located upstream of monobasic or dibasic residues. Although the presence of a pair of basic residues was hypothesized to be important for the proteolytic cleavage of the RALF preprotein (Pearce et al. 2001b), closer examination of RALF sequences found in *A. thaliana*, as well as of our ScRALFs, indicates that only 59% of AtRALFs and 40% of the ScRALFs described here share this motif. Monobasic cleavage sites have also been found in proteins from vertebrates and insects (Veenstra 2000), suggesting that other motif(s) surrounding the mono- or dibasic amino acid residues might be important for cleavage site selection. These remain to be determined in plants. One such motif might be the presence of an acidic region found immediately upstream of the basic residues as well as the YIXY motif found immediately downstream of the predicted cleavage site.

Conserved domains of the putative active polypeptide are a YIXY domain followed by a highly conserved C-

terminal domain composed of a stretch of amino acids ending 2 residues after the last conserved cysteine (Fig. 1). The mature polypeptide also contains two conserved cysteine pairs involved in the formation of disulfide bridges. The ScRALFs share high sequence similarity with the mature tobacco RALF peptide and contain most of the classic features associated with RALF amino acid sequences. In addition, we noticed that the conserved C-terminal tail was composed mainly of charged and polar amino acids, which may affect the tertiary structure of the polypeptide. This region is presumably exposed to the surrounding environment and therefore available for protein-protein interactions.

Modulation of the expression level of ScRALFs following treatment with stress-related hormones or wounding was very subtle. The weak variation observed over a wide array of treatments supports the idea that RALF-like peptides play a developmental role rather than a defense-related role, a hypothesis suggested previously by other authors (Haruta and Constabel 2003; Olsen et al. 2002; Pearce et al. 2001b). Expression of the five ScRALFs seemed to be more influenced by physiological or developmental cues. One interesting finding in our analysis is that while expression of the ScRALFs is rather weak at the time of pollination and fertilization (corresponding to 0 h and 48 h, respectively) it is induced to strong levels at various times during the fruit maturation process. In the case of ScRALF1 and ScRALF2, the peak in expression in fruit seems to occur in the placenta between 8 and 12 days after pollination, while ScRALF3 peaks at an earlier timepoint (96h to 8 days), and ScRALF4 and ScRALF5 do not actually exhibit a defined peak in expression but have a broader expression pattern, being strong from 96 h and later. Interestingly, some ScRALFs, e.g., ScRALF1 and ScRALF3, appear to be expressed almost exclusively in ovary tissues and fruits while the two others, ScRALF2, ScRALF4, together with ScRALF5, showed expression in other tissues. Expression in fruits sometimes appears identical as in the case for ScRALF1 and ScRALF2 (Fig. 3b), but remains different in other tissues, indicating that function may overlap in fruits but may differ in vegetative and other reproductive structures. Another noteworthy observation is that ScRALF2, our closest homolog to *N. tabacum* RALF (87% identity, 93% similarity), is the only one of our ScRALFs to show expression in roots. The NtRALF peptide was shown to stop root growth when supplemented to the medium (Pearce et al. 2001b). Therefore ScRALF2 could also potentially act in roots as a negative regulator of root growth.

Although no bona fide receptor has been found to interact with the small RALF peptides, they have all the characteristic features to be added to a growing pool of small putative peptide ligands in plants. Interestingly, one RALF peptide was recently found to interact with the leucine-rich repeat domain of a pollen-specific PEX protein (Bedinger et al. 2003). The leucine-rich repeat domain is also the most common motif found in ectodomains of plant protein receptor kinases (Shiu and Bleecker 2001), with which many small ligands might interact. The fact that all RALFs tested display different expression patterns suggests that each RALF peptide will exert a different physiological or developmental role, as determined for some members of the CLE peptide family, such as the CLV3 peptide active in shoot apical meristem development (Fletcher et al. 1999) and the CLE19 peptide active in root meristem growth (Casamitjana-Martinez et al. 2003). Assignment of specific roles to each member of the RALF peptide family awaits the production and screening of transgenic plants modulated in their expression level, either through overexpression or silencing.

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