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A conserved dibasic site is essential for correct processing of the peptide hormone AtRALF1 in *Arabidopsis thaliana*

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Abstract Prohormone proteins in animals and yeast are typically processed at dibasic sites by convertases. Propeptide hormones are also found in plants but little is known about processing. We show for the first time that a dibasic site upstream of a plant peptide hormone, AtRALF1, is essential for processing. Overexpression of preproAtRALF1 causes semi-dwarfism whereas overexpression of preproAtRALF1(R69A), the propeptide with a mutation in the dibasic site, shows a normal phenotype. RALF1(R69A) plants accumulate only the mutated proprotein and not the processed peptide. In vitro processing using microsomal fractions suggests that processing is carried out by a kexin-like convertase.

Keywords: Convertase; Protein processing; Prohormone

1. Introduction

Rapid alkalinization factor (RALF) is a 5 kDa ubiquitous plant peptide hormone first isolated from tobacco leaves that induces a rapid and strong alkalinating activity in cell suspension cultures and activates a mitogen-activated protein kinase (MAP-kinase) [1]. When the active peptide was applied exogenously to *Arabidopsis* seedlings it inhibited root growth and development [1]. Gene expression profiles of two RALF peptides isolated from hybrid poplar leaves and five isoforms isolated from fertilized ovule and ovary cDNA libraries of *Solanum chacoense* also suggest a developmental role [2,3]. RALF peptide regulates the extracellular pH at the root hair tip during root hair development and also mobilizes extracellular and intracellular Ca^{+2} [4,5]. The peptides are synthesized as preproproteins and when tobacco preproRALF was fused to GFP it was visualized in the ER and, later on, in the apoplast [6]. In tomato cell suspension cultures, two membrane proteins of 25 and 125 kDa are cross-linked to RALF peptide and may be part of a membrane receptor [7].

The primary structure of RALF precursors contains a conserved dibasic site upstream of the active peptide suggesting that they undergo protein processing similar to prohormones of animals and yeast [1,8]. In animals and yeast, proteases such as kexin, furin and convertases PC2, PC1/PC3, PC4, PACE4, PC5/6 and PC7, all of them members of the subtilisin family of serine proteases, are responsible for the recognition and processing of preprohormones at dibasic sites [9,10]. In plants, subtilisin-like proteinases have been isolated and characterized from several species. *Arabidopsis* exhibit 56 annotated subtilases [11], and two of them exhibit high similarity to the mammalian kexin proteases. Subtilisin-like activity similar to prohormone convertases has also been observed in leaves and plant microsomes [12–14].

To date, no evidence has been presented that dibasic sites are essential for in vivo processing of plant peptide hormone precursors. Here we report that *Arabidopsis* plants overexpressing AtRALF1 gene (35S:AtRALF1) show a semi-dwarf phenotype, and accumulate the processed peptide. On the other hand, plants overexpressing the mutated AtRALF1 precursor with an Arg to Ala substitution at the conserved dibasic site [35S:AtRALF1(R69A)] fail to exhibit the semi-dwarf phenotype. The 35S:AtRALF1(R69A) plants show normal root and leaf growth with accumulation of the mutated proprotein and nearly undetectable levels of the processed peptide. In addition, protein extracts from the microsomal fractions were able to cleave the preproAtRALF1, but not the mutated precursor preproAtRALF1(R69A). Our results demonstrate that an intact dibasic site upstream of the active peptide hormone RALF is essential for proper processing and suggest that, like in animals and yeast, this processing in plants is likely done by a kexin-like convertase.

2. Materials and methods

2.1. Plant transformation and root and leaf measurements

Arabidopsis plants (Columbia ecotype) were grown in environmental chambers at 16 h light, 22 °C and 8 h dark, 18 °C. For AtRALF1 gene overexpression, the intronless AtRALF1 gene was obtained from genomic DNA of *Arabidopsis* using PCR. The mutation of AtRALF1 to generate AtRALF1(R69A) was also performed by PCR. The primers used for cloning are available in Supplementary Table S1. For cloning strategy, details of AtRALF1 gene mutation and plant transformation see Supplementary methods. Root and leaf measurements were obtained as described [15].

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Abbreviations: RALF, rapid alkalinization factor; MAP-kinase, mitogen-activated protein kinase; 35S, cauliflower mosaic virus 35S RNA promoter

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2.2. Purification of AtRALF1(R69A) proprotein, digestion, N-terminal sequencing and isolation of active peptide

Crude protein extracts were prepared and purified with preparative slab gels as described [16] (Supplementary methods). Purified AtRALF1(R69A) protein was digested over-night at room temperature using Endoproteinase Glu-C (Boehringer Mannheim) according to manufacturer's instructions. Digested products were separated using a reversed phase C18 HPLC column (218TP54, 5-μm 4.6 × 250 mm column, Vydac) and fractions containing RALF peptides were detected using ELISA. N-terminal sequence of the peptides was obtained using Edman chemistry on an Applied Biosystems (Foster City, CA) Procise Model 492 protein sequencer.

AtRALF1 and AtRALF1(R69A) active peptides were purified from leaves of *Arabidopsis* plants as described [1] (Supplementary methods).

2.3. In vitro synthesis of labeled peptide and Arabidopsis microsomal fraction

The wild-type AtRALF1 gene and its mutated form AtRALF1(R69A) were amplified by PCR and cloned into the pGBKT7 vector (Clontech). For primer sequences see Supplementary Table S2. Precursors were produced using the TNT® coupled wheat germ extract system (Promega) according to manufacturer's instructions. The translation reaction was made in the presence of Redivue [35S] methionine (GE Healthcare Bio-sciences).

Crude microsomal membrane extracts were obtained as previously described [17] with some modifications. Cells from a 7-day-old *Arabidopsis* cell suspension culture [18] were collected by a brief spin and then pulverized in a mortar in the presence of liquid N_2. The lysate was homogenized in ice-cold buffer (50 mM Tris–HCl, pH 8.5, 5 mM EDTA) and filtered through two layers of miracloth (Clontech). The homogenate was centrifuged at 12000×g for 15 min at 4 °C and the supernatant was collected and then centrifuged at 100000×g for 1 h at 4 °C. The resulting microsomal pellet was resuspended in 100 μl of 1% Triton X-100 ice-cold solution to a final protein concentration of 1.25 μg/μl. The resulting protein concentration was measured with Bradford reagent (Sigma) and visualized by SDS–PAGE.

The proprotein processing assay was carried out in a 200 μl final volume reaction by mixing 5 μl of labeled peptides with 10 μg of total microsomal protein extract in Kex2p reaction buffer [19]. The mix was incubated for 1 h at 30 °C followed by TCA precipitation. The pellet was washed with 200 μl of cold acetone and resuspended in Laemmli sample buffer. Samples were separated in polyacrylamide gels (SDS–PAGE) that were later dried and exposed to X-ray film.

3. Results

3.1. AtRALF1 mutation and plant transformation

In order to evaluate the significance of the conserved dibasic site in the maturation of preproRALF, a mutation that replaced the second Arg for an Ala (Arg69 in AtRALF1, locus At1g02900) was introduced into the *Arabidopsis* AtRALF1 gene. Transgenic plants carrying the AtRALF1 gene or the mutated AtRALF1 transgene [AtRALF1(R69A)] under the control of the constitutive CaMV 35S promoter were obtained and overexpressors were identified based on kanamycin resistance and RNA blots. Over thirty plants of 35S:AtRALF1 and 35S:AtRALF1(R69A) independent transgenic lines were produced and they all showed a high level of expression of the transgenes. An RNA gel blot of nine selected transgenic lines is shown to illustrate the level of transcript accumulation for both 35S:AtRALF1 and 35S:AtRALF1(R69A) plants (Supplementary Fig. S1).

3.2. Phenotype of transgenic plants and quantitative analyses

All 35S:AtRALF1 plants overexpressing AtRALF1 gene showed a semi-dwarf phenotype (Fig. 1A and Supplementary Fig. S2) as opposed to the normal phenotype showed by all

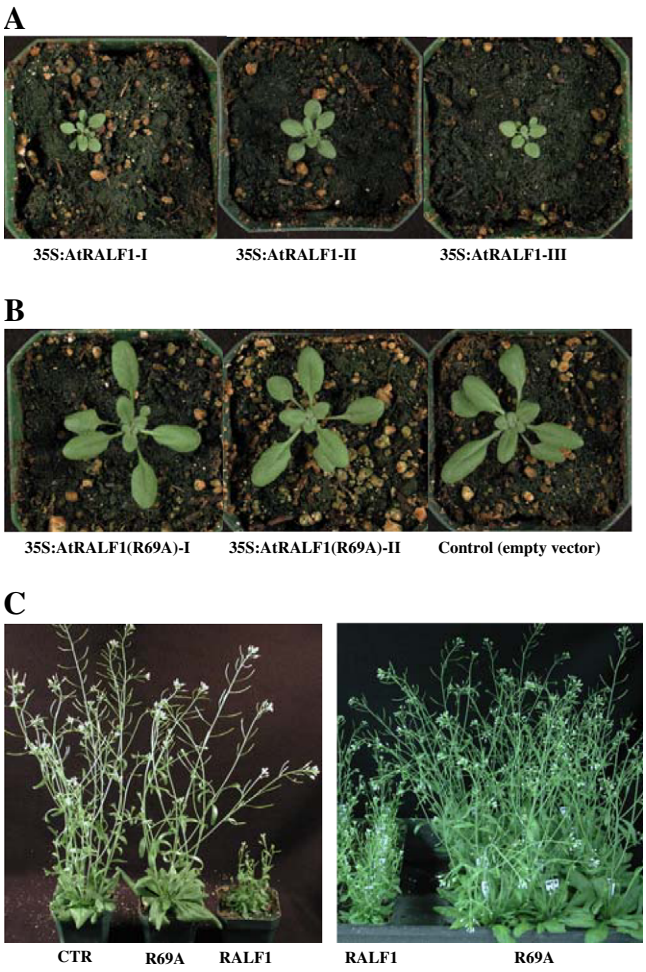

Fig. 1. Third-generation transgenic plants with high level of expression of AtRALF1 and the mutated AtRALF1(R69A). (A) Three plants overexpressing AtRALF1 (lines 35S:AtRALF1-I, II and III). (B) Two plants overexpressing AtRALF1(R69A) (lines 35S:AtRALF1(R69A)-I and II) and a control plant transformed with empty vector. (C) Phenotype comparison among control, 35S:AtRALF1 and 35S:AtRALF1(R69A) plants in advanced stage of maturation. Control and 35S:AtRALF1(R69A) plants show normal phenotypes while 35S:AtRALF1 plants show semi-dwarf phenotype.

AtRALF1(R69A) plants (Fig. 1B). AtRALF1(R69A) overexpressors were undistinguishable from wild-type or plants transformed with an empty vector (Fig. 1B). We are now using the third generation of the transgenic plants and they all show stability of both semi-dwarf and normal phenotypes. Fig. 1C shows mature control plants, 35S:AtRALF1 and 35S:AtRALF1(R69A) plants. Mature plants overexpressing AtRALF1(R69A) gene could not be set apart from control plants and could be easily separated from plants overexpressing AtRALF1. No differences in flowering time were observed in transgenic plants (data not shown).

Quantitative analyses were obtained for leaf length and width and for root growth (Supplementary Figs. S3 and S4). Evaluation of leaf growth revealed reduced leaf dimensions for 35S:AtRALF1 plants, but similar leaf sizes for both 35S:AtRALF1(R69A) and control plants (Fig. 2A). Roots of 12 days old seedlings of 35S:AtRALF1 plants are much smaller than roots of 35S:AtRALF1(R69A) plants (Fig. 2B).

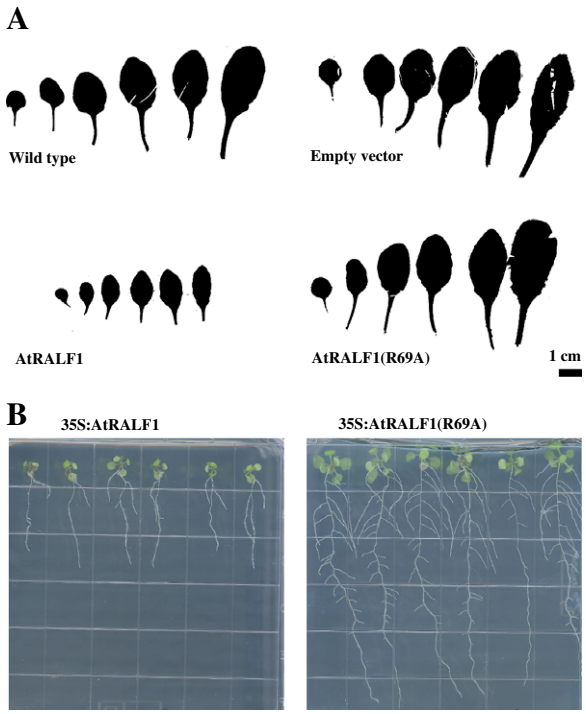

Fig. 2. Leaf and root growth of *Arabidopsis* transgenic plants. (A) Leaves from 1 to 6 of adult plants were digitalized for dimensional comparison. Plants overexpressing the AtRALF1(R69A) show normal growth when compared to reduced growth of plants overexpressing the AtRALF1 precursor. Plants carrying an empty vector and wild-type plants were used as controls. (B) Seedlings growing in plates for root measurements. Seedlings overexpressing the 35S:AtRALF1 transgene show reduced root growth when compared to seedlings overexpressing the 35S:AtRALF1(R69A) transgene.

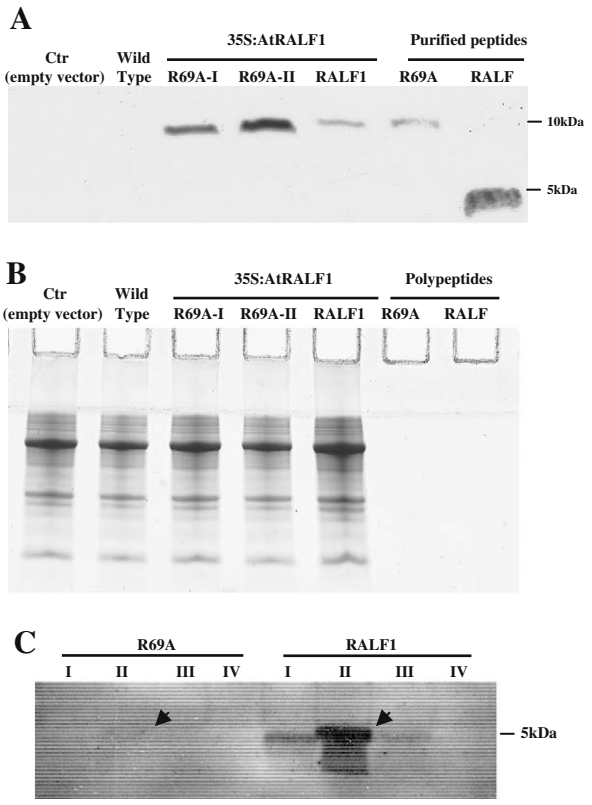

Fig. 3. Detection of AtRALF proproteins and AtRALF active peptides in leaf protein extracts. (A) Western blot analysis of crude protein extracts from plants overexpressing the 35S:AtRALF1 and 35S:AtRALF1(R69A) transgenes. Levels of endogenous AtRALF (in control and wild-type plants) are too low to be detected. (B) A Coomassie Brilliant Blue stained replicate gel is shown as a protein loading control. R69A propeptide and rapid alkalinization factor (RALF) peptide are positive controls. (C) Western blot analysis of AtRALF peptides purified from leaf protein extracts by reversed phase chromatography. Active fractions in the alkalinization assay of the AtRALF1 extract and correspondent fractions of AtRALF1(R69A) extract are numbered from I to IV. Arrows indicate bands identified using an antibody raised against RALF peptide. Standard protein bands are shown on the right.

3.3. Identification of unprocessed and processed precursors

Crude protein extracts from 35S:AtRALF1(R69A) plants separated in SDS/PAGE gels showed a strong band about twice the size of RALF peptide in a protein blot (Fig. 3A). This band has a molecular weight (M_r) of approximately 10 kDa and it is in accordance with the expected molecular weight for AtRALF1 precursor lacking the signal peptide. A weak band of the same size could also be detected in crude protein extracts from plants overexpressing AtRALF1 transgene. Neither wild-type plants nor plants transformed with an empty vector showed bands in crude protein extracts. Same amounts of protein were loaded in the gel (Fig. 3B).

We also isolated the active processed RALF peptides from leaves of *Arabidopsis* plants. Crude protein extracts from plants overexpressing the AtRALF1 and AtRALF1(R69A) transgenes were obtained and loaded in a C-18 flash column chromatography. After freeze-drying, the 60% methanol fraction yielded 67 and 66 mg of protein for AtRALF1 and AtRALF1(R69A) extracts, respectively. The materials were resuspended in appropriate buffer and loaded into a RESOURCE-3 ml HPLC reversed phase column. Fractions were tested in the alkalinization assay and RALF peptide activity was detected only in fractions 26–29 of the AtRALF1 purified extract. Fractions from 22 to 33 of AtRALF1 extract and correspondent fractions of AtRALF1(R69A) extract were combined every three fractions, lyophilized and loaded in an SDS–PAGE gel. Western blot analyses with anti-RALF antibody revealed the presence of a band in lane I (fractions 22–24), II (fractions 25–27) and III (fractions 28–30) of the AtRALF1 protein extract (Fig. 3C). A hardly noticeable band can be seen in lane II (fractions 25–27) of the AtRALF1(R69A) extract (Fig. 3C).

3.4. Mutated unprocessed precursor

Preparative slab gels were used to isolate and confirm the identity of the mutated unprocessed proprotein showed in Fig. 4A. The purified putative precursor was digested with Endo-Glu-C and ELISA was used to identify the digested fragments. AtRALF1 deduced preproprotein has six Endo-Glu-C cleavage sites and also a predicted signal peptidase cleavage site between amino acids Ala26 and Gly27 (Fig. 4A). Enzyme immunoassay (ELISA) performed in HPLC fractions of the Endo-Glu-C digested and undigested putative AtRALF1(R69A) precursor detected two peaks in the digested sample that were absent in the undigested precursor (Fig. 4B). The first peak contained a peptide resulted from Endo-Glu-C cleavage at the amino acid position Glu65. The second peak

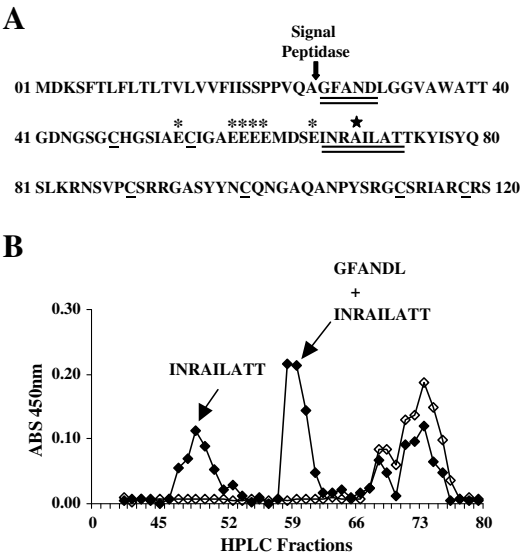

Fig. 4. Identification of the amino acid substitution (R69A) in the precursor preproAtRALF1(R69A) extracted from *Arabidopsis* leaves. (A) Primary structure of the preproAtRALF1 protein. The arrow shows the cleavage site for the enzyme Signal peptidase and the asterisks indicate possible targets of Endo-Glu-C. The substitution of the amino acid alanine at position 69 in the preproAtRALF1 is indicated by a star. Sequenced peptides are double-underlined and cysteine residues are underlined. (B) ELISA results of chromatographic fractions of preproAtRALF1(R69A) prior to digestion (empty circles) and after digestion with the enzyme Endo-Glu-C (black-filled circles). Sequenced peptides are shown above their respective peaks.

contained two peptides. One is identical to the first peak, and the second is the N-terminal sequence of the unprocessed proprotein. The two sequences eluted together generating two N-terminal sequences in a single eluting peak. The first eluting peak had a retention time on HPLC similar to RALF peptide standard (data not shown) and the N-terminal sequence obtained covered the Arg69 to Ala69 amino acid substitution (star in Fig. 4A).

3.5. *In vitro cleavage of preproAtRALF1 by Arabidopsis microsomal fraction*

Radiolabeled wild-type preproAtRALF1 and mutated preproAtRALF1(R69A) were produced by in vitro transcription and translation and then incubated with protein extracts from microsomal fractions of *Arabidopsis* cell suspension cultures. The results in Fig. 5 show that only the preproAtRALF1 was processed when incubated with the microsomal protein extract. The mutated preproAtRALF1(R69A) remained intact. The observed mass based on relative gel mobility of the band matched the expected mass of the labeled N-terminal part of the processed peptide plus the amino acids of the pGBKT7 vector, approx. 8874 Da. Both radiolabeled peptides were fully digested when incubated with trypsin.

4. Discussion

RALF peptides are located in the C-terminal region of preproproteins that share a conserved dibasic site upstream of the active peptides [1–3,20]. Dibasic residues are also conserved in the Phytosulfokine and Clavata families of peptide hormones

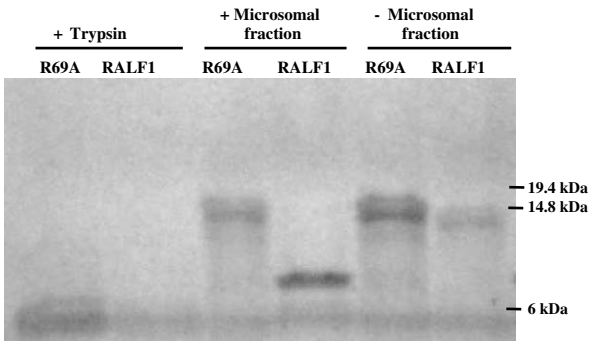

Fig. 5. In vitro digestion assay of native and mutated AtRALF precursors by microsomal extract from *Arabidopsis*. Radiolabeled [35S] methionine preproAtRALF1 and preproAtRALF1(R69A) were incubated into kex2p reaction buffer with (+) or without (−) microsomal protein extract. A prior digestion of precursors with trypsin was carried out as a control. Molecular weight standards are represented on the right.

[8,21–23]. Such evidences suggest that, like in animals and yeast, a dibasic site for processing may be essential for the maturation of plant prohormones.

We generated a mutation in the second Arg of the At1gRALF1 precursor coded by the gene At1g02900, and our results confirm that a dibasic site is essential for proper maturation and release of RALF active peptides in *Arabidopsis*. Lack of processing activity due to a single change in the dibasic site has been shown using chimeric reporter proteins in tobacco cell suspension culture [13].

Transgenic plants overexpressing the AtRALF1 gene show a semi-dwarf phenotype while plants mutated at Arg69 do not (Fig. 1). The lack of typical phenotype in 35S:AtRALF1(R69A) plants was revealed in detail by quantitative analyses of root and leaf growth (Fig. 2). It must be noted that the shortening of aerial parts of 35S:AtRALF1 plants is somehow unexpected since AtRALF1 isoform is expressed solely in roots. Possibly, ectopic overexpression of AtRALF1 is interfering in the normal perception of other (leaf) RALF isoform peptides.

The absence of the semi-dwarf phenotype in 35S:AtRALF1(R69A) plants indicates that no active AtRALF1 peptide was being produced or released from mutated precursor in the aerial parts of these plants, and protein analyses of transgenic plants confirm such hypotheses. Crude protein extracts from 35S:AtRALF1(R69A) plants revealed an accumulation of mutated precursor, but nearly undetectable levels of active peptides (Fig. 3). The presence of a signal of low intensity is probably due to endogenous conserved and ubiquitously expressed RALF isoforms [8,20].

Interesting to note that even with high levels of transgene expression, no processed AtRALF1 peptide was detected in protein extracts of 35SAtRALF1 plants. We were able to detect the active peptide when crude extracts were enriched with semi-purification of RALF peptides (Fig. 3C). This suggests a fast turnover of the active AtRALF1 peptide as a way to control its activity.

In animals and yeast, the group of kexin-like serine proteases is a branch of the subtilisin family that recognizes a more specific site generally composed by RR or KR [24]. In plants, a kexin-like activity has been demonstrated to play a role in protein processing [12,13]. To start elucidating the nature of the

processing enzyme of RALF peptides, we extracted proteins from microsomal fractions (RER and Golgi complex) of *Arabidopsis* cell suspension cultures and conducted experiments in conditions proper to kex2p-like enzymes. Radiolabeled prepro-roRALF1 was processed to liberate a peptide with a molecular weight equivalent to RALF active peptide, while radiolabeled preproAtRALF1(R69A) showed no alteration (Fig. 5). This result demonstrates that the processing enzyme may be a convertase or kex2p-like subtilisin, similar to processing enzymes of animals and yeast prohormones.

Our results set the beginning of a search for the processing enzymes of propeptide hormones in plants. There is a large number of RALF and RALF-like peptides in *Arabidopsis* and it would be interesting to investigate how plants dealt with specific processing considering that only two convertase-like enzymes have been identified in the *Arabidopsis* genome [11].

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.08.025.

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