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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

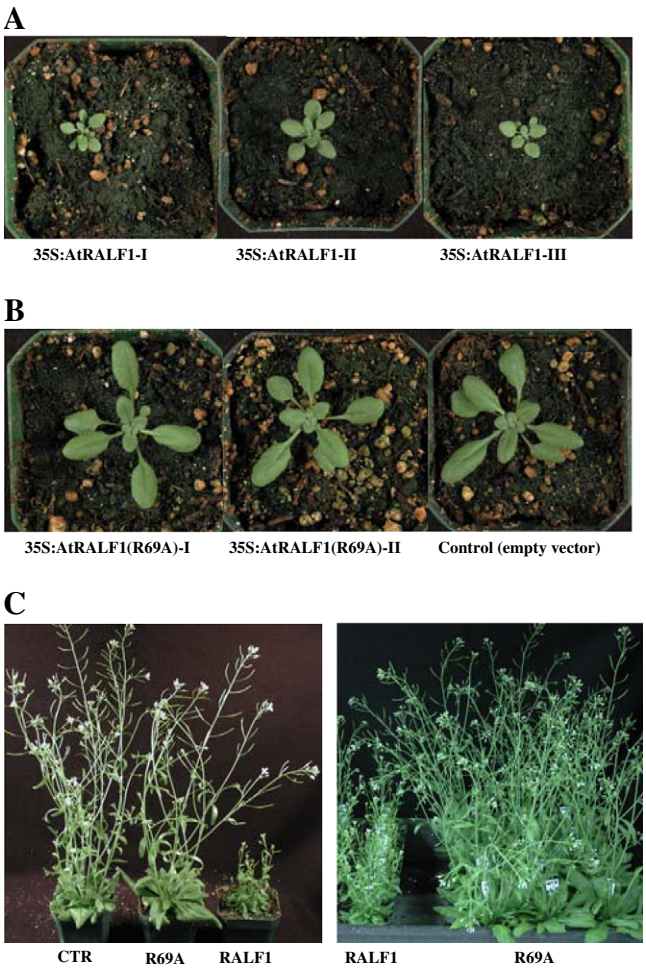

Fig. 1. Third-generation transgenic plants with high level of expression of AtRALF1 and the mutated AtRALF1(R69A). (A) Three plants overexpressing AtRALF1 (lines 35S:AtRALF1-I, II and III). (B) Two plants overexpressing AtRALF1(R69A) (lines 35S:AtRALF1(R69A)-I and II) and a control plant transformed 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).