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Ghrelin in plants: What is the function of an appetite hormone in plants?

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ABSTRACT

In the present work, we provide compelling evidence for the expression of a ghrelin-like peptide hormone that has only been associated with animals, in various plant tissues. Ghrelin, the appetite stimulating hormone, has been identified from a number of different species including humans, rat, pig, mouse, gerbil, eel, goldfish, bullfrog and chicken. The study here was conducted using an immunohistochemistry assay to screen whether plants have any ghrelin immunoreactivity. In this respect, *Prunus x domestica* L. and *Marus alba* were examined. Immunohistochemistry results showed that there is a strong human ghrelin immunoreactivity substance in the parenchyma cells of these plants. This was entirely unexpected since this hormone was considered to be present solely in animals. Thus, this study is the first to report the presence of a peptide with ghrelin-like activity in plants, a finding that has only been observed in the animal kingdom. RIA analysis confirmed that these plants contain significant amounts of this substance. Furthermore, reverse-phase HPLC analyses of plant extracts showed an elution characteristic of the peptide identical to that of human ghrelin. In general, fruit from both plants had higher levels of the peptide than the vegetative parts.

1. Introduction

Ghrelin (G-HH), an endogenous ligand for the growth hormone (GH) secretagogue receptor (GHSR) was characterized recently from extracts of rat stomach by Kojima et al. [10] and they named it "ghrelin" based on its role as a "Growth Hormone Releasing Peptide", with allusion to the Proto-Indo-European root *ghre* meaning "grow". Ghrelin is a 28-amino acid peptide hormone in which the third amino acid, usually a serine but in some species a threonine, is modified by a fatty acid; this modification is essential for ghrelin's activity. In this respect, ghrelin is the first known example of a bioactive peptide modified by an acyl acid. This small peptide hormone is expressed primarily by the stomach and secondarily by lower parts of the gastrointestinal

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tract [1]. A recent study by our group and others [7] also showed the presence of ghrelin in the salivary glands of human subjects, making the assay for this hormone highly practical [2].

G-HH, considered the counterpart of the hormone leptin, acts as an appetite stimulator. It increases prior to and decreases after a meal. While the effect of ghrelin on appetite and food intake in man has not been determined, it has been shown that this hormone promotes increased food intake, weight gain and adiposity in rodents [1]. People suffering from the eating disorder ''anorexia nervosa'', however, appear to have high plasma levels of ghrelin. Also, G-HH levels are found to be increased in patients who have cancer-induced cachexia [5]. We also showed that gastric and mucoepidermoid tumors displayed no immunoreactivity for ghrelin, while noncancerous gastric and salivary gland tissues were intensely labeled with anti-ghrelin antibodies [3].

Since the discovery of this hormone, research has mainly been focused on the stimulatory effect of ghrelin on the food intake and its role in the modulation of energy expenditure [1,4,13–15]. To date, ghrelin has only been identified in tissues of animal origin as that of humans, rat, pig, mouse, gerbil, eel, goldfish, bullfrog and chicken [11]. Having a highly conserved amino acid sequence (especially at N-terminal region) across all animal species studied, ghrelin makes an excellent example of biopetides with diverse functions. Given the ubiquity of ghrelin in all animals studied and its probable evolutionary function, the present study was concerned with whether plants also expressed this multifunctional hormone. Various tissues of *Prunus x domestica* L. and *Marus alba* were studied using highly sensitive immmunohistochemical, radioimmunoassay and reverse-phase HPLC.

2. Methods

2.1. Kits and reagents

Standard (pure) ghrelin and associated reagents (e.g. in rabbit anti-ghrelin (human) antibody) for ghrelin assay were obtained from Phoenix Europe (Karlsruhe), Germany. All other reagents, as amino ethyl carbazole, were from Lab Vision Corporation (Cat: TA-125-HAS), Fremont, CA, USA. Radioimmunoassay analysis was carried out by monitoring the radioactivity (I^{125}) using a 20-well multichannel gamma counter (Multigamma model 1261; LKB-Wallac, Turku 10, Finland).

2.2. Sample preparation

Ghrelin presence (if any) assay (the immunohistochemical reactivity of anti-human ghrelin) in *P. x domestica* L. was carried on fruit, leaf and leaf petiole. RIA analysis of ghrelin-like activity was carried out in fruit and leaf petiole of *M. alba*. From each plant species ripped fruits, leaf and leaf petioles were collected and transferred to pathology and biochemistry laboratories. The homogenate was prepared first by finely slicing 1 g of fresh tissue and then crushing with an iron mold. Crushed samples were then homogenized in PBS (5%, w/v) using a stainless-steel mortar. Homogenized samples were subsequently centrifuged at 9800 rpm for 20 min at 4 °C and the resulting supernatant was stored at −20 °C until use.

2.3. Immunohistochemistry

Immunohistochemistry (IHC) analysis was carried out using streptavidin–avidin–biotin–peroxidase complex (ABC) procedure [9] with minor modification [2]. First, microtome (Reichert Jung Microtome Model Histoslide 2000, Germany) sliced 4-μm thick sections were mounted on silanized slices, de-waxed in xylene, dehydrated with alcohol and then immersed in 3% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase activity and immunostained by the ABC procedure. ABC was prepared according to the protocol provided by manufacturer (Lab Vision Corporation). Plant tissue sections were then placed in citrate buffer (pH 6.0) incubated in a 650 W microwave for 5 min, and washed in 0.01 M phosphate buffer pH of 7.4 (PBS). Blocking reagent was applied at this point in the assay for 10 min to remove nonspecific antibody binding. Next, the sections were incubated in rabbit anti-ghrelin (human) antibody® (which was generated against Gly-Ser-Ser(Octanoyl)-Phe-Leu-Ser-Pro-Glu-His-Gln-Arg-Val-Gln-Gln-Arg-Lys-Glu-Ser-Lys-Lys-Pro-Pro-Ala-Lys-Leu-Gln-Pro-Arg) at 38°C for 30 min, washed in PBS, incubated with biotinylated goat anti-polyvalent (Lab Vision Corporation), at 38 °C for 15 min and washed again in PBS. Streptavidin–biotin–peroxidase complex was applied to each tissue section for 10 min and then the tissues were washed twice in PBS for 5 min. Amino ethyl carbazole (AEC) was applied as a chromogen for 10 min. Finally, the sections were counterstained with Mayer's hematoxylin for 1–2 min, washed with distilled water, dried and mounted. For negative controls, the primary antibody was replaced with PBS. Positive controls were performed using normal human salivary gland tissue. Slides were examined under a light microscope.

2.4. Assay of ghrelin in plant tissues

Ghrelin immunoreactive in plant tissues was performed using a human ghrelin RIA Kit (Phoenix, Europe, Karlsruhe, Germany) according to Manufacturer's Manual. Our preliminary studies showed that, the lowest detectable level of ghrelin with this method was about 5 pg/mg tissue, making the assay as a highly sensitive one. Ghrelin measurements were made in triplicates of three independent experiments. PBS buffer was used as a zero control.

2.5. High performance liquid chromatography (HPLC)

Fresh samples were smashed in a mortar and then 1 g fruit paste was made to a total of 5 ml with 0.1 M Tris–HCl buffer, pH 7.5. The mixture was centrifuged at 10,000 rpm at 4 °C for 20 min. The supernatant fraction was immediately used for the HPLC analysis, which was performed according to the method of Dawes and Dawes (SGE Choromatography Products Catalog, 2000, p. 182). Separations were carried out at room temperature with a Cecil liquid chromatography system (Series 1100) consisting a sample injection valve (Cotati 7125) with a 20 μl sample loop, an ultra-violet (UV) spectrophotometric detector (Cecil 68174) set at 215 nm, Integrator (HP 3395) and a SGE Walkosil 11 5Cl8 RS column (150 × 4.6 i.d., 5 μm particle and 120 Å pore size) packed with 50 mM $NaClO_4$ in 0.1% H_3PO_4 as a mobile phase at 1.1 ml min^{-1} flow rate.

3. Results

The immunohistochemical ghrelin localization in various plant tissues is given in Figs. 1–6. It is apparent that ghrelin localization is mainly in parenchyma cells (PC), where a heavy labeling with anti-human ghrelin antibody is observed. Also, immunoreactive ghrelin localization was determined in phloem, which is made from less specialized and nucleate parenchyma cells. Furthermore, reverse-phase HPLC analyses of plant extracts revealed almost identical elution characteristics of standard human ghrelin (Fig. 7). Both plant extracts and the standard (pure human ghrelin) showed an elution time in around 7.2 min. The presence of ghrelin activity was confirmed through radioimmunoassay and highly specific immmunohistochemical analysis that utilized a human anti-ghrelin antibody. Ghrelin concentration in plant tissue extracts was determined using a standard curve generated by commercially available human ghrelin, a

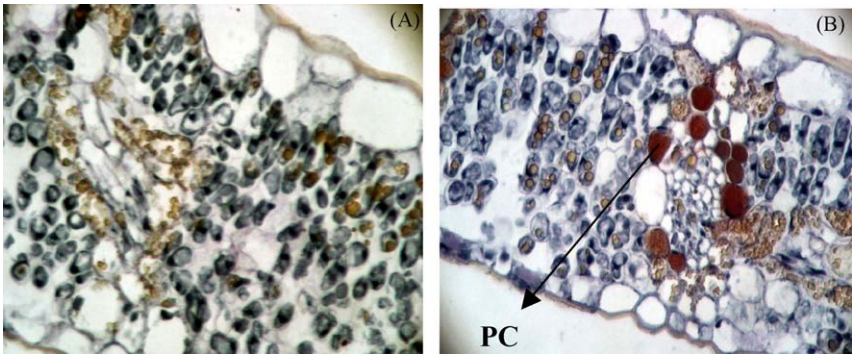

Fig. 1 – Immunohistochemical reactivity of ghrelin in leaf cells from *Prunus x domestica* L. (A) Negative control; (B) immunoreactive parenchyma cells (PC) heavily labeled with anti-human ghrelin antibody. ×200.

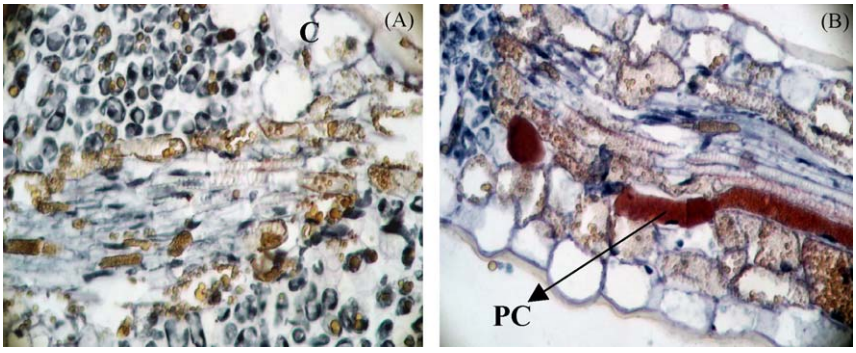

Fig. 2 – Immunohistochemical reactivity of ghrelin in leaf cells (the longitudinal section) from *Prunus x domestica* L. (A) Negative control; (B) immunoreactive parenchyma cells (PC) where ghrelin localization is apparent through binding of anti-human ghrelin antibody. ×200.

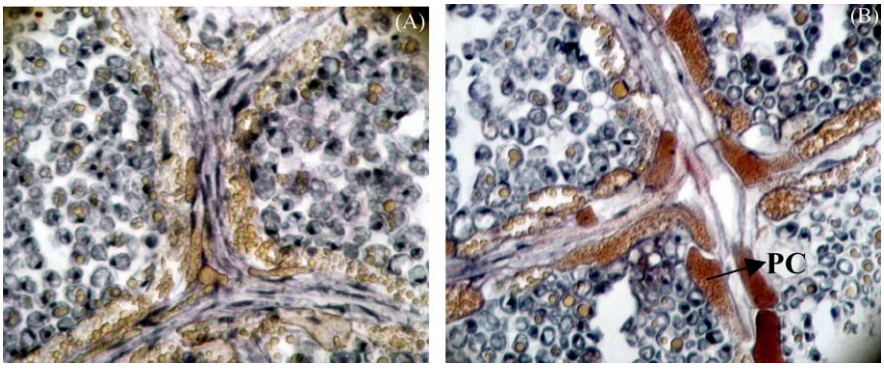

Fig. 3 – Immunohistochemical reactivity of ghrelin in leaf cells (the horizontal section) from *Prunus x domestica* L. (A) Negative control; (B) immunoreactive parenchyma cells (PC) where ghrelin immunolocalization is apparent. ×200.

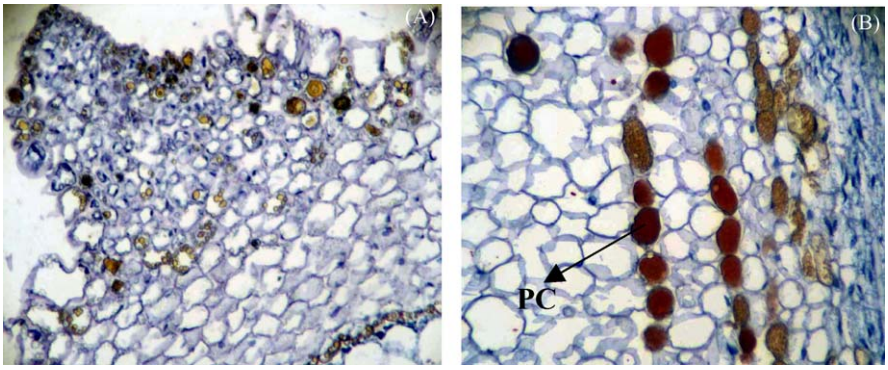

Fig. 4 – Immunohistochemical reactivity of ghrelin in fruit cells (the cross-section) from *Prunus x domestica* L. (A) Negative control; (B) immunoreactive parenchyma cells (PC) where ghrelin immunolocalization is apparent. ×200.

solid phase synthesized peptide. Each value is the average of three independent experiments with ±S.D. (σ_{n-1}). In general, fruit from both plants had higher level of ghrelin than the vegetative parts as leaf or leaf petiole. Fruit from *P. x domestica* L. had slightly higher ghrelin levels than that of from *M. alba*. Given the interference of other cellular compounds, a method for hormone analysis should not only be specific but also highly sensitive. In the present study, ghrelin immunoreactive in plant tissues was performed using a human ghrelin RIA kit. Our preliminary studies showed that, the lowest detectable level of ghrelin with this method was about 5 pg/mg tissue, making the assay as a highly sensitive one. The RIA analysis of ghrelin in fruit from *P. x domestica* L. and *M. alba* revealed 16.55 (±3.32) and 11.05 (±0.78) pg ghrelin per milligram of fresh tissue, while these values for leaf petiole from *P. x domestica* L. was 10.24 (±1.08) and *M. alba* was 12.23 (±0.75) pg per milligram of fresh tissue.

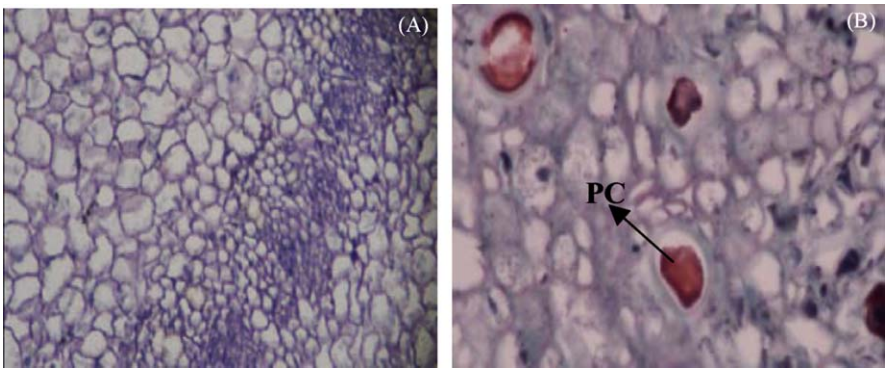

Fig. 5 – Immunohistochemical reactivity of ghrelin in branch cells (the cross-section) from *Morus alba*. (A) Negative control; (B) immunoreactive parenchyma cells (PC) where ghrelin immunolocalization is apparent. ×200.

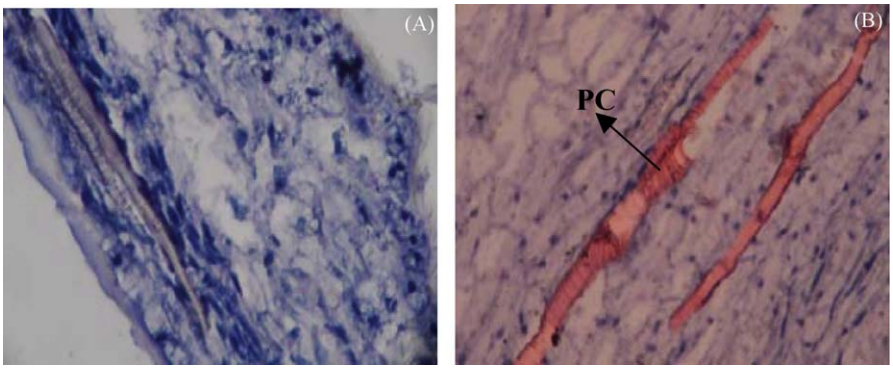

Fig. 6 – Immunohistochemical reactivity of ghrelin in fruit cells (the longitudinal section) from *Morus alba*. (A) Negative control; (B) immunoreactive parenchyma cells (PC) where ghrelin immunolocalization is apparent through heavily labeling for ghrelin antibody. ×200.

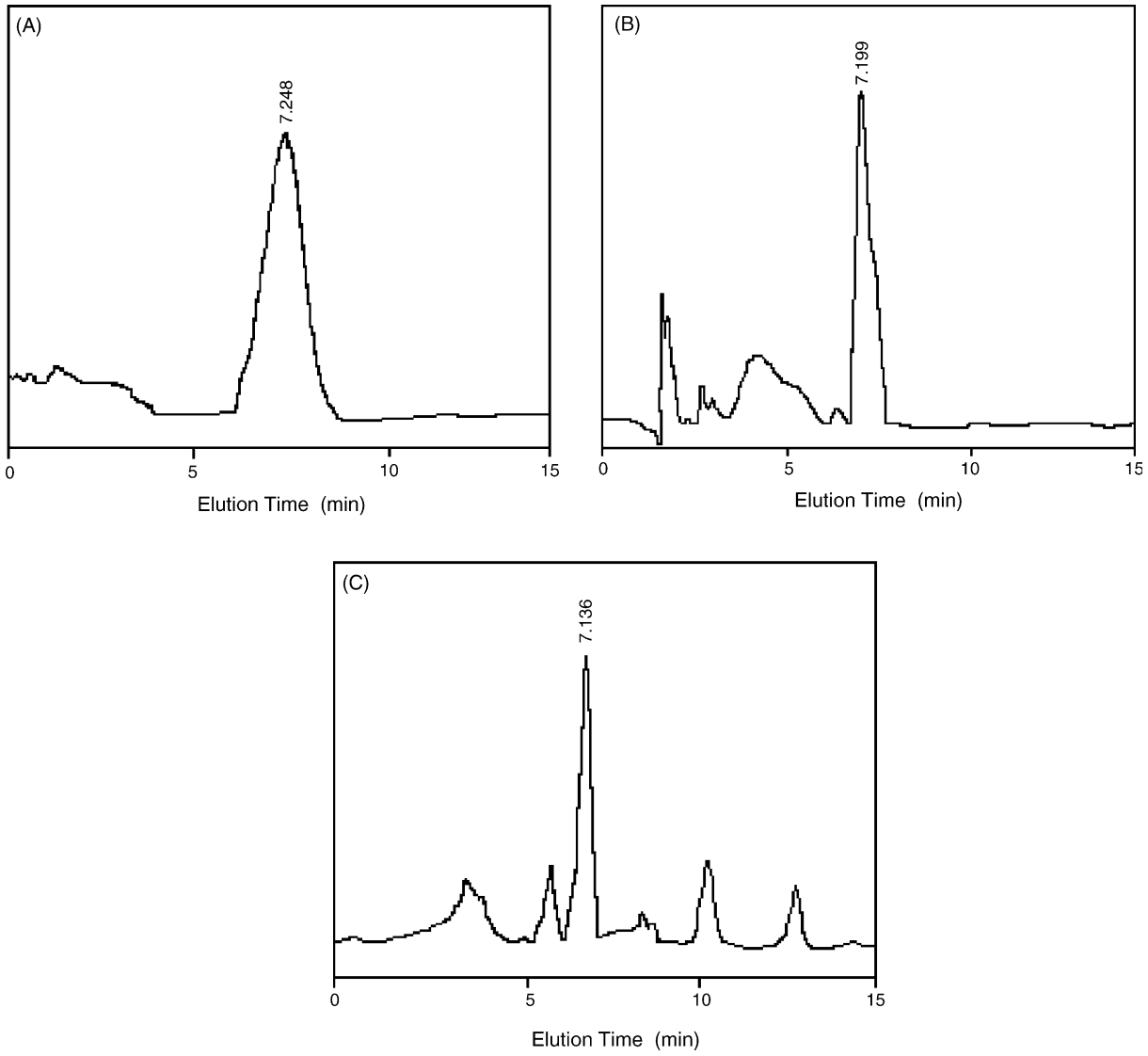

Fig. 7 – Reverse-phase HPLC analyses of plant extracts. (A) Standard human ghrelin (33.3 μg/ml); (B) 20 μl *Morus alba* fruit (ca. 50 μg/ml); (C) 20 μl *Prunus x domestica* L. fruit extract (50 μg/ml). Elution time for both the standard (human ghrelin) and samples is apparent around 7.2 min.

4. Discussion

In this study we made an attempt to determine whether plants had the recently discovered [10] multifunctional peptide hormone, ghrelin, which has only been determined in species from animal kingdom. G-HH is a growth related hormone expressed in almost all tissues of human [6] and other animals studied from man to mouse. Having a highly conserved (>90%) amino acid sequence across the species as diverse as man and mouse indicates an evolutionary role for this hormone, similar to that of melatonin which was determined to be not present not only in animals but in plants. Various tissues from *P. x domestica* L. (common plum) and *M. alba* (mulberry) were used for their content of ghrelin. To our surprise, both plants showed a substantial level of ghrelin immunoreactivity in their fruits and other vegetative parts as leaf and leaf petiole. This observation was puzzling, since for the first time immunoreactive ghrelin localization was determined outside the animal kingdom. Furthermore, the presence of ghrelin activity in these plants was also verified through a ghrelin-specific radioimmunoassay. Our preliminary studies with commercially obtained human ghrelin showed that the RIA kit used mainly for human plasma ghrelin levels could well be used for determining the level of this hormone in plant tissue extracts. This RIA method utilized here has been shown by HPLC to measure the intact peptide in the type of plasma samples being analyzed [12]. Although, this method is mainly used for determining plasma ghrelin levels, the utilization of the assay for any biological fluid with a sufficient level of ghrelin was also reported [2]. Furthermore, reverse-phase HPLC analyses of plant extracts showed an elution characteristic of the peptide identical to that of human ghrelin. Both human ghrelin and the related substance from plant extracts had almost identical elution characteristics. Both molecules

showed a reverse-phase HPLC elution time of about 7.2 min under the same conditions. The level of ghrelin-like peptide hormone in plant extracts, however, was more than one order of magnitude lower than in plasma ghrelin levels [2,13]. From the calculated molecular mass of 3244.6 for the 28-amino acid sequence of the peptide [8], these concentrations are substantially lower than the average ghrelin level (0.1 pmol/ml or about 324 pg/ml) in plasma of fasting humans, while they are similar to that of immunoreactive acylated ghrelin levels (5.4 fmol/ml or about 17.61 pg/ml) existing in human plasma [13]. We tentatively assume that this observation of ghrelin activity in parenchyma cells and phloem is suited for plant growth regulation (as is for growth hormone in animals) through interaction with one or more plant growth hormones, especially with auxins, which are mainly transported in stream of fluid in phloem vessels. Furthermore, this localization of ghrelin-like substance is well suited for nutrient uptake, an expected function from a hormone of this kind.

In conclusion, ghrelin is mainly produced in the stomach and its main roles are the regulation of energy balance and growth hormone release in humans and animals. To the best of our knowledge, the present work is the first to report the presence of this hormone activity in plants. The identification has been accomplished by immunohistochemical, HPLC and radioimmunoassay analysis. Although, the physiological relevance of this finding is yet unclear, it strongly suggests that ghrelin might have highly been conserved across the species and probably does have further essential biological functions than previously assumed. Thus, further research on ghrelin will contribute to our understanding of its physiological functions in such diverse life forms as plants and animals and it can be foreseen that important new insights and contributions will be provided in the future.

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