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Title

Effects of 5-aminolevulinic acid on growth and amylase activity in the radish taproot

Authors

Masakazu Hara[1*], Ikuo Takahashi[1], Michiyo Yamori[1], Toru Tanaka[2], Shigeyuki Funada[2], Keitaro Watanabe[2]

[1] Faculty of Agriculture, Shizuoka University,
836 Ohya, Suruga-ku, Shizuoka,
422-8529, Japan

[2] Research and Development Center,
Cosmo Oil Co., Ltd.,
1134-2, Gongendo, Satte-Shi, Saitama
340-0193, Japan

Name and address for editorial correspondence

Masakazu Hara
Faculty of Agriculture, Shizuoka University,
836 Ohya, Suruga-ku, Shizuoka,
422-8529, Japan
Telephone & FAX: +81-54-238-5134
E-mail address: amhara@ipc.shizuoka.ac.jp

Abstract 5-Aminolevulinic acid (ALA) promotes the growth of plants by enhancing their photosynthetic activities, but there is little information on how ALA influences the metabolism of sugars produced by photosynthesis. Here, we report the effects of ALA on tissue growth, sugar content, and amylase activity in the radish taproot. 5-Aminolevulinic acid was applied with a foliar spray (5.3-13,500 μM), and application at concentrations of 53, 530, and 2,700 μM enhanced the fresh weight of the taproot. Glucose is a major soluble sugar of the radish taproot. 5-Aminolevulinic acid slightly increased the glucose content but did not influence the fructose, sucrose, or starch contents. Radishes have β-amylase (RsBAMY1), which is expressed in the taproot. 5-Aminolevulinic acid enhanced both the amylase activity and the RsBAMY1 protein accumulation. These results suggest that ALA may control starch accumulation by increasing the RsBAMY1 expression in the radish taproot. The relationship between taproot growth and free sugar accumulation by ALA is also discussed.

Keywords 5-Aminolevulinic acid · β-Amylase (EC 3.2.1.2) · Plant growth · *Raphanus sativus*

Abbreviations

ALA, 5-aminolevulinic acid

DAS, days after sowing

RsBAMY1, *Raphanus sativus* β-amylase 1

Introduction

5-Aminolevulinic acid (ALA) is a biosynthetic precursor of porphyrins such as chlorophyll and heme (Von Wettstein et al. 1995). Recently, various biological activities of ALA, including agricultural and medical applications, have been studied (Sasaki et al. 2002; Fukuda et al. 2005). In agricultural applications, ALA was first noted as a biodegradable herbicide (Hotta et al. 1997a) that is effective when used at high concentrations of more than 10 mM. Such high-dose applications of ALA promote the accumulation of an excess amount of protoporphyrin IX, which produces a large amount of reactive oxygen species by a photodynamic mechanism (Chakraborty and Tripathy 1992). Inversely, it has been reported that the application of ALA at lower concentrations accelerates the growth of many species of plants, especially the edible parts of root crops such as radishes, potatoes, and garlic (Sasaki et al. 2002). This growth

promotion is probably related to the photosynthesis-enhancing effects of ALA, which have been demonstrated in various plant species. However, further mechanisms of the growth-promoting effects of ALA have not been elucidated.

Radishes have been used to investigate the growth-promoting effects of ALA. The application of ALA to radishes increased the weight of the taproot, which is a sink organ of the radish (Hotta et al. 1997b). In this case, CO_2 fixation in light was promoted and CO_2 release in darkness was suppressed by ALA (Hotta et al. 1997b). Here, we report the effects of ALA on tissue growth, sugar content, and amylase activity in the radish taproot. We also discuss the role of amylase in the promotion of taproot growth by ALA.

Materials and methods

Plant materials

Radish seeds (*Raphanus sativus* L. cv. Comet, Takii, Kyoto, Japan) were sown in a plastic pot (100 cm^2 x 10 cm) containing Peatban (Sakata Seed, Yokohama, Japan) in a greenhouse at Shizuoka University, Japan, in 2007, 2008, and 2009. The plants (3 plants per pot) were watered with Hyponex solution (500 times dilution) (Hyponex, Tokyo, Japan) every week and harvested on the 42nd day after sowing (DAS). During cultivation, the ALA solution was sprayed on the leaves 3 times. The first, second, and third sprays were on the 7th DAS, 21st DAS, and 28th DAS, respectively. The harvest periods and spray timings were slightly different among cultivations, because the growth depended on the climate. The volume of solution sprayed was 1.7 ml per pot. Concentrations of the ALA solution were 0, 5.3, 53, 530, 2,700, and 13,500 μM, respectively. After harvest, each radish plant was divided into the aerial part and the taproot, and the parts were then weighed. The taproots were maintained at -20°C until use.

Determination of sugars

The glucose, fructose, and sucrose contents were measured as described previously (Hara et al. 2003). The ethanol extract of a radish taproot was subjected to an enzyme-linked assay by using hexokinase plus glucose-6-phosphate dehydrogenase, glucose-6-phosphate isomerase, and invertase. The starch content was measured as described previously (Hara et al. 2009). The ethanol-insoluble material was extracted by potassium hydroxide solution. After the extract was

neutralized, the soluble starch was digested by amylase and amyloglucosidase. Glucose formation was determined by the hexokinase and glucose-6-phosphate dehydrogenase system.

Crude enzyme extraction

Radish taproots (2 g fresh weight) were ground by a chilled steel musher until they became a paste. The paste was centrifuged at 10,000 g for 15 min at 4°C. The supernatant was a crude enzyme extract that was kept at -20°C until use. The amylolytic activity in the enzyme extracts was stable during storage at -20°C for 6 months. The antigenicity for the anti-*Raphanus sativus* β-amylase 1 (RsBAMY1) antibody in the extracts was also maintained during storage at -20°C for 6 months.

Amylase activity measurement

The 3,5-dinitrosalicylic acid (DNSA) method (Hara et al. 2009) was applied to determine glucan-hydrolyzing activities in the radish extracts. An enzyme solution (4 μl) was combined with a substrate solution (36 μl) consisting of 20 μl of 1% soluble starch and 16 μl of 100 mM sodium acetate buffer (pH 4.8). After incubation at 37°C for 5 min, 40 μl of the DNSA reagent containing 44 mM DNSA, 1 M sodium potassium tartrate, and 0.4 M sodium hydroxide was added to the reaction mixture. The solution was heated at 100°C for 5 min, and the absorbance was read at 540 nm. Calibration curves were produced using maltose solutions.

Protein determination

Protein quantification was done using the Quick Start Bradford Protein Assay (Bio-Rad, Tokyo, Japan) with bovine γ-globulin as a standard. Assays were performed according to the manufacturer's instructions.

Immunoblot analysis

Protein samples were resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis, the proteins were blotted onto a nitrocellulose membrane filter (Hybond-ECL, GE Healthcare, Tokyo, Japan) with a Mini Trans-Blot (Bio-Rad). A blocked filter was incubated with a primary antibody, i.e., a rabbit polyclonal anti-RsBAMY1

antibody (Hara et al. 2009). Horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (GE Healthcare) was used as a secondary antibody. Positive signals were detected by a chemiluminescence technique with the ECL Western Blotting Detection System (GE Healthcare). The signals were detected by an LAS-4000 Image Analyzer.

Statistical analysis

Statistical significance was determined by Dunnett's test ($P < 0.05$ vs. control) to compare means.

Results and discussion

5-Aminolevulinic acid was applied to the radishes at concentrations of 5.3, 53, 530, 2700, and 13,500 μM three times intermittently during cultivation, as described in the materials and methods section. Preliminary experiments indicated that the intermittent application provided sufficient promotion of taproot growth by ALA (data not shown). After harvest, the plants were divided into the aerial part and the taproot. The aerial part contained leaves and petioles. The taproot is a swollen storage organ consisting of the hypocotyl and the upper part of the main root. The fresh weight of each part is shown in Fig. 1. The growth of the aerial part was not influenced by ALA administration except that the highest concentration of ALA (13,500 μM) reduced the growth of the aerial part (Fig. 1A). Taproot growth increased when ALA was applied at concentrations of 53, 530, and 2,700 μM (Fig. 1B). However, the application of ALA at 13,500 μM reduced the taproot growth. This indicates that the ALA application affected the growth of the taproot more than the growth of the aerial part. The concentrations of ALA that were effective in increasing the growth of the taproot ranged from 53 to 2700 μM. The highest-dose application of ALA (13,500 μM) damaged the plants. The error bars attached to the columns of the 13,500 μM ALA treatment are large (Figs. 1A, B) because the degrees of damage and growth deterioration were highly different, even between plants in the same treatment. This damage is likely due to the ALA's herbicidal effect, which is noted in the introduction section (Hotta et al. 1997a). Further analyses were done using plants treated with 0, 53, and 2,700 μM ALA.

We determined the total sugar contents in the taproot. Figure 2A shows the results of the measurement of free sugars, i.e., glucose, fructose, and sucrose. More glucose was accumulated

in the taproot than fructose and sucrose. 5-Aminolevulinic acid enhanced the glucose content (Fig. 2A, dark columns), but the fructose and sucrose contents were not affected by ALA application (Fig. 2A, gray and white columns). Determination of the starch content indicated that ALA did not cause the starch content in the taproot to change (Fig. 2B).

Our previous study has shown that the radish taproot has respectable amylolytic activity due to a taproot-specific RsBAMY1 (Hara et al. 2009). We measured the amylase activity and RsBAMY1 protein accumulation in plants treated with ALA and plants not treated with ALA (Fig. 3) and found that the amylase activity (U/g fresh weight) and the specific activity (U/mg protein) were enhanced by ALA application (Figs. 3A, B), whereas soluble protein content was not affected by ALA (Fig. 3C). Immunoblot using the anti-RsBAMY1 antibody showed that the amount of RsBAMY1 protein increased following ALA application (Fig. 3D). This finding shows that the increase of the RsBAMY1 protein amount was not the result of an elevation of the total protein level in the taproot. 5-Aminolevulinic acid may directly regulate the expression of RsBAMY1 proteins.

The *RsBAMY1* gene is an ortholog of *Arabidopsis bmy1* (At4g15210) (Hara et al. 2009), which is likely to involve starch degradation, because the *Arabidopsis* T-DNA insertion lines for the *bmy1* gene showed a starch-excess phenotype (Kaplan and Guy 2005). In the radish taproot, the glucose contents were approximately 7 to 10 mg/g fresh weight, but the starch contents were approximately 0.4 to 0.6 mg/g fresh weight, indicating that the glucose contents overwhelmed the starch contents. This suggests that RsBAMY1 may participate in starch degradation to maintain the balance between the high soluble sugars content and the low starch content in the radish taproot. Since it was demonstrated that ALA enhances CO_2 fixation by photosynthesis (Hotta et al. 1997b), we assumed that a higher quantity of mobile sugars may be introduced into the taproots of radishes treated with ALA. As a result, larger amounts of both free sugars and starch can accumulate in the taproot. However, enhancement of the RsBAMY1 production by ALA may promote glucose accumulation while maintaining the low starch content. The glucose accumulation may enhance turgor pressure, which is a driving force in the formation of vast parenchyma cells in the taproot. Moreover, glucose is degraded through glycolysis and the citric acid cycle to produce adenosine 5'-triphosphate and nicotinamide adenine dinucleotide, which are energetic sources for cell division. All of this suggests that glucose accumulation may play a crucial role in the growth of parenchyma cells in the taproot. The putative mechanisms of the taproot growth regulated by ALA are shown in Fig. 4.

It has been reported that the application of ALA can enhance the stress tolerance of plants. 5-Aminolevulinic acid promoted salinity stress tolerance in cotton (Watanabe et al. 2000),

spinach (Nishihara et al. 2003), potatoes (Zhang et al. 2006), date palms (Youssef and Awad 2008), and rice (Wongkantrakorn et al. 2009), and ALA enhanced cold tolerance in rice (Hotta et al. 1998), melons (Wang et al. 2004), and peppers (Korkmaz and Korkmaz 2009). In the case of melons, enhancement of the soluble sugar content by ALA may be responsible for the stress tolerance (Wang et al. 2004). Modulation of the expression of sugar-related enzymes by ALA may provide such enhancement of the soluble sugar content.

Generally it is believed that the growth-enhancing effects of ALA are simply due to the promotion of photosynthesis by ALA. In this study, however, we found that ALA may regulate starch-degrading enzyme(s) to enhance the soluble sugar contents in plants. Starch mobilization may be one of the basic mechanisms by which ALA promotes the growth of plants.

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Figure Legends

Fig. 1 Effect of ALA on growth of the aerial parts (**a**) and taproots (**b**) of radishes. Values and bars represent the means and SE of 4 plants, respectively. * Significant difference at the $P < 0.05$ level. To determine *, the value of the leftmost column (0 μM ALA) in each graph was standardized.

Fig. 2 Effects of ALA on the contents of free sugars (**a**) and starch (**b**) in radish taproots. The free sugars tested in this study were glucose (dark columns), fructose (gray columns), and sucrose (white columns) (**a**). Values and bars represent means and SE of 3 plants, respectively. To determine *, the value of the leftmost column (0 μM ALA) in each item was standardized.

Fig. 3 Effects of ALA on amylase activity in radish taproots. Amylase activity (**a**), its specific activity (**b**), and soluble protein content (**c**) are shown. Values and bars represent the means and SE of 3 plants, respectively. To determine *, the value of the leftmost column (0 μM ALA) in each graph was standardized. Immunoblot analysis (**d**) is shown. A constant amount of protein (3 μg each per lane) was loaded in the immunoblot. Lanes 1, 2, and 3 represent 0, 53, and 2700 μM of ALA, respectively. An arrow represents RsBAMY1.

Fig. 4 Putative mechanism of the growth promotion of radish taproots by ALA. Photosynthesis, free sugars content, β-amylase activity, and taproot growth were positively affected by ALA application (solid arrows). Broken lines indicate the directions of metabolic flows. The enhancement of free sugars content likely involves the promotion of taproot growth (open arrow).

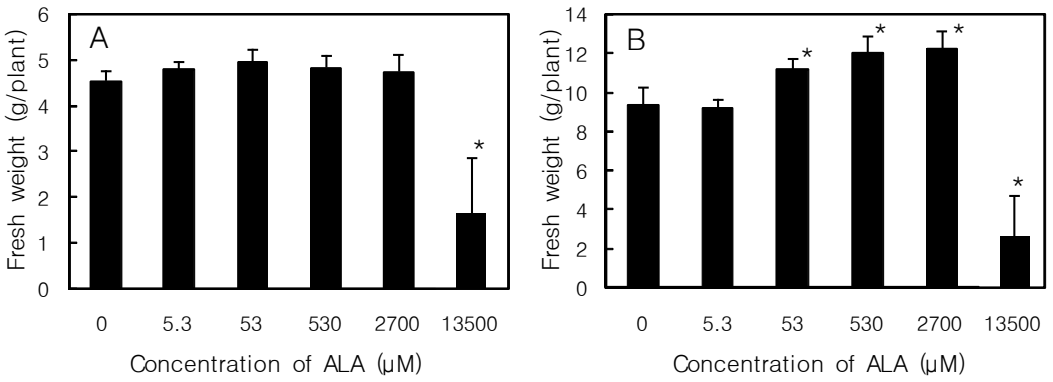

Fig. 1 Hara et al.

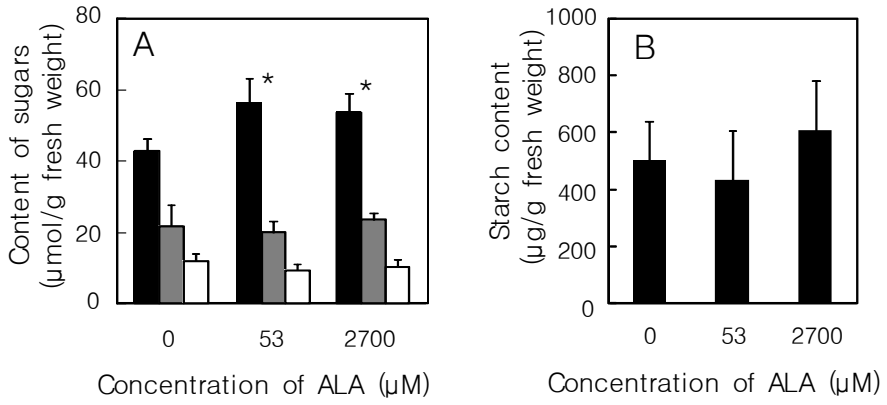

Fig. 2 Hara et al.

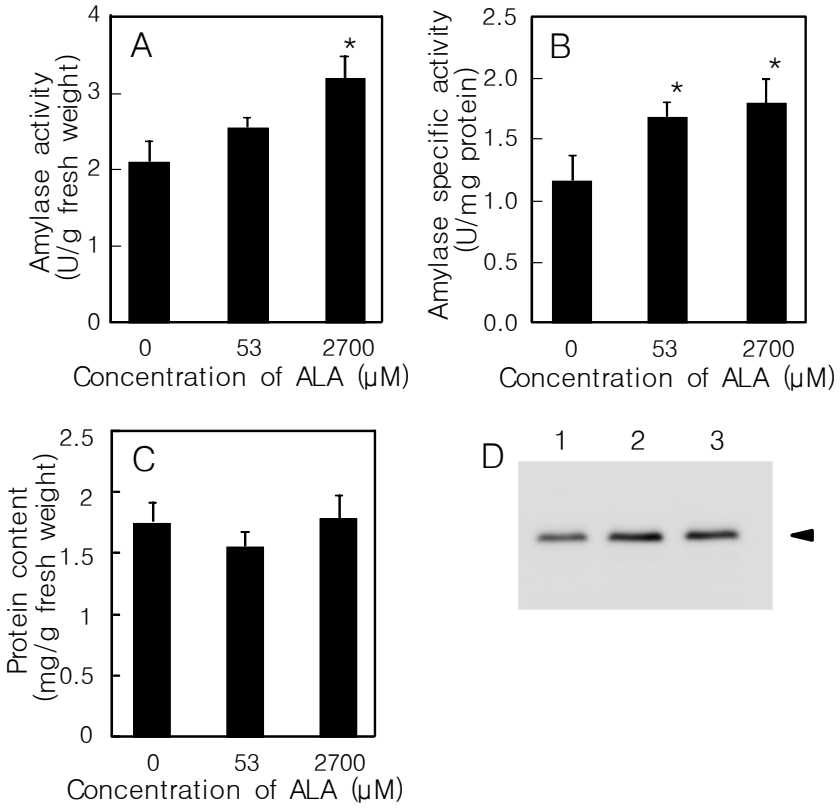

Fig. 3 Hara et al.

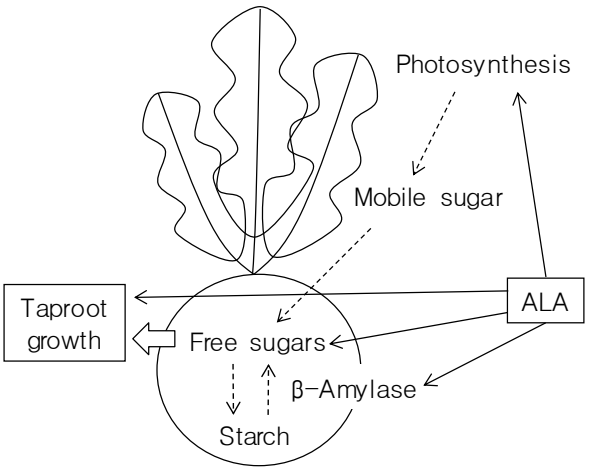

Fig. 4 Hara et al.