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Sucrose Metabolism for the Development of Seminal Root in Maize Seedlings

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Abstract : The objective of this study was to elucidate the roles of sugar in the formation of root systems. Several parts of the seminal root were investigated to determine their sucrose, glucose and fructose contents, and the activity and the *in situ* localization of the activities of two kinds of metabolic enzymes, invertase and sucrose synthase, which hydrolyze sucrose. The sucrose, glucose and fructose concentrations in the 0-1 cm section from the root apex were three to five times those in the other sections. The invertase and sucrose synthase activities were also higher in the apical section. The *in situ* localization of invertase activity was detected in the cell elongation zone of the seminal root using histochemical method. The sucrose synthase activity was detected in the cell elongation zone of the seminal root and the root apices of lateral roots. These results suggested that sucrose is transported to the root elongation zone and the surrounding tissue of the lateral root primordia, and is cleaved into glucose, fructose, and UDP-glucose by invertase or sucrose synthase. This suggested that sucrose contributes to root formation by serving as the energy source, the carbon source for cell wall synthesis, and as a compatible solute for cell elongation.

Key words : Enzyme localization, Fructose, Glucose, Invertase, Root development, Sucrose, Sucrose synthase, *Zea mays*.

In higher plants, root growth is regulated by internal and external factors. Sugar is an important factor, since it is used not only as an energy source but also for constructing structural components of cells and cell walls (Jarvis, 1986). In addition, it is used as a compatible solute for osmotic adjustment in roots (Sharp et al., 1990; Rodrígues et al., 1997; Ogawa and Yamauchi, 2006). External application of sugar stimulated root growth. Sattelmacher and Tomas (1989; 1991) hypothesized that the proliferation of lateral roots occurs in response to an increase in phloem solute import generated by an increase in sink demand for carbohydrates. They argued that an increase in respiratory activity, and consequently in sink demands for carbohydrate, would increase lateral root initiation. Bingham et al. (1997; 1998) studied the pattern of lateral root initiation in seminal roots of wheat, and the location, scale, and time-course of adjustment of initiation after a change in glucose supply. They proposed that proliferation of lateral roots might be signaled by an increase in the sugar content of the tissue. In adventitious roots of *Arabidopsis*, elongation was stimulated by external application of sucrose, glucose and fructose introduced into the medium (Takahashi et al., 2003). Additionally, Kurata and Yamamoto (1997) showed that white-light irradiation of a whole *Arabidopsis* seedling stimulated root elongation, that DCMU, an inhibitor of photosynthesis, completely inhibited this stimulation, and that excised roots lost their responsiveness. These results also suggested that sugar is a factor regulating root growth.

In most plants, sucrose is the major end product of photosynthesis and the major form of carbohydrate transported in the phloem to non–photosynthetic organs such as the root system (Trouverie et al., 2004). The transported sucrose cannot be used for metabolic processes, but must be cleaved into hexose by invertase (β-fructosidase, EC 3.2.1.26) or sucrose synthase (UDP-D-glucose:D-fructose 2-α-glucosyltransferase, EC 2.4.1.13) before entering into carbohydrate metabolism. Invertase catalyzes the irreversible reaction to convert sucrose into glucose and fructose. Sucrose synthase catalyzes the reversible reaction that converts sucrose into fructose and UDP-glucose. Although the reaction is reversible, it is thought to be involved primarily in the breakdown of sucrose (Kruger, 1990; Huber and Huber, 1996). Invertase and sucrose synthase are the key enzymes in carbohydrate metabolism.

Invertase belongs to a multi-enzyme family with various forms classified in accordance with cellular localization, optimum pH, and solubility properties: two soluble forms (soluble acid invertase in the vacuole and neutral invertase in the cytoplasm) and a particulate form (cell-wall-bound invertase in the cell wall) (Doehlert and Felker, 1987). Little is known about the roles of neutral invertase, whereas acid invertases (soluble acid invertase and cell-wall-bound

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invertase) have been well characterized (Trouverie et al., 2004). Although the predominant role of acid invertases is to provide glucose for cell energy production (Karuppiah et al., 1989), they have several other physiological functions depending on their cellular localization. For example, invertases play a major role in sucrose partitioning and long-distance transport by modulating the sucrose gradient between the phloem and the surrounding cells, and in the control of the relative sink strength of plant tissues (Roitsch et al., 1995; Godt and Roitsch 1997; Roitsch 1999). Invertases are involved in cell elongation through the maintenance of cell turgor via osmotic potential control (Pfeiffer and Kutsuchera, 1995). In addition, invertases play a role in the regulation of genes related to photosynthetic enzymes (Herbers et al., 1996).

Sucrose synthase plays a role in the respiratory pathway (Xu et al., 1989), in energy production (Fukuda et al., 2008), in cell wall synthesis where membrane-associated callose synthase and cellulose synthase use UDP-glucose as a substrate (Delmer and Amor, 1995), and for starch biosynthesis (Chourey and Nelson, 1976; Claussen et al., 1985; 1986; Doehlert, 1990). The extent of sugar import into many plant organs correlated with sucrose synthase activity (Claussen, 1983; Sung et al., 1988; Nguyen-Quoc et al., 1990).

Sugar is an important factor in root system formation; it is used as an energy source and for constructing structural components of cells and cell walls (Jarvis, 1986). Although Kim et al. (2000) showed the invertase activity in the whole seminal root and the localization of invertase in the basal zone of seminal root in maize seedling, how sucrose is metabolized , and how it contributes to root system formation remain unknown. The objective of this study was to determine the roles of sugar in the formation of the root system. We investigated sucrose, fructose, and glucose content in several parts of the seminal root in maize seedling. Additionally, the activities and the *in situ* localization of invertase and sucrose synthase were also investigated. Although immunocytological methods are available for detecting enzyme protein, the presence of an enzyme does not prove that it is active *in situ*; enzymes might be activated/inactivated by proteolysis, by phosphorylation/dephosphorylation, or by other regulatory mechanisms (Sergeeva and Vreudenhil, 2002). A histochemical method is available for detecting the presence of an active enzyme. In this study, the *in situ* localizations of invertase and sucrose synthase activities were detected using a histochemical method, and a biochemical method was also used for the routine determination of enzyme activity.

Materials and Methods

1. Plant culture

Seedlings of *Zea mays* L. (cv. White Pop, Sakata Seed Corporation, Yokohama, Japan) were germinated and grown in the dark at 28ºC in petri dishes for 3 days. During this time, the seminal root elongated approximately 10 mm. These seedlings were transplanted onto nets floating on nutrient solution in a one-liter beaker. Twelve seedlings were planted in one beaker. The nutrient solution contained 6.0 mM KNO_3, 4.0 mM $Ca(NO_3)_2$, 1.0 mM $NH_3H_2PO_4$, 2.0 mM $MgSO_4$, 26.8 μM Fe-EDTA, 4.6 μM $MnCl_2$, 23.1 μM H_3BO_3, 0.38 μM $ZnSO_4$, 0.16 μM $CuSO_4$, and 0.015 μM $(NH_4)_6Mo_7O_{24}$. Sufficient and continuous aeration of the nutrient solution with small air bubbles was provided with an HPα10000 aerator (NISSO, JAPAN). The bubbles did not reach the root directly, and the growth of the root system was not inhibited by using this procedure (Ogawa et al., 2005). The nets and the beaker were covered with aluminum foil to provide darkness and to stimulate growth of the root system. The plants were exposed to a 12-hour photoperiod in a growth chamber (MLR-350H, SANYO). The photon flux density of photosynthetically active radiation (PAR, 400–700 nm) was 320 μmol m^{-2} s^{-1}. The chamber was maintained at 28ºC with 80% relative humidity during the day and night. Seedlings were harvested 3 days (d 3), 4 days (d 4) and 5 days (d 5) after transplanting.

2. Measurement of sugar content

The primary seminal roots were excised from the seedlings and were cut into 10 mm segments. Ten root segments were sealed in a plastic tube and plunged into liquid N_2. Three tubes were used for sugar analysis. The sample tubes were stored in a freezer at –30ºC prior to measurement.

Samples (0.05–0.2 g fresh weight) were rapidly frozen in liquid nitrogen, ground to a powder, and then extracted as described by Tobias et al. (1992). The glucose, fructose and sucrose contents of the samples were assayed by the coupled enzymatic assay method, which measures the increase in A_{340}, as described by Guglielminetti et al. (1995). The samples and standards were incubated at 37ºC for 30 minutes, and mixed with the reaction mixture (1 mL). The mixture was then incubated at 37ºC for 30 minutes, and the increase in A_{340} was recorded. The composition of the reaction mixture for the glucose assay was Tris-HCl (120 mM, pH 7.6), $MgCl_2$ (3 mM), ATP (2 mM), NADP (0.6 mM), 1 U hexokinase (Wako), and 1 U glucose 6-phosphate dehydrogenase (Wako). Fructose was assayed using the reaction mixture of the glucose assay plus 2 U phosphoglucose isomerase (Sigma). Sucrose was hydrolyzed using 85 U invertase in 15 mM sodium acetate (pH 4.6) (Sigma), and then the glucose and fructose were assayed as described above.

3. Extraction and activity determination of enzymes

Invertase and sucrose synthase activities were measured as descried by Appeldoorn et al. (1997) and Pelleschi et al. (1997) with some modifications.

On each sampling day, root sections were collected from 0–1 cm from the root apex (including the cell division and the cell elongating zones, Ogawa and Yamauchi, 2006), 3–4 cm (lateral roots had not emerged), 6–7 cm (lateral roots had begun to emerge), 9–10 cm and 12–13 cm from the root apex (lateral roots were already elongated). Root segments were frozen and stored in the same way as the samples for sugar analysis (see "Measurement of sugar content") and three tubes, each with ten segments sealed within, were used for enzyme analysis.

Extracts for assays of enzymes were prepared from samples (0.05–0.2 g fresh weight) in 1.2 mL of extraction buffer [50 mM Hepes-KOH (pH 7.4), 5 mM $MgCl_2$, 1 mM EGTA, 1 mM Na-EDTA, 10% glycerol, 0.1% bovine serum albumin, 5 mM dithiothreitol (DTT) and 2% polyvinylpyrrolidone 40 (PVP)]. After centrifugation (5 min, 15000 *g*) and removal of the low-molecular-weight compounds on an Econo-Pac 10DG column (Bio-Rad), the extract was stored at –80 °C until used. The whole extraction procedure was carried out at 4°C.

Soluble acid invertase was assayed by incubating 50 μl of extract in 250 μl of mixture [25 mM sucrose with 25 mM citrate-phosphate buffer (pH 5.2)] at 37°C. After 45 min the reaction was stopped by immersion in a boiling-water bath (4 min). The glucose content of the reaction mixture was measured enzymatically as the soluble acid invertase activity by the method descried in "Measurement of sugar content".

Neutral invertase activity was measured using the same method as the soluble acid invertase assay; however, the mixture used was [25 mM sucrose with buffer (1 mM Na-EDTA and 2 mM DTT with 50 mM Hepes/NaOH (pH 7.5))]. The glucose content of the reaction mixture was measured enzymatically in the same manner as the neutral invertase activity.

Cell-wall-bound acid invertase was assayed after extraction of the pellets obtained by centrifugation of the homogenized tissue. The extraction procedure entailed washing the pellets three times with extraction buffer (DTT and PVP omitted), overnight incubation of the washed pellets in 1.0 mL of 20 mM 2-(N-morpholino)ethanesulphonic acid (Mes-KOH; pH 6.0) and 1.0 M NaCl, and centrifugation of the pellets (15 min, 15000 *g*). The extraction procedure was performed at 4°C. A sample of the supernatant (50 μL) was assayed for cell-wall-bound invertase activity as described for soluble acid invertase. The glucose content of the reaction mixture was measured enzymatically as the cell-wall-bound invertase activity.

Sucrose synthase activity was measured using the same method as the soluble acid invertase assay; however, the mixture used was [1mM UDP and 25 mM sucrose with buffer (1 mM Na-EDTA and 2 mM DTT with 50 mM Hepes-NaOH (pH 7.5))]. The fructose content of the reaction mixture was measured enzymatically in the same manner as the sucrose synthase activity using the method described in "Measurement of sugar content."

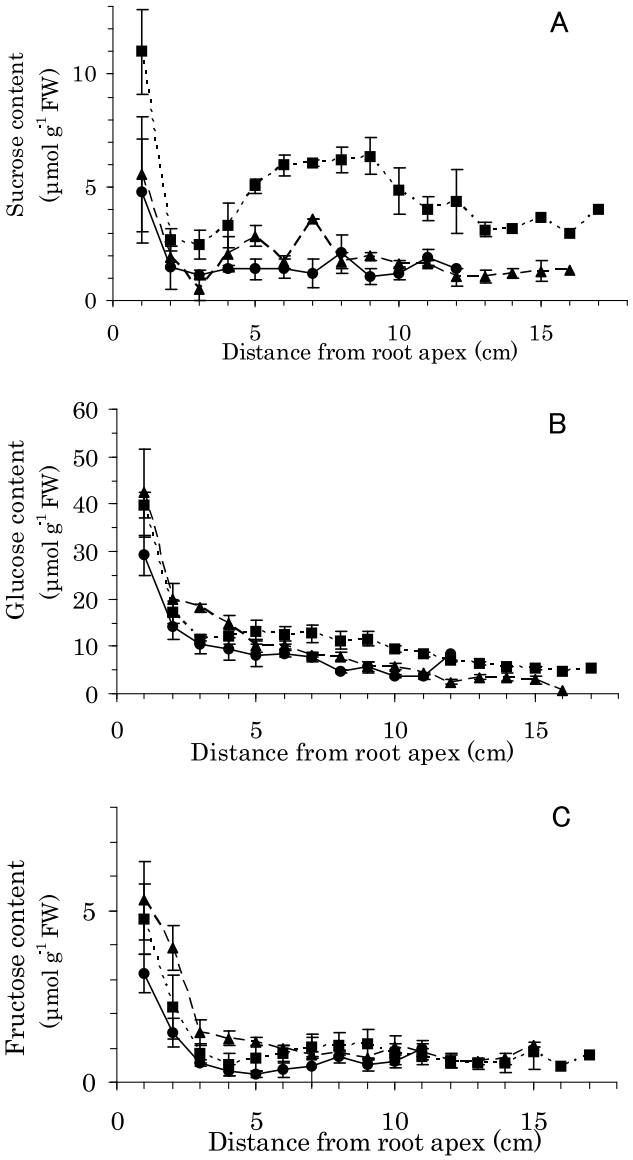

Fig. 1. The concentration of sucrose (A), glucose (B), and fructose (C) in the 10 mm segments of the seminal root sampled successively along the root axis at d 3 (●), d 4 (▲) and d 5 (■). Each value is the mean of three replicates ± the standard error.

4. Localization of enzyme activity

The localizations of invertase and sucrose synthase activities were visualized using the Nitro blue tetrazolium (NBT) reaction as described by Wittich and Vreugdenhil (1998) and Serggeva and Vreugdenhil (2002), with some modifications. Sections

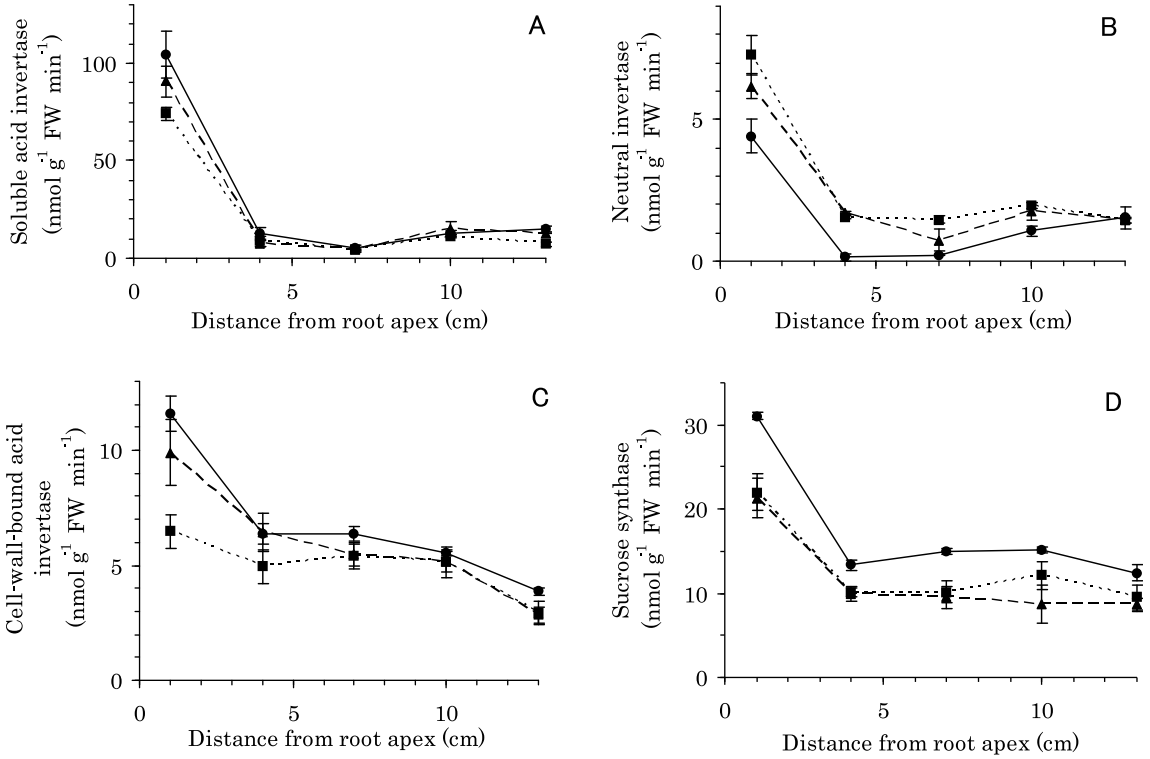

Fig. 2. The activities of soluble acid invertase (A), natural invertase (B), cell-wall-bound invertase (C), and sucrose synthase (D) in the following sections starting from the root apex: 0-1 cm (including the cell division and cell elongation zones), 3-4 cm (no lateral root emergence), 6-7 cm (lateral roots began to emerge), 9-10 cm and 12-13 cm sections (lateral roots were already elongated) on each sampling day. For the symbols, see the legends for Fig. 1. Each value is the mean of three replicates ± the standard error.

of the root, which were 0–1 cm, 3–4 cm, 6–7 cm, and 12–13 cm from the root apex, were collected on each sampling day. The tissues were immediately fixed in 2% paraformaldehyde with 2% PVP and 0.001 M DTT, pH 7.0, at 4ºC, and placed under a vacuum for 1 h. After fixation, tissues were rinsed overnight in water at 4ºC, which was refreshed at least five times to remove the soluble carbohydrates, and stored at 4ºC for 2 days.

In order to detect the localization of invertase activity, tissues were incubated in the following medium [25 U glucose oxidase (Wako), 0.024% NBT, 0.014% phenazine methosulfate, and 1% sucrose with 38 mM PBS (pH 6.0)] for 2 h at 30ºC in the dark. Sucrose was omitted for the negative control. After incubation, the tissues were rinsed in distilled water.

The localization of sucrose synthase activity was visualized by incubating the tissues at 30ºC overnight under dark conditions in a reaction medium which contained 50 mM HEPES-NaOH buffer (pH 7.4), 5 mM MgCl, 1 mM Na-EDTA, 0.1% BSA, 1 mM EGTA, 1 mM NAD, 1 U phosphoglucomutase from rabbit muscle (Wako), 1 U glucose 6-phosphate dehydrogenase (Wako), 20 μM glucose-1,6-bisphosphate, 1 U UDPG-pyrophosphorylase (Sigma), and 0.03% NBT. The reaction was started by adding the substrate solution (containing sucrose, UDP and pyrophosphate) in proportions resulting in a final concentration of the substrates in the incubation medium of 3.6 mM, 71 μM, and 71 μM, respectively. Sucrose was omitted for the negative control. After incubation, the tissues were rinsed in distilled water.

These stained tissues were observed with a stereoscopic microscope (C-PS 160, Nikon) with a charge coupled device (CCD) camera (Disital Sight DS V1, Nikon).

Results

The glucose, sucrose and fructose contents of the 10 mm segments of the seminal root, which had been sampled successively along the root axis, were examined (Fig. 1). Although the sugar contents were different along the seminal root axis, the changes in content of the sucrose (Fig. 1A), glucose (Fig. 1B), and fructose (Fig. 1C) were similar. The glucose content was about three times higher than the sucrose content, and about four times higher than the fructose content in most portions of the seminal root. The contents of all sugars increased acropetally. In the 0–1 cm portion, the sucrose content was three to five times, glucose content was three to twenty times, and fructose three to ten times that in the other sections of the root. The sucrose content decreased in the 1–3 cm portion, but increased again in the 5–10 cm portion, especially in samples from d 5. These sugar contents tended

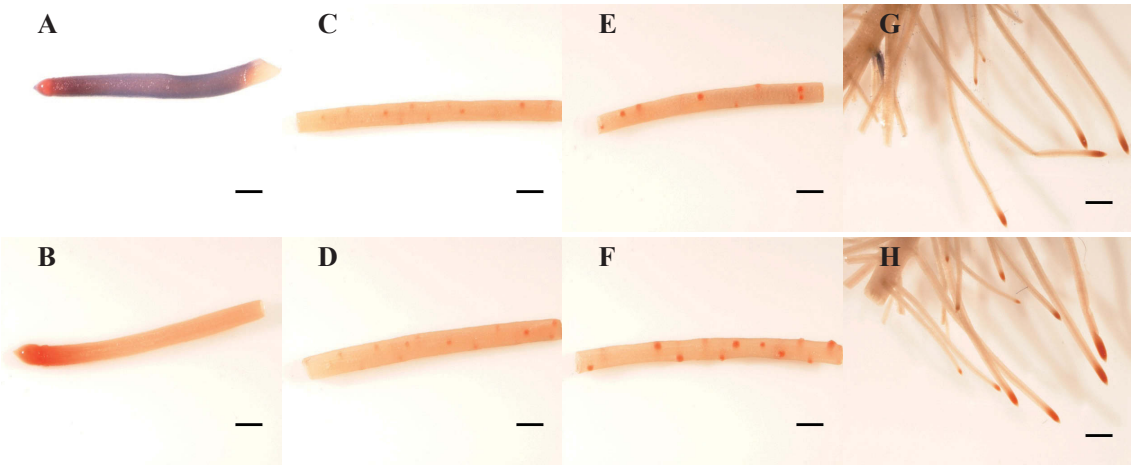

Fig. 3. The *in situ* localization of invertase activity visualized using the NBT reaction in the 0–1 cm section (A and B), 3–4 cm section (C and D), 6–7 cm section (E and F), and the lateral roots elongated from the 12–13 cm section (G and H) sampled on d 5. For the negative control (B, D, F, and H), sucrose was omitted from the NBT reaction. Bars = 200 μm.

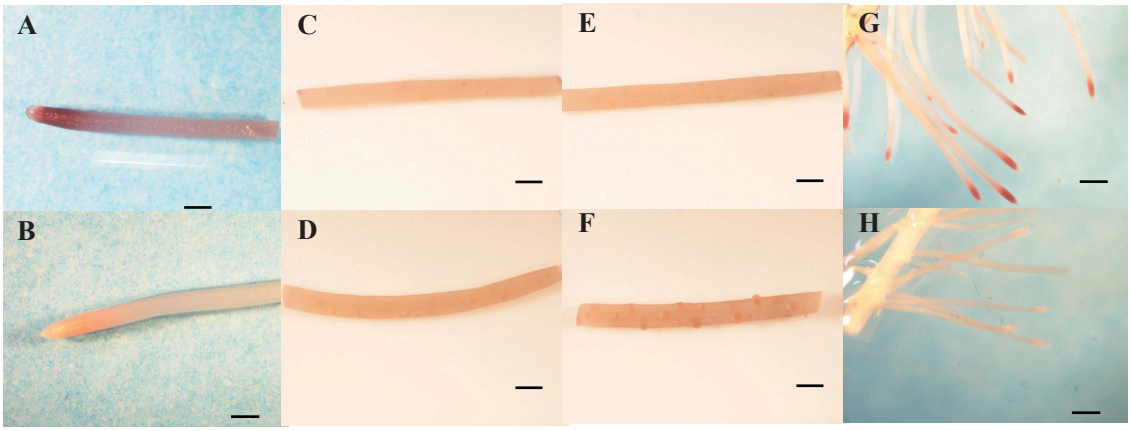

Fig. 4. The *in situ* localization of sucrose synthase activity visualized using the NBT reaction in the 0–1 cm section (A and B), 3–4 cm section (C and D), 6–7 cm section (E and F), and the lateral roots elongated from the 12–13 cm section (G and H), sampled on d 5. For the negative control (B, D, F, and H), sucrose was omitted from the NBT reaction. Bars = 200 μm.

to increase from d 3 to d 5 in most portions of the seminal root.

The activities of the three kinds of invertase and the sucrose synthase changed along the seminal root axis (Fig. 2), having their highest values in the 0–1 cm portion. The activities of the soluble acid invertase, neutral invertase, cell-wall-bound invertase, and sucrose synthase in this section were about 10, 5, 2, and 2.5 times those in the other portions, respectively. In the invertase group, the activity of the soluble acid invertase was about 10 times that of the other invertase enzymes. The activities of the soluble acid invertase, cell-wall-bound invertase, and sucrose synthase decreased 30%, 50%, and 30%, respectively, and the activity of neutral invertase increased 30% from d 3 to d 5.

The *in situ* localization of invertase activity at d 5 was visualized (Fig. 3). In the 0–1 cm section, the segment was stained violet compared with the negative control, and invertase activity was localized in this part, although the apical 0–1 mm and basal 2 mm parts were not stained (Fig. 3A). Although the primordia of the lateral roots in the 6–7 cm section (Fig. 3E) and the lateral root apices (Fig. 3G) were stained orange, invertase activity may not be localized in these parts because these parts in the negative control were also stained orange (Fig. 3F, H), and because the invertase activity is stained violet by NBT. In the 3–4 cm section (Fig. 3C), the 6–7 cm section (Fig. 3E), and in the lateral roots elongated from the 12–13 cm section (Fig. 3G), invertase activity was not detected. There were no differences in the staining with the sampling day (data not shown).

The *in situ* localization of sucrose synthase activity

was visualized (Fig. 4). The 0–1 cm section was stained violet, as compared with the negative control, except for the 0–1 mm and the basal 2 mm parts (Fig. 4A). The localizations of sucrose synthase activities were also detected in the root apices of the lateral roots which had elongated from the 12–13 cm section (Fig. 4G). Although the primordia of the lateral roots in the 6–7 cm section (Fig. 4E) were stained orange, sucrose synthase activity may not be localized in this part because this part in the negative control was also stained orange (Fig. 4F), and because sucrose synthase activity is stained violet by NBT. In the 3–4 cm sections (Fig. 4C) and the 6–7 cm sections (Fig. 4E), no sucrose synthase activity was not detected. There were no differences in the staining with the sampling day (data not shown).

Discussion

The elongation of roots requires sugar as an energy source and as a substrate for the synthesis of cellular components and the cell wall. In addition, it is used as a compatible solute for osmotic adjustment in roots (Sharp et al., 1990; Rodrígues et al., 1997; Ogawa and Yamauchi, 2006). Sugar is important for lateral root initiation (Bingham et al., 1997; 1998; Takahashi et al., 2003). Bingham et al. (1997) showed that when glucose was fed to the root zone of wheat, lateral root primordia frequency increased within 15 hours, and primordia in addition to those at the start of the treatment were initiated in the tissue located 0–20 mm behind the apex. Furthermore, when glucose was fed to a part of the root system using the split root method, the number of lateral root primordia also increased in the tissue located 0–30 mm behind the apex (Bingham et al., 1998). Takahashi et al. (2003) showed that the addition of sucrose, glucose, and fructose to the medium stimulated the induction of adventitious roots in *Arabidopsis* seedlings. These results showed that the exogenous sugar supply stimulated root growth. In this study, we showed that the content of sucrose and metabolized substances (glucose, fructose) was higher in the apical root section (Fig. 1). Albrecht and Mustroph (2003) also showed that the root apex contained sucrose and hexose at the highest concentration. These results suggested that sucrose and its metabolites played important roles in root system formation.

The highest activities of the invertases and the sucrose synthase were detected in the apical portion (the 0–1 cm portion form the seminal root apex) of the seminal root (Fig. 2). Histochemical NBT staining detected the invertase activity in the 0–1 cm section of the seminal root, but it was not detected in the apical 1 mm or the basal 2mm parts of the section (Fig. 3). Histochemical NBT staining revealed sucrose synthase activity in the 0–1 cm section of the seminal root, except for the apical 1 mm and basal 2 mm parts, and in the root apex of the lateral root (Fig. 4). The apical 0–1 cm zone, except for the apical 1 mm, is the cell elongation zone of the seminal root (Ogawa and Yamauchi, 2006). In previous studies, the higher activity of invertase was detected in the apical zone of maize root (Duke et al., 1991) and the gradient of the sucrose synthase activity from the root tip to the base was observed in wheat seedling (Albrecht and Mustroph, 2003). The *in situ* localization of invertase activity was detected using a histochemical method in root of *Arabidopsis* (Sergeeva et al., 2006) and maize (Duke et al., 1991; Kim et al., 2000). The localization of sucrose synthase activity was detected using a histochemical method in wheat root (Albrecht and Mustroph, 2003). Kim et al. (2000) showed that invertase activity was detected as the modulated cell wall signal and the weak intercellular signal in the epidermis, cortex, and central cylinder. Albrecht and Mustroph (2003) showed that increased sucrose synthase activity was observed in the tip region and stele of root in wheat subjected to the O_2 deficit condition. In this study, we examined the localization of invertase and sucrose synthase activities using the histochemical method and we measured the activities of these enzymes in some sections of the seminal root; our results were in agreement with theirs. These results suggested that sucrose is transported to the root elongation zone and the surrounding tissue of the lateral root primordia. There, it was cleaved into glucose, fructose, and UDP-glucose by invertase or sucrose synthase. Glucose, fructose, and UDP-glucose were probably distributed to the cell division and cell elongation zones of the seminal and lateral roots. Glucose and fructose are used as an energy source and as a compatible solute for cell elongation. UDP-glucose is used for cell wall synthesis and as a substrate for root system formation.

The sugar content and the activity of the invertases and sucrose synthase changed with the number of days after transplantation (Figs. 1, 2). If the amount of sucrose transported from shoot did not change from d 3 to d 5, then this result illustrated that the decline in enzyme activity led to the increment of sugar accumulation. Kim et al. (2000) used 15-day-old maize seedlings for investigation of the diurnal changes in the sugar content and the expression of invertases and sucrose synthase in seminal root. They showed that the contents of sucrose and hexose and the enzyme activity did not change. In this study, the sugar content and enzyme activity showed diurnal changes; these results differ from those reported by Kim et al. (2000) because we used young 3- to 5-day-old seedlings with high metabolic activities, and because the sugar content and enzyme activity were examined in different parts of the seminal root axis, not in the whole root.

In conclusion, the sucrose transported to the root system was distributed to the root apex zone and the

lateral root emergence zone. Then the sucrose was cleaved into glucose, fructose, and UDP-glucose by invertase or sucrose synthase. It is suggested that they contribute to root system formation as an energy source, a compatible solute, and as a substance for cell wall synthesis.

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