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Inhibition of the Formation of Adventitious Roots on Cucumber Hypocotyls by the Fractions and Methoxybenzylglutamine from Xylem Sap of Squash Root

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In studies of the functions of roots in the development of aboveground organs, the butanol fraction of xylem sap collected from squash root was found to have inhibitory activity against the formation of adventitious roots on the hypocotyls of cucumber in a culture of shoot cutting. The inhibitory activity was fractionated with reverse phase column chromatographies, and an inhibitory fraction was recovered with a single peak of absorbance at 280 nm, which contained a novel amino acid, N^5-(4-methoxyphenyl) methyl-L-glutamine (methoxybenzylglutamine) as a major component (Inouye *et al.* 1998). Chemically synthesized methoxybenzylglutamine (5 mM) inhibited the formation of adventitious roots and also inhibited the growth of first leaf and cotyledons in a culture of shoot cuttings. On the basis of the results obtained, discussed is possible regulation of the developmental events on the aboveground organs by the roots through xylem sap.

Key words: Adventitious root — Cucumber (*Cucumis sativus*) — Inhibition — Methoxybenzylglutamine [N^5-(4-Methoxyphenyl)methyl-L-glutamine] — Squash (*Cucurbita*) — Xylem sap

Growth of higher plants is thought to depend on interactions among different organs such as roots, shoots and leaves. For example, flower formation (McDaniel 1996, Satoh 1996) and sex expression (Chailakhyan and Khryanin 1978) were known to be controlled by the root in some plant species. One of the major routes for interaction between the root and aboveground organs is xylem in the vascular system. Xylem is mainly composed of xylem vessels, which are a kind of apoplastic space. Organic nutrients such as amino acids, sugars and organic acids, as well as water and inorganic nutrients, are known to be transported in xylem to aboveground organs (Zornoza *et al.* 1996, Schurr and Schulze 1995). Moreover, cytokinin, abscisic acid and other growth substances are known to be synthesized in the root and transported to the aboveground organs through xylem. They are known to control aspects of physiological and developmental events of aboveground organs, such as stomatal responses (Liang *et al.* 1997, Else *et al.* 1995), the senescence of leaves (Soejima *et al.* 1992, Noodén *et al.* 1990), the development of lateral buds (Bangerth 1994, Beveridge *et al.* 1997) and the formation of flower buds (Kinet *et al.* 1993). In recent years, the presence of polyamines (Friedman *et al.* 1986) and macromolecules such as proteins (Biles and Abeles 1991, Satoh *et al.* 1992), oligo- and polysaccharides (Campbell *et al.* 1995, Satoh *et al.* 1992) in xylem sap was also reported.

For the elucidation of the physiological functions of these organic substances in xylem sap, we analyzed the biological activities of xylem sap substances using a shoot culture of cucumber. When the hypocotyl of cucumber seedlings was cultured in the fractions of xylem sap collected from squash roots, both promoting and inhibiting influences on the formation of adventitious roots from the hypocotyl were observed.

According to reports, the formation of adventitious roots is promoted by various organic substances including auxins (Went 1939, Basu *et al.* 1969, Shibaoka 1971, Mensualisodi 1995). There are, however, few reports on the inhibition of the formation of adventitious roots by chemicals, even those which are endogenous or exogenous compounds (Heide 1965, Mitsuhashi *et al.* 1969a, b). The formation of adventitious roots on the stems and hypocotyls in vivo is thought to be controlled negatively by the inhibitors produced by roots in addition to the promotion by the substances produced by shoots such as auxins (Torrey 1959, Libbert 1956). Therefore, we focused on the inhibitory activity of xylem sap. As a result, we identified a novel amino acid, methoxybenzylglutamine [N^5-(4-methoxyphenyl)methyl-L-glutamine], in the inhibitory fraction of xylem sap (Inouye *et al.* 1998). The chemically synthesized methoxybenzyl-L-glutamine inhibited the formation of adventitious roots and also the development of first leaf and cotyledons of cucumber in a culture of shoot cuttings. The physiological meaning of the organic sub-

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Abbreviations: MS medium, Murashige and Skoog's medium; SD, standard deviation

stances in xylem sap, especially the derivative of amino acid in plant development, will be discussed.

Materials and Methods

Plant materials and the collection of xylem sap

Seeds of interspecific hybrids of squash (*Cucurbita maxima* Duchesne×*C. moschata* Duchesne; cv. Shintosa-ichigou), commonly used commercially as the root stock for cucumber, and seeds of a cucumber cultivar (*Cucumis sativus* cv. Shimoshirazu-jibai) were obtained from Sakata Seed Co. (Kanagawa, Japan).

Shintosa-ichigou squash plants were grown in the field for 2-3 months (May to August) and stems were cut off 15-30 cm above soil level. After the first 2-3 drops of exudate from the cut surface on the root side had been discarded, the cut surface was washed with distilled water and the exudate was collected in flasks on ice and stored at −30 C as xylem sap.

Fractionation of xylem sap

Xylem sap (1 L) was mixed with four volumes of ethanol and allowed to stand for one week at 4 C. The solution was centrifuged at 7,700×g for 30 min. The supernatant (80% ethanol soluble fraction) was evaporated at 40 C and was lyophilized. The precipitate (80% ethanol insoluble fraction) was dried *in vacuo*. Both samples were homogenized in 50 ml of water with a glass-Teflon homogenizer, and then each homogenate was sonicated with an ultrasonic disruptor (output, 3; duty, 50; 20 times; Tomy Seiko, Tokyo). Because the 80% ethanol insoluble fraction could not be completely solubilized, a suspension of the fraction was used for the assay. The 80% ethanol soluble fraction was sequentially partitioned with 50 ml of ethylacetate and then butanol. Each partition was repeated 3 times and combined. The ethylacetate, butanol and aqueous phases were separately evaporated at 40 C, lyophilized and solubilized in 50 ml of water with the sonication described above.

Fractionation of the inhibitory activity

The 80% ethanol soluble fraction (dissolved in 50 ml of water) of xylem sap (2 L), obtained as described above, was partitioned with 50 ml of butanol 4 times and the combined butanol phases were evaporated at 40 C, lyophilized and dissolved in 20 ml of water. The sample was then subjected to reverse phase chromatography on a Sep-Pack C_{18} cartridge column (Waters, Milford, MA, USA), washed with 10 ml of water and pooled as a flow-through fraction. The bound substances were eluted sequentially with 15 ml of 20, 50 and 100% (v/v) acetonitrile and the samples were evaporated to completely remove acetonitrile.

An aliquot (100 μl) of 20% acetonitrile eluate which had inhibitory activity was further subjected to reverse phase chromatography on an ODS-80Tm column (4.6 mm I.D.×15 cm; Tosoh, Tokyo) with a high performance liquid chromatography system (Tosoh) and eluted with a linear gradient of acetonitrile (from 0 to 60% (v/v) in 30 min) at a flow rate of 0.5 ml/min with monitoring absorbance at 280 nm. The pooled fractions were evaporated at 40 C to remove acetonitrile and used for the assay. The eluate from 18 to 22 min with the inhibitory activity was then applied to an ODS-120T column (4.6 mm I.D.×25 cm; Tosoh) with the same conditions as mentioned above and the pooled fractions were used for the assay after evaporation. The inhibitory fraction (from 18 to 22 min) was finally re-chromatographed on the same column and with the same conditions for a check of purity.

Identification of methoxybenzylglutamine

The eluate from the ODS-120T column from 18 to 22 min was evaporated and lyophilized, and 0.2 mg of solid material was obtained. The major component of the solid was identified as a novel amino acid, N^5-(4-methoxyphenyl) methyl-L-glutamine (methoxybenzylglutamine), by UV spectrum, high resolution mass spectrum, $ESI^{+/-}$-mass spectrum, ^{1}H-NMR spectrum, amino acid analysis, and by chemical synthesis. The details on the determination of the structure were described elsewhere (Inouye *et al.* 1998).

Induction of the formation of adventitious roots

Shimoshirazu-jibai cucumber seedlings were grown in artificial soil (Kurehakagaku, Tokyo) for 6 days under white fluorescent light (15 μmol $m^{-2}s^{-1}$) with a 16-h photoperiod at 28 C. The hypocotyl of each seedling was cut off with a razor blade at a site 4 cm below the cotyledons and cultured with 1 ml of the solution to be tested with 1/20 strength Murashige and Skoog's medium (MS medium; Murashige and Skoog 1962) without sucrose in a plastic microcentrifuge tube (2 ml; Sorenson, Salt Lake City, UT, USA) under white fluorescent light (15 μmol $m^{-2}s^{-1}$). Because the active formation of adventitious roots was observed in the presence of 1/20 strengh MS medium, the inhibitory effects of the fractions on the formation of adventitious roots was tested in the presence of 1/20 strengh of MS medium. After 5 days in culture, the adventitious roots that had emerged from the epidermis of the hypocotyl were counted by using a loupe. Five to nine plants were used for each assay and all experiments were repeated at least twice.

Results and Discussion

Squash plants are widely and commercially used as root stock for cucumber plants to ensure protection from soil-borne disease. This means that aboveground parts of the cucumber plant can normally grow and develop on the root of squash, at least to some extent. Some physiological phenomena such as delay of the formation of flower buds owing to squash root stock were observed in the development of cucumber scion (Satoh 1996). Therefore, cucumber shoots were used for assays and squash roots were used as the source of xylem sap for the study of the interactions between root and shoot. The squash plant used in the present study was a cultivar (Shintosa-ichigou) used for grafting, and a large amount of xylem sap could be collected from plants over a long period of time (300-3,000 ml/plant in 2 days). Intensive formation of adventitious roots can be

Table 1. Effects of various fractions of xylem sap from squash roots on the formation of adventitious roots on cucumber hypocotyls

Treatment	No. of adventitious roots per hypocotyl (SD)	
	H_2O	1/20 MS medium
None	2.6 (1.8)	13.3 (3.3)
Toxal xylem sap	16.2 (4.2)	19.5 (3.4)
80% ethanol-insoluble	12.8 (2.6)	18.8 (3.3)
80% ethanol-soluble	13.5 (2.1)	15.7 (2.3)
ethylacetate-phase	12.1 (2.1)	17.2 (4.9)
butanol-phase	1.0 (1.1)	8.5 (2.8)
H_2O-phase	21.4 (4.4)	16.9 (4.1)

The xylem sap collected from squash root was added with ethanol up to 80% (v/v) and then the soluble fraction was further partitioned sequentially with ethylacetate and butanol. Total xylem sap which was prepared by lyophilization of the whole xylem sap, and the each fractions which were evaporated and lyophilized, were solubilized in water and subjected to the assay with or without 1/20 strength MS medium. The concentrations were adjusted to be equal to those in the original xylem sap.

Table 2. Fractionation of the inhibiting activity on the formation of adventitious roots of butanol-phase of xylem sap on Sep-Pack C_{18} column

Fractions	No. of adventitious roots per hypocotyl (SD)
None	16.6 (2.6)
flow-through	17.0 (3.3)
20% acetonitrile	6.5 (2.1)
50% acetonitrile	17.2 (2.3)
100% acetonitrile	16.8 (4.2)

The butanol phase of xylem sap was applied to Sep-Pack C_{18} column and eluted sequentially with 20, 50 and 100% acetonitrile and subjected to the assay in the presence of 1/20 strength MS medium. The concentrations were adjusted to 10 times of those in the original xylem sap.

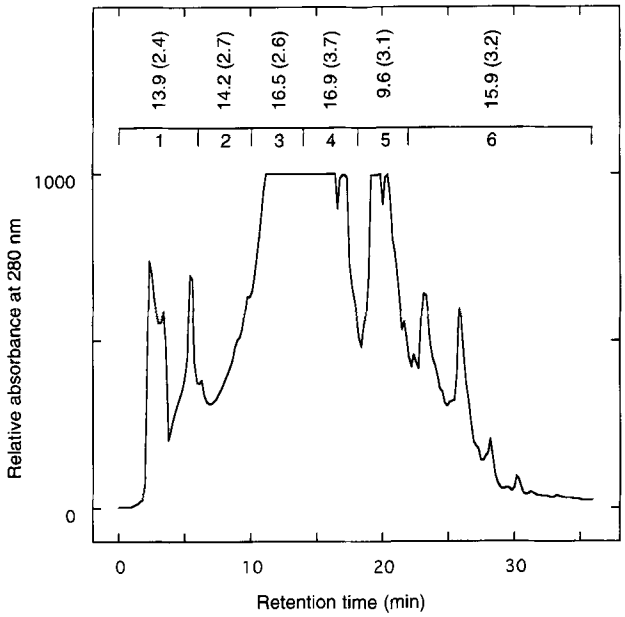

Fig. 1. Fractionation of the inhibitory activity against the formation of adventitious roots with reverse phase chromatography on an ODS-80Tm column. The 20% acetonitrile eluate from Sep-Pack C_{18} cartridge column of butanol phase partitioned from 80% ethanol soluble fraction of xylem sap collected from squash roots was applied to the ODS-80Tm column with high performance liquid chromatography system and eluted with a linear gradient of acetonitrile (from 0 to 60% in 30 min) and absorbance at 280 nm was monitored. The pooled fractions (No. 1-6) were evaporated and used for the assay in the presence of 1/20 strengh MS medium. The numbers above the elution profile of 280 nm indicate the mean number (SD) of adventitious roots formed on the hypocotyls of cucumber. The concentrations were adjusted to 20 times of those in the original xylem sap. The number of adventitious roots formed without the fractions was 14.3 (3.0).

readily induced on the hypocotyl of cucumber.

Promotion of the formation of adventious roots on hypocotyls was observed when hypocotyls of cucumber shoots were cultured in water with total xylem sap, H_2O phase fraction of xylem sap collected from squash root and the other fractions (except the butanol fraction of 80% ethanol soluble fraction) (Table 1). Some parts of these promotions may be due to the effects of borate on the cell division and maintenance of meristems (Hemberg 1951, Clarkson 1980). As shown in Table 1, various substances in the fractions should act positively on the formation of adventitious roots with a supply of water only.

Only the butanol fraction showed slight inhibition in water; it showed clear inhibition against control in the presence of nutrition with 1/20 strength MS medium. Therefore, the butanol fraction was further subjected to reverse phase chromatography on a Sep-Pack C_{18} cartridge column. As shown in Table 2, an inhibitory effect was observed only in 20% acetonitrile eluate. The eluate was further subjected to reverse phase chromatography on an ODS-80Tm column with a high performance liquid chromatography system and the inhibitory activity was detected in the fraction from 18 to

Table 3. Effects of synthetic methoxybenzyl-L-glutamine and L-glutamine on the formation of adventitious roots on cucumber hypocotyls in the absence of 1/20 strength MS medium

Treatments		No. of adventitious roots per hypocotyl (SD)
H_2O		10.0 (3.8)
methoxybenzyl-L-glutamine	0.5 mM	12.0 (3.6)
	5.0 mM	3.5 (2.4)
L-glutamine	5.0 mM	11.4 (3.4)

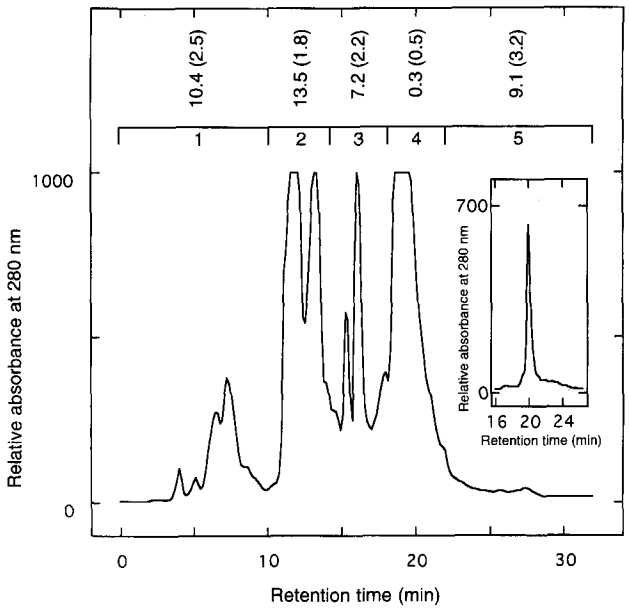

Fig. 2. Fractionation of the inhibitory activity against the formation of adventitious roots with reverse phase chromatography on an ODS-120T column. The inhibitory fraction No. 5 (from 18 to 22 min) from an ODS-80Tm column was applied to an ODS-120T column and eluted with the same condition as mentioned in Fig. 1. The inhibitory fraction No. 4 (from 18 to 22 min) was re-chromatographed with the same condition and the elution profile at 280 nm was shown in the parenthesis. The pooled fractions (No. 1-5) were evaporated and used for the assay in the presence of 1/20 strength MS medium. The numbers above the elution profile of 280 nm indicate the mean number (SD) of adventitious roots formed on the hypocotyls of cucumber. The concentrations were adjusted to 40 times of those in the original xylem sap. The number of adventitious roots formed without the fractions was 11.7 (2.9).

22 min (Fig. 1). The inhibitory fraction was then subjected to reverse phase chromatography again on an ODS-120T column and the inhibition was observed in the fraction from 18 to 22 min (Fig. 2). When this fraction was re-chromatographed in the same condition, a single peak of absorbance at 280 nm was detected (parenthesis in Fig. 2).

The final fraction of ODS-120T contained approximately 0.2 mg of solid material, which was subjected to identification of chemicals with UV spectrum, high resolution mass spectrum, $ESI^{+/-}$ mass spectrum, ^1H-NMR spectrum, amino acid analysis, and chemical synthesis (Inouye *et al.* 1998). As a result, a novel amino acid, N^5-(4-methoxyphenyl) methyl- L-glutamine (methoxybenzylglutamine), was identified as a major component.

When the chemically synthesized methoxybenzyl-L-glutamine was applied to the hypocotyl of shoot of cucumber, the inhibition of adventitious root formation in the absence of 1/20 strength MS medium was observed at a concentration of 5 mM but not at 0.5 mM (Table 3). L-Glutamine, as a control, showed no effect even at 5 mM, but the inhibition of methoxybenzyl-L-glutamine was not observed in the presence of 1/20 strength MS medium. Moreover, in the culture of cucumber shoots with 5 mM methoxybenzylglutamine, an inhibition of the growth of the first leaf and cotyledons was observed (data not shown).

The real concentration of methoxybenzylglutamine in plants is not yet known, but even if the recovery of the substance in the purification is assumed to be 1%, the concentration of methoxybenzylglutamine can be estimated to be 10 mg/L (4×10^{-6} M) in the xylem sap. Although the substances in xylem sap can be concentrated in the aboveground organs by transpiration at several hundred times, this concentration is far from effective in the present bioassay. This result suggests that methoxybenzylglutamine may not be involved in the control of the formation of adventitious roots in plant.

On the other hand, the butanol fraction of xylem sap showed an inhibitory effect on the formation of adventitious roots at the concentration adjusted to be equal to those in the original xylem sap, and the strong inhibition of the formation of adventitious roots was also observed in the fraction from 18 to 22 min on ODS-120T column at the concentration adjusted to 40 times of that in the original xylem sap (Fig. 2). Moreover, methoxybenzylglutamine inhibited the formation of adventitious root only in the absence of 1/20 strength MS medium, whereas the fractions of xylem sap showed its inhibitory effect even in the presence of 1/20 strength MS medium. These results suggest that the inhibitory activity detected in the fractions of xylem sap might not be due to methoxybenzylglutamine and there should be the other stronger inhibitors for the formation of adventitious roots in the fractions of xylem sap, in which methoxybenzylglutamine was finally identified as a major component. It is possible that those unknown inhibitors may be involved in the regulation of the formation of adventitious roots in plant. Identification of the inhibitors will be required for the elucidation of the control mechanism of the formation of adventitious roots in plant.

Methoxybenzylglutamine is a derivative of an amino acid, glutamine; glutamine is known to be one of the major trans-

porters of nitrogen from root to shoot through xylem sap in some plants (Schurr and Schulze 1995). In many plants, ammonium and nitrate in soil are known to be absorbed by roots and metabolized to glutamine there. The glutamine is then transported to various organs and further metabolized to the other amino acids by a glutamine synthetase-glutamate synthase system (Lam *et al.* 1996, Temple *et al.* 1998). Presence of the other derivative of glutamine, N^5-(4-hydroxylphenyl)methylglutamine, was reported in seeds of buckwheat (*Fagopyrum esculentum*) (Koyama *et al.* 1973) and in seeds and seedlings of *Sinapsis* species (Larsen *et al.* 1984), but its physiological function is not known. It is possible that methoxybenzylglutamine may be involved in the metabolism of glutamine in plant. Further physiological and biochemical analysis will be required for the elucidation of the roles of methoxybenzylglutamine in plant.

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