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Involvement of Ethylene and Gibberellin Signalings in Chromosaponin I-Induced Cell Division and Cell Elongation in the Roots of *Arabidopsis* Seedlings

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Chromosaponin I (CSI), a triterpenoid saponin isolated from pea, stimulates the growth of roots in *Arabidopsis thaliana* seedlings on wetted filter paper in the light for 14 d. The growth rates of roots in Columbia (Col) and Landsberg erecta (Ler) wild-types were 0.92 and 0.26 mm d^{-1}, respectively, and they were accelerated to 3.46 (Col) and 2.20 (Ler) mm d^{-1} by treating with 300 μM CSI. The length of mature epidermal cells was increased by 1.8-fold (Col) and 2.81-fold (Ler) compared with control and the number of epidermal cells was increased by a factor of 1.65 (Col) and 2.12 (Ler). Treatment with 2-aminoethoxyvinylglycine (AVG), an inhibitor of ethylene biosynthesis, also increased cell length but not cell number. The effects of CSI on root growth were not detected in the ethylene-insensitive mutant *ein2-1*. CSI did not inhibit ethylene production but stimulated the growth of roots in *ctr1-1*, the constitutive triple response mutant for ethylene, indicating that CSI inhibits ethylene signaling, especially downstream of CTR1. In the GA-insensitive mutant *gai* and the mutant *spy-3*, in which the basal level of GA signaling is activated, CSI did not increase cell number, although both CSI and AVG stimulated cell elongation in these mutants. These results suggest that the inhibition of ethylene signaling is the cause of CSI-induced cell elongation. A possible involvement of both GA and ethylene signalings is discussed for the CSI-induced cell division.

Key words: *Arabidopsis thaliana* — Cell division — Chromosaponin I — Ethylene — GA — Root growth.

Application of saponins to plants has been reported to influence some biological processes as reviewed by Geuns (1978). Saponins stimulated the growth of wheat (Balansard and Pellissier 1943) and pea embryos (Helmkamp and Bonner 1953). Germination of fenugreek seeds was inhibited by endogenous saponin-like substances (Zambou et al. 1993). A spinostanol saponin induced callose synthesis in carrot cells (Messiaen et al. 1995). Root growth was stimulated by a γ-pyronyl-triterpenoid saponin (chromosaponin I, CSI) in a variety of plants (Tsurumi and Wada 1995). A glycosidic triterpenoid saponin inhibited diguanylate cyclase, the key regulatory enzyme of the cellulose synthesizing apparatus of *Acetobacter xylinum* (Ohana et al. 1998). Although physiological importance of these diverse actions of saponins is not yet established, saponins may be potential growth regulators. We show in the present paper that CSI may be an useful tool to study the mechanism of root growth.

CSI was isolated from pea (Tsurumi et al. 1991, 1992) and other leguminous plants (Kudou et al. 1992, 1993, Massiot et al. 1992), and characterized as an amphipathic natural reductant (Tsujino et al. 1995). The typical effects of CSI on the growth of roots in lettuce were the increase in the mechanical extensibility of root cell walls, the increase in cell length and the reduction in cell diameter (Tsurumi et al. 1996, Tsurumi and Ishizawa 1997). These effects on the shape of root cells are very similar to those of GA in stem cells (Shibaoka 1994). However, treatment with GA_3 did not show any significant effect on the growth of roots in lettuce. Tanimoto (1987, 1988) proposed that roots require much less GA than shoots for normal growth. This concept was supported by the facts that promotive effects of exogenous GA on the growth of roots were observed only when the endogenous level of GA was reduced by GA-synthesis inhibitors and that the lowest concentration of GA to promote root elongation was one ten-thousandth of that for shoot elongation (Tanimoto 1987, 1988). In the roots treated with GA-biosynthesis inhibitors or those of mutants deficient in GA-synthesis, application of GA changed the shape of root cells to longer and narrower ones (Tanimoto 1987, 1988, Barlow et al. 1991, Baluska et al. 1993). The above concept is the most reasonable explanation for the absence of significant effects of exogenous GA on the growth of wild-type roots.

Tsurumi and Ishizawa (1997) found that CSI reduced

Abbreviations: AVG, L-α-(2-aminoethoxyvinyl)glycine; Col, Columbia; CSI, chromosaponin I; Ler, Landsberg erecta; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; QC, quiescent center.

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both the sensitivity of roots to ethylene and the maximal effects of ethylene in lettuce, and we proposed that CSI stimulates the growth of roots by inhibiting ethylene signaling. Ethylene is thought to affect the mode of growth by altering orientations of both cortical microtubule and newly deposited cellulose microfibril from transverse to longitudinal direction (Steen and Chadwick 1981, Eisinger 1983, Roberts et al. 1985). Gibberellins exert opposite effects on the shape of cells and on the orientations of microtubules and cellulose microfibrils in stems (Shibaoka 1994) and counteract the inhibitory action of ethylene in the growth of subhook region of pea epicotyls (Stewart et al. 1974). Therefore, it is reasonable to raise the possibility that GA, in addition to ethylene signaling, may be involved somehow in the CSI action in stimulating root growth.

Arabidopsis is a very useful plant material for investigating the mechanism of actions of plant hormones. The roots of *Arabidopsis* are more sensitive to CSI compared with other plants (unpublished results) and several mutants related to ethylene and GA are available (Ecker 1995, Ross et al. 1997). Hence, we have been studying the effects of CSI on the growth of roots in *Arabidopsis* seedlings using wild-type (Col and Ler), ethylene-insensitive mutant *ein2-1*, ethylene overproduction mutant *eto1-1* (Guzmán and Ecker 1990), GA-insensitive mutant *gai* (Koornneef et al. 1985) and the mutant *spy-3*, in which the basal level of GA signal transduction is activated (Jacobsen and Olszewski 1993). The *spy-3* was isolated as a mutant resistant to GA-biosynthesis inhibitor paclobutrazol. In our preliminary experiments, the promotive effect of CSI on the growth of *Arabidopsis* roots in the light was unexpectedly great, so that they could not be explained solely by the idea that CSI may inhibit ethylene signaling. Although the physiological roles of CSI are not yet known, the great effects of CSI on root growth prompted us to analyze the CSI action. In the present paper we show that CSI increases the cell number as well as cell length in stimulating the growth of *Arabidopsis* roots, and also show that inhibition of ethylene signaling may be the cause of the CSI-induced cell elongation. As to the CSI-induced cell division the requirement of normal level of signalings of both GA and ethylene is discussed.

Materials and Methods

Plant material—Wild types of *Arabidopsis thaliana* (L.) Heynh, ecotype Columbia (Col) and Landsberg erecta (Ler), and the GA-insensitive mutant *gai* (Koornneef et al. 1985) which was derived from ecotype Ler were obtained from Sendai *Arabidopsis* Seed Stock Center (Sendai, Japan). Seeds of the mutant insensitive to ethylene, *ein2-1* (Guzmán and Ecker 1990), the ethylene overproduction mutant *eto1-1* (Guzmán and Ecker 1990), the constitutive triple response mutant of ethylene, *ctr1-1* (Kieber et al. 1993), and a mutant resistant to GA biosynthesis inhibitor paclobutrazol, *spy-3* (Jacobsen and Olszewski 1993), were obtained from *Arabidopsis* Biological Resource Center (Ohio, U.S.A.). The ecotype of these mutants is Col. Seeds were propagated as follows; seeds were sown on vermiculite fertilized with Hyponex nutrient solution (Hyponex Corp., Ohio, U.S.A.), placed at 4°C for 4 d, and then allowed to grow at 23°C under 16 h photoperiod cycle. Light source was two 40 W white fluorescent lamps (FLR 40SW/M36, Hitachi, Tokyo, Japan) and the intensity at plant level was about 10 W m^{-2}. The nutrient solution was added every week and the ripened seeds were harvested in order of siliques into full maturity.

Chemicals—CSI was extracted from 7-day-old etiolated pea seedlings (*Pisum sativum* L. cv. Alaska) with aqueous methanol and purified by HPLC as described previously (Tsurumi et al. 1992). The purified CSI was dried to white powder and kept under N_2 at −80°C. AVG was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and other chemicals were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Growth test—Test solutions were prepared by dissolving chemicals in 20 mM MOPS buffer (pH 6.6). The buffer was made of 5 mM KNO_3, 2 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, 1 mM KH_2PO_4 and 20 mM MOPS. The pH of the buffer was adjusted with KOH. *Arabidopsis* seeds were placed in 2.6 cm Petri dish on filter paper (Advantec no. 2; Toyo Roshi Kaisha, Ltd., Tokyo, Japan) wetted with 300 μl of the buffer. Two or 4 d after cold treatment at 4°C under nearly saturating humidity in the dark, seeds were irradiated for one or two days with white fluorescent lamps (FL 20SS-BRN/18, Toshiba, Tokyo, Japan) at an irradiance of about 1.6 W m^{-2}. The irradiated seeds were transferred with forceps to another 2.6 or 3.5 cm Petri dish on filter paper wetted with 300 or 600 μl of test solution, respectively, and allowed to grow in the light at 23°C for 3, 7 or 14 d. The test solutions were exchanged with fresh ones 7 d after the start of incubation. With *eto1-1*, test solutions were renewed by fresh ones 3 d after the incubation. For ethylene treatment, the dishes containing irradiated seeds were placed in a sealed 140-ml plastic cylinder. Ethylene was injected with a syringe into each cylinder through a small side hole as described previously (Tsurumi and Ishizawa 1997). The length and width of roots were measured under a microscope. The diameter of roots and the length of mature epidermal cells were measured at the longitudinal midpoint of roots. In each case the mean (±SE) for 10–15 seedlings was calculated. Each assay was repeated at least three times.

Measurement of cell number in one epidermal cell file of roots—The cell number in one epidermal cell file, from the junction between the cap and the quiescent center (QC) to the basal end of root, and the average cell lengths along the cell file were obtained as follows. A root was divided into several zones, two zones of about 50 μm each in the meristematic region, several zones of 100–300 μm in the elongation zone and of 500–5,000 μm in the mature zone. While cell length increased gradually, the length of zone was relatively short and after cessation of cell elongation the length of zone was long (Fig. 1). In the meristematic region, the cell number in one epidermal cell file from the QC to 50 μm and that of next proximal 50 μm zone were counted, and the average cell length was calculated by dividing the length of the zone by the cell number. In the elongation and mature zones, the average cell length was obtained from 10 epidermal cells at the central part of each zone, and the cell number was calculated by dividing the length of the zone by the average cell length. Since non-hair epidermal cells are longer than hair forming cells (Dolan et al. 1994), 10 epidermal cells were selected from at least four cell files to include evenly both types of epidermal cells. The total cell number in one epidermal cell file of a root was

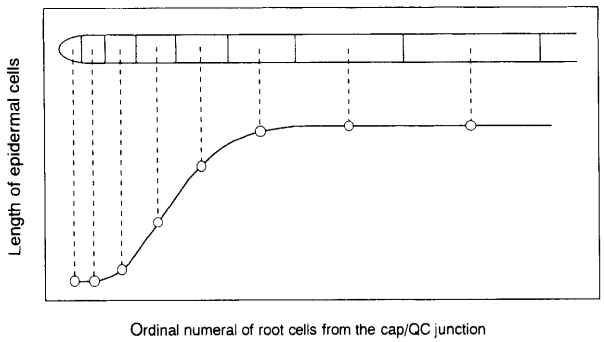

Fig. 1 Plotting the mean length of epidermal cells obtained from each zone of a root against the ordinal numeral of cells from the cap/QC junction. A root was divided into several zones: the length of zone was about 50 μm in the meristem, 100–300 μm in the elongation zone and 500–5,000 μm in the mature zone.

the summation of cell numbers of all successive zones. The average of total cell number was obtained from 8 seedlings. Each assay was repeated at least three times.

When the mean length of epidermal cells from each zone of a root is plotted against the ordinal numeral of cells from the cap/QC junction, the slope of the resulting line could represent the rate of cell elongation if the rate of cell division were constant (Fig. 1) (Burström 1969, Tsurumi and Wada 1995).

Measurement of ethylene production—To investigate the effect of CSI on ethylene production, we used the ethylene overproduction mutant *eto1-1*. The irradiated 100 seeds of *eto1-1* were placed in a 20-ml conical flask on a filter paper wetted with 400 μl of test solution. The flask was kept under nearly saturating humidity in a plastic container in the light at 23°C for 2 d and then sealed with a rubber stopper. Accumulation of ethylene was measured every 24 h from day 3 to 7. Before resealing the flask, the test solution and the air inside were refreshed everyday. Ethylene concentration in the flask was measured with a GC-14A gas chromatograph (Shimadzu Co., Kyoto, Japan) as described earlier (Hoson et al. 1990, Tsurumi and Ishizawa 1997) except a 2.0 ml gas sample was taken out from each flask with a syringe after injecting a 2.0 ml of fresh air. Fresh weight of the seedlings was measured on day 7. The experiment was repeated at least 4 times.

Results

CSI increases cell number as well as cell length in stimulating the growth of roots in wild-type Arabidopsis—Application of CSI stimulated the growth of roots in wild-type *Arabidopsis* seedlings (Col and Ler) (Fig. 2A), and the optimum concentration of CSI was 300 μM for both the wild types. CSI also increased the length of mature epidermal cells (Fig. 2B) and decreased the root diameter (Fig. 2C). The increase in cell length and the decrease in root diameter were saturated at around 100 μM CSI. These effects of CSI on *Arabidopsis* roots are quite similar to those on lettuce roots (Tsurumi and Ishizawa 1997), but the optimum concentration of CSI was lower in *Arabidopsis* than in lettuce. The average growth rates of roots in Col and Ler wild-types were 0.92 and 0.26 mm d^{-1}, respectively (Fig. 3). Treatment with 300 μM CSI accelerated the growth rates to 3.46 (Col) and 2.20 (Ler) mm d^{-1} and these stimulations in growth were constant for 14 d. While the promotive effects of CSI on root growth were 3.7-fold (Col) and 8.5-fold (Ler) compared with control, the CSI-induced increase in the length of epidermal cells was only 1.8-fold (Col) and 2.8-fold (Ler), respectively. Therefore, CSI-induced elongation of roots cannot be ascribed solely to its action on cell elongation. We measured the cell number of an epidermal cell file from the cap/QC junction to the basal end of roots using seedlings incubated for 7 d (Table 1). CSI increased the cell number

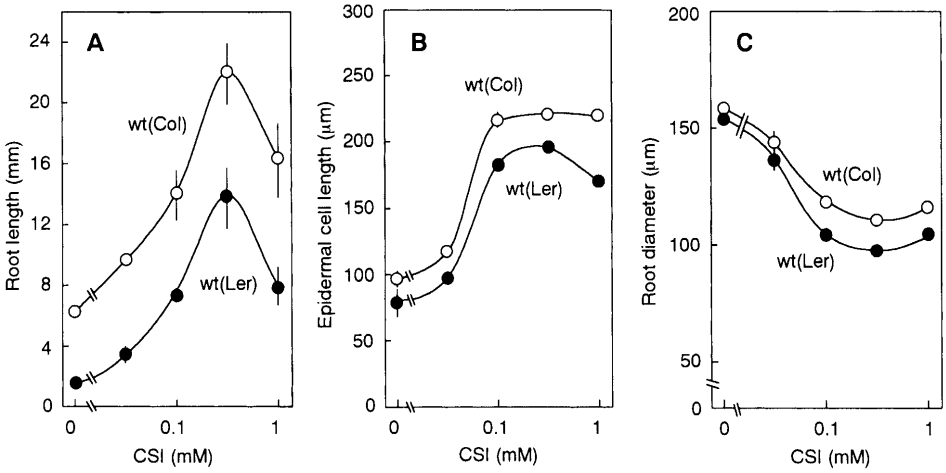

Fig. 2 Effect of CSI on the length of roots (A), the length of epidermal cells (B) and the root diameter (C) in wild-type *Arabidopsis*, ecotype Col (○) and Ler (●). *Arabidopsis* seedlings were incubated in the buffer (pH 6.6) with various concentrations of CSI (0, 30, 100, 300 and 1,000 μM) in the light for 7 d. The length of mature epidermal cells and the diameter of roots were measured at the longitudinal midpoint of roots. The length of epidermal cells for an individual seedling was the average from 10 cells. Data are the averages from 10 to 15 seedlings (±SE).

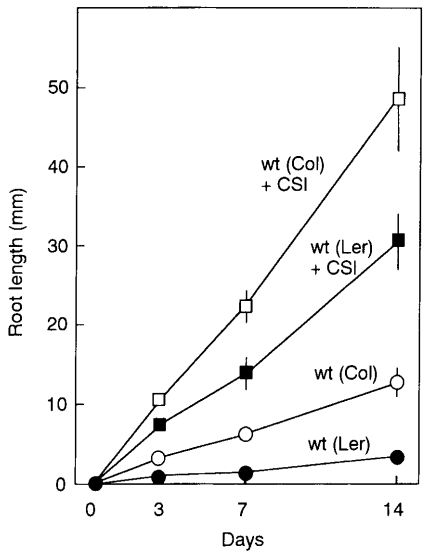

Fig. 3 Effect of CSI on the growth of roots in wild-type *Arabidopsis*, ecotype Col (□, ○) and Ler (■, ●). *Arabidopsis* seedlings were incubated in the buffer (pH 6.6) with (□, ■) or without (○, ●) 300 μM CSI in the light for 14 d. Data are the averages from 10 to 15 seedlings (±SE).

up to 1.65-fold (Col) and 2.12-fold (Ler), respectively. When the length of epidermal cells is plotted against the ordinal numeral of cells from the cap/QC junction (Fig. 4, 5), the lines representing the increase in cell length were steeper in the presence of CSI than in control in both the wild types, suggesting that CSI stimulated the rate of cell elongation (Tsurumi and Wada 1995). These results indicate that the actions of CSI on root growth involve the increase in cell number as well as the increase in cell length.

AVG stimulates cell elongation but not cell division in the roots of wild-type Arabidopsis—To investigate the involvement of ethylene in CSI actions, we compared the effect of CSI with that of AVG, an ethylene biosynthesis inhibitor (Yang and Hoffman 1984) (Table 1, Fig. 4, 5). Application of AVG to the wild types (Col and Ler) stimulated the growth of roots but the stimulation was smaller than that by CSI. The optimum stimulatory effect of AVG was obtained at 3 μM in both the wild types and the effect of AVG was fully reversed by applying 10 μl liter^{-1} ethylene (data not shown). AVG at 3 μM stimulated the elongation of epidermal cells to the same level as CSI in Col and to a slightly greater extent in Ler, and also reduced the root diameter in both the wild types as CSI did. However, no increase in cell number was observed in AVG-treated

Table 1 Effects of CSI on the length of roots, the cell number of an epidermal cell file, the length of mature epidermal cells and the root diameter in *Arabidopsis* seedlings

	Root length (mm)	Cell number	Length of mature cells (μm)	Root diameter (μm)
wt (Ler)	1.82±0.22	55.2± 4.2	61.2±3.5	139.3±2.3
wt (Ler)+CSI	14.61±2.17	117.1±15.5	172.0±5.1	96.2±1.8
wt (Ler)+AVG	4.74±0.13	53.9± 2.3	199.1±5.0	108.0±1.5
gai	1.57±0.34	39.8± 4.7	64.7±7.5	145.8±3.2
gai+CSI	2.71±0.35	35.5± 2.7	174.1±9.2	118.1±2.6
gai+AVG	2.35±0.18	37.8± 1.7	146.0±5.6	124.0±2.8
wt (Col)	7.19±0.62	93.0± 6.5	113.5±4.3	158.7±3.6
wt (Col)+CSI	25.51±1.37	154.0± 6.1	205.5±2.9	115.7±1.9
wt (Col)+AVG	14.86±1.91	108.2±11.1	208.7±7.8	125.0±3.3
ein2-1	15.11±3.06	110.1±17.8	195.8±5.0	113.2±2.2
ein2-1+CSI	14.62±1.30	108.3± 6.9	182.1±5.8	109.5±1.8
eto1-1	1.08±0.18	46.8± 5.4	39.4±2.4	163.2±3.6
eto1-1+CSI	8.47±0.51	90.6± 5.8	138.9±3.1	110.9±2.1
eto1-1+AVG	5.04±0.98	61.9± 9.4	164.7±9.6	115.0±2.3
spy-3	7.34±0.69	113.8± 5.7	92.4±5.8	156.9±5.1
spy-3+CSI	12.30±2.30	109.1±16.1	172.1±8.8	121.0±2.5
spy-3+AVG	9.84±1.44	104.4±10.8	166.8±2.3	124.0±3.1

Arabidopsis seedlings were incubated in the buffer (pH 6.6) without additives (control) or with 300 μM CSI and 3 μM AVG in the light for 7 d. The cell number of an epidermal cell file is the summation from the cap/QC junction to the basal end of roots. The diameter of roots was measured at the longitudinal midpoint of roots. Data were obtained from 8 seedlings.

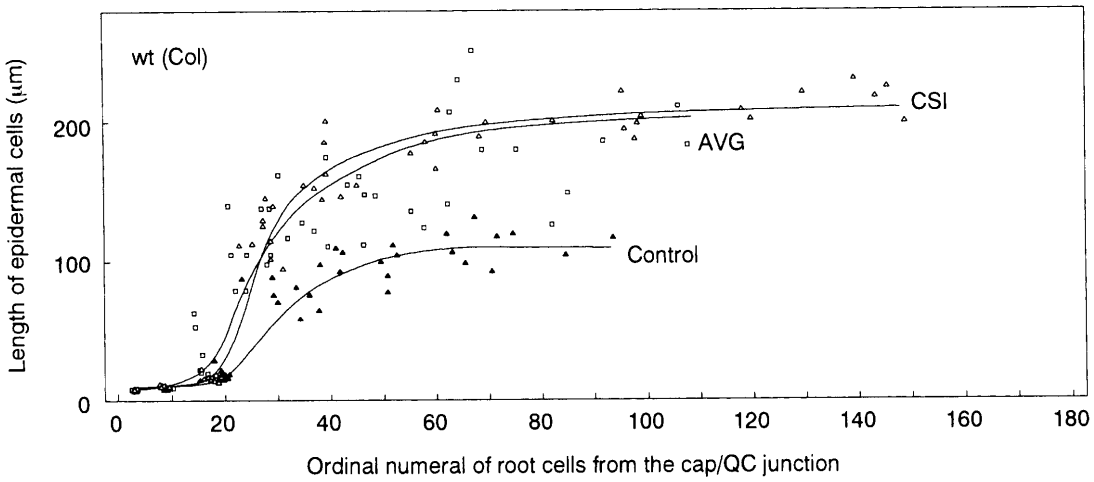

Fig. 4 CSI and AVG increase cell length but only CSI increases cell number in the epidermis of Col wild-type roots. *Arabidopsis* seedlings were incubated in the buffer (pH 6.6) without additives (control, ▲) or with 300 μM CSI (△) and 3 μM AVG (□) in the light for 7 d. Data were obtained from 8 seedlings.

wild type roots. In terms of cell shape the effects of AVG were similar to those of CSI, but with respect to cell division AVG did not mimic the effect of CSI.

Effect of CSI on ethylene mutants ein2-1 and eto1-1—The root of ethylene-insensitive mutant *ein2-1* (Guzmán and Ecker 1990) grew longer than that of wild type and the length of epidermal cells was longer as well (Table 1, Fig. 6). The line in Fig. 6 indicating the elongation rate of epidermal cells of *ein2-1* root was steep as observed in AVG-treated wild type roots (Fig. 4). Application of CSI did not induce any significant effect on the growth of *ein2-1* roots, including cell length, cell number or root diameter (Table 1 and Fig. 6). This result indicates that the presence of ethylene signaling is required for CSI actions.

The ethylene overproduction mutant *eto1-1* (Guzmán and Ecker 1990) has a very short root in which the length of epidermal cells was shorter than that in wild type and the cell number was fewer as well (Table 1, Fig. 7). The treatment with 3 μM AVG increased the epidermal cell length by a factor of 4.18 and cell number by a factor of 1.32. In CSI-treated roots the cell length was increased by a factor of 3.53 and the cell number was increased by a factor of 1.94. The effects of AVG on root growth was mainly due to the increment in cell elongation, but in contrast to Col wild-type a slight stimulation in cell number was induced by AVG. CSI increased both the cell elongation and cell number as observed in wild type (Fig. 7).

Effects of CSI and AVG on gibberellin mutants gai and spy-3—In the GA-insensitive mutant *gai* (Koornneef et al. 1985) and the mutant *spy-3*, in which the basal level of GA signaling is activated (Jacobsen and Olszewski 1993), both CSI and AVG increased cell length as observed in wild

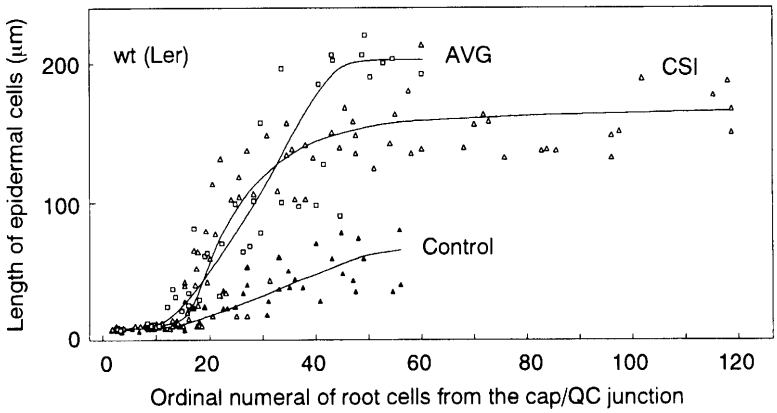

Fig. 5 CSI and AVG increase cell length but only CSI increases cell number in the epidermis of Ler wild-type roots. *Arabidopsis* seedlings were incubated in the buffer (pH 6.6) without additives (control, ▲) or with 300 μM CSI (△) and 3 μM AVG (□) in the light for 7 d. Data were obtained from 8 seedlings.

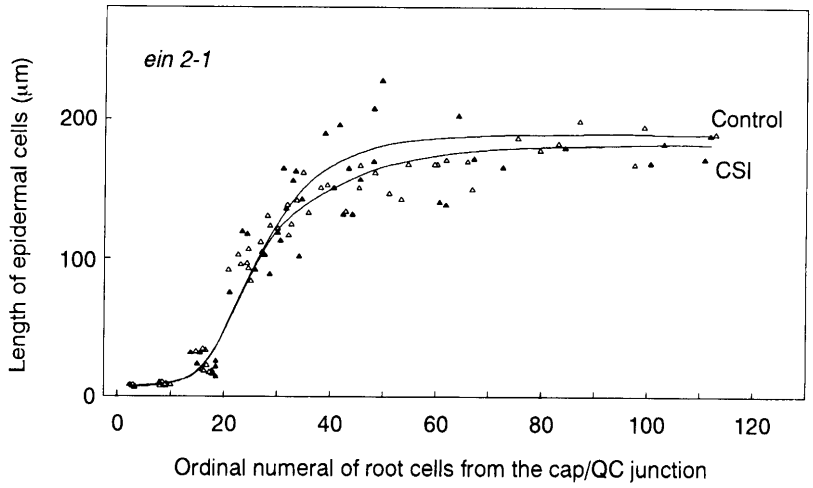

Fig. 6 CSI has no effect on cell number and cell length in the epidermis of the ethylene-insensitive mutant *ein2-1* roots. *Arabidopsis* seedlings were incubated in the buffer (pH 6.6) with (△) or without (▲) 300 μM CSI in the light for 7 d. Data were obtained from 8 seedlings.

types (Table 1 and Fig. 8, 9). However, CSI-induced increase in cell number was not detected in these mutants. These results indicate that the mechanism of CSI action in stimulating cell elongation is different from the mechanism of CSI action in increasing cell number and suggest that the normal level of GA signaling as observed in wild-type is required for the CSI action in increasing cell number.

Effects of CSI and AVG on ethylene production— Since the effect of CSI on cell elongation was very similar to that of AVG as described above, there is a possibility that CSI may inhibit ethylene synthesis as AVG. To clarify this possibility we investigated the effect of CSI on ethylene production using the ethylene overproduction mutant *eto1-1*, because of a high rate of ethylene production in this mutant and a good response to CSI. Ethylene production of *eto1-1* is 6–10 times greater than that of Col wild-type (data not shown), and CSI-induced stimulation in the growth of roots was 7.8-fold in *eto1-1* (Table 1). To measure ethylene production, a hundred *eto1-1* seedlings were incubated in a 20-ml flask in the presence or absence of CSI and AVG for 7 d. Ethylene accumulation for every 24 h was measured from day 3 of incubation to day 7 and on the last day the fresh weight of 100 seedlings was determined. Effect of CSI was expressed in two ways as shown in Table 2, the average of ethylene production rate per day and the ethylene evolution per mg fresh weight at day 7. CSI has no effect on ethylene production in both the cases whereas AVG inhibited ethylene synthesis to a great

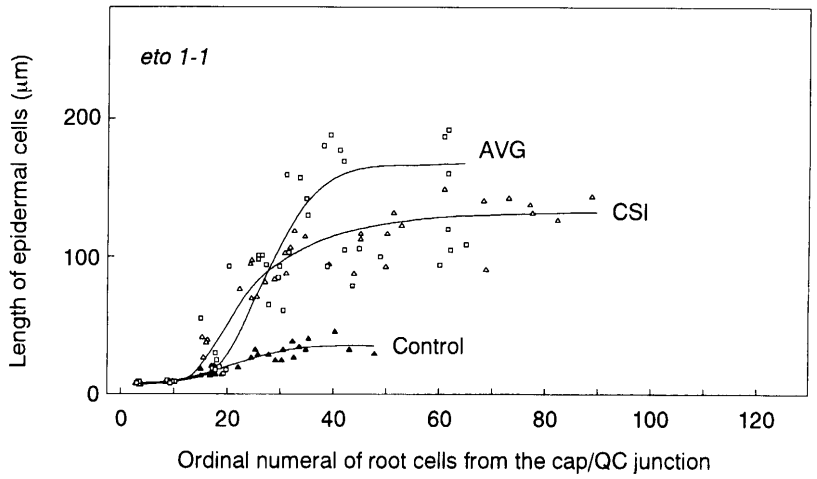

Fig. 7 CSI and AVG increase cell number as well as cell length in epidermis of the ethylene-overproduction mutant *eto1-1* roots. *Arabidopsis* seedlings were incubated in the buffer (pH 6.6) without additives (control, ▲) or with 300 μM CSI (△) and 3 μM AVG (□) in the light for 7 d. Data were obtained from 8 seedlings.

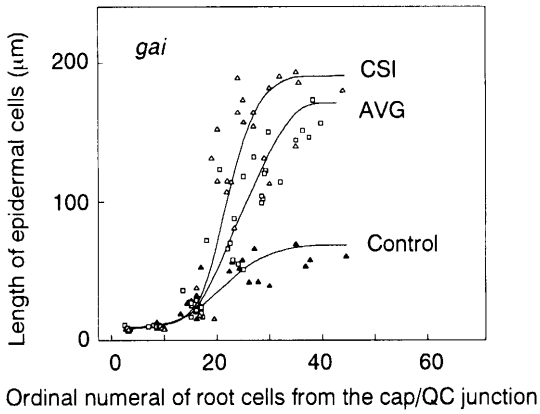

Fig. 8 CSI and AVG increase cell length but not cell number in the epidermis of the GA-insensitive mutant *gai* roots. *Arabidopsis* seedlings were incubated in the buffer (pH 6.6) without additives (control, ▲) or with 300 μM CSI (△) and 3 μM AVG (□) in the light for 7 d. Data were obtained from 8 seedlings.

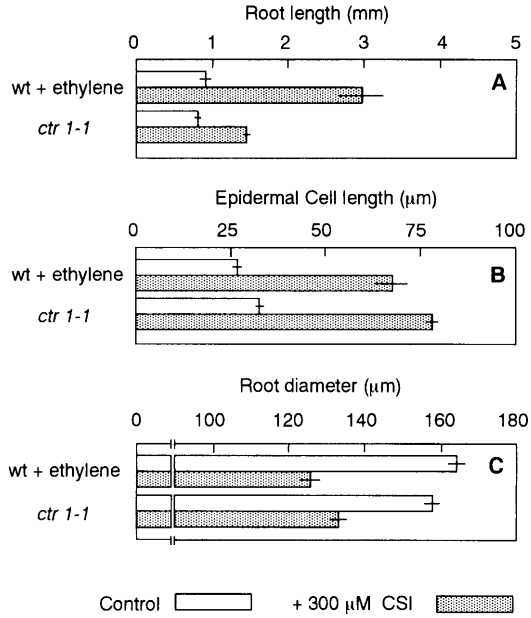

Fig. 10 Effects of CSI on the length of roots (A), the length of epidermal cells (B) and the root diameter (C) in Col wild-type grown under ethylene and in the constitutive triple response mutant *ctr1-1*. *Arabidopsis* seedlings were incubated in the buffer (pH 6.6) with or without 300 μM CSI in the light for 3 d. Col wild-type seedlings were grown under 100 nl liter^{-1} ethylene. Other explanation as in Fig. 2.

extent. This result clearly differentiates the mode of action of CSI from that of AVG and suggests that CSI may inhibit ethylene signaling.

Effect of CSI on ctr1-1 mutant—The above idea on CSI action prompted us to investigate the effect of CSI on the constitutive triple response mutant *ctr1-1* (Keiber et al. 1993). The mutant seedlings were grown with or without CSI for 3 d. As shown in Fig. 10, CSI stimulated root elongation and epidermal cell elongation, and inhibited radial expansion of roots. The effects of CSI on *ctr1-1* mutant were compared with those on Col wild-type grown in the presence of 100 μl liter^{-1} ethylene (Fig. 10). Although CSI-induced stimulation of growth was smaller in *ctr1-1* mutant than that in the wild-type grown under ethylene, the effects of CSI on cell elongation and root diameter were similar. This result gives us an idea that CSI may act downstream of CTR1 in the ethylene signal transduction pathway.

Discussion

Application of CSI to wild-type *Arabidopsis* seedlings increased the rate of root growth up to 3.7-fold in Col and to 8.5-fold in Ler. Such great effects of saponins on the

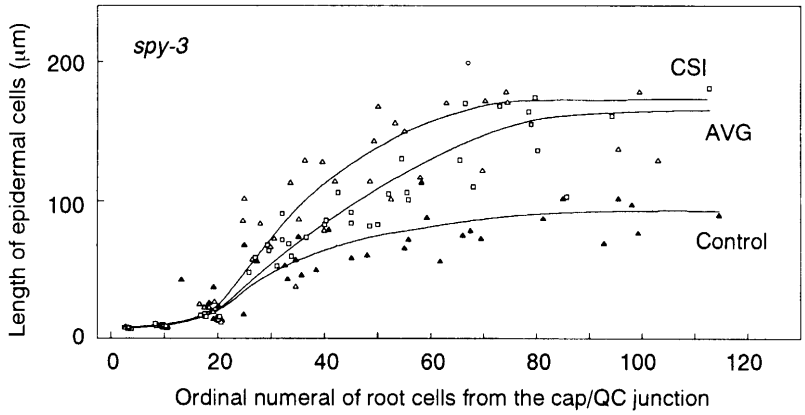

Fig. 9 CSI and AVG increase cell length but not cell number in the epidermis of the mutant *spy-3* roots. *Arabidopsis* seedlings were incubated in the buffer (pH 6.6) without additives (control, ▲) or with 300 μM CSI (△) and 3 μM AVG (□) in the light for 7 d. Data were obtained from 8 seedlings.

Table 2 Effects of CSI and AVG on ethylene production in *eto1-1* seedlings

	Ethylene accumulation per 100 seedlings per d (nl)	Ethylene accumulation per mg fresh weight (nl mg^{-1})
eto 1-1	5.39±0.27	0.254±0.011
eto 1-1+CSI	5.36±0.64	0.288±0.009
eto 1-1+AVG	1.94±0.29	0.052±0.005

Hundred *eto1-1* seedlings were placed in a 20-ml conical flask and incubated in the buffer (pH 6.6) without additives (control) or with 300 μM CSI and 3 μM AVG in the light for 7 d. Ethylene accumulation was measured every 24 h from day 3 to 7 after the start of incubation and on day 7 fresh weight of seedlings was determined. Effects of CSI and AVG were expressed in two ways, the average of ethylene production rate per day and the ethylene evolution per mg fresh weight at day 7.

growth of plants have never been reported. *Arabidopsis* roots are the organ, most sensitive to CSI among we have tested. The stimulatory effect of CSI on the growth of roots can be separated into two components, i.e. stimulation of cell elongation and increase in cell number (Table 1). With regards to cell elongation CSI and AVG showed the similar stimulatory effect in all *Arabidopsis* roots shown in Table 1 except the ethylene-insensitive mutant *ein2-1*. Instead of the similarity of the two compounds in regulating cell elongation, CSI did not inhibit ethylene production (Table 2). These results are consistent with the previous idea that CSI may inhibit ethylene signaling (Tsurumi and Ishizawa 1997). The idea was further supported by the facts that the effects of CSI were not detected in the ethylene-insensitive mutant *ein2-1* (Table 1 and Fig. 6) and that CSI stimulated the growth of roots in *ctr1-1*, the constitutive triple response mutant for ethylene (Fig. 10). Ethylene signaling in *Arabidopsis* has been extensively studied and the Raf kinase homolog CTR1 is predicted to negatively regulate the ethylene response pathway through a MAP kinase cascade (McGrath and Ecker 1998). The reasonable expectation is that CSI inhibits ethylene signaling downstream of CTR1. On the other hand, the action of CSI to increase cell number can not be explained by this idea because of the inability of AVG to increase cell number in wild-types roots.

The cell number of an epidermal cell file in *gai* roots was less than that of Ler wild-type (Table 1), suggesting that endogenous GA is involved in root cell division. The stimulatory effects of GA on cell division in roots have been reported in tomato and other plants (Barlow et al. 1991). The effect of CSI on cell division was not detected in the mutant *gai*, suggesting that GA may be involved in the CSI action to increase cell number. The effect of CSI on cell number was not detected in *spy-3* as well. Jacobsen and Olszewski (1993) proposed that *spy* mutations increase the basal level of GA signal transduction. These results suggest that the normal level of GA signaling, as expressed in wild-type plants, may be required for the CSI action. If the action of CSI in stimulating cell division was independent of its action on ethylene signaling, CSI is expected to increase cell number in *ein2-1* roots. However, the stimulatory effect of CSI on cell number was not found in *ein2-1* roots (Table 1, Fig. 6), suggesting that both ethylene signaling and the normal level of GA signaling might be required for the CSI action in stimulating cell division.

Ethylene has been shown to inhibit cell division in pea and maize roots (Apelbaum and Burg 1972, Barlow 1976). However, Barlow (1976) suggested that ethylene is not a natural regulator of either DNA synthesis or cell division in the root apex because of the requirement of a high concentration of ethylene, i.e. 100 nl $liter^{-1}$, to cause a slight increase in the duration of the mitotic cycle. This notion is consistent with our results that treatment with AVG did not increase cell number in wild-type roots but increased slightly in the ethylene-overproduction mutant *eto1-1* (Table 1).

Both CSI and AVG increased cell length and inhibited radial expansion of roots (Table 1). The reduction in root diameter is due to the reduction in cell diameter (data not shown). Since these effects on cell shape are very similar to that of GA on hypocotyl cells, CSI and AVG are likely to change the polarity of cell growth. Both the CSI- and AVG-induced changes in cell shape were observed in the GA-insensitive mutant *gai* (Fig. 8), and CSI changed the cell shape in *ctr1-1* roots as well (Fig. 10). These results clearly indicated that ethylene signaling controls the direction of cell growth. The epidermal cell length of *ein2-1* roots is longer than that of wild-type control (Fig. 6) and similar to that in AVG-treated wild-type roots (Fig. 4), indicating that endogenous ethylene regulates cell elongation. These results are not always inconsistent with the role of endogenous GA in regulating cell elongation, which has been shown in lettuce and pea (Tanimoto 1987, 1988), tomato (Barlow et al. 1991) and maize (Baluska et al. 1993). Since the requirement of GA in roots has been proposed to be very low (Tanimoto 1987, 1988, Barlow et al. 1991) and the mutant *gai* is not completely insensitive to

GA (Koornneef et al. 1985), the low level of GA signaling activity present in *gai* may be enough for cell elongation in roots.

Although the physiological importance of CSI is not yet established, the stimulatory effects of CSI on cell division and cell elongation in *Arabidopsis* roots may suggest some of its possible role in leguminous plants. More detailed works are required to prove the physiological roles of CSI in plants and to clarify the mechanism of CSI action in stimulating root growth.

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