# Phototropism in Hypocotyls of Radish[1]

## II. ROLE OF cis- AND trans-RAPHANUSANINS, AND RAPHANUSAMIDE IN PHOTOTROPISM OF RADISH HYPOCOTYLS

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## ABSTRACT

When etiolated radish (*Raphanus sativus* var. *hortensis* f. *gigantissimus* Makino) hypocotyls were subjected to a continuous unilateral illumination with white-fluorescent light (0.1 watt per square meter), the growth rate at the lighted side was strongly inhibited for the first 2 hours, while that at the shaded side showed no change. After 2.5 hours growth on the lighted side recovered gradually, while that on the shaded side was slightly inhibited. The neutral growth inhibitors, *cis*- and *trans*-raphanusanins and raphanusamide, were determined in the lighted and shaded sides from 1 hour before until 2 hours after the start of unilateral illumination. In the lighted side, *cis*- and *trans*-raphanusanins increased by 0.5 hour after the start of illumination, reached 3 to 3.5-fold greater concentrations than in the shaded side after 1 hour, and then decreased gradually. Raphanusamide increased in the lighted side to a 3-fold greater concentration than that in the shaded one 2 hours after the start of the illumination. Unilateral applications of *cis*- and *trans*-raphanusanins and raphanusamide suppressed the growth of the hypocotyl on the applied side more than that on the opposite one, causing the hypocotyls to bend towards the site of application. The data suggest that phototropic curvature in radish is caused by the light-induced synthesis of growth-inhibiting *cis*- and *trans*-raphanusanins, and raphanusamide at the site of illumination.

In the preceding paper (6), we reported that three growth inhibitors were isolated from the fraction showing higher inhibiting activity for radish hypocotyl growth in the lighted sides of phototropically responding radish hypocotyls than in the shaded ones and identified as *cis*- and *trans*-raphanusanins and 6-methoxy-2,3,4,5-tetrahydro-1,3-oxazepin-2-one (raphanusamide) by spectrometric analyses.

In this paper, the role of *cis*- and *trans*-raphanusanins and raphanusamide in the phototropism of radish hypocotyls is investigated. Changes in the amounts at the lighted and shaded sides of radish hypocotyls during phototropic response are analyzed and the bending response to the unilaterally applied substances is followed.

## MATERIALS AND METHODS

**Plant Materials.** Sakurajima radish seeds (*Raphanus sativus* var. *hortensis* f. *gigantissimus* Makino) were germinated in ver-

miculite moistened with water in large trays ($37 \times 60 \times 14$ cm$^3$), in the dark at 25°C. About 3 d later, uniform seedlings were transplanted under dim green light into small trays ($8.5 \times 17.5 \times 3.5$ cm$^3$) containing moist vermiculite, and kept in the dark at 25°C for 1 d.

**Phototropic Experiments.** Etiolated seedlings (hypocotyl length, about 4 cm) were placed at 25 cm distance from white fluorescent tubes (Natural Daylight, Toshiba Corp.) in a nonreflecting black box with 5 layers of black cheese cloth and with a 3-cm wide, horizontal slit through which the seedlings were illuminated unilaterally. The illumination was given over the whole of the seedlings. Incident energy was 0.1 W·m$^{-2}$ (optimum energy for the first-positive phototropic curvature of radish hypocotyls) at plant distance. To measure the growth rates at the lighted and shaded sides of the hypocotyls during phototropic stimulation, ion exchange resin beads, Amberlite CAD, smeared with lanolin, were positioned on the lighted and shaded sides from 0 to 3 cm below the hook of 3 seedlings under dim green light. Photographs were taken at 30 min intervals to determine the growth rates at both sides. Three etiolated seedlings were photographed under dim green light. The distances between the upper and lower beads on both sides were measured using a map-measuring device.

**Determination of cis- and trans-Raphanusanins, and Raphanusamide.** The phototropically stimulated seedlings were harvested every hour. The parts of the hypocotyls almost equal to the distance between the upper and lower beads were excised and bisected into the lighted and shaded sides with a razor under dim green light. The bisected hypocotyls were immediately fixed in 10 ml of cold acetone (0°C) and stored at −20°C until extraction of growth inhibitors. Frozen plant materials were ground with pieces of dry ice in a mortar and extracted with 50 volumes of 70% cold acetone (v/v, 0°C). The extract was filtered through Toyo No. 1 filter paper and the residue rinsed with 30 volumes of 70% cold acetone (0°C). The filtrate was evaporated *in vacuo* at 35°C. After evaporation, ¹/₁₀ volume of K-phosphate (pH 7.8, 1 M) was added to the aqueous residue, and the solution was partitioned three times against an equal volume of ethyl acetate. The neutral fraction was dried over anhydrous Na$_2$SO$_4$ and then evaporated to dryness *in vacuo* at 35°C. The crude material was chromatographed on a Sep-Pak C$_{18}$ cartridge column (Waters) with a water-methanol solvent system by increasing the methanol concentration in a series of 10% steps (10 ml/step). The *cis*- and *trans*-raphanusanins were eluted with 20 to 30% methanol and raphanusamide 20 to 40%; then the 20 to 40% methanol eluate was evaporated to dryness *in vacuo* at 35°C. Next, this was chromatographed on a Sep-Pak silica gel cartridge column (Waters) with an ethyl acetate-*n*-hexane solvent system by increasing the ethyl acetate in a series of 10% steps (10 ml/

step). The cis- and trans-raphanusanins and raphanusamide were eluted with 20 to 30% ethyl acetate, then the 20 to 30% ethyl acetate eluate was evaporated to dryness in vacuo at 35°C. This was finally separated by HPLC ($\mu$Bondapak $C_{18}$, water:methanol, 6:1, v/v, 2 ml·$min^{-1}$, 271 nm detector [$\lambda_{max}$ of cis- and trans-raphanusanins and raphanusamide, 271 nm]). Retention times of cis- and trans-raphanusanins and raphanusamide were 19.41, 13.87, and 6.83 min, respectively (retention time of solvent peak, 1.85 min). After the areas of the peaks were determined, the amounts of endogenous cis- and trans-raphanusanins and raphanusamide were calculated from standard curves. Known amounts of genuine cis- and trans-raphanusanins and raphanusamide were added to the other half of each sample during extraction in order to determine losses during the purification procedures. Overall recovery of them added to the samples was between 75 and 85%. All data have been corrected for losses.

**Unilateral Applications of cis- and trans-Raphanusanins and Raphanusamide.** Attempts to induce curvature by unilateral applications of cis- and trans-raphanusanins and raphanusamide were done using uniform 4-d-old etiolated radish seedlings. Ten, 1, 0.1, or 0 $\mu$g of genuine cis- and trans-raphanusanins and raphanusamide in 2 mg lanolin was unilaterally applied along the length from 0.3 to 2.7 cm below the hook of 3 seedlings. Simultaneously, ion exchange resin beads were positioned on the smeared and opposite sides from 0 to 3 cm below the hook. Seedlings with or without growth inhibitors were incubated in the dark at 25°C for 5 h. The bending responses were photographed at 1 h intervals and the distance between the upper and lower beads on the applied and opposite sides was measured using a map-measuring device.

All experiments were repeated at least twice.

## RESULTS

**Changes in the Growth Rates of the Lighted and Shaded Sides during Unilateral Illumination.** The growth rates at the lighted and shaded sides of radish hypocotyls in relation to a continuous unilateral illumination were measured. As shown in Figure 1, the growth of control seedlings in darkness proceeded undisturbed. Upon phototropic stimulation, the most noticeable change was a marked inhibition of the growth on the lighted side of the hypocotyls. Especially from 0.5 to 2.0 h after the start of the illumination, this growth was completely suppressed. The maximal curvature of hypocotyls was observed during a 2 to 2.5 h period, the amount of curvature being about 90° at the light intensity used. The growth rate at the shaded side showed little change 0 to 2 h after the start of unilateral illumination, but was slightly inhibited after 2.5 h unilateral illumination. The recovery of the growth rate on the lighted side after 3 h unilateral illumination might result from gravistimulation. The growth inhibition on the shaded side after 2.5 h unilateral illumination may result from interference of photostimulation by gravistimulation and autotropic straightening.

**Lateral Distribution of cis- and trans-Raphanusanins, and Raphanusamide during Phototropic Response.** The lateral distribution of endogenous cis- and trans-raphanusanins, and raphanusamide was determined in etiolated hypocotyls and the lighted and shaded halves of hypocotyls from 1 h before unilateral illumination until 2 h after the start of illumination when the interference of gravistimulation did not yet appear. Three separate experiments were carried out. In the halves at the illuminated side, an apparent difference between cis- and trans-raphanusanins, and raphanusamide was observed during phototropic response (Fig. 2). The amounts of cis- and trans-raphanusanins increased 0.5 h after the start of unilateral illumination, reached a maximum 1 h after the start and then decreased gradually. The maximum amounts of cis and trans-raphanusanins in the lighted halves were 3 to 3.5-fold greater than those in the shaded one.
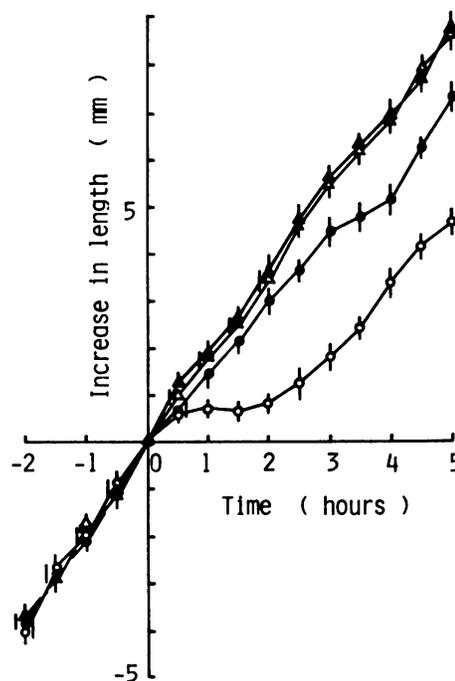


FIG. 1. Growth rates at the lighted and shaded sides of radish hypocotyls before and after phototropic stimulation with a continuous white light. O, Lighted side; ●, shaded side; △, ▲, right or left side of etiolated hypocotyls. Average values of 3 seedlings. Bars represent ± SE.

In the case of raphanusamide, the amount in the lighted side started to increase 1 h after the start of the unilateral illumination, and became 3 times that in the shaded one 2 h after the start. The amounts of cis- and trans-raphanusanins and raphanusamide in the etiolated halves decreased gradually or maintained the initial level during incubation, while those in the shaded side showed a small increase. The small increase in the shaded side may play a role in the slight growth inhibition of that side after 2 h. Invariably, the illuminated halves contained considerably more cis- and trans-raphanusanins and raphanusamide than the shaded ones during the normal phototropic response.

**Bending Responses to Unilaterally Applied cis- and trans-Raphanusanins and Raphanusamide.** It was determined whether unilaterally applied cis- and trans-raphanusanins and raphanusamide could induce bending toward the site of application (Fig. 3 and Table I). The growth rate of the side treated with plain lanolin was the same as that of the opposite side, the lanolin treatment itself being ineffective. Inhibition of growth also occurred at the opposite side with amounts of 1 $\mu$g and more of cis- and trans-raphanusanins and raphanusamide per seedling, after 2 to 3 h incubation. This may be caused by diffusion of the penetrated cis- and trans-raphanusanins and raphanusamide from the treated side to the opposite one. Significant differences in growth rates between treated and opposite sides were revealed 2 to 3, 3 to 4, and 5 h after application of 10, 1, and 0.1 $\mu$g inhibitor per seedling, respectively.

The curvature of the hypocotyls toward the site of inhibitor application 2 h after the start of treatment is shown in Table I. A gravitropic effect was not yet apparent in the experiments. Bending because of local growth inhibition always occurred, the two isomers, cis- and trans-raphanusanins being much more effective than raphanusamide.

## DISCUSSION

A unilateral illumination suppressed the growth rate at the lighted side of radish hypocotyls, whereas it hardly affected the
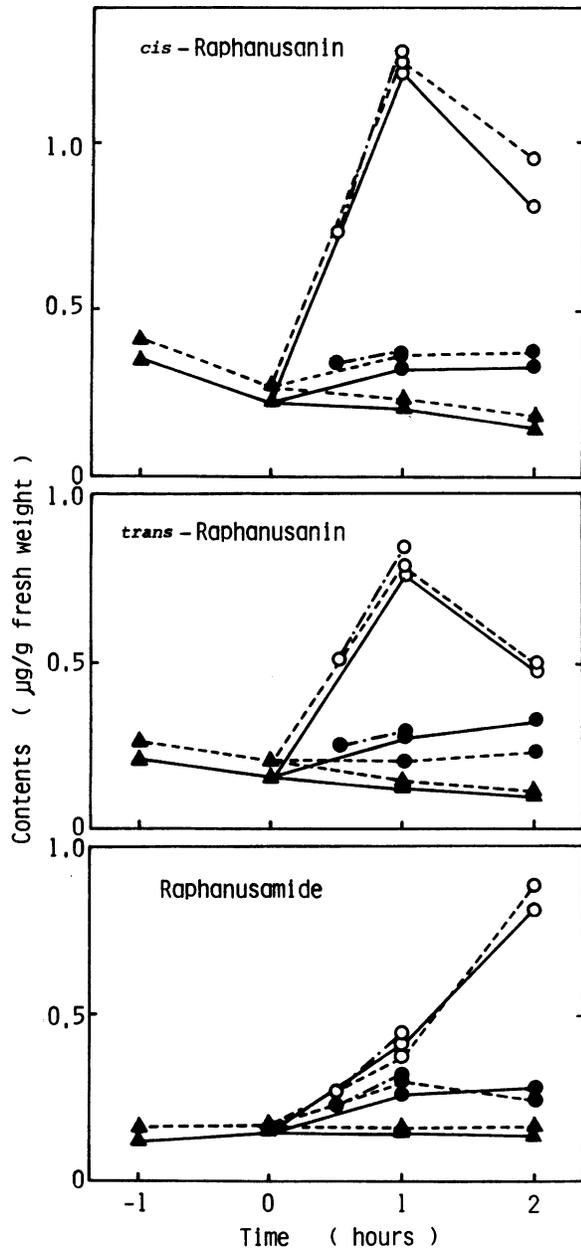
FIG. 2. Lateral distribution of *cis* and *trans*-raphanusanins and ra-phanusamide in etiolated radish hypocotyls and the lighted and shaded halves of radish hypocotyls from 1 h before until 2 h after the start of unilateral illumination. O, Lighted halves; ●, shaded halves; ▲, halves of etiolated hypocotyls. The different lines indicate independent experiments.
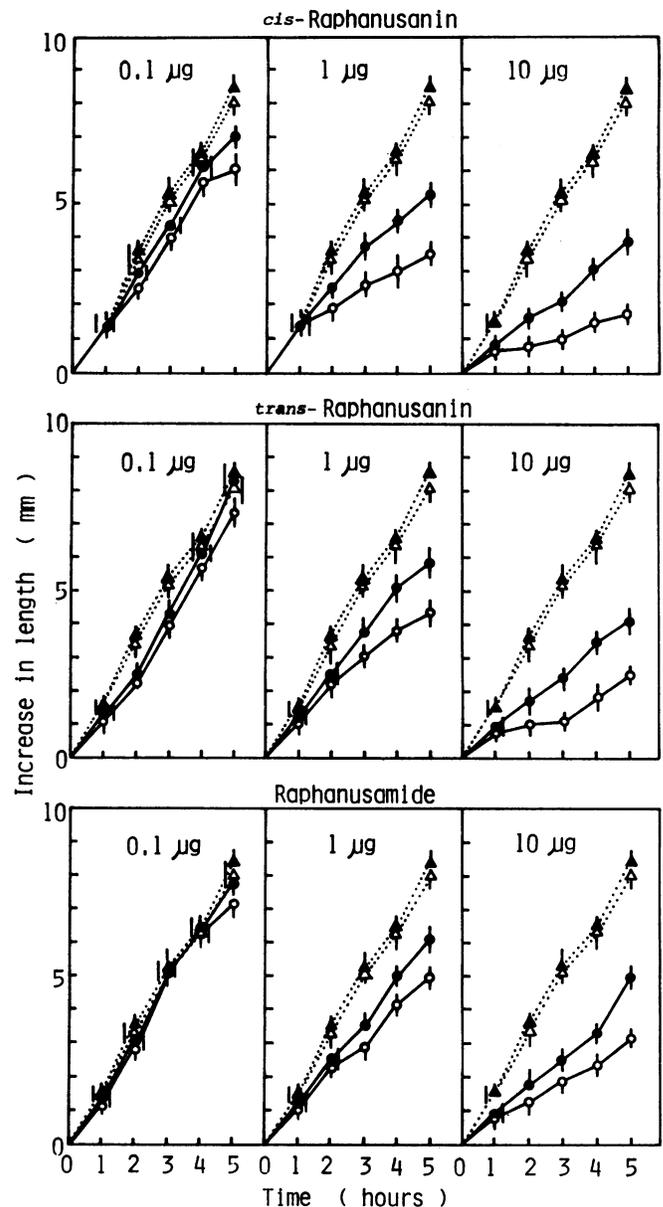


FIG. 3. Effects of *cis*- and *trans*-raphanusanins and raphanusamide applied to one side of etiolated radish hypocotyls on the growth rates of the applied and opposite sides. O, Side applied with inhibitor; ●, opposite side to one applied with inhibitor; △, side applied with plain lanolin; ▲, opposite side to one applied with plain lanolin. Average values of 3 seedlings. Bars represent ± SE.

Table I. *Degree of Bending in Continuous Darkness of Etiolated Radish Hypocotyls, 2 Hours after Unilateral Application of 0.1, 1, or 10 µg Inhibitor per Seedling*

Average data from experiments with 3 seedlings per sample.

| Inhibitor | 0.1 | 1.0 | 10 µg |
|---|---|---|---|
| *cis*-Raphanusanin | 3.3 ± 2.0 | 12.5 ± 5.1 | 33.1 ± 5.5 |
| *trans*-Raphanusanin | 2.5 ± 1.6 | 10.3 ± 4.3 | 29.3 ± 6.3 |
| Raphanusamide | 1.0 ± 0.5 | 2.1 ± 1.0 | 5.2 ± 2.1 |

growth rate at the shaded side, until a subsequent autotropic straightening and gravitropic reaction appeared (Fig. 1). That the cessation of growth at the lighted side is a major factor causing seedlings to bend towards the light source has also been reported for other dicotyledonous plant species (5, 7). Moreover, in phototropically responding, green sunflower seedlings the absence of a lateral gradient in extractable or diffusable indole-3-acetic acid was established (2, 4). Therefore, it should be proposed that in accordance with the Blaauw theory (1, 3), a light-promoted, growth-inhibiting factor is involved in the control of phototropic response. Franssen and Bruinsma (4) demonstrated that the neutral growth inhibitor, xanthoxin, plays a role in the phototropic response of sunflower seedlings. However, the endogenous

xanthoxin level was less than the total amounts of neutral growth inhibitors which Thompson and Bruinsma (8) previously determined in sunflower hypocotyls using the cress bioassay. Moreover, exogenous xanthoxin, applied unilaterally to etiolated seedlings, failed to cause bending towards the site of application (4),

probably because of its lability. Therefore, other growth inhibitor(s) may also play a role as regulator in phototrophic responses.

In Sakurajima radish seedlings, we (6) recently isolated three neutral growth inhibitors from the fraction which showed higher inhibiting activity in the lighted sides of phototropically responding radish hypocotyls than in the shaded ones by radish hypocotyl bioassay, and identified as cis- and trans-raphanusanins and 6-methoxy-2,3,4,5-tetrahydro-1,3-oxazepin-2-one (raphanusamide), respectively. These quantitative changes in the lighted and shaded sides during phototropic response, were determined by physicochemical assay. The endogenous contents of cis- and trans-raphanusanins and raphanusamide, presented in Figure 2, showed good correlative changes to the phototropic reaction. Particularly cis- and trans-raphanusanins accumulated at the irradiated side during the normal phototropic response. These distinct differences between the amounts of growth inhibitors in the lighted and the shaded sides may well be caused by the enhanced biosynthesis upon unilateral illumination, rather than by lateral transport from the shaded side to the lighted one. Their total amounts in the lighted and shaded halves greatly outmeasured those of the dark controls which only gradually decreased (Fig. 2). Furthermore, when cis- and trans-raphanusanins, and raphanusamide were applied exogenously to one side of hypocotyls, they could induce the hypocotyls to bend toward the treated side. This clearly demonstrates that a lateral gradient of endogenous cis- and trans-raphanusanins and raphanusamide, as induced by phototropic stimulation, will cause curvature toward the light source. From these results, it is suggested that cis- and trans-raphanusanins and raphanusamide increase at the lighted side upon unilateral illumination, strongly suppress hypocotyl growth at that side, and thus cause the differential growth that induces the radish seedlings to bend phototropically.

In further studies, for the purpose of investigating the involvement of the three neutral growth inhibitors in phototropism of radish hypocotyls, influences of light quality and energy on the production, and distribution of the inhibitors have to be studied. Furthermore, whether these inhibitors play a role in the phototropic response of other plant species should also be investigated.

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