PERGAMON

Phytochemistry 54 (2000) 275–279

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Phototropic stimulation induces the conversion of glucosinolate to phototropism-regulating substances of radish hypocotyls

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Received 10 September 1999; received in revised form 17 February 2000

Abstract

The distribution of natural growth inhibitors, the raphanusanins (isomers of 3-(methylthio)methylene-2-pyrrolidinethione) and their precursors (4-methylthio-3-butenyl glucosinolate (MTBG) and 4-methylthio-3-butenyl isothiocyanate (MTBI)), between illuminated and shaded halves of radish hypocotyls during phototropic curvature was analyzed using a physicochemical assay. Phototropic stimulation rapidly decreased MTBG content, and abruptly increased contents of MTBI and raphanusanins in the illuminated halves of radish hypocotyls within 30 min after the onset of unilateral illumination. Content in the shaded halves was similar to that in dark controls. When MTBG, MTBI, and raphanusanins at endogenous levels were applied unilaterally to etiolated hypocotyls, MTBI and raphanusanins caused hypocotyls to bend but MTBG showed no activity. Blue illumination promoted myrosinase (thioglucosidase) activity, which releases MTBI from MTBG, in hypocotyls after 10 min, although enzyme activity in dark controls did not change. These results suggest that phototropic stimulation promotes myrosinase activity in the illuminated side of radish hypocotyls, releasing bioactive MTBI from inactive MTBG and simultaneously producing bioactive raphanusanins.

Keywords: *Raphanus sativus*; Cruciferae; Radish hypocotyl; Growth inhibitor; Myrosinase; Glucosinolate; Raphanusanin; 3-(Methylthio) methylene-2-pyrrolidinethione; 4-Methylthio-3-butenyl isothiocyanate; Phototropism

1. Introduction

Evidence that phototropism is not regulated by a lateral auxin gradient, but instead where unilateral illumination induces the unequal distribution of growth inhibitors of auxin action, has been obtained for many plant organs in species commonly used for phototropic studies — oats, maize, radish, sunflower, and pea (Bruinsma et al., 1975; Feyerabend and Weiler, 1988; Hasegawa and Sakoda, 1988; Sakoda et al., 1988; Bruinsma et al., 1989; Bruinsma and Hasegawa, 1989; Hasegawa et al., 1989; Bruinsma and Hasegawa, 1990; Togo and Hasegawa, 1991; Hasegawa and Yamada, 1992; Yokotani-Tomita et al., 1999).

In radish (*Raphanus sativus*) hypocotyls, the distribution of endogenous auxin (indole-3-acetic acid, IAA) in illuminated and shaded sides of phototropically stimulated hypocotyls was measured using a physicochemical assay. The IAA was evenly distributed over illuminated and shaded sides in either the first or the second phototropic curvature, and no net exchange of IAA between peripheral and central cell layers was observed in these curvatures (Sakoda and Hasegawa, 1989). As candidates for growth inhibitors involved in phototropism of radish hypocotyls, raphanusanins were isolated and identified from light-grown radish seedlings (Hasegawa et al., 1986). Raphanusanins accumulated at the illuminated side of hypocotyls

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0031-9422/00/$ - see front matter
PII: S0031-9422(00)00080-7

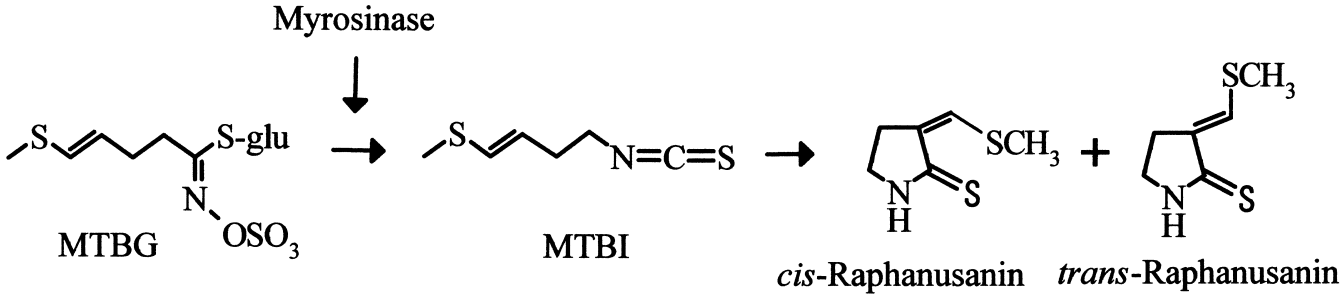

Fig. 1. MTBG conversion to MTBI and natural raphanusanins.

stimulated phototropically and inhibited growth of the illuminated side (Noguchi et al., 1986; Noguchi and Hasegawa, 1987; Hasegawa et al., 1987). Lateral application of these substances caused differential growth, resulting in bending toward the applied side (Noguchi et al., 1986). Raphanusanins were first proposed as isomers of 3-methoxy-4-methylthio-2-piperidinethione (Hasegawa et al., 1986), but were revised as (3*R*∗,6*R*∗)- and (3*R*∗,6*S*∗)-3-[methoxy(methylthio)-methyl]-2-pyrrolidinethione based on X-ray analysis (Harada et al., 1991). Attachment of the methoxy group at the methylene functionality of 2-pyrrolidinethione resulted during methanol extraction (Kosemura et al. 1993). Natural raphanusanins were determined to be isomers of 3-(methylthio) methylene-2-pyrrolidinethione (Fig. 1) (Sakoda et al., 1990; Kosemura et al., 1993). If phototropism of radish hypocotyls is regulated by natural growth inhibiting raphanusanins, previous experiments with substituted raphanusanins must be reexamined. Kosemura et al. (1993) suggested that 4-methylthio-3-butenylisothiocyanate (MTBI), the precursor of raphanusanins, is released from 4-methylthio-3-butenyl glucosinolate (MTBG) via myrosinase (thioglucosidase) action, so some MTBI is spontaneously converted to raphanusanins (Fig. 1).

We studied the lateral distribution of MTBG, MTBI, and raphanusanins during phototropism induced by unilateral blue light on radish hypocotyls and changes in myrosinase activity, which releases MTBI from MTBG.

2. Results and discussion

The phototropic curvature of etiolated radish hypocotyls was measured under continuous, unilateral blue illumination (Fig. 2). Hypocotyls began to bend toward the light source between 30 and 60 min after the onset of phototropic stimulation, with curvature peaking 90 min after onset, then decreasing.

The lateral distribution of raphanusanins and precursors MTBG and MTBI during phototropism of radish hypocotyls was studied using a physicochemical assay (Fig. 3). The MTBG content in illuminated halves of hypocotyls decreased abruptly, reaching a minimum 60 min after the start of phototropic stimulation while the phototropic curvature was still developing (Fig. 2), then gradually recovered; MTBG in

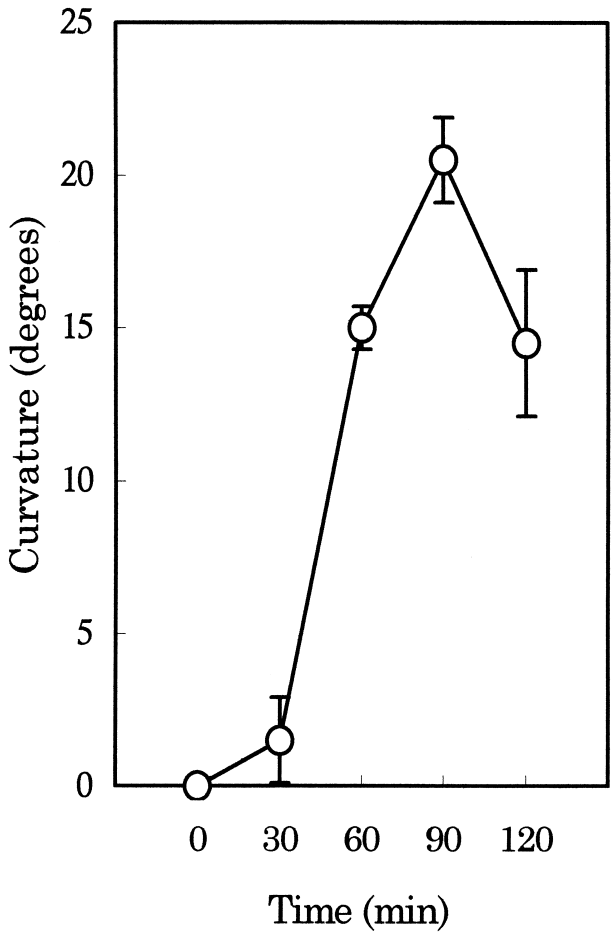

Fig. 2. Time course of phototropic response of etiolated radish hypocotyls to continuous, unilateral blue illumination. Values are means of 10 seedlings ± SE.

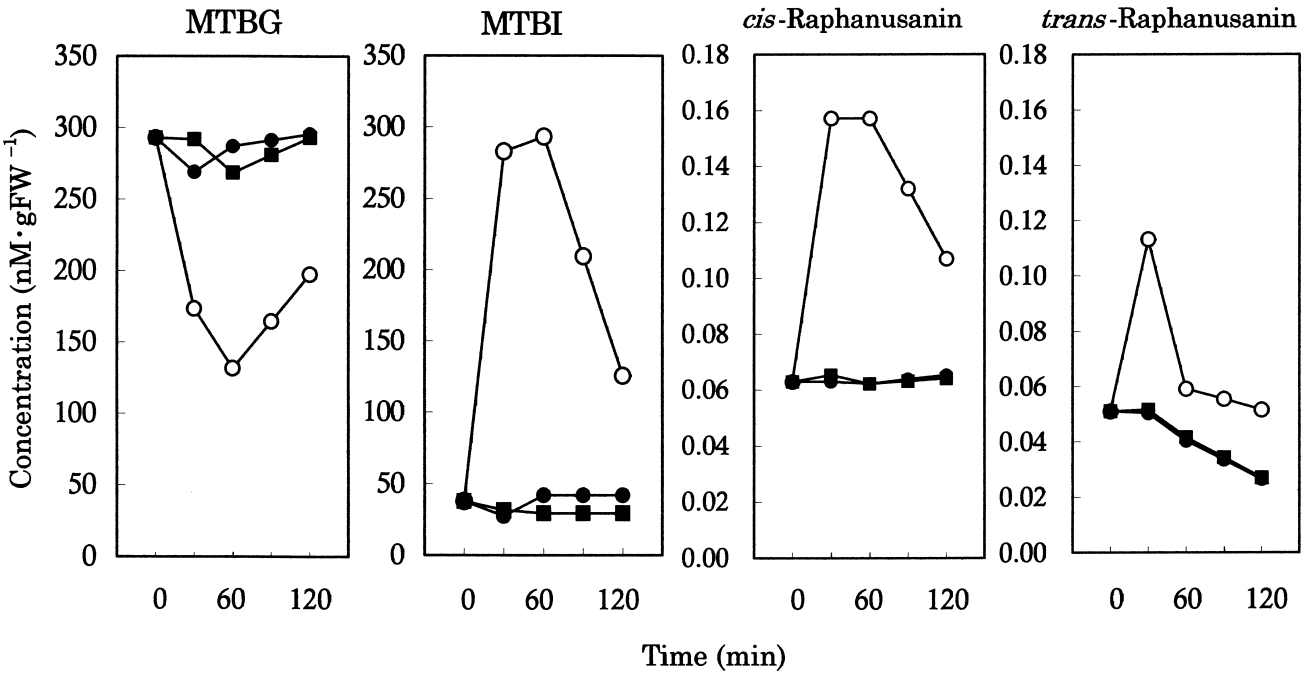

Fig. 3. Lateral distribution of MTBG, MTBI, and *cis*- and *trans*-raphanusanins in etiolated radish hypocotyls up to 2 h after onset of unilateral illumination. ○, illuminated halves; ■, shaded halves; ●, etiolated hypocotyls. This experiment was repeated three times with similar results.

both shaded halves and dark controls maintained initial levels. In response to decreasing MTBG, MTBI rapidly increased in illuminated halves, peaking after 60 min, then decreased. However, MTBI in shaded halves and dark controls did not change. The amount of *cis*-raphanusanin in illuminated halves markedly increased at 30 min, then gradually decreased after 60 min; a similar profile was observed with *trans*-raphanusanin. The amounts of *cis*- and *trans*-raphanusanins in shaded halves and dark controls remained essentially unchanged. The levels of raphanusanins in illuminated halves of hypocotyls at 30 min was about one-thousandth of that of MTBI — comparable to the ratio (about 0.1%) of the in vitro conversion of MTBI to raphanusanins in H_2O at 25°C for 30 min, suggesting that a very small percent of MTBI is converted to raphanusanins on the illuminated side over a 30 min period.

We tested whether the unilateral application on etiolated hypocotyls of exogenous raphanusanins and their precursors at endogenous levels resulted in their unequal distribution, thus causing hypocotyls to bend toward the site of application. MTBI clearly induced hypocotyls to bend, raphanusanins being less active than MTBI (Table 1). MTBG was inactive. These results indicate that phototropic stimulation induces conversion of inactive MTBG to active MTBI and a very small percent of MTBI is spontaneously converted to active raphanusanins.

We then studied the activity of myrosinase (thioglucosidase), which releases MTBI from MTBG, in radish hypocotyls during blue illumination (Table 2), which markedly promoted myrosinase activity in hypocotyls even 10 min after onset of illumination, peaking after 30 min, then decreasing. The enzyme activity in dark controls remained unchanged.

Table 1
Degree of bending of etiolated radish hypocotyls in continuous darkness, 30 and 60 min after unilateral application of MTBG, MTBI, and *cis*- and *trans*-raphanusanins at endogenous levels. Endogenous amounts of MTBG, MTBI, and *cis*- and *trans*-raphanusanins in etiolated hypocotyl halves were 1.5 μg, 0.1 μg, 0.2 ng, and 0.16 ng. Values are means of seven seedlings ± SE. This experiment was repeated three times with similar results

Compound	Dosage	30 min	60 min
MTBG	0.0 μg	0.0 ± 0.1	0.0 ± 0.1
	0.5	0.0 ± 0.0	0.0 ± 0.0
	1.0	0.1 ± 0.1	−0.1 ± 0.1
	10.0	0.0 ± 0.1	0.1 ± 0.1
MTBI	0.1 μg	2.4 ± 0.1	4.2 ± 0.2
	0.5	3.8 ± 0.2	6.2 ± 0.1
	1.0	6.2 ± 0.1	10.9 ± 0.3
	10.0	7.1 ± 0.1	12.5 ± 0.4
cis-Raphanusanin	0.1 ng	0.4 ± 0.1	1.0 ± 0.1
	0.2	0.9 ± 0.1	1.4 ± 0.1
	0.5	1.3 ± 0.2	2.0 ± 0.1
	1.0	2.0 ± 0.2	2.4 ± 0.1
trans-Raphanusanin	0.1 ng	0.8 ± 0.1	1.3 ± 0.1
	0.2	1.2 ± 0.1	1.7 ± 0.2
	0.5	2.0 ± 0.1	2.8 ± 0.1
	1.0	3.0 ± 0.2	4.8 ± 0.2

Unilateral illumination thus induces the conversion of inactive MTBG to active MTBI by enhancing myrosinase activity, simultaneously producing physiologically active raphanusanins. This, in turn, suppresses the growth of the illuminated side of radish hypocotyls, causing a phototropic response.

Sakoda et al. (1991) reported that raphanusanins are widespread in the genus *Raphanus*, but not detectable in other plant species examined — including the *Brassica* genus belonging to the same Cruciferae family. The mechanism we propose thus may be restricted to *Raphanus*. Other growth inhibitors — candidates involved in phototropism — include benzoxazolinone analogues for maize coleoptiles and 8-epixanthatin for sunflower hypocotyls (Hasegawa et al., 1992; Yokotani-Tomita et al., 1999). We therefore concluded that different growth inhibitors are involved in plant response to phototropic stimulation.

Further work is in progress to determine whether phototropic stimulation induces myrosinase gene expression or myrosinase activation.

3. Experimental

3.1. Phototropic experiment

Four-day-old, etiolated radish (*Raphanus sativus* var. *hortensis* f. *gigantissimus* Makino) seedlings (hypocotyl: *ca* 4 cm long) were unilaterally illuminated with blue light (half band width: 43 nm; λ_{max}: 448 nm) for 120 min at 25°C. Incident energy was 0.46 μmol/m^2/s at the plant level. Photographs were taken at 30 min intervals to determine the curvature of hypocotyls under a photomorphogenetically inactive intensity (0.03 μmol/m^2/s) of dim green light. Experiments were repeated three times.

3.2. Determination of MTBG, MTBI, and raphanusanin levels

After onset of phototropic stimulation, 25 phototropically stimulated hypocotyls were harvested at 0, 30, 60, 90, and 120 min. Hypocotyl sections from 0 to 3 cm below the hook of seedlings were excised and bisected into illuminated and shaded halves with a razor under dim green light. Bisected hypocotyls were immediately frozen in liquid N_2 and stored at −40°C until use. Frozen materials were homogenized in 50 vol. of ethyl acetate using a homogenizer, and the resulting suspension was centrifuged at 3000 rpm to separate the ethyl acetate layer from residue. Resulting ethyl acetate solubles were dried over anhydrous Na_2SO_4 and evaporated to dryness in vacuo at 35°C. The material was taken up in 10 ml of 10% ethyl acetate in *n*-hexane, then applied to a silica Sep-pak cartridge column (Waters) eluted first with 10 ml of 10% ethyl acetate in *n*-hexane; the resulting MTBI fraction was evaporated to dryness in vacuo at 35°C. The Sep-pak column was eluted with 10 ml of 50% ethyl acetate in *n*-hexane and the raphanusanin fraction evaporated to dryness in vacuo at 35°C. The pellet following centrifugation was extracted with cold H_2O saturated with phenol and centrifuged at 3000 rpm; the resulting aqueous layer was partitioned with $CHCl_3$ and centrifuged at 3000 rpm. The upper layer (MTBG fraction) was evaporated to dryness in vacuo at 35°C. The MTBI fraction was subjected to HPLC (TSK gel Silica-60, Tosoh, Japan, eluted with 5% ethyl acetate in *n*-hexane at 1.2 ml/min with detection at 271 nm); the retention time for MTBI was 6.2 min. The raphanusanin fraction was applied to an HPLC column (TSK gel ODS-80Ts, Tosoh, Japan, eluted with 20% CH_3CN in H_2O at 1.2 ml/min with detection at 325 nm). The retention time of *trans*-raphanusanin was 10.3 and that of *cis*-raphanusanin was 17.7 min. The MTBG fraction was subjected to HPLC (TSK gel ODS-80Ts, Tosoh, Japan, eluted with 15% MeOH in H_2O (0.01% TFA) at 1.0 ml/min with detection at 340 nm); the retention time of MTBG was 19.8 min. MTBG, MTBI, and raphanusanins were calculated from standard curves; overall recovery was about 80% and all data was corrected based on this. Each experiment was repeated three times.

Table 2
Myrosinase activity of radish hypocotyls 0, 10, 30, and 60 min after blue illumination as indicated by production of MTBI from added MTBG. Values are means of three experiments ± SE

Time (min)	Myrosinase activity in hypocotyls (MTBI ng/ gFW)	
	De-etiolated	Etiolated
0	3.75 ± 0.75	3.75 ± 0.75
10	14.70 ± 3.68	3.83 ± 0.45
30	43.50 ± 3.75	4.28 ± 0.83
60	7.20 ± 1.28	3.38 ± 0.60

3.3. Unilateral application of MTBG, MTBI, and raphanusanins

MTBG, MTBI, *cis*- and *trans*-raphanusanins were isolated from fresh radish root based on the procedure of Kosemura et a1. (1993). MTBG, MTBI, *cis*- and *trans*-raphanusanins smeared with 0.5 mg lanolin at endogenous levels were unilaterally applied length wise from 0 to 2 cm below the hook of seven uniform, 4-day-old etiolated radish seedlings. Treated seedlings were incubated in the dark at 25°C. Photographs were taken at 30-min intervals using an infrared camera, with the degree of bending measured using a photo-

graphic enlarger. Experiments were repeated three times.

3.4. Myrosinase assay

Myrosinase activity was measured by the production of MTBI from MTBG. Four-day-old etiolated radish seedlings were exposed to blue illumination (0.46 μmol/m^2/s), and 15 hypocotyl sections from 0 to 3 cm below the hook of seedlings harvested 0, 10, 30, and 60 min after onset of illumination under dim green light and frozen in liquid N_2. Frozen materials were ground with 10 mM imidazole–HCl buffer (6 ml, pH 6.2) in a mortar. The crude extract was centrifuged for 15 min at 10,000 rpm at 4°C and the supernatant ultrafiltered with Mol-cut (Millipore, M_r cut off: 5000) at 4°C. The resulting residue ($> M_r$ 5000, crude enzyme fraction) was resuspended in phosphate buffer (33 mM, 0.5 ml, pH 7.0) and incubated with MTBG (0.28 mg) at 30°C for 10 min. The resulting solution was partitioned with an equal vol. of ethyl acetate twice, and the ethyl acetate layer (MTBI fraction) was dried under N_2. MTBI content was determined as above, and all experiments were repeated three times. Myrosinase activity was measured as the liberation of glucose with a GOD-Perid assay as described by Bones and Slupphaug (1989); results were comparable to those of the MTBI production assay.

Acknowledgements

The authors wish to thank the Ministry of Education, Science and Culture of Japan for financial support.

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