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Raphanusanin-induced genes and the characterization of *RsCSN3*, a raphanusanin-induced gene in etiolated radish hypocotyls

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ABSTRACT

Raphanusanin is a light-induced growth inhibitor involved in inhibition of hypocotyl growth in response to unilateral blue light illumination in radish seedlings. To understand better the role of raphanusanin in growth inhibition, we randomly analyzed raphanusanin-induced genes using a modified DD-RT-PCR (differential display RT-PCR) approach. The differential expression RT-PCR approach resulted in identification of four known candidate genes, of which three encoded functional proteins known to be related to responsiveness to diverse environmental stimuli. One of these genes appeared to be an essential element in the inhibition of hypocotyl growth, and was named *RsCSN3* (a homologue of subunit 3 of the COP9 signalosome). During the growth inhibition that was observed within minutes of irradiation, the expression of the *RsCSN3* gene was increased by phototropic stimulation, as well as by raphanusanin treatment, suggesting that this gene is involved in light-induced growth inhibition. In addition, down-regulation of the *RsCSN3* transcript, that is specifically expressed at 60 min after the onset of stimulation under blue light, green light, and raphanusanin treatment, shows a functional correlation with the phototropic response.

1. Introduction

Raphanusanin (3-methylthio-methylene-2-pyrrolidonethione) (**1**) can be isolated from radish seedlings grown under illumination, and plays a role in the light-induced inhibition of hypocotyl growth (Hasegawa et al., 1982). In radish hypocotyls, the level of raphanusanin **1** is increased in the illuminated areas during both the first and second positive phototropic curvatures (Sakoda and Hasegawa, 1989). When applied unilaterally, it suppresses the growth of the hypocotyl on the treated side more than on the opposite side, causing a differential growth gradient, and causing the hypocotyl to bend towards the side of application (Noguchi et al., 1986). The biosynthetic pathway of raphanusanin **1** is shown in Fig. 1. Phototropic stimulation promotes myrosinase activity on the illuminated side of radish hypocotyls, releasing bio-active 4-methylthio-3-butenyl isothiocyanate (4-MTBI) (**2**) from bio-inactive 4-methylthio-3-butenyl glucosinolate (4-MTBG) (**3**), and simultaneously producing bio-active compound **1** (Hasegawa et al., 2000; Yamada et al., 2003). Sakoda et al. (1991) demonstrated that IAA-mediated transverse microtubule reorientation is significantly inhibited by the simultaneous addition of raphanusanin **1** analogues. Moreover, Nakajima et al. (2001) showed that a raphanusanin 1 inhibited apical dominance in pea seedlings. There are undoubtedly additional ways in which raphanusanin **1** may allow a plant to adapt to prevailing light environments, and these may be discovered through further photophysiological, cellular, biochemical and genetic testing of the effects of raphanusanin **1**. In order to integrate these observations and understand better the role of raphanusanin **1** in growth inhibition, and the correlation between growth inhibition and phototropic curvature, we started an extensive analysis of its physiological roles in differential growth curvature. In the present study, we first analyzed raphanusanin **1**-induced genes by a modified differential display-reverse transcriptase-polymerase chain reaction (DD-RT-PCR) to obtain candidate genes that play a role in growth inhibition; we then analyzed expression of these genes. We also report upon the structural and functional analysis of one of these candidate genes.

2. Results

2.1. Raphanusanin (*1*)-induced dose-dependent curvature of radish hypocotyls

To determine the dose-dependence of raphanusanin (**1**)-induced growth inhibition that results in hypocotyl curvature, 4-d-old etiolated radish hypocotyls were exogenously treated with a

Abbreviations: BL, blue light; GL, green light; RL, red light; 4-MTBI, 4-methylthio-3-butenyl isothiocyanate; 4-MTBG, 4-methylthio-3-butenyl glucosinolate; CSN3, subunit 3 of the cop9 signalosome; IAA, indole-3-acetic acid; RLM-RACE, RNA ligase-mediated rapid amplification of cDNA ends; DD-RT-PCR, differential display-reverse transcriptase-polymerase chain reaction; ORF, open reading frame; EST, expressed sequence tag.

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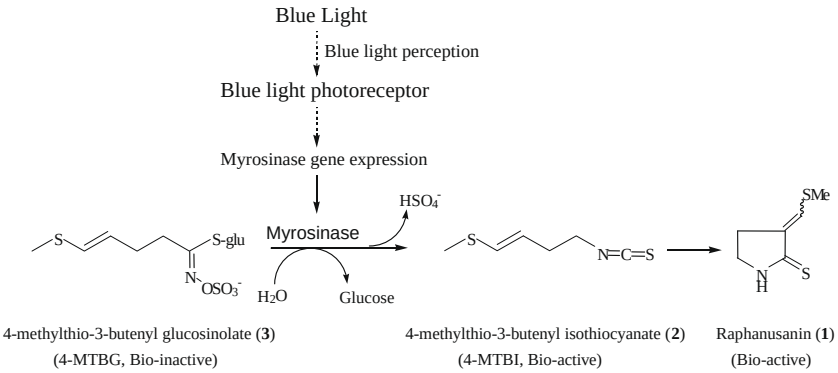

Fig. 1. Formation of raphanusanin (**1**) growth inhibitor by hydrolysis of 4-MTBG (**3**) by myrosinase. Phototropic stimulation promotes myrosinase activity on the illuminated side of radish hypocotyls, releasing bioactive, 4-MTGI (**2**) from inactive compound **3** and simultaneously producing bioactive compound **1** (modified from Yamada et al., 2003).

unilateral application of it at various doses (Fig. 2A and B). The magnitude of curvature is regulated in a dose-dependent manner, and the curvature at 15 ng of exogenous raphanusanin **1** was less than at 50 and 100 ng. Although no significant difference was observed between 50 ng and 100 ng within a time point of 30, 60, and 120 min, 50 ng is likely to be a maximum dosage of induced curvature.

*2.2. Differential expression of genes in raphanusanin (**1**)-treated and control radish seedlings*

To identify genes involved in the raphanusanin (**1**)-mediated photo-inhibition pathway, a modified DD-RT-PCR was performed with RNAs isolated from exogenous by supplied raphanusanin **1**-treated and control seedlings. Two pairs of arbitrary primers were used in this experiment. Typical results showed a differential display of bands in agarose gels, and four differentially expressed bands were recovered from these gels for further sequence analysis (Fig. 2C). According to a homology search for gene function, all four differentially expressed ESTs obtained from this DD-RT-PCR were homologous to genes encoding functional proteins with known functions (Table 1). We named these four EST clones as raiA, raiB, raiC, and raiD (raphanusanin induced clones A–D), respectively. The differential expression of the four ESTs was confirmed by RT-PCR (Fig. 2D). Our data demonstrate that raiA (subunit 3 of the COP9 signalosome, CSN3, *A. thaliana*), raiC (01P13-1, *Brassica rapa*), and raiD (Ethylene responsive binding factor-related, *Brassica oleracea*) were up-regulated and raiB (myrosinase, *Brassica juncea*) was down-regulated during the first 10 min after the onset of treatment. Further analysis of the characteristics and functions of these genes might provide valuable mechanistic information for our understanding of the molecular basis of growth inhibition.

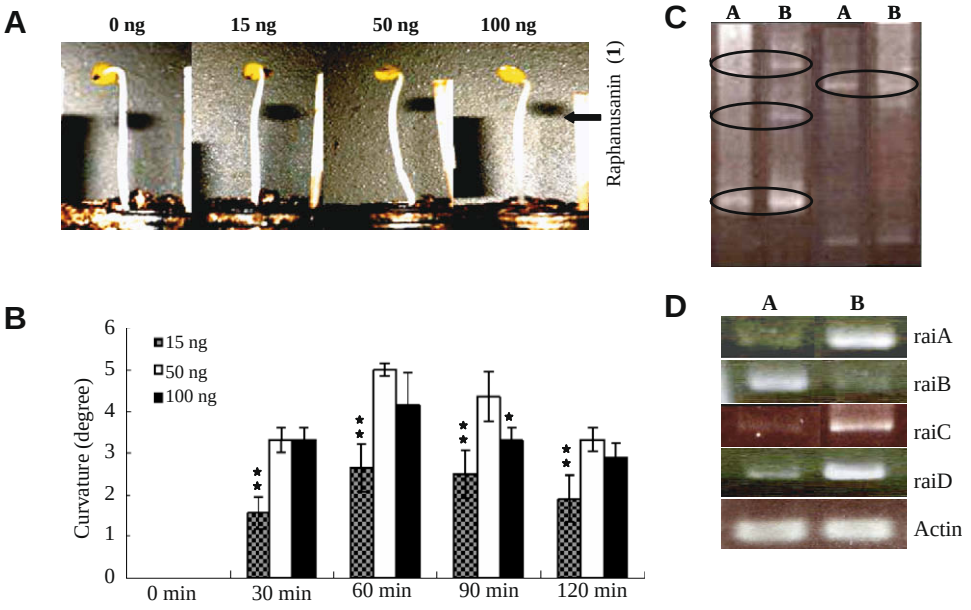

Fig. 2. Dose-dependency and time course of curvature of 4 d-old etiolated radish hypocotyls. Panels A and B; raphanusanin (**1**)-induced growth curvature of etiolated radish hypocotyls after unilateral application of lanolin paste (0.5 mg) containing the indicated concentrations of compound **1** to hypocotyls. Degree of curvatures were measured from digital images as described in the Experimental section. Arrow head shows the side of treatment. Ten seedlings from three replicate experiments were used to generate each mean value in panel B. Error bars represent SD of the mean. ★$P < 0.05$, ★ ★$P < 0.01$ when compared with 50 ng within a time point (student's *t*-test). The levels of curvature between 50 ng and 100 ng were not significantly changed in 30, 60, and 120 min time points. Panels C and D; differentially expressed genes between control and compound **1**-treated hypocotyls 10 min after application. A and B represent control and treatment, respectively. (C) A typical agarose gel. The circles indicate the differentially expressed bands. (D) Confirmation of the differential expression of four ESTs between the control and treated hypocotyls by semi-quantitative RT-PCR. Primers for the four ESTs described in experimental are specific to the cDNA regions of each respective clone.

Table 1
Differentially expressed genes between raphanusanin (**1**) treated and control radish hypocotyls

Clone	Highest hit of BLASTX	Gene expression level in treatment
raiA	COP9 signalosomc subunil 3 [*Arabidopsis thaliana*]	Up
raiB	Myrosinasc [*Brassica juncea*]	Down
raiC	01 PI 3-1 [*Brassica rapa* sub sp. Pckincnsis]	Up
raiD	Ethylene responsive clcmenl binding factor-rcltaed [*Brassica oleracea*]	Up

2.3. Analysis of RsCSN3 (clone raiA) during raphanusanin (*1*) treatment

The product of the clone raiA is a homologue of subunit 3 of the COP9 signalosome (CSN3) (Peng et al., 2001), and was analyzed in more detail (hereafter raiA is referred as *RsCSN3*). CSN3 is a conserved protein, and was originally identified as a negative regulator of photomorphogenic development in *Arabidopsis* (Wei and Deng, 1992; Wei et al., 1994a,b; Chamovitz et al., 1996). Moreover, a recent report by Pedmale and Liscum (2007) showed that there are links between *phot*1, NPH3, and E3 base-ubiquitin-dependent proteosome degradation in phototropic signaling. Since the physiological role of CSN3 appears to be conserved as an E3 mediator, the *RsCSN3* gene may be involved in light-mediated processes. Therefore, we characterized the *RsCSN3* gene in more detail. We first examined the structure of *RsCSN3*. The sequence of the full length cDNA generated by RLM-RACE was extended upstream using the genomic sequence to obtain the entire structure of the *RsCSN3* gene (Fig. 3). Although the transcription start site has not been determined as of yet, no further extension of the ORF is possible past 2.5 Kb. The ORF is divided into 10 exons by 9 introns, and predicts a protein of 420 amino acids (Fig. 3A). The deduced *RsCSN3* protein contains an imperfect leucine zipper domain at its N-terminal region (amino acids 32–66) that is also present in AP1 transcription factors, and is involved in responsiveness to growth factors, extracellular signals, or stress stimuli as important regulators (Milde-Langosch, 2005; Waetzig and Herdegen, 2003). The radish CSN3 protein also contains a PCI domain for interaction with components of the plant COP9-complex (Hofmann and Bucher, 1998), and is involved in light signaling via its C-terminal region (amino acids 175–346) (Fig. 3B). The radish CSN3 shares 59%, 30%, 33%, and 32% identity with its *Arabidopsis*, *Drosophila*, human, and ape counterparts, respectively (Fig. 3C). The CSN3s of radish and *Arabidopsis* are more closely related to each other than to any other CSN3 orthologue from other eukaryotes (Fig. 3D). Based on the high sequence conservation between them, the two proteins may have similar functions.

We then analyzed the expression of the *RsCSN3* gene in the early response to raphanusanin **1** treatment by semi-quantitative RT-PCR, in order to validate the differential expression levels of *RsCSN3* between treated samples and controls over time (Fig. 4A). Total RNA was isolated from treated and control hypocotyls, and the amount of mRNA was normalized using actin as an internal control. Semi-quantitative RT-PCR indicated that *raiA* expression levels after raphanusanin **1** treatment were the highest from 1 min to 10 min after the onset of treatment, and declined thereafter. The expression results were confirmed by northern blot analysis (Fig. 4B).

2.4. Analysis of RsCSN3 during light response

In order to obtain information regarding the strict specificity of *RsCSN3* gene involvement in light-mediated growth inhibition, we analyzed the expression of *RsCSN3* after stimulation with light of varying wavelengths. Irradiation with light rapidly inhibits stem growth within minutes of irradiation (Meijer, 1968; Gaba et al., 1984; Spalding and Cosgrove, 1989). Before analyzing the expression of *RsCSN3*, we first checked whether BL, GL or RL induce growth inhibition leading to curvature on the irradiated side after unilateral irradiation at a low-fluence rate for a 2 h period (Figs. 5 and 6). After BL illumination, the hypocotyls began to bend towards the light source at 30 min after the onset of light stimulation, and the curvature increased thereafter (Fig. 5A and B). Unilateral illumination with RL and GL also induced hypocotyl bending in radish seedlings, but there was less curvature than was observed under BL (Fig. 6A and B).

To determine if the *RsCSN3* gene is associated with the mechanism of light-mediated growth inhibition, the expression levels of *RsCSN3* were analyzed after early responses to BL, GL and RL. Fig. 5 shows that both semi-quantitative RT-PCR and northern blotting analyses indicated that after BL treatment, the abundance of *RsCSN3* transcripts also increased from 1 min to 10 min after the onset of treatment, and declined thereafter (Fig. 5C and D). Treatment with GL showed similar transcript profiles to those found under BL and raphanusanin **1** treatment during a period from 1 min to 60 min after the onset of treatment (Fig. 6A and C). However, RL treatment led to a significant reduction in the mRNA levels (Fig. 6C). The similar expression profiles of *RsCSN3* during the early response to BL and GL treatment is likely to be mediated by *phot 1*, the primary photoreceptor for both growth inhibition and phototropism involved in the perception of BL and GL (Correll and Kiss, 2005; Folta and Spalding, 2001). The constant down-regulation of *RsCSN3* expression after RL treatment appears to be mediated by *phyA* (Parks and Spalding, 1999; Hisada et al., 2000; Kircher et al., 2002).

2.5. Analysis of RsCSN3 after application of plant growth substances

In order to determine whether *RsCSN3* is actually involved in the growth inhibition that leads to curvature on the treated side of the hypocotyl, we first examined the expression levels of *RsCSN3* in response to the exogenous unilateral application of the known growth inhibitor, 4-MTBI (**2**) (Hasegawa et al., 2000). Fig. 7 shows that when it **2** is exogenously applied at a level approximately equal to endogenous levels (100 ng), the induced growth curvature occurs towards the applied side, indicating that the growth inhibition occurs on the applied side (Fig. 7A). The mRNA levels of *RsCSN3* abruptly increased at 1 min after the onset of application, and then returned back to the control level (Fig. 7B).

In addition, we also tested the response of *RsCSN3* during curvature induced by the phytohormone, IAA (Fig. 8). Unilateral application of exogenous IAA to the hypocotyls induced bi-directional curvature in a dose-dependent manner (Watahiki and Yamamoto, 1997; Collett et al., 2000). Application of 1 μM of exogenous IAA caused hypocotyls to bend towards the opposite side, indicating growth promotion on the applied side (Fig. 8A and B). In contrast, 3 μM and 5 μM applications of IAA caused hypocotyl bending to the applied side, indicating growth inhibition on the applied side. 10 μM IAA did not cause significant bending on either side of the hypocotyl, but caused a dramatic reduction in hypocotyl size. These results indicate that 1 μM of exogenous IAA induces the optimal level of growth promotion in the etiolated seedlings, whereas additional exogenous IAA leads to stress levels of IAA and causes growth inhibition. The expression patterns of the *RsCSN3* gene between the differential growth patterns induced by growth promotion (1 μM IAA) and growth inhibition (3 μM IAA) were analyzed. The expression level of *RsCSN3* did not prominently change between the control and unilateral application of 1 μM IAA, although treatment with 3 μM IAA-induced a temporary up-regulation of *RsCSN3* over a period of 1 min after treatment (Fig. 8C).

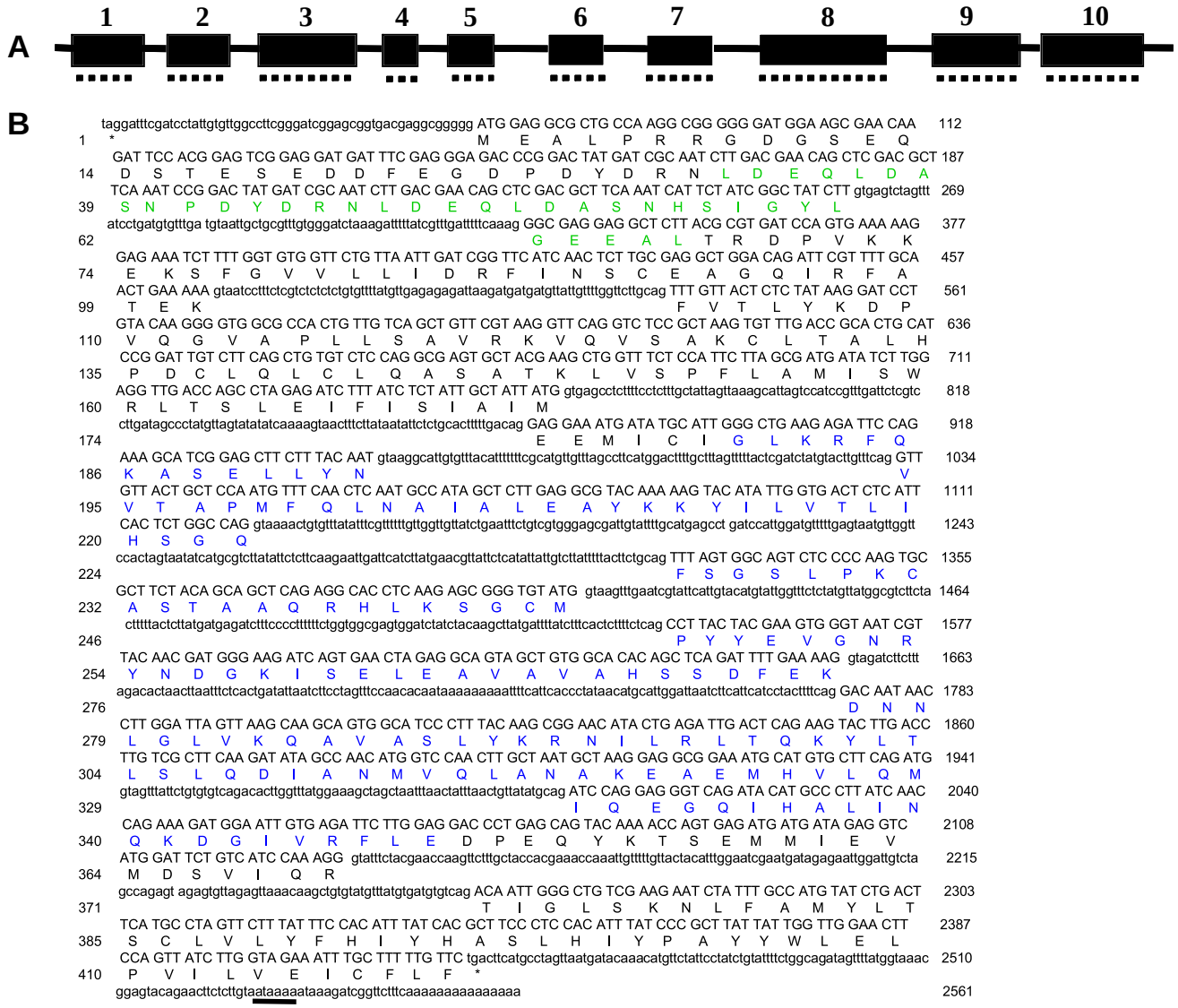

Fig. 3. Structure of the *raiA* (*RsCSN3*) gene. (A) Genomic structure of the *RsCSN3* gene. The 10 exons are numbered and the protein coding regions of the exons are shown as black boxes. Thin lines indicate untranslated regions. Dashed lines indicate the region of the probe for northern blot analysis. (B) Nucleotide (upper) and deduced amino acid sequences (lower) are shown for the entire region illustrated in (A). Lower case letters indicate untranslated sequences. The positions of nucleotides starting from the initiation codon are indicated on the right, and those for amino acids, on the left. The stop codon is marked by an asterisk, and a predicated polyadenylation signal downstream of the coding sequence is underlined. The N-terminal leucine zipper domain and the C-terminal PCI domain are represented by green and blue letters, respectively. (C) Sequence alignment of radish *RsCSN3* and CSN3 homologues from *Arabidopsis*, human, ape, and *Drosophila.* Numbers on the right indicate the positions of the amino acid residues. Yellow boxes indicate positions with identical aa. Gene bank Accession numbers are AB355980 for *Raphanus,* AF361759 for *Arabidopsis*, AF098109 for human (*Homo sapiens*), XM511309 for ape (*Pan troglodytes*), and AF071313 for *Drosophila.* (D) Phylogram with *R. sativus* and CSN3 genes from *A. thaliana*, *O. sativa, D. discoideum, D. melanogaster, C. elegans, M. musculus, P. troglodytes,* and human. The phylogram was produced using the Neighbor-joining method with the Clustal X program. Numbers at the branch points indicate the bootstrap values with 1000 replicates.

Therefore, these results show that the *RsCSN3* gene is involved in growth inhibition during initiation of treatment.

3. Discussion

It has previously been shown that unilateral illumination suppresses the growth rate of the illuminated side of radish hypocotyls, whereas it hardly affects the growth rate of the shaded side until the appearance of a subsequent autotropic straightening and gravitotropic reaction (Noguchi et al., 1986). In other dicotyledonous plant species, the retardation of growth of the illuminated side during the period of light-mediated development has been reported as one of the prominent factors that cause seedlings to bend towards the light source (Franssen et al., 1981). Moreover, growth inhibitors, such as raphanusanin (**1**) in radish hypocotyls (Hasegawa et al., 1986), 8-epixanthatin in sunflower hypocotyls (Yokotani-Tomita et al., 1999), uridine in oat coleoptiles (Hasegawa et al., 2000), MBOA and DIMBOA in maize coleoptiles (Hasegawa et al., 1992), indole-3-acetonitrile in cabbage hypocotyls (Yamamura and Hasegawa, 2001) and in *Arabidopsis* coleoptiles (Hasegawa et al., 2004) have all been shown to act as light-induced growth inhibiting substances that may play a role as regulators in light-mediated development. As described above, although several light-induced growth inhibiting substances have been isolated from various plant species, the molecular mechanisms underlying this phenomenon are still largely obscure. This study examines the mechanism of growth inhibition during a short period of light irra-

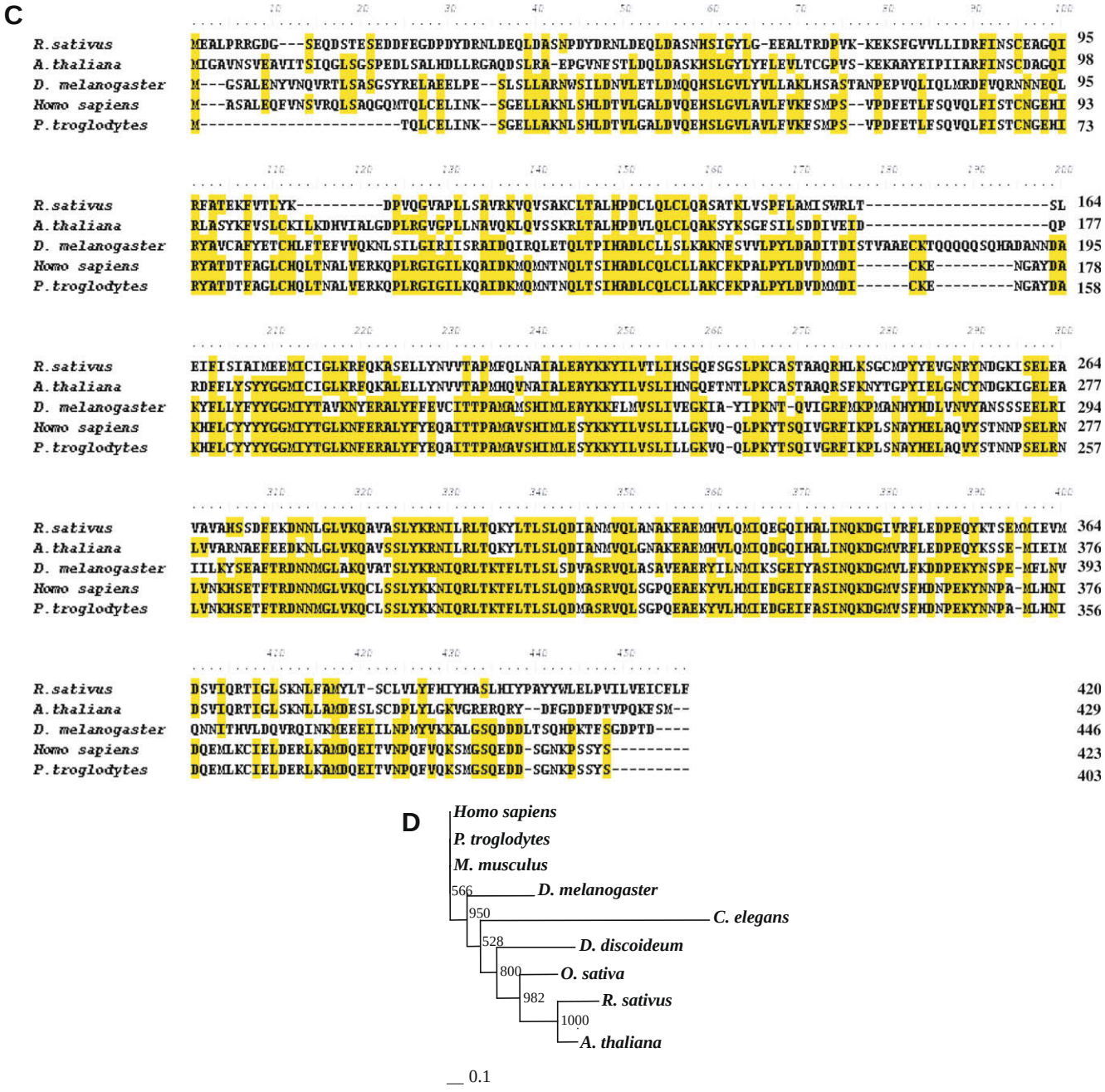

Fig. 3 (*continued*)

diation in radish hypocotyls using physiological and molecular approaches. The study of the dose-dependent curvature of radish hypocotyls induced by multiple stimuli is an important step in the comprehensive analysis of the physiological basis of growth inhibition, since the differential growth of plant parts that can be influenced by both endogenous and environmental factors is a complex phenomenon.

3.1. Irradiation at a low-fluence rate and plant growth substances induce hypocotyl bending towards the side of treatment

The amounts of light intensity, exposure time, and photoreaction are linked, and lead to complexities in plant morphology and signaling pathways. Therefore, the effects of irradiation were studied at low light intensities for only 2 h in these experiments. Our results showed that after the onset of unilateral irradiation with BL, RL and GL at 0.1 μmol m^{-2} s^{-1}, etiolated radish seedlings exhibit growth inhibition and phototrophic bending. Growth inhibition and phototropic curvature are maximal in BL-treated seedlings (Christie and Briggs, 2001; Folta, 2004). As expected, the phototropic bending in radish seedlings is most pronounced under BL when compared to RL and GL irradiation at the same intensity. GL between the wavelengths of 500–550 nm has been reported to retard growth and induce phototropic bending in dicotyledonous plants, such as *Arabidopsis*, lettuce and cockscomb seedlings (Atkins, 1936; Steinitz et al., 1985). Therefore, the wavelength of 525 nm used in this experiment is consistent with the induction of both growth inhibition and hypocotyl bending. The observed GL induced inhibition have produced result in agreement with other light conditions studied, from UV to Far-red, each rapidly in-

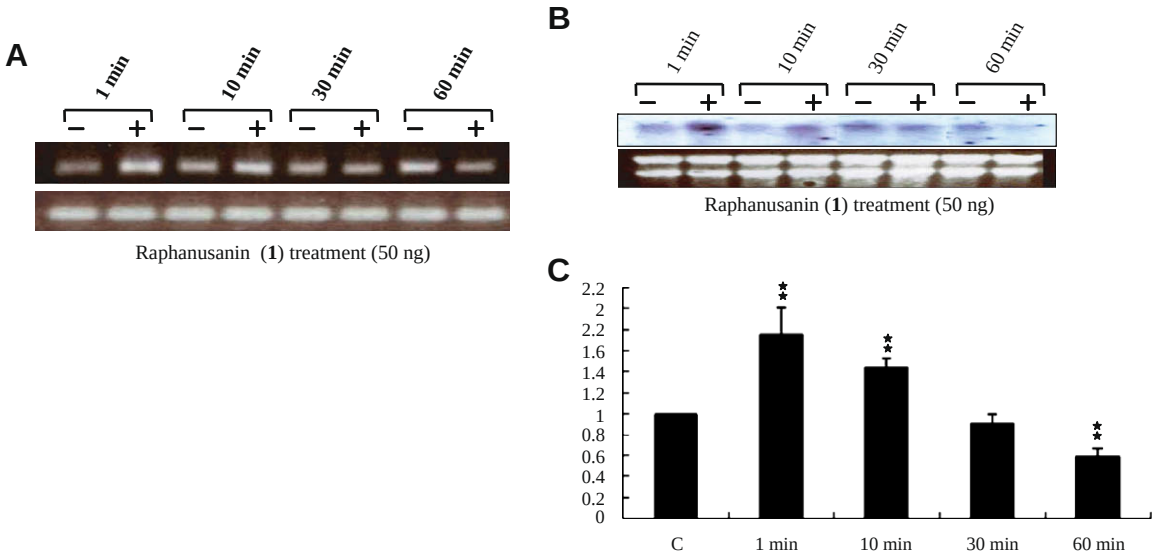

Fig. 4. Expression analysis of the *RsCSN3* gene. Panel A; RT-PCR analysis of the time course expression levels of *RsCSN3* relative to the control following treatment with raphanusanin (**1**). The bottom panels show the actin loading control. Primers for *RsCSN3* are specific to transcripts from the respective cDNA. Panels B and C; the result of a single experiment of time course expression analysis by northern hybridization of *CSN3* relative to control following treatment with compound **1** (B) and the combined data from three independent experiments normalized to each respective control as raphanusanin **1** (C). The data represent the mean ± ranges of triplicate samples. ★ ★$P < 0.01$ when compared with the control (student's *t*-test). The bottom panel shows ethidium bromide staining of rRNA. (+), treatment; (−) or C, control. The full length cDNA of *CSN3* was used as the probe for northern hybridization.

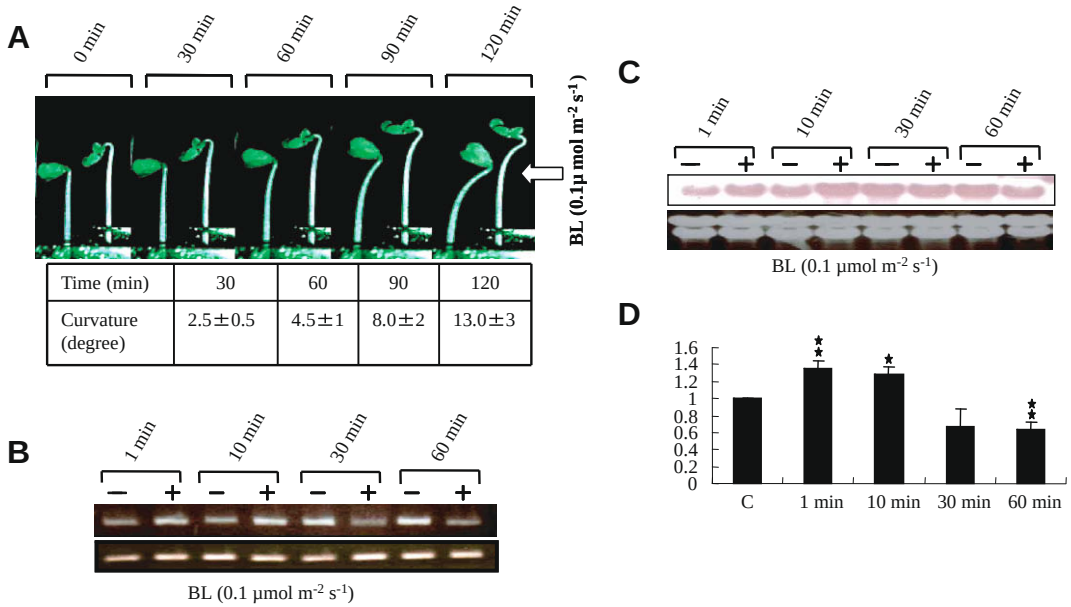

Fig. 5. Induction of hypocotyl curvature and expression analysis of the *RsCSN3* gene after unilateral illumination with blue light (BL). Panels A; 4-d-old etiolated radish seedlings were exposed to unidirectional BL (0.1 μmol m−2 s−1) for 2 h. The bottom panel shows degree of curvatures after the indicated time points. The phototropic curvatures were measured from digital images as described in the Section 5. Ten seedlings from three replicate experiments were used to generate each mean value in the bottom panel. Arrow head shows the illuminated side. Panel B; RT-PCR analysis of the time course expression levels of *RsCSN3* relative to the control following treatments with BL. The bottom panels show the actin loading control. Primers for *RsCSN3* are specific to transcripts from the respective cDNA. Panels C and D; the result of a single experiment of time course expression analysis by northern hybridization of *RsCSN3* relative to control following treatment with BL (C) and the combined data from three independent experiments normalized to each respective control as 1 (D). The data represent the mean ± ranges of triplicate samples. ★ ★$P < 0.01$, ★$P < 0.05$ when compared with the control (student's *t*-test). The bottom panel shows ethidium bromide staining of rRNA. (+), treatment; (−) or C, control. The full length cDNA of *RsCSN3* was used as the probe for northern hybridization.

hibit stem growth within minutes of irradiation (Meijer, 1968; Gaba et al., 1984; Spalding and Cosgrove, 1989). More likely, inhibition is simply triggered by the conserved role of their respective photoreceptors. To the contrary, a report by Folta (2004) showed that constant irradiation at low-fluence or a short-single pulse of GL induced growth promotion, and only at high-fluence of GL led to transient inhibition of growth rate in *Arabidopsis*. One possibility is that the response in etiolated radish is more sensitive and rapid than in *Arabidopsis*, leading to inhibition at low-fluence of GL. Based on previous reports on various effects of GL that inhibition of seedling mass (Went, 1957), inhibition of plant cell culture growth (Klein, 1979), retardation of elongation during gravitropic

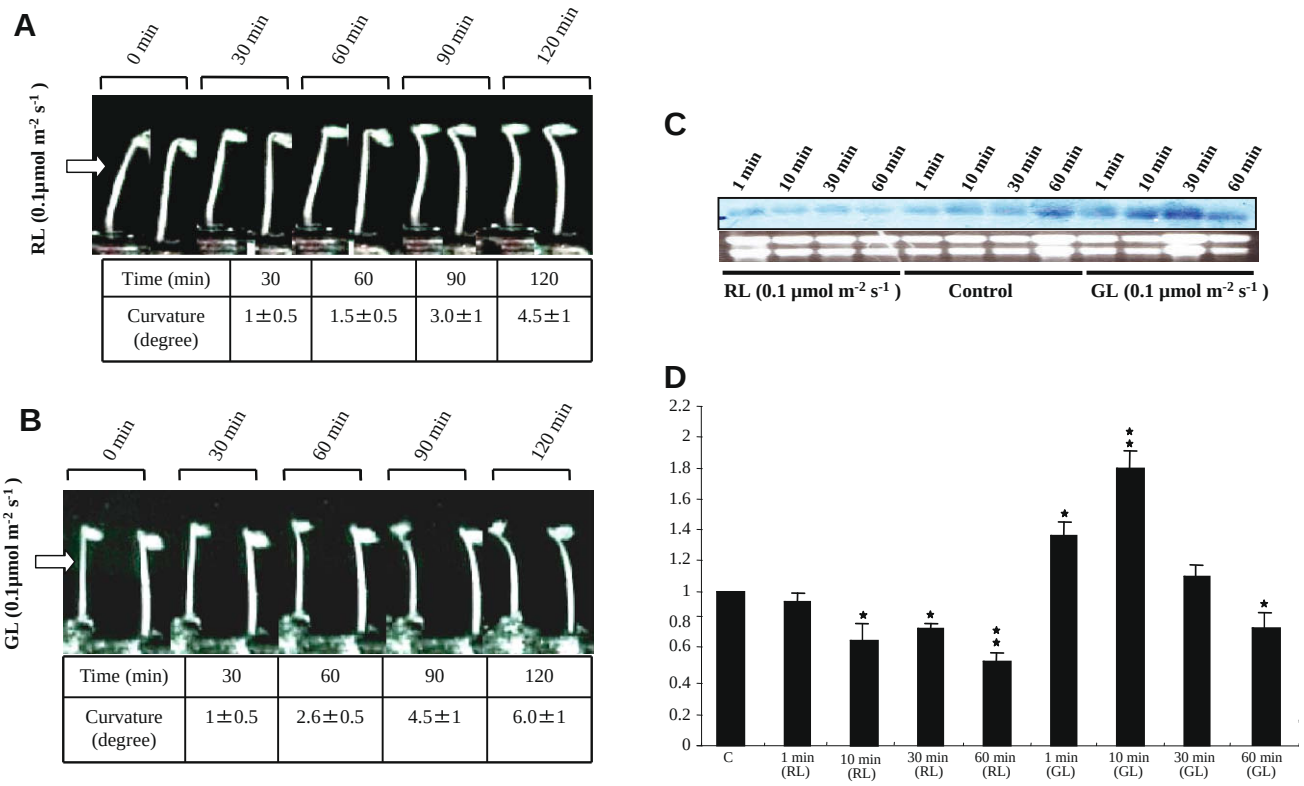

Fig. 6. Induction of hypocotyl curvature and expression analysis of the *RsCSN3* gene after unilateral illumination with red light (RL) or green light (GL). Panels A and B; 4-d-old etiolated radish seedlings were exposed to unidirectional GL or RL (0.1 μmol m−2 s−1) for 2 h. Bottom panels show degree of curvatures after the indicated time points. The phototropic curvatures were measured from digital images as described in the experimental section. Ten seedlings from three replicate experiments were used to generate each mean value in the bottom panels. Arrow heads show the illuminated sides. Panels C and D; the result of a single experiment of time course expression analysis by northern hybridization of *RsCSN3* relative to control following treatment with RL or GL (C) and the combined data from three independent experiments normalized to each respective control as 1 (D). The data represent the mean ± ranges of triplicate samples. ★$P < 0.05$, ★★$P < 0.01$ when compared with the control (student's *t*-test). The bottom panel shows ethidium bromide staining of rRNA. (+), treatment; C, control. The full length cDNA of *RsCSN3* was used as the probe for northern hybridization. Error bars represent SE of the mean.

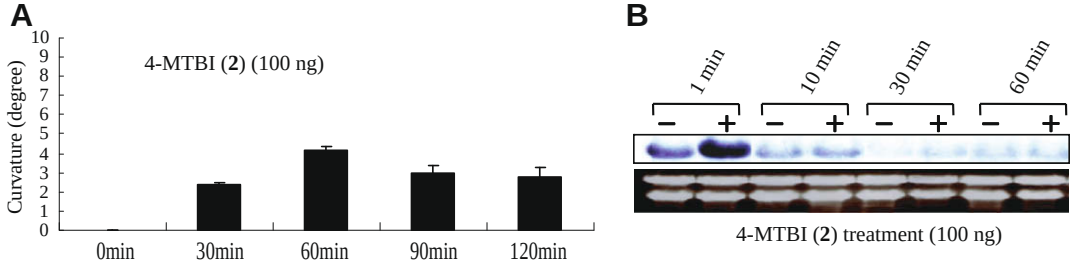

Fig. 7. Time course of hypocotyl curvature and expression analysis of the *RsCSN3* gene after 4-MTBI (**2**) treatment. Panel A; 4-MTBI (**2**) induced growth curvature of etiolated radish hypocotyls after unilateral application of lanolin paste (0.5 mg) containing the indicated concentrations of compound **2**. Curvatures were measured from digital images as described in the Section 5. Ten seedlings from three replicate experiments were used to generate each mean value in panel A. Error bars represent SE of the mean. Panel B; the result of time course expression levels as visualized by northern hybridization of *RsCSN3* relative to control following treatment with compound **2** at the indicated concentrations. The bottom panel shows ethidium bromide staining of rRNA. (+), treatment; (−), control. The full length cDNA of *RsCSN3* was used as the probe for northern hybridization.

stimulation (Klein, 1979), its reversible activity of blue and UV-B induced stomatal opening (Frechilla et al., 2000; Eisinger et al., 2003; Talbott et al., 2003), stimulation of early stem elongation (Folta, 2004), and adjustment of plastid transcripts during early photomorphogenic development (Dhingra et al., 2006), it is necessary to be widely cautious about using green safelights in photobiological studies. Moreover, the result RL induced curvature in radish seedlings is also consistent with phytochrome-mediated RL phototropism studies in the hypocotyls of cress, *Celosia cristata,* d other dicotyledonous plants, as well as the maize seedling shoots (Atkins, 1936; Iino et al., 1984a,b).

Under these conditions, when 4-d-old etiolated radish seedlings were treated with a unilateral application of raphanusanin **1** at given concentrations, growth inhibition was primarily induced in the hypocotyls, resulting in bending only towards the side where treatment had been applied. This is consistent with former report, where the extent of hypocotyl curvature is dependent on the degree of inhibition on the side of application (Sakoda et al., 1991). In addition, the application of endogenous levels of the bioactive growth inhibitor 4-MTBI (**2**) (endogenous level of 100 ng) induces differential growth curvature towards the applied side, although the degree of curvature was much less. Hasegawa et al. (2000) reported that a

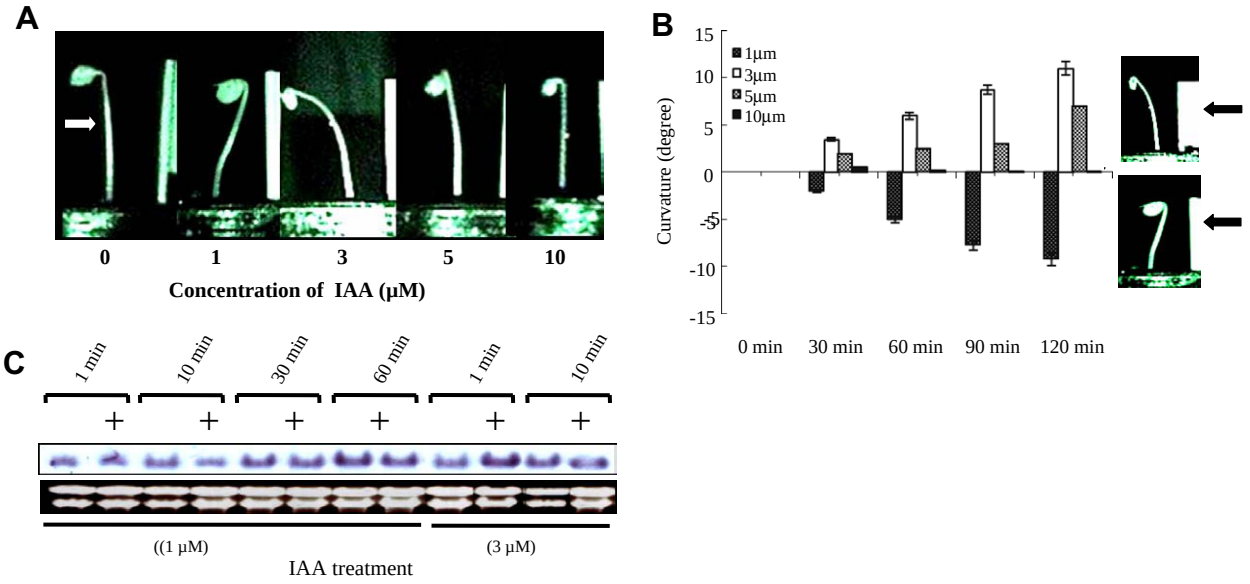

Fig. 8. Dose-dependent hypocotyl curvature and expression analysis of the *RsCSN3* gene after IAA treatment. Panels A and B; IAA-induced growth curvature of 4-d-old etiolated radish hypocotyls after unilateral application of lanolin paste (0.5 mg) containing the indicated concentrations of IAA. Curvatures were measured from digital images as described in the Section 5. Ten seedlings from three replicate experiments were used to generate each mean value in panel B. Error bars represent SE of the mean. Arrow heads show the sides of treatment and the vertical axis indicates mean bending to the applied sides (+) and to the opposite sides (−), respectively. Panel C; time courses of expression levels of *RsCSN3* relative to control following treatment with IAA at the indicated concentrations by northern blot hybridization. The bottom panel shows ethidium bromide staining of rRNA. (+), treatment; (−), control. The full length cDNA of *RsCSN3* was used as the probe for northern hybridization.

significant curvature could be induced by higher concentrations of 4-MTB1 **2**. However, the use of the optimal level of 4-MTB1 **2** is important in order to determine its proper role in growth inhibition. We also investigated the IAA-induced differential growth of hypocotyls. Our results indicate that IAA can induce hypocotyl bending in both directions. Unilateral application of 1 μM exogenous IAA-induced growth promotion, causing the hypocotyl to bend towards the side opposite to application. The level of auxins in wild-type seedlings is optimal for elongation and growth, but additional exogenous auxin inhibits hypocotyl growth (Watahiki and Yamamoto, 1997; Collett et al., 2000) as well as leaf growth (Keller et al., 2004). Moreover, auxin is able to promote elongation in plants where the auxin levels or auxin sensitivity have been reduced (Lincoln et al., 1990; Leyser et al., 1993; Smalle et al., 1997; Jensen et al., 1998). Therefore, these results can be explained by assuming that the observed growth promotion upon exogenous application of 1 μM IAA is due to the sub-optimal levels of auxin in the dark. As expected, at high doses of 3–5 μM IAA, elongation was inhibited, resulting in hypocotyl bending towards the side where IAA had been applied. More remarkably, at a concentration of 10 μM inhibition occurred on both sides of the hypocotyl, resulting in a shorter hypocotyl when compared with controls, which did not bend in any direction. This observation is consistent with former reports that hypocotyls are abnormally short in the dark in the presence of exogenous auxin (Boerjan et al., 1995; Celenza et al., 1995; King et al., 1995; Barlier et al., 2000). The results obtained in this study clearly show that the curvature of the treated side is due to growth inhibition induced by stress levels of auxin. Additional exogenous auxin does not appear to increase or decrease endogenous auxin levels under optimal growth conditions, but promotes hypocotyl growth inhibition instead.

3.2. Raphanusanin (*1*) induces genes to respond to diverse environmental stimuli

This is the first report of genes in radish seedlings induced by the light-induced growth-inhibitory substance raphanusanin (**1**). In order to identify the genes associated with its, inhibition of growth, a modified DD-RT-PCR method was used to compare differences in gene expression between control and treated seedlings, thereby allowing for the identification of candidate genes involved in growth inhibition. Four known genes encoding functional proteins were found to be differentially expressed in the treated samples versus the lanolin base control. In fact, three of the four genes (clone raiA, raiB and raiD) are genes that respond to diverse environmental stimuli, specifically as regulators during defense signaling (Bones and Rossiter, 1996; Rask et al., 2000; Glazebrook, 2001; Liu et al., 2002; Onate-Sanchez and Singh, 2002). These findings led us to question how environmentally responsive genes are relevant to the mechanism of raphanusanin **1**-mediated growth inhibition.

3.3. RsCSN3 may be involved in light-mediated signaling

As a first target of the functional analysis of the candidate genes, we investigated the particularly interesting gene *RsCSN3* (clone raiA). Expression analysis was used to evaluate the responsiveness of this gene to various stimuli. In general, phototropism and hypocotyl growth inhibition are modulated by the coordinated action of different light photoreceptors and their signaling pathways. The inhibition of hypocotyl elongation that begins within 30 s of irradiation and persists for approximately 30 min is initially activated by phototropin 1 (*PHOT1*) excitation (Parks et al., 1998; Folta and Spalding, 2001). The upregulation of the *RsCSN3* gene noted in this study within 1 min after the onset of all stimuli indicates that the product of this gene may act as a short-lived regulator in response to growth inhibition. It then persists for 10 min after compound **1**, BL, and GL treatments, as summarized in Table 2. Since all of these stimuli induce growth inhibition at the time of initiation, we suggest that the differential transcript profiles observed under different light and growth inhibition conditions may represent different pathways involved in the inhibition process in which *RsCSN3* participates. Generally, hypocotyl bending was initiated within 30 min after the onset of all stimuli. The expression of *RsCSN3* showed that transcripts were downregulated at 60 min in response

Table 2
Summarized results of the expression levels of raiA (*RsCSN3*) in response to multiple stimuli during the early period of treatment

Treatment	Time (min)	
	1	10
Raphanusanin (**1**)	Up	Up
BL	Up	Up
GL	Up	Up
RL	Down	Down
4-MTBI (**2**)	Up	NC
IAA (1 nM)	NC	NC
IAA (3 uM)	Up	NC

Up: up-regulation; Down: down-regulation; NC: expression is no different between treatment and control.

to raphanusanin **1**, BL, and GL. This is an expected result, given that *RsCSN3* may also participate in the bending process. Accordingly, the *RsCSN3* expression profiles observed after BL and GL treatment were similar to those after raphanusanin **1** treatment. Therefore, we speculate that *RsCSN3* expression is specifically induced by raphanusanin **1** in response to light. Regarding the identities of primary photoreceptors that could be specific for both phototropism and hypocotyl growth inhibition, *PHOT1* has been reported to play a role in the perception of both BL and GL at a low-fluence rate (Steinitz et al., 1985; Batschauer, 1999; Sakai et al., 2000; Folta and Spalding, 2001), whereas both *PHYA* and *PHYB* are regulated by RL at a low-fluence rate (Hisada et al., 2000; Liscum and Stowe-Evans, 2000; Kircher et al., 2002). The similar expression profiles of *RsCSN3* found after raphanusanin **1**, BL and GL treatments are thought to be activated by the excitation of *PHOT1*-mediated signaling. Due to the fact that *RsCSN3* is both upregulated and downregulated after treatment, *RsCSN3* may have a dual role, and may be involved in both the inhibition and bending processes. CSN3 is known as a multi-faceted protein in developmental processes, and can act as a repressor of light mediated development (Wei and Deng, 1992) or as a regulator of the light signaling pathway by mediating E3 base-ubiquitin-dependent proteosomal degradation in the model plant *Arabidopsis* (Chamovitz et al., 1996; Lyapina et al., 2001). Moreover, because of the function of CSN3 at the interface between signal transduction and ubiquitin-dependent proteolysis, the respective developmental processes that many different SCF E3 ligases regulate may be triggered during diverse functions (Lyapina et al., 2001; Schwechheimer et al., 2001). Therefore, the reason that *RsCSN3* plays a different role in different processes may be explained by assuming that the conserved function of CSN3 in multi-faceted developmental processes ranging from plant to animal systems (Deng, 2000) is also conserved in the radish system. To obtain direct evidence for this assumption, analysis of null mutants in specific pathways may clarify the direct or indirect involvement of these processes. Accordingly, we tried to generate its null mutants in the radish system; however, several initial attempts have been unsuccessful. Although raphanusanin **1** is a specific compound involved in some *Brassica* species, but not in Arabidopsis (Fahey et al., 2001), as CSN3 and its regulation is very conserved, a unique promoter or a conserved protein may be activated by raphanusanin **1** and as such be potentially active in Arabidopsis. The question whether either the complicated or simple relationships between the radish and Arabidopsis arise from raphanusanin **1** response or arise from different application assay given to the small hypocotyls of Arabidopsis, which could result in different responses, needs further elucidation. However, the identification of other raphanusanin **1**-induced genes and the genetic links between these genes will be essential in order to elucidate it role in the growth inhibition of radish hypocotyls.

4. Concluding remarks

Raphanusanin (**1**) has been identified as light-induced growth inhibitor and characterized as a phototropism-regulating substance in radish seedlings (Hasegawa et al., 1982; Sakoda and Hasegawa, 1989). Although physiological evidence for it role in inhibition of hypocotyl growth, microtubule orientation, and inhibition of apical dominance have been demonstrated (Noguchi et al., 1986; Sakoda et al., 1991; Nakajima et al., 2001), the molecular mechanisms underlying this phenomenon are still obscure. With the application of RCR-based differential display, the present study identified and characterized the first genes induced by raphanusanin **1** in radish hypocotyls. It **1** induced the defense-associated genes that respond to diverse environmental stimuli. The differential expression of these genes in response to raphanusanin **1** indicates the relationship between this compound and defense system. Moreover, one of these genes, *RsCSN3*, appeared not only to be an essential element in the inhibition of hypocotyl growth, but also to be correlated with phototropic signaling. However, the effects of raphanusanin **1** on these signaling cascades currently remain elusive. Finally, our data shows the correlations of it **1** to diverse processes (light signaling, defense signaling, and growth inhibition). The occurrence and biological relevance of raphanusanin **1**-related genes in their specific pathways needs to be investigated and defined.

5. Experimental

5.1. Plant materials

Sakurajima radish (*Raphanus sativus* var. *hortensis* f. *gigantissimus* Makino) seeds were germinated in vermiculite moistened with water in large trays (37 × 60 × 14 cm) in absolute darkness at 25 °C. About 3 d later, uniform seedlings were transplanted to small trays (8.5 × 17.7 × 3.5 cm) containing moist vermiculite under extremely dim-green 'safelight' (<0.01 μmol m^{-2} s^{-1}, below the detectable level), and kept in the dark at 25 °C for 1 d.

5.2. Light sources and treatments

Light treatments were taken using one of two sources, LED array (NSPB 520 S and NSPG 520S; NICHIA for blue light (BL) and green light (GL), and GL5URzK/GL5UR3K; SHARP for red light (RL)) or acrylic filter (PARAGLAS 302K, Kuraray, Japan) with a combination of a white fluorescent tube (FL 15N, Toshiba, Japan), each producing similar results. Etiolated seedlings (with a hypocotyl length of about 4 cm) were unilaterally illuminated with BL (LED array: λ_{max}: 470 nm; half band width: 20 nm or acrylic filter: λ_{max}: 445 nm; half band width: 30 nm), RL (LED array: λ_{max}: 660 nm; half band width: 20 nm or Acrylic filter: λ_{max}: 660 nm; half band width: 30 nm) or GL (LED array: λ_{max}: 525 nm; half band width: 20 nm or acrylic filter: λ_{max}: 525 nm; half band width: 30 nm) for 120 min at 25 °C. The incident energy was 0.1 μmol m^{-2} s^{-1} at the plant level. Fluence rates were assessed with a LI-COR LI-189 photometer. The images of hypocotyl growth were taken using an digitalized infrared camera (DCR-TRV9 NTSC, SONY, Japan), and the degree of curvature was measured at the angle between the hypocotyl's original orientation and the post-treatment orientation at 10 min intervals using photographic enlargement. Experiments for BL, GL, or RL were repeated three to six times.

5.3. Unilateral application of raphanusanin (*1*), IAA, and 4-MTBI (*2*)

Raphanusanin (**1**) and 4-MTBI (**2**) were isolated from fresh radish roots based on the procedures described by Kosemura et al.

(1993). 15, 50 or 100 ng of raphanusanin **1**, 100 μg of compound **2** or 1, 3, 5, or 10 μM of IAA was mixed with 0.5 mg lanolin and unilaterally applied to the hypocotyls in a lengthwise manner from 0 to 2 cm below the hook of uniform 4-d-old etiolated seedlings. Control seedlings were also treated with 0.5 mg lanolin. Treated seedlings were incubated in absolute darkness at 25 °C. All manipulations were performed under safelight. The images of hypocotyl growth and degree of bending were performed on the same way as mentioned above. Experiments were repeated three times.

5.4. Sample collection

Etiolated 4 day old seedlings were unilaterally illuminated with BL, RL, or GL (0.1 μmol m^{-2} s^{-1} each), or treated with a unilateral application of compound **1** (50 ng), IAA (1 μM and 3 μM), or compound **2** (100 ng) using the same procedure as mentioned above. The seedlings were harvested and immediately submerged into liquid nitrogen with minimal exposure to 'safelight' (>0.01 μmol m^{-2} s^{-1}) after the indicated time periods. For 15, 30, or 60 min time course, applied samples were immediately replaced in the dark box until they were to be harvested. For 1 min time course, each round of control and treated samples were harvested at 5 s interval to be a precise time point. A larger size of radish hypocotyl is convenient in handling under 'safelight' and sampling quickly. Ten replicates (both control and treated samples) were harvested directly into liquid nitrogen after the following treatments.

5.5. RNA preparation

Total cellular RNA was extracted using a plant RNeasy Mini kit (QIAGEN, Germany) according to the manufacturer's instructions. This was followed by the removal of contaminating genomic DNA with an RNase-Free DNase Set (QIAGEN, Germany). To ensure the high purity of polyadenylated RNA (polyA$^+$), mRNA was then obtained by purification using the Oligotex™-dT30 (Super) kit, and the purification procedure was repeated three times (Takara, Japan).

5.6. First strand cDNA synthesis and mRNA differential display

The prepared mRNA was used as a template for first strand cDNA synthesis with ThermoScript RNase H$^-$ RT (Invitrogen, USA) using an Oligo $(dT)_{12-18}$ primer (Invitrogen, USA). mRNA differential display PCR (DD-PCR) was performed using a modification of the procedure described by Sambrook and Russel (2001). To avoid anchoring primers annealing to regions of primary transcripts that contain tracts of A residues (Luce and Burrows, 1998), double standard DNA was synthesized with pairs of arbitrary primers in the absence of radio-labeled anchoring primers. In order to improve reproducibility and specificity, longer arbitrary primers with high annealing temperatures were used (Zhao and Barnstable, 1996). The arbitrary primers used in this experiment were A1: 5′-TGACCGGCAGCAAAATG-3′, A2: 5′-GGAAACAGCTATGA CCATG-3′, and B1: 5′-ATCTATGCAGCCATCTATG-3′, B2: 5′-ATGCAT CAGCAAGCCTCTT-3′. The PCR reactions contained 2 μl of reverse transcription products, 1.25 U ExTaq polymerase (Takara, Japan), 0.4 μM of each sense and antisense arbitrary primer, and 20 μM dNTPs. Manual hot start PCR was performed under the following conditions: 95 °C for 3 min and then 30 cycles of 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 2 min. A final extension was carried out at 72 °C for 5 min. In order to avoid missing rare mRNAs in the target cell, a second round of PCR was performed with 3 μl of the first round PCR product as the template, using the same PCR conditions as for the first round of PCR. The PCR products were screened by 0.9% w/v agarose gel electrophoresis.

5.7. Re-amplification, cloning, and sequencing of differentially expressed cDNA fragments

The differential bands were excised from the gel, and the excised DNA was eluted using the Wizard®SV Gel and PCR Clean-Up System (Promega, USA). The eluted DNA was used as the template to re-amplify the fragment by PCR, with the same primer set and conditions used for the above DD-RT-PCR reaction. The purified DNA was ligated into the pCR® 4-TOPO vector (Invitrogen, USA), and sequenced using a DNA sequencer (ABI 310 Applied Biosystems, USA) according to the manufacturer's protocols.

5.8. RT-PCR detection

The primers were designed according to the sequences of four newly identified ESTs. BLASTX searches were performed against sequence databases in order to confirm the gene specificity of the primer sequences. The sense and antisense primers for the compound **1**-induced clone, raiA (hereafter clone raiA is referred as *RsCSN3* as it is a homolog of CSN3 gene) clones were 5′-TGG CCAGTTTAGTGGCAGTCT-3′ and 5′-GTAAAGGGATGCCACTGCTTG-3′; 5′-GGAGAAGGGTGTCAACGTGT-3′ and 5′-CTTGAGCGGAGGAA AATCTTG-3′ for raiB; 5′-TGACCAGCAAGAAGGGAATC-3′ and 5′-AG CAACGCCTCTTTTAGCA G-3 for raiC; and 5′-TGATTGTTTGTGTGCTG CAA-3′ and 5′-CGGCTAGACAGCTTCCAAGT-3′ for raiD. The total RNA of three independent PolyA$^+$ mRNA samples was pooled to eliminate sampling variation. One microgram of total RNA was used as the template for first stand cDNA synthesis with ThermoScript RNase H$^-$ RT (Invitrogen, USA) using an Oligo $(dT)_{12-18}$ primer (Invitrogen, USA). The PCR amplification was carried out in a 20 μl reaction volume containing 1 μl of reverse transcription products as the template, 1 × PCR buffer, 20 μM dNTPs, 2.5 mM $MgCl_2^+$, 0.4 μM primer, and 0.5 U of Taq polymerase (Takara, Japan). Manual hot start PCR was performed under the following conditions: 95 °C for 3 min and then 27 cycles of 94 °C for 30 s, 58 °C for 1 min and 72 °C for 2 min. A final extension was carried out at 72 °C for 5 min. 10 μl of each PCR product was electrophoresed in a 1.3% w/v agarose gel. Actin was used as a control gene, and was amplified with a sense primer (5′-TGGCATCACACTTTCTAC-3′) and an antisense primer (5′-TGAGACACACCATCACCA-3′), which were generated from the conserved region of the *ACTIN* gene from the sequences of the Brassica family, using the same templates as for the experiments described above.

5.9. Molecular cloning using 5′ and 3′ rapid amplification of cDNA ends (RACE) and genomic DNA sequencing of RSCSN3

cDNA for RLM-RACE was synthesized from radish hypocotyls 10 min after the application of compound **1**. 5′ RACE and 3′ RACE were performed with a Gene Racer™ Kit (Invitrogen, USA) according to the manufacturer's instructions. The forward and reverse GSP primers were 5′-TGGCCAGTTTAGTGGCAG TCT-3′ and 5′-GGAT AAATGTGGAGGGAAGCGTGAT-3′. The touch-down PCR conditions for the GSP products were 94 °C for 2 min, followed by 5 cycles of 94 °C for 30 s and 72 °C for 2 min, followed by 5 cycles of 94 °C for 30 s and 70 °C for 2 min, followed by 20 cycles of 94 °C for 30 s, 65 °C for 30 s, and 68 °C for 2 min, and finally elongation for 10 min at 68 °C. The entire structure of the *RsCSN3* gene was obtained by PCR amplification of the genomic region between the region upstream of the ORF and downstream of the polyA$^+$ tail. The fragments were subcloned into the pCR® 4-TOPO vector (Invitrogen, USA), and vectors containing fragments were transformed into One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen, USA). The plasmids were purified with a Wizard® Plus SV Minipreps DNA Purification System (Promega, USA), as described by the manufacturer.

5.10. Determination of nucleotide sequences

Nucleotide sequences were determined with a DNA sequencer (ABI 310 Applied Biosystems, USA) using a Big-Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Sequencing reactions were performed by standard procedures. All sequencing reactions involved either the standard M13 forward or reverse primers, and both the 5′ and 3′ ends of each cDNA were obtained. The sequence text files were edited to remove the vector sequences and ambiguous base calls.

5.11. Northern hybridization analysis

Total RNA was extracted from control and treated radish hypocotyls using a Plant Total RNA Extraction System (Viogene, USA) according to the manufacturer's instructions. Northern blot hybridization was performed by conventional procedures (Sambrook et al., 2001). All treatments, harvesting, labeling, and hybridizations were performed independently on three independent sets of seedlings. The samples placed on the working bench and in the dark for 1 h were checked if our safe light have biological effect and change gene expression. No difference was observed. The signals were quantified and analyzed by image J tool.

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