Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 6314–6318, July 1995
Plant Biology

Location of light-repressible, small GTP-binding protein of the *YPT/rab* family in the growing zone of etiolated pea stems

(gene expression/photomorphogenesis/phytochrome/*ras*-related gene/vesicular transport)

YUKIO NAGANO*, YOICHIRO OKADA*, HIROSHI NARITA†, YUKIKO ASAKA†, AND YUKIKO SASAKI*‡§

*Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Kyoto 606-01, Japan; and †Department of Food Science, Kyoto Women's University, Kyoto 605, Japan

Communicated by Winslow R. Briggs, Carnegie Institution of Washington, Stanford, CA, March 15, 1995

ABSTRACT ***YPT/rab* proteins are *ras*-like small GTP-binding proteins that serve as key regulators of vesicular transport. The mRNA levels of two *YPT/rab* genes in pea plants are repressed by light, with the process mediated by phytochrome. Here, we examined the mRNA expression and the location of the two proteins, *pra2*- and *pra3*-encoded proteins, using monoclonal antibodies. The *pra2* and *pra3* mRNA levels were highest in the stems of dark-grown seedlings. The corresponding proteins were found in the cytosol and the membranes of the stems. Most of the *pra2* protein was in the growing internodes, especially in the growing region, but the *pra3* protein was widespread. These results suggest that the *pra2* protein is important for vesicular transport in stems, possibly contributing to stem growth in the dark, and that the *pra3* protein is important for general vesicular transport. The amounts of *pra2* and *pra3* proteins decreased with illumination. The decrease in these proteins may be related to the phytochrome-dependent inhibition of stem growth that occurs in etiolated pea seedlings.**

Small GTP-binding proteins are molecular switches that are turned on by GTP and off by the hydrolysis of GTP to GDP. These proteins regulate diverse cell functions, including cell proliferation, cytoskeleton organization, and intracellular traffic in various eukaryotes (1). On the basis of their structural and functional properties, the family of genes coding for small GTP-binding proteins has been divided into five subfamilies: *ras*, *rho*, *YPT/rab*, *arf*, and *Ran* (2). In yeasts and mammals, members of the *YPT/rab* family are associated with the endoplasmic reticulum, the intermediate compartment, the Golgi apparatus, the plasma membrane, and early and late endosomes (3) and control docking and fusion during intracellular transport. Several recent findings about plant homologues of the *YPT/rab* family suggest that the genes have important roles for cellular activities in plants as well. The rice *rgp1* gene is related to dwarfism (4). The *Arabidopsis* homologue, *rha1*, is expressed primarily in developing guard cells (5). The legume homologues are involved in the development of the peribacteroid membrane compartment in effective symbiosis (6) and may be involved in vesicle-mediated transport and secretion.

We have cloned from peas 11 cDNAs that belong to the *YPT/rab* subfamily (7). The expression of two of the genes, *pra2* and *pra3*, is down-regulated by light in a process mediated by the red-light receptor phytochrome (8), which suggests that the two genes are important in intracellular traffic in dark-grown seedlings. Phytochrome may modulate certain developmental processes in such seedlings by regulating the expression of the two corresponding small GTP-binding proteins.

One of the most striking responses of dark-grown seedlings to light is the photoinhibition of stem growth after germination (9). The photoinhibition is associated with the transition from a seedling that is dependent on food reserves and adapted for rapid growth upward through the soil to a young plant with photosynthetic leaves. Etiolated pea seedlings have an epicotyl consisting of a stem, a hook, and the shoot apex. The epicotyl grows rapidly by extending its stem with minimal leaf development in the dark. When there is illumination, the extension of the stem is inhibited and young leaves start to expand by first unfolding the hook. This photoinhibition of stem extension is mediated by at least two photoreceptors, a blue-light receptor and phytochrome. Immediate and transient inhibition is caused by blue light, and delayed but prolonged inhibition is caused by red light (10, 11). Because *pra2* and *pra3* mRNA levels are decreased after a short period of irradiation with red light (8), inhibition of stem growth by red light may be accompanied by a decrease in *pra2*- and *pra3*-encoded proteins.

To elucidate the role of the light-repressible, small GTP-binding proteins *pra2* and *pra3* in etiolated pea seedlings, we examined the expression of these two genes and the location of the proteins they encode. We found that most of the *pra2* protein was in the growing region of the stem in dark-grown seedlings and disappeared with illumination. The *pra3* protein was more widespread than the *pra2* protein and disappeared more gradually on illumination.

MATERIALS AND METHODS

Plant Material. Pea seedlings (*Pisum sativum* cv. Alaska; Snow Brand Seed, Sapporo, Japan) were grown in the dark for 5, 6, or 10 days at 23°C ± 1°C. For continuous white light irradiation, white-light fluorescent tubes (National, FL40SS.W/37) were used at an intensity of about 100 microeinsteins·m^{-2}·sec^{-1}.

Expression of Recombinant Proteins in *Escherichia coli*. For expression of the *pra2* gene, a DNA fragment containing most of the *pra2* sequence (7) was cloned into a vector, pIH889 (New England Biolabs), harboring the *malE* gene and the factor Xa protease recognition site, by the manufacturer's protocol. The fusion protein expressed in *E. coli* was purified with amylose resin and digested with factor Xa by the same protocol. The protein mixture was separated by SDS/PAGE and the recombinant *pra2* protein was electroeluted from the gel. The protein solution was cooled and the SDS was removed by centrifugation. The recombinant *pra3* protein was expressed with vector pET-16b (Novagen), which has a histidine tag, and the protein was purified by the manufacturer's protocol. The recombinant *pra6* protein was prepared as described elsewhere (7) and purified on a DEAE-cellulose column and by SDS/PAGE.

Preparation of Monoclonal Antibodies. Two groups each of three female BALB/c mice 9 weeks old were immunized separately with the recombinant *pra2* and *pra3* protein. Hybridomas producing monoclonal antibodies against one of the

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‡Present address: Laboratory of Plant Molecular Biology, School of Agricultural Sciences, Nagoya University, Nagoya, 464-01, Japan.
§To whom reprint requests should be sent at the present address.

antigens were established as described (12). The anti-*pra2* protein antibody obtained was IgG1 (κ) and the anti-*pra3* protein antibody obtained was IgG2a (κ). Electrophoretically pure antibodies were prepared from ascitic fluids with protein A columns and used in this study.

Isolation of RNA and Analysis of RNA Gel Blots. Total RNA was extracted from the buds, stems (about 3 cm long from the top) including the hook, and roots of pea seedlings, and poly(A)$^+$ RNA was prepared as described (8). Template activities of the poly(A)$^+$ RNAs were measured in an *in vitro* protein-synthesizing system prepared from wheat germ (13) to confirm that the mRNA was intact. Northern blotting was done as described (14). The *pra2* and *pra3* probes were C-terminal-specific fragments (7), and the 1.5-kb *Not* I fragment of pea actin cDNA was used.

Extraction of Protein and Immunoblotting. Total protein was extracted by maceration of the plant organs with sand together with a same volume of a buffer equal to that of the tissue containing 200 mM Tris·HCl (pH 6.8), 6% SDS, and 20% glycerol in a mortar and pestle at room temperature. Maceration was done rapidly. The mixture was heated at 95°C for 3 min and centrifuged. The supernatant proteins were separated by SDS/PAGE, blotted onto a nitrocellulose membrane (15), probed with monoclonal IgGs against the *pra2* and *pra3* proteins (1 μg/ml) and goat anti-mouse IgG conjugated to peroxidase (Bio-Rad), and developed with an ECL kit (Amersham). The protein concentration was measured with a protein assay kit (Bio-Rad) with bovine serum albumin as the standard. Cytosolic and membrane-bound proteins from stems were prepared as follows. The top 3 cm of 6-day-old seedlings grown in the dark was macerated with sand and a one-half volume of a buffer containing 100 mM Tricine·KOH (pH 8), 0.25 mM EDTA, 50 mM NaCl, 2 mM benzamidine, 5 mM ε-aminocaproic acid, and 0.5 mM phenylmethylsulfonyl fluoride, and the mixture was centrifuged at 35,000 rpm for 30 min in a 100.3 rotor of a TL-100 ultracentrifuge (Beckman). The supernatant was the cytosol fraction and the pellet was the membrane fraction. The membrane fraction was washed with either 0.5 M NaCl, 5 M urea, 0.1 M Na_2CO_3 (pH 11), or 1% Triton X-100 and suspended in 5% SDS, heated at 95°C for 3 min, and centrifuged. The proteins in the supernatant were analyzed by SDS/PAGE.

RESULTS

Organ-Specific Location and Effect of Light. To identify an organ-specific expression of the two light-repressible genes, *pra2* and *pra3*, we used RNA gel blotting and examined their expression in 6-day-old seedlings grown in the dark (Fig. 1). The levels of expression of *pra2* and *pra3* were highest in the stems. *pra2* mRNA was not detected in buds and roots under the conditions we usually used, although a small amount was detected with a longer exposure time (data not shown). *pra3* mRNA was in low concentrations in roots and buds. When 5-day-old seedlings grown in the dark were illuminated for 1 day with white light, the expression of *pra2* and *pra3*, especially *pra2*, decreased in the stem. Actin mRNA as a control was expressed in all the samples, although its level decreased in the stem with irradiation and increased slightly in the roots. These results are consistent with our previous results (8) and indicate that light-dependent changes in the expression of the two genes occurred mainly in the stems.

To determine the location of *pra2* and *pra3* proteins, we prepared monoclonal antibodies against recombinant *pra2* protein, named PRA2, or recombinant *pra3* protein, named PRA3. The monoclonal antibody to PRA2 did not cross-react with PRA3 and the antibody to PRA3 did not cross-react with PRA2 (Fig. 2*a*). Neither antibody reacted with recombinant PRA6. These results suggested that the antibodies did not recognize highly conserved sequences (7) such as GTP-binding domains. Using these monoclonal antibodies as probes, we surveyed *pra2* and *pra3* proteins in total protein extracted from etiolated stems (Fig. 2*a*). The anti-PRA2 antibody reacted with a protein with the apparent molecular weight of 26,000, and the anti-PRA3 antibody reacted with a protein with the apparent molecular weight of 24,000, values that agreed roughly with the calculated molecular weights of the two proteins (7). Bands at these locations were not found when the electroblotted sheets were probed with these antibodies incubated first with excess amounts of the appropriate antigen, evidence that the observed bands were of the *pra2* and *pra3* proteins. There was about 1 ng of the *pra2* protein in 30 μg of total protein from stems and there was about 1 ng of the *pra3* protein in 15 μg of total protein when their recombinant proteins were used as the standard, so these proteins accounted for 0.003–0.006% of the total protein.

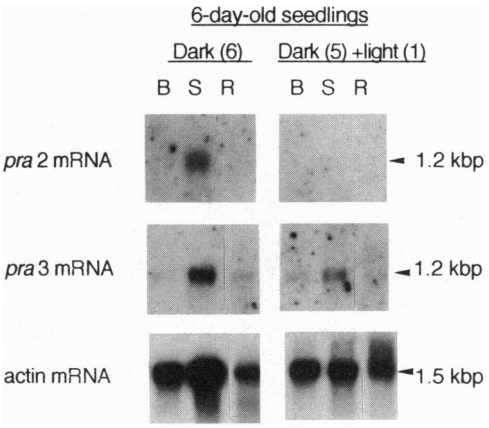

FIG. 1. RNA gel blotting of *pra2* and *pra3* mRNAs. The organ-specific expression of seedlings grown in the dark for 6 days or in the dark for 5 days and in the light for 1 day was examined. Each lane contained 2 μg of poly(A)$^+$ RNA. Exposure time was 36 h for the *pra2* and *pra3* probes and 3 h for the actin probe. B, buds; S, stems; R, roots.

The *pra2* protein was abundant in the stems but was not detected in the buds or roots under the conditions used (Fig. 2*b*). With longer exposure of the immunoblots, a small amount of *pra2* protein was detected in the buds and roots (data not shown). As with its mRNA, the *pra2* protein was not found after 1 day of white-light irradiation. In dark-grown plants, the *pra3* protein was abundant in the stems and present in small amounts in the roots. When plants were illuminated, the *pra3* protein level did not change in the roots, but it decreased in the stems and was detected for the first time in buds. These results were obtained from proteins made soluble with a buffer containing SDS, so that soluble and membrane-bound forms were present.

Small GTP-binding proteins are synthesized as soluble precursors that are modified posttranslationally by isoprenylation and anchored on a membrane, where they function as a molecular switch. The *pra2* and *pra3* proteins have C-terminal consensus sequences for isoprenylation (7) and probably function as membrane-bound proteins. To locate them, we examined the cytosol and membrane fractions from etiolated stems for these proteins (Fig. 2*c*). The *pra2* and *pra3* proteins were found in both fractions. The cytosol and membrane forms of the proteins had the same electrophoretic mobility. The location of these proteins in both fractions and the similar mobility for membrane and soluble forms have been found for the proteins of the *YPT/rab* family of yeasts and mammals (16, 17), and the *pra2* and *pra3* proteins probably cycle between the cytosol and membranes. Washing of the membrane fraction with a solution with a high salt concentration, high pH, or urea did not dissociate the proteins from the membranes, but washing with 1% Triton X-100 did, indicating that these

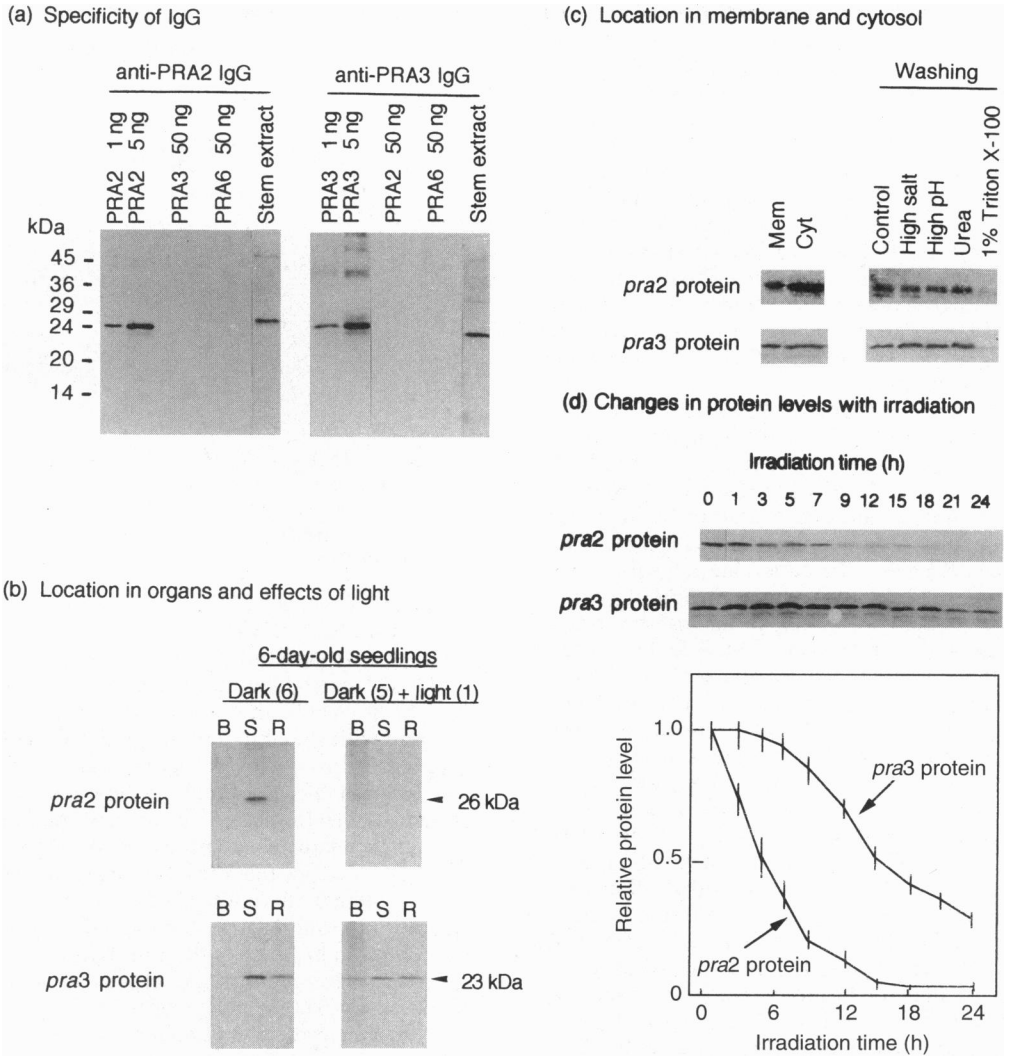

FIG. 2. *pra2* and *pra3* proteins detected by immunoblotting in various organs. (*a*) PRA2, PRA3, and PRA6 were recombinant proteins. Stem extracts from 6-day-old etiolated seedlings were used; 30 μg of protein of the stem extract was used for anti-PRA2 IgG and 15 μg was used for anti-PRA3 IgG. (*b*) Proteins from seedlings grown in the dark for 6 days or from seedlings in the dark for 5 days and in the light for 1 day were probed with anti-PRA2 IgG or anti-PRA3 IgG. Each lane contained 30 μg of total proteins. B, buds; S, stems; R, roots. (*c*) Membrane-bound (Mem) and cytosol (Cyt) proteins from 6-day-old stems were probed with anti-PRA2 or anti-PRA3 IgG. The membrane fraction was washed with the indicated solutions. Each lane contained 30 μg of protein. (*d*) Pea seedlings 6 days old were illuminated with continuous white light for various times and the total stem proteins were probed with anti-PRA2 or anti-PRA3 IgG. The same amount of protein (30 μg) was put in each lane. Relative protein level was measured by a densitometer. The mean value of four measurements was calculated, the value at 0 h being taken to be 1.

proteins were associated tightly with membranes, most likely by isoprenyl groups.

The *pra2* mRNA level decreases to a minimum within 3 h after the start of continuous white-light irradiation (8). To examine changes in the protein levels caused by irradiation, we monitored the proteins by immunoblotting of total stem protein (Fig. 2*d*). The *pra2* proteins gradually decreased. When the immunoblot was evaluated by densitometry, the protein level was half the initial value after 5 h. The decrease was less rapid than the decrease in the mRNA levels, a result that suggests that this protein does not turn over very rapidly. The *pra3* mRNA level decreases to half of the baseline value after 6 h of irradiation (8). The corresponding protein decreased to half of the baseline value during 15 h of irradiation.

Protein Levels in Different Internodes. The growth of dark-grown pea seedlings takes place in the apical portion of the epicotyl. As one internode is finishing growth, the next one is starting (18). Ten-day-old seedlings have four internodes (Fig. 3*a*). The first and second internodes have already stopped elongation, the third internode has almost stopped elongation, and the fourth internode is still rapidly elongating. To find which internode of 10-day-old seedlings has the largest amounts of *pra2* and *pra3* proteins, we compared the levels of these proteins in the four internodes. The first and second internodes contained no detectable *pra2* protein, the third internode contained some, and the fourth internode contained much, so the *pra2* protein was abundant in the growing internode. The *pra3* protein was abundant in the fourth internode but was found in all four internodes, suggesting that the *pra3* protein was not limited to the internode that was growing.

To examine the effect of a light period on stem growth and the abundance of these proteins, etiolated seedlings 5 days old were illuminated with white light for 1 day and then returned to the dark. Elongation of the second internode was inhibited by the illumination, and young leaves started to develop. When these seedlings were again returned to the dark and left for 1 day, the second internode did not elongate and the *pra2* protein was not detected (data not shown). When these seedlings were left in the dark for 3 more days, the second internode did not elongate but the third and fourth internodes elongated. We compared *pra2* and *pra3* protein levels in these

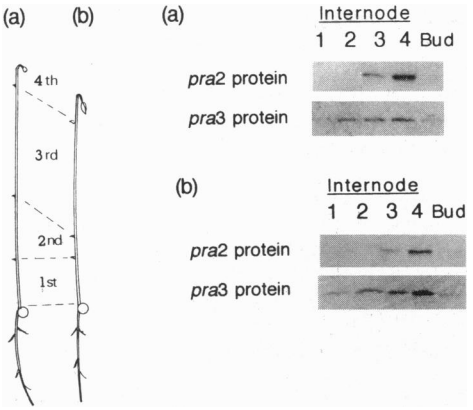

FIG. 3. *pra2* and *pra3* proteins in different internodes. (*a*) Proteins from the internodes and buds of 10-day-old seedlings grown in the dark were probed with anti-PRA2 or anti-PRA3 IgG. The same amount of protein (20 μg) was put in each lane. (*b*) Proteins of 10-day-old seedlings grown for 9 days in the dark with 1 day in the light on the fifth day were probed as in *a*. The stem was about 28–33 cm long.

10-day-old seedlings. Results were similar to those in Fig. 3*a*; the *pra2* protein was abundant in the fourth internode, which was growing, and was not found in the first and second internodes, but the *pra3* protein was widespread. These results indicate that the *pra2* proteins, having declined in the second internode during light treatment, apparently do not reaccumulate substantially during a subsequent 4 days in darkness.

Relationship Between the *pra2* Protein Level and Stem Growth. To find if there was a relationship between the *pra2* protein level and stem growth in 6-day-old seedlings, we measured *in vivo* extension of the stem for 6 h, from 3 h before to 3 h after the time of protein extraction, and the extension was plotted (Fig. 4). The stem of 6-day-old seedlings had three internodes as shown in Fig. 4. The third internode did not elongate under these conditions. The upper part of the second internode was growing, but growth of the lower part of the second internode and the first internode was undetectable. The growing region was from 0.3 cm below the hook to about 2.3 cm. The level of *pra2* protein was highest in the section that was 0.75–2.25 cm from the top, and the concentration was lower in the lower parts. The *pra3* protein was found in all parts, although its concentration was highest in the upper part of the second internode. These results suggest that there is a relationship between the *pra2* protein level and stem extension. When the stem of an etiolated pea seedling grows, cell division and cell elongation occur. The growth up to 1 cm from the top is mainly by cell division with little elongation (18). That the *pra2* protein was at a higher concentration at 0.75–2.25 cm from the top than at 0–0.75 cm from the top suggests that the *pra2* protein is correlated with cell elongation rather than cell division. Thus, the *pra2* protein was abundant in the stem zone of rapid growth but the *pra3* was found in more uniform concentrations everywhere.

DISCUSSION

YPT/rab proteins act as molecular switches that can flip between two conformational states—an active, membrane-localized state with GTP bound and an inactive, soluble state with GDP bound—and they function as regulators of vesicular transport. *YPT/rab* proteins operate in a cycle that typically depends on two auxiliary components: a guanine-nucleotide-releasing protein to catalyze exchange of GDP for GTP and a GTPase-activating protein to trigger the hydrolysis of the bound GTP. Hence the activity of *YPT/rab* protein is regulated by various factors. Although the activity is not always dependent on the absolute amount of this protein, the protein level

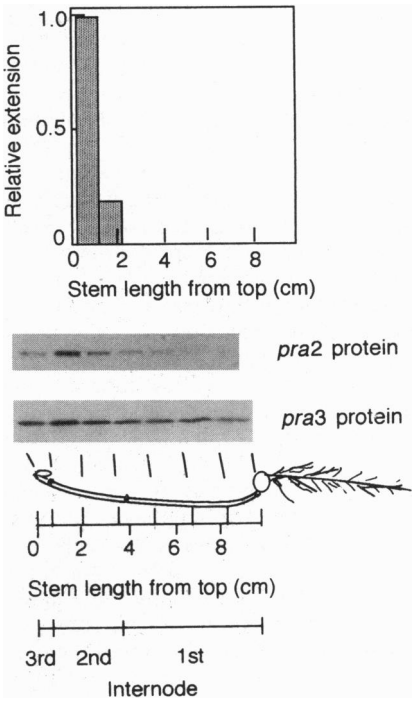

FIG. 4. Relationship between stem extension and *pra2* and *pra3* protein levels of 6-day-old seedlings. Stem extension in 6-day-old seedlings was measured for 6 h. Starting 0.3 cm below the hook, the stem was marked every 1 cm with India ink 3 h before protein extraction was done. Six hours later, the stem length of each marked section was measured with a ruler. The 1-cm measurement zones on the graph correspond to the start of the 6 h. The mean of the results from 24 measurements was calculated. The maximum extension value was taken as 1 and each value was plotted against the marked position at the start. The first 0.3 cm from the hook did not extend. Six-day-old stems grown in the dark were cut into seven parts starting below the hook as shown. The part with the third internode was about 0.75 cm long. The other parts were 1.5 cm long, as indicated. Total proteins from these parts were extracted and probed with anti-PRA2 or anti-PRA3 IgG. The same amount of protein (30 μg) was put in each lane.

is still one of the important factors for its function in regulation. The changes in *YPT/rab* protein levels may have some important consequences for vesicular transport.

Most of the *YPT/rab* genes identified so far seem to be expressed at all times (19), but several genes show organ specificity of expression (5, 20, 21). These *YPT/rab* proteins are in a restricted subcellular compartment and regulate distinct vesicular transport events at the level of membrane docking and fusion (22). Our results showed that the light-repressible *pra2* protein, which belongs to the *YPT/rab* family, is most abundant in the region of rapid growth in etiolated stems and that the protein disappears when the seedlings are illuminated. To our knowledge, this protein is the first small GTP-binding protein to be found to be at its highest concentration in the growing region of etiolated stems and to decrease in relation to total protein with irradiation. These findings suggest that the *pra2* protein may regulate vesicular transport that is active in etiolated stems, especially in the growing zone, and that such transport is inhibited by illumination. The vesicular transport in which the *pra2* protein is involved is most likely that related to stem growth because it is limited to growing regions in etiolated seedlings. Usually plant growth is an irreversible increase in cell volume by a factor of 10 to >100 times, accompanied by vacuole enlargement and changes in the cell wall. Rapid changes in cell-wall and vacuole components are essential for the elongation of etiolated stems. Cell-wall components such as polysaccharides are synthesized in the Golgi

apparatus and transported into the cell wall (23, 24). Vacuole enlargement is caused by increases in the concentration of the osmotically active solutes that are transported into the vacuole. The *pra2* protein may be a molecular switch needed for etiolated stems to transport certain components into the cell wall or vacuoles, leading to cell growth.

The protein with the most sequence identity with the *pra2* protein in the present data bases, such as National Biomedical Research Foundation/Protein Identification Resource, Swiss-Prot, and GenBank, is the *pra3* protein and that of the *pra3* protein is a small GTP-binding protein of rice, *rgp1* protein. The *rgp1* gene was identified by differential screening of a cDNA library of dwarf rice; this gene is expressed in lower concentrations in dwarf plants than that in the wild type and is related to dwarfism (4). The sequence identity of *rgp1* protein with the *pra3* and *pra2* proteins was 82% and 69%, respectively. Even the C-terminal hypervariable region of the *pra3* protein is about 60% the same as that of the *rgp1* protein (7), and *pra3* is likely to be a counterpart of *rgp1*, probably related to dwarfism. Expression of the *pra2* and *pra3* genes was different; both genes were expressed mainly in the stems of dark-grown seedlings, but the *pra3* gene was expressed also in other organs and in illuminated stems. Although the sequence identity of the *pra2* and *pra3* proteins is 72%, the sequences were identical in about 180 amino acids of the N-terminal end; a sequence about 40 residues long of the C-terminal hypervariable region of the *pra2* protein is completely different from that of the *pra3* protein (7). The C-terminal region of *YPT/rab* proteins is responsible for their unique cellular locations (25) and probably regulates the assembly of docking–fusion complexes at different steps of transport (26). That the *pra3* protein is found throughout much of the seedlings suggests that it has general functions in biosynthetic and secretory pathways that the *pra2* protein does not.

The findings that mRNA levels of *pra2* and *pra3* are down-regulated by phytochrome (8) and that the *pra2* and *pra3* proteins decreased gradually with illumination suggest that a decrease in these proteins may be related to the photoinhibition of stem growth mediated by phytochrome. However, at present it is too early to suggest a causal relationship between photoinhibition or stem growth and the loss of these proteins from these results. Photoinhibition of stem growth by red light is at a maximum 2 h after the start of irradiation (11). Under the conditions we used, inhibition by continuous white light occurred within 3 h (data not shown), which was more rapid than the decrease of *pra2* and *pra3* proteins. Even the *pra2* protein existed after the cessation of stem elongation. However, the activity of *YPT/rab* protein is controlled by several components. Possibly a light-dependent change in membrane association, in which *pra2* and *pra3* proteins are involved, may be more rapid and may be more related to the photoinhibition than the loss of the protein itself. The loss of the protein may bring the prolonged photoinhibition without reversibility. Total phytochrome in etiolated pea stems is abundant in the upper part of the top internode (27), where the *pra2* protein is located, although we do not know which molecular species of the phytochrome family is involved in the phytochrome-dependent expression of *pra2*. Perhaps phytochrome inhibits stem growth of dark-grown seedlings partly by regulating the expression of *pra2* and *pra3*. It is important for plants to develop long internodes rapidly in the soil where it is dark, in order to expose leaf primordia to sunlight. It is then important to reduce stem elongation when apical buds come out of the ground and start photosynthesis. Such important steps may be carried out partially by regulation of these small GTP-binding proteins by phytochrome.

We thank Mr. N. Murai and T. Tsunokawa for their technical assistance and Dr. R. Matsuno for encouragement. This work was supported by Grants-in-Aid for Scientific Research on Priority Areas (06278211 and 06259210) from the Ministry of Education, Science, and Culture, Japan.

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