

# Phytochrome-regulated expression of the genes encoding the small GTP-binding proteins in peas

(gene expression/light regulation/light-repressible gene/*ras*-related gene/red/far-red reversibility)

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**ABSTRACT** We examined the effect of light on the mRNA levels of 11 genes (*pra1*–*pra9A*, *pra9B*, and *pra9C*) encoding the small GTP-binding proteins that belong to the *ras* superfamily in *Pisum sativum*. When the dark-grown seedlings were exposed to continuous white light for 24 hr, the levels of several *pra* mRNAs in the pea buds decreased: *pra2* and *pra3* mRNAs decreased markedly; *pra4*, *pra6*, and *pra9A* mRNAs decreased slightly; the other 6 *pra* mRNAs did not decrease. We studied the kinetics of mRNA accumulation for *pra2*, *pra3*, and *pra9B* in detail during white light illumination and compared them with those of the phytochrome gene and the small subunit gene of ribulose biphosphate carboxylase: mRNA levels of *pra2* and *pra3* decreased in a manner similar to that of phytochrome while that of the small subunit increased as was expected. The decreases were triggered by a 2-min monochromatic red light (660 nm) irradiation. The effect of red light was reversed by subsequent exposure to far-red light, indicating an involvement of phytochrome as a photoreceptor in this light-regulated event. This work reports negative regulation of mRNA levels of small GTP-binding proteins by light, mediated by phytochrome.

Small GTP-binding proteins that belong to the *ras* superfamily are molecular switches that are turned on by GTP and off by hydrolysis of GTP to GDP (1–4). Although their precise functions remain unclear, small GTP-binding proteins have been shown to participate in cell proliferation and differentiation, vesicular transport, cell polarity and cytoskeleton integrity, and scaffolding of actin microfilaments in yeast and animal cells (1–4). Studies on small GTP-binding proteins in plant cells are not as far advanced, although several proteins (5, 6) and several genes (7–13) have been found in various higher plants. These genes probably regulate various cellular activities, as has been shown in yeast and animal cells, but little is known about their function except that a rice gene, *rgp1*, has been shown to be related to dwarfism (10, 14). One approach to understanding their function is to study the signals affecting their gene expression. Among several environmental factors known to affect plant gene expression, light is a critical environmental signal and only a few photoreceptors are involved in the reception of the signal: the phytochrome family for red light and separate photoreceptors for blue/UV-A and UV-B light. Light absorbed by all three photoreceptor systems triggers alterations in gene expression (15, 16). Among them, the phytochrome family is well characterized and mediates expression of a number of genes in positive and negative manners (17, 18).

We have recently cloned and characterized 11 cDNAs (*pra1*–*pra9A*, *pra9B*, and *pra9C*) encoding small GTP-binding proteins from pea (*Pisum sativum*) leaves and found a diversity of their expression in roots and leaves (19). These

11 cDNAs as well as the genes reported in plants all belong to the *YPT/rab* subfamily of the *ras* superfamily (19). Several *YPT/rab* genes are known to be involved in intracellular traffic or cell proliferation in animals and yeast (20–22), but the function of plant *YPT/rab* genes is still unknown. In the present study, we examined the effect of light on the expression of the genes encoding the small GTP-binding protein in etiolated pea buds and found that light regulates the changes in steady-state levels of a few mRNAs and that phytochrome mediates the changes in a negative manner.

## MATERIALS AND METHODS

**Plant Materials and Light Treatment.** Seeds of *P. sativum* cv. Alaska (Snow Brand Seed, Sapporo, Japan) were imbibed in darkness at 25°C for 12 hr and were sown on irrigated vermiculite in a plastic container (100 seeds per 10 × 15 cm plane of a container) under dim green light. They were kept in complete darkness at 21°C ± 1°C for 5 days and then subjected to light treatment.

For continuous white light irradiation, white light fluorescent tubes (Hitachi; FL2DSSD/18-G) were used at an intensity of 22.3 W/m<sup>2</sup>. For brief red light irradiation, monochromatic light with peak emission at 660 nm was exposed for 2 min at an intensity of 30.5 μmol·m<sup>-2</sup>·sec<sup>-1</sup> (measured by Li-cor quantum sensor LI-190SB). For brief far-red light irradiation, monochromatic light with peak emission at 750 nm was exposed for 5 min at an intensity of 36.5 μmol·m<sup>-2</sup>·sec<sup>-1</sup> (measured by MIR-100Q thermopiles; Mitsubishi Oil Chemicals, Tokyo). A pair of modified slide projectors (Cabin III) each equipped with a 300-W halogen lamp was used as a source. This light was filtered through a combination of a red-interference filter (DIF-BPF-2; λ<sub>max</sub> = 660 nm; Vacuum Optics, Tokyo) and a heat-cut filter (CF-B) to obtain red light and was filtered through a far-red interference filter (DIF-BPF-2; λ<sub>max</sub> = 750 nm) and a long-wavelength heat-cut filter (CF-A) to obtain far-red light.

After various light treatments, apical buds were harvested, frozen immediately in liquid nitrogen, and stored at –80°C until RNA extraction. All manipulations after sowing, except for experimental light treatment, were done in complete darkness with the use of an IR scope (Hamamatsu Photonics, Hamamatsu, Japan, C2550) equipped with a long-wavelength far-red illuminator (>850 nm; Toshiba IR-D cut-off filter was used) if necessary. The temperature was kept at 21°C throughout the experiments.

**Preparation of RNA and Measurement of mRNA Abundance.** Total RNA was extracted from ≈100 apical buds and poly(A)<sup>+</sup> RNA was isolated by chromatography on oligo(dT)-cellulose as described (23). For measurement of the level of specific mRNA, RNA was denatured with glyoxal and dimethyl sulfoxide and slot-blotted onto nylon membranes (BNRG, Pall). A series of 4, 2, and 1 μg of the same poly(A)<sup>+</sup> RNA was loaded on membranes for the measurement of *pra*

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mRNAs, and 1, 0.5, and 0.25  $\mu\text{g}$  of the poly(A)<sup>+</sup> RNA or 2, 1, and 0.5  $\mu\text{g}$  of total RNA was loaded in series on membranes for the measurements of *phy* and *rbcS* mRNAs. For RNA gel blot analysis, 1 or 3  $\mu\text{g}$  of poly(A)<sup>+</sup> RNA was subjected to electrophoresis in 1% agarose/formamide gels and blotted onto nitrocellulose membranes. The membranes were then baked *in vacuo* at 80°C for 2 hr.

3'-End-specific probes were prepared as described (19). The probes for *phy* and *rbcS* were the 2-kb *Hind*III fragment of *phy* cDNA (A609) from pea (24) and the 0.75-kb *Cla* I fragment of *rbcS* cDNA (pGR407) from pea (25). The probes labeled with [ $\alpha$ -<sup>32</sup>P]dCTP using a T7 Quick Prime kit (Pharmacia) were purified through a Quick Spin column (Sephadex G-50; Boehringer Mannheim), heat-denatured, and added to hybridization buffer containing 6 $\times$  standard saline citrate (SSC), 50% formamide, 5 $\times$  Denhardt's solution, 0.25 mg of sonicated salmon sperm DNA per ml, and 0.3% SDS. The hybridization solutions were incubated with membranes at 42°C for 3 hr to eliminate the DNA fragments that nonspecifically bind to the membranes. The solutions were collected, heat-denatured again, and then used for hybridization. The RNA-loaded membranes were prehybridized and hybridized for 12 hr at 42°C each. After hybridization, the membranes were washed four times in 2 $\times$  SSC/0.1% SDS at room temperature for 5 min each, and then two times in 0.1 $\times$  SSC/0.1% SDS at 42°C for 15 min each. The dried membranes were autoradiographed for an appropriate time.

The values for specific mRNA were obtained by slot-blot analysis and scanning of the autoradiograms with a Shimadzu CS-930 densitometer. After confirming the linearity of the values for 0–4  $\mu\text{g}$  of RNA, the mean of the results from six to nine measurements was calculated for each RNA preparation. The standard error of the results from each RNA preparation was within 10%. The mean value from two independent RNA preparations was plotted.

## RESULTS

### Effect of White Light on Steady-State Levels of *pra* mRNAs.

To examine the effect of white light on mRNA level of the genes encoding small GTP-binding proteins, 5-day-old pea seedlings grown in darkness were exposed to white light for 24 hr, and poly(A)<sup>+</sup> RNAs were prepared from the apical

buds. Steady-state levels of *pra* mRNAs before (Fig. 1, lanes E) and after (lanes L) white light treatment were compared with that of the dark control (lanes D) by RNA gel blot hybridization using 3'-end-specific probes for *pra1*–*pra9A*, *pra9B*, and *pra9C*. We used the small subunit gene of ribulose biphosphate carboxylase (*rbcS*) and the phytochrome gene (*phy*) as a positive and a negative control of gene expression responsive to light, respectively.

A single band with a molecular mass of  $\approx$ 1-kb RNA was observed for each *pra* probe (Fig. 1), suggesting the presence of mature mRNA for each gene. In etiolated 5-day-old buds, all 11 cDNAs were expressed (lanes E), and the mRNA levels did not change after further 24-hr dark treatment (lanes D). Upon white light irradiation (lanes L), mRNA levels of *pra2* and *pra3* decreased as markedly as that of *phy*; that of *pra4*, *pra6*, and *pra9A* slightly decreased, and the other 6 *pra* mRNA levels remained unchanged. mRNA levels of *rbcS* and *phy* in the same RNA preparations changed as reported elsewhere (26, 27). Thus, the effect of white light differed among *pra* genes, and expression of a few mRNAs encoding small GTP-binding proteins was markedly repressed by continuous white light.

To obtain an appropriate band intensity, the indicated amount of poly(A)<sup>+</sup> RNA and exposure time was used (Fig. 1). The relative amounts of mRNA could be estimated from these values and the band intensity, although an exact comparison cannot be made because other factors such as specific activity of the probe and its length also affect the band intensity. The *rbcS* mRNA level was most abundant, and the *phy* mRNA level was lower than that of *rbcS* but more abundant than those of *pra* genes. *pra* mRNA levels were less than  $\approx$ 1/20th that of *phy*. The *pra* mRNA was not detectable in the total RNA preparations because of its low abundance, but it could be detected in poly(A)<sup>+</sup> RNA preparations.

**Changes in mRNA Levels During Continuous White Light Irradiation.** Because mRNA levels of *pra2* and *pra3* decreased markedly after 24 hr of continuous white light irradiation, the time course of mRNA levels in a fixed amount of poly(A)<sup>+</sup> RNA was followed by slot-blot analysis for 24 hr. For comparison, the mRNA level of *pra9B*, which did not change after 24 hr of irradiation (Fig. 1), and those of *rbcS* and *phy* were also measured. To determine whether mRNA levels in a fixed

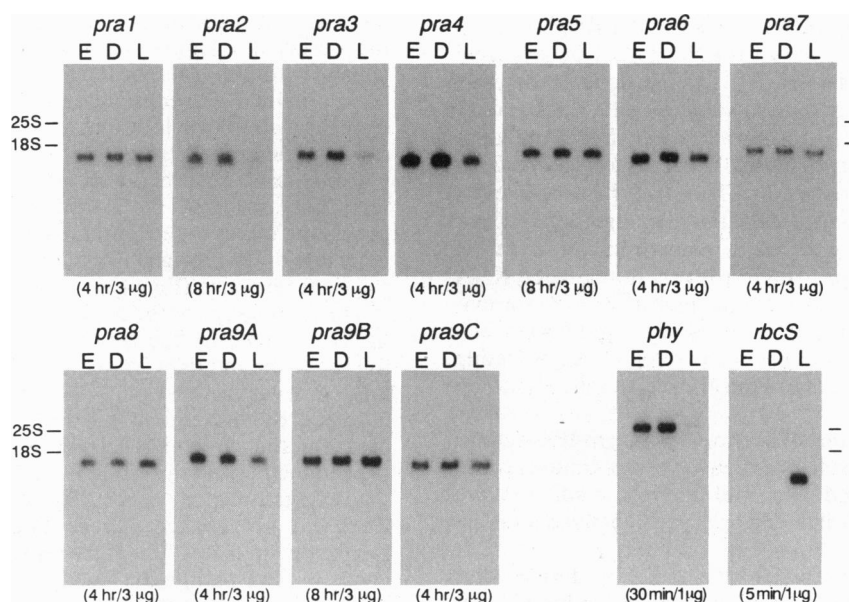


FIG. 1. RNA gel blot analyses of effect of continuous white light on *pra* mRNAs. Five-day-old etiolated seedlings (lanes E) were exposed to continuous white light for 24 hr (lanes L) or kept in further darkness for 24 hr (lanes D). *pra* mRNA accumulations in poly(A)<sup>+</sup> RNA extracted from the buds were analyzed with 3'-end-specific probes. Analyses were also done for *phy* and *rbcS* for reference. The conditions used were the same except for exposure time for autoradiography and the amounts of poly(A)<sup>+</sup> RNA, which are indicated in parentheses.

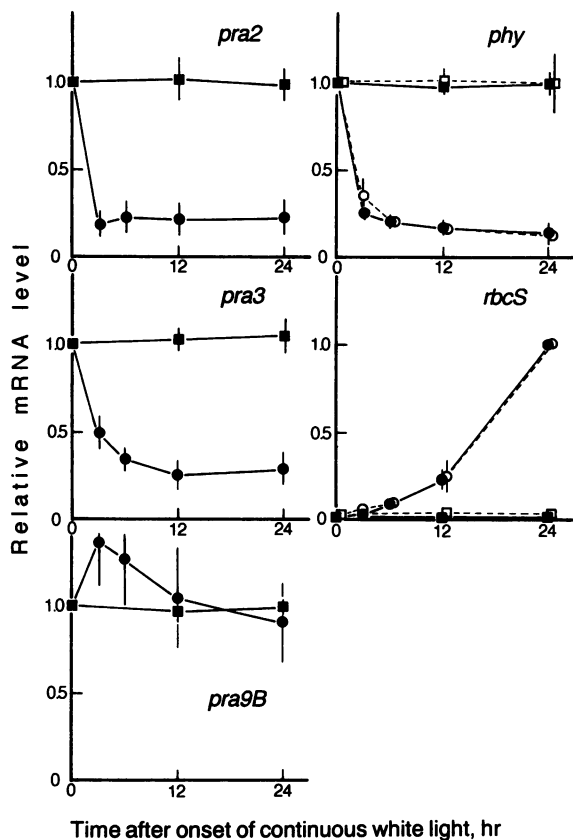


FIG. 2. Time course of the steady-state levels of *pra* mRNAs during continuous white light irradiation. Five-day-old etiolated seedlings were exposed to continuous white light (● and ○) or kept in darkness (■ and □) for the indicated time. The steady-state levels of mRNA in total RNA (○ and □) and poly(A)<sup>+</sup> RNA (● and ■) were measured by slot-blot analysis and expressed relative to the level before white light illumination except for *rbcS*. The level of *rbcS* mRNA was expressed relative to the level after 24-hr irradiation. Each plotted value represents the mean of the results from two independent RNA preparations, and error bars indicate deviation.

amount of poly(A)<sup>+</sup> RNA reflect those in total RNA, we also measured *rbcS* and *phy* mRNA levels in total RNA.

Upon white light irradiation, mRNA levels of *pra2* and *pra3* decreased markedly (Fig. 2). In particular, the *pra2* mRNA level reached a minimum value of ≈20% of the dark control within 3 hr and changed in a manner similar to that of *phy*. The *pra3* mRNA level gradually decreased and reached the minimum value after 12 hr. The *pra9B* mRNA level, which increased slightly within the first 6 hr and then reached the dark control level, showed a minor fluctuation during illumination. *rbcS* and *phy* mRNA levels in the same RNA preparation changed in the same manner as reported previously (26, 27). The profiles obtained by use of poly(A)<sup>+</sup> RNA were the same as those obtained using total RNA, indicating that the observed profiles of *pra* mRNAs reflect the alteration in total RNA.

**Changes in mRNA Levels After Brief Red Light Irradiation.** To examine whether the changes caused by continuous white light (Fig. 2) are triggered by a brief red light irradiation, we measured the mRNA level after a brief red light irradiation by slot-blot analysis.

The *pra2* mRNA level decreased to 20% of the dark control within 3 hr, and then gradually recovered to the initial level (Fig. 3). *pra3* mRNA decreased to 50% of the dark control within 3 hr and *pra9B* slightly increased for the first several hours. *phy* and *rbcS* mRNAs responded to light as was expected (27, 28). The *phy* mRNA in a fixed amount of

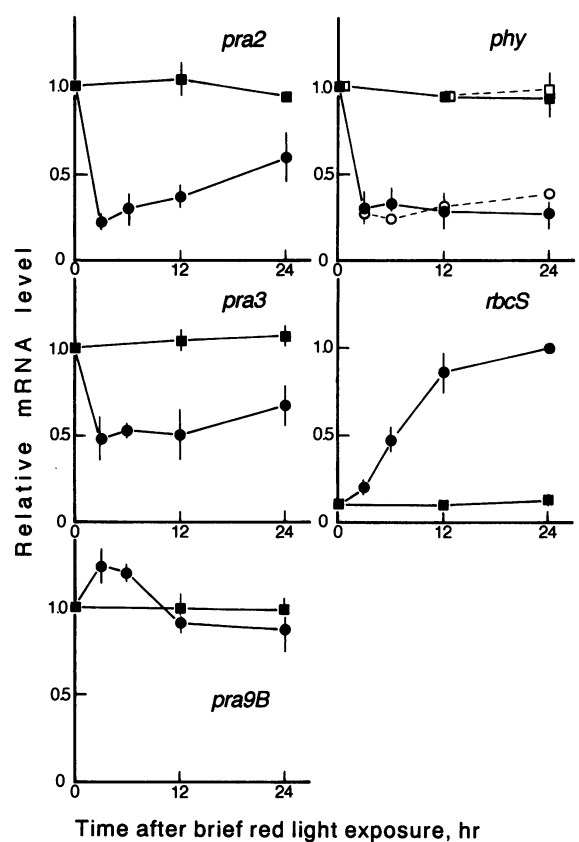


FIG. 3. Time course of the steady-state levels of *pra* mRNAs by brief red light irradiation. Five-day-old etiolated seedlings were exposed to red light for 2 min and then returned to darkness (● and ○) or were kept in darkness (■ and □) without exposure. Total RNA and poly(A)<sup>+</sup> RNA were prepared from the buds at the indicated time. The steady-state levels of mRNA in total RNA (○ and □) and poly(A)<sup>+</sup> RNA (● and ■) were measured by slot-blot analysis and expressed relative to the level before irradiation except for *rbcS*. The level of *rbcS* was expressed relative to the level 24 hr after irradiation. Each plotted value represents the mean of the results from two independent RNA preparations, and error bars indicate deviation.

poly(A)<sup>+</sup> RNA and of total RNA showed a similar profile and the observed changes for *pra* mRNA levels reflected the change in total RNA. These profiles were basically similar to those obtained with continuous white light, indicating that the changes caused by white light are triggered by a brief red light and suggest an involvement of phytochrome.

**Red/Far-Red Reversibility of *pra* mRNA Levels.** If phytochrome mediates the light-regulated expression of *pra* genes, the effect of red light must be reversed by subsequent exposure to far-red light. We examined the reversibility of the red light response (Fig. 4).

The repressions of the mRNA levels of *pra2* and *pra3* were partly reversed by the far-red light irradiation immediately following the red light irradiation. The repression of the *phy* mRNA level in the same RNA preparation was similarly reversed by subsequent exposure to far-red light. The increase in the *rbcS* mRNA level, which is known to be a red light-inducible and far-red light-reversible response, showed the reported responses (28). By contrast, the *pra9B* mRNA level did not change. These findings indicate that phytochrome mediates the light-repressed change in mRNA levels of *pra2* and *pra3*.

## DISCUSSION

The small GTP-binding proteins possess intrinsic GTPase activity and function as molecular switches that are active when GTP is bound to them and inactive in the GDP-bound

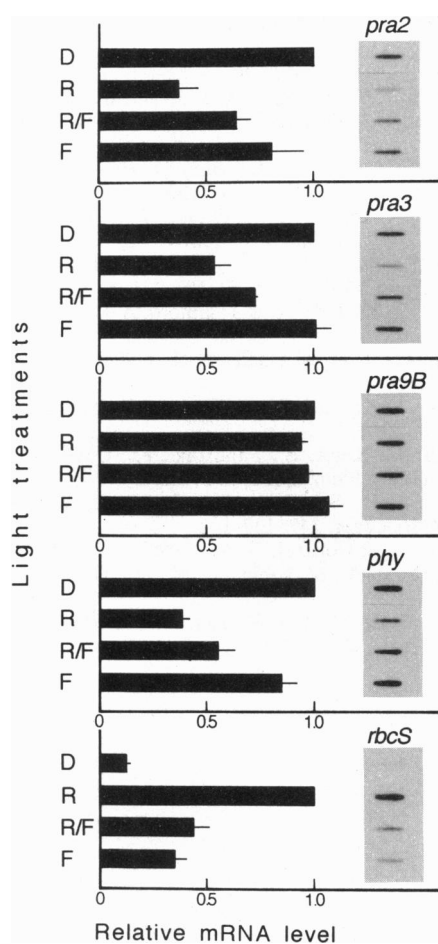


FIG. 4. Red/far-red reversibility of the change in the steady-state levels of *pra* mRNAs. Five-day-old etiolated seedlings were exposed to 2 min of red light (660 nm;  $3.7 \times 10^3 \mu\text{mol}$  of photon per  $\text{m}^2$ ) and/or 5 min of far-red light (750 nm;  $1.1 \times 10^4 \mu\text{mol}$  of photon per  $\text{m}^2$ ), and then kept in darkness for 12 hr. The steady-state level of mRNA was measured by slot-blot analysis and expressed relative to the level of the dark control except for *rbcS*, which is expressed relative to the level of red light treatment. One example of slot-blot analysis is shown in the right column. Each plotted value represents the mean of the results from two independent RNA preparations, and error bars indicate deviation. Red/far-red reversibility for *pra2*, *pra3*, *phy*, and *rbcS* mRNA levels was observed for each RNA preparation. D, dark control; R, red light treatment; R/F, far-red light treatment immediately after red light treatment; F, far-red light treatment.

form (1–4). To understand their biological function, it is important to study cellular factors that interact with these proteins. However, studies on its gene expression are also important to understand the function. The mRNA levels of two tobacco genes encoding these proteins were not affected by light (12). In the present report, we show evidence that light decreased the steady-state levels of a few mRNAs encoding these proteins in peas. This work reports that mRNA levels of these proteins are regulated by light. Although we do not know the precise function of *pra2* and *pra3* genes, various cellular activities regulated by these gene products probably are affected by the alteration of the amounts of *pra2* and *pra3* mRNAs upon illumination.

One of the most rapid cases of transcriptional down-regulation reported for any higher eukaryotic gene is *phy* (29). Transcription of the *phy* gene in oats is rapidly and directly regulated by the phytochrome signal transduction chain (29). In the case of *pra2* and *pra3* genes, the amounts of their mRNA are so scarce that we could not examine the transcription activity in isolated nuclei. It remains to be deter-

mined whether the light-regulated changes in mRNA levels of *pra2* and *pra3* reflect a transcriptional response and whether the changes are directly regulated by the phytochrome signal transduction chain. However, the fact that *pra2* and *pra3* mRNAs decreased in a profile similar to that of *phy* (Figs. 2 and 3) suggests a possibility that the *pra* genes also belong to rapidly responsive genes like *phy*.

The red/far-red reversibility of the response of *pra2* and *pra3* genes (Fig. 4) indicates that phytochrome is a photoreceptor of this response. Light absorbed by phytochrome affects expression of many genes in a positive manner and that of a few genes in a negative manner by way of unknown signal transduction pathways (15, 18). The negatively regulated genes reported are *phy* itself in pea (30), oat (29), and rice (31); protochlorophyllide reductase in barley (32); asparagine synthetase in pea (33); and three genes of unknown function in *Lemna* (34). There are several genes negatively regulated by light whose receptors are unknown. Two kinds of the transcripts encoding putative protein kinases possibly involved in signal transduction decrease on white light illumination (35). Whether the *pra2* and *pra3* gene products are involved in the signal transduction pathway remains unknown, but the regulation of expression of the protein kinase genes and the *pra* genes could play key roles for light-induced changes in various cellular activities leading to photomorphogenesis.

Among the 11 genes, only the mRNA levels of *pra2* and *pra3* showed a marked response to light. We successfully cloned these two cDNAs from a leaf library (19), which might not be cloned from a library of seedlings irradiated with continuous light. The structural features of *pra3* protein discussed in our previous report (19) are similar to those of other plant ras-related proteins reported, but those of the *pra2*-encoded protein are different in the domain involved in the GTP/GDP-binding and the C-terminal motif. The organ-specific expression of the *pra* genes reported previously (19) shows that the *pra2* mRNA level is more abundant in leaves than in roots, while most *pra* mRNAs are more abundant in roots than in leaves or almost the same amounts of mRNA are present in both organs. These findings taken together with the present findings suggest that *pra2* and *pra3* play different roles for cellular regulation, with *pra2* playing a leaf-specific role.

We previously found several GTP-binding proteins in pea leaves, outer envelope membrane of chloroplasts, and membrane fractions of leaves (6), but the cDNA encoding the respective protein has not yet been identified. Our continuing studies are aimed at characterizing the localization of *pra* gene products as well as searching for proteins that interact with *pra2*- and *pra3*-encoded proteins.

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