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Phytochrome Activities and Biological Activities of Genes for Phytochrome A of Horseradish in Horseradish Hairy Roots.

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Abstract

Full-length cDNAs of *ArPHYAs* were fused with the 35S promoter of cauliflower mosaic virus (CaMV35S*ArPHYAs*) and introduced into horseradish hairy roots. The phytochrome levels in hairy roots that had been transformed with CaMV35S*ArPHYAs* were about 2-3 times higher than those in normal hairy roots. Though ArphyAs were photochemically active in horseradish hairy roots, the efficiency of ArphyAs on shoot formation was different among them. The efficiency of light-induced formation of adventitious shoots in horseradish hairy roots was greatest for ArphyA3 and smallest for ArphyA2. These results indicated that *ArPHYAs* isolated from horseradish encoded photochemically active phytochromes and that they were biologically active in horseradish.

Key words: adventitious shoot, hairy root, horseradish, light induction, phytochrome A.

Introduction

Light serves as an environmental signal and energy source, and regulates growth and differentiation of plants during processes such as seed germination, greening and flowering. In plants, light signals are known to be received by different photoreceptors, i. e. phytochromes, cryptochromes and phototropins (Chory, 1997; Huala *et al.*, 1997; Kagawa *et al.*, 2001). Recent progress of molecular biology proved the presence of phytochrome genes in a variety of plants and at least five different genes (phytochrome A-E) in higher plants (Sharrock and Quail, 1989; Clack *et al.*, 1994). The functions of the major two members, phytochrome A (phyA) and phytochrome B (phyB), have been well studied using mutants and transformants of *Arabidopsis* (Quail *et al.*, 1995). PhyA is abundant in etiolated tissues, and decreases drastically under light illumination. The abundance of phyB, on the other hand, is constant in etiolated and green tissues although its amount is one fifth of that of phyA in etiolated tissues.

In horseradish, light induces the formation of adventitious shoots from various organs, for example, cultured roots and leaves, roots of whole plants and hairy roots, that are types of adventitious roots formed by infection with *Agrobacterium rhizogenes*. As hairy roots of horseradish are more sensitive to light compared to other organs (Saitou *et al.*, 1992; Kamada *et al.*, 1995), we investigated the effects of light on adventitious shoot formation in horseradish. Since the action spectrum of this phenomenon has peaks in the red and the blue regions and the induction is reduced by subsequent irradiation with far-red light, we proposed the involvement of phytochromes in the induction of adventitious shoots (Saitou *et al.*, 1993). We isolated genes for phytochrome A (*ArPHYAs*) and characterized their expression (Saitou *et al.*, 2000) by screening and selection of cDNA library comprised of the proximal areas of hairy roots in which the extent of light-induced formation of adventitious shoots was higher than other areas. In previous work (Saitou *et al.*, 1999a), a gene for phytochrome A of *Arabidopsis thaliana* was expressed in horseradish hairy roots. Photochemically

active phytochrome was detected in the hairy roots and increased levels of phytochrome increased the extent of light-induced formation of adventitious shoot. In this report, we introduced *ArPHYAs* to horseradish hairy roots to investigate whether these genes have photochemical activity and biological activity of phytochrome.

Materials and Methods

Plant materials and transformation with Agrobacterium

Leaves and petioles of horseradish (*Armoracia rusticana* Gaert., Mey. et Scherb.) were sterilized according to the method described by Saitou *et al.* (1992) and axenic plants were propagated by culture of explants from leaves, petioles and roots on phytohormone-free Murashige and Skoog's (MS) medium (Murashige and Skoog, 1962) semi-solidified with Gelrite (0.2%; Wako Pure Chemical Industry Ltd.) with 16 h of light and 8 h of darkness daily (light intensity, 1.9 Wm^{-2}) at 25 ℃. Adventitious shoots formed on these explants were transferred to fresh semi-solidified MS medium and cultured under the same conditions. These axenic plants were infected directly with *Agrobacterium tumafaciens* strain R1000 harboring various kinds of plasmids using a needle. Independent lines of hairy roots selected by kanamycin were cultured on phytohormone-free MS medium with kanamycin ($50\ mgl^{-1}$) in darkness at 25 ℃ (Saitou *et al.*, 1999a).

Record of the extent of shoot formation

Distal ends (1 cm) of hairy roots were cultured for 16 weeks in darkness on the same medium with kanamycin. The dark-cultured hairy roots were subjected 16 h of light and 8 h of darkness daily (light intensity, 3.0 Wm^{-2}) at 25 ℃. The frequency of light-induced formation of adventitious shoots is equal to the number of explants forming shoots divided by the total number of explants, and the average number of shoots is equal to the total number of shoots divided by the total number of explants.

Fusion of ArPHYAs with the 35S promoter of cauliflower mosaic virus

Full-length cDNAs of *ArPHYA1, ArPHYA2* or *ArPHYA3* (Saitou *et al.*, 2000) in pBluescript II SK^+ (Stratagene) were excised with both *Bam*HI and *Sac*I, and the fragments were cloned in the sense direction (CaMV35S*ArPHYAs*) with pBI121 (Clontech) which had also been digested with both *Bam*HI and *Sac*I. The resultant plasmids (pBITS35S*ArPHYAs*) were transferred to *E. coli* (HB101) and then transferred to *A. tumefaciens* strain R1000 that harbored pRiA4b by triparental method with *E. coli* (pRK2013) as the helper. *A. tumefaciens* strain R1000 harboring pBITS35S-*ArPHYAs* or pBI121 was used to infect axenic leaves and petioles of horseradish.

Spectrophotometric measurements of phytochrome in tissues

Distal ends (1 cm) of hairy roots were cultured in darkness for 16 weeks and about 0.5 gram of the segments excised from various areas of the hairy roots were pooled and put into a custom-made cylindrical cuvette (diameter 10 mm). This was then placed in a custom-made cuvette holder set in a Hitachi spectrophotometer 557. Irradiation of the sample in the cuvette was performed *in situ* with a hand-made mirror-operated apparatus. The sample was exposed to actinic red and far-red light supplied from a 250 W tungsten-iodine lamp filtered through 5 cm water and a 660 and 740 nm interference filter (Japan Vacuum Optics Co.), to give flux rates of 50 and 60 Wm^{-2}, respectively (Saitou *et al.*, 1999b). After the measurements, the fresh weight of the sample was measured. To quantitatively determine the amount of phytochromes in the tissues, the concentration of phytochrome(s) in the tissues was measured and expressed as Δ (Δ A) [absorption unit per gram fresh weight (au gfw^{-1})], where Δ (Δ A) is the absorption difference between absorption at the Pr (red-absorbing form of phytochromes) and the Pfr (far-red-absorbing form of phytochromes) peaks of an absorption difference spectrum normalized to one gram fresh weight of the tissues. All procedures were performed under dim green safety light obtained by either 40 W fluorescent tubes (Toshiba FL 40S W) or portable incandescent lamps wrapped with two layers of dark green and one layer of Italian blue plastic films (4421C and 4515C, Nakagawa Chemical Co.).

Result and Discussion

Phytochrome level in transformed hairy roots with CaMV35SArPHYAs

We obtained more than 21 clones of hairy roots that had been transformed with CaMV35S*ArPHYAs*. Phytochrome levels were measured photochemically in both control hairy roots (transformed with pBI121) and hairy roots transformed with CaMV35S*ArPHYAs* (**Fig. 1**). Distal ends of hairy roots (1 cm) were cultured for 16 weeks in darkness on MS medium with kanamycin. Since phytoch-

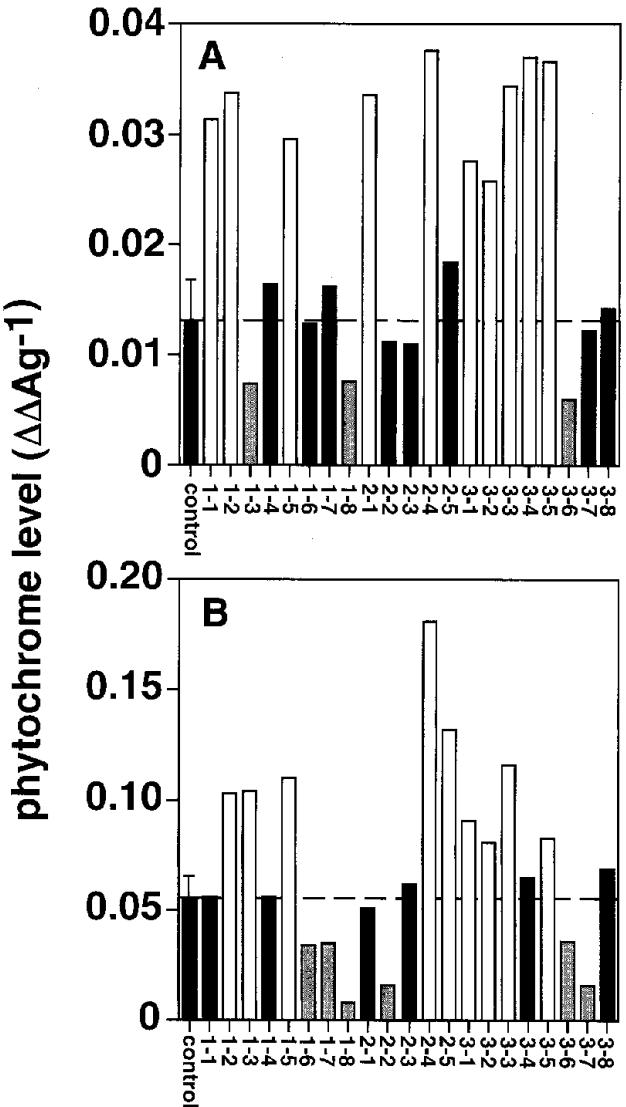

Fig. 1 Phytochrome levels in independent hairy root lines transformed with CaMV35S*ArPHYAs*. Distal ends (1 cm) of hairy roots were cultured in darkness for 16 weeks and distal areas (3 cm; A) or proximal areas (3 cm; B) were used for measurement of phytochrome levels. Hairy root lines whose names start with 1, 2 or 3 were transformed with CaMV35S*ArPHYA1*, CaMV-35S*ArPHYA2* or CaMV35S*ArPHYA3*, respectively. Open bars, solid bars or shaded bars showed higher, similar or lower levels of phytochrome than that in the control hairy roots, respectively. Experiments were repeated more than twice. Bars in the control samples show means ± SE.

rome levels varied along the hairy roots with maximum and minimum levels in proximal and distal areas, respectively (Saitou *et al.*, 1999b), proximal areas (3 cm) and distal areas (3 cm) of dark-cultured hairy roots were used for measurements. The levels of phytochrome in several lines of hairy roots transformed with each CaMV35S*ArPHYA* were about 2-3 times higher than those in control hairy roots in both distal (**Fig. 1A**) and proximal areas (**Fig. 1B**). These results indicate all three *ArPHYAs* encode photochemically active phytochrome.

On the other hand, the levels of phytochrome in a few lines transformed with each CaMV35S*ArPHYA* were lower than those in control hairy roots. The levels of phytochrome in these hairy roots were about half of those in the control hairy roots at the distal areas and about a half to a quarter at the proximal areas (**Fig. 1**). The hairy roots with lower levels of phytochrome were observed in the hairy roots transformed with three CaMV35S*ArPHYAs*.

The variety of phytochrome levels among hairy roots transformed with *ArPHYAs* was supposed to be caused mainly by that of mRNA levels transcribed from *ArPHYAs* owing mainly to the fact that in horseradish hairy roots phytochrome levels increased with increased levels of mRNA (Saitou *et al.*, 1999b, 2000). In addition, since transcription of a gene in a plant is sometimes suppressed when it is transformed to a plant, it was suggested that phytochrome genes might be suppressed in hairy roots that showed lower phytochrome levels than control hairy roots.

Formation of adventitious shoots in hairy roots transformed with CaMV35SArPHYAs

The same hairy root lines that were used for measurement of phytochrome levels were transferred to light conditions with a cyclic illumination comprising of 16 h light and 8 h dark. Both the frequency of adventitious shoot formation and the average number of shoots in both control hairy roots and hairy roots transformed with CaMV35S*ArPHYAs* increased with increased exposure of the culture to light (**Fig. 2**). Among hairy root lines overexpressing phytochrome in the proximal areas and/or in the distal areas, those showing a larger number of shoots than the control hairy roots were plotted in **Fig. 2B** (the same lines were used in **Fig. 2A**). Other hairy root lines with high levels of phytochrome showed similar light-induced shoot formations to control hairy roots (data not shown). The difference in frequencies between phytochrome-overexpressing and control hairy roots was small (**Fig. 2A**) because the frequency in the control hairy roots was more than 70% one week after the start of the culture exposed to light which close to the 100% level. The extent of light-induced formation of adventitious shoots was highest at the proximal areas in both phytochrome-overexpressing and control hairy roots. Furthermore, adventitious shoots were also formed nearby the proximal areas

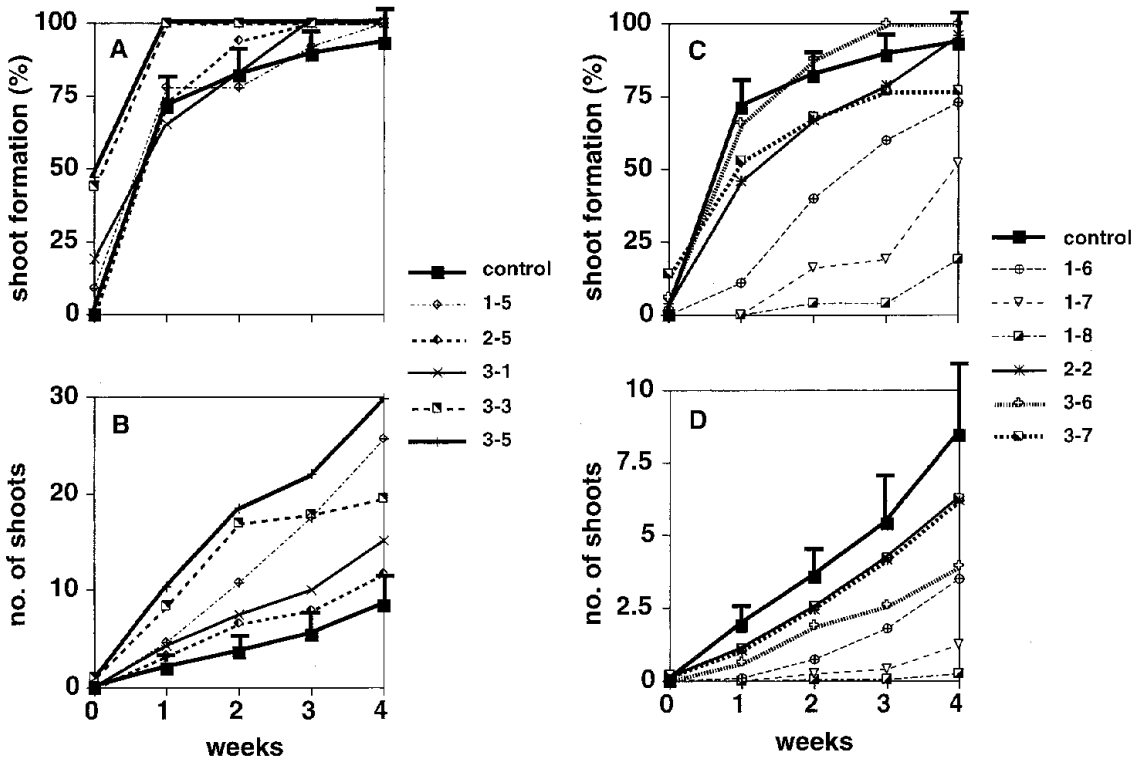

Fig. 2 The extent of light-induced formation of adventitious shoots in hairy roots transformed with CaMV35S*ArPHYAs*. The same hairy root lines used in **Fig. 1** were transferred to light and hairy roots with higher levels of phytochrome (A, B) or lower levels of phytochrome (C, D) and control hairy roots were cultured for 4 weeks. A and C, shoot formation (%) = total number of explants forming shoots / total number of explants; B and D, average number of shoots = total number of shoots / total number of explants. Hairy root lines whose names start with 1, 2 or 3 were transformed with CaMV35S*ArPHYA1*, CaMV35S*ArPHYA2* or CaMV35S*ArPHYA3*, respectively. Experiments were repeated more than twice with more than 10 samples. Bars in control samples show ± SE.

on hairy roots overexpressing phytochrome whereas adventitious shoots rarely formed in control hairy roots (data not shown).

In several lines of hairy roots, levels of phytochrome were lower than those in the control hairy roots (**Fig. 1**). In the hairy roots with lower levels of phytochrome, the extent of light-induced formation of adventitious shoots was less than that in the control hairy roots (**Fig. 2C, D**). In addition, adventitious shoots were formed only at the proximal ends in hairy roots with lower levels of phytochrome (data not shown). Furthermore, hairy roots and control with similar levels of phytochrome (**Fig. 1**) showed similar extents of shoot formation (data not shown).

Efficiency of increased phytochrome levels in hairy roots transformed with CaMV35SArPHYAs to the extent of shoot formation

Hairy root lines overexpressing *ArPHYA1* or *ArPHYA3* apparently showed higher extents of light-induced formation of adventitious shoots than the control hairy roots. On the other hand, hairy roots overexpressing *ArPHYA2*, especially hairy roots named 2-4 that showed highest levels of phytochrome among hairy roots transformed with CaMV35S*ArPHYAs*, did not form more adventitious shoots than the control hairy roots (**Fig. 1, 2**).

To compare the efficiency of ArphyAs on the extent of light-induced shoot formation in horseradish hairy roots, relative numbers of the extents with respect to the control hairy roots were compared among hairy root lines overexpressing phytochrome (**Fig. 3**). A hairy root line (3-5) transformed with *ArPHYA3* showed the highest efficiency to shoot formation. In addition, another line (1-5) transformed with *ArPHYA1* and the others (3-1, 3-3) transformed with *ArPHYA3* showed moderate efficiency. On the other hand, both hairy root lines (2-4, 2-5) transformed with *ArPHYA2* showed lower efficiency (**Fig. 3B**). The differences in shoot efficiencies among hairy roots transformed with CaMV35S*ArPHYAs* are most clearly illustrated by comparing the relative number of average shoots. These results suggested that ArphyA3 might be highly active to form adven-

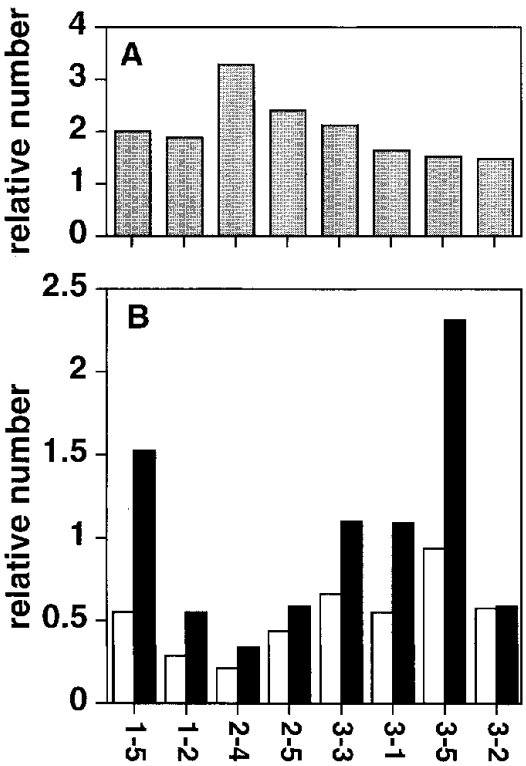

Fig. 3 The efficiency of phytochrome to shoot formation. The hairy root lines which showed increased levels of phytochrome in the proximal areas were analyzed in the extent of increased levels of phytochrome (A) and in the efficiency of phytochrome to the extent of shoot formation (B). Phytochrome levels shown in **Fig. 1B** were transformed to relative number where control hairy roots was designated as 1. Relative number of the frequency of shoot formation 1 week after the start of light irradiation (B, □) and relative number of the average number of shoots 4 weeks after the start of light irradiation (B, ■). Relative number of shoot formation = (frequency of shoot formation / that of control hairy roots) / relative number of phytochrome level. Relative number of average number of shoots = (average number of shoots / that of control hairy roots) / relative number of phytochrome level. Hairy root lines whose names start with 1, 2 or 3 were transformed with CaMV35S*ArPHYA1*, CaMV35S*ArPHYA2* or CaMV35S*ArPHYA3*, respectively.

titious shoots on horseradish hairy roots and that ArphyA1 or ArphyA2 might be moderately or low active among them, respectively.

Though ArPHYA1, ArPHYA2 and ArPHYA3 had the same amino acid length (1122 amino acid) and were very similar throughout the peptide, they showed some amino acid substitutions among them (Saitou *et al.*, 2000). For example, the 103th Ala, the 337th Leu or the 353th Pro in ArPHYA1 and ArpPHYA3 were substituted with Ser, His or Thr in ArPHYA2, respectively. These substitution might be involved in lower activity of shoot formation in hairy roots overexpressing ArPHYA2.

Meanwhile, hairy root lines (1-2, 3-2) transformed with CaMV35S*ArPHYA1* or CaMV35S*ArPHYA3*, respectively, also showed higher levels of phytochrome but not the higher extent of light-induced formation of adventitious shoots. These results suggested that in some lines of hairy roots expressing higher levels of phytochrome than the control samples, photochemical activities of other types of phytochrome might be reduced. This is owing to the fact that plants have phytochromes other than phyA and these phytochromes are involved in the light-induced formation of adventitious shoots in horseradish, such as phyB (Saitou *et al.*, 1999b). In addition, phytochromes are thought to share the common chromophore with other phytochromes (Quail *et al.*, 1995). Overexpression of apoprotein of phyA without chromophore increased levels of phyA but might have reduced photochemical activities of other types of phytochromes in hairy roots expressing high concentration of phyA if chromophore were not sufficient to apoprotein the phytochromes.

To clarify the reason why hairy roots overexpressing ArphyA2 showed the lower extent of shoot formation we might need to produce more hairy root lines with moderately overexpressing ArphyA2 and to use another promoter weaker than CaMV35S if the latter hypothesis were correct. On the other hand, it might be necessary to substitute some amino acids in ArphyA1 or ArphyA3 to investigate the effect of substitution in ArphyA2 to shoot formation if the former hypothesis were correct.

With the same system, we have previously reported the overexpression of *Arabidopsis PHYA* (*AtPHYA*) in horseradish hairy roots (Saitou *et al.*, 1999a). In horseradish hairy roots, AtphyA was photochemically active and increased levels of phytochrome resulted in increased shoot formation. The efficiency of AtphyA on adventitious shoot formation was supposed to be similar to that of ArphyA1 or ArphyA3 in horseradish hairy roots.

Relationships between levels of phytochrome and the extent of light-induced formation of adventitious shoots

Independent lines of hairy roots transformed with CaMV35S*ArPHYAs* differed both in the level of phytochrome (**Fig. 1**) and the extent of light-induced formation of adventitious shoots (**Fig. 2**). In addition, since the efficiency of each ArphyA on shoot formation varied in horseradish hairy roots,

relationships between phytochrome levels and the extent of light-induced formation of adventitious shoots were investigated with hairy roots transformed with each CaMV35S*ArPHYA* (**Fig. 4**). Phytochrome levels in proximal areas were plotted against the frequency of shoot formation (**Fig. 4A**) and the average number of shoots (**Fig. 4B**) 1 or 4 weeks after the start of irradiation, respectively. Since the extent of shoot formation of hairy roots transformed with CaMV35S*ArPHYA2* increased slightly with increased levels of phytochrome, phytochrome levels in those hairy roots showed weaker correlation to the frequency of shoot formation (r = 0.26) and the average number of shoots (r = 0.31). On the other hand, phytochrome levels in hairy roots transformed with CaMV35S*ArPHYA1* showed stronger correlation with the frequency of shoot formation (r = 0.85) and the average number of shoots (r = 0.69). Furthermore, phytochrome levels in hairy roots transformed with CaMV35S*ArPHYA3* showed moderate correlation with the frequency of shoot formation (r = 0.47) and the average number of shoots (r = 0.49). Though correlation between phytochrome levels and the extent of shoot formation was stronger in hairy roots transformed with CaMV35S*ArPHYA1* than that in hairy roots transformed with CaMV35S*ArPHYA3*, the efficiency of phytochrome on the extent of shoot formation was higher in hairy roots transformed with CaMV35S*ArPHYA3* than that in hairy roots transformed with CaMV35S*ArPHYA1*.

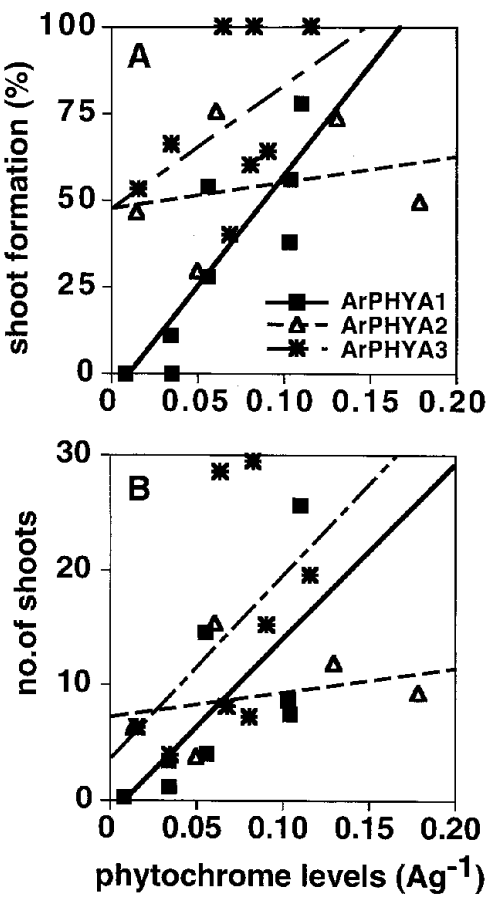

Fig. 4 Relationships between phytochrome levels and the extent of formation of shoots. Phytochrome levels shown in **Fig. 1B** and either the frequency of shoot formation 1 week after the start of light irradiation (A) or the average number of shoots 4 weeks after the start of light irradiation (B) were plotted. The lines were fitted by regression analyses, r = 0.85 (A, *ArPHYA1*), 0.26 (A, *ArPHYA2*), 0.47 (A, *ArPHYA3*), 0.69 (B, *ArPHYA1*), 0.31 (B, *ArPHYA2*) or 0.49 (B, *ArPHYA3*). *ArPHYA1*;—■—, *ArPHYA2*;- -△- -, *ArPHYA3*;-·✳-·

Meanwhile, phytochrome levels in distal areas showed weaker correlation with the frequency of light-induced formation of adventitious shoots and the average number of shoots than those in the proximal areas (data not shown). The difference might be caused partly because the extent of shoot formation in the distal areas were very low with respect to that in proximal areas and that the effects of reduced levels of phytochrome in the distal areas might not have affected the extent of shoot formation in the distal area. Furthermore, though the increased levels of phytochrome increased the extent of shoot formation in the distal areas, similar levels of phytochrome showed less shoot formation in distal areas than in proximal areas (Saitou *et al.*, 1999a, b). Nevertheless, if the extent of shoot formation were investigated with both distal areas and whole hairy roots, phytochrome levels in the distal areas would show higher correlation values with respect to shoot formation. Though both the proximal and the distal areas are thought to be useful in investigating phytochrome levels and the extent of shoot formation, the proximal areas can be more useful when the effect of decreased levels of phytochrome to the extent of shoot formation is investigated.

In this report, we demonstrated that ArphyAs were photochemically active phytochromes and that ArphyA3, ArphyA1 or ArphyA2 showed strong, moderate or weak activity in light-induced formation of adventitious shoots in horseradish hairy roots. In addition, the shapes of action spectra were similar to those of the phyA-mediated VLFR (Saitou *et al.*, 1993, Shinomura *et al.*, 1996). Nevertheless, this phenomenon was reversibly regulated by red and far-red light and required photons with intensities in the range of photoreversible LFR (Saitou *et al.*, 1992a, 1993) mediated by phyB (Shinomura *et al.*, 1996; Frankhauser and Chory, 1997). These results suggest that phyB may also be involved in this phenomenon. We are trying to

isolate other phytochrome genes from horseradish, namely, *PHYB*, *C*, *D* and *E* to analyze both levels of transcripts and the effects of their overexpression on light-induced formation of adventitious shoots from horseradish hairy roots.

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