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BRIEF COMMUNICATION

Phase- and age-related differences in protein tyrosine phosphorylation in *Sequoia sempervirens*

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Abstract

Apical and basal halves of 3 cm long apical segments of *in vitro* cultured juvenile, adult and rejuvenated *Sequoia sempervirens* shoots were analyzed for total and tyrosine phosphorylated proteins. The latter was detected by a phosphotyrosine specific antibody. Younger tissues, or the apical halves of shoot terminals, showed larger amounts of 36, 44, 46 kDa proteins and lesser amounts of 29 kDa proteins. These are proposed as age-related changes. Phase-related proteins were also evident. Adult tissues contained more of the 34 and 36 kDa proteins than juvenile and rejuvenated shoots. Western blotting with a phosphotyrosine specific antibody revealed more of 25, 39, and 54 kDa protein in the younger tissues. In addition, tyrosine phosphorylated proteins of 25 and 34 kDa were higher in the adult, than in juvenile or rejuvenated tissues. Our findings showed that protein tyrosine phosphorylation, or the signal transduction pathway, is involved in phase- and age-related processes.

Additional key words: aging, rejuvenation, signal transduction.

Protein tyrosine phosphorylation is crucial in many cellular regulatory mechanisms (Cans *et al.* 2000). The phosphorylation of specific tyrosine residues on proteins could determine its function. These proteins play central roles in a variety of signal transduction pathways responding to extracellular environments, as well as those directing the cell cycle, cell growth and differentiation. The occurrence of tyrosine phosphorylation is determined by balance between the actions of protein tyrosine kinases and protein tyrosine phosphatases. It has been reported that basal tyrosine kinase activity in aged rats was higher than in young rats (Majumdar *et al.* 1989). In addition, the pattern of tyrosine kinase activity in young and aged rats after injury was found to be different (Fligiel *et al.* 1994). Recently, a reduction with age in the phosphorylation of growth factor receptor at tyrosine residues was reported (Palmer *et al.* 1999). These studies suggest that protein tyrosine phosphorylation may play an important role in aging.

Rejuvenation, indicated by a restoration of rooting competence in *Sequoia sempervirens* can be achieved by a repeated grafting of adult shoot tips onto juvenile root stocks *in vitro* (Huang *et al.* 1992, 1995, Huang *et al.* 1996). Complete restoration of rooting competence was achieved after grafting five times. Patterns of proteins as revealed by two-dimensional PAGE were different in juvenile or rejuvenated and adult *Sequoia* shoots (Huang *et al.* 1992). Protein patterns were also different between growth phases in *Sequoiadendron* (Bon and Monteuuis 1991) and *Betula* (Brand and Lineberger 1992). We also reported that, in *Sequoia*, phosphorylation of a 32 kDa protein was evident only in adults, and that a 31 kDa phospho-protein was only detectable in juvenile cells. Differences of mitochondrial kinase activities were also studied and found to decline greatly in adult shoots (Kuo *et al.* 1995).

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Relationships between protein tyrosine phosphorylation and plant embryogenesis have been studied in carrot (Barizza *et al.* 1999) and coconut (Islas-Flores *et al.* 1998). Different tyrosine-phosphorylated proteins were observed in carrots and coconut of different embryo stages. Moreover, the embryogenesis of carrots was blocked *in vivo* by a tyrosyl-phosphorylation inhibitor. These data have unraveled the strong correlations between protein tyrosine phosphorylation and plant development. In this study, we tried to compare the tyrosine-phosphorylated protein patterns of crude extracts of whole cells from the adult, juvenile, and rejuvenated *Sequoia sempervirens* shoot tips of different ages.

Tissue samples were obtained from *in vitro* cultures of adult, juvenile and rejuvenated *Sequoia sempervirens* shoots. The shoots were maintained in stock by subculturing 2 - 3 cm terminal sections every 2-months in MS-based medium (Murashige and Skoog 1962), 3 % sucrose, 0.25 % Gelrite^{TM}, and 0.1 mg dm^{-3} thiamine, 0.5 mg dm^{-3} nicotinic acid, 0.5 mg dm^{-3} pyridoxine, and 2 mg dm^{-3} glycine. The cultures were maintained at 25 °C and 12-h photoperiod with irradiance of 18 μmol m^{-2} s^{-1} (fluorescent lamps). Age differences in protein composition were studied by dividing 3-cm-long shoot tips into apical and basal halves, the apical half being considered the younger of the two halves. Phase differences were examined by comparing segments of adult, juvenile and rejuvenated shoots. The adult shoots originated in a 60-year-old tree and the juvenile shoots were established from recently germinated seedlings. The rejuvenated shoots were obtained by repeatedly grafting, five times, the shoot apices of the adult onto rooted juvenile shoots *in vitro* (Huang *et al.* 1992). Shoot segments were ground in a mortar with liquid nitrogen, then resuspended in freshly prepared cold extraction buffer (50 mM Tris-HCl, pH 7.4, 250 mM sucrose, 10 mM Na_3VO_4, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 5 mg dm^{-3} leupeptin, 5 mg dm^{-3} antipain). Protein concentrations were determined according to Bradford (1976). Protein samples were denatured by boiling 8 min in standard sample buffer [150 mM Tris-HCl, pH 6.8; 15 % β-mercaptoethanol; 6 % sodium dodecyl sulphate (SDS), 30 % glycerol; 0.05 % bromophenol blue]. Equal microgram of proteins (about 30 μg) were separated on 10 % standard SDS-polyacrylamide gels. Total proteins were detected by staining with Coomassie blue (*Bio-Rad*, Hercules, USA). Tyrosine phosphorylated proteins were detected by immunoblotting with phosphotyrosine specific antibody according to Barizza (1999). Gels were blocked and washed in blocking solution (200 mM Tris-HCl, pH 7.5, and 250 mM NaCl) with 1 % bovine serum albumin and 0.1 % *Tween-20*. Immunodetection was revealed in a dark room by exposing membranes on the *Hyperfilm*

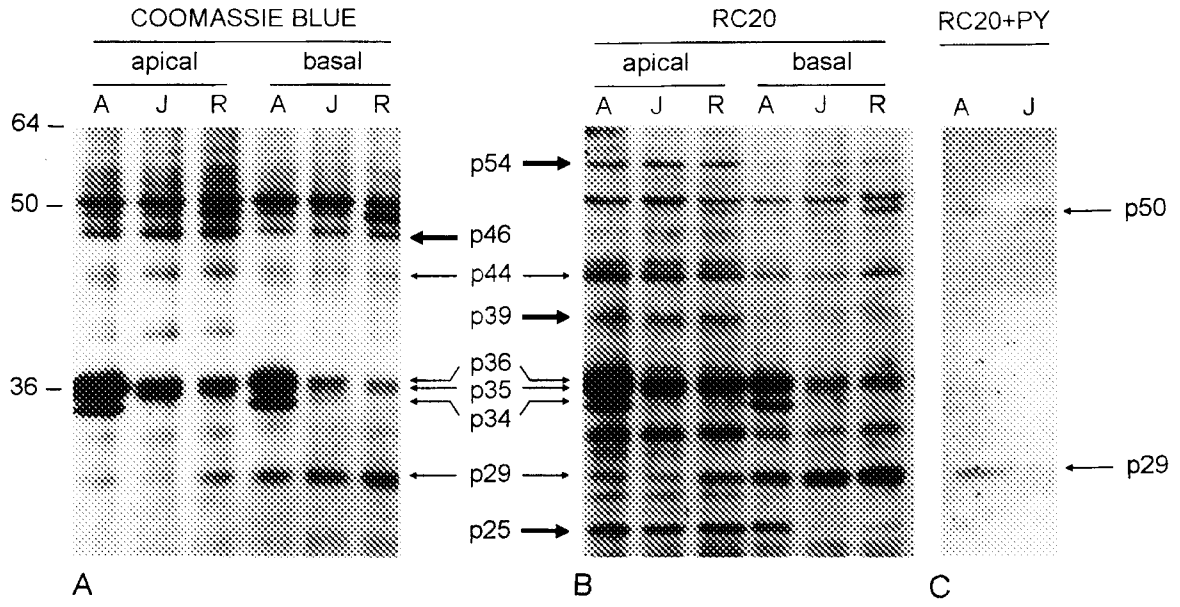

Fig. 1. The *Sequoia* protein and tyrosine phosphorylation patterns of adult, juvenile, and rejuvenated shoots of different segments. Equal amounts of proteins extracted from the apical halves of adult (A), juvenile (J), and rejuvenated (R) shoots terminals as well as the basal halves of adult (A), juvenile (J), and rejuvenated (R) shoots were separated by standard 10 % SDS-PAGE and stained with Coomassie blue (*A*) or subjected to immunoblotting with an anti-phosphotyrosine antibody (RC20) against the phosphotyrosine residues (*B*). Extracts of the adult and juvenile apical halves were also immunodetected using the anti-phosphotyrosine antibody (RC20) with the present of 2 mM phosphotyrosine (PY) added to the antibody preparation to block the antiphosphotyrosine antibody by free phosphotyrosine. Molecular mass is indicated in the left margin. *Arrowheads* indicate the protein bands that varied. The amount of protein per lane was about 30 μg.

(*Amersham Bioscience*, Little Chalfont, UK) for 2 h. Differences in total protein as well as tyrosine-phosphorylated proteins were observed between apical and basal halves of shoots and between adult and juvenile or rejuvenated shoots. All experiments were repeated at least two times. Similar results and identical trends were obtained each time.

Differences between apical and basal halves can be considered to reflect age-related differences. The basal halves represented the older tissues, consisting of more highly differentiated cells. On the other hand, differences between adult and juvenile or rejuvenated shoots are most likely to be the result of their developmental phase.

Among the Coomassie blue stained proteins, younger (*i.e.*, apical) tissues displayed more 36, 44, 46 kDa protein as judged by heavier staining. In contrast older tissues contained more 29 kDa protein (Fig. 1*A*). Immunoblotting revealed more 25, 39, and 54 kDa tyrosine phosphorylated protein in the apical halves, or younger tissues (Fig. 1*B*). Moreover, tyrosine phosphorylated proteins of 25 and 34 kDa were higher in the adult, than in juvenile or rejuvenated tissues.

Adult tissues contained more of the 34 and 36 kDa proteins than juvenile or rejuvenated tissues (Fig. 1*A*). The two proteins of adult shoots also showed more intense fluorescence on immunoblotted membranes (Fig 1*B*), indicating that there were more tyrosine phosphorylated proteins of the corresponding sizes in adult shoots, as a proportion of total protein.

To determining the specificity of detection, we pre-incubated antibody with phosphotyrosine to establish the specificity of the phosphotyrosine antibody. Only two proteins of 29 and 50 kDa were apparent when the antibody was pre-incubated with phosphotyrosine, indicating that the other proteins were tyrosine phosphorylated (Fig. 1*C*).

These findings are consistent with the view that tyrosine phosphorylated proteins are fundamental to plant developmental processes. Differentiation, an age dependent process, is clearly associated with changes in these proteins. The pattern with respect to tyrosine-phosphorylated proteins also reinforces our earlier findings of differential protein phosphorylation in adult, juvenile and rejuvenated *Sequoia sempervirens* shoots (Kuo *et al.* 1995). How these proteins regulate differentiation and developmental phase change remains unclear. Several kinases and phosphatases that phosphorylate and dephosphorylate tyrosine residues have been identified (Xu *et al.* 1998), and most of them play pivotal roles in signal transduction pathways. Studies are needed to identify specific signal transduction components, especially their variance with age and phase.

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