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General paper

Antibodies to phase related proteins in juvenile and mature *Prunus avium*

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Abstract

In the search for biochemical and molecular markers of juvenility in trees proteins have been identified which are preferentially or differentially expressed in either the juvenile or the mature phases of *Prunus avium* cv. Stella. These fall into two classes: those which are phase-related and those which may be affected by root-shoot distance. N-terminal amino acid sequence data from some of these proteins were used to produce polyclonal antibodies to corresponding synthetic peptides in order to determine if they could be used as markers of phase state in woody plants. Western blot analysis was performed on proteins extracted from three sources; juvenile trees, mature trees and rooted cuttings from mature trees. The results showed that the antibodies recognised differentially-expressed proteins. In particular, one antibody to a juvenile specific protein cross-reacted with a polypeptide of approx. 28 kDa which was present in greater amounts in shoot tips of juvenile *P. avium* cv. Stella seedlings compared with rooted cuttings of mature plants placed in the same growth environment.

Introduction

As woody plants grow they undergo a developmental change from the juvenile to the mature state, a process known as "phase change" or "maturation" [10]. This process is believed to involve stable changes in apical meristems which lead to altered patterns of gene expression in the tissues which subsequently derive from them [16]. Maturation can be wholly or partially reversed during sexual reproduction or by chemical or cultural treatments, a process often referred to as "rejuvenation".

We are interested in identifying biochemical and molecular markers of phase state in trees. Relatively little is known about differences between juvenile and mature tissues at the biochemical and molecular levels. Changes in protein composition associated with phase change have been observed in several species. A 16 kDa membrane-associated protein was detected in shoot apices of juvenile *Sequoiadendron giganteum*. The protein was absent from apices of the mature tree [7]. Differences in 2-D PAGE profiles of leaves from seedlings, adult trees and repeatedly grafted adult shoots of *Sequoia sempervirens* have been found [13]. Protein differences have also been observed in a comparison of *in vitro* shoots derived from stump and crown shoots of the same 90 year old *S. sempervirens* tree [8], and between basal and crown shoots of a mature *Castanea sativa* tree [1]. An immunological study in *Citrus* spp. showed that a 60 kDa protein was present in greater quantities in leaves of mature plants [18]. At the molecular level information is very limited. A phase-specific difference in anthocyanin accumulation in *Hedera helix* has been shown to be due to differences in gene expression [16]. The gene for dihydroflavonol reductase, an enzyme in the anthocyanin biosynthesis pathway, could be induced in juvenile leaf laminae but not in laminae of mature plants. Reversible modifications of mitochondrial DNA sequences have been found to occur in juvenile, mature and rejuvenated *Sequoia sempervirens* [14], though the significance of this rearrangement is not yet clear.

We are using *Prunus avium* as a model to study the biochemical and molecular mechanisms which control phase state. The advantages of this species include a short juvenile period, small genome size [2] and the

availability of material of defined genetic background as a result of years of controlled crossing and breeding for fruit tree production. We have found differences in the amounts of specific polypeptides and mRNA transcripts in shoot tips of juvenile and mature *P. avium* cv. Stella [4]. Here we describe the production and initial assessment of antibody markers of phase state in *P. avium*.

Materials and methods

Plant material

In order to compare genetically similar material, we obtained self-pollinated seedlings and rooted cuttings of shoots from the same mature (circa 20 year-old) *Prunus avium* cv. Stella tree. Cuttings were produced as follows: leafy summer shoots were dipped in a solution of 2500ppm IBA in 50% acetone to a depth of 8 mm for 5 sec. The cuttings were then placed in pots of peat/granulated bark medium (80/20 v/v) on a sand bed with bottom heat (minimum temperature 20 °C) in a "wet fog" high-humidity propagating system. The rooted cuttings continued to show mature phase characteristics, e.g. flowering and plagiotrophic growth, for at least three years after rooting. They were used in this study two years after rooting. Twelve plants of each type in pots were placed in a controlled environment cabinet in an alternating checkerboard array at the beginning of the growing season. Growing conditions were 16 h photoperiod and irradiance of 250 μmol m^{-2} s^{-1} (PAR), humidity 70% and temperatures of 16 °C light/11 °C dark. Under these conditions the development of the photosynthetic apparatus in leaves is the same in juvenile and mature plants [5]. Samples were also taken in April from the mature parent tree growing in the field at East Malling Research Station, Kent, UK. Samples were taken approximately 3–4 h after the beginning of the photoperiod. Entire shoot tips were harvested, comprising the shoot apex plus surrounding leaf primordia and developing leaves up to 2 cm in length. Tissue was weighed and flash-frozen in liquid N_2.

Antibody production

For the production of polyclonal antibodies, a membrane enriched polypeptide fraction was extracted and separated by 2-D PAGE [4]. Briefly, frozen shoot tip samples (c.1 g) were ground in liquid N_2 and protein extraction buffer (50 *mM* Tris-HCl, pH 8.0; 200 *mM* sucrose; 10 *mM* NaCl; 1 *mM* phenylmethylsulphonyl fluoride; 1%(v/v) 2-mercaptoethanol) [19] was added. The suspension was squeezed through 2 layers of Miracloth and the filtrate was left at −20 ° overnight then centrifuged at 13000rpm for 20 min. After washing in extraction buffer and recentrifugation, the pellet was resuspended in ice-cold acetone and allowed to precipitate at −20 °C for 2–3 h. The samples were then centrifuged as before and the pellet was air-dried before being resuspended in a minimal volume of CHAPS buffer. First dimension isoelectric focusing and second dimension PAGE were carried out as described [4]. Individual polypeptides of interest were cut from polyacrylamide gels and extracted into buffer (50 *mM* Tris-HCl, 1%SDS, 4*M* urea, pH 8.0). It was necessary to combine extracts from replicate gels to obtain sufficient material for sequencing. Three polypeptides were isolated, two which were found in higher amounts in juvenile shoots (labelled J1 & J2) and one which was expressed in larger amounts in the mature tree in the field (labelled M1). N-terminal polypeptide microsequencing was carried out on them [15]. Synthetic peptides corresponding to the N-terminal regions of the extracted polypeptides were produced commercially (Neosystem Laboratoire, France): J1 peptide, DPEADTAPGTQSLSKVLLD; J2 peptide, EEESTIGAGPGENA; M1 peptide, EPNQGPDQGDTN. These were used for the production of polyclonal antibodies in rabbits after conjugation with the carrier protein purified protein derivative (PPD) from tuberculin [6].

Western blotting

Frozen shoot tip samples (c.300 mg) were ground in liquid N_2 and a membrane enriched protein fraction was extracted as described above except for the addition of 0.1%(w/v) NaN_3 to the extraction buffer. The final pellet was vacuum-dried and solubilized by boiling for 3 min in loading buffer (50 *mM* Tris-HCl, pH 7.4; 2% (w/v) SDS; 10% (v/v) glycerol; 1% (v/v) 2-mercaptoethanol) for gel electrophoresis. Polypeptides were separated by electrophoresis on a discontinuous gradient of SDS-polyacrylamide consisting of a 15% (w/v) acrylamide resolving gel and a 3% (w/v) stacking gel. Protein content of extracts was determined using the Bio-Rad protein assay [9] and samples were loaded on an equal protein basis. Western blotting and immunostaining were as described [3]. Shoot samples chosen at random from each plant type on three

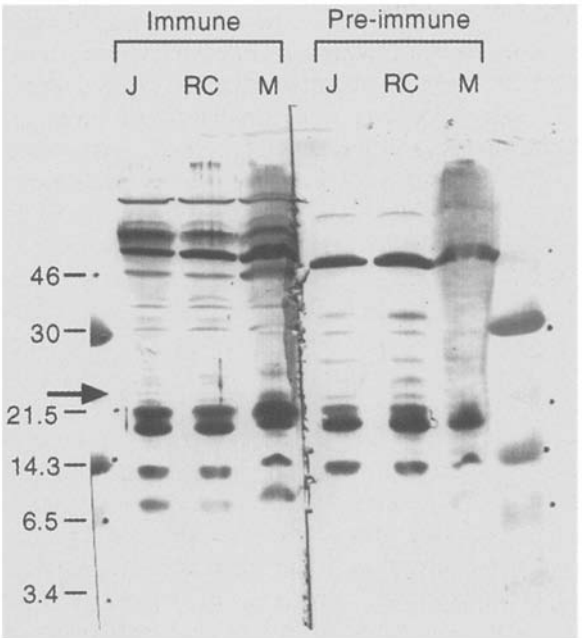

Figure 1. Western blot of protein extracts from shoot tips of *Prunus avium* cv. Stella, probed with antiserum raised to the N-terminal sequence peptide of a juvenile-specific protein J1 (immune) or with pre-immune serum. Lanes loaded on an equal protein basis. Numbers indicate molecular weight markers. Arrow indicates a 23 kDa polypeptide which is found only in the juvenile state. J: seedling, RC: rooted cutting (mature) – both growing in controlled environment cabinet, M: mature tree – growing in the field.

separate dates were extracted and analyzed and typical Western blots are presented.

Results

Three peptide antibodies were produced which recognised differentially expressed polypeptides in *P. avium* cv. Stella. The polyclonal antibodies also recognised polypeptides at other molecular weights which were either present at the same levels in all the tissues tested or which were also found in pre-immune serum. For each antibody however, a polypeptide band could be identified which reproduced the expression pattern shown by the polypeptide used for sequence analysis and peptide antibody production. The J1 antibody recognised a low-abundance band at mol. wt. c.23 kDa which was only present in polypeptide extracts from juvenile shoots (Figure 1). However, multiple banding against *P. avium* polypeptides was also observed. Much of this appeared to be non-specific as it bound equally to components of the pre-immune serum.

Antiserum to the M1 polypeptide from mature tree shoot tips recognised a c.45 kDa molecular weight polypeptide which was strongly expressed in the mature tree growing in the field, but a comparison with seedlings and rooted mature cuttings grown together in a controlled environment cabinet showed that the amounts of this polypeptide were greatly reduced and apparently not phase-specific (Figure 2(A)). Two other polypeptides greater than 46 kDa also reacted with this antibody in all three sample types.

The antibody to juvenile polypeptide J2 cross-reacted with a polypeptide of mol. wt. approx. 28 kDa in juvenile shoot tips which was barely detectable in mature shoot tips of cv. Stella plants in the same growth environment. This polypeptide was not detected in polypeptide samples from the mature tree in the field (Figure 2(B)). The level of this polypeptide was therefore strongly correlated with the phase state of the source plants. An immunoreactive polypeptide of approx. 46 kDa was present in the mature tree in the field, but at comparatively low levels in the rooted mature cuttings and seedlings. A further polypeptide of mol. wt. greater than 46 kDa gave a signal of approximately equal intensity in all three tissue extracts.

Discussion

Polyclonal antibodies have been raised to synthetic peptides corresponding to N-terminal sequences of polypeptides showing differential expression in juvenile and mature *P. avium* and it has been demonstrated that the antibodies cross-react with individual polypeptides which also show differential expression. This confirmed the findings of our earlier study, that phase change is associated with changes in the amount of specific polypeptides in shoots of *P. avium* [4]. The results are also in agreement with studies on other tree species [1, 7, 8, 13].

Both the J1 and J2 peptide antibodies detected polypeptide markers of the juvenile phase of growth. The J2 antibody cross-reacted with relatively few *P. avium* polypeptides, producing a simple pattern of bands. Of these, a 28 kDa mol. wt. polypeptide was found in consistently greater amounts in seedling shoot tips. Although the polypeptide was present in mature rooted cuttings and the mature tree in the field, detection in these tissues often required prolonged development of the blots (data not shown). Phase-related quantitative differences in proteins have been observed in cherry and other tree species [4, 8]. This antibody

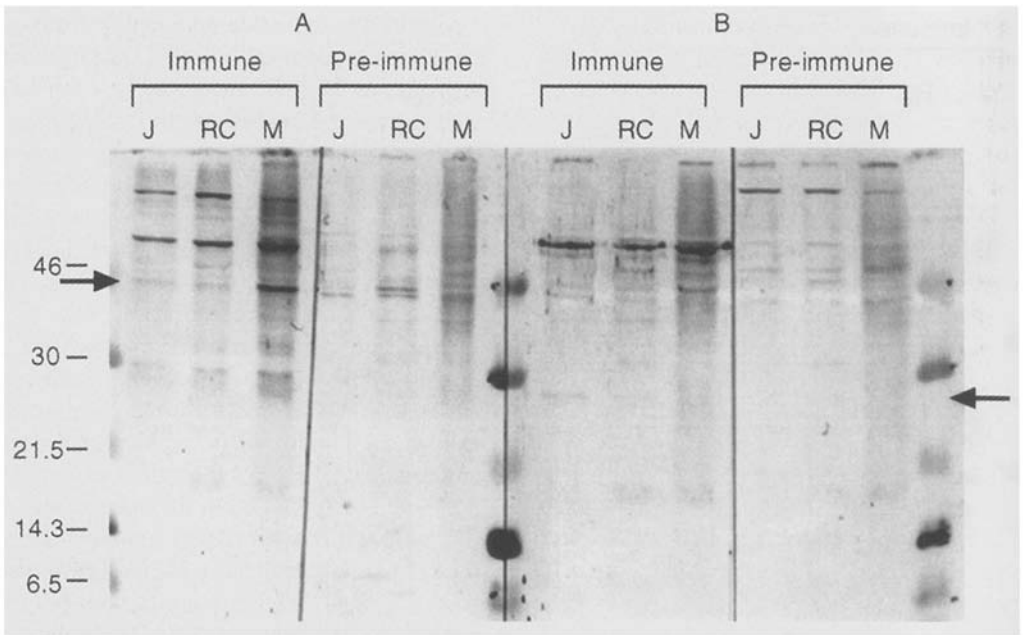

Figure 2. Western blot of protein extracts from shoot tips of *Prunus avium* cv. Stella probed with antiserum (immune) or with pre-immune serum (pre-immune). Lanes loaded on an equal protein basis. Numbers indicate molecular weight markers. (A) Antiserum to a peptide sequence present in a protein expressed most strongly in the mature tree in the field (M1). Arrow indicates a 45 kDa polypeptide which is differentially expressed. (B) Antiserum to a peptide sequence present in a protein expressed in greater amounts in juvenile shoots (J2). Arrows indicate a 28 kDa polypeptide which is differentially expressed. J: seedling, RC: rooted cutting (mature) – both growing in controlled environment cabinet, M: mature tree – growing in the field.

shows potential for the determination of phase state in *P. avium*.

The antibody raised to a sequence in the J1 polypeptide detected a low abundance 23 kDa polypeptide which appeared to be specific to the juvenile phase. However many other *P. avium* polypeptides cross-reacted in a non-specific manner with antisera from the rabbit inoculated with the J1 peptide sequence, making discrimination of the 23 kDa band difficult. This antiserum would therefore be of limited use in studies of phase-related protein expression.

The antibody raised to the M1 peptide sequence detected a 45 kDa polypeptide which was differentially expressed though apparently not specifically phase-related. The polypeptide was present in large amounts in the original mature tree in the field, but in low amounts in both juvenile and mature plants in the controlled environment. A similar pattern of expression was observed for a polypeptide of approx. 46 kDa which was recognised by the J2 antibody. This pattern could be due to differences in growth conditions or root/shoot distance between the mature tree and the plants in the controlled environment. Root/shoot distance has been suggested as a factor which influences phase state [12]. Any biochemical changes which resulted from reduction in the root-shoot distance of the mature rooted cuttings did not appear to cause reversion of phase state as the plants retained competence to flower for at least 2 years after rooting.

All three polyclonal antibodies produced more than one band in Western blot analysis of polypeptide extracts from *P. avium* cv. Stella. This may represent cross-reactive binding to antigens other than the peptides used to produce the antibodies. This is a characteristic of heterogeneous polyclonal antisera [11]. This was particularly apparent in the case of the J1 antibody, where a strong signal was obtained from several cherry polypeptides when challenged with serum taken from the rabbit before immunisation (Figure 1: pre-immune serum). The other pre-immune sera gave a much lower response in both the number and degree of non-specific binding to *P. avium* polypeptides. The other immune sera cross-reacted strongly with one (J2) or two (M1) high-molecular weight polypeptides which did not appear to be phase-specific. These may be unrelated polypeptides which share a common epitope, or the same polypeptide present in different molecular weight forms.

Biochemical markers of phase state would be useful in tree improvement programmes, where *in vivo* and *in vitro* treatments are sought to rejuvenate characteristics such as rooting, apical dominance and growth rate in selected mature trees. Such markers would be especially useful in species such as *P. avium* where morphological markers are not available. Studies with the antibodies described in this paper are in progress to determine the expression of marker proteins during treatments which induce apparent rejuvenation of juvenile characteristics in mature tissues.

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