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A simplified procedure for the subtractive cDNA cloning of photoassimilate-responding genes: isolation of cDNAs encoding a new class of pathogenesis-related proteins

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Abstract

Transgenic tobacco plants (ppa-1) constitutively expressing *Escherichia coli* pyrophosphatase behind the 35S CaMV promoter accumulate high levels of soluble sugars in their leaves [27]. These plants were considered a tool to study adaptation of leaves to photoassimilate accumulation at the molecular level. By differential hybridization of a subtractive library enriched for transcripts present in the transgenic plants 12 different cDNAs were isolated. By sequence analysis four cDNAs could be identified as 1-aminocyclopropane-1-carboxylate-oxidase and as three different pathogenesis-related proteins (PR-1b, PR-Q and SAR 8.2). Two cDNAs were homologous to a calmodulin-like protein from *Arabidopsis* and a human ribosomal protein L19 while six cDNA clones remained unknown. One of these clones (termed PAR-1 for photoassimilate-responsive) displayed features similar to pathogenesis-related proteins: Hybridizing transcripts, 1.2 and 1.0 kb in length, were strongly inducible by salicylate and accumulated in tobacco plants after infection with potato virus Y (PVY) both in infected and uninfected systemic leaves. PAR-1 transcripts also accumulated in wildtype leaves upon floating on glucose and sucrose whereas sorbitol and polyethylene glycol had no effect. Rescreening of the ppa-1 cDNA library with the PAR-1 cDNA as probe resulted in 25 hybridizing cDNAs which by homology were found to fall into three classes (PAR-1a, b, c). The cDNAs coding for PAR-1a and b were 90.6% homologous on the DNA level while both were less related to the PAR-1c cDNA (70.5% and 75.2% homologous, respectively). One open reading frame was identified in all three PAR-1 cDNA classes. Translation would result in proteins with a theoretical molecular mass of about 20 kDa. The N-terminal amino acid sequences resemble a signal peptide which would direct the proteins to the secretory pathway. Using selective 3′ hybridization probes of the three PAR-1 cDNAs it was possible to discriminate the different transcripts. Both PAR-1a and PAR-1c mRNAs are induced in plants treated with PVY.

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X83853 (PAR-1a), X83851 (PAR-1b) and X83852 (PAR-1c).

Introduction

Plants are confronted with sugar accumulation in their leaf mesophyll cells if their photoassimilate production exceeds their utilization in sink tissues. This situation may arise when they are exposed to high light, elevated levels of atmospheric CO_2 and/or when they suffer from sink limitations as under nitrogen deficiency or cold stress [34]. As a result of sugar accumulation the rate of sucrose synthesis decreases, starch accumulates and photosynthesis is inhibited. In contrast to the well studied biochemical mechanisms in response to sugar accumulation the underlying molecular mechanisms are largely unknown. Systems used to cause an increase of assimilates in photosynthetically active plant cells are girdling (cold or hot wax collar, respectively) of source leaves, the removal of sink tissues and sugar feeding experiments using detached leaves, cell suspension cultures or protoplasts [6, 12, 13, 24]. All these experimental systems, however, are restricted to investigations of the short-term response to sugar accumulation. An alternative to study long-term adaptation of plant cells to elevated levels of sugars would be the use of transgenic plants.

Transgenic tobacco plants expressing *E. coli* pyrophosphatase behind the constitutive CaMV 35S promoter (ppa-1 plants) accumulate high levels of carbohydrates in their source leaves [27, 9]. These biochemical changes have been explained to be due to increased sucrose synthesis and inhibited loading of sucrose into the phloem [27, 15, 5]. In addition to the expected biochemical alterations in plant metabolism caused by the *E. coli* transgene a number of other phenotypical changes were observed in the transgenic tobacco plants. The high sugar levels in the source leaves lead to an increase in turgor pressure by a factor of about 9 in the transgenic as compared to wild-type plants while the water potential was essentially not changed [28]. Presumably as a consequence of the elevated turgor, the mesophyll cell enlarged two-fold as compared to wild-type cells and cell walls were found to contain 14% and 43% more pectin and uronic acids, respectively [28].

To study the long-term adaptation of gene expression of plants that are continuously confronted with high levels of endogenous soluble sugars we have chosen the transgenic ppa-1 plants as a tool. Here we describe the cloning and description of cDNAs from a subtractive library enriched for transcripts present in the sugar-accumulating ppa-1 plants. An efficient and simplified procedure for the establishment of subtractive cDNA libraries will be presented. One of the cDNAs that could not be identified by sequence homology will be characterized in more detail and is suggested to code for a new class of pathogenesis-related proteins (PR proteins).

Materials and methods

Recombinant DNA techniques and bacterial strains

Standard procedures were used for recombinant DNA work [23]. Bacterial transformation were done into *E. coli* XL1-blue (Stratagene, La Jolla, CA).

RNA preparation

Total RNA was extracted from frozen plant material [16]. Poly(A) RNA was isolated by means of oligo(dT) cellulose type 7 (Pharmacia) according to the manufacturer's instructions.

Preparation of cDNA libraries

Poly(A) RNA was prepared from source leaves of wildtype and ppa-1 plants. 5 μg were transcribed into double-stranded cDNA using the cDNA synthesis kit from Pharmacia. *Eco* RI-*Not* I adaptors (Pharmacia) were ligated to the cDNAs which were then cloned into *Eco* RI-digested λZAP II vector arms (Stratagene). After *in vitro* packaging using the Gigapack II packaging extract (Stratagene) recombinant lambda DNA was transfected into *E. coli* XL1-Blue cells (Strategene) and the titer of the cDNA libraries determined by counting the plaques. Thereafter the

cDNA libraries were amplified according to the amplification protocol of Stratagene.

Preparation of a subtractive cDNA library

The preparation of the subtractive library is schematically drawn in Fig. 1. The wild-type and the ppa-1 cDNA libraries prepared as described above were *in vivo* excised according to the *in vivo* excision protocol of Stratagene. Obtained bacterial colonies were amplified and their recombinant plasmid DNA isolated using standard procedures. 200 μg of the wild-type plasmid cDNA library was digested with *Not* I while plasmids of the ppa-1 cDNA library were cut with *Eco* RI. cDNA fragments were then separated in agarose gels and fragments between 0.5 kb and 1.5 kb eluted. Wild-type cDNA was photobiotinylated (Clontech) according to the protocol by Strauss and Ausubel [30]. Photobiotinylated wild-type cDNA and ppa-1 cDNA fragments were mixed in a ratio 10:1, denatured at 95 °C and renatured by slowly decreasing the temperature to 25 °C. Avidin (Vectrex Avidin) was added to the mixture and wild-type and hybrid cDNA molecules removed by centrifugation. PPa-1 enriched cDNA fragments were cloned into *Eco* RI-digested λZAP II vector arms, packaged, transfected into XL1-blue cells, the titer determined and the subtractive cDNA library amplified as described above.

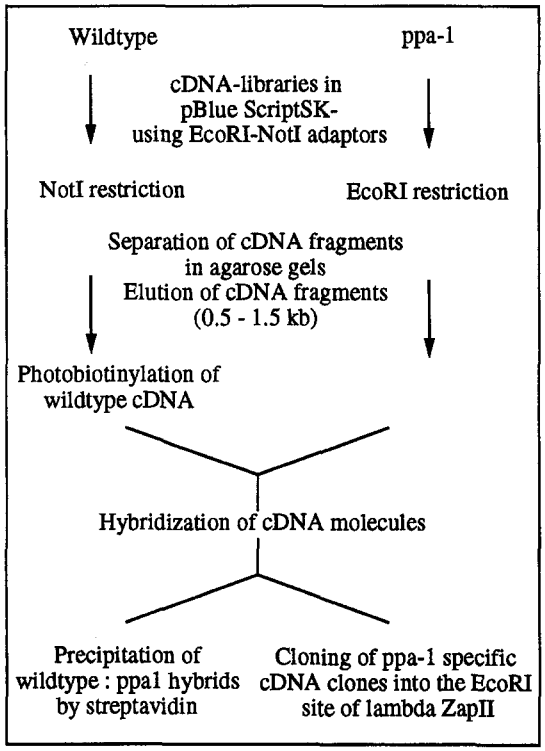

Fig. 1. Procedure for the generation of a subtractive cDNA library by means of restriction site exclusion (for details see Materials and methods).

Differential screening of the subtractive library

Screening of cDNA libraries followed the DNA screening protocol by Stratagene. DNA of 2×10^4 plaque-forming units of the subtractive library were transferred onto nylon filters (Hybond N, Amersham Buchler) and hybridized to radioactively labelled wild-type cDNA. Hybridizing phage DNAs were visualized by autoradiography before the filters were hybridized to radioactively labelled ppa-1 cDNA. After autoradiographic exposure the two autoradiographies were compared to identify phage DNAs specific to ppa-1. Purified clones were *in vivo* excised and characterized by sequencing.

Plant material

Nicotiana tabacum L. cv. Samsun NN was obtained from Vereinigte Saatzuchten AG (Ebstorf, Germany). The transgenic ppa-1 tobacco plants have been described [27]. Plants in the greenhouse were maintained in soil under a light-dark regime of 16 h/8 h (mean temperatures 25 °C/20 °C; mean irradiance 300 μmol m^{-2} s^{-1}) at 60% humidity.

Floating experiments

Tobacco plants used for floating experiments had 14 to 18 leaves. Leaf discs were derived from

source leaves of 18–25 cm. All experiments were started 6 h after illumination and if not otherwise stated incubations were done for 24 h in the greenhouse. Concentrations of plant hormones used: 1 mM indoleacetic acid (IAA), 1 mM gibberellic acid (GA_3), 10 μM methyl jasmonate and 50 μM abscisic acid. The hormones were dissolved as follows: IAA and GA_3 at 100 mM in 100% EtOH; ABA at 50 mM in 100% EtOH, methyl jasmonate at 10 mM in dimethylformamide.

Application of ethephon

Ethephon (0.1% v/v in 0.1% Tween-80) was sprayed onto leaves of tobacco plants in the greenhouse. 24 h later, samples were taken from the sprayed leaves. Leaves of control plants were sprayed with 0.1% Tween-80 only.

Northern blot experiments

Total RNA was denatured in 40% formamide, subjected to agarose gel electrophoresis (1.5% agarose, 15% formaldehyde) and blotted onto nylon membranes (Hybond N; Amersham Buchler, Braunschweig, Germany).

Labelling of probes and hybridization of membranes

If not otherwise stated, membranes were hybridized at 42 °C in a buffer containing polyethylene glycol and 25% formamide [1]. Radioactive labelling of DNA probes was performed using a random-primed DNA labeling kit (Boehringer, Mannheim, Germany). Filters were washed three times for 20 min at 42 °C with 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate). Radioactive probes for screening the subtractive library were prepared as follows: 0.5 μg mRNA of wild-type and ppa-1 plants, respectively, were transcribed into double-stranded cDNA (cDNA synthesis kit, Pharmacia). This cDNA was radioactively labelled using the random primed DNA labeling kit as described above.

Infection of tobacco leaves with potato virus Y (PVY^N)

PVY^N was obtained from the Bundesanstalt für Züchtungsforschung an Kulturpflanzen (Aschersleben, Germany). Leaves of infected tobacco plants were homogenized in 100 mM potassium phosphate buffer, pH 7.0 (ca. 1 g leaf material in 20 ml buffer) to obtain viral extract. Leaves to be infection were dusted with carborundum (SiC) and the viral extract applied by gently rubbing the upper face of the respective leaves with a pistil. A few minutes later the treated leaves were rinsed with water. Mature source leaves were used to apply the viral extract. 8 to 12 days after infection symptoms appeared on the plants.

Immunological detection of virus coat protein

Plant material was homogenized with PBS buffer [23] containing 0.05% Tween-20, 2% polyvinylpyrollidone 25000, 0.2% bovine serum albumin (1 g plant material/20 ml buffer). Serial dilutions of homogenized extract were analysed by means of the 'Double Antibody Sandwich' test using monoclonal antibodies against PVY (Bioreba, Reinach, Schweiz). The ELISA procedure was performed according to the protocol by Bioreba.

Results

Preparation of a ppa-1 subtractive cDNA library by restriction site exclusion

In order to get ppa-1-specific cDNA clones a subtractive library enriched for transcripts present in ppa-1 plants was prepared (Fig. 1). RNA was isolated from ppa-1-10 plants which is the best characterized ppa-1 plant line [27]. To avoid multiple cycles of subtraction with subsequent PCR amplification as developed by Straus and Ausubel for genomic subtraction [30], the subtractive cDNA library was made by only one cycle

of subtraction with subsequent selective cloning of transcripts from ppa-1 plants (Fig. 1). To this end the recombinant plasmids of the original cDNA libraries made from mRNA of wild-type and ppa-1 plants, respectively, were digested with different restriction enzymes, i.e. wild-type cDNA with *Not* I and ppa-1 cDNA with *Eco* RI. The respective cDNAs were eluted from agarose gels and the wild-type cDNA fragments were then photobiotinylated and hybridized to the ppa-1 cDNA. After addition of avidin wild-type as well as hybrid cDNAs were removed by centrifugation. The remaining cDNA fragments – already enriched for ppa-1 clones – were inserted into the *Eco* RI restriction site of the λZAP II vector thereby excluding the cloning of *Not* I-digested wild-type cDNAs. By hybridizing the same number of plaque-forming units of the subtractive library and the original ppa-1 cDNA library with the *E. coli* pyrophosphatase DNA (present only in the transgenic ppa-1 plants) it was found that this procedure had resulted in a ten-fold increase of ppa-1-specific clones in the subtractive library. The main advantage of this method is that amplification of the library *in vivo* largely excludes artifacts which are commonly found in PCR-amplified cDNA libraries. One step of subtraction was performed in order not to lose cDNA clones which are only moderately induced in ppa-1 plants.

Isolation of ppa-1-specific cDNA from the subtractive library by differential screening

The subtractive library was successively hybridized to radioactively labelled cDNA prepared from mRNA of wild-type and ppa-1 plants. 12 differentially hybridizing clones were purified and sequenced. By homology to known sequences four cDNAs could be identified as coding for 1-aminocyclopropane-1-carboxylic acid oxidase [7] and for three different pathogenesis-related proteins (PR proteins), PR-1b [4], PR-Q [20] and SAR8.2 [35]. The DNA sequence of one cDNA clone shares homology with a calmodulin-like protein from *Arabidopsis* [2] and one is homologous to the ribosomal protein L19 from man [14] while the remaining six could not be identified by sequence homologies in the databanks. Because of their responsiveness to sugar accumulation in the transgenic ppa-1 plants these cDNAs were designated PAR (1 to 6) which stands for photoassimilate-responsive genes.

Induction of the PAR-1 cDNA by sucrose, glucose and salicylate

To get a clue about possible functions we started to analyse the expression pattern of one of the isolated unknown cDNA clones (PAR-1). In ppa-1-10 plants the steady-state RNA levels hybridizing to this unidentified cDNA increased in the order from sink to source leaves (Fig. 2b, lanes 1 to 6) which are characterized by increasing sugar levels in the same order [27]. Much weaker hybridization signals were detected in source leaves of wild-type plants (Fig. 2a, lane 6). Signal strengths in leaves of ppa-1 plants were approximately four times more intense than in the corresponding leaves of wild-type plants as deter-

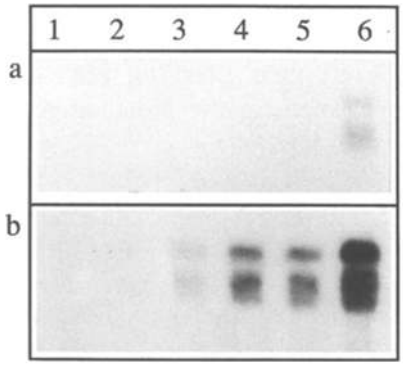

Fig. 2. Northern blot analysis of total RNAs from a leaf gradient of wild-type (a) and ppa-1 plants (b) probed with PAR-1a cDNA at 42 °C using a formamide (25%) containing buffer. Plants were of the 14-leaf stage at the time of harvest. Leaves of different sizes (length) were analyzed: 1–2 cm (lane 1), 4–5 cm (lane 2), source leaves in the middle part of the plants (lane 3), fully developed source leaves with starting ppa-1 phenotype (lane 4) and with fully developed ppa-1 phenotype (lane 5), primary leaves (lane 6). Primary leaves were considered the first two leaves developing after the cotyledons. 15 μg total RNA was loaded per lane.

mined using a phosphoimager. The results were confirmed by analysing RNA from two other independent ppa-1 lines (ppa-1-5 and ppa-1-30) [27]. The unknown cDNA which was 950 bp in length hybridized to two mRNA transcripts being ca. 1.2 kb and 1.0 kb in length.

Because the accumulation of transcript levels appeared to be correlated with corresponding steady-state levels of soluble sugar (Fig. 2) we wondered whether the mRNA transcripts would accumulate in wild-type leaves upon incubation with soluble sugars. Leaf discs from mature leaves of wild-type tobacco plants were therefore floated on water, glucose and sucrose (50, 100, 200, 300, 400, 500 mM) for 24 h and the RNA was analysed (Fig. 3). The PAR-1 mRNA transcripts accumulated in response to sucrose at a concentration of 200 mM and levels increased with higher concentrations of sucrose (Fig. 3, lanes 8–14). Floating on glucose at concentrations of 200 mM to 500 mM also lead to accumulating levels of PAR-1 mRNA although less strongly (Fig. 3, lanes 1–7). At a glucose concentration of 500 mM levels of PAR-1 transcripts had increased 12-fold while floating on sucrose resulted in an increase of about 29-fold compared to the water control as quantified by phosphoimaging.

There are multiple ways by which sugars may possibly exert their effect on gene expression. They might mediate the inducing response by being the effector molecules themselves. Alternatively, the water household may be perturbed (osmotic potential, water potential and resulting turgor pressure) or metabolic changes such as alterations in the C/N ratio may occur in response to sugar accumulation. Alternatively, sugars might induce a state of general stress resulting in changed levels of phytohormones which would regulate gene expression. To study whether the accumulation of PAR-1 transcripts was due to osmotic effects caused by an increase in the levels of soluble sugars, leaf discs were incubated in different osmolytes and the RNA was analysed. Sorbitol at concentrations of 80 and 250 mM (measured to correspond to 98 and 260 mmol/kg), did not cause PAR-1 transcript accumulation in floating experiments (Fig. 4, lanes 3 and 4). These concentrations were used as they were about equal in osmolality to 50 mM and 200 mM glucose (measured to be 84 and 247 mmol/kg, respectively). As expected, floating on 200 mM glucose resulted in the accumulation of PAR-1 transcripts (Fig. 4, lane 2). No induction was observed after floating leaf discs on 7% polyethylene glycol 6000 (Fig. 4, lane 5). Altogether the

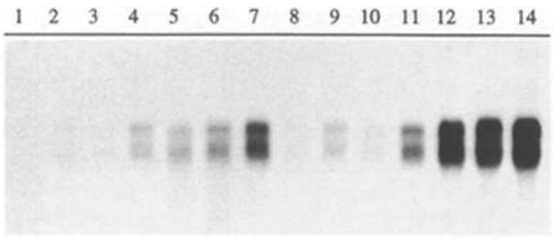

Fig. 3. Expression analysis of PAR-1 transcripts in leaf discs of wildtype plants floated on water (lanes 1, 8) glucose (lanes 2 to 7) and sucrose (lanes 9 to 14) for 24 in the dark. 15 μg total RNA was loaded per lane. Each RNA is a pool from samples of 6 mature source leaves (18 to 25 cm in length) taken from different plants. Plants used had 18 leaves. Concentrations used: 50 mM (lanes 2, 9), 100 mM (lanes 3, 10), 200 mM (lanes 4, 11), 300 mM (lanes 5, 12), 400 mM (lanes 6, 13), 500 mM (lanes 7, 14).

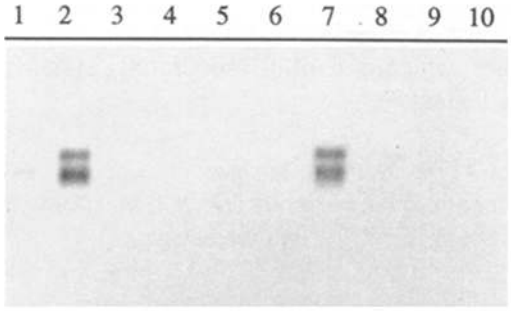

Fig. 4. Expression analysis of PAR-1 transcripts in leaf discs of wildtype plants floated on different solutes (lanes 1 to 5), glutamine (lane 6), different plant hormones (lane 7 to 9) and water (lane 10) for 24 h in the dark. 15 μg total RNA was loaded per lane. Each RNA is a pool from samples of 6 mature source leaves (18 to 25 cm in length) taken from different plants. Plants used had 18 leaves. Leaves were floated on glucose at concentrations of 50 mM and 200 mM (lanes 1 and 2, respectively), on sorbitol at concentrations of 80 mM and 250 mM (lanes 3 and 4, respectively), on 7% polyethylene glycol 6000 (lane 5) and on 100 mM glutamine (lane 6). Plant hormones: 1 mM salicylate (lane 7), 10 μM methyl jasmonate and 50 μM abscisic acid (lanes 8 and 9, respectively).

data suggest that the induction of the PAR-1 transcripts by glucose and sucrose cannot be caused by osmotic changes alone. Glutamine used in floating experiments to perturb the cellular C/N ratio by increasing the level of amino acids did not lead to enhanced levels of PAR-1 transcripts either (Fig. 4, lane 6).

As cDNAs coding for PR-proteins had been isolated from the ppa-1-specific library supporting the hypothesis that sugar accumulation in leaves would result in a general stress response we wondered whether PAR-1 also possessed any features of stress-related genes. To this end the expression of the PAR-1 transcripts was studied in response to hormones associated with stress in plants by means of floating experiments (Fig. 4). No substantial differences in transcript levels were observed after floating leaf discs on water, methyl jasmonate and abscisic acid (Fig. 4, lanes 8 to 10) whereas levels of PAR-1 transcripts rose significantly as a result of floating leaf discs on 1 mM salicylate for 24 h (Fig. 4, lane 7).

Floating leaves on gibberellic acid and indole acetic acid as well as spraying plants with ethephon did not induce PAR-1 transcripts (data not shown).

PAR-1 transcripts are constitutively expressed in sepals of open flowers

RNA analysis of different tobacco organs showed a low level of expression of PAR-1 transcripts in source leaves of wild-type plants (see Fig. 2a, lane 6). About the same basal levels of PAR-1 transcripts were detectable in flower buds (green, white, coloured) and flowers (closed, open) (data not shown). Absolutely no hybridization signals were detectable in stems and roots as well as in petals, anthers, pistils and ovaries of open flowers while significant amounts of PAR-1 transcripts were present in sepals of open flowers (data not shown). It remains to be shown whether the constitutive expression in sepals of open flowers and the inducible expression in leaves are mediated by the same or different genes.

Local and systemic induction of the PAR-1-specific transcripts in tobacco plants after infection with potato virus Y (PVY)

Because of their induction by salicylate the levels of mRNA transcripts corresponding to the PAR-1 cDNA were analysed in response to virus infection. To this end tobacco plants cv. Samsun NN were infected with PVY which on this cultivar gives rise to systemic infections (unpublished data). First symptoms of infected leaves are the browning of veins which is followed by a crinkly and finally necrotic appearance of the leaves. In plants with 6 to 8 leaves PVY spreads systemically to all leaves above the directly infected one while in elder plants – depending on the number of leaves at the time of infection – the first to the fourth above the infected leaf does not get infected (unpublished observation).

The expression of PAR-1 transcripts was studied after treatment of tobacco plants with PVY from 2 to 12 days after infection (dpi). Samples were taken for RNA analysis as well as for detection of PVY by the ELISA technique from the directly infected as well as from the systemic non-infected and infected leaves. Four days after mechanical treatment of leaves with PVY a weak induction of the PAR-1 transcripts could be observed (Fig. 5a, lane 3). Transcript levels increased up to 9 dpi (Fig. 5a, lanes 3–8). A moderate accumulation of transcripts was also found in the systemically uninfected leaves (Fig. 5b, lanes 7–9). At no time virus could be detected in these leaves using monoclonal antibodies against PVY coat protein or by hybridizing northern blots with cDNA encoding PVY coat protein (data not shown). Systemic leaves which got infected accumulated high levels of PAR-1 transcripts starting 8 dpi (Fig. 5c, lanes 7–9). PVY coat protein could be detected 2 dpi after PVY treatment in locally infected leaves and 6 dpi in systemically infected leaves. The data prove that the PAR-1 transcripts are inducible by virus both locally and systemically. The expression of the PAR-1 transcripts in response to PVY occurred co-ordinately with the expression of mRNAs encoding PR-Q and SAR8.2 (data not shown).

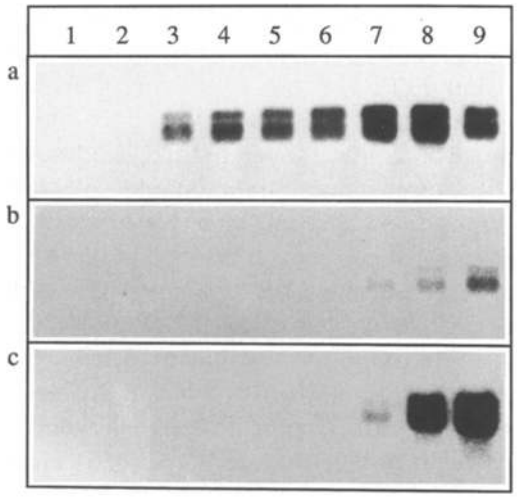

Fig. 5. Accumulation of PAR-1 transcripts in tobacco leaves infected with PVY. Samples were taken from the directly infected (a), upper uninfected (b) and upper infected (c) leaves. Each RNA (15 μg total RNA was loaded per lane) is a pool of eight different plants. Samples were taken before (lane 1) and 2, 4, 5, 6, 7, 8, 9, 12 days (lanes 2–9) after infection.

Isolation of different classes of PAR-1 cDNAs

To find out whether the two differently sized PAR-1 transcripts were due to the expression of homologous genes the original ppa-1 cDNA library was screened with the PAR-1 cDNA fragment. Out of several hundred hybridizing plaques 25 independent clones were purified and after *in vivo* excision analysed by restriction and sequencing. According to their homology the cDNAs (between 0.6 and 1 kb in length) could be grouped into three different classes (termed PAR-1a, b, c). The first two classes (a and b) shared 90.6% homology on the DNA level, while both groups were less related to class c (75.2% and 70.5% homology, respectively). The three different cDNAs contained one common open reading frame which would code for a protein with a theoretical molecular mass of ca. 20 kDa. Comparisons between the putative coding regions of the three PAR-1 proteins essentially reflected the degree of similarity found on the DNA level. PAR-1a and PAR-1b proteins would be identical to 90.3% while they would share only 78.1% and 79% identical amino acids with the PAR-1c protein, respectively. The amino acid sequences of the proteins deduced from the open reading frames of the three different PAR cDNAs have been aligned in Fig. 6. The DNA homology in the putative coding region of the three PAR proteins is significantly higher than in the surrounding untranslated regions (data not shown) which additionally argues for this open reading frame as being the coding region. All three PAR proteins have the same hydrophilic profile with a hydrophobic stretch of 28 amino acids at the N-terminus which resembles a signal peptide. It seems likely that the three PARs are targetted into the secretory pathway.

Differential expression of PAR mRNAs after virus infection

To discriminate between the differently sized PAR-1 transcripts DNA fragments were isolated from the putative 3′-untranslated regions of the three PAR-1 cDNAs. These were used as probes in northern blots to analyse the accumulation of PAR-1 mRNAs in infected and uninfected leaves of PVY-treated tobacco plants. As shown in Fig. 7, PAR-1a (lanes 5–8) and PAR-1c cDNA fragments (lanes 9–12) differentially hybridized to the 1.2 kb and 1.0 kb mRNA transcript, respectively. For comparison, the same blot was hybridized with the full-length PAR-1a probe

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A  MVASFHSF-TLAIVACALVFCVQVTLGSITCENLNKDSCTFAISSTGKRC
B  ......N.-.............................A..........
C  .-.....LK......L.ISYF......G.A.....E...A.....N....

A  VLEKHLRRSGEEVYACKTLEIEADKLKNWIETDQCIQACGVDRNTLGISS
B  ....R.Q.......T...S.........D.................F....
C  ............G.T.R.S.........D.....E..E.......A.....

A  DSLLECHFTQKLCSPQCYKHCPNIVDLYFNLAAGEGVYLPRLCEKQGGNA
B  .......................I..................E.....
C  .A...SR..N.....A.......................K..AE...K.

A  RRGMAEIKNSGIVAPAPESGVKPVNFMITPAMAPAS          185
B  ........S...L..S...E.NSA...........F          185
C  ..EI...RS..L...G-..E...S....A...-.PF          183
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Fig. 6. Alignment of the amino acid sequences deduced from the three different classes of PAR-1 cDNAs. A, B, C represent the amino acid sequences of PAR-1a, PAR-1b and PAR-1c, respectively. Gaps marked by dashes were introduced for optimal alignment. Identical amino acids are marked by dots.

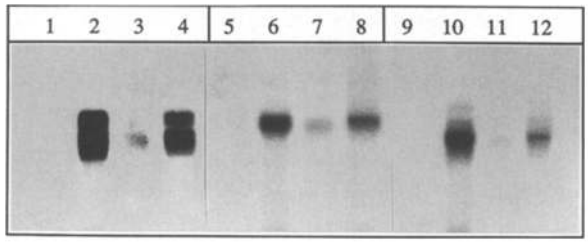

Fig. 7. RNA analysis of PVY-infected tobacco plants using selective PAR-1 3′ probes. RNAs loaded (15 μg loaded per lane) were prepared from leaves of non-infected plants (lanes 1, 5, 9), from the mechanically infected leaves (lanes 2, 6, 10) and from non-infected upper (lanes 3, 7, 11) and systemically infected leaves (leaves 4, 8, 12) of PVY-treated tobacco plants 9 days after infection. Each RNA is a pool from 8 different plants. Lanes 1–4 were hybridized with a full length PAR-1a cDNA probe in a buffer containing 25% formamide while lanes 5–8 and 9–12 were hybridized with PAR-1a and PAR-1c specific 3′ cDNA probes, respectively, in a buffer containing 50% formamide. The 3′ probes were 229 bp (PAR-1a), 156 bp (PAR-1b) and 175 bp (PAR-1c) in length and displayed at best 62% homology to the cDNAs of the other classes. Allowed mismatches under the hybridization conditions used were calculated to be about 14%.

under low-stringent conditions (Fig. 7, lanes 1–4). The PAR-1b cDNA fragment resulted in a very weak constitutive signal after 2 days exposure. PAR-1a and c transcripts were induced both in infected and uninfected leaves of PVY-treated plants (Fig. 7).

Discussion

Plants are faced with photoassimilate accumulation in their cells in a number of natural conditions like elevated levels of atmospheric CO_2, nitrogen deficiency, freezing, cold and osmotic stress. To study long-term adaptation of gene expression to sugar accumulation we used transgenic photoassimilate-accumulating tobacco plants (ppa-1) [27] as a model system.

The strategy was to isolate cDNA clones by differential hybridization of a subtractive library enriched for transcript present in the sugar-accumulating ppa-1 plants. In order to avoid cDNA artifacts frequently resulting from PCR amplifications during the establishment of a subtractive library a novel strategy was employed (Fig. 1). cDNA libraries were prepared from mRNAs of wild-type and ppa-1 plants allowing the *in vivo* amplification of the respective cDNAs. The cDNA library prepared from mRNA of ppa-1 plants was cut with the same restriction enzyme later used for establishment of the subtractive library. On the other hand, the cDNA of the library prepared from mRNA of wild-type plants was excluded from cloning because a different restriction enzyme was used. The exclusion of wild-type cDNA cloning allowed for only one round of subtraction with an enrichment for ppa-1-specific cDNAs by 10-fold. Thus this efficient and simple procedure eliminated the need for multiple rounds of hybridizations/subtractions and subsequent PCR amplifications.

By differential screening of the subtractive library 12 different cDNAs were isolated of which four could be identified as encoding 1-aminocyclopropane-1-carboxylate-oxidase (ACC oxidase) and three different PR-proteins (PR-1b, PR-Q and SAR8.2). Two cDNAs were found to be homologous to a Calmodulin-like protein from *Arabidopsis* and the human ribosomal protein L19 [17]. The remaining six did not show similarities to sequences in the databanks.

One of these unknown cDNAs (termed PAR-1 for photoassimilate-responsive) hybridized to mRNAs of about 1.0 and 1.2 kb. The steady-state levels of PAR-1 transcripts in leaves increased in the order from upper to lower leaves both in wild-type and ppa-1 plants thus being correlated with the levels of endogenous sugars (Fig. 2) [27, 9]. In ppa-1 plants which accumulate much higher levels of soluble sugars as compared to wild-type plants [27, 9], PAR-1 transcripts also accumulated to higher levels. These correlations strongly indicate that the induction of PAR-1 mRNA may be mediated by an increase in soluble sugars in the plant cells. This assumption has further been supported by the finding that wild-type leaves floated on different concentrations of glucose and sucrose also accumulated high levels of PAR-1 transcripts (Fig. 3). Thus the accumulation of PAR-1 cDNA by

soluble sugars appears to be the cause for its isolation as a photoassimilate-responding gene.

By rescreening the ppa-1 cDNA library using PAR-1 as a probe 25 independent cDNAs were isolated and characterized. By comparison of their respective DNA sequences the cDNAs could be grouped into three different classes of which the first two (PAR-1a and b) were 90.6% homologous while they were less related to the third-group PAR-1c (75.2% and 70.5% homologous, respectively). PAR-1a and PAR-1c transcripts were found to be strongly inducible by salicylate in floating experiments and were furthermore shown to accumulate in uninfected and infected leaves of tobacco plants treated with PVY (Fig. 4, 5, 7). By these characteristics the PAR-1 cDNAs may be classified as cDNAs encoding a novel class of pathogenesis-related proteins. The PAR-1 cDNAs would code for proteins with a theoretical molecular mass of 20 kDa which could be targetted into the secretory pathway due to an N-terminal signal peptide (Fig. 6).

Different sets of PR proteins are known to be induced under a number of stress conditions like viral infection, pathogen invasion, injury, UV light, ozone, chemical treatment, salicylic acid, ethylene and other plant hormones ([3]; reviewed in [19]). Thus the accumulation of at least four different PR proteins under conditions where the plant cells permanently meet high levels of sugars and osmotic stress might have been expected. However, PR proteins have attracted attention mainly for their roles in host/pathogen relationships, particularly for their possible involvement in inducing resistance against further development of virus disease in tobacco leaves. Less consideration has been attributed to PR protein function in response to other physiological conditions like osmotic stress. Nevertheless, Pierpoint *et al.* [22] and Ohashi and Matsuoka [18] reported on the induction of PR proteins under mannitol-induced osmotic stress. Osmotin, a protein which belongs to the group 5 of PR proteins, had first been studied in cultured tobacco cells osmotically adapted to low water potential [25, 26]. Under comparable osmotic conditions, namely floating leaves on sorbitol and PEG, accumulation of PAR-1 mRNAs was not induced (Fig. 4). Thus the induction of PAR-1 transcripts in wildtype leaves upon floating on osmolytes cannot be solely attributed to osmotic effects. Penetrating and possibly metabolizable solutes such as sucrose and glucose (Fig. 3) may be required for the response.

The question arises how the induction of the PAR-1 mRNAs is mediated and whether there might be induction mechanisms common to other PR protein genes in physiological situations where plants accumulate photoassimilates in their cells. Hypothetically, even the induction of PR proteins by pathogens might follow the same mechanism. Viruses have been described to cause severe perturbations in carbohydrate metabolism in leaves leading to the accumulation of starch [32 and references therein] and/or soluble sugars [36, 29]. Sturm and Chrispeels [31] found that bacterial infection caused rapid induction of extracellular invertase which hydrolyses extracellular sucrose. Glucose and fructose may be taken up which might trigger the induction response. Sugars have also been reported to induce other stress-related proteins, such as proteinase inhibitor II [10, 11], cathepsin D inhibitor and leucine aminopeptidase from potato [unpublished data] and chalcone synthase [33]. Thus, as already suggested by Jang and Sheen [8], there might be a common mechanism of sugar sensing in the repression of photosynthetic genes and the activation of stress-related/pathogenesis-related genes. As the induction of PAR and PR protein-specific transcripts in the ppa-1 plants might be mediated by salicylate we have determined salicylate levels in these plants. There was no difference in the levels of salicylic acid or its conjugate between ppa-1 and wild-type plants (Ph. Meuwly, J.-P. Métraux, unpublished data). This indicates that the accumulation of PR protein-specific transcripts by soluble sugars follows a salicylate-independent pathway. Other or additional mechanisms for the induction of PR proteins in the photoassimilate-accumulating plants cannot be ruled out. For instance, the accumulation of the mRNA coding for ACC oxidase might indicate a role of ethylene in the induction process. This is supported by the

finding that carbohydrates stimulate ethylene production in tobacco leaf discs [21].

Current experiments are devoted to studies on the signal transduction mechanisms of stress-related as well as photosynthetic genes in ppa-1 and other transgenic sugar-accumulating plants (manuscript in preparation).

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