Plant Molecular Biology **28**: 885–900, 1995.

Stress responses in alfalfa (*Medicago sativa* L.) XIX. Transcriptional activation of oxidative pentose phosphate pathway genes at the onset of the isoflavonoid phytoalexin response

Theo Fahrendorf, Weiting Ni[1], Basil S. Shorrosh[2] and Richard A. Dixon*
*Plant Biology Division, The Samuel Roberts Noble Foundation, 2510 Sam Noble Parkway, Ardmore, OK 73401, USA (*author for correspondence); [1] Present address: USDA-ARS, Department of Agromony and Genetics, University of Minnesota, St Paul, MN 55108, USA; [2] Present address: Michigan State University, Department of Botany and Plant Pathology, Plant Biology Building, East Lansing, MI 48824-1312, USA*

Received 22 February 1995; accepted in revised form 2 May 1995

Key words: alfalfa, elicitation, glucose 6-phosphate dehydrogenase, isoflavonoids, pentose phosphate pathway, 6-phosphogluconate dehydrogenase

Abstract

We have isolated cDNA clones encoding the pentose phosphate pathway enzymes 6-phosphogluconate dehydrogenase (6PGDH, EC 1.1.1.44) and glucose 6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) from alfalfa (*Medicago sativa* L.). These exhibit extensive nucleotide and amino acid sequence similarity to the corresponding genes from bacteria, *Drosophila* and mammals. Transcripts encoding both enzymes are expressed at high levels in roots and nodules. Exposure of alfalfa suspension cells to an elicitor from yeast cell walls results in co-ordinated increases in transcription rates for both genes, followed by increased steady state transcript levels but only slightly increased extractable enzyme activities, at the onset of accumulation of isoflavonoid phytoalexins. Levels of NADPH and NADP remain relatively constant in alfalfa cells following elicitation. The rapid transcriptional activation of 6PGDH and G6PDH does not therefore appear to be a response to altered pyridine nucleotide redox state. These genes appear to respond to early events in elicitor-mediated signalling rather than to subsequent elicitor-induced changes in secondary metabolism. Hydrogen peroxide, a potential signal for elicitation of anti-oxidative genes in biologically stressed plant cells, did not induce 6PGDH or G6PDH transcripts or enzymatic activity.

Introduction

Elicitation of isoflavonoid phytoalexin synthesis in intact tissues or cell cultures of leguminous plants involves the coordinated induction of a complex set of biosynthetic enzymes for the conversion of *L*-phenylalanine to antimicrobial pterocarpans or isoflavans [4]. Increases in the activi-

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers U18238 (alfalfa glucose 6-phosphate dehydrogenase) and U18239 (alfalfa 6-phosphogluconate dehydrogenase).

ties of enzymes involved in the biosynthesis of pterocarpan phytoalexins are preceded by increases in steady-state levels of the corresponding transcripts, which in turn appear to result from *de novo* transcriptional activation [12, 31]. Elicitor-responsive increases in transcript levels are not, however, restricted to enzymes directly involved in secondary metabolism. Thus, in alfalfa, elicitor induces striking increases in transcripts encoding acetyl CoA carboxylase, the enzyme which forms malonyl CoA as co-substrate for the first committed step of flavonoid biosynthesis [50], and *S*-adenosyl *L*-methionine (SAM) synthetase, the enzyme that forms the methyl group donor for isoflavone methylation [18], at the onset of accumulation of the phytoalexin medicarpin. Likewise, in parsley cell cultures, elicitation of methylated furanocoumarin phytoalexins is associated with increases in transcripts encoding SAM synthetase and *S*-adenosyl *L*-homocysteine hydrolase [26]. DAHP synthase, the first enzyme of the shikimic acid pathway of aromatic amino acid synthesis, is induced in elicited parsley cell cultures [35] and in bacterially infected *Arabidopsis* leaves [27]. Elicitation of defense-related secondary metabolism would therefore appear to require increased enzymatic capacity from primary metabolism, which may be provided by de novo enzyme synthesis.

The formation of one molecule of medicarpin from *L*-phenylalanine requires six molecules of NADPH (Fig. 1). The oxidative pentose phosphate pathway is a major source of NADPH in the cytoplasm of plant cells [46]. This pathway also provides the erythrose 4-phosphate that, along with phosphoenolpyruvate formed from glycolysis, serves as a precursor for phenylalanine biosynthesis via the shikimic acid pathway. Several studies have suggested that the pentose phosphate pathway is the source of reducing equivalents for phenylpropanoid biosynthesis, and that its activity is increased under conditions of increased flux into the phenylpropanoid pathway. Thus, ^{14}C-glucose labelling experiments and measurement of the activity of glucose 6-phosphate dehydrogenase (G6PDH) indicated a high activity of the pentose phosphate pathway during lignification in stems of *Coleus blumei*, sunflower, and pea [40, 41]. G6PDH activity increases during synthesis of pterocarpan phytoalexins in bean and chickpea cell suspension cultures [10, 43], and soybean hypocotyls [7].

NADPH is produced by the first two steps of the oxidative pentose phosphate pathway, catalyzed by G6PDH and 6-phosphogluconate dehydrogenase (6PGDH). Cytoplasmic and plastidic isoforms, with very similar kinetic properties, exist for both enzymes in plants [22, 23, 46, 51]. The activities of the two forms of both enzymes are regulated by NADPH:NADP ratio, as NADPH is a potent competitive inhibitor with respect to NADP [11, 23]. Both forms of G6PDH are inactivated by light via a redox-regulated reaction [2]. Phenylpropanoid pathway intermediates have been shown to inhibit both tobacco 6PGDH isoenzymes [1], although the physiological significance of this is not clear. It is not known whether the increased activities of the pentose phosphate pathway enzymes measured in crude extracts from elicited cells reflect transcriptional, post-transcriptional or metabolic regulation.

In this paper we report the characterization of cDNA clones for 6PGDH and G6PDH from alfalfa, the developmental expression of these genes, and their transcriptional activation in elicited cell cultures. Increased activities of these two enzymes during isoflavonoid phytoalexin synthesis are preceded by rapid transcriptional activation of their corresponding genes. Comparison of the kinetics of transcriptional activation with NADPH levels in elicited cells suggests that the signals for initial activation of these two pentose phosphate pathway genes are not generated by elicitor-induced changes in metabolic flux, a conclusion further supported by the observation that treatment of elicited cells with the phenylalanine ammonia-lyase (PAL) inhibitor *L*-α-aminooxy-β-phenylpropionic acid (AOPP) failed to block transcriptional activation of G6PDH and 6PGDH.

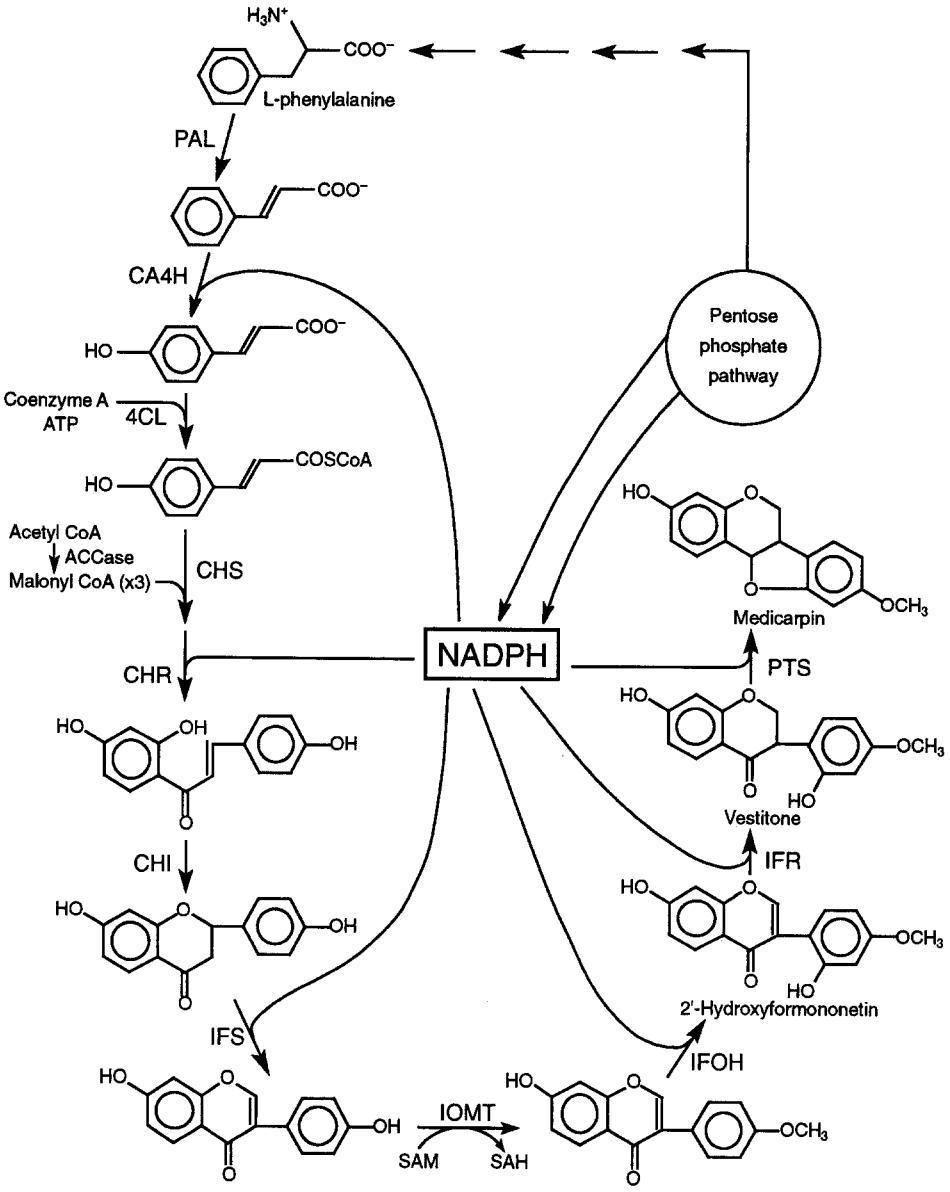


Fig. 1. Phytoalexin biosynthesis in alfalfa, showing inputs from primary metabolism. The enzymes are: PAL, *L*-phenylalanine ammonia-lyase; CA4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate: CoA ligase; CHS, chalcone synthase; CHR, chalcone reductase; ACC, acetyl CoA carboxylase; CHI, chalcome isomerase; IFS, isoflavone synthase; IOMT, isoflavone O-methyltransferase; IFOH, isoflavone 2′-hydroxylase; IFR, isoflavone reductase; PTS, pterocarpan synthase.

Materials and methods

Library screening

A full length potato glucose-6-phosphate dehydrogenase cDNA clone [20] was used to screen a λZAP II (Stratagene) cDNA library constructed from poly(A)$^+$ RNA isolated from alfalfa suspension cells 2, 3 and 4 h after exposure to fungal elicitor, using the procedure of Mierendorf *et al.* [36]. A positive clone was used to re-screen the library and obtain apparently full-length clones. An alfalfa 6-phosphogluconate dehydrogenase (6PGDH) cDNA fragment (see

Results) was likewise used to screen the same library for isolation of an apparently full-length 6PGDH clone.

DNA sequence analysis

cDNA inserts were initially subjected to double-stranded sequence analysis on an Applied Biosystems Model 373A DNA sequencer using a fluorescent dye-labelled dideoxy terminator kit (ABI), *Taq* polymerase (Cetus), and forward and reverse M13 primers. Subsequent sequencing reactions were primed with synthetic oligonucleotides based on the emerging sequence of the inserts. Deduced amino acid sequences were aligned by the CLUSTAL program [21].

Growth and elicitation of cell cultures

Cell suspension cultures of alfalfa (*Medicago sativa* L.) cv. Apollo were initiated and maintained in a modified Schenk and Hildebrandt medium as described [9]. Cells were treated with yeast elicitor [47] at a final concentration of 50 μg glucose equivalents/ml, frozen in liquid N_2, and stored at −80 °C.

Growth of plants

Nodulated alfalfa plants were grown, and tissues harvested, as described previously [18, 19].

Analysis of RNA and DNA

Total RNA and DNA were isolated from alfalfa tissues as described previously [49], and subjected to northern and Southern blot analyses according to the method of Church and Gilbert [8]. Final washing conditions for homologous probes were 40 mM sodium phosphate pH 7.2, 1 mM EDTA, 1% SDS at 65 °C for 4 × 10 min. The alfalfa cinnamic acid 4-hydroxylase cDNA probe used for northern analysis was the *Bam* HI to *Sal* I fragment of clone W2a described previously [15]. The *Arabidopsis* tubulin gene [33] was used as a control for loading/transfer efficiency of northern blots.

Nuclear transcript run-on analysis

The following buffers were used: Honda buffer (25 mM Tris-HCl pH 7.8, 0.44 M sucrose, 5 mM $MgCl_2$, 2.5% Ficoll Type 400 (Sigma), 5% Dextran T40 (Sigma), 10 mM 2-mercaptoethanol, 2 mM spermine); nuclei washing buffer (NWB, 50 mM Tris-HCl pH 8.5, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 20% v/v glycerol); nuclei resuspension buffer (NRB, 50 mM Tris-HCl pH 8.5, 5 mM $MgCl_2$, 10 mM mercaptoethanol, 50% v/v glycerol). Sucrose, mercaptoethanol and spermine were added immediately before use. All solutions were prepared using DEPC-treated water.

Cells (5–10 g) were ground in liquid N_2 to a fine powder with a mortar and pestle. The powder was transferred to 25–50 ml Honda buffer and stirred on ice for 5 min. After filtration through 4 layers of pre-wetted Miracloth, the nuclei were pelleted by centrifugation (1000 × ***g*** for 5 min) and resuspended in 20 ml Honda buffer. Interfering starch grains were removed by centrifugation at 100 × ***g*** for 5 min. The resuspended nuclei were centrifuged again (1000 × ***g*** for 5 min) and resuspended in 20 ml NWB. After an additional centrifugation (1000 × ***g*** for 5 min), the white to slightly grayish-looking nuclei (as seen under the microscope) were resuspended in 20 ml NRB. After a final centrifugation at 1000 × ***g*** for 5 min, the nuclei were resuspended in NRB (final vol 0.5–1 ml), divided into 0.1 ml aliquots and frozen in liquid N_2.

Nuclei (50 μl) were thawed on ice and centrifuged for 10 s at 4 °C. To 25 μl of the supernatant was added 10 μl 1 M $(NH_4)_2SO_4$, 4 μl 100 mM $MgCl_2$, 1 μl 100 mM phosphocreatine, 4 μl 0.25 mg/ml phosphocreatine kinase, 12.5 μl RNAsin, 31.5 μl H_2O, 2 μl NTP mix (500 mM ATP, CTP, GTP) and 10 μl UTP (200 mCi, 3000 Ci/mmol). The solution was incubated at

30 °C, and the reaction stopped with 1% SDS, 10 mM EDTA, after 10 to 20 min. Labelled transcripts were extracted as described previously [38], and subsequently used to probe membranes that had been saturated with up to 5 μg denatured cDNA probe per slot. The PAL cDNA probe was the 471 bp *Hind* III fragment of the alfalfa pAPAL1 cDNA described previously [19]. Hybridization and washing procedures were identical to those used for northern blots.

Analysis of NADPH and NADP levels

Freshly harvested alfalfa cells (0.5 g) were immediately homogenized in 5 ml 0.1 M NaOH (for determination of NADPH) or 0.1 M HCl (for determination of NADP), incubated at room temperature for 30 min, and adjusted to pH 7.8 with 1 M HCl (NADPH) or pH 7.2 with 1 M NaOH (NADP). Extracts (100 μl) were added to reaction mixtures containing 50 mM Tris/HCl pH 8.0, 30 mM glucose 6-phosphate, 7.5 mM dichlorophenolindophenol (DCPIP, Sigma), 3.7 mM phenazine-methosulfate (Sigma), and 5 units G6PDH (Boehringer, grade I) in a final volume of 1.0 ml. The absorbance of the cycling assay was recorded at 625 nm. Internal standards of NADP or NADPH between 50 and 400 pmol were included in the assay.

Assay of G6PDH and 6PGDH activities

Cells were extracted in 50 mM Tris-HCl pH 8.0, 300 mM NaCl, 0.1 mM benzamidine and 0.1 mM phenylmethylsulfonylfluoride (PMSF). G6PDH activity was measured at 30 °C in 0.1 M Tris-HCl pH 8.0, 0.4 mM NADP and 2 mM glucose-6-phosphate by measuring the change in absorbance at 340 nm [20]. 6PGDH activity was similarly measured at 30 °C in 0.1 M Hepes pH 7.5, 0.4 mM NADP and 3 mM 6-phosphogluconate (Boehringer).

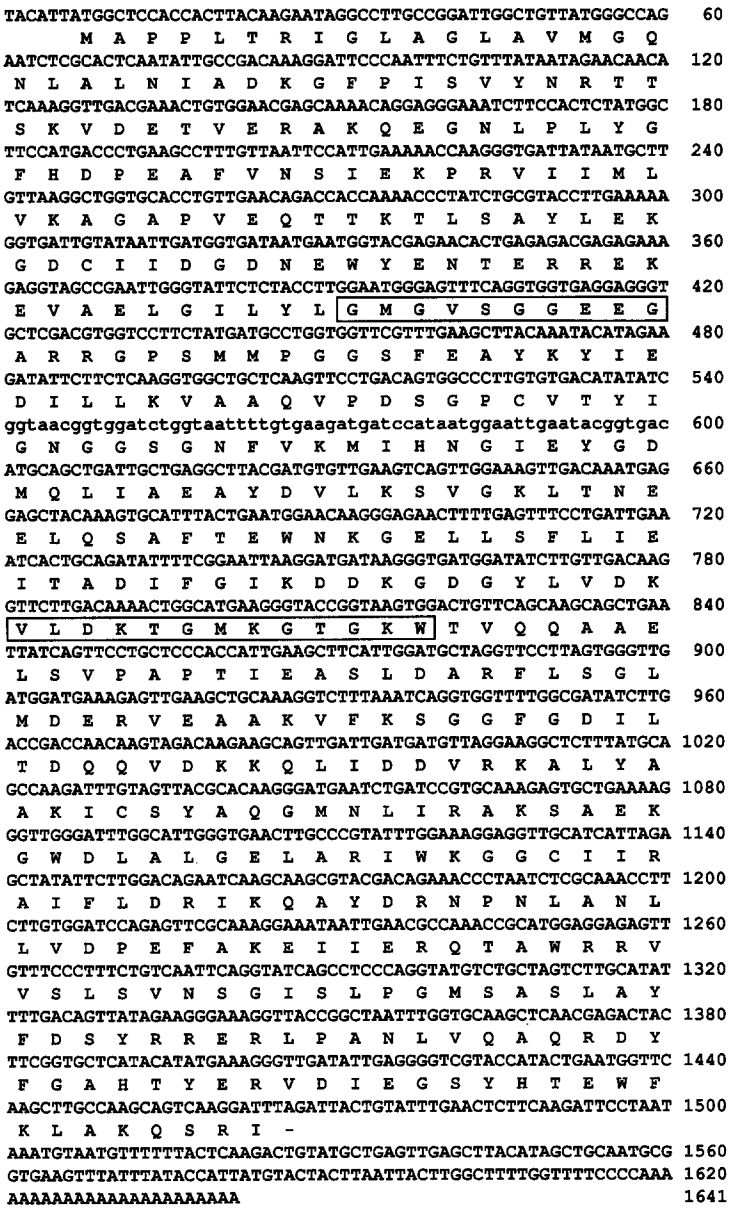
Results

Molecular characterization of alfalfa 6PGDH and G6PDH

In screening our cDNA library from elicited alfalfa cells for acetyl CoA carboxylase sequences, we discovered an acetyl CoA carboxylase clone that artifactually contained a second cDNA insert. On sequence analysis, this second insert showed strong similarity to bacterial, mammalian, and *Drosophila* 6PGDHs [3, 48, 52]. Rescreening the library with this insert resulted in the isolation of a large number of positive clones (ca. 0.3% of the library). Sequence analysis of more than 10 independent clones revealed identical 5′ and 3′ sequences, suggesting that most of the clones represent a single class of transcript which is highly expressed in the elicited cultures. The longest cDNA clone isolated (O19) encoded a polypeptide of deduced M_r 53625, pI 5.1, with 50 to 60% overall amino acid sequence identity to the 6 PGDHs from sheep [52] and *Escherichia coli* [3] (Figs. 2 and 3). Although the O19 clone clearly lacks most of the 6PGDH 5′-untranslated region, it probably contains the complete coding sequence, based on alignment with other 6PGDH sequences and the transcript size (see below). Among the blocks of highly conserved sequence were a potential dinucleotide binding site (consensus GxGxxGxxxG) at amino acid positions 129–138, and the proposed 6-phosphogluconate binding site with consensus [L/I/V/M]-x-D-x-x-[G/A]-[N/Q/S]-K-G-T-G-x-W, based on the comparison of all known bacterial and mammalian 6PGDH sequences [42], at amino acid positions 260–272 (Figs. 2 and 3). The alfalfa 6PGDH has a methionine residue in place of the consensus [N/Q/S] at position 266.

Screening the cDNA library with a potato G6PDH cDNA clone [20] resulted in the isolation of a single partial alfalfa G6PDH clone which was used to re-screen the library, again yielding a large number (ca. 0.3%) of positive plaques. As with the 6PGDH clones, all the G6PDH clones appeared to be of the same class, based on 5′ and 3′ sequencing of six independent clones. The

A



longest alfalfa G6PDH clone (G10) encoded a polypeptide with a deduced M_r of 58 923, pI 5.6, which exhibited 90% amino acid sequence identity to the potato G6PDH and 50 to 60% identity to G6PDHs from bacterial and mammalian sources [24, 44, 45] (Figs. 2 and 3). G10 contains the full N-terminal coding sequence of alfalfa G6PDH, as two in-frame stop codons are found upstream of the ATG translation initiation codon.

Neither the G6PDH nor the 6PGDH cDNA sequences appeared to contain plastidic signal sequences, suggesting that they encode the cytoplasmic forms of the corresponding enzymes. Southern blot analysis (Fig. 4) was consistent with both enzymes being encoded by small gene families in the alfalfa genome. However, as alfalfa is a hetero-tetraploid, the multiple bands observed on genomic blots could also be the result of alleic variation. We do not know whether chloroplastic forms of the enzymes would be detected in the

B

```
CACCAGATATAATTAAGTAGATCAGAGTAGAAGAAGATGGGAACAAATGAATGGCATGTA   60
                                    M  G  T  N  E  W  H  V
GAAAGAAGAGATAGCATAGGTACTGAATCTCCTGTAGCAAGAGAGGTACTTGAAACTGGC  120
 E  R  R  D  S  I  G  T  E  S  P  V  A  R  E  V  L  E  T  G
ACACTCTCTATTGTTGTGCTTGGTGCTTCTGGTGATCTTGCCAAGAAGAAGACTTTTCCT  180
 T  L  S  I  V  V  L  G  A  S  G  D  L  A  K  K  K  T  F  P
GCACTTTTTCACTTATATAAACAGGAATTGTTGCCACCTGATGAAGTTCACATTTTTGGC  240
 A  L  F  H  L  Y  K  Q  E  L  L  P  P  D  E  V  H  I  F  G
TATGCAAGGTCAAAGATCTCCGATGATGAATTGAGAAACAAATTGCGTAGCTATCTTGTT  300
 Y  A  R  S  K  I  S  D  D  E  L  R  N  K  L  R  S  Y  L  V
CCAGAGAAAGGTGCTTCTCCTAAACAGTTAGATGATGTATCAAAGTTTTTACAATTGGTT  360
 P  E  K  G  A  S  P  K  Q  L  D  D  V  S  K  F  L  Q  L  V
AAATATGTAAGTGGCCCTTATGATTCTGAAGATGGATTTCGCTTGTTGGATAAAGAGATT  420
 K  Y  V  S  G  P  Y  D  S  E  D  G  F  R  L  L  D  K  E  I
TCAGAGCATGAATATTTGAAAAATAGTAAAGAGGGTTCATCTCGGAGGCTTTTCTATCTT  480
 S  E  H  E  Y  L  K  N  S  K  E  G  S  S  R  R  L  F  Y  L
GCACTTCCTCCTTCAGTGTATCCATCCGTTTGCAAGATGATCAAAACTTGTTGCATGAAT  540
 A  L  P  P  S  V  Y  P  S  V  C  K  M  I  K  T  C  C  M  N
AAATCTGATCTTGGTGGATGGACACGCGTTGTTGTTGAGAAACCCTTTGGTAGGGATCTA  600
 K  S  D  L  G  G  W  T  R  V  V  V  E  K  P  F  G  R  D  L
GAATCTGCAGAAGAACTCAGTACTCAGATTGGAGAGTTATTTGAAGAACCACAGATTTAT  660
 E  S  A  E  E  L  S  T  Q  I  G  E  L  F  E  E  P  Q  I  Y
CGTATTGATCACTATTTAGGAAAGGAACTAGTGCAAAACATGTTAGTACTTCGTTTTGCA  720
 R  I  D  H  Y  L  G  K  E  L  V  Q  N  M  L  V  L  R  F  A
AATCGGTTCTTCTTGCCTCTGTGGAACCACAACCACATTGACAATGTGCAGATAGTATTT  780
 N  R  F  F  L  P  L  W  N  H  N  H  I  D  N  V  Q  I  V  F
AGAGAGGATTTTGGAACTGATGGTCGTGGTGGATATTTTGACCAATATGGAATTATCCGA  840
 R  E  D  F  G  T  D  G  R  G  G  Y  F  D  Q  Y  G  I  I  R
GATATCATTCCAAACCATCTGTTGCAGGTTCTTTGCTTGATTGCTATGGAAAAACCCGTT  900
 D  I  I  P  N  H  L  L  Q  V  L  C  L  I  A  M  E  K  P  V
TCTCTCAAGCCTGAGCACATTCGAGATGAGAAAGTGAAGGTTCTTGAATCAGTACTCCCT  960
 S  L  K  P  E  H  I  R  D  E  K  V  K  V  L  E  S  V  L  P
ATTAGAGATGATGAAGTTGTTCTTGGACAATATGAAGGCTATACAGATGACCCAACTGTA 1020
 I  R  D  D  E  V  V  L  G  Q  Y  E  G  Y  T  D  D  P  T  V
CCGGACGATTCAAACACCCCGACTTTTGCAACTACTATTCTGCGGATACACAATGAAAGA 1080
 P  D  D  S  N  T  P  T  F  A  T  T  I  L  R  I  H  N  E  R
TGGGAAGGTGTTCCTTTCATTGTGAAAGCAGGGAAGGCCCTAAATTCTAGGAAGGCAGAG 1140
 W  E  G  V  P  F  I  V  K  A  G  K  A  L  N  S  R  K  A  E
ATTCGGGTTCAATTCAAGGATGTTCCTGGTGACATTTTCAGGAGTAAAAAGCAAGGGAGA 1200
 I  R  V  Q  F  K  D  V  P  G  D  I  F  R  S  K  K  Q  G  R
AACGAGTTTGTTATCCGCCTACAACCTTCAGAAGCTATTTACATGAAGCTTACGGTCAAG 1260
 N  E  F  V  I  R  L  Q  P  S  E  A  I  Y  M  K  L  T  V  K
CAACCTGGACTGGAAATGTCTGCAGTTCAAAGTGAACTAGACTTGTCATATGGGCAACGA 1320
 Q  P  G  L  E  M  S  A  V  Q  S  E  L  D  L  S  Y  G  Q  R
TATCAAGGGATAACCATTCCAGAGGCTTATGAGCGTCTAATTCTCGACACAATTAGAGGT 1380
 Y  Q  G  I  T  I  P  E  A  Y  E  R  L  I  L  D  T  I  R  G
GATCAACAACATTTTGTTCGCAGAGACGAATTAAAGGCATCATGGCAAATATTCACACCA 1440
 D  Q  Q  H  F  V  R  R  D  E  L  K  A  S  W  Q  I  F  T  P
CTTTTACACAAAATTGATAGAGGGGAGTTGAAGCCGGTTCCTTACAACCCGGGAAGTAGA 1500
 L  L  H  K  I  D  R  G  E  L  K  P  V  P  Y  N  P  G  S  R
GGTCCTGCAGAAGCAGATGAGTTATTAGAAAAAGCTGGATATGTTCAAACACCCGGTTAT 1560
 G  P  A  E  A  D  E  L  L  E  K  A  G  Y  V  Q  T  P  G  Y
ATATGGATTCCTCCTACCTTATAGAGTGACCAAATTTCATAATAAAACAAGGATTAGGAT 1620
 I  W  I  P  P  T  L  -
TATCAGGAGCTTATAAATAAGTCTTCAATAAGCTTGTGAAATTTTCGTTATAATCTCTCT 1680
CATTTTGGGGTGTATATCAAGCATTTAAGCGCGTGTTTGACACAGTTTGTGTAATAGATT 1740
TGGCTCTGAATGAAAATAAACGGGAATTGTTTCTTTTTGTTTTAAAAAAAAAAAAA     1796
```

Fig. 2. DNA and deduced amino acid sequences of alfalfa 6PGDH cDNA clone O19 (**A**, previous page) and G6PDH clone G10 (**B**). Nucleotides are numbered from the first base of the cDNA insert. The deduced amino acid sequence for the alfalfa 6PGDH and G6PDH are indicated below the nucleotide sequence in single-letter code. A potential NADP-binding site (GXGXXGXXXG), that is fully conserved in all 6-phosphogluconate dehydrogenases, and the substrate-binding sites for each clone, are boxed.

Southern hybridization (perhaps as the weakly hybridizing bands in Fig. 4), and therefore whether the total number of genes encoding G6PDH and 6PGDH is higher than indicated here.

Development expression of 6PGDH and G6PDH transcripts

Total RNA was isolated from different organs of 6-week old alfalfa plants. Northern blot analysis

A

```
Alfalfa  - MAPPLTRIGLAGLAVMGQNLALNIADKGFPISVYNRTTSKVDETVERAKQEGNLP -55
E.coli   - MSK--QQIGVVGMAVMGRNLALNIESRGYTVSIFNRSREKTEEVI--AENPGKK- -50

Alfalfa  - LYGFHDPEAFVNSIEKPRVIIMLVKAGAPVEQTTKTLSAYLEKGDCIIDGDNEWY -110
E.coli   - LVPYYTVKEFVESLETPRRILLMVKAGAGTDAAIDSLKPYLDKGDIIIDGGNTFF -105

Alfalfa  - ENTERREKEVAELGILYLGMGVSGGEEGARRGPSMMPGGSFEAYKYIEDILLKVA -165
E.coli   - QDTIRRNRELSAEGFNFIGTGVSGGEEGALKGPSIMPGGQKEAYELVAPILTKIA -160

Alfalfa  - AQVPDSGPCVTYIGNGGSGNFVKMIHNGIEYGDMQLIAEAYDVLKSVGKLTNEEL -220
E.coli   - AVAEDGEPCVTYIGADGAGHYVKMVHNGIEYGDMQLIAEAYSLLKGGLNLTNEEL -215

Alfalfa  - QSAFTEWNKGELLSFLIEITADIFGIKDDKGDGYLVDKVLDKTGMKGTGKWTVQQ -275
E.coli   - AQTFTEWNNGELSSYLIDITKDIFTKKDEDGN-YLVDVILDEAANKGTGKWTSQS -269

Alfalfa  - AAELSVPAPTIEASLDARFLSGLMDERVEAAKVFKSGGFGDILTDQQVDKKQLID -330
E.coli   - ALDLGEPLSLITESVFARYISSLKDQRVAASKVLSGRQ-----AQPAGDKAEFIE -319

Alfalfa  - DVRKALYAAKICSYAQGMNLIRAKSAEKGWDLALGELARIWKGGCIIRAIFLDRI -385
E.coli   - KVRRALYLGKIVSYAQGFSQLRAASEEYNWDLNYGEIAKIFRAGCIIRAQFLQKI -374

Alfalfa  - KQAYDRNPNLANLLVDPEFAKEIIERQTAWRRVVSLSVNSGISLPGMSASLAYFD -440
E.coli   - TDAYAENPQIANLLLAPYFKQIADDYQQALRDVVAYAVQNGIPVPTFSAAVAYYD -429

Alfalfa  - SYRRERLPANLVQAQRDYFGAHTYERVDIEGSYHTEWFKLAKQSRI -486
E.coli   - SYRAAVLPANLIQAQRDYFGAHTYKRIDKEGVFHTEWLD       -468
```

revealed that 6PGDH transcripts (the probe detected a single transcript of 1.8 kb) were highly expressed in roots and nodules, with only very weak expression in leaves and growing points (Fig. 5). The highest level of expression of both G6PDH (1.8 kb transcripts) and 6PGDH was in young but fully differentiated nodules (3 weeks); this decreased as nodules matured until, after 8 weeks, the expression level was the same as in unnodulated root tissue (Fig. 6). In contrast, the level of cinnamic acid 4-hydroxylase transcripts (encoding the first NADPH-utilizing enzyme of phenylpropanoid synthesis) remained relatively constant during nodule development and maturation.

Transcriptional activation of pentose phosphate pathway genes at the onset of the phytoalexin response

Treatment of alfalfa cells with yeast elicitor results in the accumulation of up to 300 nmol/g fresh weight of the pterocarpan phytoalexin medicarpin [12]. Medicarpin accumulation begins about 6 to 8 h after elicitation, and is maximal around 40 h after elicitation. Northern blot analysis revealed striking induction of G6PDH and 6PGDH transcripts in elicitor-treated alfalfa cell suspension cultures (Fig. 7). Transcript levels were strongly induced by 4 h after elicitation, followed by a gradual decline to control levels over the next 10 to 20 h. The high transcript levels were

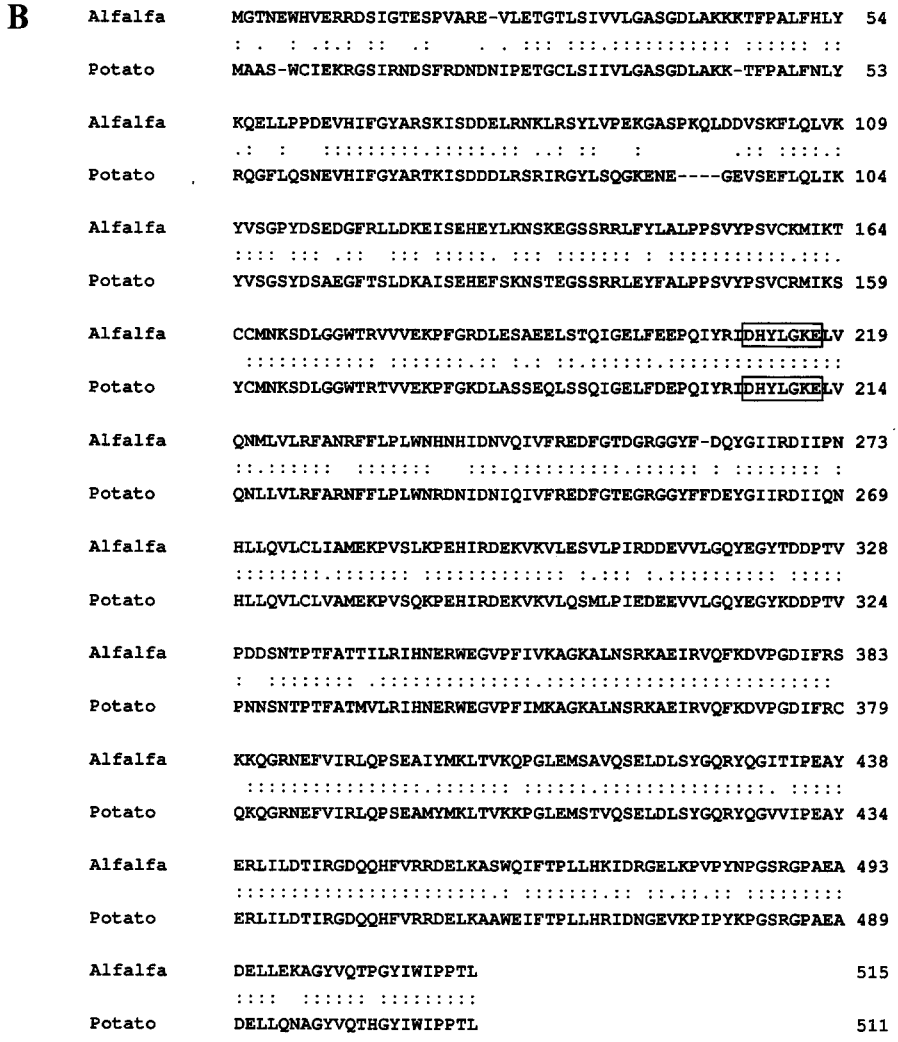


Fig. 3. Comparison of the deduced amino acid sequences for the alfalfa clones O19 (6-phosphogluconate dehydrogenase, **A**) and G10 (glucose-6-phosphate dehydrogenase, **B**) with the deduced amino acid sequences for *E. coli* 6PGDH [40] and potato G6PDH [20]. Identical amino acids are indicated by (:), similar amino acids by (.). The resumed binding sites for 6-phosphogluconate and glucose 6-phosphate are boxed.

consistent with the high proportion of G6PDH and 6PGDH clones in the elicited alfalfa cDNA library. The increased transcript levels resulted in small but significant increases in the extractable activities of the two enzymes, reaching maximum levels (ca. 30% increase) by 4 h after elicitation (Fig. 8). The increased activity declined to control levels by 12 h in the case of 6PGDH, but G6PDH activity remained elevated for at least 18 h.

To determine whether the elicitor-induced increases in steady-state transcript levels reflected transcriptional activation of the G6PDH and 6PGDH genes, nuclear transcript run-on analysis was performed. Changes in 6PGDH transcription rates compared to those of PAL were determined in three independent experiments using different alfalfa cell batches, and G6PDH transcription was measured in two of these experiments. The data in Fig. 9 summarize these studies. Transcription of G6PDH and 6PGDH genes was rapidly activated following exposure to elicitor. Although the absolute timings of the changes in transcription rate varied considerably between

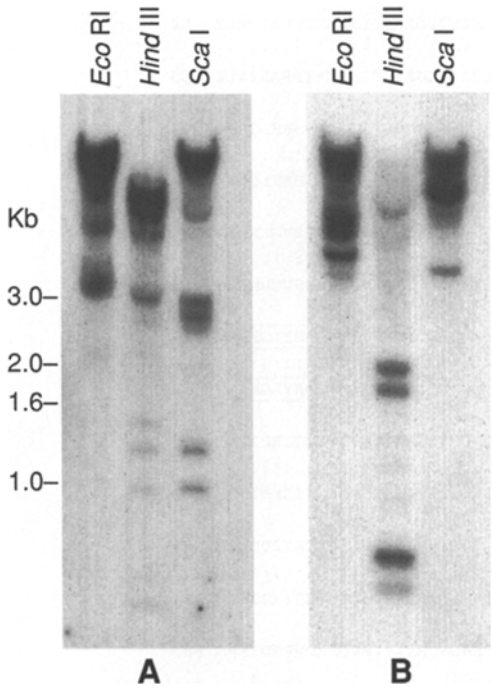


Fig. 4. Southern blot hybridization of alfalfa genomic DNA to G6PDH and 6PGDH sequences. Genomic DNA was digested with *Eco* RI, *Hind* III and *Sca* I, electrophoresed on 1% agarose gels, blotted onto nylon membranes and probed with full-length O19 (6PGDH, **A**) or G10 (G6PDH, **B**) inserts.

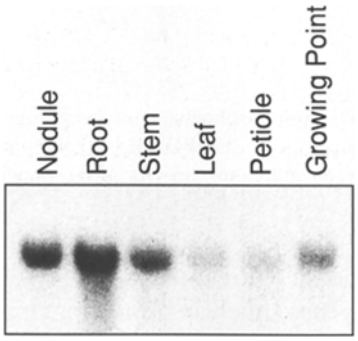


Fig. 5. Northern blot analysis of 6PGDH mRNA levels in various organs of 6 week old alfalfa plants. Total RNA (10 μg/lane) was separated by electrophoresis, blotted onto a nylon membrane and hybridized with the labelled 6PGDH clone.

different batches of elicited cells, transcription of the two pentose phosphate pathway enzyme genes was closely co-ordinated over the first 30 to 45 min after elicitation. Increases in transcription

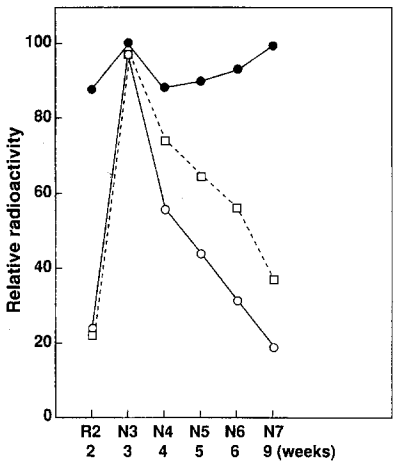


Fig. 6. Relative levels of 6PGDH (□), G6PDH (○) and CA4H (●) mRNAs during nodule development. For each time point 10 μg of total RNA was separated by electrophoresis, blotted onto a nylon membrane and hybridized with the different probes. N3 to N6 represent nodule mRNAs from 3–6-week old plants, and N7 represents mRNA from old, senescing nodules harvested from 9-week old alfalfa plants. R2 represents mRNA from 2-week old alfalfa-roots; at this early stage of development, the young nodules could not yet be harvested because of their microscopic size. The blots were quantitated with a PhosphorImager. The strongest signal for each probe during the developmental time course was normalized to 100%.

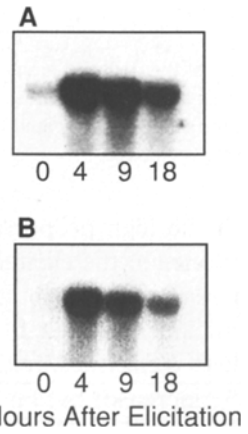


Fig. 7. Accumulation of G6PDH (**A**) and 6PGDH (**B**) transcripts in alfalfa suspension cells upon elicitation with yeast elicitor. Cells were harvested at different time points after elicitation, and total RNA (10 μg/lane) was separated by electrophoresis, blotted onto nylon membranes and probed with the labelled full-length clones for 6PGDH and G6PDH.

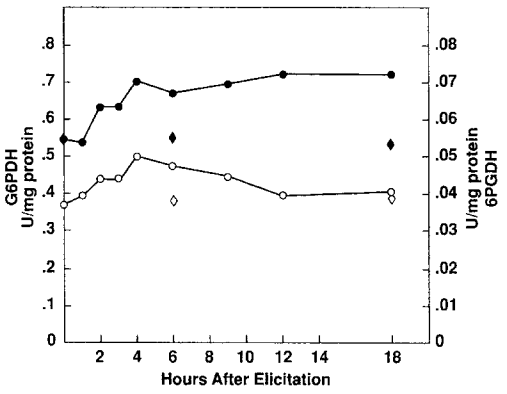


Fig. 8. Increase in 6PGDH (o) and G6PDH (●) enzyme activities in alfalfa suspension cells after elicitation with yeast extract. Corresponding activities in unelicited cells are shown by ◇ and ◆ respectively. One unit of enzyme activity is defined as 1 mmol NADPH produced per minute.

of G6PDH and 6PGDH were slower than that of PAL in the early stages of induction. Thus, in the experiment shown if Fig. 9A, increased transcription of PAL was measurable 10 min after elicitation, whereas increased transcription of 6PGDH was not initiated until 20 to 30 min after elicitation. Similar results are seen for both pentose phosphate pathway genes in relation to PAL induction in the experiment shown in Fig. 9B, in which maximum transcription rates were attained at one hour post-elicitation for all three genes. In the experiment shown in Fig. 9C, increased transcription of G6PDH and 6PGDH was measurable earlier than in the two previous experiments, although the initial rate was still lower than for PAL.

Signals for activation of pentose phosphate pathway genes in elicited cells

As the above results indicated that elicitation leads to increased pentose phosphate pathway enzyme activities via transcriptional activation, we asked whether this was brought about directly through elicitor-mediated signal pathways or was a consequence of the cells' sensing of the requirement for increased production of NADPH to support the synthesis of elicitor-induced isoflavonoids. We first measured NADPH and NADP

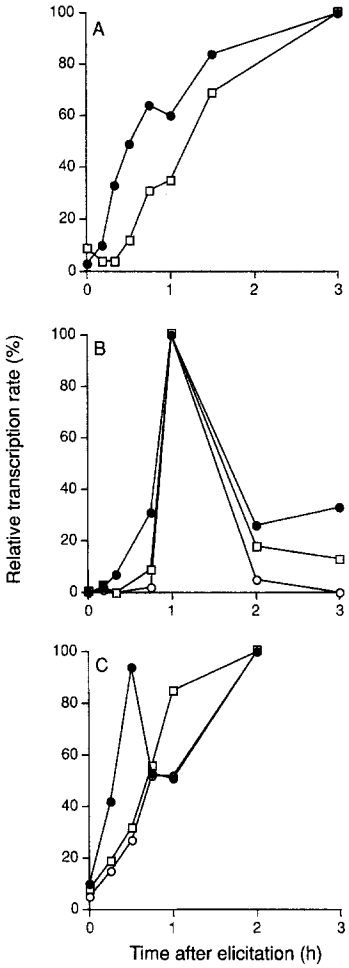


Fig. 9. Transcriptional activation of G6PDH, 6PGDH and PAL genes upon elicitation. Three different batches of alfalfa suspension cells were treated with yeast elicitor, cells from different time points were harvested, and nuclei isolated for nuclear transcript run-on analysis. Transcripts synthesized by the isolated nuclei *in vitro* were hybridized to excess immobilized cDNA probes. Each batch of *in vitro* transcripts was hybridized to a membrane strip containing all three probes. Transcription induction kinetics are shown for the three different experiments (**A**, **B**, **C**). Values from PhosphorImager analysis are presented, normalized to the highest value for each transcript as 100%. Kinetics of G6PDH (○) and 6PGDH (□) transcription are compared to those of PAL (●), the first enzyme of the phenylpropanoid pathway.

levels in the cells (Fig. 10). Elicitation had no significant effect on the levels of NADPH (20–25 nmol/g fresh weight) or NADP (2–5 nmol/g fresh weight). The levels of NADPH and NADP remained constant during the period of early in-

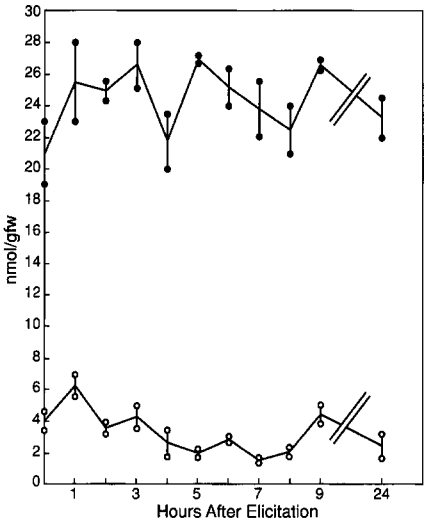


Fig. 10. Changes in NADPH and NADP levels in alfalfa suspension cells upon elicitation. Cells were harvested at different times after elicitation, and immediately assayed for NADPH (●) and NADP (○) levels.

duction of phytoalexin biosynthetic enzyme activities (which consume NADPH) [12] and during the later increase in G6PDH and 6PGDH activities (which produce NADPH). It is therefore unlikely that NADPH level itself could act as a signal for pentose phosphate pathway gene activation.

L-α-aminooxy-β-phenylpropionic acid (AOPP) is a potent inhibitor of PAL activity *in-vitro* and *in vivo*, and effectively blocks synthesis of phenylpropanoid derived compounds in alfalfa cell cultures [28]. AOPP-treated cells can respond to elicitor-mediated signals [34], but are blocked at the level of flux into metabolites synthesized from cinnamate, the product of the PAL reaction. Treatment of elicited alfalfa cells with AOPP at a concentration known to inhibit alfalfa PAL *in vivo* led to a one hour delay but only a 27.5% decrease in maximum transcription rate for both genes (data not shown). In contrast, PAL transcription was more rapid and was 250% higher in AOPP-treated than in control elicited cultures (data not shown), consistent with previous results which have shown super-induction of PAL transcription and activity associated with inhibition of flux into the phenylpropanoid pathway [5, 34].

The above results suggest that transcriptional activation of pentose phosphate pathway genes is the result of early elicitor-mediated signal transduction events rather than subsequent metabolic events dependent on induction of the phenylpropanoid pathway. Hydrogen peroxide has recently been demonstrated to act as a diffusible signal for the elicitation of genes of antioxidative metabolism, such as glutahione *S*-transferase and glutathione peroxidase, in soybean cell cultures [32]. However exogenously supplied H_2O_2 (2 mM) failed to induce either G6PDH or 6PGDH transcripts in the alfalfa cell cultures (data not shown).

Discussion

Using the recently described potato G6PDH cDNA clone [20] as a probe, we isolated corresponding cDNAs from an elicited alfalfa cell suspension library. These clones, which were highly represented in the elicited cell culture library, appeared to represent a single class of G6PDH transcript. This alfalfa G6PDH was over 90% identical to that from potato at the amino acid level. Neither alfalfa nor potato G6PDH clones contain a characteristic chloroplast targeting sequence or the conserved cysteine residues characteristic of light/dark redox-regulated cyanobacterial G6PDHs [20, 45]. The alfalfa G6PDH cDNA therefore most likely corresponds to a cytoplasmic form of the enzyme involved in the oxidative pentose phosphate pathway. The lack of a chloroplast target sequence suggests the same is true for the 6PGDH, the sequence of which has not been previously reported from plants. These findings are consistent with the lack of functional chloroplasts in the non-photoautotrophic alfalfa cell cultures.

A comparison of 6PGDH sequences from *Drosophila*, bacterial, ovine and porcine sources revealed two highly conserved sequence motifs [30]. In the alfalfa 6PGDH sequence these are located at amino acid positions 129–138 and 259–271, and represent the NADP and 6-phosphogluconate binding sites. The deduced M_r of 53625 is consistent with previous reports of

6PGDHs from soybean, spinach, and castor bean having an overall M_r of 110000 or a subunit M_r of 55000 [22, 46, 51].

The highest levels of pentose phosphate pathway enzyme transcripts in alfalfa plants were observed in root nodules and non-nodulated root tissue. In soybean, the high activity of 6PGDH and corresponding flux through the pentose phosphate pathway in the plant fraction of root nodules has been proposed to be associated with the requirement for synthesis of purine ribonucleotides (via ribulose 5-phosphate), for production of ureides, the transport form of fixed nitrogen in soybean [22, 30]. However, alfalfa nodules, which transport fixed nitrogen in the form of amides, have similar overall pentose phosphate pathway activity to soybean nodules [30], suggesting the need for alternative explanations of the high pentose phosphate pathway activity. In pea roots, 6PGDH and G6PDH activities have been implicated in the production of reducing power for nitrite reduction in the plastids [14, 39]. Our analyses did not distinguish between plastidic and cytosolic activities.

Both G6PDH and 6PGDH have been reported to be higher in leaves of *Citrus* species resistant to infection by the bacterial pathogen *Xanthomonas campestris* cv. *citri* than in susceptible species [25]. This may be associated with increased levels of pre-formed antimicrobial phenolic compounds in the resistant species.

The activities of both G6PDH and 6PGDH increase at the onset of phytoalexin accumulation in elicited alfalfa cell suspension cultures, preceded by increased steady-state transcript levels and transcriptional activation rates. The relatively small overall increases in extractable activities (compared to those of several of the enzymes of the phenylpropanoid/isoflavonoid pathway [12]) are perhaps surprising in view of the extent of the increase in steady state transcript levels, but are similar to the changes in G6PDH reported in elicited bean and chickpea cell suspensions [10, 43]. In soybean, fungal elicitation was reported to have no effect on G6PDH activity in cell cultures, although the enzyme was induced in hypocotyls, but not significantly in roots, following infection with *Phytophthora megasperma* f. sp. *glycinea* [6, 7, 29].

Is induction of the pentose phosphate pathway necessary to support the synthesis of isoflavonoid phytoalexins in elicited alfalfa cells? Medicarpin synthesis involves 6 enzymatic activities requiring NADPH (Fig. 1). The total NADPH requirement for phytoalexin synthesis from *L*-phenylalanine is ca. 1800 nmol/g fresh weight over the first 20 h after elicitation, based on accumulation of 300 nmol/g fresh weight total medicarpin (aglycone plus conjugate) over this time period [12]. Literature values for the K_ms for NADPH for some of these enzymes are 17 μM (CHR), 20–50 μM (IFR) and 40–45 μM (PTS) [4, 16, 17, 53, 54]. Thus, the concentration of NADPH in unelicited cells is poised at around the K_m values for this set of enzymes. As other NADPH-consuming reactions will also be occurring in the cells, replenishment of cytoplasmic NADPH may be a metabolic priority which can only be achieved by increased enzyme production, and the small changes (ca. 30%) reported here may be sufficient. It will be interesting in the future to determine the reason for the apparent discrepancy between transcript levels and enzyme activity for G6PDH and 6PGDH in the elicited cells. This may reflect a low rate of recruitment of the transcripts into polysomes, or the synthesis of new enzyme which is either inactive or subsequently inactivated.

Elicitor-mediated transcriptional activation of genes encoding early enzymes of isoflavonoid biosynthesis (e.g. PAL and CHS) is highly co-ordinated [31], suggesting response to a common signal transduction pathway. The initial transcription kinetics of G6PDH and 6PGDH are mutually co-ordinated, but these genes are activated somewhat later than the PAL group. However, the timing of induction of the two pentose phosphate pathway genes, and the still significant activation of both genes in cells in which flux into the phenylpropanoid pathway is inhibited with AOPP, suggest that downstream metabolic sensing of requirement for NADPH production is not the signal for pentose pathway gene activation. It appears, from the induction kinetics, that both

genes respond to a secondary signal generated during the first 10 min post-elicitation. This signal does not appear to be hydrogen peroxide, which has been implicated as a diffusible elicitor of anti-oxidative enzymes at the onset of induction of phytoalexin synthesis in soybean cells [32].

Acknowledgements

We thank Kerstin Graeve for providing the potato G6PDH cDNA, Alex Levine for the GST cDNA, Bob Gonzales, Valerie Graves and Ann Harris for DNA sequencing and synthesis of oligonucleotides, Vincent Sewalt and Marilyn Roossinck for critical reading of the manuscript, and Cuc Ly and Ty Myers for artwork. This work was supported by the Samuel Roberts Noble Foundation.

References

1. Al-Quadan F, Wender SH, Smith EC: The effect of some phenolic compounds on the activity of 6-phosphogluconate dehydrogenase form tobacco tissue cultures. Phytochemistry 20: 961–964 (1981).
2. Anderson LE, Duggan JX: Light modulation of glucose-6-phosphate dehydrogenase. Plant Physiol 58: 135–139 (1976).
3. Barcak GJ, Wolf Jr RE: Comparative nucleotide sequence analysis of growth-rate-regulated *gnd* alleles from natural isolates of *Escherichia coli* and from *Salmonella typhimurium* LT-2. J Bact 170: 372–379 (1988).
4. Bless W, Barz W: Isolation of pterocarpan synthase, the terminal enzyme of pterocarpan biosynthesis in cell suspension cultures of *Cicer arietinum*. FEBS Lett 235: 47–50 (1988).
5. Bolwell GP, Mavandad M, Millar DJ, Edwards KJ, Schuch W, Dixon RA: Inhibition of mRNA levels and activities by *trans*-cinnamic acid in elicitor-induced bean cells. Phytochemistry 27: 2109–2117 (1988).
6. Bonhoff A, Loyal R, Ebel J, Grisebach H: Race: cultivar-specific induction of enzymes related to phytoalexin biosynthesis in soybean roots following infection with *Phytophthora megasperma* f.sp. *glycinea*. Arch Biochem Biophys 246: 149–154 (1986).
7. Börner H, Grisebach H: Enzyme induction in soybean infected by *Phytophthora megasperma* f.sp. *glycinea*. Arch Biochem Biophys 217: 65–71 (1982).
8. Church GM, Gilbert W: Genomic sequencing. Proc Natl Acad Sci USA 81: 1991–1995 (1984).
9. Dalkin K, Edwards R, Edington B, Dixon RA: Stress responses in alfalfa (*Medicago sativa* L.) I. Induction of phenylpropanoid biosynthesis and hydrolytic enzymes in elicitor-treated cell suspension cultures. Plant Physiol 92: 440–446 (1990).
10. Daniel S, Tiemann K, Wittkampf U, Bless W, Hinderer W, Barz W: Elicitor-induced metabolic changes in cell cultures of chickpea (*Cicer arietinum* L.) cultivars resistant and susceptible to *Ascochyta rabiei*. Planta 182: 270–278 (1990).
11. Dennis DR, Miernyk JA: Compartmentation of nonphotosynthetic carbohydrate metabolism. Annu Rev Plant Physiol 33: 27–50 (1982).
12. Dixon RA, Choudhary AD, Dalkin K, Edwards R, Fahrendorf T, Gowri G, Harrison MJ, Lamb CJ, Loake GJ, Maxwell CA, Orr J, Paiva NL: Molecular biology of stress-induced phenylpropanoid biosynthesis in alfalfa. In: Stafford HA, Ibrahim RK (eds) Phenolic Metabolism in Plants, pp. 91–138. Plenum Press, New York (1992).
13. Dixon RA, Harrison MJ: Activation, structure and organization of genes involved in microbial defense in plants. Adv Genet 28: 165–234 (1990).
14. Emes MJ, Fowler MW: The supply of reducing power for nitrite reduction in plastids of seedling pea roots (*Pisum sativum* L.) Planta 158: 97–102 (1983).
15. Fahrendorf T, Dixon RA: Molecular cloning of the elicitor-inducible cinnamic acid 4-hydroxylase cytochrome P450 from alfalfa. Arch Biochem Biophys 305: 509–515 (1993).
16. Fischer D, Ebenau-Jehle C, Grisebach H: Phytoalexin synthesis in soybean: purification and characterization of NADPH: 2′-hydroxydaidzein oxidoreductase from elicitor-challenged soybean cell cultures. Arch Biochem Biophys 276: 390–395 (1990).
17. Fischer D, Ebenau-Jehle C, Grisebach H: Purification and characterization of pterocarpan synthase from elicitor-challenged soybean cell cultures. Phytochemistry 29: 2879–2882 (1990).
18. Gowri G, Bugos RC, Campbell WH, Maxwell CA, Dixon RA: Stress responses in alfalfa (*Medicago sativa* L.) X. Molecular cloning and expression of *S*-adenosyl-*L*-methionine: caffeic acid 3-*O*-methyltransferase, a key enzyme of lignin biosynthesis. Plant Physiol 97: 7–14 (1991).
19. Gowri G, Paiva NL, Dixon RA: Stress responses in alfalfa (*Medicago sativa* L.) XII. Sequence analysis of phenylalanine ammonia-lyase (PAL) cDNA clones and appearance of PAL transcripts in elicitor-treated cell cultures and developing plants. Plant Mol Biol 17: 415–429 (1991).
20. Graeve K, von Schaewen A, Scheibe R: Purification, characterization, and cDNA sequence of glucose-6-phosphate dehydrogenase from potato (*Solanum tuberosum* L.). Plant J 5: 353–361 (1994).
21. Higgins DG, Sharp PM: Clustal: a package for performing multiple sequence alignments on a microcomputer. Gene 73: 237–244 (1988).

22. Hong ZQ, Copeland L: Isoenzymes of 6-phosphogluconate dehydrogenase from the host fraction of soybean nodules. J Plant Physiol 139: 313–319 (1992).
23. Ishida S, Ashihara H: Isoenzymes of phosphogluconate dehydrogenase (decarboxylating) from suspension-cultured *Catharanthus roseus* cells. Phytochemistry 33: 1307–1311 (1993).
24. Jeffery J, Barros-Soderling J, Murray L, Wood I, Hansen R, Szepesi B, Jornvall H: Glucose 6-phosphate dehydrogenase. Characteristics revealed by the rat liver enzyme structure. Eur J Biochem 186: 551–556 (1989).
25. Jiao HJ, Wang SY, Civerolo EL: Enzymatic activities of citrus leaves from plants resistant and susceptible to citrus bacterial canker disease. Environ Exp Bot 32: 465–470 (1992).
26. Kawalleck P, Plesch G, Hahlbrock K, Somssich IE: Induction by fungal elicitor of *S*-adenosyl-*L*-methionine synthetase and *S*-adenosyl-*L*-homocysteine hydrolase mRNAs in cultured cells and leaves of *Petroselinum crispum*. Proc Natl Acad Sci USA 89: 4713–4717 (1992).
27. Keith B, Dong X, Ausubel F, Fink GR: Differential induction of 3-deoxy-*D-arabino* heptulosonate 7-phosphate synthase genes in *Arabidopsis thaliana* by wounding and pathogenic attack. Proc Natl Acad Sci USA 88: 8821–8825 (1991).
28. Kessmann H, Edwards R, Geno PW, Dixon RA: Stress responses in alfalfa (*Medicago sativa* L.) V. Constitutive and elicitor-induced accumulation of isoflavonoid conjugates in cell suspension cultures. Plant Physiol 94: 227–232 (1990).
29. Kochs G, Welle R, Grisebach H: Differential induction of enzymes in soybean cell cultures by elicitor or osmotic stress. Planta 171: 519–524 (1987).
30. Kohl DH, Lin J-J, Shearer G, Schubert KR: Activities of the pentose phosphate pathway and enzymes of proline metabolism in legume root nodules. Plant Physiol 94: 1258–1264 (1990).
31. Lawton MA, Lamb CJ: Transcriptional activation of plant defense genes by fungal elicitor, wounding and infection. Mol Cell Biol 7: 335–341 (1987).
32. Levine A, Tenhaken R, Dixon RA, Lamb CJ: H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell 79: 583–593 (1994).
33. Marks MD, West J, Weeks DP: The relatively large beta-tubulin gene family of *Arabidopsis* contains a member with an unusual transcribed 5′ noncoding sequence. Plant Mol Biol 10: 91–104 (1987).
34. Mavandad M, Edwards R, Liang X, Lamb CJ, Dixon RA: Effects of *trans*-cinnamic acid on expression of the bean phenylalanine ammonia-lyase gene family. Plant Physiol 94: 671–680 (1990).
35. McCue KF, Conn EE: Induction of 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase activity by fungal elicitor in cultures of *Petroselinum crispum*. Proc Natl Acad Sci USA 86: 7374–7377 (1989).
36. Mierendorf RC, Percy C, Young RA: Gene isolation by screening lgt11 libraries with antibodies. Meth Enzymol 152: 458–469 (1987).
37. Nasoff MS, Baker HVII, Wolf REJ: DNA sequence of the *Escherichia coli* gene, *gnd*, for 6-phosphogluconate dehydrogenase. Gene 27: 253–264 (1984).
38. Ni W, Trelease RN: Post-transcriptional regulation of catalase isozyme expression in cotton seeds. Plant Cell 3: 737–744 (1991).
39. Oji Y, Watanabe M, Wakiuchi N, Okamoto S: Nitrite reduction in barley-root plastids: dependence on NADPH coupled with glucose-6-phosphate and 6-phosphogluconate dehydrogenases, and possible involvement of an electron carrier and a diaphorase. Planta 165: 85–90 (1985).
40. Pryke JA, ap Rees T: Activity of the pentose phosphate pathway during lignification. Planta 132: 279–284 (1976).
41. Pryke JA, ap Rees T: The pentose phosphate pathway as a source of NADPH for lignin synthesis. Phytochemistry 16: 557–560 (1977).
42. Reizer A, Deutscher J, Saier MH Jr, Reizer J: Analysis of the gluconate (gnt) operon of *Bacillus subtilis*. Mol Microbiol 5: 1081–1089 (1991).
43. Robbins MP, Bolwell GP, Dixon RA: Metabolic changes in elicitor-treated bean cells. Eur J Biochem 148: 563–569 (1985).
44. Rowley DL, Wolf RE Jr: Molecular characterization of the *Escherichia coli* K-12 *zwf* gene encoding glucose 6-phosphate dehydrogenase. J Bact 173: 968–977 (1991).
45. Scanlan DJ, Newman J, Sebaiaha M, Mann NH, Carr NG: Cloning and sequence analysis of the glucose 6-phosphate dehydrogenase gene from the cyanobacterium *Synechococcus* PCC 7942. Plant Mol Biol 19: 877–880 (1992).
46. Schnarrenberger C, Oeser A, Tolbert NE: Two isoenzymes each of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in spinach leaves. Arch Biochem Biophys 154: 438–448 (1973).
47. Schumacher HM, Gundlach H, Fiedler F, Zenk MH: Elicitation of benzophenanthridine alkaloid synthesis in *Escholtzia* cell cultures. Plant Cell Rep 6: 410–413 (1987).
48. Scott MJ, Lucchesi JC: Structure and expression of the *Drosophila melanogaster* gene encoding 6-phosphogluconate dehydrogenase. Gene 109: 177–183 (1991).
49. Shorrosh BS, Dixon RA: Molecular cloning of a putative plant endomembrane protein resembling vertebrate protein disulfide-isomerase and a phosphatidylinositol-specific phospholipase C. Proc Natl Acad Sci USA 88: 10941–10945 (1991).
50. Shorrosh BS, Dixon RA, Ohlrogge JB: Molecular cloning, characterization, and elicitation of acetyl-CoA carboxylase from alfalfa. Proc Natl Acad Sci USA 91: 4323–4327 (1994).
51. Simcox PD, Dennis DT: 6-Phosphogluconate dehydrogenase isoenzymes from the developing endosperm of *Ricinus communis* L.. Plant Physiol 62: 287–290 (1978).
52. Somers D O'N, Medd SM, Walker JE, Adams MJ: Sheep

6-phosphogluconate dehydrogenase. Biochem J 288: 1061–1067 (1992).

53. Tiemann K, Hinderer W, Barz W: Isolation of NADPH: isoflavone oxidoreductase, a new enzyme of pterocarpan phytoalexin biosynthesis in cell suspension cultures of *Cicer arietinum*. FEBS Lett 213: 324–328 (1987).

54. Welle R, Grisebach H: Isolation of a novel NADPH-dependent reductase which coacts with chalcone synthase in the biosynthesis of 6′-deoxychalcone. FEBS Lett 236: 221–225 (1988).