Arabidopsis thaliana NADPH Oxidoreductase Homologs Confer Tolerance of Yeasts toward the Thiol-oxidizing Drug Diamide*

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To isolate new plant genes involved in the defense against oxidative stress, an Arabidopsis cDNA library in a yeast expression vector was transformed into a yeast strain deficient in the YAP1 gene, which encodes a b-Zip transcription factor and regulates general stress response in yeasts. Cells from ~10^5 primary transformants were subjected to a tolerance screen toward the thiol-oxidizing drug diamide, which depletes the reduced glutathione in the cell. Four types of Arabidopsis cDNAs were isolated. Three of these cDNAs (P1, P2, and P4) belong to a plant â-crystallin family and P3 is an Arabidopsis homolog of isoflavonoid reductases. As such, all four isolated cDNAs are homologous to NADPH oxidoreductases. P1, P2, and P3 steady-state mRNAs accumulated rapidly in Arabidopsis plants under various oxidative stress conditions, such as treatment with paraquat, tert-butylhydroperoxide, diamide, and menadione. The data suggested that proteins encoded by the isolated cDNAs play a distinct role in plant antioxidant defense and are possibly involved in NAD(P)/NAD(P)H homeostasis.

The formation of oxygen radicals by partial reduction of molecular oxygen is an unfortunate consequence of aerobic life. Active oxygen species (AOS), such as superoxide anion (O2-), hydrogen peroxide (H2O2), and hydroxyl radicals (OH·) are natural by-products of metabolism and also result from exposure to free radical-generating compounds (natural quinones, xenobiotics, and pollutants) (1). AOS are highly reactive and damage DNA, proteins, lipids, and carbohydrates (1, 2).

Aerobic organisms have evolved a number of enzymic and nonenzymic antioxidant defense mechanisms, which counteract the harmful effects of AOS and maintain the cellular steady-state of pro-oxidants and antioxidants (1, 3). Nonenzymic antioxidants, such as ascorbic acid, glutathione, carotenoids, and â-tocopherol scavenge AOS directly through chemical mechanisms. The major enzymes of importance in O2- scavenging are superoxide dismutases, which disproportionate superoxide anions to dioxygen and hydrogen peroxide, and the latter being detoxified by catalases, glutathione peroxidases, and ascorbate peroxidases (1, 3–5).

Adverse environmental conditions often lead to the disruption of the pro-oxidant/antioxidant homeostasis. A disbalance in favor of pro-oxidants, potentially leading to cell damage, has been defined as oxidative stress (3). The activity of antioxidant defenses often correlates positively with aging or senescence, disease development, or ability to adapt to changing environment (4). Furthermore, hereditary defects in antioxidant protection have multiple pleiotropic effects both in prokaryotic and eukaryotic organisms. For example, mutations in the gene coding for the copper/zinc superoxide dismutase are associated with familiar amyotrophic lateral sclerosis in humans (6) and yeast mutants in mitochondrial manganese superoxide dismutase are hypersensitive to oxygen (7). The increase in activity of AOS-scavenging enzymes by gene engineering, for instance, prolongs the life span of Drosophila (8) and improves stress tolerance of plants (9).

The importance of the antioxidant defense mechanisms is mirrored by their complexity, and new antioxidant proteins are continuously described. The reversion of mutant phenotypes associated with defects in antioxidant defense by screening for extragenic suppressors or by gene overexpression has been found to be a suitable approach toward better understanding pro-oxidant/antioxidant homeostasis (9–12).

Following such studies in yeasts, we became interested in examining whether the random overexpression of plant cDNAs in yeast cells would allow by-pass of phenotypes associated with a deficiency of antioxidant defense mechanisms. This approach allows the characterization of hitherto unidentified genes, the isolation of which would be not amenable by conventional methods.

The yeast mutant deficient in YAP1 was chosen in the present study. The YAP1 gene encodes a transcription activator of the b-Zip family of DNA-binding proteins and was originally isolated as a yeast homolog of mammalian jun (13). In later studies, however, the YAP1 gene was isolated by different research groups as a gene, which when overexpressed on multicopy plasmids, confers tolerance to different toxic compounds (14–16). Direct expression analysis and DNA binding studies have shown that YAP1 plays an important role in stress-induced transcriptional activation of many yeast genes, and more
specifically of antioxidant genes, including those encoding thioredoxin, TRX2 (17), and γ-glutamylcysteine synthetase, GSH1 (18). The levels of antioxidant defense enzymes, such as superoxide dismutase, glutathione peroxidase, and glucose-6-phosphate dehydrogenase are lower in yap1 mutants (16). The role of YAP1 in regulation of the antioxidant defense is reflected in the increased sensitivity of yap1 mutants to hydroperoxides, thiol oxidants, and redox-cycling drugs (17).

In this paper, we present experiments aimed to isolate Arabidopsis cDNAs, of which the overexpression improves the survival of yeast cells deficient in YAP1 function in the presence of the thiol-oxidizing drug diamide (azodicarboxylic acid bis(dimethylamide), which depletes intracellular glutathione in vivo (1) and oxidizes cysteine residues in proteins in vitro (19)). The expression of antioxidant genes, such as the thioredoxin gene TRX2 in yeasts and manganese superoxide dismutase in Escherichia is strongly activated in diamide-treated cells (17, 20). Yeast, diamide-stimulated up-regulation of the TRX2 gene is mediated through YAP1 activation (17). Furthermore, the YAP1 gene, when present on a high copy number plasmid, confers high tolerance of yeast cells to diamide (17). In Escherichia, the diamide-induced manganese superoxide dismutase gene activation is mediated through the superoxide anion sensor soxRS (20).

MATERIALS AND METHODS

Yeast and Escherichia coli Strains—Nearly isogenic Saccharomyces cerevisiae strains (DY, MATa his3 can1-100 ade2 leu2 trp1 ura3::3Sx540AP1:1acZ), and WYT, MATa his3 can1-100 ade2 leu2 trp1 ura3 yap1::TRP1) were used in this study (17). Yeast transformation was carried out according to Dohmen et al. (21). Strains were grown either on nutrient-rich medium (1% yeast extract, 2% bactopeptone, 2% glucose) supplemented with 50 mg liter⁻¹ of adenine or on minimal SD medium (0.67% yeast nitrogen base (Difco, Detroit, MI), 2% glucose). Yeast strains were grown in stationary or logarithmic phases were grown in SD medium at 30°C, with shaking at 200 rpm. Cells suspensions were serially diluted in 0.3% NaCl and 10 μl of dilutions were spotted on SD plates supplemented with drugs. After 3 days of incubation at 30°C, growth was assessed. Dilution series grown on plates without drugs were used to compare original cell density of different cultures. For the WYT strain, the following concentrations of drugs were used: 1 mM diamide, 0.2 and 0.3 mM t-BOOH, 0.2 mM cycloheximide, 100 μM CdCl². The diamide tolerance of DY strains was tested on plates with 1.5 mM diamide.

DNA and RNA Work—DNA was extracted from Arabidopsis plants according to Shirzadegan et al. (27). Genomic DNA, extracted by the method of Motter (28), was additionally purified by CsCl isopnic centrifugation in the presence of 10 μg ml⁻¹ ethidium bromide. Gene copy number and possible RFLP were estimated by DNA gel blot analysis using Arabidopsis DNA digested with different restriction endonucleases according to standard procedures (22). Analysis of mRNA was carried out by glyoxal RNA gel blot analysis (22) with modifications according to Mironov et al. (29). For the DNA and RNA hybridizations on nylon membranes (Hybond N; Amersham, Aylesbury, United Kingdom) the buffer (0.25 m sodium phosphate, pH 7.2, 7% sodium dodecyl sulfate, 1% bovine serum albumin, 1 mM EDTA) of Church and Gilbert (30) was used. Radioactively labeled DNA probes were made by the random priming method according to the manufacturer’s instructions (QuickPrimer kit; Pharmacia, Uppsala, Sweden). The aph cDNA was isolated in our laboratory and was identical to the cDNA published by Kubo et al. (31).

Plasmid Construction—The expression cassettes containing cDNA clones designated P1 and P3 were excised from pFL61 as BglII fragments, which, after filling in DNA ends were ligated into the blunt SphI site of pGAD424 (Clontech, Palo Alto, CA). The shuttle vector pGAD424 has the LEU2 gene for selection in yeasts, which permitted the isolation of yeast strains with both pFL61 (URA3 selection) and pGAD234 based vectors.

Sequence Analysis—DNA sequencing was performed according to protocols provided by U. S. Biochemical Corp. Vector primers, GTTTCAGCTTTTATAGTC and AGGGTAGAAAGTGGG, were used to obtain initial sequences. To complete the sequences on both strands, gene-specific primers were synthesized on an oligonucleotide synthesizer (Applied Biosystems, Foster City, CA). The DNA and amino acid sequence analyses were done with a software package by Genetics Computer Group Inc. (Madison, WI).

RESULTS

Isolation of Arabidopsis cDNAs Conferring Diamide Tolerance of yap1 Yeast—Yeast cells lacking a functional YAP1 gene were transformed with an Arabidopsis cDNA library in the yeast expression vector pFL61 (24). Approximately 10⁸ uracil prototrophic (URA3⁺) colonies were selected in 11 independent transformation experiments. Forty thousand and 200,000 cells of pooled primary transformants were plated onto SD plates containing 1 and 1.5 mM diamide, respectively. Forty-eight colonies, which grew after 4-7 days of selection, were tested for diamide tolerance, by spotting 10-μl aliquots of diluted stationary phase cultures on plates containing 1 mM diamide; 23 doses showed confluent growth at a dilution of 1/50 on 1 mM diamide and were further analyzed in this study.

Partial DNA sequencing of the rescued plasmids was used to identify the clones. Four types of clones, designated P1, P2, P3, and P4 were found. Fourteen cDNA clones of eight different lengths contained the P1-type cDNA. The P2 (4 clones), P3 (3 clones), and P4 (2 clones) were represented each as two different length cDNAs. This shows the consistency in the recovery of Arabidopsis cDNAs in the described screen.

The longest cDNAs of each type were retransformed into the yap1⁻ mutant strain, WYT, and the diamide tolerance of two independent transformants for each cDNA was assessed in a semi-quantitative dilution series test, as shown in Fig. 1. WYT (pFL61) cells from stationary phase culture are sensitive to 1

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The longest P2 cDNA is 1206 bp, and RNA gel blot analysis showed a transcript of 1.24 kilobases. The isolated P2 cDNA was not able to improve the survival of yeast cells on plates supplemented with t-BOOH, cycloheximide, and cadmium (data not shown).

The isolated Arabidopsis cDNAs were then tested for their ability to improve the diamide tolerance of the nearly isogenic to WYT, YAP1 strain DY. As shown in Fig. 2A, YAP1 strain DY showed better survival on 1.5 mM diamide, when it expressed P1, P2, P3, and P4 cDNAs. However, P1, P2, P3, and P4 clones were less efficient in rescuing cells from diamide toxicity as compared to the clone D3, which was isolated in a similar screening with a DY strain.2

If the mechanisms of protection from yeast cells from diamide toxicity by the isolated clones were different, we may expect additive effects for the diamide tolerance, when several cDNAs are co-expressed. To test this hypothesis, we recloned the P1 and P3 expression cassettes into another yeast-Escherichia shuttle vector, which has a LEU2 gene as a selectable marker. Yeast strains containing the two plasmids were selected on SD medium plates lacking uracil and leucine. When assessed in a dilution test, none of the tested combinations showed strong additive effects for the diamide tolerance, when several cDNAs were co-expressed.

The two isolated P4 cDNAs, of which the longest was 2175 bp, were different in one base pair at the 5' end and 12 bp at the 3'-nontranslated regions (not counting the polyadenylation tail). A 2.4-kilobase transcript was detected by RNA gel blot analysis using P4 as a probe (Fig. 3D). The P4-encoded polypeptide of 630 amino acids is preceded by an in-frame stop codon.

Fig. 4 shows that a number of proteins identified in the EMBL data base have strong amino acid sequence similarity to P1 and P2. The best scores (Table I) were found to the rabbit protein AdRab-F, which is expressed only in the intestine of adult rabbits and not in newborn animals. Lower homology was found to z-crystallins from guinea pigs, trypanosome Leishmania amazonensis, and to the plant cDNA TED2, originally isolated as a transcript up-regulated during in vitro xylem differentiation of zinnia mesophyll cells (Fig. 4). The actual function of these proteins in vivo is not well defined, although lens z-crystallin have been shown to possess NADPH:quinate oxidoreductase activity in vitro (35).

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Fig. 5 presents the sequence of the amino terminus of the P4 polypeptide. In this sequence, 250 amino acids are similar to those of a family of short-chain dehydrogenases. It shares, for example, 58.9% similarity and 38% identity with human NAD-
dependent 15-hydroxyprostaglandin dehydrogenase, 53% similarity and 28% identity with NADP-dependent glucose dehydrogenase from Bacillus megaterium, and 50% similarity and 30% identity with the Arabidopsis 3-oxoacyl (acyl carrier protein) reductase. The amino acid residues, which are important for the enzymic activity of short-chain dehydrogenases (39,40), are well conserved in the P4 polypeptide (Fig. 5).

The carboxyl-terminal part of the P4 polypeptide, starting from the 284th amino acid, shows similarity with the P1 polypeptide (Table I). The highest similarity can be found to the L. amazonensis ζ-crystallin, which is followed by the TED2 cDNA, the guinea pig lens ζ-crystallin, and the rabbit protein AdRab-F (Fig. 4 and Table I). Therefore, P4 is most probably a two-domain protein. Specific peptidesequences for targeting to different cell compartments could not be identified in P1, P2, or P4, suggesting that the proteins reside in the cytosol.

The gene copy numbers for P1, P2, and P4 were estimated on DNA gel blots of Arabidopsis genomic DNA digested with Dral, SacI, EcoRV, PstI, and HindIII. DNA extracted from two different land races of Arabidopsis was compared to find RFLP. The pattern of bands hybridizing with the P1 and P2 probes is highly similar, as predicted from the high DNA sequence identity of these two cDNAs (Fig. 3, A and B). Since single hybridizing bands of the same molecular weight were detected with P1 and P2 probes in DNA digested with SacI and PstI, these two genes are likely to be tightly linked on the chromosome. Additionally, weakly hybridizing bands were also detected that may indicate the presence of divergent genes in the Arabidopsis genome. The P4 gene is apparently unique in the Arabidopsis genome, because a simple hybridization pattern was observed (Fig. 3D). The P4 locus is polymorphic and an RFLP polymorphism was found, with four out of the five used restriction endonucleases tested, between Arabidopsis ecotypes Columbia and Landsberg erecta. Using the RFLP polymorphism obtained with Dral and DNA extracted from 98 Arabidopsis inbred recombinant lines (23), the P4 gene was mapped in the middle of chromosome 1 close to the RFLP marker m213 (mapping data are available from the laboratory of Dr. C. Dean, John Innes Institute, Norwich, UK).

The highest levels of P1, P2, and P4 steady-state mRNAs were detected in leaves and less in stems. Weak expression occurred in flowers and very weak expression in roots of Arabidopsis plants (Fig. 3, A, B, and D). From the sequence comparison, it was clear that the cDNAs P1, P2, and P4 corresponded to a rather divergent family of plant proteins, which were tentatively named together with zinnia TED2, plant ζ-crystallins.

P3 Encodes a Putative Isoflavonoid Reductase—The longest P3 cDNA was 1116 bp in length and RNA gel blot analysis showed a transcript of 1.2 kilobase (Fig. 3C). The P3-homo-
Response to Oxidative Stress Conditions—The isolated cDNAs render yeast more resistant to diamide. Our hypothesis is that in plants these genes play a role in oxidative stress tolerance. To test this, the levels of the respective mRNAs in plants exposed to oxidative stress were analyzed by RNA gel blot analysis. As a positive control, the mRNA levels of the cytosolic ascorbate peroxidase (apx) were analyzed in the same RNA samples. Ascorbate peroxidases are major H$_2$O$_2$-scavenging enzymes in chloroplasts and cytosol (44). The apx mRNA levels and enzyme activities have been shown to increase in plants exposed to stress conditions, such as drought, salt stress, and treatment with paraquat, which result in a pro-oxidant state in cells (45).

Oxidative stress was imposed by the following treatments: the hydroperoxides, hydrogen peroxide (H$_2$O$_2$), and t-BOOH were used to induce direct oxidation of cell constituents. Treatment with diamide was used to induce pro-oxidant conditions through depletion of intracellular glutathione. The herbicide paraquat (methyl viologen) and the quinone, menadione, are redox-cycling drugs. In the cell they serve as efficient electron acceptors from electron transport chains. Reduced paraquat and semi-quinones are readily oxidized by oxygen, leading to the formation of O$_2^-$ anions. Then, the cycle of reduction-oxidation is repeated, a process called redox cycling (1). Furthermore, potent antioxidants such as reduced glutathione, diethiothreitol (DTT), and N-acetylcysteine, may cause the activation of plant oxidative stress defense genes (46). Therefore, we analyzed the expression of the isolated genes after the infiltration of plants with DTT.

The chemicals mentioned above were applied by infiltration into Arabidopsis plants. Although infiltration by itself caused activation of apx, P1, P2, and P4 genes, this method was used to eliminate problems of drug uptake through roots or epidermis.

As shown in Fig. 7, apx mRNA levels steadily increased up to 8 h after infiltration of water. The strongest response was observed in response to paraquat treatment; accumulation of mRNA was detected after 15 min of treatment and reached a plateau after 30 min, which lasted at least 8 h. Transiently higher steady-state RNA levels (compared to control) were observed in plants treated with t-BOOH, diamide, and menadione, with the highest mRNA levels approximately 1 h after infiltration. The apx gene was also induced by DTT and levels of mRNA detected in H$_2$O$_2$-infiltrated plants after 8 h were already observed after 15 min following DTT infiltration. No differences were observed between control and H$_2$O$_2$-infiltrated plants.

P1 and P2 genes responded similarly, although, considering the high sequence homology, the detected hybridization signals might be derived from P1 and P2 mRNA together. Like apx, P1 and P2 mRNAs were induced by water infiltration. Rapid in-

Table 1  

| Similarity between z-crystallins |
|---|
| P1 | P2 | P4 | L.c | L.a | Zeta | TED2 |
|---|---|---|---|---|---|---|
| P1 | 1.00 | 0.92 | 0.85 | 0.64 | 0.65 | 0.60 | 0.60 |
| P2 | 0.92 | 1.00 | 0.90 | 0.60 | 0.61 | 0.60 | 0.60 |
| P4 | 0.85 | 0.90 | 1.00 | 0.50 | 0.50 | 0.50 | 0.50 |
| L.c | 0.64 | 0.60 | 0.50 | 1.00 | 0.75 | 0.75 | 0.75 |
| L.a | 0.65 | 0.61 | 0.50 | 0.75 | 1.00 | 0.75 | 0.75 |
| Zeta | 0.60 | 0.60 | 0.50 | 0.75 | 0.75 | 1.00 | 0.75 |
| TED2 | 0.60 | 0.60 | 0.50 | 0.75 | 0.75 | 0.75 | 1.00 |

% similarity
treatments lowered the a/b methyl viologen had little effect on P4 gene expression.

The same membranes were also hybridized with a tobacco cDNA which encodes the antioxidant protein, ascorbate peroxidase (45).

The original interest in z-crystallin stems from the fact that a mutation in the z-crystallin gene is associated with formation of an autosomal dominant congenital cataract in guinea pigs (49). Since cataract development is thought to result from oxidative damage of lens proteins (1), it was logical to presume that high levels of z-crystallin is a species-specific adaptation to control the formation of active oxygen species. It was further found that detoxification of hydrogen peroxide is more efficient in lens of guinea pigs compared to rat lens, which have only small amounts of z-crystallin (49). The expression of z-crystallins is also induced in rat lens under oxidative stress conditions (50).

z-Crystallin specifically binds NADPH and is a novel NADPH:quinone oxidoreductase (35, 49). Based on biochemical data, Rao and Zigler (49) hypothesized that one of the important functions of z-crystallins is an initiation of NADPH/NADP redox cycling, which in turn is coupled to the up-regulation of the hexose monophosphate shunt. Hexose monophosphate shunt is the principal source of NADPH in cells and one of the components of antioxidant defense, because NADPH is needed for the reduction of oxidized glutathione and ascorbic acid, whose formation greatly increases under oxidative stress (51–53).

The P3 gene was slower in response to paraquat and \( \text{H}_2\text{O}_2 \) and was not induced by water infiltration. Considering, the importance of \( \text{H}_2\text{O}_2 \) metabolism in processes of cell wall cross-linking and lignin biosynthesis (54) and possible xylem-specific expression of the P3 gene, its product may play a role in AOS homeostasis in specific cell types. The P3 clone is highly homologous to isoflavonoid reductases. IFRs are believed to be specifically involved in the biosynthesis of isoflavonoid phytoalexins in Leguminosae (55). However, IFR cDNA homologs have been isolated from several species, which do not synthe-

**DISCUSSION**

In this work we have isolated four Arabidopsis cDNAs which improve the tolerance of yeast cells to the thiol oxidizing drug diamide. Plant \( \zeta \)-crystallins and IFR-like cDNAs were consistently recovered in our screening for diamide tolerance of the yap1 yeast mutant. Furthermore, we have found that the cDNAs isolated also improved the survival of YAP1 yeast strain, DY which has a normal response to oxidative stress. The mechanism of protection is apparently rather specific for diamide. Efforts to find cross-tolerance to other drugs were not successful, however, the choice of stresses as well as possible growth conditions were not exhaustively tested in this study.

This observation is not in disagreement with the data obtained by multicopy plasmid screening in yeasts. Significant cross-tolerance was usually observed when genes encoding regulatory proteins, like YAP1 or YAP2 were overexpressed (17, 47, 48). The tolerance provided by enzymes is rather limited to the particular stress agent (17, 48).

Although we cannot entirely exclude the direct detoxification of diamide by \( \zeta \)-crystallins or IFR, our expression analysis suggest that the isolated cDNAs encode enzymes with a distinct role in stress response. The genes encoding P1 and P2 were rapidly induced by drugs which induce redox cycling, and in general their expression is very similar to the expression of the well characterized plant antioxidant protein, ascorbate peroxidase (45).

**FIG. 5.** The amino-terminal part of the P4 cDNA is homologous to short-chain dehydrogenases. The amino-terminal part of the ORF encoded by the P4 cDNA (A.t. P4) was aligned to the amino acid sequences of human 15-hydroxyprostaglandin dehydrogenase (15prost) (36), Arabidopsis 3-oxoacyl (acyl carrier protein) reductase (loxacyl) (37), and glucose dehydrogenase from B. megaterium (glucose) (38). The amino acids, which are identical in P4 and other short-chain dehydrogenases, are indicated by dots and boxed. The amino-terminal part of the oxacyl dehydrogenase is a chloroplast transit peptide. The amino acid residues, important for the enzymic activity of the short-chain dehydrogenases are indicated with lozenges. The arrowhead above the P4 sequence shows the beginning of the P4 domain with homology to the \( \zeta \)-crystallins. To improve alignment, gaps indicated by dashes have been introduced.

**FIG. 6.** The amino acid sequence comparison of the P3-encoded ORF. A.t. P3 corresponds to the ORF encoded by cDNA P3. Other sequences are tobacco isoflavonoid reductase, such as cDNA A622 (N.t. A622) (41), isoflavonoid reductases from chickpea (C.a. IFR) (42), and alfalfa (M.s. IFR) (43). Amino acids, which are identical between P3 ORF and other plant proteins, are indicated by dots and boxed. Gaps introduced to optimize the alignment are shown by dashes.

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size isoflavonoid phytoalexins and the tobacco IFR-like protein expressed in E. coli did not show any enzymatic activity (41, 56). The IFR activity of legume proteins suggests that a whole IFR-like family are oxidoreductases utilizing NAD(P)H as a co-factor and the use of isoflavonoids as electron acceptors is likely to be an acquired enzymatic activity in legumes.

The expression of the P4 gene was only elevated in control infiltration with water; furthermore, its expression was suppressed by diame and t-BOOH. Apparently, P4 protein, which is composed of two domains has a rather specific function in legumes. The screening most is composed of two domain which is likely to be an acquired enzymatic activity in legumes.

In this work, we have identified 4 new Arabidopsis cDNAs, which, when expressed under control of a yeast promoter on a multicopy plasmid provide better tolerance of yeasts to thiol oxidizing drug diamide. It might not be coincidental that plant ζ-crystallin homologs were isolated by such a screen. Loss of ζ-crystallin activity in guinea pigs leads to cataract formation and this effect can be mimicked by diame (57). The putative NAD(P)H oxidoreductase activities of proteins encoded by the isolated cDNAs suggest the importance of NAD(P)H homeostasis in plant cell antioxidant defense. The use of other drugs and chemicals in similar functional screening of plant cDNA libraries may provide an important tool for the identification of plant genes involved in stress response and defense, which would not be amenable to isolation by conventional screening.

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FIG. 7. Analysis of the mRNA levels in Arabidopsis plants under oxidative stress conditions. Three-week-old in vitro grown Arabidopsis plants were infiltrated with water (H2O) alone, or water solutions of hydrogen peroxide (H2O2), diame, menadione, methyl viologen, or DTT at the indicated concentrations. RNA was extracted from treated plants at different time points, as indicated above the lanes either in minutes () or in hours. To check equal RNA loading, membranes were stained with methylene blue, the staining of the 28 S rRNA is shown in the column rRNA. The levels of mRNAs homologous to probes made from P1, P2, P3, P4, and apx cDNAs are shown in vertical columns. For each treatment, the same membrane was rehybridized with different probes.
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