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Annexin-like protein from *Arabidopsis thaliana* rescues Δ*oxyR* mutant of *Escherichia coli* from H_2O_2 stress

(oxidative stress/plant defense/salicylic acid/acquired resistance)

XAVIER GIDROL*, PAOLO A. SABELLI, YIP SOO FERN, AND ANIL K. KUSH†

Institute of Molecular and Cell Biology, National University of Singapore, 10 Kent Ridge Crescent, Singapore-0511, Republic of Singapore

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ABSTRACT **Reactive oxygen species are common causes of cellular damages in all aerobic organisms. In *Escherichia coli*, the oxyR gene product is a positive regulator of the *oxyR* regulon that is induced in response to H_2O_2 stress. To identify genes involved in counteracting oxidative stress in plants, we transformed a Δ*oxyR* mutant of *E. coli* with an *Arabidopsis thaliana* cDNA library and selected for clones that restored the ability of the Δ*oxyR* mutant to grow in the presence of H_2O_2. Using this approach, we isolated a cDNA that has strong homology with the annexin super-gene family. The complemented mutant showed higher catalase activity. mRNA expression of the annexin gene in *A. thaliana* was higher in roots as compared with other organs and was also increased when the plants were exposed to H_2O_2 stress or salicylic acid. Based on the results presented in this study, we propose a novel physiological role for annexin in counteracting H_2O_2 stress.**

Oxidative stress, resulting from the deleterious effects of reduced oxygen species (ROS), is an important phenomenon in many biological systems. As a by-product of normal aerobic metabolism, molecular oxygen undergoes successive single electron transfers that result in the generation of partially ROS such as superoxide radical (O_2^-), hydroxyl radical (·OH), and hydrogen peroxide (H_2O_2). ROS are involved in lipid peroxidation, protein denaturation, and DNA damage which eventually results in various mutations (1). These cellular damages can lead to the development of a range of pathological symptoms observed, both through apoptosis and necrosis, in diseases like cancer, arthritis, muscular dystrophy, and various genetic and nervous disorders like Trisomy 21 syndrome and amylotrophyc lateral sclerosis (2, 3).

In response to increased concentration of H_2O_2 more than 30 proteins are induced in bacteria, most of which play preventive and reparative defensive roles against the stress. This response is defined as the peroxide-mediated stress response. The regulation of this defense is better understood in the bacteria *Escherichia coli* and *Salmonella typhimurium* (4) in which the OxyR transcriptional regulator activates the expression of defense genes in response to peroxide stress (5). The genes induced by OxyR upon exposure to H_2O_2 include *katG*, which encodes catalase, and *ahpCF*, which encodes an alkyl hydroperoxide reductase. The expression of the *oxyR* gene is self-regulated. During oxidative stress there is an increased influx of H_2O_2, resulting in the oxidation of OxyR, which in turn activates the transcription of *oxyR* regulon genes (6). Hence, this regulon plays a dual role both in sensing and transducing peroxide stress signals. Although the exact mechanism by which OxyR activates transcription has not been clearly elucidated, evidence for an involvement of conformational changes of these proteins in response to the redox state is accumulating (7).

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High ROS concentrations are also generally considered to be detrimental to plant cellular functions. These molecules are normally generated during metabolism, and their production and destruction are regulated cellular phenomena (8). Plant defense response to pathogens often depends on the plant's ability to recognize the pathogen early in the infection process. One of these early events, characteristic of the hypersensitive response (HR), is the striking release of ROS, known as the oxidative burst (9). The oxidative burst seems to play an important role in strenthening the plant cell wall in the event of pathogen attack. In addition to the HR, plants respond to necrotic pathogens by developing enhanced resistance to the same or other unrelated pathogens throughout the plant body over a period of time. This response is called the systemic acquired resistance (SAR) (10–12). Treatment with salicylic acid (SA), which is known to induce SAR, also leads to accumulation of H_2O_2 by down-regulating catalase (11). Hence, H_2O_2 appears to play a key defensive role both in the HR and SAR.

Our current understanding of the physiological functions of antioxidative enzymes has been greatly influenced by the use of well-characterized bacterial null mutants. Among the most intensively null mutants studied are those for MnSOD and FeSOD (encoded by the *sodA* and *sodB* genes), glutathione reductase (*gor* gene), and two forms of catalases (*katG* and *katE* genes). Very little is known about other antioxidant enzymes and their regulation in plants. We used *Arabidopsis thaliana* as a model plant to investigate the mechanism by which plants respond to H_2O_2 stress. We describe here the cloning and characterization of a cDNA from *A. thaliana* which rescues Δ*oxyR* mutants of *E. coli* from H_2O_2 stress by functional complementation. The encoded protein has high homology with the family of annexin proteins. We present evidence for a potential role of this annexin-like protein in response to oxidative stress in bacteria and plants.

MATERIALS AND METHODS

Bacterial Strains and cDNA Library. The genotypes of the bacterial strains used are shown in Table 1. The *E. coli* Δ*oxyR* mutant (strain TA4112), a deletion mutation of the *oxyR* gene, was derived from the strain RK4936 (5) and was obtained from Spencer Farr (Harvard School of Public Health, Boston). UM120 (*kat*E::Tn10) and N7900 (K-12 *kat*G::Tn10) (13, 14) were gifts from Gisela Storz (National Institutes of Health).

Seeds of *A. thaliana* ecotype Columbia (Col-0; Lehle Seeds, Tucson, AZ) were planted in 10-cm-diameter plastic pots in a growth chamber. Seedlings were grown under a 10-h photoperiod (100–150 μ $Em^{-2}\cdot s^{-1}$ of light) at 24°C and 95% relative

Abbreviations: ROS, reduced oxygen species; SA, salicylic acid.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U28415).

*Present address: Xenometrix, Inc. 2860 Wilderness Place, Suite 150, Boulder, CO 80301.

†To whom reprint requests should be sent at the present address: Indo-American Hyb Seeds, P.O. Box 7099, K.R. Road, 17 Cross, 2A Main, BSKII Bangalore-560070, India.

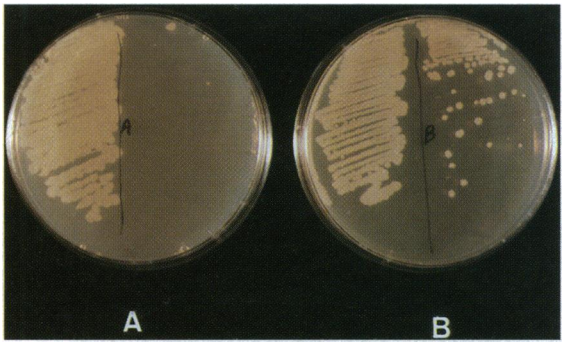

FIG. 1. Rescuing Δ*oxyR* mutant of *E. coli*. (*A*) H_2O_2 sensitivity of *E. coli* strains. Growth of RK4936, the parental *E. coli* strain on the left half of the plates, and TA4112, the Δ*oxyR* mutant strain on the right half of plates containing M-9 medium with 350 μM H_2O_2 after 48 h at 30°C. (*B*) Annexin cDNA rescues Δ*oxyR* mutant. Same as in *A*, but on the right half is the mutant (TA4112) rescued by poxy5 cDNA from an *A. thaliana* cDNA library.

humidity. Plants were watered on alternate days with nutrient medium (15). A cDNA library from 4-week-old seedlings in λZapII was obtained from Stratagene.

Screening for Transformants Resistant to H_2O_2. The Δ*oxyR* mutant (*E. coli* strain TA4112) is unable to grow in minimal medium containing H_2O_2 in excess of 100 μM, whereas the parent strain (RK4936) is able to grow in media containing up to 350 μM of H_2O_2. Phagemid DNA was prepared from the *A. thaliana* cDNA library and used to transform the Δ*oxyR* mutant using a Bio-Rad gene pulser as per the manufacturer's instructions. Transformants were selected for growth on M-9 minimal agar plates containing 100 μg/ml ampicillin and 350 μM H_2O_2. Plasmid was extracted from each individual colony, rescued and used to retransform Δ*oxyR* for selection with increasing concentration of H_2O_2. One isolate of such a selection scheme was a transformant designated as AKGX5 containing the plasmid poxy5 (Table 1), which was found to grow in the presence of up to 600 μM H_2O_2 .

Enzyme Activity. Cell extracts were prepared by sonication, from both TA4112 and RK4936 strains grown to stationary phase in Luria–Bertani medium (16). Catalase activity was assayed by back titration of the remaining H_2O_2 with 0.5 M K_2MnO_4 in 50 mM KH_2PO_4-KOH (pH 7.0) and 60 mM sodium perborate and was expressed as mkat/mg of protein as described (17).

Peroxidase activity of the *E. coli* (using glutathione *S*-transferase fusion technique) and *Arabidopsis* (using antiannexin antibody affinity column) purified annexin was measured in the presence or absence of antiannexin antibody and compared with standard horseradish peroxidase (Sigma) as control (18, 19). Five microliters of 10^{-4} dilution of antibody was used per microgram of the purified protein for immunoprecipation, and supernatant was used to test enzymatic activity as described (18, 19). *E. coli* stains UM120 and N7900 having Tn10 insertions in the *kat*E and *kat*G genes, respectively, were complemented with poxy5, and restoration of their ability to grow in the presence of H_2O_2 was tested on a H_2O_2 concentration gradient (0–600 μM) M-9 plate. The gradient plate was prepared by making a gradient of M-9 medium containing 600 μM of H_2O_2 by slanting the square Petri dish, followed by over layering with the medium without H_2O_2 to level the surface.

Table 1. Strains and plasmids

Strain or plasmid	Description	Source or ref.
Strains		
XL-1 blue	*rec*A, *end*A1, *gyr*A96, *thr1*, *hsd*R17, *Sup*E44, *rel*A1 (F′.*pro*AB, *lac*I, Tn10-tetr)	Stratagene
RK4936	*ara*D139, (*arg*F-*lac*)205, *flb*B5301, non-9*gyr*A219, *rel*A1, *rps*L150, *met*E70, *btu*B::Tn0	5
TA4112 Δ*oxy*R	*oxy*Δ3[*oxy*Δ(*oxy*RbutB)3]	5
AKGX5	TA4112 containing poxy5	This study
UM120	*kat* E :: Tn10	13
N7900	*kat* G:: Tn 10	14
Plasmids		
poxy5	Annexin cDNA in pBluescript	This study

DNA and Protein Sequence Analyses. DNA sequencing and analysis of the encoded amino acid sequence were carried out according to standard procedures.

Primer Extension. A ^{32}P-labeled oligomer (5′-CTG TGA GCC AAG ATT GAT ATG ATC AAG TCC TCG TTC GTA CCC C-3′) complementary to the cDNA sequence from the position +79 to +121 was hybridized to 40 μg of RNA. Extension with reverse transcriptase was carried out as described (20).

Protein Purification and Antibody Production. Glutathione *S*-transferase gene fusion was used to express and purify the fusion protein in *E. coli* as per the manufacturer's instructions (Pharmacia), except that annexin was cleaved from the fusion protein while the fusion protein was still bound to the glutathione Sepharose matrix using the factor "Xa" (21). Antibod-

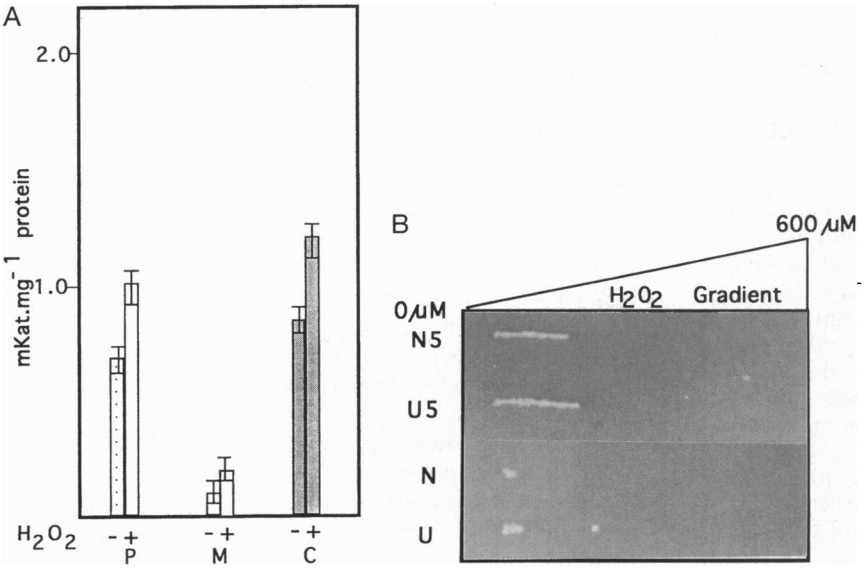

FIG. 2. Catalase activity and H_2O_2 stress. (*A*) The catalase activities of the parent (lanes P), Δ*oxyR* (lanes M), and AKGX5 (lanes C) were assayed in the absence (−) or presence (+) of 50 μM H_2O_2 in triplicate. Error bars represent standard deviation from the mean. (*B*). Growth of *E. coli* UM120 and N7900 strains without (rows U and N) and with poxy5 (rows U5 and N5), respectively, on a gradient (0–600 μM) of H_2O_2 in M-9 medium.

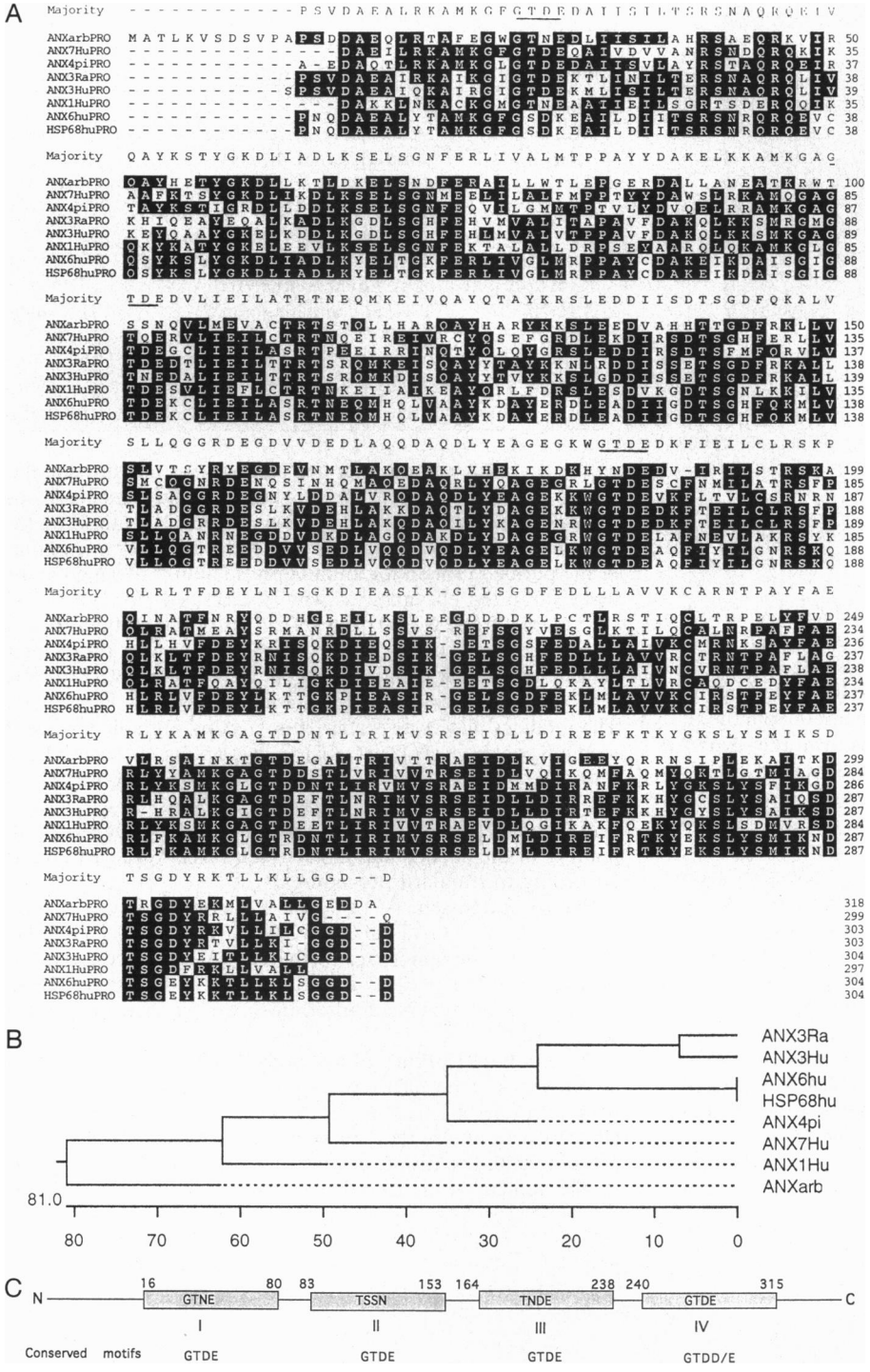

FIG. 3. Sequence analysis of poxy5. (*A*) poxy5 encodes an annexin-like protein. Shown is an amino acid comparison of annexin from *A. thaliana* (ANXarb) identified in this study with annexins from human (ANXHu), rat (ANXRa), and pig (ANXpi). The amino acid sequence numbers are shown on the right. Identical residues are shaded and the consensus sequence is shown by the row labeled "Majority." The four GTDE repeats are underlined. The fourth conserved motif can be either GTDE or GTDD. (*B*) Evolutionary relationships of annexins. The horizontal bar indicates the percentage of homology in the amino acid sequences in the evolutionary tree. (*C*) Annexin repeats. The conserved repeat cores I, II, III, and IV and the variations in the GTDE motifs within are indicated.

ies against the purified protein were raised in rabbits. The plant annexin was purified from 1-month-old *A. thaliana* using antiannexin antibody affinity column as described (19).

***In Vitro* Transcription and Translation.** The *Arabidopsis* cDNA, cloned in pBluescript, was transcribed *in vitro* using either T3 or T7 RNA polymerase to generate antisense or sense RNA strands, respectively, as per the manufacturer's instructions (Stratagene). The RNA was translated using the rabbit reticulocyte lysate system (Promega) and was immunoprecipitated by using the antiannexin polyclonal antibodies, prepared as described above. The *in vitro* translated and immunoprecipitated product was analyzed on a 10% SDS/PAGE and fluorographed using Amplify (Amersham).

Northern and Southern Analysis. Standard protocols were used for RNA blots and restriction enzyme digestion (20). Total RNA was extracted from roots, leaves, stems, flowers, and whole seedlings using guanidium thiocynate as described (20). Total RNA (20 μg) from each tissue was electrophoresed and transferred onto a Hybond-N membrane. For stress analysis, 1 ml of either 1 mM H_2O_2 or 10 mM SA was sprayed on 4-week-old seedlings. Control plants were sprayed with 1 ml of water and total RNA was extracted after 0, 12, 24, and 48 h of treatment. A cDNA encoding βATPase from tobacco was used as an internal control (22).

High molecular weight genomic DNA was extracted from 2-week-old *A. thaliana* seedlings as described (20) and digested with various restriction enzymes. An *Eco*RI–*Xho*I (1.2 kb) full-length cDNA of annexin-like insert from poxy5 was labeled with ^{32}P-αdCTP by random priming.

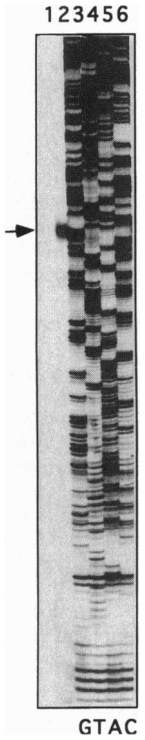

FIG. 4. Primer extension analysis. A ^{32}P end-labeled primer was hybridized to 40 μg of RNA from *A. thaliana* (lane 2) and with 10 μg of *E. coli* tRNA (lane 1) as negative control. The arrow indicates the extension product. Its size was determined by comparison with the sequencing gel (lanes 3, 4, 5, and 6 represent G, T, A, and C lanes, respectively).

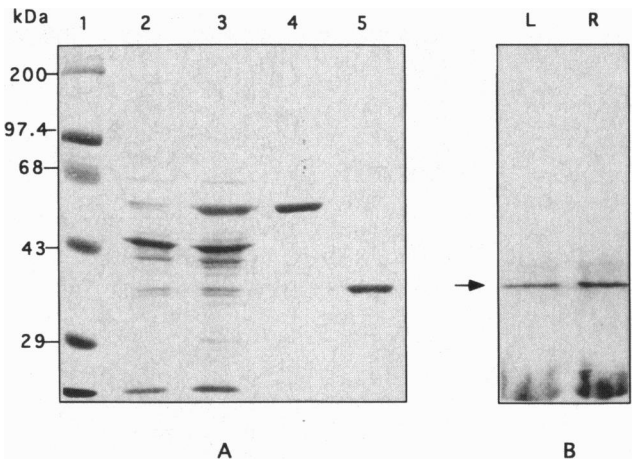

FIG. 5. Recombinant annexin. (*A*) Purification of recombinant annexin. The annexin cDNA from *Arabidopsis* was expressed in *E. coli* as a fusion protein and purified by affinity chromatography as described. Protein were separated by SDS/PAGE (10% acrylamide). Lanes: 1, molecular mass marker; 2, uninduced crude protein extract; 3, induced protein extract; 4, fusion protein; 5, purified annexin. (*B*) *In vitro* transcription and translation of annexin. The arrow indicates the T3 RNA polymerase products after *in vitro* transcription and translation before (lane L) and after immunoprecipitation (lane R), using polyclonal antibodies against the protein purified from *E. coli*.

RESULTS

Rescuing of *E. coli* Δ*oxyR* Mutant. The H_2O_2 sensitivity of the Δ*oxyR* mutant (strain TA4112) was verified to optimize the screening procedure for complementing *Arabidopsis* cDNA clones. This mutant was unable to grow in the presence of concentration of H_2O_2 higher than 100 μM, whereas the parent strain (RK4936) could tolerate up to 350 μM of H_2O_2 (Fig. 1*A*). From $\approx 10^6$ independent clones excised from an *A. thaliana* cDNA library and introduced into the Δ*oxyR* mutant, we obtained 42 clones that restored the ability of Δ*oxyR* to grow in the presence of 350 μM H_2O_2 (Fig. 1*B*). Individual colonies from the transformed bacteria were patched on increasing concentrations of H_2O_2 to estimate their levels of H_2O_2 tolerance. After a second round of screening, we selected one clone, designated AKGX5, which could tolerate up to 600 μM H_2O_2 and which contained the plasmid poxy5 with a cDNA insert of about 1.2 kb. To further elucidate this, we compared catalase activity of various cell types (Fig. 2*A*). Catalase activity was higher in AKGX5 cells as compared with RK4936 and about 5-fold higher than that of Δ*oxyR* mutant. Upon H_2O_2 treatment, catalase activity further increased (Fig. 2*A*). This suggested that a peroxidase-like activity may be involved in rescuing Δ*oxyR* from H_2O_2. This was further supported by our observation that in catalase-deficient UM120 and N7900 strains, poxy5 could at least partially restore the ability to grow in the presence of otherwise lethal doses of H_2O_2 (Fig. 2*B*)

poxy5 cDNA Encodes an Annexin-Like Protein. The 1.2-kb cDNA insert of poxy5 contains an open reading frame encoding a protein of 317 amino acids with a high degree of homology to the annexin protein super-family (Fig. 3*A*), the highest homology being with the human annexin I (Fig. 3*B*). The full-length nature of the annexin cDNA was confirmed by the size of the corresponding mRNA (see Fig. 7) and by primer extension analysis (see Fig. 4). The four repeated domains characteristic of annexin proteins were also present in the *A. thaliana* annexin homolog (Fig. 3*C*). However, in the *Arabidopsis* sequence, the glycine residue at position 23 of each domain, which is highly conserved in all repeats of the annexin sequences published so far, was not present in domains 2 and 3. Similarly, the highly conserved motif GTDE (residues 23–26) was absent from domains 2 and 3.

Primer extension data indicated that the most probable transcription start site is located 33 bp upstream of the start codon (Fig. 4). The full-length cDNA encodes for a 36.2-kDa protein with an isoelectric point of 5.34. This molecular mass is consistent with the apparent molecular mass (34 kDa) of the protein transcribed and translated *in vitro* (Fig. 5). This protein could be immunoprecipitated using polyclonal antibodies raised against the recombinant annexin (Fig. 5*B*).

A data base search using only 30 residues at the N terminus of the *Arabidopsis* annexin-like protein revealed significant similarity with the heme-binding domain of plant peroxidases, which are enzymes that bind H_2O_2 via the heme group and catalyze its break-down and detoxification (Fig. 6*A*). In peroxidases, this domain is highly conserved and binding to the heme iron is through a histidine residue (the proximal histidine) (28), which is also conserved in the annexin protein. Interestingly, in our preliminary assays for peroxidase activity, we did notice peroxidase-like activity both from cloned cDNA product as well as plant purified annexin (Fig. 6*B*).

Expression of Annexin and Oxidative Stress. Considerably higher expression of annexin mRNA was observed in *Arabidopsis* roots as compared with stems, leaves or flowers (Fig. 7*A*). A similar pattern of expression was also observed in tobacco and wheat using the *Arabidopsis* cDNA as a probe (data not shown). The annexin mRNA levels increased in response to treatment with H_2O_2 in 4-week-old *A. thaliana* seedlings. This increase was observed 12 h after treatment (Fig. 7*B*) and was followed by a decrease after 3 days (results not shown). Since SA is known to cause increased accumulation of H_2O_2 (12), we tested the effect of SA on the expression of annexin mRNA. The transcript level was indeed increased in response to SA (Fig. 7*C*). No significant changes in the level of expression of annexin mRNA were noticed in the control plants sprayed with H_20 only. Taken together these results indicate that the expression levels of annexin message is increased when plants undergo oxidative stress and suggest that annexin may play a role in plant defense against various types of biotic as well as abiotic stresses.

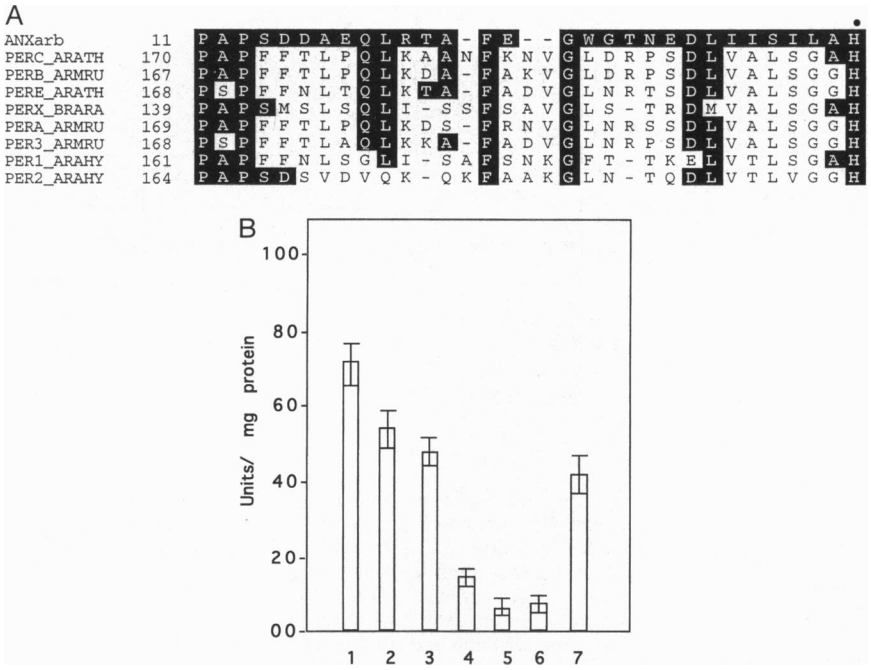

FIG. 6. Annexin and peroxidases. (*A*) Homology between N terminus of *Arabidopsis* annexin and various peroxidases. Amino acid comparison of the N-terminal region of the *Arabidopsis* annexin (ANXarb) with the heme-binding domain of plant peroxidases. Identical residues between ANXarb and peroxidases from *Arabidopsis* (ARATH) (23), horseradish (ARMRU) (24, 25) turnip (BRARA) (26), and peanut (ARAHY) (27) are indicated by shaded boxes. Gaps (indicated by dashes) have been introduced to maximize the alignment. Amino acid positions are shown on the left. The heme-binding proximal histidine in the peroxidase sequences is indicated by a dot. (*B*) Annexin and peroxidase activity. Peroxidase activity of standard horseradish peroxidase (lane 1), *E. coli*-produced and purified annexin (lane 2), and *Arabidopsis* immunopurified annexin (lane 3) before and after (lanes 4, 5, 6, respectively) immunoprecipitation with the anti-annexin antibodies. Lane 7 is the same as lane 6 except preimmune serum is used for immunoprecipation. The experiment was done in triplicate, and error bars represent standard deviation from the mean.

Annexin-Like Protein Is Encoded by a Single Gene in *A. thaliana*. Southern analysis of *Arabidopsis* genomic DNA, digested with various restriction enzymes, and probed with the 1.2-kb annexin cDNA is shown in Fig. 8. Among the enzymes tested, the cDNA has a single internal restriction site for *Hin*dIII and three sites for *Rsa*I. The other enzymes have no internal sites in the cDNA. The restriction patterns obtained are consistent with the restriction map of the cDNA sequence and suggest the presence of one gene copy of annexin per haploid genome of *A. thaliana*.

DISCUSSION

To investigate the possible mechanism by which plants counteract H_2O_2 stress, we used the well-characterized *E. coli* system. The Δ*oxyR* mutant was chosen for complementation experiments with an *Arabidopsis* cDNA library as this mutant had little probability of reversion. However, the restoration of the ability of this mutant to grow in the presence of H_2O_2 could stem from either complementation by plant *oxyR* homologs or any other downstream gene which is controlled by the *oxyR* gene product. Interestingly, the sequence of poxy5 cDNA had no homology with *oxyR* but had strong homology with the annexin gene family.

Annexins constitute a ubiquitous family of more than 15 structurally related, calcium-dependent, membrane-binding proteins present in eukaryotic cells (29). Annexins are amphipathic proteins and are distinct from soluble and integral membrane proteins, although they share limited homology with both. Despite the fact that annexin proteins have been found in a range of eukaryotic organisms, their exact physiological roles have not been completely elucidated.

To the best of our knowledge this is the first report on a possible role for annexins in counteracting oxidative stress. Although the exact mechanism of action of annexin in counteracting H_2O_2 stress is not very clear, its N-terminal sequence similarity with plant peroxidases suggests that annexin may be involved in heme binding and, therefore, in contributing some of the functions normally performed by peroxidase enzymes (Fig. 6*A*). This is supported by the *in vivo* increase in catalase activity of the annexin tranformed Δ*oxyR* mutant (Fig. 2*A*), peroxidase-like activity of the *E. coli* and plant purified annexin (Fig. 6*B*), and ability of catalase deficient mutants to grow in the presence of H_2O_2 after complementation with poxy5 (Fig. 2*B*).

The presence of annexins in plants has been recently reported, based upon the amino acid homology of sequences of tryptic fragments or partial cDNAs from tomato (30), cotton (31) alpha-alpha (*Medicago sativa*) (32), and pea (33) with mammalian annexin sequences. Here we report a full-length cDNA encoding annexin from *A. thaliana*. This allows us to compare the plant annexin with similar proteins from mammals more precisely. With the exception of eight repeats in annexin VI, all members of the annexin family have primary sequences composed of four repeats, which correspond to four distinct domains in the molecule (34, 35). These repeats are highly conserved both within the same protein and among different annexins. By contrast, the amino termini of annexins are variable in length and sequence. In addition to these four characteristic domains, strategic locations of crucial amino

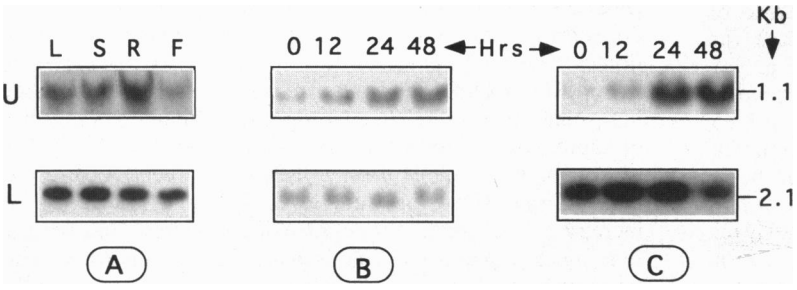

FIG. 7. Annexin expression and stress. (*A*) Annexin expression in different plant organs in *A. thaliana*. Total RNA was isolated from 4-week-old seedlings (L, leaf; S, stem; R, root; and F, flower). (*B*) Annexin expression and H_2O_2 stress. Four-week-old seedlings were treated with 1 mM H_2O_2 and total RNA was extracted after 0, 12, 24, and 48 h of treatment. (*C*) Annexin expression and SA. Northern blot analysis was done as explained in *B*, but H_2O_2 treatment was replaced with 10 mM SA. Water treated plants were used as control. The blots were first hybridized with annexin (*Eco*RI–*Xho*I fragment of poxy5) probe (row U) and then were stripped and reprobed with a cDNA for βATPase from tobacco as an internal control to account for any difference in loading of RNA samples (row L).

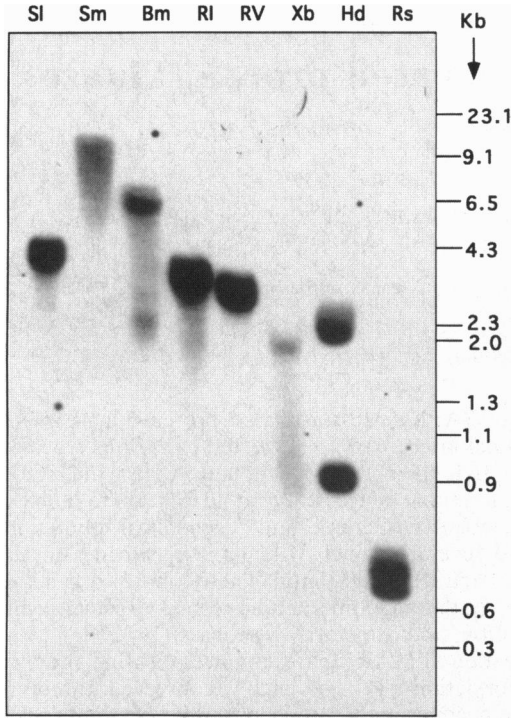

FIG. 8. Genomic Southern analysis. *A. thaliana* genomic DNA (20 μg per lane) was digested with *Sal*I (Sl), *Sma*I (Sm), *Bam*HI (Bm), *Eco*RI (RI), *Eco*RV (RV), *Xba*I (Xb), *Hind*III (Hd), and *Rsa*I (Rs), separated by gel electrophoresis, and blotted onto Hybond-N (Amersham) filters. The blot was hybridized to annexin cDNA (1.2-kb *Eco*RI–*Xho*I fragment). The size markers are indicated in kb on the right.

acids make up the signature of the annexin protein superfamily (34, 35). Among these, Glu-95 and Gly-23 are of particular relevance. Glu-95 is crucial for its role as a voltage channel regulator in annexin V. Although Glu-95 is well conserved in plant annexins, Gly-23, which is conserved in all other annexin repeats (35), is not present in domains 2 and 3 of the *Arabidopsis* annexin (Fig. 3*C*). This suggests that the plant annexin might be an ancestor of mammalian genes in which duplication from two repeats to four had not yet occurred. This is in agreement with the evolutionary tree previously proposed for the annexin superfamily (35).

Annexin mRNA was found to be constitutively expressed in *Arabidopsis*, tobacco, and wheat roots. This may be related to the phenomenon of hypoxia which roots and seeds often encounter (36). Higher expression levels of antioxidant enzymes may be a result of an adaptive defense mechanism that protects them from oxygen toxicity (37–39). Annexin mRNA can also be induced in response to H_2O_2 treatment, thus supporting the hypothesis that it may play an important role in counteracting H_2O_2 stress. Plants treated with SA were found to have higher levels of annexin transcript. Previous observations have documented the accumulation of H_2O_2 in SA-treated plants (12) and this does not appear to be in agreement with the peroxidase-like activity of annexin here reported. However, the level of induction of annexin or its peroxidase-like activity may not be sufficient to compensate for the down-regulation of catalase resulting from SA treatment. Annexin-like proteins are mutifunctional (40), and we are further exploring their DNA binding properties and their possible role in signal transduction.

Recent experiments on transgenic plants indicates that although SA is required in the signal transduction pathway, it is not the translocated signal responsible for inducing the SA response (12). This raises the possibility of exploring further the role of H_2O_2 in the signal transduction pathway. In terms of plant defense mechanisms, ROS may have a dual function. They may act extracellularly by killing the pathogens via oxidative cross-linking of cell wall proteins (9), and intracellularly as signal intermediates in gene expression and possibly as toxic molecules leading to the death of the host cells. This proposed ROS dual role is reminiscent of their destructive and gene-regulatory roles during the immune response in mammals (3). In plants, the molecular machinery for the generation of ROS, the nature of the coupling receptors, and their role in the regulation of diverse cellular processes related to defense are only beginning to be elucidated.

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