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Stress induces peroxisome biogenesis genes

Eduardo Lopez-Huertas[1,2], Wayne L.Charlton[1], Barbara Johnson[1], Ian A.Graham[3] and Alison Baker[1,4]

[1]Centre for Plant Sciences, Leeds Institute for Biotechnology and Agriculture, University of Leeds, Leeds LS2 9JT and [3]Centre for Novel Agicultural Products, Department of Biology, University of York, York YO1 5DD, UK

[2]Present address: Puleva Biotech, 66 Camino de Purchil, Granada 18004, Spain

[4]Corresponding author
e-mail: a.baker@leeds.ac.uk

Peroxisomes are the cellular location of many antioxidants and are themselves significant producers of reactive oxygen species. In this report we demonstrate the induction of peroxisome biogenesis genes in both plant and animal cells by the universal stress signal molecule hydrogen peroxide. Using *PEX*1–*LUC* transgenic plants, rapid local and systemic induction of *PEX*1–luciferase could be demonstrated *in vivo* in response to physiological levels of hydrogen peroxide. *PEX*1–luciferase was also induced in response to wounding and to infection with an avirulent pathogen. We propose a model in which various stress situations that lead to the production of hydrogen peroxide can be ameliorated by elaboration of the peroxisome compartment to assist in restoration of the cellular redox balance.

Keywords: hydrogen peroxide/oxidative stress/PEX genes/plant pathogenesis/wounding

Introduction

Peroxisomes are subcellular respiratory organelles that carry out a wide range of functions in eukaryotic cells, including β-oxidation of fatty acids, glyoxylate metabolism and metabolism of reactive oxygen species (van den Bosch *et al*., 1992). In recent years >20 genes involved in peroxisomal protein import and peroxisome biogenesis (*PEX* genes) have been identified (Distel *et al*., 1996). *pex* mutants lack peroxisomes or mislocalize peroxisomal proteins. In many cases the function(s) of the corresponding proteins is unknown, as is the mechanism by which peroxisomes import proteins into the organelle (Subramani, 1998).

*PEX*1 is a peroxisome biogenesis gene that encodes a member of the AAA (ATPases associated with diverse cellular activities) superfamily of ATPases. Proteins of this family are involved in a wide range of cellular functions (Patel and Latterich, 1998). In humans, mutations in *PEX*1 are responsible for complementation group 1 (CG1), which comprises the most severe of the peroxisome biogenesis disorders, such as Zellweger syndrome. CG1 cells have abnormal peroxisomes that mislocalize peroxisomal proteins (Portsteffan *et al*., 1997). In yeasts, *pex*1 null mutants contain small peroxisomes with very reduced matrix content. This suggests that these mutants contain peroxisomes with a competent protein import machinery but are unable to grow (Heyman *et al*., 1994; Kiel *et al*., 1999). In *Pichia pastoris*, Pex1p and its interacting partner, another AAA ATPase, Pex6p, are mainly found associated with vesicles distinct from peroxisomes and only a small amount of these proteins are peroxisome associated (Faber *et al*., 1998). In contrast, in *Hansenula polymorpha* most Pex1p and Pex6p cofractionates with peroxisomes (Kiel *et al*., 1999). Pex1p and Pex6p have high sequence identity with proteins thought to facilitate membrane fusion events in mammalian and yeast cells, like NSF/Sec18p and p97 /Cdc48p (Patel and Latterich, 1998 and references therein). Consequently, these proteins have been suggested to mediate lipid and/or membrane addition to peroxisome membranes, allowing the peroxisomes to grow (Faber *et al*., 1998; Kiel *et al*., 1999).

Peroxisomes are sensitive to external signals and are able to proliferate. In yeasts, where peroxisomes are the sole site of β-oxidation, many peroxisomal enzymes and *PEX* genes are induced by fatty acids, notably oleate, and repressed by glucose. In *Saccharomyces cerevisiae*, oleate-inducible genes contain an oleate response element (ORE) within the promoter that binds a transcription factor Oaf1p/Oaf2p (Karpichev *et al*., 1997). In mammalian cells, expression of a range of genes involved in lipid homeostasis, including all the peroxisomal β-oxidation genes, is controlled by the peroxisome proliferator activator receptor α isoform (PPARα), which binds to the peroxisome proliferator response element (PPRE) in the promoters of these genes (Lemberger *et al*., 1996). A broad range of medium and long chain fatty acids and a diverse class of compounds, including clofibrate, which act as hypolipidemic drugs are all ligands for PPARα (Isseman and Green, 1990). Administration of clofibrate results in up-regulation of PPARα target genes, including acyl-CoA oxidase, but does not affect *PEX* gene expression (Okumoto *et al*., 1998a; Shimuzu *et al*., 1999). In plants, proliferation of peroxisomes has been reported in early post-germinative growth (Mansfield and Briarty, 1996) in response to herbicides (de Felipe *et al*., 1988), xenobiotics (Palma *et al*., 1991) and ozone (Morre *et al*., 1990), and during senescence (Pastori and del Rio, 1997), but the mechanism is not known.

In recent years reactive oxygen species, particularly hydrogen peroxide, have been implicated in a number of signal transduction pathways. These include activation of the transcription factor NF-κB in mammalian cells (Schreck *et al*., 1991), gene regulation in bacteria (Demple, 1991) and plant stress. Hydrogen peroxide has

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been shown to act as a signal in osmotic stress (Guan *et al*., 2000), ABA-mediated guard cell closure (Pei *et al*., 2000), stress caused by excess light (Karpinski *et al*., 1999) and in response to infection by avirulent pathogens leading to a hypersensitive response (Alvarez *et al*., 1998). Nitric oxide is also required for the hypersensitive response (Delledonne *et al*., 1998; Durner *et al*., 1999) and nitric oxide synthase has recently been discovered in plant peroxisomes (Barroso *et al*., 1999). Peroxisomes contain antioxidant molecules, such as ascorbate and glutathione, and also a battery of antioxidant enzymes, including superoxide dismutase, ascorbate peroxidase, dihydro- and monohydroascorbate reductase, glutathione reductase and the cell's principal H_2O_2-degrading enzyme, catalase. Changes in activities of these enzymes are correlated with many situations in which plants experience stress (del Rio *et al*., 1998 and references therein). Accordingly, peroxisomes have been suggested to play important roles in defence against abiotic and biotic stress in plants (Willekens *et al*., 1997; del Rio *et al*., 1998; Barroso *et al*., 1999) and in altered redox status associated with ageing and a variety of disease states in mammals (Masters and Crane, 1995). However, the signal(s) or substrates that trigger peroxisome proliferation as a result of stress have not been identified. In addition, it is not known whether peroxisomes are induced as a result of the stress or as a consequence of the metabolic events that occur in stress situations such as lipid degradation, protein losses or damage of organelle structures.

In this study we provide the first demonstration that *PEX* genes in both plant and animal cells are induced by the universal stress signal H_2O_2. Using *Arabidopsis* plants transformed with a *PEX*1 promotor–luciferase reporter (*PEX*1–*LUC*) we show induction of *PEX*1 expression *in vivo* in naturally occurring stress situations such as pathogen attack and wounding.

Results

PEX1 from Arabidopsis

We cloned a *PEX*1 cDNA from *Arabidopsis*. A composite sequence (DDBJ/EMBL/GenBank accession No. AF275382) was assembled from two clones that are identical in 189 bp of overlap. The 5′-end of the cDNA sequence and ~995 bp of genomic sequence are shown in Figure 1. There are two in-frame ATG motifs (underlined). The more probable start site is the second ATG as it possesses a strong Kozak consensus GTGATGG (Kozak, 1997). Assuming that translation starts at this ATG, the deduced amino acid sequence of *Arabidopsis* Pex1p comprises 1117 amino acids with a predicted molecular weight of 122 305 Da. Two ATP-binding motifs (P-loops) are predicted between amino acids 604 and 611 and 889 and 896, and an AAA family signature between 985 and 1003. A protein database search with AtPex1p identified human Pex1p as the most similar sequence in the database ($E = 1 \times 10^{-79}$), closely followed by Pex1p from the yeasts *Yarrowia lipolytica*, *H.polymorpha* and *P.pastoris*. AtPex1p shares identity with known Pex1 proteins ranging from 27.6% with *S.cerevisiae* to 29.3% with *P.pastoris*. AtPex1p can be immunoprecipitated by antibodies raised against *P.pastoris* Pex1p (data not shown). The *PEX*1

A

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-973                            5'- atggagccgccgtggatattctcaccaac
-944 ccatttaattacggttaagtatctaacttatcccttgttttacatttttttaatcaaat
-884 gtttcgttttagcttagttgagagcattttaacttgaaatggtttgattttgttttctt
-824 ttattgtttgtaggattcgaagtgacggatagagggtgttgtggagtagggagaaataga
-764 ggagaaataacgtgtctgccattagcggtaccatgcgctttcagagaccgacatgtgttc
-704 tgggatgcatttcatccaacacaagcttttaatctcataattgctctcagagcctttaat
-644 ggttccaaatccgattgttaccccattaatctttctcaactttctcgtctctaaaccctc
-584 aacaaatccttctcttttgtttatgtattttgttactttaattttgtagacttgtagt
-524 agtactattgcatatttcttcgcattatctttttggttgtgatttggttccaatttgaat
-484 gtggacctctaatgattacattatcgttaactgcaaacatatacgtagaaaatgagtcga
-424 tcgagaaattgtagaatctttttctattattcttggtttcacaaattaatggtagagact
-364 ttcatgcatagtgatgacataagatgttgcatattgatatggcggattgttcatagtttt
-304 tttttgtttttttttatgacataaaatgttgcatgttggcatgttgcggactaattcata
-244 gattattattagtccatagatgtattatgtactgtatatacatcatttctctttttttgtt
-184 acatgtttgaacttaaacaaaaataagtaactaactataggtttaaaaggcccaattagg
-124 aaattagacaggcccaaatataattattgaagcccaaaatgaggctagcaattcgaacaa
-64  aaaagataagcttaggaaattgtaccaatgtctcgcaatcggcaaatcatcatccc

                                                            c
  gacgtgatggaaacagaagcggtggttaataccgtcgccggcgtcgactgtttcgtctca
        M  E  T  E  A  V  V  N  T  V  A  G  V  D  C  F  V  S
  ctccctcgtcaattactacacgcgcttcaatccaccagctcctctcctctccctccgctt
   L  P  R  Q  L  L  H  A  L  Q  S  T  S  S  S  P  L  P  P  L
  ctccctgtcgagctacgctccggtgaccgccgttggtcagttgcttggtccggctctagc
   L  P  V  E  L  R  S  G  D  R  R  W  S  V  A  W  S  G  S  S
  tcctcctcttctgcgatcgaggtttctatccctctctatatttgatccagtttattcaaa
   S  S  S  S  A  I  E
  tccagtaatcgactactttggttttggaattctgattactcttacattcccctgtttttg
  attttgattcttctaataagatcgctcgagtgtttgcggaaagcatttcgttgccggatg
                      I  A  R  V  F  A  E  S  I  S  L  P  D
  gtacagttgttaaagttcgtgttcttcctaatgtgcccaaggctactttagtaacagtcg
  G  T  V  V  K  V  R  V  L  P  N  V  P  K  A  T  L  V  T  V
   aaccagagactgaagatgactgggaagttcttgagctcaatgctgaacttgccgaggca
   E  P  E  T  E  D  D  W  E  V  L  E  L  N  A  E  L  A  E  A
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B

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                                    NcoI site
cca gag act gaa gat gac tgg gaa gcc atg gaa gac gcc aaa aac ata aag
 P   E   T   E   D   D   W   E   A   M   E   D   A   K   N   I   K
---------PEX1--------------------|  ---------luciferase---------
                                 |
                               unique
                             amino acid
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Fig. 1. (**A**) At*PEX*1 promoter and N-terminus of coding region. Italics show the additional 5′ sequence derived from the 215 bp partial cDNA. The two possible ATG start codons are underlined. The amino acid sequence deduced from the cDNA clone is shown below the nucleotide sequence for the first and part of the second exon. The DDWE motif where the fusion to luciferase was made is shown in bold. (**B**) The fusion junction between *PEX*1 and luciferase.

promoter does not contain any obvious ORE or PPRE-like elements.

AtPEX1 expression in seedlings

Figure 2 shows the expression profile of At*PEX*1 and At*PEX*5 (Brickner *et al*., 1998), which encodes Pex5p, the receptor for PTS1-targeted proteins (Dodt *et al*., 1995). Both genes are expressed at a low level in imbibed seeds and increase rapidly during the first 2 days of post-germinative growth before falling to a lower steady-state level by day 3, which is maintained to day 8. At this time (0–3 days post-imbibition), peroxisome size and number increase considerably in the cotyledon cells of *Arabidopsis* (Mansfield and Briarty, 1996). Malate synthase, a unique enzyme of the glyoxylate cycle, is maximally expressed at 1–2 days post-imbibition, which coincides with the maximal rate of lipid breakdown (Mansfield and Briarty, 1996), but thereafter declines to almost undetectable levels (Eastmond *et al*., 2000). The up-regulation of PEX1 and PEX5 expression at days 1–2 is consistent with their involvement in peroxisome biogenesis, and their continued expression at a lower level after day 2 presumably reflects the requirement for continued peroxisome biogenesis as cells divide and organelles turn over.

PEX1 induction by hydrogen peroxide

Different stress situations yield hydrogen peroxide as a signal molecule that triggers gene expression of various

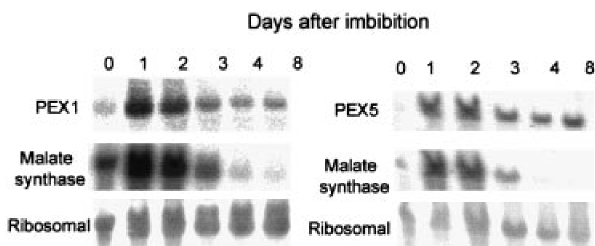

Fig. 2. Expression profile of At*PEX*1 and At*PEX*5 in seedlings. RNA from seedlings at the days indicated was hybridized with probes for At*PEX*1, At*PEX*5 and At malate synthase.

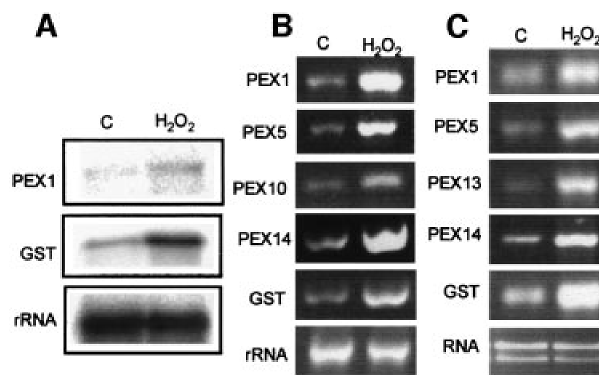

Fig. 3. H_2O_2 induces plant and animal peroxisome biogenesis genes. RNA used in (**A**) (northern blot) and (**B**) (RT–PCR) was obtained from control and 1 mM H_2O_2-treated *Arabidopsis* leaves, whereas in (**C**) (RT–PCR) the RNA is from control and 1 mM H_2O_2-treated chinese hamster ovary cells.

cellular protectants (Levine *et al*., 1994; Mittler and Zilinkas, 1994; Willekens *et al*., 1994a; Karpinski *et al*., 1999; Guan *et al*., 2000). As proliferation of peroxisomes and induction of some peroxisomal genes in response to oxidative stress has been reported, the effect of the stress signal molecule H_2O_2 on the expression of *PEX* genes was investigated. Northern blots showed that At*PEX*1 was induced ~10-fold in detached leaves by H_2O_2 (Figure 3A), similar to the level of induction of glutathione *S*-tranferase (GST), a well-characterized H_2O_2-responsive gene (Levine *et al*., 1994). The same result was obtained by semi-quantitative RT–PCR (Figure 3B) with gene-specific primers against At*PEX*1, GST, At*PEX*5, At*PEX*14 (J.Oh, E.Lopez-Huertas, W.Charlton and A.Baker, in preparation) and At*PEX*10 (Schuman *et al*., 1999). As peroxisomes may also be important in protecting animal cells against oxidative stress, the effect of H_2O_2 treatment on *PEX* gene expression in CHO cells was investigated (Figure 3C). Semi-quantitative RT–PCR showed that *PEX*1, *PEX*5, *PEX*13 and *PEX*14 are all induced to a comparable or greater extent than GST in cells treated with 1 mM H_2O_2 for 1 h.

Response of AtPEX1 to stress in whole plants

The *PEX*1 promoter was linked to the luciferase (*LUC*) gene to create transgenic plants (*PEX*1–*LUC*) that would report upon activation of *PEX*1 in response to stress. Figure 1 shows the fragment used. A translational fusion was made at the sequence DDWE, which is conserved in all known *PEX*1 genes. Depending on which start codon is used *in vivo*, a translational fusion with either 107 or 113 amino acids of the N-terminus of Pex1p fused to luciferase will be expressed.

All experiments were carried out on T2 progeny from two lines, 5ci and 1ci, which were recovered from independent transformations. Because the T2 plants of the two lines are segregating for the transgene, the absolute level of expression varies between plants and quantitative comparisons cannot be made. However, qualitative results are extremely consistent both within and between lines. No luciferase expression was seen in plants transformed with a promoter-less luciferase construct (data not shown). In untreated *PEX*1–*LUC* plants, *PEX*1–luciferase is mainly expressed in the meristems and young developing leaves, whereas there is little expression in mature leaves (controls in Figures 4A–C, 5B and 6B). These results suggest that *PEX*1 expression and peroxisome biogenesis are highest in actively dividing tissue and much lower in tissues where organelle maintenance (rather than biogenesis) is required. When *PEX*1–*LUC* plants were sprayed with 1 mM H_2O_2, *PEX*1–luciferase expression increased rapidly and dramatically throughout the plants (Figure 4A). This is consistent with the northern and RT–PCR results obtained with H_2O_2-treated wild-type plants (Figure 3A and B). To verify that increased *PEX*1 expression reflects increased peroxisome biogenesis, plants were subjected to two stress conditions in which peroxisomes have previously been described to proliferate, clofibrate treatment (Palma *et al*., 1991) and natural senescence (Pastori and del Rio, 1997). Incubation of *PEX*1–*LUC* plants with clofibrate resulted in a significant increase in the expression of *PEX*1–luciferase (Figure 4B). Compared with a young (20-day-old) plant, naturally senescent plants (60-day-old) also showed increased expression of *PEX*1–luciferase, which was no longer restricted to meristems and young tissue but was high in light yellow senescent leaves (Figure 4C).

Hydrogen peroxide induces PEX1 systemically

Hydrogen peroxide acts as a mobile signal (Alvarez *et al*., 1998; Karpinski *et al*., 1999). Opposite leaves of *PEX*1–*LUC* plants (Figure 5A, arrows) were incubated with a 1 mM H_2O_2 solution or a 1 mM H_2O_2 solution containing catalase (control) (Figure 5A). After 10 min incubation, a strong induction of *PEX*1–luciferase was detected throughout the treated leaf, but no photon emission was detected in the control leaf. By 30 min, induction of *PEX*1–luciferase in distant leaves, including the opposite (control) leaf, was observed.

Plant tissues rapidly break down exogenously applied H_2O_2 and it is difficult to estimate the resulting intracellular concentration. However, treatment of cell suspensions with the H_2O_2-regenerating system glucose (2.5 mM) and glucose oxidase (2.5 U/ml) (G/GO) initiates a steady and prolonged production of >5 μM H_2O_2. This mimics the level of H_2O_2 generated by the oxidative burst during pathogen attack (Delledonne *et al*., 1998). One leaf of a young *PEX*1–*LUC* plant was incubated with the G/GO H_2O_2-regenerating system (Figure 5B). *PEX*1–luciferase expression was induced locally and systemically within minutes of the start of the treatment, especially in vascular

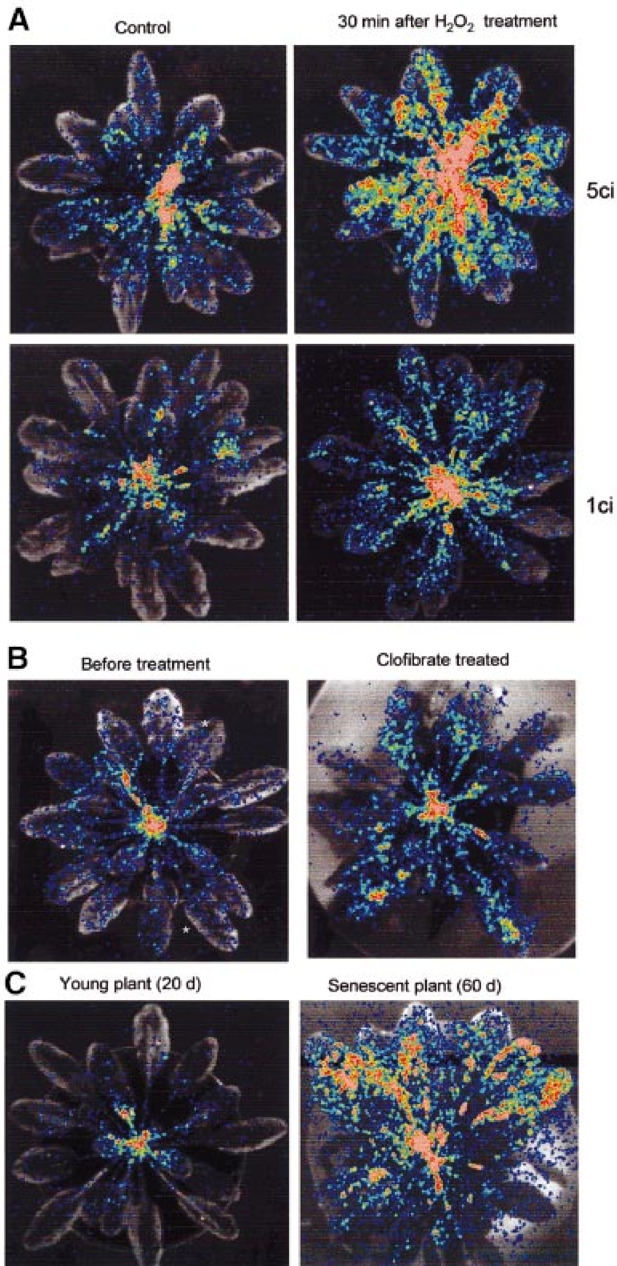

Fig. 4. H_2O_2 rapidly induces At*PEX*1–LUC gene expression. (**A**) Control plants (left) were sprayed with 1 mM H_2O_2 and light emission measured after 30 min (right). Clofibrate treatment (**B**) and senescence (**C**) were used as positive controls for peroxisome proliferation.

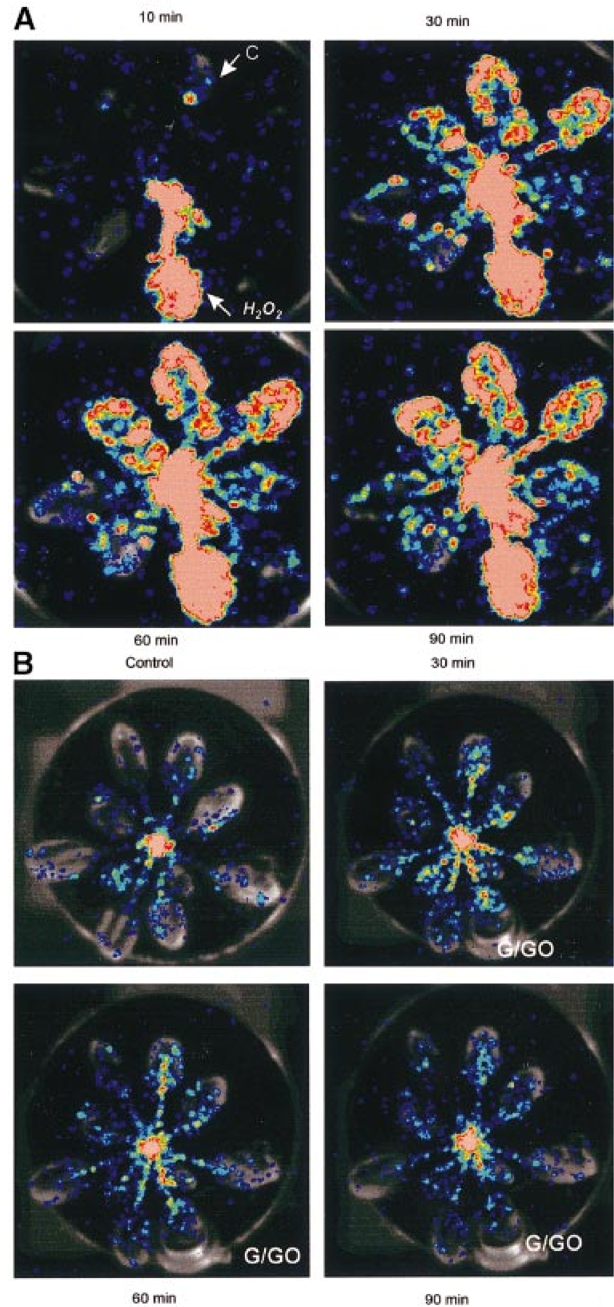

Fig. 5. H_2O_2 induces *PEX*1–LUC systemically. (**A**) Opposite leaves (arrows) were incubated with either 1 mM H_2O_2 (bottom leaf) or 1 mM H_2O_2 plus catalase (upper leaf) for 15 min. (**B**) The bottom leaf was incubated with the H_2O_2-regenerating system G/GO. Light emission was measured at the indicated times.

tissue. Note that photon emission from the treated leaf is much reduced, as it is submerged in G/GO solution.

The pathogen-induced hypersensitive response rapidly results in PEX1 induction

The ability to reproduce *PEX*1–luciferase induction with an authentic avirulent pathogen was tested. *PEX*1–*LUC* plants were inoculated in one leaf with *Pseudomonas syringae* pv. tomato, which induces an oxidative burst and hypersensitive response. An adjacent (control) leaf in the same plant was mock infiltrated. Photon emission in the inoculated and control leaves was monitored for 4 h immediately after inoculation (Figure 6A). Soon after infiltration of the pathogen, increased *PEX*1–luciferase expression was clearly seen in the treated leaf compared with expression in the control leaf and with the treated leaf before inoculation. Expression in the inoculated leaf was maximal at ~2 h. Although the pathogen was inoculated at the tip of the leaf, gene expression was mainly localized around the vascular tissue, similar to when a leaf was incubated with 10 μM H_2O_2-regenerating solution (Figure 5B); however, the response to pathogen was localized rather than systemic.

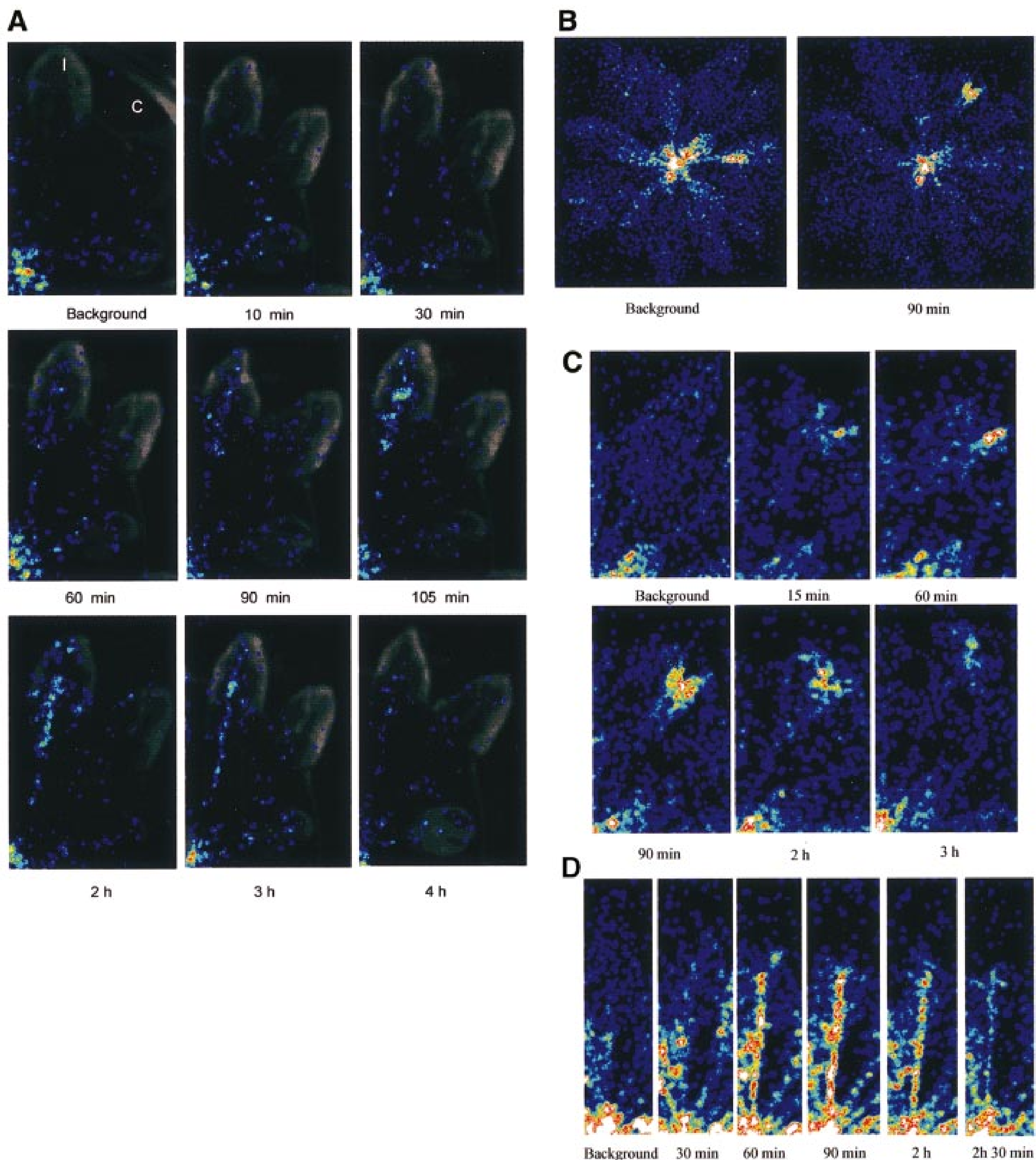

Fig. 6. *PEX*1–LUC is induced by pathogen attack and wounding. (**A**) *Pseudomonas syringae* pv. tomato was inoculated (I) into the indicated leaf and the effect on *PEX*1–LUC expression compared with a control leaf inoculated with buffer only. Light emission was measured at the indicated times. (**B**–**D**) Wounded plants. (B) Whole plant before (left) and 90 min after wounding (right). (C) Close up of a leaf of the plant shown in (B). (D) Close up of leaf before and after wounding; different plant.

PEX1 is induced by wounding

Plants respond to wounding and pathogen attack by distinct signalling mechanisms, although these pathways interact in a complex and incompletely understood manner (Malek and Dietrich, 1999). Photon emission was measured before wounding and then after excision of one or two leaves at the indicated times (Figure 6B–D). *PEX*1–luciferase expression was induced in the wounded leaf, but, as with pathogen treatment, the response remained localized. In the wounded leaves, *PEX*1–luciferase expression was observed mainly around the vascular tissues in the veins and the periphery of the leaf. In five independent experiments (two of which are shown) expression typically peaked between 45 and 120 min and then declined. Expression of *PEX*1 in the detached portion of the leaf continued to increase throughout the time course of the experiment, with expression increasing from the cut surface towards the tip of the leaf (data not shown). This may be the combined result of wounding and senescence responses.

Discussion

Up-regulation of PEX genes by H_2O_2

In this paper we provide the first demonstration that peroxisome biogenesis is directly responsive to the

major cellular stress signal H_2O_2. Peroxisomes are significant sources of active oxygen species as well as the site of important antioxidant enzymes and molecules. Consequently, peroxisomes are likely to play an important role in cellular redox balance. Steady-state mRNA levels of both plant and animal peroxisome biogenesis genes increase rapidly in response to 1 mM exogenous H_2O_2 treatment (Figure 3). *PEX*5 encodes the import receptor for PTS1-targeted proteins (Dodt *et al*., 1995), including catalase. *PEX*13 and *PEX*14 comprise the docking site for PTS1 and PTS2 import receptors and as such are central components of the peroxisomal import machinery (Gould *et al*., 1996; Albertini *et al*., 1997). *PEX*10 is a peroxisome membrane protein with a RING type zinc finger, which when mutated severely compromises the import of peroxisomal matrix proteins (Okumoto *et al*., 1998b).

AtPEX1 gene expression in whole plants in response to stress

Promoter–luciferase fusions are ideal for detecting rapid real time changes in gene expression with fidelity and high sensitivity (Millar *et al*., 1992). Using transgenic plants containing a *PEX*1–*LUC* transgene, exogenous application of 1 mM H_2O_2 resulted in the appearance of luciferase activity in the treated leaf within 10 min and the *PEX*1 promoter was activated systemically within 30 min (Figure 5A). This very rapid response argues strongly for activation of transcription of *PEX*1 being a direct consequence of the application of H_2O_2. The systemic induction is consistent with the known properties of H_2O_2 as a signal molecule (Levine *et al*., 1994). Sustained production of low micromolar concentrations of H_2O_2 by G/GO resulted in similar kinetics of induction of the *PEX*1 promoter (Figure 5B), demonstrating that this is a response to physiological levels of H_2O_2.

Infection with an avirulent pathogen results in the well-characterized production of H_2O_2 *in vivo* (Levine *et al*., 1994; Alvarez *et al*., 1998) and the induction of *PEX*1 by pathogen treatment is consistent with this (Figure 6A). This result demonstrates that peroxisome biogenesis is up-regulated when H_2O_2 is produced in a natural situation, thus it is a biologically meaningful response. However, the response is slower and not systemic. This may be because in plants time is required to generate the H_2O_2 and other mechanisms come into play to modulate the response.

Hydrogen peroxide is cytotoxic to the pathogen and to plant cells in the immediate vicinity, leading to a hypersensitive response. Protection of adjacent cells is achieved by induction of antioxidant defences, but as many of these are located in peroxisomes, elaboration of the peroxisome compartment might also be required early on to permit the uptake of newly synthesized antioxidant enzymes and molecules. The physiological importance of peroxisomal catalase as a sink for H_2O_2 has been demonstrated (Willekens *et al*., 1997). Of the three catalase genes in tobacco, Cat 2, which is preferentially expressed in vascular tissue (Willekens *et al*., 1994b), shows the highest induction by stress (Willekens *et al*., 1994a). Our results (Figures 4–6) show that *PEX*1–luciferase expression in response to various stresses is highest around vascular tissue. It is tempting to speculate that Cat 2 represents a stress-induced catalase that is imported into peroxisomes that are themselves elaborated in response to oxidative stress. In addition to protecting surrounding cells, peroxisomal antioxidants could also be responsible for turning off the H_2O_2 signal.

Wounding also produced a transient and localized response (Figure 6B). Wounding results in the induction of specific sets of genes, including those required for synthesis of jasmonates (Creelman and Mullett, 1997). Synthesis of jasmonic acid requires three cycles of (presumably) peroxisomal β-oxidation. Wounding also strongly induces one of the four acyl-CoA oxidases of *Arabidopsis*, AtACX1 (>10-fold induction within 1 h; Hooks *et al*., 1998). Either the fatty acids released from the phospholipids, jasmonic acid itself or the H_2O_2 produced by the wound-induced acyl-CoA oxidase could be responsible for induction of *PEX*1 upon wounding. The *PEX*1–*LUC* plants should be useful in testing these models.

A model for the control of peroxisome biogenesis by oxidative stress

Our results suggest a model whereby diverse stresses that generate H_2O_2 as a signalling molecule result in peroxisome proliferation via the up-regulation of components (*PEX* genes) required for biogenesis of the organelle and import of proteins. Previous work (Palma *et al*., 1991) has demonstrated that clofibrate treatment of pea plants results in elevated H_2O_2 and increased peroxisome number. The data presented here indicate that there is a causal relationship between these two events and suggest a mechanism for it. Additionally, two other stresses, wounding and pathogen attack, which also generate H_2O_2 as a signalling molecule, were demonstrated to induce *PEX*1 expression. Our model would predict that other stress conditions which elevate H_2O_2, such as drought (Pei *et al*., 2000), osmotic stress (Guan *et al*., 2000) and excess light (Karpinski *et al*., 1999), would also result in *PEX* gene induction and peroxisome proliferation. Peroxisome number and morphology has been reported to increase as a function of light intensity (Ferreira *et al*., 1989).

Induction of *PEX* genes by H_2O_2 is not confined to plants. Expression of all mammalian *PEX* genes tested was significantly increased by exposure to H_2O_2, suggesting that this may be a common mechanism for dealing with oxidative stress. Induction of peroxisome biogenesis by this general oxidative stress signal fits nicely with the presence of so many antioxidant enzymes within peroxisomes and appears to be an evolutionarily ancient response of cells to stress.

Materials and methods

Biological material

Arabidopsis thaliana ecotype Colombia was used for all experiments. Wild-type seeds were sown on 1/2 MS, 0.7% agar plates containing 500 μg/ml carbenicillin (Melford Laboratories), and 2.5 μg/ml amphotericin B (Sigma) and incubated in 16 h light, 8 h dark at 18°C. Transgenic seeds were sown in pots containing a 9:1 compost:sand mixture and grown in a Sanyo growth chamber at 18°C with 9 h light (240 $\mu mol/m^2/s$), 15 h dark. Experiments with transgenic plants were carried out with T2 seeds from two independent transgenic lines (5ci and 1ci) and were repeated between two and six times. CHO cells were obtained from the European Collection of Cell Cultures (Salisbury, Wiltshire) and were cultured in Ham's F12 nutrient mix (Gibco BRL, Paisley, UK) supplemented with 2 mM L-glutamine, 10% (v/v) fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C with 5% CO_2.

Isolation of a PEX1 cDNA clone

A tBLASTn search with *S.cerevisiae* Pex1p identified EST T43478 as a possible *Arabidopsis* orthologue (Kaplan, 1996) and a BAC clone MAH20 (DDBJ/EMBL/GenBank accession No. AB006697) from chromosome 5 was found that showed a highly significant identity ($P = 5.6 \times 10^{-160}$) to the EST. An 899 bp genomic DNA probe was amplified by PCR and used to screen a fractionated *Arabidopsis* 3 day seedling hypocotyl cDNA library (CD4-16, size range 3–6 kb; ABRC DNA Stock Centre, Ohio State University). The longest clone (3.5 kb) was sequenced commercially (MWG Biotech). A partial cDNA clone of 215 bp, which extends the sequence 5′, was isolated from a 2 day seedling library.

Isolation of the PEX1 promoter

A fragment of 1436 bp comprising 441 bp of coding sequence and 995 bp of 5′ sequence was amplified by PCR (Figure 1). Clones from three independent PCRs were sequenced and verified against the genomic sequence. The fragment was cloned as a *Pst*I–*Nco*I fragment into pGRT10 (pUC18 into which the luciferase gene had previously been inserted) to produce p*PEX*1-GRT. The *PEX*1–luciferase cassette was excised with *Not*I (blunted) and *Sac*I and inserted into *Sma*I–*Sac*I-digested pBI101. To produce a promoterless control, luciferase was excised from pGRT10 with *Hin*dIII and *Sac*I and ligated with *Hin*dIII–*Sac*I-digested pBI101. Transformation of *Arabidopsis* was according to the protocol of A.Bent (University of Illinois at Urbana, http://www.cropsci.uiuc.edu/~a-bent/protocol.htm).

RNA analysis

For H_2O_2 treatments, leaves from young plants were detached and immediately submerged in 1 mM H_2O_2 $\pm$ excess catalase (Sigma) for 1 h at room temperature, then frozen in liquid nitrogen and stored at –80°C. CHO cells were treated with 1 mM H_2O_2 solution *in situ* for 1 h at room temperature with gentle shaking. RNA was extracted using the Qiagen RNeasy Midi kit (catalogue no. 75142). Total RNA (10 µg) was separated in a formaldehyde gel and transferred to Hybond NX membrane (Amersham Pharmacia Biotech UK). Probes were amplified by PCR from the corresponding cDNA clone (*PEX*1); from a 2 day seedling cDNA library and sequenced (*PEX5*); and from an EST (GST, clone ATTS1553; ABRC, Ohio State University). Probes were labelled using a Rediprime kit and unincorporated label removed using Nick columns (both from Amersham Pharmacia Biotech UK). Hybridization was as described by Hooks *et al*. (1999). RT–PCRs were carried out using the Reverse-iT One Step kit from the AB gene following the manufacturer's instructions with equal amounts of RNA from control and H_2O_2-treated samples.

Plant treatments

For H_2O_2 treatments of whole plants a solution of H_2O_2 (1 mM in 0.9% NaCl, 10 mM Tris–HCl pH 7.5) was sprayed from above. For leaf incubations, one leaf was submerged either in 50 ml of 1 mM H_2O_2 in 0.9% NaCl, 10 mM Tris–HCl pH 7.5 for 15 min or in a 5–10 µM H_2O_2 solution and an H_2O_2 regenerating system consisting of glucose (2.5 mM) and glucose oxidase (2.5 U/ml). For clofibrate treatment, 25 µl of a 100 mM solution were placed on opposite leaves of the plant. For pathogen inoculation and wounding experiments, 3- to 5-week-old plants that had not initiated flowering were used. *Pseudomonas syringae* pv. tomato (Pst) strain DC3000 carrying the *avrRpm1* gene was grown overnight from a plate in King's B Medium containing rifampicin (50 µg/ml) and kanamycin (25 µg/ml), washed twice with 10 mM $MgCl_2$, and ~5–10 µl of a 10^6–10^7 c.f.u./ml suspension in 10 mM $MgCl_2$ were gently inoculated into the underside of an intact leaf using a 1 ml sterile plastic syringe without a needle. Control inoculations were carried out with 10 mM $MgCl_2$. For wounding experiments, one or two leaves were cut with scissors. The wounding and pathogen experiments were conducted with plants that had been kept in the dark overnight. Wounding and G/GO treatments were carried out in the camera to avoid exposing the plants to light between recording of the background image and data capture.

In vivo imaging of luciferase bioluminescence

An intensifier camera (VIM) and photon counting image processor (ARGUS-50) (Hamamatsu Photonics UK) were used for all experiments. *Arabidopsis* plants were sprayed with 5 mM D-luciferin solution (Analytical Bioluminescence Laboratories, San Diego, CA) for 12 h (Millar *et al*., 1992) and then again 20 min before photon emission measurements were taken. Plants were left within the camera case for at least 5 min in complete darkness to allow auto-luminescence to decay before measurements were taken. A reference measurement (control) was always taken prior to treatments.

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