Supplementary text 1.

Where excess H_2O_2 is present, but a reducing substrate is absent, peroxidases undergo suicide inactivation. There is experimental evidence for several possible H_2O_2-mediated inactivation pathways [S1, S2]. In the so-called "three-pathway model" [17], catalytic competition exists in the presence of excess H_2O_2, with three possible outcomes: (a) formation of the dead-end Compound III, (b) a catalase-type reaction, and (c) complete inactivation. The first and second outcomes are enzyme survival routes [17]. If excess H_2O_2 persists, the survival pathway is abandoned and the enzyme progressively inactivates, resulting in the formation of inactive verdohaemochrome P670 [S3]. The Compound I - peroxide complex is the critical point. A transient intermediate (P965) is formed from Compound I and the peroxide substrate. Decay of this intermediate, via two competing reactions (survival or inactivation), determines the enzyme's fate [S4]. One can measure the ratio of turnover to inactivation [S3].

HRP A2's increased H_2O_2 tolerance has been attributed to the formation of a modified Compound III. Normally, a water molecule is weakly bound to Compound III; however, the altered Compound III replaces this H_2O molecule with a tightly bound O_2 ● radical, affording increased stability. The Compound III alternative may be kinetically disfavoured during HRPC's catalytic cycle [S3].

Supplementary text 2

The RZ (A_{403}/A_{280}) values of recombinant peroxidases frequently do not match that of good-quality plant-derived commercial HRP (typically 3.0). For instance, Morawski et al. [S5] reported RZ values ranging 0.4 – 0.6 for recombinant HRPC expressed in *Pichia pastoris* and purified by three chromatography steps (hydrophobic interaction, gel filtration and ion exchange). In general, recombinant apo-peroxidase is easily produced but reactivation (incorporation of heme prosthetic group) is not straightforward [S6]. Heme-free apo-protein, wherever present, will absorb at 280 nm but not at 403 nm; this will adversely affect A_{403}/A_{280} ratio and, hence, the RZ value.

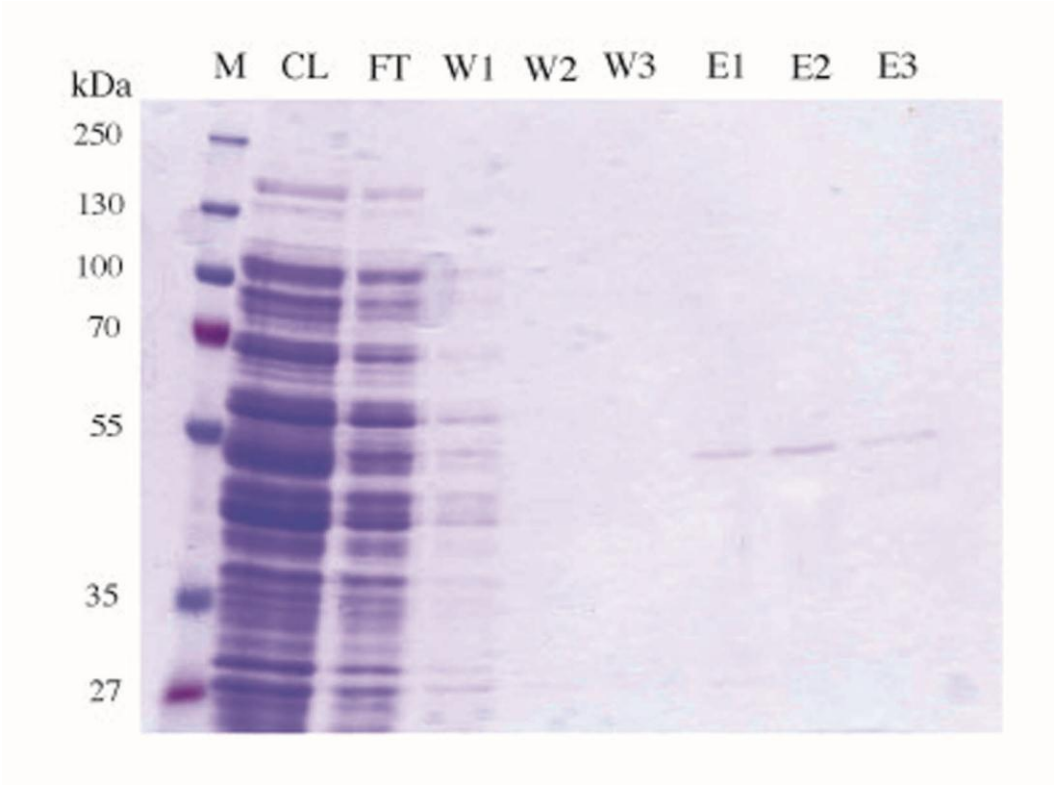

Figure [S1]: Purification of recombinant ancestral plant peroxidase. Monomer (~54 kDa) present in elution fractions (E1-E3). Lane M: molecular weight standards in kDa, CL: cell lysate, FT: flow through, W: wash fraction, E: elution fraction.

Supplementary text 3

Known functions of plant peroxidases include: control of levels of the growth hormone indole-3-acetic acid, cell elongation, cell wall formation, differentiation and lignin synthesis. They also play a role in wound healing and in defence against plant pathogens [S7, S8].

Plant peroxidases in general may be even older than previously thought: the discovery of putative peroxidase genes in algae [S8, S9] undermines the prevailing belief that the emergence of peroxidases coincided with plants' colonization of land (ms. ref. [8]). Algal Class III peroxidases could be worthwhile targets for protein engineering precisely because they represent direct evolutionary precursors of extant HRP C (and of other commercially-exploited peroxidases) [S8]. Similar reasons apply to the study of 110 MYO GP, the most likely ancestral sequence of HRP A2, HRP C and SBP, representing the most likely ancestral stability and other properties at that ancestral node. Thus, we hypothesize that GP may hold a desirable mixture/complement of these extant protein characteristics. GP is the most likely ancestral sequence but the chance of it being the **exact** ancestral sequence is quite small given the accumulation of errors across nodes and sites. However, the likelihood of reconstructing ancestral *phenotypes* is far higher than precise *sequences* and so we can consider this ancestral phenotype reasonably accurate [S10].

A highly supported phylogeny is fundamental to accurate estimation of ancestral states and the reduced plant peroxidase phylogeny of ms. ref. [8] provides reassurance in this regard. We generated the GP ancestral sequence using maximum likelihood (ML) methods and Bayesian statistics. This approach determines the most likely sequence at the node on the phylogenetic tree where the extant soybean (SBP) and horseradish (HRPC) peroxidases last shared a common ancestor.

Arg38 and the distal His42 are key catalytic residues in HRPC's active site, with Asn70 hydrogen-bonded to the latter. GP has identical residues at the corresponding sites in the aligned sequences.

Each enzyme has a sole Trp at position 117. At the active site entrance, HRPC has a Phe142-Phe143 pair; Phe142 is thought to play a key role in aromatic substrate binding. GP also has Phe143 but Thr occupies position 142. In the proximal region, His170 and the adjacent Thr171 occur in both GP and HRPC and GP; in HRPC, His170 positions the heme group and keeps it in the penta-coordinated state. Also in the proximal region, HRPC's Phe179 (ref. [7] in ms.) is replaced by Ser in GP.

GP's thermal stability lies within the range displayed by extant plant peroxidases (albeit at the less-stable end of the spectrum). Outside of extreme habitats, thermal stability is not necessarily under selective pressure (although a threshold molecular stability is linked with a polypeptide's ability to tolerate potentially-beneficial mutations leading to enhanced or new function [S11]).

GP's greater H_2O_2 tolerance may be due to a catalase-type reaction selected over Compound III formation (ms. ref. [10]), but this remains to be tested. The property of peroxide resistance may not, however, reside exclusively in the active site. In the case of HRP C, for instance, we previously reported (ms. ref. [10]) H_2O_2-stabilizing mutations (notably T110V, K232N, K241F), all of which are remote from the active site. (Interestingly, a GP-HRPC sequence alignment reveals that HRPC residues Thr110 and Lys232 are replaced in GP by Val and Ser respectively, while Lys241 occurs in both. Valine lacks the hydroxyl group found in threonine, and so should be less prone to oxidation. Val 110, however, also occurs in soybean peroxidase, which has unremarkable peroxide tolerance. Although serine (GP position 232) is hydroxylated, it may be less vulnerable to oxidative degradation than is lysine.) Atmospheric oxygen levels are known to have increased from 100 million years ago, when angiosperm diversity expanded under warm conditions [S12]. Possibly, a peroxidase with greater tolerance of oxidizing conditions could have conferred some selective advantage on its host plant at higher oxygen levels. Alternatively, GP's peroxide tolerance could simply be serendipitous.

Supplementary text 4.

Peroxidases have actual or potential applications in 5 major areas. These include (i) as pharmaceutical or antimicrobial agents, (ii) in bleaching or detergents, (iii) in polymerization or depolymerization processes, (iv) in the transformation of organic molecules and (v) in analytical and medical applications [S6]. More specifically, HRP C has been used in (a) the oxidation of amines and of catechols, (b) hydroxylation reactions and (c) the nitration of aromatic rings [S13]. It also has uses in polymer synthesis/ modification [S14] and in the breakdown/ transformation of environmental pollutants [S15].

Low operational stability, due to low stability in the presence of H_2O_2, is the major constraint on the large-scale application of peroxidases [S15]. Quite low H_2O_2 concentrations (0.5 – 10 mM) can lead to oxidative inactivation of peroxidases [S6]. Peroxide tolerance well above this inactivation threshold would likely be beneficial for industry. (Recall that GP's C_{50} value is 40 mM H_2O_2.)

Our T110V HRPC mutant (ref. [10] in ms.) retained 50% activity after 30 min exposure to 425 mM peroxide, a 25-fold improvement over wild type. Soybean peroxidase is stable for 12h at 70^{o}C and retains activity for up to 2.5h 85^{o}C (ref. [11] in ms.); this degree of thermal stability is of industrial interest. If these sorts of naturally-occurring or previously-attained stability characteristics could be combined in a single peroxidase enzyme, it would have considerable industrial applicability.

An ideal industrial biocatalyst would possess (i) thermal stability up to 120-130^{o}C, (ii) activity in water-organic co-solvent mixtures 100- to 10,000-fold greater than present levels, (iii) increased productivity of 10- to 100-fold and (iv) a lifetime durability of months to years [S16]. Clearly, these characteristics represent ultimate goals rather than incremental improvements that could, nevertheless, benefit present-day actual, or prototype, processes.

Supplementary text 5.

Ferulic acid, a phenolic constituent of plant cell walls, acts as a substrate for Class III peroxidases. HRPC Compounds I and II are similarly reactive to ferulic acid [S17], while quantum mechanics/ molecular mechanism calculations highlighted the importance of His42 (and of a water molecule) in a proton-coupled electron transfer step that begins both oxidation processes [S18]. In barley peroxidase 1, ferulic acid enhanced the rate of Compound I formation [S19]. Young soybean seedlings treated with millimolar ferulic acid showed increased peroxidase activities, both soluble and cell wall-bound [S20]. Given these various effects of a physiological substrate with extant peroxidases, an investigation of GP's action with ferulic acid would be instructive. Similarly, GP's reactivity with veratryl alcohol would bear study, as both HRP and SBP can oxidize veratryl alcohol to the corresponding aldehyde, with some interesting effects noted [S21, S22].

Circular dichroism (CD) is a valuable technique for probing the folding and molecular stability of proteins, including plant peroxidases such as HRP C [S23-S25] and HRP A2 [S26]. It is possible to investigate the tertiary and secondary protein structure at near- and far-UV wavelengths respectively, and to probe the active site region at Soret visible wavelengths. GP is expected to have (i) a high degree of alpha-helical secondary structure and (ii) conserved disulphide bonds. These two features are known to give strong CD signals, as do the aromatic residues in the near-UV region, including the sole Trp (which residue in HRPC shows pronounced fluorescence changes upon unfolding [S23]). In addition, differences in the Soret-region CD spectra of dead-end Compound III, formed under conditions of excess peroxide, and that of ground-state enzyme [S1] could elucidate GP's enhanced peroxide tolerance. Thus, a CD study of GP could yield valuable insights into this ancestral protein's structure, its molecular folding and stability, and its oxidative stability.

Supplementary text 6.

The observation that 13 α-helices and 2 β-strands are characteristic of Class III peroxidases, and that sequence variability within Class III is confined to certain "hot spots" in the molecule, including the substrate access channel and part of the region surrounding the interaction site of the aromatic donor molecule [S27], led us to conclude [12] that "*The Class III peroxidases have evolved to retain their structural helices and active site, [indicating] that structure and function are tightly associated in this family …. Retention of such structures suggests that these enzymes have a very ancient function consistent with their modern day one. … On the other hand, peripheral regions of a protein outside the helices are free to change their sequence (and folding pattern) entirely [S28]*."

The Fig. 1 (b) sequences of HRP C and of SBP were taken from PDB codes **1ATJ** [S27] and 1FHF [S29] respectively. The HRP A2 amino acid sequence is that posted at Uniprot P80679 [S30].

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