Plant thioredoxins and glutaredoxins: identity and putative roles

Yves Meyer, Lionel Verdoucq and Florence Vignols

Thioredoxins and glutaredoxins are ubiquitous proteins that reduce disulphide bridges of oxidized target proteins in vitro. In contrast to the situations in other organisms, phylogenic analysis has indicated that plant thioredoxins and glutaredoxins are present as multigenic families, and that thioredoxins have several subclasses. Thioredoxins and glutaredoxins are probably involved in similar physiological events – the major challenge is to identify their specific targets and establish the function of these proteins in vivo.

Thioredoxins are small (12–14 kDa) proteins that have an extremely reactive site, Trp-Cys-Gly-Pro-Cys, which is able to reduce disulphide bridges of target proteins. The first thioredoxin was discovered in *E. coli*, and found to act as a potent hydrogen donor for ribonucleotide reductase (RRase), an essential enzyme for DNA synthesis[1]. Ribonucleotide reductase obtains the energy needed to reduce ribonucleotides from a pair of cysteines that switch from a dithiol state to a disulphide bridge. At a later stage, the electrons are used for the reduction of ribonucleotides, and the disulphide bridge in the ribonucleotide reductase is reformed. The energy needed for the reduction of ribonucleotide originates from NADPH, and is transferred through a cascade of three proteins by dithiol–disulphide exchanges. First the flavoprotein, NADPH-dependent thioredoxin reductase (NTR), collects the electrons from NADPH, and uses them to reduce the disulphide bridge of its own active site. This dithiol then reduces the disulphide bridge of the oxidized thioredoxin, allowing the thioredoxin to reduce RRase (Fig. 1).

A second route, involving the transfer of energy from NADPH to RRase using a dithiol disulphide cascade, has since been discovered. The first acceptor is glutathione reductase (GR), a flavoprotein with similarity to NTR. GR reduces disulphide glutathione (GS-SG) to a reduced form of glutathione (GSH), which is followed by the reduction of glutaredoxin by GSH. In the final step, the glutaredoxin reduces RRase. Although thioredoxin and glutaredoxin do not share sequence similarity, they do have similar folding[2], with a prominent redox active site and partly overlapping functions.

In plants, three variants of thioredoxins have been well characterized. They were discovered in photosynthetic cells during a detailed analysis of the photo-activation of some chloroplastic enzymes involved in sugar metabolism[3]. Thioredoxins *m* and *f* are efficient *in vitro* reductants in the redox regulation of malate dehydrogenase and fructose-1,6-biphosphatase, respectively[3,4]. Both thioredoxins *m* and *f* are reduced by a heterodimeric ferredoxin-dependent thioredoxin reductase (FTR), which obtains its reducing power from ferredoxin. Interestingly, chloroplast thioredoxins are not involved in an energetic flux but act through structural modifications of the target proteins. The thioredoxin-dependent regulation of chloroplastic enzymes is reviewed in detail in Ref. 5. In addition to chloroplast thioredoxins, higher plants have a cytosolic thioredoxin system. This pathway involves thioredoxins *h*, which are reduced by an NADPH-dependent thioredoxin reductase[6]. All higher plant *m*, *f* and *h* thioredoxins are nuclear encoded.

The discovery that thioredoxins have a regulatory function was seminal and this was subsequently confirmed by further studies in plants and animals. The interest that followed this discovery has yielded an impressive amount of new information on thioredoxins in plants, which has been sustained largely by molecular, genomic and structural studies. Here we summarize these recent results and we also present, as a comparison, some information on glutaredoxins. Glutaredoxins might have overlapping functions with the thioredoxins in plants, as is the case in bacteria.

A flood of thioredoxins

Over the past ten years, the screening of cDNA and genomic libraries, and the systematic sequencing of ESTs and genomes, have led to the discovery that all three types of plant thioredoxins are encoded by a multigene family. To date, five thioredoxins *h* (Ref. 7), four thioredoxins *m* (D. Mestres and Y. Meyer, unpublished) and two thioredoxins *f* have been described in *Arabidopsis*. A survey of both monocot and dicot genome databases indicates a similar diversity of thioredoxin families, including the multigene characteristics. In addition to these three types of thioredoxins, at least 15 other sequences showing homology to thioredoxins, but which do not fall within the previously described groups, are present in the *Arabidopsis* database. Interestingly, database searches reveal that such thioredoxin-like proteins have also been found in other plants. In addition, other proteins exhibiting thioredoxin domains have also been described. The disulphide isomerases (PDIs), which play an important role in the

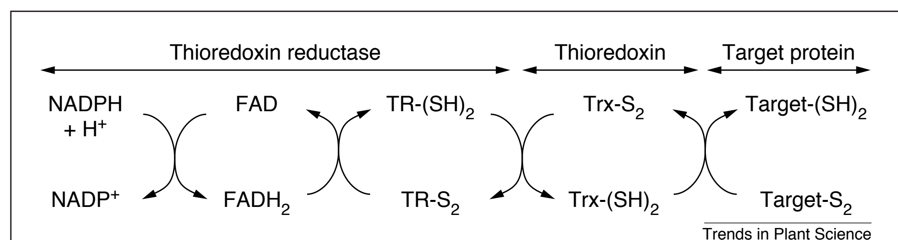

Fig. 1. Mechanism of NADPH-dependent protein disulphide reduction by the thioredoxin system. The NADPH-dependent thioredoxin reductase is a 70 kDa flavoprotein containing two subunits strongly associated to two FAD molecules. Each subunit presents fixation sites for both FAD and NADPH and a disulphide bridge within its active site. During thioredoxin reduction, the electrons are transferred from NADPH to the disulphide bridge of the thioredoxin reductase active site through the flavoprotein. Hydrogen ions are then exchanged with disulphides in the thioredoxin molecule. Abbreviations: TR, thioredoxin reductase; Trx, thioredoxin.

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PII: S1360-1385(99)01475-2

conformation of secreted proteins, possess two or three thioredoxin domains (reviewed in Ref. 8). Another protein described in monocots, and whose function has been related to pollen self-incompatibility, also has a thioredoxin domain in its C-terminal region[9]. In the tomato chloroplast, a 32 kDa protein of unknown function, but which is induced by drought stress, possesses two thioredoxin domains in the C-terminal region[10]. In maize, a 69 kDa nuclear protein containing three thioredoxin domains has also been described[11]. Both the tomato protein and the maize protein are encoded by genes whose homologues are known in *Arabidopsis*. Three other genes encoding chloroplast adenylphosphosulphate (APS) reductases that exhibit a C-terminal thioredoxin domain have also been studied[12,13]. The current genomic sequencing projects have so far revealed other genes coding for non-PDI thioredoxin-containing proteins.

Whether these thioredoxin-like and thioredoxin-containing proteins are efficient thioredoxins capable of disulphide reductase activity remains to be determined, and an exhaustive analysis of these proteins, including tests for disulphide reductase activity, will be necessary. Nevertheless, among higher eukaryotes, this multitude of thioredoxins is peculiar to plants. This contrasts with the human genome, which encodes only one cytosolic and one mitochondrial thioredoxin, and with the *E. coli* genome in which sequencing indicates the presence of only two thioredoxins and three glutaredoxins[14]. This raises the question of the specificity and physiological role of thioredoxins in plants.

Another striking observation is the opposite situation concerning the NTR and FTR proteins that reduce the oxidized thioredoxins. Only two NTR-encoding genes[15] and one copy encoding each subunit of FTR (Ref. 16) have so far been identified in *Arabidopsis*.

The occurrence of glutaredoxins in plants resembles that of thioredoxins. *Arabidopsis* has only two divergent genes encoding classical glutaredoxins, but at least 20 additional genes encode proteins with homology to glutaredoxins. One cDNA and one gene encoding glutaredoxins have also been described in rice[17] and for most dicots.

Structure of thioredoxins and thioredoxin reductases

In recent years, 3-D structures have been established for thioredoxins and corresponding thioredoxin reductases by crystallography or NMR (reviewed in Ref. 4). These analyses have revealed the structure of all types of thioredoxins: from *E. coli*, *Chlamydomonas m* and *h*, human and plants, and they all highlight the prominence of the active site that is responsible for thioredoxin activity. Crystal structures have also been obtained for *E. coli* and *Arabidopsis* NTRs (Refs 18,19), and *Synechocystis* FTR (P. Schürmann, pers. commun.).

A better understanding of the thiol-based reductive cascades has been obtained from 3-D thioredoxin structures. For example, *Arabidopsis* and *E. coli* NTR show high sequence similarity. Nevertheless, *E. coli* NTR poorly reduces plant thioredoxins, and in turn *Arabidopsis* NTR is a poor reductant of *E. coli* thioredoxin[15]. A careful examination of the 3-D structure of both proteins indicates that the overall structures are similar except in the loops and the relative position of FAD and NADPH domains. These structural differences between the two proteins are probably responsible for the species specificity of their activity. The 3-D structure of FTR shows a flat protein interacting with ferredoxin on one face and thioredoxin on the other face. Based on 3-D structures and sequence information, several studies have been devoted to the characterization of domains or specific amino acids within thioredoxins and their putative targets that are involved in their specific activity[20–22].

Furthermore, 3-D structures have also been obtained for plant glutaredoxin[2]. In spite of the lack of homology between primary sequences, glutaredoxin structure is similar to that of thioredoxin, except for a β1 sheet and an α-helix, which are absent in the glutaredoxin molecule.

Looking for the function of thioredoxins and glutaredoxins

Cellular localization

The cellular localization of plant thioredoxins, particularly their subcellular compartmentation, provided the first indications about their function. Thioredoxins *m* and *f* were isolated from chloroplasts of spinach and pea. Analysis of the sequences available in plant databases indicate that they all have an N-terminal sequence that could be a transit peptide compatible with their chloroplastic localization. The two FTR subunits that are necessary for the reduction of *m*-type and *f*-type thioredoxins also have a typical chloroplastic transit peptide.

In contrast with animals and *Saccharomyces cerevisiae*, no mitochondrial thioredoxin sequence has been characterized in higher plants, although mitochondrial disulphide reductase activities have been detected[23]. These activities might result from proteins that are unrelated to thioredoxins or from one or more of the thioredoxin homologues that are presently sequenced, but for which neither enzymatic function, nor cellular localization has been established.

Thioredoxins *h* do not appear to posses a transit peptide and are assumed to be cytosolic proteins. Nevertheless, it has been demonstrated in rice, and in numerous other plants, that thioredoxin *h* is also present in phloem sap[24,25]. In the absence of a secretion signal in thioredoxin sequences, it is thought that thioredoxins are transported through the plasmodesmata because of their small size[26]. This is reminiscent of human thioredoxin, which is massively secreted into the blood. These studies suggest that thioredoxins have the capacity to act as messengers in transduction pathways.

Like thioredoxins *h*, glutaredoxins do not have a transit peptide, indicating that they are probably not imported into chloroplasts or mitochondria. Glutaredoxins have also been encountered in the phloem sap together with thioredoxins[24,25], reinforcing the concept of overlapping functions for these reductants. However, in contrast with the thioredoxin system, glutathione reductase isoforms are present in the cytosol, as well as in chloroplasts and in mitochondria[27]. This is in accordance with the multiple functions of reduced glutathione, which are not limited to the reduction of glutaredoxins.

Chloroplast thioredoxins

Chloroplast light-regulated proteins implicated in sugar metabolism were the first targets identified in plants, and this led to the discovery of thioredoxin *m* and *f* (for a detailed review, see Ref. 5). Recently, some research has been devoted to the characterization of cysteine residues within the targets that are responsible for the interaction with thioredoxin[28,29]. Because of the multiple isoforms of chloroplast thioredoxins *m* and *f*, and their possible targets, the characterization of the physiological reductant for each target remains a complex and time-consuming process.

Nevertheless, an important gap in our understanding of sulphate assimilation in plants has been solved. Two groups independently isolated a cDNA encoding a chloroplast bipartite protein showing homologies with thioredoxin[12,13]. The N-terminal domain appears to be a 5′ APS reductase, indicating that plants directly transfer APS to sulphide without a 3′-phosphate adenosine 5′-phosphosulphate (PAPS) intermediate. The C-terminal region of this bipartite protein is a thioredoxin-like domain that is able to transfer energy to the APS domain. Because of its chloroplastic character, it had been hypothesized that this new thioredoxin-like protein would obtain its reducing power from the ferredoxin–FTR system, the same process that reactivates thioredoxins *m* and *f*.

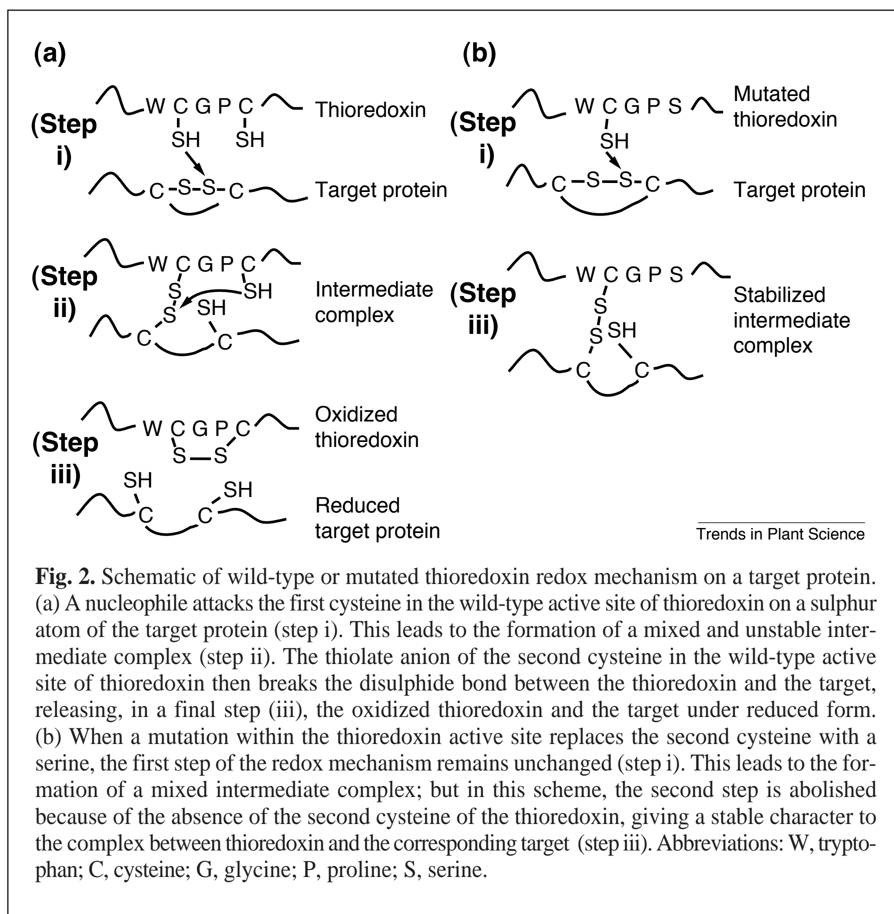

Fig. 2. Schematic of wild-type or mutated thioredoxin redox mechanism on a target protein. (a) A nucleophile attacks the first cysteine in the wild-type active site of thioredoxin on a sulphur atom of the target protein (step i). This leads to the formation of a mixed and unstable intermediate complex (step ii). The thiolate anion of the second cysteine in the wild-type active site of thioredoxin then breaks the disulphide bond between the thioredoxin and the target, releasing, in a final step (iii), the oxidized thioredoxin and the target under reduced form. (b) When a mutation within the thioredoxin active site replaces the second cysteine with a serine, the first step of the redox mechanism remains unchanged (step i). This leads to the formation of a mixed intermediate complex; but in this scheme, the second step is abolished because of the absence of the second cysteine of the thioredoxin, giving a stable character to the complex between thioredoxin and the corresponding target (step iii). Abbreviations: W, tryptophan; C, cysteine; G, glycine; P, proline; S, serine.

Surprisingly, the domain is unable to obtain energy from this system. Instead it is reduced by reduced glutathione as is glutaredoxin[30]. The possibility that other thioredoxin homologues are reduced *in vivo* by glutathione should be considered in the analysis of the numerous thioredoxins that are presently identified only by their sequence.

Cytosolic thioredoxins

Recent results have suggested different roles for the cytosolic thioredoxins *h*. One of the putative functions is related to seed maturation and germination. Several studies have demonstrated that the NADP–thioredoxin system specifically reduces intramolecular disulphide bonds of specific seed proteins, such as thionins, gliadins and glutenins, or α-amylase and trypsin inhibitor proteins (reviewed in Ref. 4). In addition, thioredoxin has been found to reduce, and thereby activate a serine protease, thiocalsin from wheat[31]. In all cases, disruption of disulphide bridges by thioredoxins during early germination appears to be necessary to block the activity of the inhibitors, to relax the conformation of the storage proteins, and to activate hydrolases implicated in nitrogen and carbon release. However, as with chloroplast thioredoxins, the identity of the interacting partners remains unsolved.

More recently, two groups have suggested a role for thioredoxins or thioredoxin-like proteins in reproductive processes in plants: a gene encoding a thioredoxin domain that maps to the self-incompatibility locus of *Phallaris*[9], and a cDNA encoding a thioredoxin from *Ricinus* pistil[32]. This thioredoxin *h* was revealed during a two-hybrid screen using the kinase domain of an *S*-locus receptor kinase as bait.

The observation that a thioredoxin is able to interact with a receptor kinase[32], and the presence of thioredoxins in the phloem sap[24–26], strongly suggest that thioredoxin might also act as a messenger in a signalling pathway. Thioredoxins could act as signalling molecules in plant tissues by means of their redox-regulation properties, by interacting with specific receptors.

The latest news on thioredoxin functions

As already mentioned, the main questions regarding thioredoxins are:

- Are all thioredoxins redundant and able to comply indiscriminately with all the putative functions described to date?
- Is there any specificity of both thioredoxins and their targets?

To date, no evidence has been obtained *in planta* that suggests that the different plant thioredoxins isoforms are implicated in specific reactions. Nevertheless, some evidence has been obtained in answer to the first question[33] based on the use of heterologous complementation in *S. cerevisiae*, which has three thioredoxin genes, one encoding a mitochondrial protein, and two encoding cytosolic thioredoxins. The cytosolic proteins perform similar functions because single mutants have no obvious phenotype. By contrast, the double mutant (EMY63) is impaired in sulphate assimilation, and requires methionine or cysteine for growth. EMY63 also reduces methionine sulphoxide (MetSO) poorly, is hypersensitive to H_2O_2, and has a long S phase and no G1 phase in its cell cycle[34]. Different plant thioredoxins of both chloroplastic- and cytosolic-type have been introduced into this mutant[33]. The five thioredoxins *h* from *Arabidopsis* (AtTRX1 to AtTRX5) expressed in EMY63 confer a normal cell cycle and the ability to use MetSO as a sole sulphur source with different efficiencies. Only AtTRX2 enables growth on sulphate, but does not confer H_2O_2 tolerance. In turn, AtTRX3 confers H_2O_2 tolerance but not sulphate assimilation. The two chloroplast thioredoxins tested (AtTRXf2 and AtTRXm5) only confer the ability to use MetSO, with a lower efficiency than cytosolic thioredoxins, and are unable to restore a normal cell cycle. This demonstrates that both chloroplastic and cytosolic thioredoxin have different potentials to interact with the different targets in *S. cerevisiae*, and strongly suggests that they have specific functions in plants.

To date, the most promising strategy to answer the question 'is there any specificity of both thioredoxins and their targets?' is the idea that once the target of a thioredoxin has been revealed and identified, it becomes possible to further analyse the cascade of events dealing with the specific interaction. Several methods can be used to identify thioredoxin-interacting proteins. They are all based on the principle of thioredoxin action on a putative target through its active site (Fig. 2). In the classical scheme, thioredoxin reduces the disulphide of the target protein in two fast steps, such that the lifetime of the intermediate complex between thioredoxin and its target is extremely short. Consequently, both the reduced target and the oxidized thioredoxin are released. It is possible to block the second step of the reaction (Fig. 2) and stabilize the intermediate complex using a subtle mutation of the second cysteine in a serine residue within the thioredoxin molecule. The mutation leads to a stable thioredoxin–target complex. This strategy has been successfully tested *in vitro* for *E. coli* and human thioredoxins[35,36] and is thought to be applicable to all the thioredoxins from other origins.

In our opinion, this promising strategy will also help to isolate thioredoxin–target complexes *in vivo* and so elucidate thioredoxin functions. This system has recently been used to isolate an *in vivo* thioredoxin–target complex from yeast using a plant mutated thioredoxin as a bait[37]. This was achieved by producing a His-tagged mutated form of AtTRX3 in the yeast EMY63 (Ref. 34) *in vivo*, and purifying the His-tagged mixed (AtTRX3–target) intermediate complex. The target protein appears to be a new type of thioredoxin-dependent peroxidase, forming a new family of peroxiredoxins[37] found in yeast, animals and plants[38]. This protein and the *Arabidopsis* homologues can reduce H_2O_2 in the presence of NADPH, thioredoxin reductase and thioredoxin *h* (Ref. 37). In the case of *Arabidopsis*, the NADPH–thioredoxin reductase–thioredoxin cascade acts as an energetic flux for the peroxidase, rather than by a conformation change. Nevertheless, because of the role of H_2O_2 and other reactive oxygen species in signal transduction pathways, it is probable that thioredoxin plays a regulatory role in this anti-oxidant function.

Another method that can be applied is the yeast two-hybrid system. As already mentioned, an interaction has been shown between a *Ricinus* thioredoxin and the kinase domain of an S-locus receptor kinase[32]. To our knowledge, this is the only example of such an interaction involving a thioredoxin using this method. However, because the kinase domain was used as the bait, it is not known at present whether the thioredoxin is the substrate of the kinase, or whether the kinase needs to be reduced by the thioredoxin. Further refinements to the method are clearly needed and should be developed to identify thioredoxin targets.

Glutaredoxins

According to database searches, glutaredoxins are present in all prokaryotic and eukaryotic organisms. However, little is known about their function in plants. Neither the characterization of glutaredoxin-encoding cDNAs (Ref. 17), nor the first identification and localization of a glutaredoxin in spinach leaves[39] brought substantial information about their function in the cell. The first indication was obtained with the detection of glutaredoxins in phloem sap with thioredoxins[24–26].

Since this discovery, no more information on glutaredoxin function in plants has been obtained. The most recent published data[30] indicate that the *Arabidopsis* genome encodes a bipartite 5′-adenylsulphate reductase containing a domain in its C-terminal region that functions as a redox cofactor glutaredoxin, as is the case for thioredoxin-like proteins. On the basis of the sequence, this C-domain is more homologous to thioredoxin. However, this domain is active in the GSH-dependent reduction of ribonucleotide reductase and is able to complement an *E. coli* mutant in a glutaredoxin gene[30]. These data suggest a functional similarity between this bipartite protein from plants and the *E. coli* glutaredoxin.

Origin of the thioredoxins

According to the endosymbiotic theory, eukaryotes originated from a first endosymbiose between an archebacteria-related organism and a eubacteria related to an alpha-proteobacteria – the ancestor of the mitochondria. Plant evolution originates from a second endosymbiosis with a cyanobacteria. In modern eukaryotes, most of the genes implicated in the transcription and translation of nuclear genes show greater homology with archebacterial genes than with eubacterial genes, and are thus supposed to derive from the archebacterial ancestor. On the basis of sequence similarity, the origin of the nuclear genes encoding the chloroplast Calvin cycle proteins, which includes some thioredoxin targets, has been determined[40]. Some of these genes are of cyanobacterial origin whereas others are of 'mitochondrial' origin. An additional interesting point is that the cytosolic counterparts appear to have a mitochondrial or cyanobacterial rather than an archebacterial origin.

Where do plant thioredoxins come from? A phylogenic tree (Fig. 3) constructed with the DARWIN program[41], which includes most of the plant thioredoxins and homologues, indicates that higher plant thioredoxins *m* are clustered. In addition, they are close to the chloroplast-encoded thioredoxins of the red alga and to the thioredoxin encoded by the open reading frame slr0623 from *Synechocystis*. Thus we propose that thioredoxins *m* have a cyanobacterial origin. By contrast, thioredoxins *f* and *h* form a separate group with the animal thioredoxins, suggesting a common eukaryotic origin. This has been further sustained by the position of an intron, which has been shown to be the same in the human and in plant *h* and *f* thioredoxins[42].

Where do the eukaryotic-type thioredoxins come from? Clearly these eukaryotic thioredoxins are far from the eubacterial thioredoxins. The first sequenced archebacterial genomes (*Methanococcus jannaschii* and *Thermotoga maritima*) do not encode thioredoxin. More recently, six divergent thioredoxin sequences from archebacterial genomes (*Archaeoglobus fulgidus*, *Halobacterium* and *Methanobacterium thermoautotrophicum*) have been characterized, and all originate between the animal and eubacterial thioredoxins. Thus in the present state of our knowledge, thioredoxins *h* and *f* could share a common archebacterial ancestor. Interestingly, the thioredoxin domain of APS is also rooted in the same region of the tree. The thioredoxin domain of the chloroplast APS could be the most well conserved vestige of a thioredoxin encoded by the archebacterial ancestor. Nevertheless, the branches between the archebacterial sequences and their first node in the tree largely indicate some uncertainty concerning the position of the node. More data will probably give more confidence to the exact rooting of these sequences.

Prospects

Over the past two years a lot of new information concerning plant thioredoxins has been obtained. But although some new targets for cytosolic and chloroplastic thioredoxins have been found, we are far from having a complete understanding of the role of thioredoxins and glutaredoxins in plants. In addition, the presence of multiple genes for each thioredoxin and glutaredoxin type, as well as the presence of numerous homologues has raised the question of their function: is there redundancy or do they have partially overlapping or totally independent roles?

A clear analysis of the expression pattern of each gene and the eventual detection of each protein during normal development is needed. Particular conditions including stress and during pathogen attack should also be analysed. However, expression pattern analysis is not an easy task because thioredoxin mRNA and the corresponding proteins are not abundant. The second approach that has been developed for thioredoxins and for some thioredoxin-like or homologous proteins, is the isolation of *Arabidopsis* knock-out mutants. The same work should be done for glutaredoxin, and for thioredoxin- and glutaredoxin-corresponding reductases. In mouse, loss of the unique cytosolic gene is embryo-lethal[43]. By contrast, *E. coli* thioredoxin or glutaredoxin mutants manage to maintain a balanced supply of ribonucleotides by modulating the expression of the genes implicated in both pathways[14]. The current experience with the *Arabidopsis* T-DNA mutant collection established in Versailles, France demonstrates that only one gene knocked out in ten gives a directly visible phenotype. Thus, we anticipate the absence of a phenotype for most of the thioredoxin gene mutants in *Arabidopsis*. Nevertheless, we are convinced that it will be particularly informative to look at the expression of related genes in such mutants to define overlapping functions, to know whether there could be some compensation phenomena, and eventually to prepare plants mutated in multiple genes that are implicated in thiol–disulphide cascades.

Fig. 3. DARWIN tree of all available thioredoxins from non-photosynthetic and photosynthetic organisms. Eubacterial sequences have also been introduced, particularly the four divergent *Synechocystis* (cyanobacteria family) thioredoxins and the unique thioredoxin of *Rickettsia* (bacterium that is most closely related to the ancestor of the mitochondria). EMBL or Swiss Prot (SWP) accession number and codes are indicated below. Archebacteria – *Methanobacterium thermoautotrophicum* (AE000880), *Halobacterium* TrxA (AF016485), *Archaeoglobus fulgidus* (1 = AE000983, 2 = AE001051, 3 = AE001015, 4 = AE000956); Photosynthetic bacteria – *Chlorobium limicola* (SWP P10472), *Chromatium vinosum* (SWP P09857), *Rhodobacter sphaeroides* (M33806), *Rhodospirillum rubrum* (SWP P10473); Cyanobacteria – *Anabaena* sp. (1 = M14736, 2 = M22997), *Synechocystis* sp. PCC6803 (1 = sll1057, 2 = slr0233, 3 = slr0623, 4 = slr1139), *Anacystis nidulans* (J04475); Green alga – *Chlamydomonas reinhardtii* (m = X62335, h = X78822); higher plants – *Arabidopsis thaliana* (trx1 = Z14084, trx2 = Z35475, trx3 = Z35474, trx4 = Z35473, trx5 = Z35476, f1 = AF144385, f2 = AF144386, m1 = AF095749, m2 = AF095750, m3 = AF095751, m4 = AF095752, p = AF095753, Thl1 = AF144387, Thl2 = AF144388, Thl3 = AF144389, Thl4 = AF144390, Thl5 = AF144391), *Brassica napus* (h1 = U59379, h2 = U59380, f = AF018074), *Lilium longiflorum* (L18909), *Mesembryanthenum crystallinum* (AF069314), *Nicotiana tabacum* (h1 = U59379, h2 = Z11803), *Oryza sativa* (h = D26547, f = AF022741), *Pisum sativum* (f = X63537, m = X76269), *Ricinus communis* (Z70677), *Spinacia oleracea* (f = X14957, m = X51462). The thioredoxin domain of the *Arabidopsis* adenylphosphosulphate (APS) reductase[12,13] is also given as APSred. As a comparison, thioredoxins from non-photosynthetic organisms are also mentioned: *Escherichia coli* (1 = P00274, 2 = AE000344), *Rickettsia prowazeki* (AJ235270), *Homo sapiens* (X77584), *Bos taurus* (D87741). Grey shading indicates distinct groups of thioredoxin. The

The first *in vivo* complex between a plant thioredoxin and a target in yeast (YLR109) has been isolated recently[37]. The conclusions concerning this target have been confirmed by two other studies describing the function of YLR109 (Refs 44,45). Although the method will probably not be directly applicable to higher plants, the use of thioredoxins mutated in the second cysteine to stabilize the intermediate complexes in the yeast two-hybrid method should help in the characterization of new plant targets. Proof that such a

system could function has been obtained from two-hybrid experiments involving mammalian wild-type or mutated thioredoxin[46,47], and from the isolation of a yeast target of the yeast mutated *yTRX1* thioredoxin gene (Y. Meyer, L. Verdoucq and F. Vignols, unpublished). Finally, this strategy should also help to isolate glutaredoxin targets, and thus to discriminate between the physiological events in which plant thioredoxin and glutaredoxin are involved.

Finally, in animal cells, several examples of *in vitro* protein–protein interaction have been shown recently between a thioredoxin and various proteins[46,48,49] whose counterparts are supposed to exist in plants. The innovative strategies being developed in these studies open the perspectives of developing the technique to isolate similar proteins *via* an interaction with plant thioredoxins.

Acknowledgements
We would like to thank P. Schürmann for communicating unpublished results on the crystal structure of *Synechocystis* FTR. We would also like to thank Richard Cooke for critical reading of the manuscript.

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Yves Meyer*, Lionel Verdoucq and Florence Vignols are at the Laboratoire de Physiologie et de Biologie Moléculaire des Plantes, UMR 5545, Université de Perpignan, Avenue de Villeneuve, 66025 Perpignan, France.

*Author for correspondence (tel +33 4 6866 2225; fax +33 4 6866 8499; e-mail ymeyer@univ-perp.fr).

Flavonoids and isoflavonoids – a gold mine for metabolic engineering

Richard A. Dixon and Christopher L. Steele

Flavonoid-derived plant natural products have long been known to function as floral pigments for the attraction of insect pollinators, as signal molecules for beneficial microorganisms in the rhizosphere, and as antimicrobial defense compounds. New functions for flavonoid compounds continue to be found, particularly in plant–microorganism signaling, and there has been an explosion of interest in flavonoids and isoflavonoids as health-promoting components of the human diet. The flavonoid and isoflavonoid pathways are probably the best characterized natural product pathway in plants, and are therefore excellent targets for metabolic engineering. Manipulation of flavonoid biosynthesis can be approached via several strategies, including sense or antisense manipulation of pathway genes, modification of the expression of regulatory genes, or generation of novel enzymatic specificities by rational approaches based on emerging protein structure data. In addition, activation tagging provides a novel approach for the discovery of uncharacterized structural and regulatory genes of flavonoid biosynthesis.

Flavonoids are a diverse group of plant natural products synthesized from phenylpropanoid and acetate-derived precursors, which play important roles in growth and development, and in defense against microorganisms and pests. These compounds often possess antioxidant activity, and the potential health benefits of fruit, vegetables, green tea and red wine might partly be because of this property of flavonoids and other phytochemicals[1–3]. In addition, the isoflavonoids, which are limited primarily to the Leguminosae, exhibit estrogenic and anti-cancer activity[4,5], and, in common with the flavonoids, are also receiving considerable attention as health-promoting 'nutraceuticals'.

Although the genetic manipulation of plants to improve the composition of health-promoting phytochemicals has been proposed, a major limitation has been the lack of knowledge regarding the complete biosynthetic pathways needed for the synthesis of most biologically active plant natural products. However, because of recent advances, metabolic engineering strategies for quantitatively and qualitatively modifying the plant's ability to synthesize flavonoid and isoflavonoid bioactive natural products for human, animal and plant health can now be considered.

Biological activities of flavonoids that impact on plant and animal health

Because of their *in vitro* antimicrobial activity, specific classes of flavonoid and isoflavonoid compounds have long been thought to play a role in plant–microorganism interactions as part of the host plant's defensive arsenal[5,6]. But, in spite of an extensive literature, direct proof of the 'phytoalexin hypothesis' remains to be found. This could now be obtained by pathway engineering approaches. Genetic manipulation of flavonoid biosynthesis will permit a better appreciation of the roles of flavonoids as inducers of the nodulation genes of symbiotic *Rhizobium* species[7] in natural rhizospheres. It will also allow an assessment to be made concerning the importance of the suppression of host defense responses[8] for the successful establishment of both rhizobial and mycorrhizal symbioses.

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