Roles of N-Terminal Fatty Acid Acylations in Membrane Compartment Partitioning: Arabidopsis h-Type Thioredoxins as a Case Study

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N-terminal fatty acylations (N-myristoylation [MYR] and S-palmitoylation [PAL]) are crucial modifications affecting 2 to 4% of eukaryotic proteins. The role of these modifications is to target proteins to membranes. Predictive tools have revealed unexpected targets of these acylations in Arabidopsis thaliana and other plants. However, little is known about how N-terminal lipidation governs membrane compartmentalization of proteins in plants. We show here that h-type thioredoxins (h-TRXs) cluster in four evolutionary subgroups displaying strictly conserved N-terminal modifications. It was predicted that one subgroup undergoes only MYR and another undergoes both MYR and PAL. We used plant TRXs as a model protein family to explore the effect of MYR alone or MYR and PAL in the same family of proteins. We used a high-throughput biochemical strategy to assess MYR of specific TRXs. Moreover, various TRX–green fluorescent protein fusions revealed that MYR localized protein to the endomembrane system and that partitioning between this membrane compartment and the cytosol correlated with the catalytic efficiency of the N-myristoyltransferase acting at the N terminus of the TRXs. Generalization of these results was obtained using several randomly selected Arabidopsis proteins displaying a MYR site only. Finally, we demonstrated that a palmitoylatable Cys residue flanking the MYR site is crucial to localize proteins to micropatching zones of the plasma membrane.

INTRODUCTION

Protein lipidation is an important modification involving a significant part of the eukaryotic proteome, up to 4% of all proteins (Meinnel and Giglione, 2008a). During the last two decades, the functions of two types of protein lipidation affecting the N termini of proteins, N-myristoylation (MYR) and S-palmitoylation (PAL), have been identified as essential regulatory components of signal transduction networks in many eukaryotes (Resh, 1999). The function of these two acyl groups is to ensure protein activity, usually by properly targeting the protein to membranes. Indeed, for many N-terminally acylated proteins, it has been shown that these two lipidations often dynamically regulate effective localization to the correct cell membrane system and/or plasma membrane (PM) nanodomains, such as lipid rafts. Such localization promotes interaction with functional partners and protein activity (Lingwood and Simons, 2010).

MYR is catalyzed by N-myristoyltransferase (NMT), an enzyme found in all eukaryotes. NMT possesses a function essential to fungi, animals, and plants (Duronio et al., 1989; Ducker et al., 2005; Yang et al., 2005; Pierre et al., 2007). MYR consists of the irreversible addition of a 14-carbon fatty acid, myristate, to an N-terminal Gly residue of a protein via an amide bond (Johnson et al., 1994). The N-Gly is usually unmasked by the N-terminal Met excision (NME) process (Giglione et al., 2004). Both NME and MYR are early cotranslational modifications performed as the first 10 amino acids emerge from the ribosomal tunnel (Wilcox et al., 1987; Glover et al., 1997). Consequently, targeted N-myristoylated (MYRed) proteins show specific amino acids within the first eight to 10 residues of the protein sequence that are specifically recognized by NMT (Pellman et al., 1985b; Towler et al., 1987; Boisson et al., 2003). All NMTs are composed of two domains, one involved in myristoyl-CoA binding and the other for N-terminal protein substrate recognition (Bhatnagar et al., 1998, 1999). However, whereas the nature of the first residue (Gly) is strictly required for MYR, the rules governing the following seven amino acids remain unclear, and the characterization of the subset of MYRed proteins of any proteome remains challenging. In this context, it should be stressed that it is difficult to reliably predict MYR, and actual experimental in vivo data on MYR are scarce and very difficult to obtain directly, even with state-of-the-art mass spectrometry technologies (Meinnel and Giglione, 2008b).
Prediction of MYR was initially based on a rough PROSITE pattern (PD000008). However, more recently developed bioinformatics tools allow more accurate prediction of protein MYR (Maurer-Stroh et al., 2002b; Boisson et al., 2003; Bologna et al., 2004; Podell and Gribskov, 2004). Approaches undertaken to identify the complete MYRed proteome, the so-called N-myristoylome, have suggested that plants might have more MYRed proteins than other kingdoms and that these proteins are substantially enriched at the PM (Maurer-Stroh et al., 2002a; Boisson et al., 2003; Marmagne et al., 2007; Ephritikhine et al., 2008). Using very stringent bioinformatics analysis followed by full genome annotation, a set of >400 independent entries could be early annotated at The Arabidopsis Information Resource (TAIR; http://www.Arabidopsis.org/index.jsp). These stringent data revealed that unexpected MYRed targets (never identified in fungi or animals) exist in the model plant Arabidopsis thaliana. In addition, the NMTs of animals have been proposed to display substrate specificity most similar to those of plants, rather than yeast (Pierre et al., 2007; Martinez et al., 2008).

Biophysical studies have suggested that the binding energy provided by an N-terminal myristate is relatively weak, which suggests a similarity between this modification and C-terminal prenyl groups that have a membrane affinity of $\sim 10^{-4}$ M (Peitzsch and McLaughlin, 1993; Silvius and l’Heureux, 1994; Bhattacharyya and Gordon, 1997). Thus, it is generally assumed that MYR is necessary but not sufficient to induce stable protein–membrane interactions and that more long-lasting membrane association is promoted by an additional anchor. This so-called second signal is often localized in the vicinity of the MYR site of the protein (Cadwallader et al., 1994). In this context, several second signals have been evidenced for many proteins undergoing MYR, including PAL and polybasic tracks (reviewed in Resh, 1999; Wright et al., 2010). At the proteome level, data available in Arabidopsis now associate both MYR and PAL with additional information relative to the function and putative second signal of each respective target. The present myristoylome set (419 entries in TAIR9) reveals that $\sim 37\%$ of MYRed proteins have at least one putative PAL site in the N-terminal decapeptide (Martinez et al., 2008). Although an additional 20% displays a putative polybasic track, which might serve to enhance binding to a membrane, $\sim 40\%$ of the remaining MYRed proteins do not display any further second signal (see http://www.isv.cnrs-gif.fr/tn/maturat/mystorylome2008.html). Two large families of plant-specific putative MYRed and PALed targets have been studied more specifically, including calcium-dependent protein kinases (CDPKs) (Martin and Busconi, 2000; Lu and Hrabak, 2002; Dammann et al., 2003; Leclercq et al., 2005; Benetka et al., 2008) and Calciumin B-like Ca$^{2+}$ binding proteins (CBLS) (Batistic et al., 2008, 2010). In the case of CBLS, the occurrence and roles of the two acylations were clearly assigned. In Arabidopsis, PAL is ensured by 24 palmitoyl S-transferases (PATs), most of them being located at the PM (Batistic, 2012).

Among the most unexpected protein families predicted to undergo MYR in Arabidopsis are the $h$-type thioredoxin protein family ($h$-TRX), which has been identified and shown to be conserved in all plants (Boisson et al., 2003; Traverso et al., 2008). This 10-member family in Arabidopsis is not only intriguing by itself but also because it can be used as a representative sample to study the roles of MYR and PAL in the complete proteome. Interestingly, based on the TermiNator3 predictions, we propose that all $h$-TRXs of subgroup 2 undergo only MYR, whereas all $h$-TRXs of subgroup 3 undergo both MYR and PAL independently of the plant species. Interestingly, no $h$-TRXs displayed any putative polybasic tracks. In addition, because $h$-TRXs have been described as soluble proteins, no specific membrane partners of $h$-TRXs have ever been identified.

Thus, we used plant TRXs as a model protein family to explore the meaning of having a single (MYR) or double acylation (MYR and PAL) in the same family of proteins. First, we used a high-throughput biochemical strategy employing peptide derivatives of the N-terminal sequence of each TRX predicted to be N-MYRed and the At-NMT1 enzyme to assess MYR in vitro. Our data confirm that all expected TRXs are MYRs by At-NMT1 and show that MYR efficiency depends on the peptide (S score value). Moreover, transient expression of TRX–green fluorescent protein (GFP) fusions was performed in plant cells using fragments of different lengths for each TRX and controls, such as the nonmyristoylatable and/or the nonpalmitoylatable versions. This revealed that TRXs displaying only an N-terminal myristoylatable site are primarily localized to the endoplasmic reticulum (ER)/Golgi system and that partitioning between this membrane compartment and the cytosol correlates with the MYR S value calculated for the corresponding peptide in vitro. We also showed that a PAL site adjacent to the MYR site was crucial to localize the protein to the PM. Finally, electron microscopy confirmed PM localization with clustering in specific regions of the PM for the proteins displaying both types of acylation.

RESULTS

Prediction of N-terminal Acylation of h-TRXs Reveals Phylogenetic Conservation of Four Distinct Modification Patterns

By using a combination of predictive software with in vitro assays designed in our laboratory, we predicted previously that >400 Arabidopsis proteins could undergo MYR. This number included certain plant h-TRXs, of which only one had been found to be MYRed in vitro, Arabidopsis TRXh2 (Boisson et al., 2003). We analyzed the h-TRX sequences identified in several plant genomes to uncover possible evolutionary clues about the origin of MYR in the plant h-TRX family. All Arabidopsis h-TRXs obtained from the last version of TAIR were used for alignment by BLAST with four other available complete genomes (Medicago truncatula, rice [Oryza sativa], Populus trichocarpa, and grape [Vitis vinifera]). We identified 10, 12, 7, 10, and 8 unique open reading frames within these complete genomes, respectively. In addition, some classical h-TRXs already characterized in the literature were added to this $\sim 50$ h-TRX sequence corpus (see the accession numbers for all TRX sequences considered). A phylogenetic tree built with those sequences showed that they clustered in four subgroups (Figure 1A). All TRX sequences were next submitted to the online prediction tool TermiNator (http://
Figure 1. The h-TRXs Undergo Predicted N-Terminal Protein Modifications According to Their Phylogenetic Origin.
www.isv.cnrs-gif.fr/terminator3), a program that we designed to efficiently predict several N-terminal modifications, such as NME, N-α-acetylation (NAC), MYR, and PAL (Frottn et al., 2006; Martinez et al., 2008). The result (Figure 1B) reveals that virtually all tested plant h-TRXs are predicted to undergo NME, the first irreversible proteolytic modification affecting >80% of plant proteins (Bienvenut et al., 2012). Surprisingly, we found a modification pattern associated with the subgrouping of h-TRXs that was not limited to NME but extended to N-terminal acylations, (e.g., MYR, NAC, and PAL). In-depth analysis demonstrated that all h-TRXs from subgroups 2 and 3 (red and green boxes in Figure 1) are characterized by the presence of an N-terminal extension (Figure 1B).

All h-TRXs of both subgroups showed a conserved Gly residue in position 2 (Figure 1B; see Supplement Figure 1 online), a feature indispensable for MYR. Subgroup 2 h-TRXs (red box in Figure 1) were predicted to undergo MYR only (Figure 1B). By contrast, subgroup 3 h-TRXs (green box in Figure 1) were predicted to undergo double acylation (MYR + PAL) of their N-terminal extensions. Indeed, all h-TRXs of subgroup 3 possess not only Gly-2, but also a conserved Cys residue (Figure 1B) predicted by both TermiNator and CSS Palm software (http://csspalm.biocuckoo.org online/php). Unlike h-TRXs from subgroups 2 and 3, all h-TRXs of subgroup 1 (yellow box in Figures 1A and 1B) were predicted to undergo NAC, one of the most common protein modifications (Martinez et al., 2008; Bienvenut et al., 2012). Interestingly, all h-TRXs from subgroup 1 showed a highly conserved Ala residue in position 2. This suggests that NαA, one of the N-acetyl transferases responsible for NAC, acts after NME (Frottn et al., 2006) to acylate h-TRXs from subgroup 1 (Figure 1B). Finally, subgroup 4 h-TRXs (Figure 1B) were predicted to be mainly N-terminal α-acetylated (NACed) and never acylated (MYRed or PALed).

Moreover, a majority of these proteins was predicted to retain the initial Met residue, suggesting that N-terminal acetylation is catalyzed by NatB or NatC complexes and not NatA, as is the case for subgroup 1 h-TRXs. Interestingly, the TRXs of subgroup 4 do not contain a clear N-terminal extension and consistently present an alternative monothiol active center (WCX5). Chlamydomonas reinhardtii was included in our predictive study because Gly-2 occurs in both of the h-TRXs in its genome; however, these open reading frames are predicted to be neither MYRed nor NACed (Figure 1B).

Our extensive analysis of the N-terminal sequences of all plant h-TRXs allowed us to propose that modification of the N terminus is conserved in each subgroup across species. It is noteworthy that the lipidations predicted in the TRX family are exclusively found within plant h-TRXs. No other TRXs from plants or other organisms were predicted to be MYRed because these sequences do not possess the indispensable Gly-2 residue. The amino acid sequence at the N termini of h-TRXs substantially varies, unlike the type of modification itself (Figure 1B), which indicates strong selective pressure caused by the crucial physiological role of the modification in plant h-TRXs.

Subgroups 2 and 3 h-TRXs Are N-MYRed in Vitro

TermiNator3 is a very accurate predictor of NME and NAC (Bienvenut et al., 2012), but its reliability remains limited for MYR (Martinez et al., 2008). In order to confirm our predictions about possible MYR of h-TRXs, we measured the capacity of the N-terminal octapeptides to undergo MYR in vitro in a reconstituted MYR assay (Boisson and Meinne, 2003; Traverso et al., 2013). This assay has been described to more efficiently test N-MYR using peptides than using entire proteins, most likely because this modification occurs very early during protein biosynthesis, immediately after NME (Boisson et al., 2003). For each peptide, the catalytic efficiency of the NMT enzyme (kcat/Km) was determined. This value (the S score) reflects the probability of MYR for each protein (Traverso et al., 2013). When S = 0, the sequence cannot be modified in vitro or in vivo, whereas when S > 0, the corresponding sequence is MYRed in vitro and most likely in vivo also. The higher the S value, the more likely MYR occurs in vivo.

We measured the S values for each of the peptides derived from the Arabidopsis h-TRXs predicted to be MYRed. As negative

Figure 1. (continued)

(A) All TRXs studied here are grouped in four evolutionary clusters. The phylogenetic tree uses all available h-TRXs from the five sequenced genomes included in this work: Arabidopsis (in bold, At1, At13g51030; At2, At5g39950; At3, At5g42980; At4, At1g19730; At5, At1g45145; At7, At1g59730; At8, At1g9880; At9, At3g08710; At10, At3g56420; At-CS1, At1g11530; At-CS2, At2g40790; M. truncatula, Mt1g058470; Mt2, Medtr7g038930; Mt3, Medtr5g038960; MtG, Medtr1g038910; MtH, Medtr8g005420/Medtr8g005260; MtL, Medtr8g116230; MtJ, Medtr2g010800; MtK, Medtr4g111230; MtL, Medtr2g096730, rice (OsA, Os07g08840; OsB, Os03g58630; OsC, Os07g09310; OsD, Os05g07690; OsE, Os05g41090; OsF, Os01g7376; OsG, Os04g35740). P. trichocarpa (Pla, Ppo181765; PtB, Ppo1710146; Ptc, Ppo191472; PtD, Ppo258763; PtE, Ppo420455; Pff, Ppo285426; PtG, Ppo192144; PtH, Ppo166332; PtI, Ppo164777; PtJ, Ppo177798), and grape (VwA, Vw04g12270; VwB, Vw18g11650; VwC, Vw14g15400; VwD, Vw01g09260; VwE, Vw08g05890; VwF, Vw00g27000; VwG, Vw00g25955; VwH, Vw19g05210). Other characterized h-TRXs are included here: Psb, Ps-TRXh4 (AY170651), from pea; Gmh, Gm-TRXh1 (A575954), from soybean; Nah, Na-TRXh (AA42864), from N. alata; Pch, Ps-TRXh (AF159388), from Phalaris coerulescens; and Cr1, Con-TRXh1 (CA56850), and Cr2, Con-TRXh2 (AA022058), from C. reinhardtii. Sequences from some genomes are denoted with letters (A, B, C, etc.) to avoid confusion. The phylogenetic tree was constructed using the Phylogeny.fr platform (www.phylogeny.fr). The cladogram is fully representative of the calculated phylogram. Branch support values are not displayed.

(B) N-terminal sequences (30 residues) of all h-type TRXs used to create the phylogenetic tree are shown, and critical positions are highlighted (Ala-2 in green, Gly-2 in blue, and Cys in red). TermiNator predicts that the highly conserved Ala-2 in subgroup 1 (yellow box) is α-acetylated. The conserved Gly-2 in the h-TRXs of subgroup 2 (red box) or 3 (green box) is predicted to be MYRed. Most of the TRXs within subgroup 3 are predicted to be double-acetylated (MYR + PAL).
controls, we used versions of the peptides with the G2A mutation because it is well known that the lack of a terminal Gly completely abolishes N-MYR (Meinelle and Giglione, 2008b). We also used a peptide derived from a well-characterized non-MYRed protein from Arabidopsis that possesses the critical Gly-2 residue (EXP5). As positive controls, we used several peptides derived from Arabidopsis proteins previously shown to be MYRed (SOS3, ARA6, and CDPK2) (Boisson et al., 2003). The data in Table 1 show that all peptides derived from the Arabidopsis TRXs were efficiently MYRed in vitro. By contrast, none of the corresponding G2A peptides were MYRed in vitro. Several peptides derived from h-TRXs from different plants predicted to be MYRed by our prediction tool (Poptr-825426, Gm-TRXh1, Na-TRXh, Ps-TRXh4, and Pc-TRXh) were also assayed. In line with the predictions, all these peptides were efficiently MYRed in vitro. Finally, in vitro analysis of one of the two peptides derived from h-TRXs from the algae C. reinhardtii (Cr-TRXh1) confirmed that they are not substrates of Arabidopsis NMT (Table 1).

Collectively, these data indicate that all peptides from subgroup 2 and 3 h-TRXs clearly undergo MYR in vitro. The efficiency of MYR depended on the peptide, with MYR S scores in the range of 0.6 to 4.7 (global range is 0.1 to 60). Together with the accuracy of predicting NME and NAC in plants, this study suggests that the tight correlation observed between the four subgroups and the modification is robust and relevant to the physiological role of each h-TRX type.

**h-TRXs of Subgroup 2 Are Specifically Localized to the ER/Golgi Membrane System**

Plant h-TRXs have long been considered soluble proteins due to the lack of transit peptides (Florencio et al., 1988). In the case of the subgroup 2 members, only a couple of localization experiments have been reported. These include Arabidopsis CXXS2 (Serrato et al., 2008) and P. trichocarpa TRXh2 (Gelhaye et al., 2004), which were described as soluble and mitochondrial proteins, respectively (see Discussion). Because we demonstrated that all peptides derived from the N termini of TRXs of subgroup 2 can be MYRed and N-MYR usually promotes membrane interactions by enhancing the hydrophobicity of the protein, we questioned whether MYRed Arabidopsis TRXs of subgroup 2 exhibit a specific membrane compartment distribution.

Full-length TRXs h2, h7, and h8 were fused to the N terminus of GFP (Figure 2), and the associated steady state subcellular localization was examined by confocal laser scanning microscopy in epidermal onion (Allium cepa) cells. GFP alone is a soluble protein localized in the cytoplasm and nucleus (Giglione et al., 2000; Nelson et al., 2007). Using confocal laser scanning microscopy, the cytosolic fraction of epidermal cells is mainly seen as a cortical fraction near the PM (indicated by C in Figure 2, panels V to Y) due to the presence of a fully expanded cell vacuole. In addition, the GFP signal can also be observed in the transvacuolar strands (indicated as T), which represent cytoplasmic tunnels that traverse the lumen of the continuous vacuole that are perpetually remodeled (Nelson et al., 2007).

All full-length Arabidopsis TRX-GFP fusion proteins assayed showed very different subcellular localization patterns compared with that of GFP alone. TRXh2-GFP displayed a pattern consisting of numerous 1-µm spots (white arrows in Figures 2A and 2B). These spots were merged in a weak three-dimensional network along the cortical cytoplasm that usually forms a ring structure around the nucleus (red arrows in Figures 2A and 2B). Similar results were found with other TRXs from subgroup 2: Arabidopsis TRXh7 (Figures 2E and 2F) and TRXh8 (Figures 2I and 2J). Both proteins were localized as spots (white arrows in Figures 2F and 2J) around the nucleus (red arrows in Figures 2E, 2F, 2I, and 2J). This specific subcellular localization of all TRXs from subgroup 2 was strictly dependent on the capacity of the protein to be MYRed, as all G2A substitutions in the corresponding TRX induced relocation of the GFP to the cytosol with a pattern most similar to that obtained with GFP alone (Figures 2C, 2D, 2G, 2H, 2K, and 2L). These data suggest that the MYR site on all h-TRXs from subgroup 2 was necessary to target the protein to these specific subcellular localization sites.

The morphology of the spots in which all subgroup 2 h-TRXs were targeted strongly suggested targeting to the endomembrane compartment. Indeed, coexpression of each TRX-GFP (Figure 3, column 1) with the Golgi and ER marker protein MAN1 from soybean Glycine max (Brandizzi et al., 2002; Saint-Jore-Dupas et al., 2006; Nelson et al., 2007), encoding the resident Golgi protein α-1,2-mannosidase I, fused with the fluorescent protein mCherry (Figure 3, column 2), showed clear colocalization of GFP and mCherry fusion proteins for all constructs (Figure 3, Merged).

Next, we questioned whether the N-terminal monolipidated protein sequences of all tested h-TRXs of subgroup 2 were not only necessary but also sufficient to target the protein to the ER/Golgi compartment. Thus, we fused to the N terminus of GFP several fragments of different lengths for each N-terminal extension. We then tested their ability to target GFP to the specific compartments observed with the full-length TRX versions (see Supplemental Figure 2 online). This progressive truncation analysis of the C terminus of subgroup 2 h-TRXs revealed that the first 10 amino acids from each TRX were enough to target GFP to Golgi apparatus in the same way as the full-length proteins (ph2, ph7, and ph8 in Figure 3). This indicates that the 10 N-terminal amino acids alone determine in vivo targeting of h-TRXs, fully mimicking the full-length protein steady state location. It is noteworthy that this short 10-residue stretch contains both NME and MYR recognition patterns, which respectively encompass residues 1 to 3 and 2 to 9. Both modifications are required to promote MYR in vivo.

We next studied the cellular localization of several variants with point mutations at each of the first 10 residues of Arabidopsis TRXh2 fused to GFP. We ensured that these variants were still predicted to be N-MYRed. All variants were clearly enriched in the endomembrane system (see Supplemental Figure 2 online). We also altered several positions of other Arabidopsis subgroup 2 h-TRXs with potential roles in localization. These included the negative charges displayed by TRXh7 at Asp-10 or TRXh2 at Ser-6, which is highly conserved in almost all subgroup 2 h-TRXs (Figure 1). Electrostatic interactions have been shown to be effective in supporting membrane links of MYRed proteins (Meinelle and Giglione, 2008a), and Ser residues can be involved in phosphorylation/dephosphorylation
events that could also be indirectly involved in membrane bonds (McLaughlin and Aderem, 1995). Substitutions of Ser-6 of TRX$_{h2}$ and Asp-10 of TRX$_{h7}$, both in the context of the first 10 residues and the full-length GFP fusion proteins, were analyzed by confocal microscopy. The localization patterns were identical to those obtained with the respective wild-type forms (see Supplemental Figure 3 online).

Together, the above data unambiguously indicate that MYR alone is responsible for the specific ER/Golgi localization of subgroup 2 h-TRXs and that this effect is unrelated to the sequence itself but rather to its unique capacity to undergo MYR in vivo. This explains why the first 10 amino acids are necessary and sufficient to induce proper localization, provided they are MYRed.

### Partitioning of Monolipidated Substrates for MYR between the ER/Golgi Compartment and the Cytoplasm Strongly Correlates with MYR Efficiency

Whereas primarily ER/Golgi compartment association has been detected for all h-TRXs of subgroup 2, Arabidopsis TRXh2-GFP, TRXh7-GFP, and TRXh8-GFP yielded additionally a measurable soluble fraction which varied from one fusion to another. The extent of the distribution of the protein between the ER/Golgi

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<th>Peptides (Entry)</th>
<th>N-Terminal Sequences</th>
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<td>0.42 ± 0.03</td>
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<tr>
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<td>ANHSTRIP</td>
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<table>
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<th>Plant proteins from the Arabidopsis myristoylome</th>
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<td>ARFC1 (AT3g22950)</td>
<td>GAFMSRFW</td>
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<td>2 ± 0.4</td>
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<tr>
<td>F2KP (AT1g07110)</td>
<td>GSGASKNT</td>
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<tr>
<td>RPM5-like (AT1g62630)</td>
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<td>12 ± 2</td>
<td>0.149 ± 0.02</td>
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<td>DEM1 (AT4g33400)</td>
<td>GASHSHED</td>
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<tr>
<td>DEM2 (AT3g19240)</td>
<td>GTSQSRED</td>
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<td>180 ± 32</td>
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<tr>
<td>RPT2a (AT4g29040)</td>
<td>GGOGPSGGL</td>
<td>0.75</td>
<td>&gt;200</td>
<td>&gt;0.15</td>
</tr>
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</table>

Kinetic parameters obtained from the in vitro assay of N-MYR by NMT1. n.m., nonmeasurable, which indicates a complete absence of signal even at the highest NMT concentration used.

<table>
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<th>Peptides (Entry)</th>
<th>N-Terminal Sequences</th>
<th>$k_{cat}/K_m$ ($10^3 \text{ M}^{-1} \text{s}^{-1}$)</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
</tr>
</thead>
</table>

*Annotation associated with each protein was retrieved from several databases (see Methods).

*N-terminal octapeptides (from amino acids 2 to 9) were derived from proteins of Arabidopsis, P. trichocarpa (Pt-TRXh4), soybean (Gm-TRXh1), N. alata (Na-TRXh), pea (Ps-TRXh4), and P. coerulescens (Pc-TRXh). Arabidopsis proteins SOS3, Ara6, and CDPK2 were used as positive controls because they are well recognized as MYRed proteins. The G2A substitution in any peptide prevents the MYR reaction and was used as negative control, along with the EXP5 peptide.

The $k_{cat}/K_m$ value reflects the catalytic efficiency of each peptide to be MYRed by NMT enzyme.
compartment and the cytosol was independent of the length of the fusion (see for comparison Figure 3). This strongly suggested that the partitioning was only related to the MYR signal. Quantitative ratiometric analysis of the accumulation at the endomembrane system over the cytosol of the different TRX-GFP fusion proteins clearly showed that the partitioning between the two compartments depended only on the nature of the N-terminal sequence of the subgroup 2 h-TRX analyzed (Figure 3). This analysis showed that all constructs predominantly targeted GFP to the surface of the ER/Golgi apparatus. Nevertheless, an increase of fluorescence was detected in the cytosol when we used the N terminus of Arabidopsis TRXh7 (32%) or TRXh8 (28%) compared with TRXh2 (14%), in parallel with a concomitant decrease of fluorescence in the ER/Golgi compartment: TRXh7 (68%) or TRXh8 (72%) compared with TRXh2 (86%). Immunoblot analysis of membrane and cytosolic protein fractions of the above transfected cells confirmed that all subgroup 2 h-TRXs predominantly retargeted GFP to the membrane compartment with a distribution strongly correlating the in vivo results (Figure 4).

Intriguingly, this different partitioning of the GFP between the ER/Golgi compartment and the cytoplasm strongly correlates with the in vitro S score for MYR of each h-TRX (Figure 5). A proportionally higher S value, such as that found for Arabidopsis TRXh2, was associated with augmented fluorescence at the ER/Golgi compartment, whereas the low in vitro S value of TRXh7 and TRXh8 was associated with a more balanced fluorescence distribution between the ER/Golgi compartment and the cytosol.

This tight relationship between subcellular protein partitioning and the efficiency of NMT1 to myristoylate the h-TRXs of subgroup 2 led us to question whether this could be generalized to other MYRed substrates unrelated to TRXs. In order to investigate this issue, we selected a set of six proteins unrelated to redoxins, such as TRXs, from the Arabidopsis N-myristoylome (ADP-ribosylation factor C1 [ARFC1], Fru-2,6-bisphosphatase [F2KP], defective embryo meristem protein1 [DEM1], DEM2, 26S proteasome regulatory subunit 4-A [RPT2a], CC-NBS-LRR class disease resistance protein [RPS5-like]). We were careful to select MYRed proteins that did not display any evident second signal, including palmitoylation sites in the vicinity of the MYR site, similar to subgroup 2 TRXs. We confirmed that all six octapeptides derived from the N termini of the proteins were MYRed in vitro, with efficiencies (S values) depending on the peptide. S values ranged from 0.75 to 56 (Table 1, Figure 6). When fused to GFP and expressed in epidermal cells, the six fusion proteins conferred steady state ER/Golgi compartment...
localization of GFP (Figure 6). As already observed with the TRxs of subgroup 2, the degree of ER/Golgi compartment enrichment was proportional to the efficiency of the NMT enzyme to myristoylate the given peptide (Figure 6).

We concluded that this kinetic trapping of a MYRed sequence is an essential aspect of the spatial organization of MYRed substrates devoid of any apparent second signal in the N-myristoylome of Arabidopsis.

Fluorescence Recovery After Photobleaching Experiments on ER/Golgi Compartment-Bound h-TRXs of Subgroup 2 Reveal Their Different Kinetic Recovery

We used fluorescence recovery after photobleaching (FRAP) experiments on cells transfected with subgroup 2 h-TRXs to investigate, quantify, and challenge protein mobility and exchange between free cytosolic and membrane-bound TRX protein pools in living cells. First, to determine whether the cytosolic and ER/Golgi membrane pools of h-TRXs of subgroup 2 are in dynamic equilibrium, we performed in vivo fluorochrome photobleaching on spherical Golgi stacks (transfected with ph2-GFP or ph7-GFP or ph8-GFP) treated for 30 min prior to imaging with 25 µM latrunculin B to disrupt Golgi movement (Brandizzi et al., 2002). We monitored the recovery of fluorescence into the circular region of interest (ROI) as a function of time by scanning the specimen at low laser intensity (see Supplemental Figure 4 online). The addition of latrunculin B changed neither the localization nor the partitioning of our constructs. Using a nonlinear curve-fitting approach (Martinière et al., 2012), we observed significant differences between constructs in the recovery kinetics recorded during 60 to 120 s at ~1 Hz. Extremely rapid initial recovery (within 1 s) after photobleaching is observed in the cytoplasm due to fast diffusion and cytoplasmic streaming (see Supplemental Figure 5 online). However, following photobleaching of Golgi-associated signal, we observed only a gradual

Figure 3. Full-Length Subgroup 2 h-TRXs Are Localized at the Endomembrane and the N-Terminal Decapeptides Contain Complete Information for Specific Localization.

Confocal scanning microscopy images of onion cells co-transfected with both full-length h-TRX-GFP (TRXh2-GFP, TRXh7-GFP, TRXh8-GFP) or peptides containing the first 10 amino acids of each Arabidopsis h-TRX fused to GFP (ph2-GFP from TRXh2; ph7-GFP from TRXh7; ph8-GFP from TRXh8) or control GFP alone and an mCherry-labeled Golgi marker (Glycine max MAN1, (Nelson et al., 2007)). TRX- or peptide-GFPs are visualized in green, m-Cherry is visualized in red, and co-localization is visualized in yellow (merged column). Scale bars = 15 µm.

Figure 4. Fractionation of TRX-GFP Transfected Cells into Membrane and Cyttoplasmic Constituents Confirmed the Distribution Observed in Vivo of the Different h-TRXs of Subgroup 2.

Transfected cells with peptides containing the first 10 amino acids of each Arabidopsis h-TRX fused to GFP (ph2-GFP from TRXh2, ph7-GFP from TRXh7, and ph8-GFP from TRXh8) or control GFP previously checked by confocal scanning microscopy were employed for subcellular fractionation followed by immunoblot analysis using anti-GFP or anti-H+ATPase antibodies. Representative immunoblots are shown. M, membrane constituent; S, cytoplasmic constituent.
and continuous fluorescence recovery in cells transfected with TRX-h2-GFP (Figure 7A). Quantitative analysis of several independent FRAP experiments (n = 44) revealed an average halftime (t_{1/2}) of 6.5 ± 1.2 s for the cytosolic TRX-h2-GFP to restore the membrane bleached area to a relatively high level (I_{t=0} = 72% ± 1%, r² = 0.99; Figure 7A). FRAP curves showed a two-phase-type recovery after bleaching resembling a diffusion-uncoupled behavior (Sprague and McNally, 2005). This diffusion-uncoupled FRAP recovery curve consists of a small fast recovery attributed to peripheral diffusion and a medium/slow recovery due to exchange at binding sites (Figure 7A). These data show that in 75 s, 72% of the initial level of h-TRXs of subgroup 2 can be restored to Golgi by traffic from cytosol, suggesting that most of the cytosolic TRX-h2 corresponds to MYRed protein forms and that the corresponding cytosolic and membrane pools are in dynamic equilibrium as previously shown for other MYRed proteins (Zhao et al., 2006). By contrast, recovery kinetics from FRAP experiments involving Arabidopsis TRXh7-GFP and TRXh8-GFP resembled one another but were different to those for TRXh2. The higher initial level of fluorescence occurring in the cytoplasm of cells transfected with TRXh7-GFP and TRXh8-GFP (see Supplemental Figure 4 online) gave rise to an apparent three-phase recovery process in which an extremely rapid initial recovery (within 1 s), most of which was due to cytoplasmic streaming (see Supplemental Figure 5 online), is followed by a still rapid recovery and then a plateau at only 64% for TRXh8-GFP and 54% for TRXh7-GFP of the initial Golgi fluorescence (Figures 7B and 7C).

The mobile fraction for both TRXh7-GFP and TRXh8-GFP was slightly but significantly reduced compared with TRXh2-GFP (the latter, 72%; Figure 7). This reduced protein mobility for TRXh7-GFP and TRXh8-GFP indicates that the cytosolic components of these two proteins were less efficiently targeted to the membrane compartment compared with TRXh2-GFP. Nevertheless, the high recovery at the bleached membrane for all h-TRXs of subgroup 2 suggests that the cytosolic pool of these proteins is composed mostly of MYRed proteins able to relocate to the ER/Golgi membrane thanks to the MYRed portion and a variable amount of non-MYRed counterpart unable to bind the membrane. To verify this hypothesis, we overexpressed Arabidopsis NMT1 in cells transfected with TRXh7-GFP or TRXh8-GFP. A reduction of fluorescence was observed at the level of the cytosol with a relative ER-Golgi/cytosol partitioning being increased and becoming more similar to that observed with TRXh2 (see Supplemental Figure 6 online). This is in agreement with a small proportion of the NMT targets being under-modified or incorrectly modified in vivo as a result of too low MYR catalytic efficiency.

Together, our results strongly suggest that the cytosolic fraction of TRXh7-GFP and TRXh8-GFP consists of both (1) a nondynamic pool of non-MYRed forms of these proteins, resulting from their poor MYR catalytic efficiency by NMT and (2) a significant amount of MYRed proteins being fully available for dynamic exchange with the membrane compartment. This later observation is fully in keeping with the low binding constant of the solely MYR proteins to a membrane compartment as measured previously in liposomes (see references in the Introduction).

Subgroup 3 h-TRXs Displaying N-Terminal Double Acylation Are Specifically Localized at the PM with Lateral Diffusion Features of Both MYRed and PALed Proteins

As reported above, subgroup 3 h-TRXs are not only in vitro MYRed (Table 1) but also predicted to be PALed due to the presence of conserved Cys residues in their N-terminal extensions (see predictions in Figure 1B and Supplemental Figure 1 online). The localization of both subgroup 3 h-TRXs was analyzed by confocal microscopy (TRXh9 and CXXS2; Figure 8). The fluorescence pattern was completely different from subgroup 2 h-TRXs. The localization of both TRXh9 and CXXS2 clearly showed strong enrichment at the PM (Figure 8A). Interestingly, TRXh9 was also found at plasmodesmata (Figure 8A), which was never found with CXXS2 or other TRXs. Even if the majority of TRXh9 was localized to the PM, reduced fractions of TRXh9 (15% ± 10% of the GFP fluorescence) or CXXS2 (<10% of the signal) were also located in spots (white arrows in Figure 8B) similar to those observed with subgroup 2 h-TRXs. Indeed, coexpression experiments with both TRXh9 and the mCherry-labeled Golgi marker MAN1 showed colocalization at the level of the ER/Golgi compartment membrane (Figure 8B). This enrichment likely occurred due to anterograde transport of PALed TRXh9 via the secretory pathway, as recently shown for several double-acetylated proteins in mammals (Rocks et al., 2010). Impeding MYR in both h-TRXs of subgroup 3 with the G2A mutation restored the diffuse cytosolic and nuclear distribution observed with GFP alone (Figure 8A), indicating that MYR is a prerequisite for the subcellular localization.

To assess whether the N-terminal extensions containing both MYR and PAL sites induced specific subcellular localization of these TRXs at the PM, we fused C-terminally truncated versions of TRXh9 and AtCXXS2 to the N terminus of GFP and we tested their ability to target GFP to the PM (see Supplemental Figure 2 online). As observed for the h-TRXs of subgroup 2, the first N-terminal amino acids alone faithfully mimicked full-length protein.

Figure 5. Different Partitioning of h-TRXs of Subgroup 2 between the ER/Golgi Compartment and the Cytosol Strongly Correlates with the Capacity of Each Peptide to be MYRed by the NMT Enzyme.

Quantitative ratiometric analysis of TRX-GFP accumulation at the Golgi/ER endomembrane compared with cytosolic accumulation (FI, fluorescence intensity; a.u., arbitrary units) is reported for each construct on the y axis (white columns) corresponds to S values.
localization (Figure 8C). The first 10 amino acids of TRX\(_h\)\(_9\) (p\(_h\)\(_9\)) targeted GFP mainly to the PM (Figure 8C). In the case of CXXS2, three Cys residues are present at positions 5, 10, and 11 and all are predicted to undergo PAL (see below). The study of different N-terminal fragments fused to GFP reveals that only the first 11 residues gave the same PM localization of the full-length protein (Figure 8C).

Cotransfection of TRX\(_h\)\(_9\)-GFP or CXXS2-GFP with an mCherry-labeled PM marker, based on the full-length coding region of PIP2A, a PM aquaporin (Cutler et al., 2000), confirmed that TRX\(_h\)\(_9\)-GFP and CXXS2-GFP were mainly localized to the PM (see Supplemental Figure 7 online). In agreement with the above data, immunogold labeling and transmission electron microscopy on ultrathin sections (80 nm) of epidermal onion cells transformed with \(p\)\(_h\)\(_9\)-GFP confirmed the presence of the protein at the PM (Figure 9). Interestingly, the gold particles were predominantly found in distinct patched areas regularly spaced in 107 ± 50-nm paths. This accumulation of the protein in defined sites of the PM (Figure 9) may reflect enrichment of the MYRed and PALed TRX\(_h\)\(_9\) in functional microdomains.

Finally, FRAP experiments on small regions of PM of cells transfected with \(p\)\(_h\)\(_9\)-GFP or pCXXS2-GFP showed a relatively high mobility of these proteins (see Supplemental Figure 8 online) with FRAP curves comparable to those recently reported for PM-targeted MYRed and PALed proteins, such as GPA1 (Martinière et al., 2012).

**Table 1.**

<table>
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<tr>
<th>Protein</th>
<th>k(_{cat})/K(_m) ((10^2 \text{M}^{-1} \text{s}^{-1}))</th>
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**Figure 6.** Partitioning of Randomly Chosen Monolipidated Substrates for MYR between the Endomembrane Compartment and the Cytoplasm Strongly Correlates with the MYR S Value.

Confocal microscopy images of transient expression in onion cells of peptide-GFP. Peptides contain the first 10 amino acids of the following MYRed proteins from the *Arabidopsis* myristoylome: \(p\)\(_h\)\(_2\) from TRX\(_h\)\(_2\) (control), ARFC1 (At3g22950), F2KP (At1g07110), RPS5-like (At1g62630), DEM1 (At4g33400), DEM2 (At4g19240), and RPT2a (At4g29040). Each construct was coexpressed with the mCherry-labeled Golgi marker soybean MAN1 (Nelson et al., 2007). Peptide-GFP is visualized in green, mCherry is visualized in red, and colocalization is visualized in yellow (Merged). Bars = 10 μm.

**N-Terminal S-Modified Cys Residues Are Critical in Targeting MYRed Subgroup 3 h-TRXs to the PM**

All subgroup 3 h-TRXs show conserved Cys residues close to the MYRed Gly and are predicted to be PALed by both Terminator and CSS-Palm (see Supplemental Figure 1 online). We recently established that >37% of the N-myristoylome shows conserved PALed Cys residues close to the myristoylatable Gly (Martinez et al., 2008). PAL is generally an N-MYR–dependent acylation usually described as a reversible second signal in several MYRed proteins targeting the protein specifically to the PM (Resh, 1996; Dunphy and Linder, 1998). TRX\(_h\)\(_9\) is a mono-PALed protein showing a highly conserved Cys residue at position 4 (Figure 1), next to the myristoylatable Gly. CXXS2, together with TRX\(_h\)\(_9\), displays a more complex pattern because of the occurrence of seven Cys residues predicted as PALed in the N-terminal extension.

The Cys-to-Ser substitution is known to abolish PAL; this modification only changes the sulfur atom of Cys into oxygen, abolishing PAL reactivity. To investigate whether the aforementioned Cys residues corresponded to the second signals inducing PM localization of subgroup 3 proteins, we studied Cys-4 to Ser-4 substitution in the context of both the full-length and the 10-residue version of TRX\(_h\)\(_9\) fused to GFP (\(p\)\(_h\)\(_9\)C4S or TRX\(_h\)\(_9\)C4S; Figure 10A). PM trapping was blocked by replacement of the target Cys with Ser (Figure 10A). Remarkably,
both TRX9C4S and ph9C4S mutants exhibited an enrichment in ER/Golgi structures, a pattern identical to that observed with TRXh2 (Figure 10B). Interestingly, MYR of the octapeptide variant ph9C4S was confirmed using the Teminiator software and in vitro studies (Table 1) revealing an S value slightly increased compared to the wild-type version (1.9 \times 10^3 \text{M}^{-1} \text{s}^{-1} versus 3 \times 10^3 \text{M}^{-1} \text{s}^{-1}). Both single (G2A) or double variants (G2A and C4S) of the fusions showed only soluble fluorescence signal (Figure 10C), suggesting the idea that MYR was necessary but not sufficient to induce PM localization. Similar results were observed with the mutated full-length protein.

To investigate the requirement of multiple N-terminal palmitoylatable Cys residues close to the myristoylatable site of CXXS2, we substituted the different Cys residues at positions 5, 10, and 11, making single or triple substitutions (Figure 10D). The C5S mutant showed reduced PM localization and the appearance of a Golgi/ER/cytoplasm distribution (Figure 10D). By contrast, the triple variant C5SC10SC11S showed no PM labeling and a distribution of the fluorescence between the ER/Golgi compartment and the cytoplasm, similar to that of non-PALed MYRed proteins featuring low S values (Figures 10B and 10D). The increase of non-PALed CXXS2 in the cytosol is in agreement with the reduced S value observed upon C5S substitution in the peptide compared with the wild-type version (Table 1). The fluorescence distribution induced by the double substitutions (G2A-CSS) did not show any preference for a particular membrane compartment (Figure 10C), pointing out the critical importance of these two residues in protein localization.

Previous studies have demonstrated that the reducing agent DTT mimics the effect of depalmitoylation, in which the palmitate is reversibly removed by dedicated enzymes. Addition of DTT to protein extracts induces palmitate release from PALed proteins. This induces a faster migrating protein species in highly resolving SDS-PAGE runs (Fukata et al., 2004; Batistic et al., 2008). To confirm S-acylation of subgroup 3 h-TRXs, we analyzed the protein extracts corresponding to the cells used for confocal microscopy, treated with 200 mM DTT or a control (10 mM DTT) prior to separating the proteins by high-resolution SDS-PAGE (Figure 11A). As previously reported for other MYRed and PALed proteins, we observed a partial shift for both ph9-GFP and pCXXS2-GFP treated with 200 mM DTT relative to that of GFP. In contrast with ph9-GFP, ph9C4S-GFP from M or S fractions did not display any difference of its mobility after DTT treatment. This suggests that the Cys-to-Ser substituted version of the protein is no longer acylated unlike the wild-type version (Figure 11A).

Addition of DTT to living material was already shown to remove the S-linked palmitates from PALed proteins in living cells and to result in loss of raft phase association (Tu et al., 1997; Levental et al., 2010). Similar to the effect of the Cys-4 to Ser substitution in ph9-GFP and Cys-5-, Cys-10-, and Cys-11-to-Ser substitutions in pCXXS2-GFP, we observed that in vivo DTT treatment induced immediate translocation of ph9-GFP or pCXXS2-GFP fusion proteins from the PM to the ER/Golgi compartment (Figure 11B). No alteration of protein localization pattern was observed when DTT was used on cells expressing non-PALed GFP versions, including (1) the Cys-substituted

Figure 7. Photobleaching the ER/Golgi Membrane-Bound TRXs Reveals Idiosyncratic Kinetics for Each Subgroup 2 h-TRX.

(A) Quantification of the FRAP experiments for ph2-GFP showing a two-phase type of gradual and continuous recovery during the postbleaching period with an ER/Golgi ph2-GFP recovery to relatively high level. The curve was obtained by fitting FRAP data to a double exponential corresponding to the equation reported inside the graph.

(B) Quantification of the FRAP experiments for ph8-GFP showing a three-phase recovery process in which an extremely rapid initial recovery (within 1 s) is followed by a still rapid recovery and then a plateau without recovery toward the initial level of fluorescence. The curve was obtained by fitting FRAP data to a single exponential corresponding to the equation reported inside the graph.

(C) Quantification of the FRAP experiments for ph7-GFP showing a three-phase recovery process in which an extremely rapid initial recovery (within 1 s) is followed by a still rapid recovery and then a plateau without recovery toward the initial level of fluorescence. The curve was obtained by fitting FRAP data to a single exponential corresponding to the equation reported inside the graph.

\[ f(t) = a + b(1 - e^{-ct}) \]

where:
- \( f(t) \) is the normalized fluorescence intensity at time \( t \),
- \( a \) is the fraction of protein able to relocalize within the bleached area during 60-s postbleaching,
- \( b \) is the fraction of protein able to recover within 1 s,
- \( c \) is the relative recovery rate constant,
- \( t \) is the relative time required for the fluorescence intensity to reach 50% of the maximum recovery fluorescence,
- \( D \) is the relative diffusion coefficient.

The curve was obtained by fitting FRAP data to a single exponential corresponding to the equation reported inside the graph.

\[ f(t) = a + b(1 - e^{-ct}) \]

where:
- \( f(t) \) is the normalized fluorescence intensity at time \( t \),
- \( a \) is the fraction of protein able to relocalize within the bleached area during 60-s postbleaching,
- \( b \) is the fraction of protein able to recover within 1 s,
- \( c \) is the relative recovery rate constant,
- \( t \) is the relative time required for the fluorescence intensity to reach 50% of the maximum recovery fluorescence,
- \( D \) is the relative diffusion coefficient.
versions of TRXh9-GFP, CXXS2-GFP (Figure 11C), and the various subgroup 2 h-TRXs, (2) the Golgi marker, and (3) GFP alone (see Supplemental Figure 9 online). This confirms that DTT only has a specific in vivo effect on the localization of palmi-toylatable GFP fusions.

Our results are in strong agreement with very recent published data showing by an independent targeted proteomic approach that TRXh9 indeed belongs to the pool of identified PALed proteins of Arabidopsis (Hemsley et al., 2013).

DISCUSSION
The N terminus is frequently modified and is known to regulate a number of crucial cellular processes (reviewed in Rusch and Kendall, 1995; Schatz and Dobberstein, 1996; Varshavsky, 2003; Meinnel and Giglione, 2008b; Arnesen, 2011). Several main functions are attributed to N-terminal protein modifications, such as regulation of protein activity, recognition by other molecules or degradation machinery, anchoring to membranes, integration into a specific signal transduction pathway, assistance in protein folding, or translocation to a specific cellular compartment. The earliest N-terminal modifications actually correspond to cotranslational events involving excision of the first Met residue (NME), NAC of the NME-unmasked N-terminal residue, and specific lipidations, including MYR. Although rarer, posttranslational NAC or MYR have been described on specific substrates (Zha et al., 2000; Martin et al., 2011; Bienvenut et al., 2012). Despite the remarkable advances in genomics, proteomics, bioinformatics, and cellular imaging, all these modifications have remained difficult to observe in vivo (Meinnel and Giglione, 2008b; Bienvenut et al., 2012). In this study, taking into account that protein localization is an important clue to the physiological role of any protein, we aimed to uncover the hidden relationship between MYR or MYR + PAL motifs and protein localization in plants. By combining three different approaches (in vitro MYR studies, bioinformatics, and in vivo localization investigation), we could determine the role of

Figure 8. Subgroup 3 h-TRXs with N-Terminal MYR and PAL Lipidations Are Specially Localized to the PM with a Reduced Fraction Also Located at the ER/Golgi Compartment.

(A) Confocal scanning microscopy images of the Arabidopsis full-length h-TRXs of subgroup 3 fused to GFP transiently expressed in onion cells are shown (TRXh9 and CXXS2-GFP). The effect induced also by the corresponding nonmyristoylable forms are also shown (TRXh9G2A-GFP and CXXS2G2A-GFP), which is equivalent to GFP alone. Two different magnification levels are displayed: whole-cell (columns 1 and 3) and cortical fraction (columns 2 and 4). C, cortical cytoplasm; N, nucleus; PD, plasmodesmata; T, transvacuolar strands.

(B) Confocal scanning microscopy images of onion cells cotransfected with full-length TRXh9-GFP or CXXS2 and the mCherry-labeled Golgi marker soybean MAN1 (Nelson et al., 2007). TRX-GFP is visualized in green, mCherry is visualized in red, and colocalization is visualized in yellow (Merged). The GFP-only control with the same mCherry-labeled Golgi (G) marker is also shown. Experiments and image capturing were performed according to methods. The white bar indicates the scale.

(C) Confocal scanning microscopy images of onion cells transfected with the first 10 or 11 amino acids of TRXh9 and CXXS2 (ph9; pCXXS2). Bars = 10 µm.
The main recognized role of MYR is to target proteins to membranes (Bhatnagar and Gordon, 1997). Studies involving phospholipid bilayers have nevertheless suggested that single lipid modification does not stably anchor proteins in membranes but rather acts as a facilitator of the interaction (Peltzsch and McLaughlin, 1993; Pool and Thompson, 1998). The strength of the interaction appears to depend on the length of the fatty acid chain (PAL > MYR). It was thus suggested that myristate had been selected during evolution because the hydrophobicity of a C14 group favors a very weak membrane association for MYRed proteins compared with longer acyl chains, which would serve as permanent membrane anchors in the absence of a reversible signal. Therefore, besides several exceptions, such as

the examples cited above, the dogma assumed that MYR is necessary but not sufficient to target any protein to a membrane compartment. Indeed, several additional anchors known as second signals have been observed in many proteins undergoing MYR, with PAL being the major one in animals. Nevertheless, while a complete list of proteins with only a MYR site (i.e., devoid of a second signal) does not yet exist, an important number of proteins have been identified with a single N-terminal MYR site. It is interesting to notice that, for few of them, the N-terminal MYR alone without any evident second signal has been shown to target these proteins to intracellular membrane compartments with the ER/Golgi complex being the most frequent localization observed (Resh, 2006). For instance, this is the case for proteins such as eNOS (Liu et al., 1997), GRASP (Kondylis et al., 2005), and the Arl family (Price et al., 2005; Sahin et al., 2008). Relocalization at the ER/Golgi compartment from the PM was also observed when the second signal of several known MYRed proteins was abolished, as in the Fyn and Yes protein kinases, in which the Cys residues necessary for the PAL were substituted by a Ser or the polybasic domain of Src protein kinase was made shorter (McCabe and Berthiaume, 1999). In such cases, the N-terminal peptides recapitulated the behavior of the full-length protein. Finally, similar results were obtained using MYR and PAL consensus sequences, and multiple mutants of this sequence abolished the PAL or MYR sites (Navarro-Lérida et al., 2002).

In plants, ~40% of MYRed proteins apparently do not display any putative second signal site. Among the several unexpected protein families predicted to undergo MYR only in Arabidopsis, we identified the TRX protein family of the h-type subgroup 2. TRXs are small proteins participating in redox control of many cellular processes in organisms from bacteria to mammals or plants (reviewed in Meyer et al., 2009). Unlike animals, fungi, and bacteria, which have only a few genes encoding TRXs, many genes encoding putative TRXs have been identified in plants. In the Arabidopsis genome, ~40 genes have been identified encoding TRXs or TRX-related proteins. They are classified based on primary structure, intron position, and subcellular localization. The h-cluster is the most numerous group of plant TRXs. It is made up of at least 10 members in higher plants. The h-TRXs are generally assumed to be cytosolic proteins because of the lack of a transit peptide, but alternative locations for a few of them have also been suggested (Gelhaye et al., 2004; Serrato et al., 2008). Recent data indicate that the h-TRX family should be subdivided into three subgroups on the basis of sequence similarity and biochemical properties. Subgroup 1 (h1, h3, h4, and h5) is the most extensively studied. Classical subgroups 2 and 3 h-TRXs show an extra N-terminal domain, but only subgroup 3 h-TRXs display alternative monothiol active centers (WCXXS). Members of subgroups 2 and 3 have been proposed to be N-MYRed, and several were also predicted to be PALed (Boisson et al., 2003; Traverso et al., 2008). Nevertheless, we show here, by studying the h-TRX diversity from various plant genomes (Figure 1A), that plant h-TRXs should instead be clustered into four evolutionary subgroups and that the N-terminal sequences and associated N-terminal modifications of each of these classes are conserved independently of the plant species. Indeed, using our prediction tools for N-terminal protein modifications,
TermiNator (http://www.isv.cnrs-gif.fr/terminator3), h-TRXs of subgroups 1 and 4 are consistently predicted to undergo NAC. By contrast, all members of subgroup 2 are predicted to undergo only MYR, and all proteins of subgroup 3 are predicted to undergo both MYR and PAL. Demonstration of both the occurrence and the role of MYR and PAL within this family has been missing so far. Moreover, the meaning of having a single (MYR) or double acylation (MYR + PAL) in two distinct subgroups of this family remained unclear. In this context, we studied the impact of MYR and PAL on the Arabidopsis TRX protein family, which we also used as a model protein family to investigate (1) why plants adopt MYR in such a general way and (2) what the modification alone confers to the targets compared with classic double acylations (MYR + PAL) of known targets or to non-modified targets of the same family.

In the Absence of a Second Signal, MYR Motifs Consistently Induce Stable ER/Golgi Localization in Plants

Peptide derivatives of the N-terminal sequence from each TRX predicted to be MYRed in Arabidopsis were synthesized and used to assess MYR in vitro. MYR scores, which correspond to the measured \( k_{cat}/K_m \) value, have been assessed (S values). In addition, several h-type TRX N-terminal peptides from other...
plants predicted to undergo only MYR (TRXh1 from soybean, TRXh from Nicotiana alata, and TRXh4 from pea [Pisum sativum]) or double acylation (TRXh from Phalaris coerulescens) have also been studied. Data concerning all these peptides confirm in vitro MYR, which demonstrates MYR within subgroups 2 and 3 of h-TRXs and the robustness of the prediction tool. We also demonstrated the absence of in vitro MYR in one of the two TRXs from C. reinhardtii (both predicted MYRed), suggesting MYR and PAL are restricted to TRXs of green plants. In this study, we coupled the in vitro analysis of the TRX-derived peptides to in vivo imaging of protein localization in plant cells. Fusion of the peptides and constructs of different lengths to the N terminus of GFP revealed that the single and double acylations delineate the critical parameters governing the spatial cell distribution of these protein family members.

Our data show for TRXs predicted to undergo only MYR (1) an ER/Golgi-specific localization and (2) the localization of a small amount of the TRXs in the cytosol. Extending our analysis to other proteins of the Arabidopsis myristoylome predicted to only be MYRed, we confirmed the same trend independently of the function associated with the protein (Figures 3 to 5). In addition, our work (Figure 8), as previously shown for other proteins (see above), has revealed that substitution of the PAL Cys residues to Ser in proteins displaying both MYR and PAL sites targeted the protein primarily to the ER/Golgi apparatus. Together, the data suggest that MYR alone specifically targets a protein to the ER/Golgi apparatus. Our data indicate also that this interaction is weak in vivo because there is always a variable minor part of the signal that is associated with the cytosol. However, the balance always favors the ER/Golgi compartment. To explain both the balance and ER/Golgi membrane specificity of solely MYRed proteins, several nonexclusive hypotheses can be proposed. First, the specific membrane curvature of the ER/Golgi compartment could participate in the selection (Hatzakis et al., 2009). Second, in addition to the lipid itself, aromatic residues next to the lipid could positively contribute to stabilization of the membrane interaction (see Figure 2 in Wimley and White, 1996). There was indeed a correlation between this feature and the membrane/cytoplasm partitioning with three analyzed peptides with hydrophobic residues (i.e., TRXh2, ARFC1, and RPSS-like; Figure 6). Finally, we discerned another clear relationship between the S value and the balance toward localization to the ER/Golgi compartment. This value that directly measures the efficiency with which a peptide is N-MYRed might suggest that...
some of the peptides escape MYR. Indeed, our results including biochemical analysis, in vivo partitioning between cytosol and ER/Golgi compartment, and FRAP experiments strongly suggest that the pool of h-TRX of subgroup 2 is composed mainly of MYRed protein and of a variable portion of the non-MYRed form, which is unable to bind the ER/Golgi membrane compartment.

While conducting a large survey of NACed proteins of both Arabidopsis and humans, we noticed that Terminator was quite accurate but showed weaknesses in the prediction of NAC and MYR on N-Gly residues (Bienvenut et al., 2012). This indicates that the two patterns are overlapping and compete for the corresponding modification enzymes when they emerge from the ribosome tunnel. Accordingly, it is indeed known that both MYR and NAC compete at the ribosome with the same set of substrates and that some proteins may undergo both modifications in an in vitro translational system (Utsumi et al., 2001, 2004). Therefore, if the S value decreases, meaning that MYR is less efficient, it is fully expected that NAC or even another cotranslational route might interfere, as shown in other systems already (Colombo et al., 2005; Hofmann and Munro, 2006).

Both MYR and PAL Usually Target a Protein to Defined Substructures of the Plant PM

In this study, we also showed that all double-acylated TRX N termini consistently localize the GFP to the PM. Unlike monoacylation, double lipidation of protein motifs induces long-lived anchoring to lipid bilayer membranes (Shahinian and Silvius, 1995). Moreover, in animals, double-acylated short N termini of viral proteins are sufficient to target a protein to the PM (Pellman et al., 1985a). Similar results have been obtained in plants with other protein families. This is the case with the Gs subunit of the heterotrimeric G protein (Adjobo-Hermans et al., 2006), protein phosphatase PP2C74 and PP2C52 (Tsugama et al., 2012a, 2012b), Arabidopsis disease resistance RPS5 protein, and PBS1 kinase (Takemoto et al., 2012). Like h-TRXs, CDPKs consist of a large family of 34 proteins in Arabidopsis, many of which are predicted to display both MYR and PAL (Hrabak et al., 2003). Subcellular localization of 13 plant CDPKs in various studies and species has revealed that 10 of the CDPKs with one or two Cys residues adjacent to the MYR site bind to the PM (Martin and Busconi, 2000; Lu and Hrabak, 2002; Dammann et al., 2003; Leclercq et al., 2005; Benetka et al., 2008). The solely MYRed Arabidopsis CDPK3 transiently associates with membranes, whereas the non-MYRed CDPK4 was cytosolic. These data are in line with ours and indicate that, together, both MYR and PAL aid anchoring of CDPKs to the PM. CBL proteins represent another family of 10 proteins in Arabidopsis, among which four are both predicted to undergo both MYR and PAL. Several GFP fusion proteins, including N-terminal short peptides fused to GFP, were used in plants to localize the proteins (Batistic et al., 2008, 2010; Batistic et al., 2012). Similar to the aforementioned examples, the four CBLs with both MYR and PAL localize to the PM and a MYRed but non-PALed CBL1 mutant accumulated in the ER instead of the PM. Moreover, with the CBL system and the use of brefeldin A and coexpression with a mutant gene blocking vesicle trafficking, it could be suggested that MYR of CBLs is required for initial cytoplasm-to-ER sorting of the protein (Batistic et al., 2008). At the ER, the proteins undergo PAL by an ER-localized PAT, inducing trafficking to the PM without any involvement of the Golgi organelle (see Figure 1D in Sorek et al., 2009). This resembles the Ras2p pathway of yeast (Linder and Deschenes, 2007). Similar to animals, there are 24 predicted PATs in plants (Hemsley et al., 2005; Batistic, 2012), and it is known that each of them displays a specific intracellular localization to the various membrane compartments of the cells with most proteins localized to the PM. It is remarkable that among the pool of cotranslationally predicted MYRed proteins shown recently to undergo PAL, 97% of them display a Cys at either position 3 and/or 4 (80%) or 5 and 6 for the others (see Supplemental Table 9 in Hemsley et al., 2013), confirming as a result the annotation of predicted PALed proteins in the Arabidopsis myristoylome. In this large-scale analysis, made using a targeted proteomics of PALed proteins of Arabidopsis, Hemsley et al. (2013) could also identify TRXh9 among all the aforementioned families of both MYRed and PALed proteins. Our data also support previous studies that identified the PM localization of Arabidopsis TRXh9 and possible cell-to-cell movement of the protein through plasmodesmata (Meng et al., 2010). In this report, abolishing PAL by mutation of Cys-4 into Trp, an unusual substitution to challenge S-acylation, in the full-length protein did not change protein localization despite the fact that this Cys residue was suggested to be important for the catalytic activity of the enzyme. This result is clearly different from those resulting from Cys-to-Ser substitution (Figure 10). As Trp is the amino acid that best promotes membrane association (Wimley and White, 1996), this might suggest that this aromatic amino acid confers additional affinity for the PM. However, our data show that, regardless of the length of the fusion considered, double acylation (MYR and PAL) is always required for PM targeting and specific signal transduction conditions induce de-PAL and recycling of the proteins to the endomembrane/Golgi system. Finally, many contributions using confocal microscopy have recently indicated that plant PMs are laterally compartmented (see references cited in Tanner et al., 2011). Our ultrastructural analysis of these double-acylated proteins revealed an evenly spaced clustering of GFP in specific nanodomain structures consistent with the size and stable location previously proposed for plant membrane microdomains also detected using electron microscopy (Raffaele et al., 2009).

Together, our data explain why plants’ PM proteomes contain higher levels of MYRed and PALed proteins compared with solely MYRed proteins (Marmagne et al., 2007; Ephritikhine et al., 2008). Finally, very few MYRed proteins, including h-TRXs, exhibit predicted transmembrane domains and, if any, usually only one (http://www.isv.cnrs-gif.fr/tm/maturity/myristoylome2008.html), which suggests that the proteins correspond to soluble entities that enter the PM essentially only through the lipid moieties.

Conclusion

The demonstration of in vitro MYR of h-TRXs and of other proteins of the myristoylome and the analysis of the impact of single or double acylations on whole evolutionary subgroups of protein families, such as the TRXs, are extremely relevant.
Particularly, in the case of subgroup 2 and 3 h-TRXs, we now can consider these proteins being made by two modules: (1) an N-terminal extension by which irreversible and/or reversible lipidation dictates the subcellular localization of the protein and (2) the TRX module itself, which ensures redoxin activity. Our studies will facilitate further efforts to better understand the physiological role of these proteins, their specific substrates, and their association with other redox proteins at the membranes, including the PM and the ER. Identifying the associated protein interaction networks at these membranes will be an exciting and challenging task.

METHODS

Materials

All chemicals were purchased from Sigma-Aldrich. Restriction enzymes and T4 DNA ligase were purchased from Fermentas. Oligonucleotides were synthesized by Invitrogen. DNA constructs were purified from the latest version of the Genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html) and with the help of a recently published revision (Chibani et al., 2009). For Arabidopsis thaliana sequences were obtained from published data (Meyer et al., 2006) and updated according to the TAIR database (V9; June 19, 2009; http://www.Arabidopsis.org/index.jsp). Arabidopsis h-TRX sequences were used to BLAST (<10e-10) other plant genomes (rice [Oryza sativa], Populus trichocarpa, grape [Vitis vinifera], and Medicago truncatula). Rice sequences were obtained from the latest available version of the Rice Genome Annotation Project (V6.0; March 6, 2009; http://rice.plantbiology.msu.edu/). P. trichocarpa TRXs were obtained from the latest version of the Populus genome project (V1.1; http://genome.igi-psf.org/Poptop1_1/Poptop1_1.home.html) and with the help of a blasted IMG-annotated M3.0 from the Medicago genome release version 3.0 (March, 2009; http://www.medicago.org/genome/downloads/ M3). Grape TRXs were obtained from the updated version “Genoscope 8.4x; PlantGDB” (http://www.plantgdb.org/VgGDB/). All obtained sequences were filtered. TRXs belonging to other families were excluded (m, t, x, etc.). We also excluded multidomain proteins, such as CDSP32, Arabidopsis TDx, or NTRC-like proteins, even if their TRX domain showed high sequence similarity with the h-TRX cluster. Finally, only sequences that fit well in the h-type phylogenic tree were considered. In addition, some characterized TRXs in the bibliography were added to this work. The phylogenic tree was constructed using the Phylogeny.fr platform (www.phylogeny.fr/), which uses a three-step pipeline including PHYLIP for tree rooting after alignment (Dereeper et al., 2008). The complete alignment obtained is shown in Supplemental Data Set 1 online. Midpoint rooting was used as criterion for rooting the phylogenetic tree. Branches having branch support value smaller than 50% were displayed.

Sequence Analysis in Silico

Amino acid sequences analyzed in this work were obtained from different sources. Arabidopsis thaliana sequences were initially obtained from published data (Meyer et al., 2006) and updated according to the TAIR database (V9; June 19, 2009; http://www.Arabidopsis.org/index.jsp). Arabidopsis h-TRX sequences were used to BLAST (<10e-10) other plant genomes (rice [Oryza sativa], Populus trichocarpa, grape [Vitis vinifera], and Medicago truncatula). Rice sequences were obtained from the latest available version of the Rice Genome Annotation Project (V6.0; March 6, 2009; http://rice.plantbiology.msu.edu/). P. trichocarpa TRXs were obtained from the latest version of the Populus genome project (V1.1; http://genome.igi-psf.org/Poptop1_1/Poptop1_1.home.html) and with the help of a recently published revision (Chibani et al., 2009). For M. truncatula TRXs, we blasted IMG-annotated M3.0 from the Medicago genome release version 3.0 (March, 2009; http://www.medicago.org/genome/downloads/ M3). Grape TRXs were obtained from the updated version “Genoscope 8.4x; PlantGDB” (http://www.plantgdb.org/VgGDB/). All obtained sequences were filtered. TRXs belonging to other families were excluded (m, t, x, etc.). We also excluded multidomain proteins, such as CDSP32, Arabidopsis TDx, or NTRC-like proteins, even if their TRX domain showed high sequence similarity with the h-TRX cluster. Finally, only sequences that fit well in the h-type phylogenic tree were considered. In addition, some characterized TRXs in the bibliography were added to this work. The phylogenetic tree was constructed using the Phylogeny.fr platform (www.phylogeny.fr/), which uses a three-step pipeline including PHYLIP for tree rooting after alignment (Dereeper et al., 2008). The complete alignment obtained is shown in Supplemental Data Set 1 online. Midpoint rooting was used as criterion for rooting the phylogenetic tree. Branches having branch support value smaller than 50% were displayed.

GFP Fusion Constructs

In order to control that cellular protein localization observed was not a consequence of overexpression of the protein in the cell, GFP fusion proteins were constructed in vectors pSmGFp and pSmRSGFP, as previously described (Giglione et al., 2000). Both plasmids encoded soluble highly fluorescent variants of GFP optimized for higher plants. smGFp and smRSGFP differ at amino acid position Ser-65; RSGFP displays the S65T substitution, which causes an increased fluorescence output and a higher expression level. Sequences were cloned in-frame with the GFP gene between the single XbaI and BamHI sites to generate N-terminal protein fusions with GFP. For cloning of the different full-length TRXs or fragments of different lengths, PCR-amplified bands of all constructs were obtained using specific oligonucleotides permitting incorporation into appropriate restriction sites (XbaI and BglII; see also Supplemental Table 1 online). The mutated versions were obtained by conventional PCR site-directed mutagenesis using the QuickChange site-directed mutagenesis kit (Stratagene) to introduce the different mutations at specific positions. For constructs fusing the shortest N-terminal domains consisting of 10 to 11 amino acids with GFP, both encoding DNA strands were directly assembled (see Supplemental Table 1 online). We mixed two complementary oligonucleotides at equimolar concentration and annealed them after boiling for 1 min followed by gradual cooling to room temperature. The reconstituted double-stranded DNA fragment showed cohesive extremities for cloning between the XbaI and BglII restriction sites of vectors pSmGFp and pSmRSGFP. All plasmids were sequenced to confirm the presence of the desired mutations or validate the constructs. Primers for mutagenesis were designed using the primerX website (http://www.bioinformatics.org/primers/cgi-bin/DNA_1.cgi).

Arabidopsis NMT1 Purification

The cloning of the complete cDNAs for Arabidopsis NMT1 has been described elsewhere (Boisson et al., 2003). The NMT1 open reading frame was inserted into pET16b (Novagen, Life Technologies) as an N-terminal fusion with a 6xHis tag as described (Boisson et al., 2003). NMT was produced in Escherichia coli by transforming BL21-pRares (Rosetta; Novagen, Life Technologies) cells with an appropriate plasmid. Cells were grown at 22°C for 6 h in 2X Yeast extract and Tryptone medium supplemented with 50 mg/mL of ampicillin and 34 mg/mL of chloramphenicol to an OD600 of 0.9. They were then induced with 0.4 mM isopropylthio-galactoside and incubated for another 12 h. In all cases, cells were harvested by centrifugation and resuspended in 10 to 20 mL of buffer A, consisting of 20 mM sodium phosphate buffer, pH 7.3, with 500 mM NaCl and 10 mM 2-mercaptoethanol. Samples were subjected to sonication, and cell debris were removed by centrifugation. The supernatant (5 to 15 mL) was applied to a Hi-Trap chelating HP nickel affinity column (0.7 x 2.5 cm; GE Healthcare) equilibrated in buffer A. Elution was performed at a flow rate of 0.5 mL/min in two steps: buffer B (buffer A plus 0.5 M imidazole) followed by a linear 0.3 to 5 mM/min imidazole gradient. The pool of purified protein (5 mL) was first dialyzed against buffer A for 12 h and then against buffer A plus 55% glycerol for 24 h before storage at
NMT activity was assayed at 30°C by continuously monitoring the formation of NADH by fluorescence (λexc = 340 nm; λem = 465 nm) in a coupled assay using pyruvate dehydrogenase activity, as described previously (Boisson and Meinell, 2003; Traverso et al., 2013). The assay was performed in a final volume of 100 µL in 96-well black plates (Grenier). Changes in fluorescence over time were followed using the Infinite M200 fluorimeter equipped with microinjectors (Tecan). The reaction mixture contained 50 mM Tris, pH 8.0, 1 mM MgCl2, 0.193 mM EGTA, 0.32 mM DTT, 0.2 mM triamine pyrophosphate, 2 mM pyruvate, 0.1 mg/mL of BSA, 0.1% Triton X-100, 5 mM to 1000 µM peptide, 2.5 mM NADP, 0.125 units of porcine heart pyruvate dehydrogenase (0.33 units/mg; Sigma-Aldrich), and 40 µM myristoyl-CoA (Sigma-Aldrich). The reaction mixture was preincubated for 3 min at 30°C before starting the reaction by adding 0.5 µM NMT. The kinetics of peptide MYR was followed for 15 min, and the data were fitted over a 5-min period to obtain the initial velocity. The kinetics parameters (kcat and Km) were obtained with Enzyme Kinetics module 1.2 of Sigma Plot (version 9.0) by nonlinear Michaelis-Menten equation fitting.

Confocal Laser Scanning Microscopy

Epidermal onion cells were bombarded with the different DNA constructs using the PDS-1000/He instrument (Bio-Rad) as previously described (Giglione et al., 2000). Epidermal onion cells expressing GFP and/or mCherry fusion proteins (Nelson et al., 2007) were observed and images were collected with an SP2 confocal microscope (Leica Microsystems) equipped with an Ar laser and a HeNe laser. The oil objectives used were 63 (numerical aperture 1.30), giving a maximal resolution of ~200 nm in the xy plane and 400 nm along the z axis (pinhole 1 Airy unit).

For the specimens labeled with different fluorochromes, images corresponding to each fluorochrome were acquired sequentially.

The confocal microscope images of TRX-GFP fusion proteins have been analyzed using ImageJ software (NIH Image, version 1.392). A small circular area was selected and used to quantify the relative fluorescence intensity at both the Golgi apparatus and the cytoplasm of all the confocal images. Measurement parameters, such as the mean gray values (the average of the gray values in the selected area), have been defined. The partitioning of fluorescence between the Golgi apparatus and the cytosol is calculated as the ratio between the measured fluorescence intensity of the Golgi apparatus over that of the cytosol.

FRAP Experiments

FRAP studies were performed as reported (Martinière et al., 2012) with the confocal microscope setting described above. For quantification of fluorescence, signals were sampled prior to bleaching the ROIs outlined in the figures by scanning with high-intensity illumination (100% 480-nm laser power). Recovery of fluorescence into ROI was recorded during 60 or 120 s with delay of 0.8 s between frames by scanning at 1% laser power. No bleaching effect was observed during recovery. Mobile fraction and half-life recovery analysis were performed as described (Martinière et al., 2012). For each treatment, images originating from 40 to 50 independent cells were analyzed. Significance statistics were determined using Student’s two-tailed t test.

Transmission Electron Microscopy

The same cells used for confocal analysis were supplied for the electron microscopy study. The processing of onion (Allium cepa) epidermal cell layers for transmission electron microscopy was adapted from Hawes and Satiat-Jeunemaitre (2001). Briefly, the samples were fixed overnight at room temperature with 1% glutaraldehyde and 3% paraformaldehyde in 0.1 M cacodylate, pH 6.8, and embedded in LRWhite resin using progressive low-temperature methods. Immunogold labeling was performed on 79- to 90-nm ultrathin sections (Ultracut UC6; Leica), using an anti-GFP antibody made in rabbit (BD International; 1:400), recognized by an anti-rabbit conjugated with 10-nm gold particles (BD International; 1:20). Sections were then observed using a JEOL 1400 transmission electron microscope operating at 120 kV. Images were acquired using a postcolumn, high-resolution (11 megapixels), high-speed camera (SC1000 Oris; Gatan) and processed with Digital Micrograph (Gatan). Selected pictures are representative of observations performed on 20 sections resulting from three experiments.

Subcellular Fractionation and Immunoblot Analysis

The transfected cells also used for confocal analysis were frozen in liquid nitrogen and ground in an MM 300 mixer mill (Qiagen). The resulting fine powder was dissolved in lysis buffer (40 mM Tris-HCl, pH 8.0, supplemented with protease inhibitor cocktail provided by Roche). Lysates were centrifuged at 15,000g at 4°C for 30 min. The supernatants were collected, and the pellets were resuspended in SDS sample buffer (lysis buffer plus 2% SDS and 1% Triton) and incubated at 4°C for 30 min with shaking. Membrane proteins were separated from insoluble fractions by an additional centrifugation. To remove palmitate groups from subgroup 3 h-TRXs, the corresponding membrane proteins were treated with 10 or 200 mM DTT as previously reported (Fukata et al., 2004; Batistic et al., 2008). Protein concentrations were measured with the two-dimensional Quant kit (GE-Healthcare Biosciences). After modified trichloroacetic acid/acetone precipitation (Espagne et al., 2007), the samples were brought up to equal volume and the fractions (supernatant:pellet = 1:1, ~1 mg) were resolved by long-range SDS-PAGE (14%) as previously reported (Fukata et al., 2004; Batistic et al., 2008) and analyzed by immunoblots using either anti-GFP or anti-h-ATPase antibodies (Abcam and Agrisera) as previously described (Adam et al., 2011). Band intensities were quantified with Quantity One software (Bio-Rad). Statistical analyses were run with R software (http://cran.cict.fr/).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: At-TRXh1, At3g51030; At-TRXh2, At3g513950; At-TRXh3, At3g42980; At-TRXh4, At1g18730; At-TRXh5, At1g45145; At-TRXh6, At1g59730; At-TRXh8, At1g98880; At-TRXh9, At3g06710; At-TRXh10, At3g56642; At-CXXS3, At2g40790; At-CXXS1, At1g11530, Medtr1g028610, DQ121443; DQ121442; Medtr5g08470; Medtr5g039890; Medtr5g038960; Medtr5g038910; Medtr8g005420; Medtr8g005260; Medtr8g116230; Medtr2g010800; Medtr4g111230; Medtr5g096730; Os07g08840; Os03g58630; Os07g09310; Os05g07690; Os05g40190; Os01g07376; Os04g53740; Poptr818765; Pt-TRXh1; Poptr710146, Pt-TRXh12; Poptr219472, Pt-TRXh3; Poptr258873, Pt-TRXh5; Poptr420455, Pt-TRXh2; Poptr825426, Pt-TRXh4; Poptr819244, Pt-TRXh4; Poptr663332, Pt-CXXS1; Poptr647767, Pt-CXXS1; Poptr722798, Pt-CXXS1; Vv04g12270; Vv18g11650; Vv14g15400; Vv01g09260; Vv08g05890; Vv00g2700; Vv00g25955; Vv19g05210; Ps-TRXh4, AY170651; Gm-TRXh1, AY575954; Na-TRXh, AAY42864; Pc-TRXh, AF195388; Cr-TRXh1, CA56850; Cr-TRXh2, AAO20258; and At-NMT, AF250966.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Plant h-TRXs Are Predicted to Undergo Specific N-Terminal Modifications According to Their Phylogenetic Origin.
Supplemental Figure 2. Summary of Different Constructs Used and Corresponding Subcellular Localizations Observed.

Supplemental Figure 3. Absence of Golgi/ER Localization of h-TRXs of Subgroup 2 Is Not Due to Negative Charges at the N Terminus nor Residues Involved in Phosphorylation/Dephosphorylation Events.

Supplemental Figure 4. Photobleaching of the ER/Golgi Membrane-Bound TRXs.

Supplemental Figure 5. Photobleaching of Cytosolic TRXs Is Followed by Rapid Recovery of Fluorescence Because of Cytoplasmic Streaming.

Supplemental Figure 6. Coexpression of NMT1 with pH8-GFP or pH7-GFP Induces Changes in Cytosol/Membrane Partitioning.

Supplemental Figure 7. Subgroup 3 h-TRXs Colocalize with the PIP2A PM Marker.

Supplemental Figure 8. ph9-GFP and pcXXS2-GFP Display High Lateral Mobility in PM.

Supplemental Figure 9. No Alteration of Protein Localization Pattern upon DTT Treatment Was Observed on Cells Expressing GFP Alone, the Golgi Marker, or the Solely MYRed Subgroup 2 h-TRXs.

Supplemental Table 1. Oligonucleotides Used in This Work.

Supplemental Data Set 1. Amino Acid Sequences of h-TRXs Studied in This Work.

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AUTHOR CONTRIBUTIONS


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