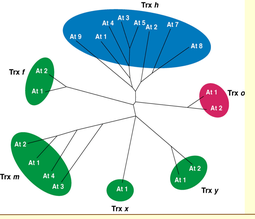



# A membrane-associated thioredoxin required for plant growth moves from cell-to-cell suggestive of a role in intercellular communication

Ling Meng, Joshua H. Wong, Lewis J. Feldman, Peggy G. Lemaux and Bob B. Buchanan
Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720, USA

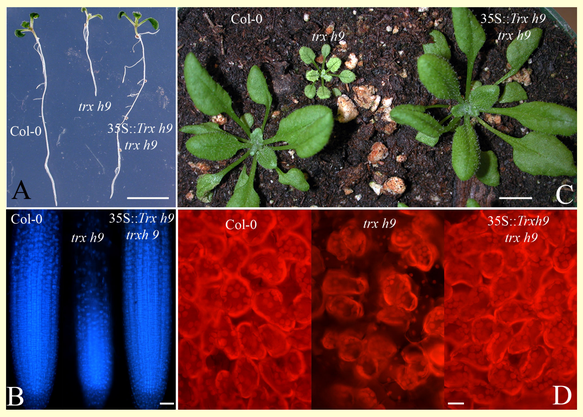
## Thioredoxin (Trx) system in plants

- Redox state is one factor determining cell fate and growth of cells during development of multicellular organisms.
- Thioredoxin (Trx), a ubiquitous disulfide regulatory protein, appears to play a role in linking redox state to development.
- Trxs are a complex family of regulatory proteins in plants with at least six distinct types in plants, *f, m, y, x, h* and *o.*
- Trxs reside in different cell compartments, functioning in many processes.
- Trx is generally reduced by ferredoxin-dependent ferredoxin-Trx reductase (FTR) or NADP-dependent NADP-Trx reductase (NTR).
- Role of chloroplast Trxs is relatively clear; function of extraplastidic *h*-type Trxs is relatively unknown.
- This work focused on one extraplastidic member,Trx *h9,* the ortholog of which is reduced by glutathione/glutaredoxin, not NTR.

## Why is Trx *h*9 so interesting?

- Trx *h*9 is highly conserved. Identities and positives, comparing protein sequences of Trxh9 in Arabidopsis versus its rice ortholog, are 63% and 82%, respectively.
- The N terminus of Trx *h*9 contains a 17-amino acid extension and an additional Cys ($Cys^4$).
- The second glycine ($Gly^2$) and fourth cysteine ($Cys^4$) in the N terminus are conserved.
- PtTrx*h*4, the othorlog of Trx h9 from poplar, provides the first example that Trx can be reduced by the Glutathione/ Glutaredoxin system.
- Wheat ortholog is involved in controlling preharvest sprouting.
- $Cys^4$ is required for the reduction by glutaredoxin of poplar PtTrxh4.


## AtTrx *h9* is required for plant growth of Arabidopsis



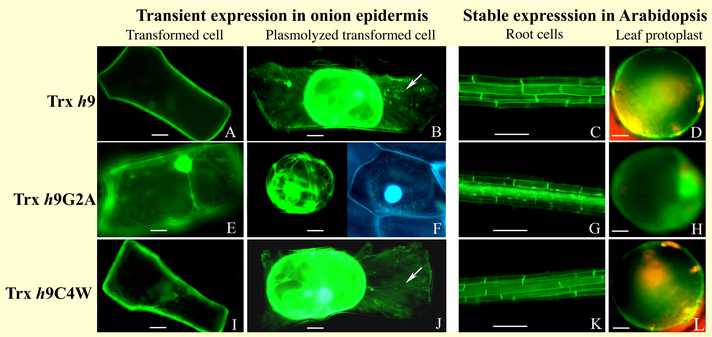

**Phenotypic and complementation analysis of *trx h9* mutation in Salk_08660 plants.**
7-day-old Arabidopsis seedlings (A) and root tips (B) grown on MS medium plus 1.0% sucrose. (C) 35-day-old Arabidopsis grown in soil. (D) Leaves from 35-day-old Arabidopsis grown in soil and viewed under fluorescence microscope. Order in all panels is the same. Bar = 1 cm in (A) and (C); 50 μm in (B) and (D).

**Root tips of 7-day-old mutant seedlings had shortened root apical meristem and were more compact with fewer elongated cells consistent with noticeably shorter roots when grown on MS medium with 1% sucrose (A) and (B). When grown in soil, mutant plants were dwarf with small yellowish leaves (C). Mesophyll cells from mutant leaves were smaller and irregularly shaped with fewer chloroplasts vs. wild type (D).**

## ABSTRACT

**Thioredoxins (Trxs) are small ubiquitous regulatory disulfide proteins. Plants have an unusually complex complement of Trxs comprised of six well-defined types (Trxs *f, m, x, y, h* and *o*) that reside in different cell compartments and function in an arrayof processes. The extraplastidic *h*-type consists of multiple members that in general have resisted isolation of a specific phenotype. In analyzing mutant lines in *Arabidopsis thaliana*, we identified a phenotype of dwarf plants with short roots and small yellowish leaves for AtTrx *h*9 (henceforth, Trx *h*9), a member of the *Arabidopsis* Trx *h* family. Trx *h*9 was found to be associated with the plasma membrane and to move from cell-to-cell. Analysis of Trx *h*9 revealed a 17-amino acid N-terminal extension in which the second Gly ($Gly^2$) and fourth cysteine ($Cys^4$) were highly conserved. Mutagenesis experiments demonstrated that $Gly^2$ was required for membrane binding, possibly via myristoylation. Both $Gly^2$ and $Cys^4$ were needed for movement, the latter seemingly for protein structure and palmitoylation. A 3D model was consistent with these predictions as well as with earlier evidence showing that a poplar ortholog is reduced by a glutaredoxin rather than NADP-thioredoxin reductase. In demonstrating the membrane location and intercellular mobility of Trx *h*9, the present results extend the known boundaries of Trx and suggest a role in cell-to-cell communication.**

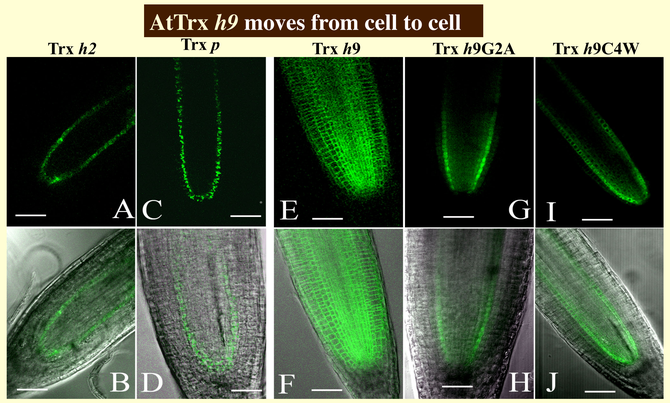
## Trx *h*9 is a plasma membrane protein




**Subcellular localization of wild-type and mutated Trx *h*9 in onion epidermal and transgenic Arabidopsis cells using GFP tagging.**
The $Gly^2$ mutation to Ala is indicated as G2A; the $Cys^4$ mutation to tryptophan (W) as C4W. Image in (F) viewed for GFP (left) and DAPI (right). Bar = 50 μm in (C), (G), and (K); 10 μm in remainder. In (B) and (J) arrows point to Hechtian strands.

**Mutation of $Gly^2$, not $Cys^4$, in conserved N-terminal extension abolishes Trx h9 membrane localization. $Gly^2$ is required for the membrane binding possibly via myristoylation**

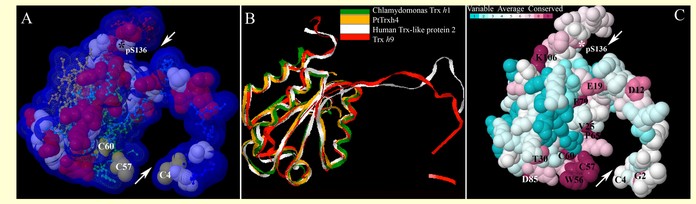
## AtTrx *h9* moves from cell to cell




**Movement analysis of Trx *h*9 protein in root tips of 7-day-old Arabidopsis (Col-0) seedlings using Arabidopsis *SCARECROW* promoter (pSCR) and GFP tag.**
pSCR directs gene expression specifically in the single endodermal cell layer of the root as shown in Trx *h*2 [At5g39950, (A) and (B)] and Trx *p* [At3G06730, (C) and (D)]. The $Gly^2$ mutation to Ala is indicated as G2A; the $Cys^4$ mutation to tryptophan (W) as C4W. Bar = 50 μm.

**Palmitoylation occurs in membranes. Thus cytosolic proteins must interact with membranes to be palmitoylated. $Gly^2$ may, therefore, be required for membrane localization of Trx *h*9 and for subsequent palmitoylation of $Cys^4$. Palmitoylated proteins, which favor specific protein-protein interactions, modulate activity of signaling cascades. The two conserved amino acids in the N-terminal extension are essential for its unusual properties: $Gly^2$ for membrane anchoring and both $Gly^2$ and $Cys^4$ for mobility.**

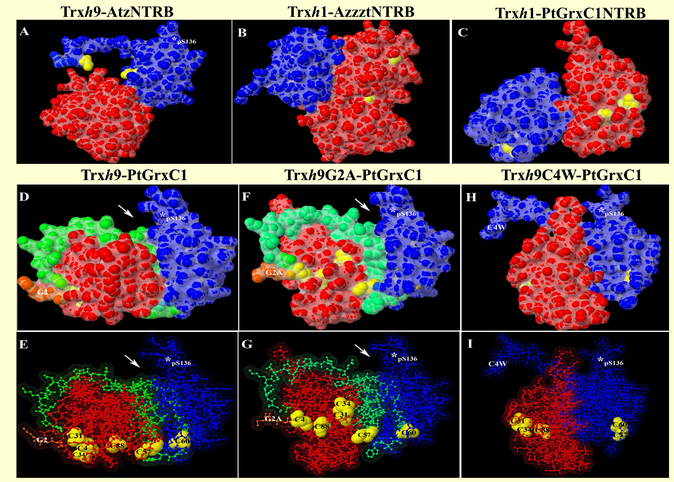
## N-terminal extension of Trx *h*9 may form a potential protein docking site




**3D models and conserved residue prediction for Trx *h*9.**
(3D model of Trx *h*9. (B) Superimposition of 3D model of Trx *h*9 (red) and the top three templates of Trx *h*9 using 3D-SS (3-Dimensional Structural Superposition) service. (C) Conserved residue analysis of Trx *h*9. Cys, Ser, and positively charged residues are shown in yellow, white, and red, respectively, as 100% of van der Waals. Remainder of residues from N- to C-terminal are shown in blue to red as 20% of van der Waals in (A). All atoms are coupled with Solvent Accessible Surface (VDW + 1.4 Angstrom) in (A). * indicates phosphorylated Ser at position 136 (pS136) at the C-terminus of Trx *h*9 in response to sucrose. Conserved amino acids with single letter abbreviations are indicated at their numbered position in (C).

**Arrows point to potential docking sites of Trx *h*9 in (A) and (C). This docking site could confer specific binding properties to Trx *h*9 in its interaction with other proteins in a manner possibly modulated by palmitoylation of $Cys^4$. In addition, the C-terminus of Trx *h*9 could form a smaller binding pocket in which $Ser^{136}$ is located at the inside surface.**

## Trx *h*9 may be reduced by Grx / GSH system, but not NTR




**Docking property analysis of Trx *h*9 and Trx *h*1.**
The $Gly^2$ mutation to Ala is indicated as G2A; the $Cys^4$ mutation to tryptophan (W) as C4W. Arrows point to potential docking sites of Trx *h*9 in (D) to (G). * indicates phosphorylated Ser at position 136 (pS136) at the C-terminus of Trx *h*9 in (A) and (D) to (I).

**Trx *h*9 appears not to interact with NADP-thioredoxin reductase, like other *h*-type Trxs [(A) vs.(B)]. Its predicted structure indicates that it might be preferentially reduced by the GSH/Grx system, as is the case for the poplar ortholog of Trx *h*9, PtTrx*h*4 (D) and (E) in an interaction dependent on $Cys^4$ but not $Gly^2$ [(H) and (I) vs. (F) and (G)].**

## Conclusions

- **An *h*-type Trx, *h*9 was shown to bind to the plasma membrane despite lacking a transmembrane domain.**
- **Using SCARECROW promoter-GFP fusions, Trx *h*9 was shown to be mobile and able to move from cell-to-cell.**
- **Two amino acids in the N-terminal extension appeared to associate with the plasma membrane (possibly for myristoylation).**
- **$Gly^2$ is required for membrane anchoring; both $Gly^2$ and $Cys^4$ are essential for mobility (seemingly for structure and palmitoylation).**
- **Modeling showed Trx *h*9 is preferentially reduced by GSH and Grx, similar to poplar ortholog, rather than by NTR as for other *h*-type Trxs.**
- **T-DNA insertion mutation showed Trx *h*9 was required for growth and development.**
- **Trx *h*9 appears to resemble Trx *h3* in bridging the Grx/Trx interface in relaying information to maintain cellular redox balance.**
- **Trx *h9* may be required for redox signaling.**

## Acknowledgements

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