Protein-Protein Interactions Involved in Water Transport in Cotton Roots

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RESEARCH PROBLEM

Down-regulation of aquaporin genes in plant roots in response to water-deficit and salt stress has been reported in many systems including corn, rice, and cotton. Since aquaporins passively aid water movement, we speculate the down-regulation of aquaporin transcripts is a means to reduce the hydraulic conductivity of roots in situations when the osmotic gradient favors water efflux. However, post-translational modification including phosphorylation or removal of aquaporins from cell membranes may also be a component of the response. The objective of the study was to identify candidate proteins involved in post-translational modification of aquaporins by identifying proteins that physically interact with selected domains of aquaporin proteins.

BACKGROUND INFORMATION

Early data from studies in pea (Guerrero et al., 1990), tomato (Fray et al., 1994), and Arabidopsis (Yamaguchi-Shinozaki et al., 1992) indicated aquaporins were up-regulated in response to water-deficit stress. However, time-course studies demonstrated transient down-regulation of aquaporin genes in the early phases of stress. For example, in cotton, seven aquaporin genes were down-regulated until 4 h after stress application but were expressed at or above pre-stress levels by 96 h, with expression recovery beginning between 4 and 24 h (Hendrix and Stewart, unpublished data). Similar reports in rice (Kawasaki et al., 2001), ice plant (Yamada et al., 1995), and corn (Wang et al., 2003) demonstrate that the down-regulation of aquaporins in roots as a result of water-deficit stress is a widespread phenomenon.

Sub-cellular relocalization of aquaporins has been reported in both ice plant (Barkla et al., 1999) and Arabidopsis (Boursiac et al., 2005) roots. In ice plant roots, three isoforms of plasma-membrane aquaporins appeared in the intercellular membrane fractions of sucrose density gradients after salt stress. In Arabidopsis, tonoplast aquaporin-GFP fusion proteins were observed in intercellular vesicles tentatively identified as

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tonoplast invaginations 2 to 4 h after salt stress, and aquaporin relocalization was coincident
with transcriptional down-regulation of aquaporin genes (Boursiac et al., 2005).

**RESEARCH DESCRIPTION**

The yeast-two hybrid technique was used to identify proteins that interact physically
with aquaporins. A GAL4 activator domain (AD)-fusion cDNA library was constructed
using equal parts of RNA extracted from polyethylene-glycol-stressed and unstressed Gossypium hirsutum ‘Siokra L23’ roots. A GAL4 binding domain (BD)-fusion construct was generated for the N-terminal domain of a cotton plasma-membrane aquaporin (TC32230) that was previously found to be down-regulated by water-deficit stress (Hendrix and Stewart, unpublished). After obtaining its DNA sequence to confirm in-frame cloning, the BD-fusion construct was transferred to yeast strain AH109 (Clontech) using lithium acetate-PEG transformation. Transformed yeast cells were selected on media lacking tryptophan, and the presence of the plasmid was confirmed using PCR. Yeast transformed with the BD-fusion construct were transformed with an aliquot of the AD-fusion library using the large-scale lithium acetate-PEG transformation technique and plated on media lacking tryptophan, leucine, histidine, and adenine to select both for the presence of the BD- and AD-fusion plasmids and for protein-protein interaction. Plasmid DNA from positive colonies was extracted, and the interactive phenotype was confirmed by repeating the sequential transformation procedure described above. The sequences of the cloned DNAs were obtained from AD plasmid colonies with a positive interaction in both experiments.

**RESULTS AND DISCUSSION**

**AD-Fusion Library**

The primary titer of the AD-fusion library was $2 \times 10^7$ cfu/ml and had an average insert size of 950 bp. The library was amplified to a titer of $1 \times 10^9$ cfu/ml, and aliquots stored in 7% DMSO at -80°C. Gene representation in the library was assessed using PCR (Fig. 1). Nine of the 10 selected cotton genes could be amplified from the fusion library.

**BD-Fusion Construct**

Cloning in the correct reading frame of the 186 bp fragment of a plasma-membrane aquaporin (TC32230) was confirmed using DNA sequencing. The cloned fragment codes for a 62-amino-acid domain with a predicted molecular mass of 6.9 kDa at the N-terminal of the aquaporin. The construct does not self-activate transcription of any of the reporter genes ($his3$, $ade$, $mell$) in yeast strain AH109.

**Interaction Screen**

In the initial library screening, approximately $2 \times 10^7$ independent clones were screened for the ability to interact with the BD-fusion construct. Ten putative interactions were identified. PCR analysis revealed the yeast colonies contained one of two
AD-plasmids with either an 850 bp fragment or a 1400 bp fragment, with the 850 bp fragment yielding the strongest interaction. Wild-type AH109 yeast sequentially transformed with the BD- and AD-fusion plasmid yielded the expected results in terms of amino acid prototrophy (Fig. 2) and displayed the same phenotype as the original isolates. DNA sequence analysis revealed that the 850 bp fragment was highly similar to the Arabidopsis protein VAP27-2 involved in vesicle fusion to membranes. The isolated clone may be an alternative splice variant of the cotton protein, TC28447. The 1400 bp fragment was the cotton tonoplast aquaporin, TC27563.

**PRACTICAL APPLICATION**

Each of the proteins identified in the interaction screening are potentially biologically relevant, though more work is needed to demonstrate that the interactions occur in cotton roots. However, identification of interaction between the BD-fusion construct of the N-terminal region of a cotton root aquaporin and a different tonoplast aquaporin raises the possibility that heterotetramerization of aquaporin proteins is possible in cotton roots. The interaction between the BD-fusion construct and the VAP protein is of special interest as re-localization of aquaporins has been recently demonstrated in Arabidopsis (Boursiac et al., 2005). The practical value of the study lies in the identification of proteins that may be involved in mechanisms by which cotton could regulate cell-membrane permeability to water. Selection of these systems through molecular breeding could increase the tolerance of cotton to water-deficit stress.

**LITERATURE CITED**


Fig. 1. Nine out of ten cotton (G. hirsutum L.) genes could be amplified from the fusion library. 1 ng of library plasmid was amplified with gene-specific primers and separated on a 1.5% agarose gel. Band intensities are proportional to clone copy number and agree well with the relative expression intensities measured via qPCR in other studies. Lane (Ln)1: 100 bp ladder; 2: vacuolar invertase; 3: neutral invertase; 4: UDP-glucose pyrophosphorylase; 5: fructokinase; 6: citrate synthase; 7: alcohol dehydrogenase; 8: aldehyde dehydrogenase; 9: tonoplast intrinsic protein 1; 10: RNA polymerase II accessory factor.
Fig. 2 The cotton proteins VAP27-2 and TIP interact with the N-terminal domain of a plasma-membrane aquaporin in yeast. The interaction restored histidine and adenine prototrophy in colonies transformed with the constructs. However, the VAP27-2 interaction is much stronger than the TIP interaction indicated by the robust growth of yeast colonies on medium lacking tryptophan, leucine, histidine, and adenine. Both the BD and AD plasmids yielded growth on the expected media and did not self-activate transcription of the his3 or ade reporter genes. Abbreviations: Media supplements listed are omitted from the media to facilitate selection. YPD – yeast peptone dextrose media (complete). pBD-SIMIP_N – N-terminal bait construct for plasma membrane aquaporin (TC32230), pAD-TIP – AD construct for tonoplast aquaporin (TC27563) isolated in the interaction screen, pAD-VAP – AD construct for VAP27-2 (TC28447) isolated in the interaction screen, pBD/AD MUT or WT – interaction control constructs coding for interacting sub-units of the λ repressor protein.