**Plasma Membrane H^+-ATPase and 14-3-3 Isoforms of Arabidopsis Leaves: Evidence for Isoform Specificity in the 14-3-3/H^+-ATPase Interaction**

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The plasma membrane H^+-ATPase is activated by binding of 14-3-3 protein to the phosphorylated C terminus. Considering the large number of 14-3-3 and H^+-ATPase isoforms in Arabidopsis (13 and 11 expressed genes, respectively), specificity in binding may exist between 14-3-3 and H^+-ATPase isoforms. We now show that the H^+-ATPase is the main target for 14-3-3 binding at the plasma membrane, and that all twelve 14-3-3 isoforms bound to the H^+-ATPase in vitro. Using specific antibodies for nine of the 14-3-3 isoforms, we show that GF14epsilon, mu, lambda, omega, chi, phi, nu, and upsilon are present in leaves, but that isolated plasma membranes lack GF14chi, phi and upsilon. Northern blotting using isoform-specific probes for all 14-3-3 and H^+-ATPase isoforms showed that transcripts were present for most of the isoforms. Based on mRNA levels, GF14epsilon, mu, lambda and chi are highly expressed 14-3-3 isoforms, and AHA1, 3, and 11 highly expressed H^+-ATPase isoforms in leaves. However, mass peptide fingerprinting identified AHA1 and 2 with the highest score, and their presence could be confirmed by MS/MS. It may be calculated that under ‘unstressed’ conditions less than one percent of total 14-3-3 is attached to the H^+-ATPase. However, during a condition requiring full activation of H^+ pumping, as induced here by the presence of the fungal toxin fusicoccin, several percent of total 14-3-3 may be engaged in activation of the H^+-ATPase.

**Keywords:** Arabidopsis — Isoforms — Plasma membrane H^+-ATPase — 14-3-3 protein.

14-3-3 proteins are small soluble proteins found in all eukaryotic organisms, and are typically encoded by relatively large gene families in multicellular organisms, whereas the genomes of unicellular organisms only harbor a few 14-3-3 genes (Rosenquist et al. 2000). Thus, there are 15 genes encoding 14-3-3s in the Arabidopsis genome (Rosenquist et al. 2001, DeLille et al. 2001), but only two genes in the Saccharomyces cerevisiae genome (van Heusden et al. 1995). The 14-3-3 proteins encoded by the S. cerevisiae BMH1 and BMH2 genes are essential for yeast growth but can be replaced by a plant homolog suggesting cross host functional activities for 14-3-3s (van Heusden et al. 1995). 14-3-3s appear as dimers and bind to particular, usually phosphorylated, motifs in other proteins (Muslin et al. 1996, Yaffe et al. 1997). Since 14-3-3s are abundant proteins and are able to bind to a large number of target proteins, they are involved in the regulation of many different processes. In plants 14-3-3s are involved in a multitude of physiological processes including signal transduction, and regulation of transcription and enzymatic activity. Perhaps the best-documented 14-3-3-regulated processes in plants are those of activation and inhibition of enzymatic activities. Thus, 14-3-3s control major metabolic pathways by regulating key enzymes such as nitrate reductase, sucrose phosphate synthase, and starch synthase, as well as key transporters, such as the plasma membrane H^+-ATPase (review, Sehnke et al. 2002).

Similar to the 14-3-3s, also the plant plasma membrane H^+-ATPase is encoded by a large gene family, with 12 genes found in the Arabidopsis genome and 10 in the rice genome (review, Arango et al. 2003). This family of H^+ pumping ATPases creates the electrochemical gradient across the plasma membrane which is the driving force for secondary active transport. The H^+-ATPase isoforms are activated by phosphorylation of the totally conserved, penultimate threonine in the C terminus and concomitant binding of 14-3-3 protein to the phosphorylated motif, resulting in displacement of a C-terminal autoinhibitory domain (Olsson et al. 1998, Fuglsang et al. 1999, Svennelid et al. 1999). A number of physiological stimuli are known to affect plasma membrane H^+-ATPase activity, and it has now been shown that blue light-induced stomatal...
openings via phosphorylation of, and 14-3-3 binding to guard cell plasma membrane H^+-ATPase. The fungus *Fusicoccum amygdali* exploits this mode of activation of the H^+-ATPase by producing the toxin fusicoccin (Ballio et al. 1964), which binds to and stabilizes the activated H^+-ATPase/14-3-3 complex (Jahn et al. 1997; Oecking et al. 1997). As a result, stomata are locked in an open position and the peach and almond trees attacked by the fungus wilt and die (Ballio et al. 1964). Although the fungus is host specific, its toxin fusicoccin has been found to stimulate H^+ extrusion from virtually all plant tissues, irrespective of origin (Marré 1979). This contradiction was recently explained by determination of the crystal structure of a complex containing a 14-3-3 protein, a phosphorylated peptide representing the last five C-terminal amino acids of a H^+-ATPase, and fusicoccin (Württele et al. 2003). The structure shows that fusicoccin is bound in the very conserved binding groove of the 14-3-3 such that the hydrophobic toxin only interacts with the hydrophobic side chain of the last amino acid (a highly conserved valine) in the C terminus of the H^+-ATPase.

Of the 15 genes encoding 14-3-3s in *Arabidopsis*, at least 13 are expressed (Wu et al. 1997, Rosenquist et al. 2001) including the recent report of a CDNA for the GF14pi isoform (gi28273162, National Center for Biotechnology Information, NCBI). Similarly, at least 11 of the 12 H^+-ATPase genes are expressed (review, Arango et al. 2003). The resulting large number of 14-3-3 and H^+-ATPase isoforms implies that expression of isoforms of both proteins may be organ-, tissue-, or cell-specific, or developmentally regulated, or only induced as a response to particular environmental cues, etc. For instance, transcript for the *Arabidopsis* 14-3-3 isoform GF14iota is only found in flowers (Rosenquist et al. 2001), and induction of a specific 14-3-3 isoform upon infection of barley with powdery mildew fungus (*Erysiphe graminis*) led to one of the initial discoveries of 14-3-3s in plants (Brandt et al. 2011). Regarding H^+-ATPase isoforms, *Arabidopsis* H^+-ATPase isoform 3 (AHAt3) is specifically expressed in phloem companion cells (DeWitt and Sussman 1995) and AHAt10 gene expression has been detected in developing seeds only (Harper et al. 1994).

The large number of 14-3-3 and H^+-ATPase isoforms also implies that specificity in binding may exist between 14-3-3 and H^+-ATPase isoforms. This is suggested by a study recording large differences in affinity between nine of the *Arabidopsis* 14-3-3 isoforms and a phosphorylated peptide representing the 14-3-3 binding motif in AHAt2 (Rosenquist et al. 2000), as well as by an vivo study indicating 14-3-3 isoform-specific interaction with the H^+-ATPase of broad bean (*Vicia faba*) guard cells (Emi et al. 2001). Furthermore, in vitro studies have indicated that the interaction between plant nitrate reductase and 14-3-3s is isoform-specific (Bachmann et al. 2000). Isoform specificity has also been described for several mammalian 14-3-3/target protein interactions, where 14-3-3 heterodimer formation as well as phosphorylation of the 14-3-3 protein itself may have a role (review, Aitken 2002). In the present work, we identify eight 14-3-3 isoforms in the *Arabidopsis* leaf as well as two plasma membrane H^+-ATPase isoforms. We show that the H^+-ATPase is the main target for 14-3-3 binding at the plasma membrane, and we show how the distribution of 14-3-3 isoforms to the plasma membrane is affected by fusicoccin treatment inducing increased binding of 14-3-3 to the H^+-ATPase. Taken together, our data suggest that there is indeed some isoform specificity in the 14-3-3/H^+-ATPase interaction in vivo.

**Results and Discussion**

**14-3-3 activation of H^+** pumping

Treatment of *Arabidopsis* leaves with fusicoccin resulted in an increase in proton pumping capacity of the plasma membrane vesicles isolated from the leaves (Fig. 1A), in agreement with previous investigations using other plant materials, such as spinach leaves (Johansson et al. 1993), radish seedlings (Rasic-Caldogno et al. 1993), oat roots (Lanfermeijer and Prins 1994), and suspension-cultured sugar beet cells (Babakov et al. 2000). The increase in proton pumping capacity was accompanied by an increased phosphothreonine level of the H^+-ATPase (Fig. 1C) and by a corresponding increase in 14-3-3 binding to the plasma membrane (Fig. 1D), whereas the amount of H^+-ATPase remained constant (Fig. 1B). A strong increase in 14-3-3 binding to the plasma membrane upon fusicoccin treatment has been observed with several materials, such as maize shoots (Jahn et al. 1997) and roots (Pullone et al. 1998), and leaves of *Commelina communis* (Oecking et al. 1997) and spinach (Olsson et al. 1998), and assumed to be due to binding to the H^+-ATPase. We now show that the H^+-ATPase is indeed the main target for 14-3-3 binding at the plasma membrane, both before (Fig. 2A) and after (Fig. 2B) fusicoccin treatment. The diffuse bands slightly above 31 kDa in Fig. 2A is probably due to binding to 14-3-3 monomers thus creating dimers. Furthermore, all 12 *Arabidopsis* 14-3-3 isoforms tested are able to bind to the H^+-ATPase, and all bind more strongly upon fusicoccin treatment, i.e. when a larger proportion of the H^+-ATPase is phosphorylated (Fig. 2; note that there is four times more protein per strip in A compared to B). That 14-3-3 binding to the H^+-ATPase is dependent upon phosphorylation of the penultimate threonine in the C terminus of the H^+-ATPase was first shown in planta using spinach leaves (Olsson et al. 1998, Svennelid et al. 1999) and then confirmed by expressing plant H^+-ATPases in yeast (Fuglsang et al. 1999, Svennelid et al. 1999, Maudoux et al. 2000). Physiological processes that involve activation of the plasma membrane H^+-ATPase by binding of 14-3-3 include blue light-induced stomatal opening (Kinoshita and Shimazaki 1999, Kinoshita and Shimazaki 2002), osmotic regulation (Babakov et al. 2000) and response to cold stress (Chelysheva et al. 1999), and this mode of regulation of the H^+-ATPase is expected to be relevant also in other physiological situations.
Isoform specificity of 14-3-3/H⁺-ATPase interactions

The 14-3-3 binding data shown in Fig. 2 suggest that all 14-3-3 isoforms bind to the H⁺-ATPase with rather similar affinity (less than 3-fold difference in Fig. 2A, and less than 2-fold in Fig. 2B), whereas previous data (Rosenquist et al. 2000) using surface plasmon resonance suggest larger differences (more than 10-fold) in binding between isoforms. To determine if there is any isoform specificity in 14-3-3/H⁺-ATPase interactions in vivo, we identified the 14-3-3 isoforms that are associated with the plasma membrane and those which remain in the soluble fraction upon plasma membrane isolation. We first used an antiserum reacting with all 14-3-3 isoforms (Fig. 3A) which indicated that the supernatant fractions contained more isoforms than the plasma membrane fractions, and that the major effect of fusicoccin was to increase the amount of 14-3-3 bound to the plasma membrane. We then used antibodies specific for nine of the 13 expressed isoforms of 14-3-3 with the same supernatant and plasma membrane fractions (Fig. 3B). All isoforms tested except GF14kappa were present in the supernatants, and the isoform patterns of the three supernatants (Sup 0 min, 30 min, and +FC 30 min) were rather similar. However, only a subset of the isoforms was bound to the control plasma membranes (PM 0 and 30 min), indicating some specificity in 14-3-3 binding in vivo. Thus, GF14epsilon, mu, lambda, omega, chi, phi, nu, and upsilon were present in supernatants, whereas control plasma membranes lacked GF14chi, phi and upsilon. By contrast, all isoforms found in supernatants, except GF14chi, were also found in the plasma membrane fraction after fusicoccin treatment.
This suggests a decrease in specificity upon fusicoccin treatment, resulting in recruitment of additional 14-3-3 isoforms to the plasma membrane. A decrease in specificity in the presence of fusicoccin is in good agreement with the data of Würtele et al. (2003), which suggest that the 14-3-3/H+-ATPase binding affinity is increased about 90-fold by the presence of fusicoccin in the 14-3-3 binding groove, which would render also low affinity 14-3-3 isoforms a high affinity.

Notably, the ‘supernatant fractions’ used are not identical to cytosol. In addition to cytosol, these fractions also contain soluble proteins from chloroplasts, mitochondria, nuclei, and other organelles that are broken during homogenization of the leaves. Although 14-3-3s are usually regarded as cytosolic proteins, they have also been shown to be present in nuclei (Bihn et al. 1997) and chloroplasts (Sehnke et al. 2000, Sehnke et al. 2001). More specifically, Arabidopsis GF14epsilon, mu, nu, and upsilon are found both in the cytosol and the chloroplast stroma (Sehnke et al. 2000). Thus, an explanation for the absence of GF14chi in the plasma membrane fractions, even in the presence of fusicoccin (Fig. 3B, PM+FC 30 min) could be that this isoform is not localized to the cytosol, but is restricted to some other compartment and therefore not accessible to the H+-ATPase. The use of fusicoccin provides an internal control for GF14phi and upsilon, which are only detected in the plasma membrane fraction upon fusicoccin treatment and hence accessible to the H+-ATPase but not bound in the absence of the toxin (Fig. 3B). Since upsilon (in contrast to phi) is present in well-detectable amounts in all supernatants, it can be concluded that GF14upsilon has a low affinity for the H+-ATPase in vivo.

The 14-3-3 isoform-specific antibodies were all raised against peptides unique to each isoform. This means that the titers of the different antisera differ and that staining intensities cannot be readily compared between isoforms, why Fig. 3B is only intended for qualitative analyses. The ~66 kDa bands seen in most of the immunoblots most probably represent dimers, since they were detected by antibodies raised against several different 14-3-3 peptides (Fig. 3B), as well as by antibodies raised against full-length 14-3-3s (Fig. 3A). Notably, lambda and phi were exclusively detected as dimers in these experiments (Fig. 3B).

Based on the staining intensities obtained using an antiserum recognizing all 14-3-3 isoforms (Fig. 3A), the relative

![Figure 3](https://academic.oup.com/pcp/article-lookup/doi/10.1093/pcp/pcp075)
amounts of 14-3-3 in the supernatant and plasma membrane fractions may be calculated. This suggests that under control conditions less than one percent of total 14-3-3 is attached to the plasma membrane; i.e. to the H⁺-ATPase (Fig. 2A). However, during a condition requiring full activation of H⁺ pumping, as induced here by the presence of fusicoccin, several percent of total 14-3-3 may be engaged in activation of the H⁺-ATPase.

Expression of 14-3-3 genes

Northern blots using isoform-specific probes for all 14-3-3 isoforms show that transcripts were present for most of the isoforms (Fig. 4A), in good agreement with reports of expressed sequence tags (ESTs; The Arabidopsis Information Resource, TAIR) for GF14epsilon, mu, lambda, omega, chi, phi, psi, and upsilon from either leaves or 'above ground' material. Based on transcript levels, GF14epsilon, mu, lambda and chi are highly expressed 14-3-3 isoforms in leaves (Fig. 4A). The isoforms for which we do not yet have specific antisera, i.e. GF14psi and the more recently identified GF14pi, omicron, and iota, only showed low levels of transcript and are therefore likely to be less abundant isoforms in leaves. Indeed, no ESTs have been reported for GF14pi, omicron, and iota (TAIR). No up- or down-regulation due to the different treatments was observed for the transcripts of any of the 14-3-3 isoforms.

Incubation with fusicoccin results in a strong hyperpolarization of the plasma membrane and probably a concomitant increase in cytosolic pH, which should be stressful conditions for the cell. The hyperpolarization is a rapid event with complete activation of the H⁺-ATPase occurring within minutes after fusicoccin application (Johansson et al. 1993). Therefore, any induced transcription of 14-3-3 genes due to hyperpolarization of the plasma membrane should have been recorded in our experiments where incubations with fusicoccin lasted for 30 min; however, no change in transcript levels was observed (Fig. 4A). This is in agreement with the data of Roberts and Bowles (1999) which only show a slow increase in 14-3-3 mRNA levels (3 h or more before induction was observed) upon exposure of excised tomato plants to fusicoccin. The slow induction seen with the 14-3-3 transcripts in cut seedlings may rather be related to the slow wilting induced by fusicoccin than to the more rapidly occurring hyperpolarization, as discussed by Roberts and Bowles (1999).

H⁺-ATPase isoforms

Most of the Arabidopsis H⁺-ATPase isoforms differ by one or more of the five C-terminal amino acids which contribute to the 14-3-3 binding motif as deduced from a crystal structure (Würtele et al. 2003). Hence, it is important to know which H⁺-ATPase isoforms are present in the Arabidopsis leaf plasma membrane, since any specificity in interaction will be determined by both partners – 14-3-3 and H⁺-ATPase. Also for the H⁺-ATPase, transcripts for most isoforms were found, although at low levels for most isoforms (Fig. 4B). An up-regulation of transcript for AHA1, 3, 4, and 11 was detected after 30 min vacuum-infiltration alone, whereas no further effect of fusicoccin was observed. However, there was no increase in total H⁺-ATPase after these 30 min as judged from the immunostaining of the H⁺-ATPase band (Fig. 1B), suggesting that 30 min was too short a time to cause any changes at the protein level. Judging from mRNA levels, AHA1, 3, and 11 should be major isoforms in leaves under unstressed conditions. However, mass peptide fingerprinting identified AHA1 and 2 with the highest score in all plasma membrane fractions, although masses corresponding to peptides unique to AHA3, 4, 8, and 11 were also recorded (data not shown). ESTs (TAIR) for AHA1 and 2 have
been reported from leaves, and for AHA1, 2, 3 and 11 from ‘above ground’ material, whereas no EST is recorded for AHA4. The low level of AHA2 transcript reported here agrees well with a previous investigation showing a weak signal for AHA2 mRNA in shoots compared to roots (Harper et al. 1990).

To positively identify H⁺-ATPase isoforms, peptides with masses unique to the respective isoforms were analyzed with MS/MS. These analyses readily gave sequence information for peptides corresponding to AHA1 and 2 (Fig. 5) but failed for the other isoforms, probably because not sufficient material was present. Thus, the MS data indicate that AHA1 and 2 are major isoforms in leaves. Additional isoforms are most probably present in leaves as indicated by both mRNA and MS data, and a recent proteomic study on the Arabidopsis leaf plasma membrane identified AHA4 and 11, as well as AHA1 and 2 (E. Alexandersson, personal communication). AHA3 has earlier been shown to be located exclusively to phloem companion cells (DeWitt and Sussman 1995), and should therefore be present in leaves but not constitute a major isoform.

Concluding remarks

The present study identifies the H⁺-ATPase as the main target for 14-3-3 binding at the plasma membrane, although it may be calculated that less than one percent of total 14-3-3 is attached to the H⁺-ATPase under ‘unstressed’ conditions – a figure which may increase to several percent under conditions requiring full activation of H⁺ pumping. 14-3-3 is known as an abundant protein. In Arabidopsis, this abundance seems to be achieved by expression of many isoforms simultaneously. Thus, transcripts for nine 14-3-3 isoforms were clearly detected in leaves of Arabidopsis, and eight isoforms were detected at the protein level using isoform-specific antibodies. Consequently, our data also suggest that only a few 14-3-3 isoforms will turn out to be organ specific. Furthermore, our data suggest that there is some specificity in the 14-3-3/H⁺-ATPase interaction in vivo. Thus, at least one 14-3-3 isoform, GF14upsilon, was only bound to the H⁺-ATPase in the presence of fusicoccin which increases binding affinity about 90-fold (Würtele et al. 2003). 14-3-3 target specificity is, hence, not absolute but only relative, as shown here by the ability of twelve 14-3-3 isoforms, including GF14upsilon, to bind to the H⁺-ATPase in
vitro. However, isoform specificity should be more pronounced in vivo than in vitro, due to competition between 14-3-3 isoforms for binding to target proteins in vivo. Future studies using immunolocalization and GFP-labelled 14-3-3s will be needed to reveal specificity at the tissue, cell or subcellular levels.

For the H+-ATPase, transcript levels for most isoforms were low, and higher levels were only observed for AHA1, 3, 4 and 11. The presence of these four isoforms, and of AHA2 and 8, was also indicated by MS identifying masses corresponding to peptides unique to these six isoforms. However, only AHA1 and 2 could be positively identified by MS/MS yielding sequence information. Interestingly, Arango et al. (2003) in a recent review divide the H+-ATPase isoforms in higher plants into five subfamilies and suggest that isoforms belonging to subfamilies I and II should be the most widely expressed. This is in good agreement with the data presented above, since in Arabidopsis, AHA1, 2 and 3 belong to subfamily II, and AHA4 and 11 constitute subfamily I.

Materials and Methods

Plant material

Arabidopsis thaliana ecotype Col-0 was grown on soil for 8 weeks at 22 °C with a 9 h light/15 h dark photoperiod (170 µE) and 70% relative humidity.

Plasma membrane isolation

Leaves (100 g) were cut into pieces and infiltrated at room temperature (ca. 21 °C) under vacuum with 170 ml of 330 mM sucrose, 10 mM MES-Tris, pH 6.0, 5 µM fusicoccin followed by 30 min incubation (‘+FC 30 min’ in figures). Controls lacked fusicoccin; one control was vacuum infiltrated as above (‘0 min’); a second control was not vacuum-infiltrated but homogenized immediately (‘0 min’) as described below. After removal of infiltration buffer from the vacuum-infiltrated samples, the leaf pieces were homogenized, using a knife blender, in 150 ml of 330 mM sucrose, 50 mM MOPS-KOH, pH 7.5, 5 mM EDTA, 0.6% (w/v) polyvinylpolypyrrolidone (PVPP), 0.2% (w/v) casein hydrolysate, 5 mM ascorbate, 5 mM dithiothreitol (DTT) (PVPP, ascorbate and DTT added immediately before use). Immediately after homogenization, phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 0.5 mM together with 1.5 ml of a protease inhibitor cocktail for plant cell and tissue extracts (P9599, Sigma, St. Louis, MO, U.S.A.) and either 5 mm or 74 mm wells. Gels were either stained with Coomassie Brilliant Blue R 250, or polypeptides were electro-photographically transferred to an Immobilon polyvinylidene difluoride (PVDF) transfer membrane (Millipore, Bedford, MA, U.S.A.) for immunostaining. After blocking in 2% (w/v) BSA, entire blots, or 5 mm strips cut from blots, were incubated with one of the following:

1. A polyclonal, rabbit antisera (1/1,000) raised against full-length 14-3-3s and recognizing all 14-3-3 isoforms.
2. Phosphothreonine-specific antibodies, affinity-purified from a polyclonal, rabbit antisera (Zymed, San Fransisco, CA, U.S.A.) used according to the manufacturer’s instructions.
3. 14-3-3 isoform-specific, polyclonal, rabbit antisera (1/10,000) produced by Bioworld (U.S.A.) using conjugated peptides derived from Arabidopsis GFI4epsilon, mu, kappa, lambda, omega, chi, phi, nu, and epsilon (P.C. Schinke and R.J. Ferl, unpublished data).
4. Twelve different His-tagged, recombinant Arabidopsis 14-3-3 isoforms in a buffer containing 20 mM HEPES-KOH, pH 7.7, 5 mM MgCl₂, 75 mM KCl, 0.1 mM EDTA, 1 mM DTT, 3% (w/v) BSA, and 0.04% (w/v) Tween 20 (Fullone et al. 1998) to produce 14-3-3 overlays. After incubation at room temperature for 3 h, the membranes were washed and incubated with a monoclonal, anti-tetrahistidine, mouse antibody (1/2,000) (VWR, Sweden).

All blots were finally incubated with horseradish peroxidase-conjugated secondary antibody (anti-rabbit or anti-mouse) and binding visualized by enhanced chemiluminescence (Amersham, U.K.).
Mass spectrometry

The H^+-ATPase band obtained after SDS-PAGE of isolated plasma membranes was digested in gel with trypsin and the resulting peptides were subjected to mass spectrometry according to Wilm et al. (1996) using a Q-tof and Masslynx software (Micromass, U.K.). Peptide fingerprinting and sequence analyses were carried out using the Mascot search engine (http://www.matrixscience.com/).

Expression and purification of 14-3-3s

The recombinant His-tagged 14-3-3 proteins from A. thaliana (GF14s) were expressed and purified on Ni^{2+} HiTrap as described in Svennelid et al. (1999).

Total RNA preparation

Leaves were harvested and a part was saved as control (‘0 min’ in figures). The remaining leaves were vacuum infiltrated for 30 min as above with a medium lacking or containing 5 μM fusicoccin (‘30 min’ and ‘-FC 30 min’, respectively). The plant material was ground in liquid nitrogen using pestle and mortar, and RNA was extracted with the conventional phenol : chloroform method. Lithium chloride (2 M) was used to precipitate the RNA.

Expression analysis using Northern blots

A denaturing 1.5% (w/v) agarose gel with formaldehyde and ethidium bromide was loaded with 20 μg total RNA per lane and run for 3 h at 5 V cm\(^{-1}\). RNA concentrations were determined spectrophotometrically. The gel was photographed in UV-light to confirm the integrity and equal loading of RNA. The RNA was blotted onto a Hybond N+-membrane (Amersham Biosciences, Sweden) using capillary transfer. UV-light was used to cross-link the RNA to the membrane. The membrane was pre-hybridized for 30 min and then hybridized with 32P-labeled probe overnight at 60°C. The membrane was pre-hybridized for 30 min and then hybridized with 32P-labeled probe overnight at 60°C using ExpressHyb (Clontech, Palo Alto, CA, U.S.A.) hybridization solution. The membranes were washed three times for 10 min at 50°C with 300 mM NaCl, 30 mM Na-citrate, pH 7.5, 0.1% (w/v) SDS and twice for 15 min at 50°C with 15 mM NaCl, 1.5 mM Na-citrate, pH 7.5, 0.1% (w/v) SDS. Hybridization was recorded using a Storm (Amersham Biosciences, Sweden).

Isoform-specific probes were designed using the first 300 bases of the 3′-UTR region in the predicted mRNAs. The probes were BLASTed against the NCBI EST database to verify the UTRs. If no EST was found, probes were chosen to be the first 300 bases. Whenever ESTs were found, probes were designed to cover the 3′-UTR region of the respective mRNAs. This resulted in probes of 240–300 bases. The probes were labeled, according to the manufacturer, using Rediprime II labeling kit (Amersham Biosciences, Sweden) and Redi- vue [γ-32P]dCTP triethylammonium (Amersham Biosciences, Sweden) mixed with 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. Probes were purified using QIAGEN Nucleotide Removal Kit (VWR, Sweden) according to the manufacturer’s instructions. Melting points for all GF14 probes were within 75–78°C, and for all AHA probes within 73–77°C (calculated at http://www.basic.nwu.edu/biotools/oligo-calc.html), which should ensure full hybridization at the temperature used, 60°C.

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References


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