The plasma membrane aquaporin NtAQP1 is a key component of the leaf unfolding mechanism in tobacco

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Summary

Epinastic leaf movement of tobacco is based on differential growth of the upper and lower leaf surface and is distinct from the motor organ-driven mechanism of nyctinastic leaf movement of, for example, mimosa species. The epinastic leaf movement of tobacco is observed not only under diurnal light regimes but also in continuous light, indicating a control by light and the circadian clock. As the transport of water across membranes by aquaporins is an important component of rapid plant cell elongation, the role of the tobacco aquaporin Nt aquaporin (AQP1) in the epinastic response was studied in detail. In planta NtAQP1-luciferase (LUC) activity studies, Northern and Western blot analyses demonstrated a diurnal and circadian oscillation in the expression of this plasma membrane intrinsic protein (PIP1)1-type aquaporin in leaf petioles, exhibiting peaks of expression coinciding with leaf unfolding. Cellular water permeability of protoplasts isolated from leaf petioles was found to be high in the morning, i.e. during the unfolding reaction, and low in the evening. Moreover, diurnal epinastic leaf movement was shown to be reduced in transgenic tobacco lines with an impaired expression of NtAQP1. It is concluded that the cyclic expression of PIP1-aquaporin represents an important component of the leaf movement mechanism.

Keywords: aquaporin, leaf movement, tobacco, NtAQP1.

Introduction

Families of Fabaceae, Maranthaceae, and Oxalidaceae use special motor organs, the pulvini, for movement of leaves or leaflets. We previously showed that the PIP2-class aquaporin SsAQP2 from rain tree (Samanea saman) is a component of this motor organ (Moshelion et al., 2002). SsAQP2-aquaporin showed expression in a diurnal and circadian manner with highest specific RNA accumulation in the morning, correlated with a changing cellular water permeability and pulvini activity. However, in numerous other plants, leaves move without any specific motor organ. In these cases, the upper and lower sides of the leaf and petiole grow with different velocities, causing a change in leaf position relative to the shoot axis. This differential growth response is quite effective with regard to leaf movement, as demonstrated for example in tobacco (Otto and Kaldenhoff, 2000). The epinastic leaf movement mechanism resembles in some instances those of the gravitropic or photonastic responses, which are also the result of differential growth. Cell growth is initiated by local cell wall loosening (Cosgrove, 1996) and osmotic driving forces, which are activated by proton pump-powered fluxes of ions (Moran et al., 1996; Philippar et al., 1999). Water simply follows the osmotic gradient that has thus been established and causes a volume change resulting in cell enlargement and a growth reaction. The leaf movement-correlated growth response is a relatively fast reaction. It most likely requires high rates of water transport, more than those achieved by passive diffusion across the lipid bilayer. Such rapid uptake of water may be facilitated by aquaporins (Javot and Maurel, 2002; Maurel, 1997; Tyerman et al., 2002). Members of this protein family may therefore play a role in the tobacco epinastic leaf movement.

We previously isolated a tobacco PIP1-type aquaporin (NtAQP1) and described its expression (Biela et al., 1999; Otto and Kaldenhoff, 2000). In these studies, elevated NtAQP1 activity was detected in the basal part of leaf petioles, and it was suggested that the aquaporin activity in these cells may be related to the daily epinastic leaf movement in tobacco. However, evidence of aquaporin function in this specific growth response is still lacking. Here, we describe several observations which, in combination, link aquaporin NtAQP1 function to epinastic leaf movement...
movement: (i) the NtAQP1-aquaporin transcriptional activity, as monitored with a luciferase (LUC) reporter gene, was positively correlated to epinastic leaf unfolding; (ii) the NtAQP1 mRNA and protein steady-state levels showed diurnal variations, and peak levels were also correlated to leaf unfolding; and (iii) the silencing of NtAQP1 expression resulted in a reduction of cellular water permeability of protoplasts obtained from petiole tissues and an inhibition of the epinastic leaf movement in tobacco plants. Together, these results indicate that PIP1-aquaporins, i.e. NtAQP1, are essential components of the epinastic growth response.

Results

Transcriptional regulation of NtAQP1

Considering that NtAQP1-facilitated plasma membrane water transport could be a significant factor of the diurnal leaf movement mechanism, NtAQP1 expression ought to coincide with the activity peaks during the day/night cycle. Modification of aquaporin activity by transcriptional regulation is one of the several steps where this membrane water permeability can be controlled. The 5’ upstream sequence of genes contains the binding sites of regulatory factors and the 1374 bp promoter region of NtAQP1, used with the LUC reporter gene, was searched for DNA sequence motifs that could serve as a recognition site for specific types of DNA-binding factors, and it turned out to be rather complex with this regard. Several distinct elements were identified (Figure 1), which relate to signaling of the phytohormones gibberellin (GA) and abscisic acid (ABA), myeloblastosis (MYB)-binding sites (Abe et al., 1997; Gubler et al., 1995; Urao et al., 1993), expression in pollen (Bate and Twell, 1998), and regulation by light (Buchel et al., 1999; Giuliano et al., 1988; Green et al., 1988; Rose et al., 1999; Terzaghi and Cashmore, 1995). In addition, a sequence motif was found that has been associated with circadian expression (Piechulla et al., 1998). In a previous publication, we were able to show that this 1374 bp upstream region is sufficient for the regulation of the NtAQP1 gene (Siefritz et al., 2001).

NtAQP1-luciferase reporter gene activity during tobacco development

In order to monitor the NtAQP1 gene activity during plant development and in relation to the epinastic leaf movement, tobacco was transformed with an NtAQP1-LUC reporter gene. Expression of the chimeric reporter gene was monitored in F1 progeny plants and that of one representative transgenic line is given. LUC activity was monitored throughout development, from germination until flowering. No LUC activity was detected in welled seeds or in the radicle during penetration of the seed coat at germination (not shown). However, after emergence of the radicle, LUC activity became detectable throughout the seedling, especially near the root tip, a site with assumed high water mobility from and to the symplast (Figure 2a). In 3-week-old seedlings, the gene activity in leaves was highest in the leaf petiole (Figure 2b), while in growing tobacco plants, activity could be detected throughout the plant. Activity was mostly associated with regions of cell expansion (Figure 2c). Figure 2 shows the activity in the inflorescence of tobacco. Images of LUC activity in flowers of increasing developmental stage again illustrate that NtAQP1 is mainly active during stages of cell elongation (Figure 2e). In mature flowers, NtAQP1-LUC activity was detectable in sepals and petals, in anther filaments (especially shortly before dehiscence), and in the pistil (Figure 2f.g). No LUC activity was detected in mature closed locules because luciferin does not penetrate these structures. However, high LUC activity was visible in mature pollen in open anthers after dehiscence and in pollen on pollinated stigmas (Figure 2f). We pollinated an untransformed tobacco with pollen from

Figure 1. Putative DNA-binding sites in the NtAQP1 promoter. Transcription start site indicated by +1. Regulatory protein-binding sites were detected by sequence similarity. MYB-binding sites (Abe et al., 1997; Gubler et al., 1995; Urao et al., 1993) are named and marked in gray, pollen-specific sites (POLLEN, LE, LAT52; Bate and Twell, 1998) were given as black arrows, root-specific sites (ROOT, MOTIF, TAPOLX; Elmayan and Tepfer, 1995) in black dotted arrows, light-regulation sites (GT1, l-boxes; Buchel et al., 1999; Green et al., 1988) are named and marked in dotted lined arrows. The circadian element are marked by a boxed arrow (CircadianLeLHC; Piechulla et al., 1998) as indicated. Orientation of the motifs corresponds to the orientation of the arrows.
Diurnal and circadian activity of NtAQP1-luciferase in leaf petioles

Diurnal oscillations in NtAQP1 gene activity could imply a link between NtAQP1 function and diurnal epinastic leaf movement. We therefore monitored the in planta activity of the NtAQP1 promoter in 3-week-old seedlings over several days (8-h light/16-h darkness illumination regime; see Experimental procedures). The images show that NtAQP1-LUC activity in most of the leaf blade is low and constant. In contrast, it was high in the petioles and showed distinct diurnal oscillations. The LUC activity peak phase coincided with leaf unfolding (Figure 3a; Fig. S1). When seedlings were entrained for 3 weeks in a 16-h light/8-h dark regime and then placed in continuous darkness, the LUC activity showed sustained oscillations in the leaf petioles over the first 24 h. Similarly, epinastic leaf movement was sustained under these conditions (Figure 3b; Fig. S2) indicating that both epinastic leaf movement and the expression of NtAQP1 are under control of the circadian clock. Quantification of the LUC-bioluminescence indicated that the activity of the NtAQP1 promoter is correlated to petiole growth (Figure 3c).

Diurnal oscillations in NtAQP1 mRNA and protein levels in leaf petioles

Although the results with the LUC reporter gene indicate diurnal oscillations in NtAQP1 transcription, it depends on the NtAQP1 RNA and protein stability whether these oscillations will be reflected at the level of NtAQP1 protein activity. We therefore also analyzed NtAQP1 RNA and protein steady-state levels in petioles at different times of the day by Northern blot analysis and in situ protein detection. Tobacco plants were subjected to a day/night (12 h/12 h) regime with a darkness period from 6:00 PM to 6:00 AM. The determination of NtAQP1 mRNA levels started at 6:00 AM with highest values as leaves were unfolding. Minimum signal intensity was obtained at 2:00 PM, which slightly increased when leaves were folding (6:00 PM) and during the subjective night (Figure 4a). Using an NtAQP1-specific antibody, the protein was detected in sections of leaf petioles taken at different times of the day. As demonstrated in Figure 4, the accumulation of NtAQP1 reaches a maximum at 8:00 AM and a minimum in the late afternoon (8:00 PM) and early morning (6:00 AM). This closely follows the maximum and minimum that were observed for NtAQP1 mRNA levels. Although the coincidence of NtAQP1 mRNA- and protein-level peak phase and the phase of leaf unfolding are remarkable, it is by itself not a conclusive evidence for a functional relation between leaf movement and aquaporin function.
Changes in cellular water permeability of petiole protoplasts

A first indication that the function of PIP1-type aquaporins could indeed play a significant role in growth-related leaf movement came from determination of petiole protoplast water permeabilities. Petioles of control plants were harvested and treated with cell wall-degrading enzymes in a way that the protoplast water permeability could be measured at times when NtAQP1 accumulation was high (8:00 AM) or low (8:00 PM). The mean of the protoplasts swelling kinetics clearly showed a difference between these two times of day (Figure 5a) in control plants. At average, a faster volume increase was obtained from material harvested in the morning, which corroborates the hypothesis that a change in membrane water permeability occurs during the day/night cycle. Evidence for the participation of NtAQP1 in that process is provided by the permeability values of protoplasts obtained from antisense plants at 8:00 AM. They showed low swelling rates, very similar to that of control protoplasts at 8:00 PM. Accordingly, the distribution of osmotic cellular water permeability peaked at higher values in the case of control protoplasts from material harvested at 8:00 AM, when compared to controls.
at 8:00 PM or antisense NtAQP1 lines at 8:00 AM. A confirmation that the expression of an NtAQP1 antisense construct did indeed affect the NtAQP1 mRNA (Siefritz et al., 2002) and protein level is given in Figure 5(c). The mRNA level was drastically reduced, and the protein was not detectable by Western blot. Taken together, the data indicate that NtAQP1 function is required for increased cellular water permeability in the morning when leaves unfold. It can also be concluded that the folding reaction at the end of the day does not require increased cellular water permeability of petiole protoplasts. Apparently, leaf folding or unfolding was based on separate mechanisms, the former not including, the latter including an increase of membrane water permeability.

Inhibition of NtAQP1 expression affects epinastic leaf movement

A synchronous expression of NtAQP1 with the epinastic reaction and a corresponding change in cellular water permeability could also reflect unrelated processes that share the same time phase. However, if NtAQP1-facilitated plasma membrane water transport is a significant factor of the diurnal leaf movement mechanism, inhibition of NtAQP1 expression should also affect epinastic leaf movement. It was therefore analyzed in plants in which aquaporin gene expression was inhibited by the introduction of an antisense NtAQP1 gene. We have previously shown that inhibition of gene activity in these antisense NtAQP1 plants is specific for PIP1-like aquaporins (Siefritz et al., 2002). Four independent NtAQP1 antisense plants were compared to control plants with regard to their capacity in leaf movement. Folding and unfolding was evaluated by measuring the angle between leaf blade and shoot axis at different times of the day. Figure 6(a) shows a time course of the leaf movement from antisense NtAQP1 lines and control plants under diurnal light/dark regime, while Figure 6(b) illustrates the results of plants that were kept under continuous dimmed light after being entrained in a day/night cycle. Data from Figure 6(c) indicate that the oscillation of NtAQP1 levels was reduced during the circadian reaction in a similar way as the leaf movement itself. A striking difference between control and antisense plants was observed, indicating an inhibition of epinastic leaf movement in the antisense plants. The graphs show that the leaf angles for control plants (left) with normal NtAQP1 expression reached a maximum of about 58° at 1:00 PM and a minimum of about 18° at 12:00 AM (40° difference in leaf angle). In contrast, the plants impaired in NtAQP1 expression show a maximum/minimum difference of only about 19°. It can therefore be concluded that expression and function of NtAQP1 is an important component of the leaf unfolding mechanism, assuring a normal diurnal leaf movement in tobacco.
Promoter elements and promoter activity throughout plant development

We have used a LUC reporter gene to investigate the expression of the NtAQP1 gene during tobacco development and to study the activity in leaf petioles in relation with epinastic leaf movement. Increased transcription rates were detected in roots and shoots, however, only after emergence of the radicle from the seed. The lack of activity in germinating seeds indicates that this aquaporin gene is not involved in water transport related to cell elongation in the radicle during germination. Possibly, other members of the aquaporin gene family in tobacco are responsible for the increase in cell volume that causes penetration of the radicle through the seed coat. Expression of NtAQP1-LUC in leaf and stem tissue during development was mainly associated with zones of cell elongation. Also, in fully expanded leaves, a low level of promoter activity remained detectable. The NtAQP1 promoter contains an element that has been implicated in pollen-specific expression of the late anther tomato (lat52 gene (Bate and Twell, 1998). This gene
is expressed during pollen development and pollen germination. Similarly, NtAQP1 promoter activity was observed in developing and germinating pollen (Figure 2f,h). Other elements that were identified in the NtAQP1 promoter suggest a possible regulation by GA, ABA, and the circadian clock. Treatment of germinated seedlings with GA did not directly affect promoter activity within 12 h, but seedlings germinated on GA showed longer zones of elongation associated with higher levels of NtAQP1 expression (not shown). Expression analysis over a period of days indicated that diurnal or circadian oscillations in NtAQP1 activity are mainly restricted to the leaf petioles. This raises the interesting question of whether this response is because of localized high concentration of clock transcription factors or other local signals that act in conjunction with a general clock signal throughout the different tissues.

Diurnal activity in leaf petioles correlated with leaf unfolding

Activity of the tobacco aquaporin NtAQP1 promoter in leaf petioles was shown to be strongly correlated with the diurnal epinastic leaf unfolding movement. The very similar oscillations in NtAQP1 mRNA and protein levels indicate a rapid turnover of both, specific messenger and protein. This results in a close link between NtAQP1 transcriptional and protein activities. It can be concluded that, at least for the leaf unfolding mechanism, a regulation of NtAQP1 activity is mainly realized by transcription. Nevertheless, an additional post-translational regulation as demonstrated for bean (Maurel et al., 1995) or spinach aquaporins (Johansson et al., 1998) cannot be excluded. However, with regard to the great amplitude in NtAQP1 transcriptional activity in petioles, it is likely that here protein modification is of minor significance.

It appeared that the mechanism of epinastic leaf response requires an increased membrane water flux at sites of high NtAQP1 expression. In situ protein analysis revealed that these sites were associated with the xylem and the epidermal as well as subepidermal layers of the petiole. The antibody signal is, however, not biased towards the abaxial or adaxial side. It seems that higher water permeability is required on both, although cells on the adaxial side should elongate comparably more, when leaves unfold. Thus, a direct quantitative relationship between NtAQP1-aquaporin concentration and growth velocity was not observed. However, significant differences between cellular water permeabilities in petioles during folding or unfolding indicate that an increased plasma membrane water flux is required only for the latter. Plants impaired in NtAQP1 expression by a 3SS CaMV promoter-driven antisense NtAQP1 construct did not show the increase in cellular water permeability at the onset of the day. The values were comparable to those from controls in the evening. Apparently, two different mechanisms operate at the folding or unfolding reaction.

The antisense lines were reduced in leaf movement, which suggests that NtAQP1 is an important component of the mechanism leading to the epinastic response. Here, the question arises, how the reduction of NtAQP1 caused such a dramatic effect. In the case of the Arabidopsis homolog aquaporins, an inhibitory effect on PIP2 activity in PIP1b antisense plants and vice versa was discussed (Martre et al., 2002). If this is also the case in tobacco petioles remains to be determined.

We were able to show in a previous paper that the antisense plants used for these experiments have a decreased specific hydraulic root conductivity as a consequence of the reduced NtAQP1 expression (Siefrizt et al., 2002). It is possible that this situation induced physiologically water stress, comparable to conditions with increased soil salt concentration. It has been shown that rapid environmentally induced changes in water status can result in a signal emitted from the roots that causes reduced leaf growth (Passioura and Munns, 2000). However, a permanent decrease in NtAQP1 expression is probably more like a long-term salt treatment, which eventually leads to comparable overall growth rates (Munns et al., 2000). Conformingly, the aquaporin-antisense plants were not smaller or showed a generally reduced leaf growth. Considering this, a direct function of NtAQP1 in the mechanism and at the sites of the epinastic leaf movement can be considered during the unfolding reaction. The diurnal and circadian leaf movements of tobacco are the effect of a differential growth reaction, which includes cell division and elongation (Poethig and Sussex, 1985). For intervenial strips, an auxin specificity of the bending reaction in tobacco was shown (Keller and Van Volkenburgh, 1998). The epinastic growth resulted from relatively greater auxin-induced growth by the adaxial epidermis and underlying palisade mesophyll than by the abaxial epidermis. However, the growth reaction does not involve acid growth (Hager et al., 1971) and is not affected by ethylene (Keller and Van Volkenburgh, 1997). Other signals, responsible for sudden changes of cell elongation rates, could be ABA (Audran et al., 1998; Davies and Zhang, 1991), pH (Bacon et al., 1998), or GA, which showed a diurnal concentration variation in leaf growing zones (Foster and Morgan, 1995). GA and ABA also regulate the NtAQP1 gene activity as indicated by consensus sequences in the NtAQP1 promoter and demonstrated by transient transformation of promoter–reporter gene constructs into tobacco protoplasts (Siefritz et al., 2001). Thus, the mechanism of epinastic growth during leaf unfolding could well be regulated via GA- and/or ABA-induced processes. These also include the activation of ion transport mechanisms across the plasmalemma (Van Volkenburgh, 1999), and it remains to be elucidated if there is a common regulation between ion and water channels and at which level it is realized.
Experimental procedures

Plant data assessment

Wild-type and transgenic *Nicotiana tabacum*, cultivar Samsun, were grown under greenhouse conditions: 16–25 °C; relative humidity (RH), 60–70%; day/night rhythm, 16 h/8 h. Plants were grown in soil and subjected to continuous dimmed light for the investigation of circadian movement of leaves.

Construction and characteristics of *NtAQ1* antisense lines were described in detail by Siefritz et al. (2002). Briefly, *NtAQ1* 1.2-kbp full-length cDNA (AJ001416.1; GI: 23853771) was cloned in an inverse orientation into the binary vector pGPTV (11 kbp) 3’ to a 35S CaMV promoter and 5’ to a Nopaline-Synthetase Terminus. The resulting plant expression vector (pNtAQ1AS) was transformed into *Escherichia coli* TOP10F. *Agrobacterium tumefaciens* LBA4404 was transformed with pNtAQ1AS by triparental mating. Transformation and regeneration of transgenic tobacco were performed following the protocol provided by Gallois and Marinho (1995), but by substituting cefotaxime by 500 mg l⁻¹ carbenicillin. The F₂ progeny of four independently transformed lines were chosen for further investigation. All lines show an overall reduction of *NtAQ1* expression levels between 80 and 95% (Siefritz et al., 2002).

Detection of *in planta* luciferase activity

The *NtAQ1* 1.4-kb promoter was cloned into the binary vector pGPTV (11 kbp) 5’ to the functional *luc* cDNA (De Wet et al., 1995) and the CaMV polyadenylation signal. The resulting plant expression vector pNtAQ1Prom::luc was transferred into *A. tumefaciens* LBA4404 by heat shock. Transformation and regeneration of transgenic tobacco were performed as mentioned above. Three independent transgenic lines were compared and they showed similar expression patterns.

The short half-life of the *luc* mRNA and protein, and especially the very limited regeneration of the LUC protein following reaction with luciferin in the absence of CoA, leads to the conclusion that *in vivo* LUC activity is very closely related to on going transcriptional activity of the promoter that drives reporter gene expression. Gene activity is monitored with a 2D-luminometer consisting of an intensified charged coupled device (CCD)-camera (C2400-77, Hamamatsu Photonics, Japan) or a nitrogen-cooled slow-scan CCD camera (512-TBK, Roper Scientific, Uianen, the Netherlands). The (semi)-continuous monitoring of LUC activity in seedlings grown under 16 h light/8 h dark regime was performed by measuring LUC activity at 10-min intervals in the dark, alternated by (during the day period) 20 min of illumination, or (during the night period) 20 min of darkness. Illumination of the plant was carried out through computer-controlled glass fiber optics. This setup allows the monitoring of LUC activity in mature large plants over a period of several days. The photon emission by LUC-expressing plants was quantified by METAPHOR software. Images of LUC activity are depicted with false color scale that increases from blue, green, yellow, red to white.

Northern blot analysis

Total RNA was isolated from tobacco petioles using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Ten micrograms of the total RNA from different preparations was size-fractionated in a 1% denaturing agarose-formaldehyde gel and transferred to a nylon membrane (Hybond N, Amersham Pharmacia, UK) by capillary blotting. The nucleic acids were UV-cross-linked and pre-hybridized in Roti-Hybi-Quick (Roth Chemikalien, Karlsruhe, Germany) solution. Hybridization to 32P-labeled probes and washing were performed at 68 °C following the manufacturers’ guidelines. The air-dried membrane was exposed to X-ray film (X-Omat, Kodak, Rochester, NY, USA) with intensifying screen at –80 °C, and the film was developed after an appropriate time period. The resulting signals were quantified by IMAGE MASTER VDS software (Pharmacia Biotech, Uppsala, Sweden). Values obtained for *NtAQ1* were related to those of the tobacco 28S rRNA after stripping and re-hybridizing with a corresponding 32P-labeled probe. The highest value obtained was set to 100%. All hybridizations were performed three times. DNA sequence used for *NtAQ1* probes was derived from the 5’ untranslated region, which is *NtAQ1*-specific and does not cross-react to other aquaporin-related sequences.

In situ protein detection

Sections were taken from petioles by hand-cutting with a razor blade. The sections were treated essentially as described by Otto and Kaldenhoff (2000), and the same *NtAQ1*-specific antibody was used. Tissues were immediately fixed in a solution of phosphate buffered saline (PBS; 58 mM Na₂HPO₄, 15 mM NaH₂PO₄, 68 mM NaCl (pH 7.4), and 6.7 mM EGTA) with 6% formaldehyde for 25 min at room temperature. After fixation, the material was stored in ethanol at 4 °C. For immunolocalization, the specimens were washed three times in PBS, 0.1% Tween 20 for 5 min and incubated at 4 °C overnight in the same buffer containing a 1 : 500 dilution of the respective serum against the *NtAQ1* N-terminus. The samples were washed in PBS, 0.1% Tween 20 and incubated with the secondary antibody, which was targeted to the serum and conjugated with alkaline phosphatase. The final washing was carried out in a buffer containing 100 mM NaCl, 50 mM MgCl₂, 100 mM Tris·HCl (pH 9.5), 0.1% Tween 20, and 1 mM Levamisol (ICN, Eschwege, Germany). Alkaline phosphatase activity was visualized by incubation in nitro benzenazulon/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) solution (Boehringer, Mannheim, Germany) as long as controls were not stained. All immunological experiments were paralleled by assays with pre-immuniserum in order to test the specificity of the reaction. The color reaction revealed no cases of substantial staining, indicating a low background of the *NtAQ1* serum. Antibodies were raised against the protein-specific hydrophilic N-terminal region of *NtAQ1*. The serum was tested for cross-reaction with other aquaporins using *in vitro* synthesized aquaporins of the PIP1, PIP2, or TIP families.

Preparation of petiole protoplasts and water uptake measurement

Petioles were harvested 2 h before water uptake measurements. Free hand-cut slices from the relevant areas were treated with an enzyme solution according to Siefritz et al. (2002). By microscopic inspection, it was confirmed that the major protoplast fraction contains principally only epidermal and cortex cells. The experimental set-up for Ph₅ determination and calculation is given by Moshelion et al. (2002) and Siefritz et al. (2002).

Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/support/TP/TP1947/TP1947sm.htm

Fig. S1. Movie of *NtAQ1* expression in petioles of tobacco.

Fig. S2. Seedlings, corresponding to Figure 3.
References


