Arabidopsis ANTR1 Is a Thylakoid Na\(^{+}\)-dependent Phosphate Transporter

**FUNCTIONAL CHARACTERIZATION IN ESCHERICHIA COLI**

Lorena Ruiz Pavón, Fredrik Lundh, Björn Lundin, Arti Mishra, Bengt L. Persson, and Cornelia Spetea

In this study, the putative anion transporter 1 (ANTR1) from *Arabidopsis thaliana* was shown to be localized to the chloroplast thylakoid membrane by Western blotting with two different peptide-specific antibodies. ANTR1 is homologous to the type I of mammalian Na\(^{+}\)-dependent inorganic phosphate (Pi) transporters. The function of ANTR1 as a Na\(^{+}\)-dependent Pi transporter was demonstrated by heterologous expression and uptake of radioactive Pi into *Escherichia coli* cells. The expression of ANTR1 conferred increased growth rates to the transformed cells and stimulated Pi uptake in a pH- and Na\(^{+}\)-dependent manner as compared with the control cells. Among various tested effectors, P\(_i\) was the preferred substrate. Although it competed with the uptake of P\(_i\), glutamate was not transported by ANTR1 into *E. coli*. In relation to its function as a P\(_i\) transporter, several physiological roles for ANTR1 in the thylakoid membrane are proposed, such as export of P\(_i\) produced during nucleotide metabolism in the thylakoid lumen back to the chloroplast stroma and balance of the trans-thylakoid H\(^{+}\) electrochemical gradient storage.

Solute and metabolite transporters play essential roles in physiological processes, including nutrient uptake, cell homeostasis, signal transduction, growth, and stress responses in every living organism. In the plant chloroplast, the photosynthetic organelle, most transporters have been identified and biochemically characterized from the envelope membrane (1, 2). Among them, there are several translocators for inorganic phosphate (P\(_i\)), all functioning as antiport systems using P\(_i\) or phosphate as well as Pi by two independent mechanisms (3, 4). Much less information is available for transport processes across the chloroplast thylakoid membrane, which is mostly studied as the site of light-driven photosynthetic reactions coupled to ATP synthesis. Only a few thylakoid transporters have been identified and functionally characterized. Examples are ATP transport across spinach thylakoid membrane into the lumenal space and a thylakoid ATP/ADP carrier identified and characterized in *Arabidopsis thaliana* (5, 6). An active nucleotide metabolism in the thylakoid lumen (5) implies the existence of additional, yet unidentified, transporters, such as those recycling P\(_i\), to the soluble stroma.

A few Na\(^{+}\)-coupled P\(_i\) transporters (NaPi) have in recent years been reported in green algae and vascular plants (7, 8). NaP\(_i\) systems are known to be mostly active in mammals, whereas H\(^{+}\)-coupled P\(_i\) transport is dominant in plants (4). Three NaPi types have been described in eukaryotes, NaPi-II and III being the main ones in mammals (9–11). NaPi-II type plays the role of an intracellular P\(_i\) accumulation system, whereas NaPi-III type has the characteristics of a housekeeping system (9). The molecular mechanisms controlling the NaPi-II and III uptake systems have recently been reviewed (11). NaPi-I represents a group of proteins for which the endogenous substrate, ionic coupling, and physiological function are still under debate. Heterologous expression of the rabbit renal NaPi-I and the human orthologue NPT1 in *Xenopus laevis* oocytes have implicated type I in Na\(^{+}\)-dependent P\(_i\) transport as well as in a channel-like conductance of organic and inorganic anions (10). The first identified vesicular glutamate transporters, VGLUT1 and VGLUT2, were initially also characterized as NaPi-I type. Most recently, VGLUTs have been shown to transport glutamate as well as P\(_i\) by two independent mechanisms (12).

In *A. thaliana*, there are six genes encoding anion transporters (ANTR1–6), sharing homology with the NaPi-I members. The ANTR1 and ANTR2 proteins have been localized to the chloroplast and the latter precisely assigned to the inner envelope using proteomics and immunodetection with a peptide-specific antibody (1, 13). The subcellular location of ANTR3–6 has not yet been addressed experimentally. Moreover, no function of the ANTRs has yet been determined.

In the present study, we show that the recombinantly expressed *Arabidopsis* ANTR1 facilitates Na\(^{+}\)-dependent P\(_i\) transport into *Escherichia coli*. This bacterium contains two H\(^{+}\)-coupled P\(_i\) uptake systems, namely the low affinity system Pit and the high affinity system Pst (14, 15). There are no known...
NaP, transporters in E. coli, which makes this organism suitable for heterologous expression and functional characterization of ANTR1. Moreover, by the use of peptide-specific antibodies we demonstrate that Arabidopsis ANTR1 is a thylakoid membrane transporter.

**EXPERIMENTAL PROCEDURES**

**Organisms—**Arabidopsis (A. thaliana cv. Columbia) plants were grown hydroponically at 120 µmol photons m⁻² s⁻¹ and 22 °C with 8-h light/16-h dark cycles. Total protein extracts from various tissues were prepared according to Ref. 13. Intact chloroplasts and pure thylakoid and envelope membranes were prepared as described (6). For heterologous expression experiments, E. coli strain TOP10F’ (Invitrogen) carrying the lacIq repressor for inducible expression using isopropyl 1-thio-β-D-galactopyranoside (IPTG) was employed.

**In Silico Analyses—**Prediction of subcellular location was performed using TargetP (17). Membrane topology was analyzed using a package of programs at the ARAMEMNON Web site and another one at PRODIV-TMHMM (18, 19). Sequence alignments were performed using ClustalW software (20).

**Cloning and Heterologous Expression of His₆-Xpress-ANTR1-FLAG Fusion Protein in E. coli—**The Arabidopsis At2g29650 (ANTR1 gene) was PCR-amplified from the RAFL09-06-K07 cDNA clone (RIKEN BioResource Center, Ref. 16) using a sense primer (5'-GAGAGACTCGAGAAGCGAGCCTCTCTTTGGC-3') flanking the Pst site (underlined) and an antisense primer (5'-GAGAGAAGCTTCTTATTTGATCGTCTTATATAATCATCGATTATTTCTCTCCGTT-3') harboring the HindIII site (underlined) and the sequence encoding the FLAG peptide, DYKDDDDK (boldface). The PCR product was inserted between the PstI and HindIII sites in the pTrcHisC plasmid (Agrisera, Umeå, Sweden) and the thylakoid LHCII protein (gift from Prof. J. Soll, Munich University) and the thylakoid LHCII protein or the fused tags. An ANTR1-specific antibody was produced in rabbit against the peptide 427–443 (CSQGTDAFSQSGLYSN), (Innovagen, Lund, Sweden). Another antibody was produced in rabbit against a peptide corresponding to the 15 residues after the cleavage site, antibodies against the envelope TIC110 protein (gift from Prof. J. Soll, Munich University) and the thylakoid LHCII protein (Agrisera, Umeå, Sweden) were employed.

**RESULTS**

**Structural Analyses of the Arabidopsis ANTR1 Protein—**Analysis of the 512-amino acid sequence of the ANTR1 protein from Arabidopsis (UniProtKB O82390) revealed the presence of a putative chloroplast transit peptide (TargetP score 0.99) with a cleavage site between amino acids 59–60 (Fig. 1A). The theoretical molecular masses for the full-length and processed forms are 56.5 and 50.7 kDa, respectively.

ANTR2 is the closest homologue among the ANTRs, showing 70% sequence identity (80% similarity) to ANTR1 (data extracted from ARAMEMNON). Although both proteins have been localized to the chloroplast (13), the transit peptide is the least conserved region between them, implying a distinct intra-chloroplast location and/or import pathway. ANTR1 ortho-
A Plant Thylakoid Pi Transporter

FIGURE 1. Sequence analyses of the Arabidopsis ANTR1 protein. A, the amino acid sequence of the full-length ANTR1 protein from Arabidopsis (UniProtKB O82390). The predicted transit peptide cleavage site is indicated by an arrow. The anion:cation symporter consensus sequence is marked by asterisks. TMDs predicted with high scores (>0.8) are highlighted, whereas TMDs predicted with low scores (<0.4) are boxed. B, topology model based on TMD prediction as in A. C, conservation of the consensus sequence for Na\(^+\)-dependent transporters in rat VGLUT2, rabbit NaPi-I, and Arabidopsis ANTR1. The consensus sequence and the identical and conserved residues are indicated below the alignment.

Logues are present in other sequenced plant species such as rice (86%) and *Populus* (88%) (data extracted from ARAMEMNON). No sequence homology was found to other known Pi transport systems from plant chloroplasts or *E. coli*. ANTR1 protein shows 30 and 36% sequence identity (50 and 60% similarity) to rabbit NaPi-I and rat VGLUT2, respectively, using another antibody raised against a common peptide for ANTR1 protein. Control experiments using antibodies for various Na\(^+\)-dependent transport systems (22), which is consistent with G342/A381X, PR391 in rabbit NaPi-I (23) and also present in rat VGLUT2 (Fig. 1C). This consensus sequence was found partially conserved in ANTR1, i.e. the leucine (non-polar) residue is changed for a glutamine (polar but uncharged) residue (Fig. 1C). Nevertheless, despite this non-conservative change the Na\(^+\) dependence of ANTR1 transport activity has been validated experimentally as described below.

Expression and Localization of the ANTR1 Protein in Arabidopsis—Western blot analysis of total protein extracts from various *Arabidopsis* tissues was performed using an ANTR1-specific antibody (see “Experimental Procedures”). A single cross-reacting band with *M* of 45 was detected and corresponds to a protein mainly expressed in photosynthetic tissues (e.g. mature leaves and flower buds), less in senescent leaves, and absent in roots (Fig. 2A), in line with microarray expression data available at Genevestigator\(^\text{®}\) data base (24).

The predicted chloroplast location of ANTR1 was experimentally confirmed using transient expression of a green fluorescent protein fusion construct (13) but not precisely assigned to any of the chloroplast membrane compartments. In this study, chloroplasts isolated from *Arabidopsis* leaves as well as thylakoid and envelope membrane subfractions, purified by sucrose density gradient centrifugation (6), were analyzed by Western blotting using the anti-ANTR1 antibody. The 45-kDa protein band was detected in chloroplasts, enriched in the thylakoid subfraction, but not found in the envelope (Fig. 2B, upper panel). No cross-reacting products were detected when the corresponding rabbit preimmune serum was used (Fig. 2A, middle panel). The topology of NaPi-I has never been addressed experimentally. The NaPi-I proteins are predicted to contain 12 transmembrane domains (TMDs) (9, 10) and an extended hydrophilic loop between TMD VI and TMD VII, as in the characteristic 6 + 6 configuration of many major facilitator superfamily members. Prediction of ANTR1 topology at ARAMEMNON (18) revealed the presence of 8–12 putative TMDs and an “in” (i.e. stroma) orientation for both the N and C termini. The most controversial hydrophobic regions (score < 0.4) correspond to TMD IV, TMD IX, and TMD X (Fig. 1, A and B). In addition, another set of topology programs at PRODIV-TMHMM (19) indicated the presence of 12 putative TMDs. The most plausible reason for the large variability between various tested software is the presence of a significant number of charged residues as well as prolines and glycines within the putative TMDs, as in the case of the thylakoid ATP/ADP carrier (6). A 12-TMD topology was resolved in the crystal structure of four different major facilitator superfamily proteins (21). Assuming that members of the same superfamily of transporters have a similar structure regardless of the type of substrate, we propose a 12-TMD model for ANTR1 in the thylakoid membrane in which both the termini and the long central loop (46 residues) connecting the two six-helix halves are exposed to the stromal side of the membrane (Fig. 1B).
leaves (a 2- to 3-fold reduction in the uptake activity was determined in transporter (6) as well as of a prokaryotic Na
processed using preimmune and anti-ANTR1 antibodies in Arabidopsis chloroplast membrane subfractions (30 μg of protein/lane): chloroplasts (C), thylakoids (T), and envelope (E). As reference, the distribution of LHCII (thylakoid marker) and TIC110 (envelope marker) are shown. C, ANTR1 and ANTR2 were immunodetected using a common peptide-specific antibody in the same fractions as in B.

Expression and Functional Characterization of ANTR1 in E. coli Cells—P, transporter mutants of yeast are widely used for functional analysis of plant P, transporters. However, previous work (13) and our own attempts showed that, although expressed, the ANTR1 protein was inactive with respect to anion transport. Instead, the bacterial system has proven successful for functional characterization of another thylakoid transporter (6) as well as of a prokaryotic Na+ -dependent P, transporter (25). Therefore, here we have expressed the full-length ANTR1 protein and studied its functional properties in E. coli cells as a recombinant His6-Xpress-ANTR1-FLAG fusion construct (Fig. 3A). A cross-reacting band with Mr of 56 was detected using the anti-FLAG antibody in the IPTG-induced transformed (EANTR1), but not in control, cells (Fig. 3B). The 56-kDa protein band was readily detected after 2 h of induction, and its level remained constant for up to 6–8 h. It corresponds to a membrane protein, as revealed by subcellular fractionation (data not shown). The 56-kDa protein was also immunodetected by the anti-ANTR1 antibodies as well as by the Xpress peptide antibody (data not shown). The preserved N terminus harboring the Xpress tag (Fig. 3A) and the size of 56 kDa for the recombinant full-length protein indicate that the E. coli system is unable to process the transit peptide (see Fig. 1A). During 8 h of IPTG induction in TB-P, medium, the EANTR1 cells grew considerably faster than the control cells (Fig. 3C), suggesting an advantage conferred by the activity of the heterologously expressed Arabidopsis protein.

Arabidopsis ANTR1 renders the transformed strain able to take up the highest levels of P, in the presence of NaCl, whereas a 2- to 3-fold reduction in the uptake activity was determined in the absence of NaCl (Fig. 4A). P, uptake in control cells both in the absence and presence of NaCl yielded ~20% of the maximal level of accumulated P, in the EANTR1 cells. The uptake activity of EANTR1 cells in the absence of NaCl was only slightly higher than that of control cells under the same conditions, a phenomenon probably due to presence of trace amounts of NaCl in the cell suspension. The specificity for Na+ versus Cl was verified by the 10-fold reduction in transport activity when NaCl was replaced by choline hydrochloride in the assay reactions (Fig. 4B). Preincubation with a Na+ ionophore, monensin, reduced the P, accumulation into the EANTR1 cells in the presence of NaCl to the level observed in control cells (data not shown), most likely due to its inability to inhibit the intrinsic H+ -coupled P, transport of E. coli (Ref. 15 and references therein). Taken together, these data indicate that, in contrast to the bacterial systems, ANTR1 can efficiently utilize the Na+ gradient created across cytoplasmic membrane for P, uptake, in line with the presence in ANTR1 of the consensus sequence for Na+ -dependent transport systems (Fig. 1C).

The induced expression of the cloned ANTR1 gene by the addition of IPTG does not appear to repress the expression of other host-encoded P, uptake mechanisms that result in the activity in the uptake of P, in non-induced cells. In the non-induced EANTR1 cells the activity was low and Na+ -independ-
A Plant Thylakoid Pi Transporter

FIGURE 4. Biochemical characterization of ANTR1 in E. coli cells. A, EANTR1 and control cells were IPTG-induced for 4 h and incubated in 25 mM Tris-succinate buffer (pH 6.5), at a concentration of 2 mg/20 µl with 100 µM [32P]orthophosphate in the absence or presence of 25 mM NaCl. After the indicated time periods, the cells were washed, and the uptake was measured by liquid scintillation spectrometry. B, phosphate uptake was measured for the indicated periods of time using 100 µM [32P] and 25 mM NaCl or choline chloride, pH 6.5. C, the uptake was carried out for 3 min in 25 mM Tris-succinate buffer at the indicated pH values in the absence (None) or presence of 25 mM NaCl. D, the uptake was carried out for 3 min in the absence or presence of 25 mM NaCl, KCl, or LiCl at pH 6.5. For B–D, the values obtained for EANTR1 cells were subtracted by those for the control strain. Data represent the mean ± S.D. of at least three independent experiments with three replicates.

ent (data not shown). After induction, the Na\(^{+}\)-dependent component was dramatically increased and showed saturation behavior, suggesting an induction of ANTR1 activity in addition to that of bacterial-encoded Na\(^{+}\)-independent Pi uptake systems. These observations suggest the advantage conferred by the ANTR1 (in the background of bacterial-derived activity) in taking up more Pi in a Na\(^{+}\)-dependent transport mechanism (see Figs. 3C and 4A). In all the forthcoming experiments, the transport activity of ANTR1 was characterized by subtracting the values in EANTR1 cells from those of the control strain.

Next, we investigated the sensitivity of ANTR1 transport activity to pH in the absence or presence of NaCl. The activity of ANTR1 in the absence of added NaCl was low, in the pH range 5.5 to 7.5, although 3-fold higher at pH 5.5 than at 6.5, and gradually increased with increasing pH only in the presence of NaCl (Fig. 4C). Thus, at pH 6.5 the activity was 15-fold higher than that observed at pH 5.5 and only 2-fold further increased at pH 7.5. A severe reduction was observed in the uptake at pH 8.5 (by 60%) as compared with 7.5, as also reported for the mammalian Na\(^{+}\)-dependent Pi transporters (26). The activity in control cells was at its optimum at pH 5.5 and showed no stimulation by the addition of NaCl (data not shown). Increased Pi transport by ANTR1 and the stimulation by NaCl at higher pH may be explained as follows. At pH below 6.0 most Pi is present as H\(_2\)PO\(_4\)\(^{-}\) and the transport does not seem to require Na\(^{+}\) ions. The activity increases dramatically with pH when the HPO\(_4\)\(^{2-}\) species becomes dominant and requires the presence of Na\(^{+}\) ions.

Among the alkali ions tested, maximum (15-fold) stimulation of Pi uptake by ANTR1 at pH 6.5 was achieved with Na\(^{+}\) followed by K\(^{+}\) (5-fold) (Fig. 4D). On the other hand, Li\(^{+}\) rather inhibited the uptake in E. coli, most likely due to indirect effects on bacterial metabolism.

The ANTR1 contribution to the Pi accumulation in the EANTR1 strain was at its optimum at pH 5.5 and showed no substantial influence on the radioactive Pi uptake, except for Na\(^{+}\) (by 60%) as compared with 7.5, as also reported for the mammalian Na\(^{+}\)-dependent Pi transporters (26). The activity increases dramatically with pH when the HPO\(_4\)\(^{2-}\) species becomes dominant and requires the presence of Na\(^{+}\) ions.

Among the alkali ions tested, maximum (15-fold) stimulation of Pi uptake by ANTR1 at pH 6.5 was achieved with Na\(^{+}\) followed by K\(^{+}\) (5-fold) (Fig. 4D). On the other hand, Li\(^{+}\) rather inhibited the uptake in E. coli, most likely due to indirect effects on bacterial metabolism.

To investigate whether ANTR1 specifically transports Pi and/or other anions, we measured the effect of various nonlabeled effectors on the Pi uptake in EANTR1 cells. The Pi concentration dependence in the range of 0 to 5 mM was virtually unchanged under these conditions, and at higher NaCl concentrations (50 mM) Pi uptake was inhibited in both strains (data not shown). The apparent K\(_{m}\) and V\(_{max}\) with respect to Na\(^{+}\) were 1.17 ± 0.36 mM and 99.15 ± 17.17 nmol mg\(^{-1}\) protein h\(^{-1}\), respectively.

Next, we have studied the effect of Pi concentration on the 32P-labeled uptake in transformed and control cells under inducing conditions. The Pi concentration dependence showed a hyperbolic behavior (Fig. 5B) with an apparent K\(_{m}\) of 78.7 ± 34 µM and a V\(_{max}\) of 161 ± 28 nmol mg\(^{-1}\) protein h\(^{-1}\), values that define ANTR1 as a high affinity Pi transporter.

To verify whether ANTR1 also transports glutamate, we have performed uptake of L-[3,4-3H]glutamate in control and EANTR1 cells. As shown in Fig. 6A, the uptake proceeded with
similar time course and extent in both types of cells, notably 60-fold lower than in the case of Pi transport (see Fig. 4A).

Taken together, the data from Fig. 6A and Table 1 indicate that glutamate can bind, but is not transported, by ANTR1. Based on the sequence alignment of VGLUT2, NaPi-1, and ANTR1, among the five TMD-located charged residues proposed to be responsible for glutamate but not for Pi transport in VGLUTs (12), only the 2 residues in the putative TMD4 (corresponding to Arg-184 and Gln-191 in VGLUT2) are conserved in ANTR1 (Fig. 6B). Poor conservation is also observed in the rabbit NaPi-1, i.e., only the residues corresponding to Arg-88 (TMD1) and Arg-184 (TMD4) in VGLUT2, which adds to the fact that there are no available indications in the literature for glutamate transport mediated by this protein. Because the structural selectivity for Pi binding and transport by NaPi are not known, we are unable at the present stage to explain using the sequence information why Pi, but not glutamate, is transported by ANTR1 as experimentally demonstrated in this work.

**DISCUSSION**

Animal NaPi-1 proteins were initially characterized as Na+-dependent phosphate transporters, but more recent studies point to organic as well as inorganic anions as substrates involved in various processes ranging from vesicular storage of the neurotransmitter glutamate to the degradation and metabolism of glycoproteins (10). *Arabidopsis* ANTRs show 30% identity to the members of the NaPi-I family, but the existence of a selectivity filter for Pi and for other potential substrates such as glutamate is far from conserved.
A Plant Thylakoid Pi Transporter

The data in this work demonstrate that the chloroplast thylakoid membrane of *A. thaliana* contains a major facilitator superfamily/anion-cation symporter member, namely the previously annotated ANTR1 (13). We show that the recombinant ANTR1 is specific for Pi transport across *E. coli* membrane in a Na⁺-dependent manner.

ANTR1 is predicted as a chloroplast protein with a 59-residue transit peptide (Fig. 1A). This protein was exclusively detected in photosynthetic tissues and localized to the chloroplast thylakoid membrane. In Western blotting experiments with two different peptide-specific antibodies, a *M* of 45 was consistently indicated (Fig. 2, B and C), which differs significantly from its theoretical size (543 residues, 50.7 kDa). Similar migration shift was observed for the envelope ANTR2 protein (Fig. 2C and Ref. 13), which has a theoretical mass of 49.9 kDa. One possible explanation could be an incorrectly predicted cleavage site. However, the *M* of 56 for the immunodetected band corresponding to the recombinant His6-Xpress-ANTR1-FLAG protein is also different from its theoretical size (554 residues, 61.2 kDa). Therefore, the most likely reason for the significant shift in the molecular weight of both the recombinant and processed forms of ANTR1 is the high content (60%) of charged and hydrophilic residues, common for this type of solute transporters.

Available information on NaPi transporters (10, 12, 26) could be compared with the uptake data for recombinant ANTR1 (Figs. 4–6). The obtained *Km* value for Pi (78 μM) is 10- to 100-fold lower than the values determined in the case of the rabbit NaPi-1 (1 mM, Ref. 10), the human NPT1 (0.29 mM, Ref. 10), and the rat VGLUT2 (10 mM, Ref. 12), indicating a high affinity in addition to the strict transport of Pi by ANTR1. The transport kinetics of ANTR1 as studied in *E. coli* have revealed a dependence of Pi transport on external Na⁺ as the driving force with an apparent affinity of 1 mM, i.e. much lower than the determined *Km* for Pi. This may indicate a stoichiometry of > 1 Na⁺:Pi, which is in agreement with the transport mechanism for other NaPi, members (26).

An important question is whether the obtained *Km* values of ANTR1 for Pi and Na⁺ in *E. coli* are physiologically relevant in chloroplast thylakoids. The answer is affirmative and explained below. (i) The *Km* value in thylakoids, at least in the case of Pi, may be the same, if not much lower, than the one obtained in the *E. coli* system, as in the case of the thylakoid ATP/ADP carrier (6). (ii) To accurately determine the Pi, concentration in organelles such as chloroplast (stroma) seems to be difficult; thus, values ranging between ≤1 and ~10 mM have been reported (27–29). (iii) When it comes to Na⁺, its physiological concentration in the stroma is ~100 mM (30). (iv) We know very little about the ionic strength of the thylakoid membrane, and the expected concentration of permeable ions (most likely K⁺, Mg²⁺, and Cl⁻) may be <10 mM (31).

The Pi transport activity of mammalian NaPi transporters is dependent on pH (10). The pH pattern obtained for the Na⁺-dependent ANTR1 activity in *E. coli* closely resembles the one reported for other NaPi-I members such as the human NPT1 (32). In our experimental conditions, when the uptake was assayed at pH 5.5, although low, the Na⁺-independent Pi transport system was dominant, and the contribution of a Na⁺-dependent system to the cellular Pi uptake activity was progressively increased with increasing pH, reaching its maximum at pH 7.5 (Fig. 4C).

The mammalian NaPi transporters use the inwardly created Na⁺ electrochemical gradient created by the Na⁺,K⁺-ATPase to drive Pi import into cells (10). Both H⁺ and Na⁺ are used as the major coupling ions in energy transduction processes in bacteria (33). Across the chloroplast thylakoid membrane an electrochemical gradient of H⁺ is generated as a result of photosynthetic electron transfer reactions. This trans-thylakoid H⁺ gradient not only powers the synthesis of ATP but also acts as a feedback regulatory component in photosynthetic events (Ref. 31 and references therein). The physiological pH in the thylakoid lumen is tightly regulated to a narrow range, 6.5 to 7.0 in darkness and 5.8 to 6.5 under growth light (34). Assuming that the obtained pH profile combined with Na⁺ dependence of ANTR1 in *E. coli* (Fig. 4C) is also valid in chloroplast thylakoids, then ANTR1 would be a H⁺-driven Pi transporter under acidic (light) conditions, whereas at pH ≥6.5 (darkness) Pᵢ transport should be essentially Na⁺-dependent. The possibility that K⁺ ions can also drive Pi transport across thylakoids, as they do in *E. coli* although with lower efficiency than Na⁺, cannot be excluded either. The direction of transport does not obligatorily depend on the orientation in the membrane (as predicted in Fig. 1B) but also on the trans-thylakoid H⁺, Na⁺, K⁺, and Pᵢ, electrochemical gradients. The reason for using a certain cation (H⁺, Na⁺, or K⁺) must be in the physiological status of the plant or in the conditions it had been experiencing in earlier stages of evolution. The physiological needs of the plant to keep the luminal pH in the narrow range (34) and the concentration of luminal ions low (31) should be taken into consideration as well as the availability of metabolic energy.

Indications about the physiological role of ANTR1 in planta are provided by data extracted from the Genesterigator® microarray data base (24). ANTR1 expression in *Arabidopsis* is 7- to 10-fold up-regulated by light of various qualities, visible light of high intensities, and programmed cell death, indicating a putative role in thylakoid biogenesis and turnover, as in the case of the thylakoid ATP/ADP carrier (6). For comparison, the envelope ANTR2 protein is not significantly affected by the above-mentioned stress conditions (data extracted from Genesterigator®) and may represent a more ubiquitous plastid housekeeping (Pᵢ) transport system. As for the physiological role(s) of the thylakoid ANTR1 in relation to a Pᵢ transport function, there are several possibilities: (i) a pathway to recycle Pi produced during nucleotide metabolism in the lumen (5) and to power the chloroplast ATPase; (ii) a pathway to get Pi into the lumen, thus lowering its levels in the stroma. ANTR1 activity in thylakoids would be yet another mechanism, in addition to envelope Pi transport and sequestration in metabolic intermediate, for Pi depletion of stroma, which has been proposed and recently demonstrated to modulate the conductivity of ATPase to protons, thus maintaining a low pH in the lumen and down-regulating photosynthetic light capture (29, 35). (iii) Balance of the trans-thylakoid H⁺ electrochemical gradient storage upon change in physiological status of the plant by interacting with cations and thus participating in ion homeostatic mechanisms (34).
Acknowledgments—We thank Prof. D. M. Kramer (Washington State University) for helpful discussions of the ionic strength of the thylakoid membrane and Prof. J. Soll (Munich University) for providing the anti-TIC110 antibody. We also thank the anonymous reviewers for constructive comments and experimental suggestions. The full-length RAFL09-06-K07 cDNA clone was provided by RIKEN BioResource Center (Japan).

REFERENCES

1. Rolland, N., Ferro, M., Seigneurin-Berny, D., Garin, J., Douce, R., and Joyard, J. (2003) Photosynth. Res. 78, 205–230
2. Weber, A. P., Schwacke, R., and Flugge, U. I. (2005) Annu. Rev. Plant Biol. 56, 133–164
3. Flugge, U. I. (1999) Annu. Rev. Plant Physiol. Plant Mol. Biol. 50, 27–45
4. Rausch, C., and Bucher, M. (2002) Planta 216, 23–37
5. Spetea, C., Hundal, T., Lundin, B., Heddad, M., Adamska, I., and Andersson, B. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 1409–1414
6. Reimer, R. J., and Edwards, R. H. (2004) Pflugers Arch. Eur. J. Physiol. 447, 629–635
7. Virkki, L. V., Biber, J., Murer, H., and Forster, I. C. (2007) Aust. J. Plant Physiol. 34, 191–199
8. Robinson, S. P., Downton, W. J., and Millhouse, J. A. (1983) Plant Physiol. 73, 238–242
9. Cruz, J. A., Sacksteder, C. A., Kanazawa, A., and Kramer, D. M. (2001) Biochemistry 40, 1226–1237
10. Miyamoto, K., Tatsuki, S., Sonoda, T., Yamamoto, H., Minami, H., Taketani, Y., and Takeda, E. (1995) Biochem. J. 305, 81–85
11. Lolkema, J. S., Speelmans, G., and Konings, W. N. (1994) Biochim. Biophys. Acta 1187, 211–215
12. Kramer, D. M., Cruz, J. A., and Kanazawa, A. (2003) Trends Plant. Sci. 8, 27–32
13. Takizawa, K., Kanazawa, A., and Kramer, D. M. (2008) Plant Cell Environ. 31, 235–243
14. Seki, M., Narusaka, M., Kamiya, A., Ishida, J., Satou, M., Sakurai, T., Nakajima, M., Enju, A., Akiyama, K., Oono, Y., Muramatsu, M., Hayashizaki, Y., Kawai, I., Carninci, P., Itoh, M., Ishii, Y., Arakawa, T., Shibata, K., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2002) Science 296, 141–145
15. Emanuellson, O., Nielsen, H., Brunak, S., and von Heijne, G. (2000) J. Mol. Biol. 300, 1005–1016
16. Schwacke, R., Schneider, A., van der Graaff, E., Fischer, K., Catoni, E., Desimone, M., Frommer, W. B., Flugge, U. I., and Kunze, R. (2003) Plant Physiol. 131, 16–26
17. Viklund, H., and Elofsson, A. (2004) Proteins 55, 239–252
18. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Nucleic Acids Res. 25, 3389–3402
19. Schwacke, R., Schneider, A., van der Graaff, E., Fischer, K., Catoni, E., Desimone, M., Frommer, W. B., Flugge, U. I., and Kunze, R. (2003) Plant Physiol. 131, 16–26