The twin-arginine translocation (Tat) system transports proteins across the chloroplast thylakoid membrane and bacterial plasma membrane. In vitro import assays have pointed to a key role for the thylakoid ΔpH in the initial assembly of the full translocon from two subcomplexes; more generally, the ΔpH is believed to provide the overall driving force for translocation. Here, we have studied the role of the ΔpH in vivo by analyzing the translocation of Tat substrates in transfected tobacco protoplasts. We show that the complete maturation of the precursor of the 23-kDa lumenal protein (pre-23K) and of a fusion of the 23K presequence linked to green fluorescent protein (pre-GFP) are unaffected by dissipation of the ΔpH. High level expression of Tat substrates in protoplasts has recently been shown to result in “translocation reversal” in that a large proportion of a given substrate is partially translocated across the thylakoid membrane, processed to the mature size, and returned to the stroma. However, the efficiency of translocation of pre-23K is undiminished in the absence of the ΔpH and/or ΔΨ, and the rate and extent of maturation of both pre-23K and pre-GFP by the lumen-facing processing peptidase is similarly unaffected. These data demonstrate that the proton motive force is not required for the functional assembly of the Tat translocon and the initial stages of translocation in higher plant chloroplasts in vivo. We conclude that unknown factors play an influential role in both the mechanism and energetics of this system under in vivo conditions.

The twin-arginine translocation (Tat) system transports proteins across the chloroplast thylakoid membrane and the plasma membranes of a wide variety of prokaryotes (reviewed in Ref. 1). The key role of this system appears to lie in the transport of globular proteins in a folded state in both chloroplasts (2, 3) and bacteria (4–8). While there are some differences between the signal peptides recognized by plant and bacterial Tat systems, almost all Tat signal peptides contain an essential invariant twin-arginine motif (9, 10). These targeting signals are recognized by a membrane-bound translocase that is encoded by three key genes in plants and Gram-negative bacteria: the homologous tatABC genes in higher plants (11–13).

The mechanism of the Tat system is poorly understood but protein purification and cross-linking studies have recently provided insights into the organization and operation of the system. Two primary Tat complexes have been characterized in membranes isolated from Escherichia coli, namely a TatABC core complex of ~370 kDa as estimated by blue native gels (14, 15) and a series of separate homo-oligomeric TatA complexes (15, 16) that are highly heterogeneous when analyzed by blue native gels (15). The situation appears to be similar, though possibly not identical in chloroplasts; a complex containing Hcf106 and cpTatC was identified using blue native gels of solubilized thylakoids, with all of the Tha4 present in separate complexes (17). Cross-linking studies have shown that substrates initially bind to a core complex (TatC-Hcf106 in chloroplasts or TatABC in E. coli (17, 18)). The subsequent events are poorly understood, but Tha4 was only found to cross-link to the core complex subunits in the presence of the thylakoid ΔpH and substrate (19), giving rise to a model in which the Tha4 complex (or TatA in bacteria) is recruited under these conditions to form the full translocation complex. A range of earlier in vitro import studies (20, 21) also showed that the full translocation of Tat substrates across the thylakoid membrane is completely ΔpH-dependent in both intact chloroplasts or isolated thylakoids.

More recently, the role of the ΔpH has been evaluated in vivo for the first time. Pulse labeling studies in the unicellular green alga Chlamydomonas reinhardtii (22) showed that several Tat substrates were converted to the mature size in the complete absence of a ΔpH, strongly suggesting that Tat-dependent translocation is not dependent on the thylakoid proton motive force. Another recent study using transfected tobacco protoplasts demonstrated another unexpected characteristic that has not been observed using in vitro assays; Tat substrates were found to be partially translocated across the thylakoid membrane but then returned to the stroma in a high percentage of instances (23). Given the emerging indications of major differences between the in vivo and in vitro characteristics of the Tat system, we have set out to study the role of the ΔpH in the translocation of substrates by the Tat pathway in tobacco protoplasts. We show that the ΔpH is not required for the partial translocation of a GFP construct across the thylakoid membrane by the Tat pathway or for the full translocation of an authentic lumenal Tat substrate.

**EXPERIMENTAL PROCEDURES**

Constructs—A chimera comprising the presequence of the pea 23-kDa lumenal photosystem II protein (23K) linked to a version of GFP termed sGFP (24) was expressed as described by Di Cola and Robinson (23). The structure of a non-processable form is described in the same report; in brief, the terminal Ala of the signal peptide was deleted, removing the consensus Ala–Xaa-Ala motif recognized by the thylakoid processing peptidase. Full-length pea pre-23K was also expressed using...
the same vector system, but the protein was modified by the addition of two methionines at the extreme C terminus to facilitate radiolabeling.

**Transient Transformation of Leaf Protoplasts**—Protoplasts were prepared from axenic leaves (4–7 cm long) of *Nicotiana tabacum* cv. Petit Havana SR1 (24). In brief, leaves were cut and incubated overnight in the presence of an enzymatic mix containing 0.2% macerozyme and 0.4% cellulase prepared in K3 medium (Gamborg’s B5 basal medium with minimal organics (Sigma), supplemented with 750 mg/liter CaCl2, 250 mg/liter NH4NO3, 136.2 g/liter sucrose, 250 mg/liter xylose, 1 mg/liter 6-benzyaminopurine, and 1 mg/liter naphthaleneacetic acid, pH 5.5). Protoplasts were subjected to polyethylene glycol-mediated transfection as described (25) and were incubated overnight at 25 °C in the dark before pulse labeling.

**In Vivo Labeling of Protoplasts, Analysis of Expressed Polypeptides, and Import Assays**—Pulse-chase labeling of protoplasts using Pro-Mix (a mixture of [35S]Met and [35S]Cys; Amersham Biosciences, Buckinghamshire, UK) was performed exactly as described in Ref. 26. In some experiments (see "Results") protoplasts were treated with 2 μM nigericin (Sigma), 2 μM valinomycin, or a combination of the two at the beginning of the pulse labeling period. KCl was added to 10 mM in each case. At the desired time points, 3 volumes of W5 medium (26) were added, and protoplasts were pelleted by centrifugation at 1000 g for 5 min. Cells were frozen in liquid nitrogen and stored at −80 °C. Homogenization of protoplasts and incubation media was performed by adding to the frozen samples 2 volumes of ice-cold homogenization buffer (150 mM Tris-Cl, 150 mM NaCl, 1.5 mM EDTA, and 1.5% (w/v) Triton X-100, pH 7.5) supplemented with complete protease inhibitor mixture (Roche Applied Science). Immunoprecipitation of expressed polypeptides was performed as described (26), using rabbit polyclonal antiserum raised against pea 23K or GFP (Molecular Probes, Eugene, OR). Immunoprecipitated proteins were analyzed by 15% (w/v) reducing SDS-PAGE and fluorography.

**Chloroplast Isolation and Fractionation**—Protoplast pellets (from 3,000,000 cells) obtained at the desired time points during pulse-chase were resuspended in 150 ml HS buffer (50 μM Hepes-KOH, 330 mM sorbitol, pH 8) and homogenized by repeated passage through a 23-gauge syringe needle. The obtained lysate was diluted to 4 ml with HS buffer and loaded on top of a 35% (v/v) Percoll pad in 5 ml 23-gauge syringe needle. The obtained lysate was diluted to 4 ml with HS and loaded on top of a 35% (v/v) Percoll pad in 5× HS and centrifuged at 1,400 × g for 9 min at 4 °C. Pellets (chloroplasts) were washed once in HS buffer, pelletted at 3,300 × g for 2 min, and resuspended in 120 μl of HS. An aliquot (30 μl) was saved and used for immunoprecipitation (chloroplast (C)), and the rest was incubated for 1 h on ice with thermolysin (Sigma 2 μM) and maintained at 20 °C. Following dark adaptation for several hours protoplasts were given a saturating pulse of white light (all saturating pulses at 3,000 μmol m−2 s−1) to record the maximum fluorescence yield, Fm. An actinic light at 400 μmol m−2 s−1 was then provided for a 30-min duration to induce non-photochemical quenching (NPQ) of chlorophyll a fluorescence. A second saturating pulse was then applied with the actinic light on to record Fm′. The actinic light was then switched off, and Fm′ values were recorded with regular saturating pulses in the dark. Dark relaxation kinetics of NPQ were then resolved into fast relaxing (NPQf) and slowly relaxing components (NPQs) according to Walters and Horton (27), using the equation Fm′/Fm′ − 1 as a measure of NPQ. When used nigericin was added at a concentration of 2 μM.

**RESULTS AND DISCUSSION**

**Maturation of Pre-GFP Is ΔpH-dependent in Chloroplast Import Assays**—One of the constructs used in this study (pre-GFP) comprises the presequence of the luminal 23-kDa photosystem II protein (from pea) linked to green fluorescent protein. GFP constructs have been successfully used as Tat-dependent carrier proteins in bacteria (28) and transgenic *Arabidopsis thaliana* (29), and we have recently shown that this construct behaves as a typical Tat substrate in standard chloroplast import assays. However, expression in transfected protoplasts gave an unexpected result: the protein was imported into chloroplasts, extensively translocated across the thylakoid membrane by the Tat pathway, and processed to the mature size, but then the majority was returned to the stroma. A similar observation was made using a natural Tat substrate (the 23-kDa protein), with about 50% of this protein also appearing as the processed, mature size form in the stroma (23). Interestingly, it was found that mutation of the terminal thylakoid-processing peptidase (TPP) cleavage site caused the intermediate form to associate strongly with the thylakoid membrane, possibly due to prolonged interaction with the Tat translocon. This non-processable mutant, which lacks the terminal Ala in the Tat signal peptide, is termed pre-GFPΔTPP in the studies described below.

The protoplast transfection system offers the opportunity to carry out pulse-chase assays on the time scales usually used for *in vitro* translocation assays, and in this study we have tested the importance of the thylakoid ΔpH for the targeting of pre-GFP. First, we tested whether this substrate exhibits the typical ΔpH dependence observed for Tat substrates in *in vitro* import assays, as shown in Fig. 1. The control panel shows the import and localization of the protein using the standard pea chloroplast import protocol, and the data show that pre-GFP is...
imported and converted to smaller products (lane C) that are protected from proteolysis of the organelles (lane C+). Fractionation of the chloroplasts shows the presence of mature size GFP in the thylakoid fraction (lane T), and this is resistant to thermolysin treatment of the membranes (lane T+) confirming a luminal location. A small amount of intermediate size protein (iGFP) is apparent in the chloroplast (lane C) and protease-treated chloroplast (lane C+) samples, and this almost certainly corresponds to stromal intermediate en route to the thylakoids. This is not detected in the stroma, probably because the protein has been fully translocated by the time the fractions are generated.

The second panel shows an import assays carried out in the presence of 2 μM nigericin. The nigericin totally inhibits transport across the thylakoid membrane and the stromal iGFP form accumulates, confirming that this substrate, like other Tat substrates, is completely dependent on the thylakoid ΔpH for transport into the lumen. The non-processable pre-GFPΔTPP form is likewise dependent on the thylakoid ΔpH, with the intermediate accumulating in the stroma in the presence of nigericin (data not shown).

Partial Translocation and Maturation of Pre-GFP Is ΔpH-independent in Transfected Tobacco Protoplasts—We studied the same substrate in vivo using transfected tobacco protoplasts under conditions that have been used previously to study the intracellular targeting of proteins by other pathways (24–26). In this assay, leaf protoplasts are transfected with plasmid DNA encoding pre-GFP, with expression driven by the strong cauliflower mosaic virus 35S promoter. After 24-h incubation, the targeting of pre-GFP was studied by pulse-chase analysis as detailed below. Assays were carried out in the absence or presence of 2 μM nigericin.

It is not possible to directly measure the ΔpH in vivo but chlorophyll fluorescence can be used as a non-invasive assay to confirm that the thylakoid ΔpH is indeed fully dissipated under these conditions. A strong light-dependent quenching of chlorophyll a fluorescence, known as NPQ, is a feature of higher plant chloroplasts (30). NPQ can be resolved into at least two key components on the basis of the kinetics of relaxation in the dark (27). A rapidly relaxing component, referred to here as NPQf, reflects the safe dissipation of excess absorbed quanta as heat. This process, which serves to regulate the efficiency of light harvesting, is dependent upon a thylakoid ΔpH for its formation. As such it can be eliminated in the presence of uncouplers such as nigericin. To test the efficacy of the nigericin treatment of tobacco protoplasts in this study, chlorophyll a fluorescence quenching analysis was used as a non-invasive, indirect assessment of the thylakoid ΔpH. Fig. 2 shows dark relaxation kinetics of NPQ in the tobacco protoplasts used in this study. In the untreated protoplasts (Fig. 2A) NPQ can be divided by linear regression into two distinct components, NPQf and NPQs. The rapidly relaxing component, NPQf, reflects the removal of the ΔpH in the dark and is complete within 5–10 min of dark treatment. In the presence of nigericin (Fig. 2B), the NPQf component is absent leaving only a single slowly relaxing component, NPQs. Since the rapidly relaxing component is completely dependent upon the ΔpH for its formation, these data demonstrate that the nigericin treatment of tobacco protoplasts in this study was successful in eliminating the ΔpH. This is to be expected, since this concentration of nigericin blocks the ΔpH-dependent targeting of Tat substrates in chloroplast import assays, where the chlorophyll concentration is higher than that used in protoplast assays. A related approach to dissipate the ΔpH was used in the study on Chlamydomonas (22).

Pulse-chase assays were used to study the requirement for the ΔpH during the targeting of pre-GFP and pre-GFPΔTPP in protoplasts, and the first data are shown in Fig. 3. The upper panel shows immunoprecipitates of labeled GFP polypeptides after a pulse of 1 h and a 3-h chase. In this experiment the protoplasts were fractionated to generate samples of intact chloroplasts (lane C), stroma (lane S), thylakoids (lane T), and thermolysin-treated thylakoids (lane T+). The data obtained for pre-GFP show the presence of intermediate and mature size GFP (iGFP, GFP) in the stroma, with only a minority of protein present in the thylakoids. In this experiment the thylakoid-associated mature GFP was calculated to be 18% of total imported protein, but it should be noted that a lower efficiency of thylakoid targeting is often observed (data not shown). In contrast, the pre-GFPΔTPP is imported and converted to the intermediate form (iGFP), most of which (70%) associates with the thylakoids. A significant proportion of this thylakoid-associated protein is resistant to digestion by thermolysin, implying a buried location within the membrane, while other molecules are converted to a smaller degradation product (DP). These data closely resemble those obtained in a recent study (23), in which a variety of controls were used to confirm that the stromal mature size GFP arose from partial translocation across the thylakoid by the Tat system, maturation by TPP, and return to the stroma.

**FIGURE 2.** Assessment of trans-thylakoid ΔpH in tobacco protoplasts. NPQ of chlorophyll a fluorescence was resolved into rapidly reversible (NPQf) and slowly reversible (NPQs) components based on their dark relaxation kinetics. NPQf represents an indirect measurement of trans thylakoid ΔpH. The left panel shows dark relaxation kinetics for untreated protoplasts; the right-hand panel shows dark relaxation kinetics for protoplasts treated with 2 μM nigericin. Prior to measurement protoplasts were placed in the dark for 3–5 h.
**ΔpH-independent Operation of the Tat System in Tobacco Protoplasts**

**FIGURE 3. Maturation of pre-GFP is ΔpH-independent in tobacco protoplasts. A**, pre-GFP and a variant containing a mutated TPP cleavage site (see “Results”) were transiently expressed in tobacco protoplasts for 24 h, after which the protoplasts were pulsed with [35S]methionine + cysteine for 1 h and chased with cold methionine for 3 h. After the chase period, chloroplasts were purified (lane C) and fractionated into stroma (lane S), thylakoids (lane T), or thermolysin-treated thylakoids (lane T+). Prior to immunoprecipitation with antibodies to GFP, mature size and intermediate forms of GFP are indicated, together with the mobility of a 30-kDa marker protein on the left. **B**, chloroplasts expressing pre-GFP were analyzed in the absence of nigericin or the presence of 2 or 5 μM nigericin using a pulse of 1 h and chase periods of 0, 2.5, or 5 h as indicated. Samples of isolated chloroplasts were subjected to immunoprecipitation with antibodies to GFP. Mature size and intermediate forms of GFP are indicated. Samples of intact chloroplasts were also treated with protease (lane C+). GFP forms were immunoprecipitated as described for A. **C**, pre-GFP and iGFP were expressed in tobacco protoplasts for 24 h, after which the protoplasts were purified (lane C), and fractionated into stroma (lane S), thylakoids (lane T), or thermolysin-treated thylakoids (lane T+). Prior to immunoprecipitation with antibodies to GFP, mature size and intermediate forms of GFP are indicated, together with the mobility of a 30-kDa marker protein on the left. Samples of isolated chloroplasts were subjected to immunoprecipitation with antibodies to GFP. Mature size and intermediate forms of GFP are indicated. Samples of intact chloroplasts were also treated with protease (lane C+). GFP forms were immunoprecipitated as described for A.

Fig. 3B shows that 2 μM nigericin does not block the maturation of pre-GFP during a 2.5- or 5-h chase period. The ratio of intermediate: mature size protein is similar in each case and is also observed in the presence of 5 μM nigericin, although this concentration is somewhat toxic and some smearing of the protein bands is apparent. Fig. 3C shows that iGFP and most of the mature size GFP is located in the stroma in both the absence and presence of nigericin, as expected given the stromal location of these forms in the absence of nigericin as shown in the upper panel. These data show that the thylakoid ΔpH is not required for the initiation of translocation by the Tat translocon and partial translocation across the membrane.

The ΔpH Is Not Required for the Complete Translocation of 23K or the Stable Insertion of Pre-GFPΔTPP—To further probe the importance of the ΔpH for Tat-dependent targeting, we studied two substrates that are transported either partially or entirely across the membrane. The first is pre-GFPΔTPP, which is transported into a thermolysin-resistant location within the thylakoid membrane as shown in Fig. 3 and other studies (23). Fig. 4A shows chloroplast fractionation tests after 1 h pulse labeling + 3-h chase in the absence of nigericin, and Fig. 4 shows that essentially identical results are obtained in each case. Approximately half of the imported intermediate form of pre-GFPΔTPP is localized in the thylakoid membrane (T) where it is largely resistant to digestion by thermolysin. Some proteolytic clipping of the stromal intermediate is observed (degradation product denoted DP). The data demonstrate that the ΔpH is not required for this partial translocation process.

A further test is shown in Fig. 4B, where we studied the authentic Tat substrate pre-23K. Note that the protein was modified by the addition of two methionine residues at the extreme C terminus; this was carried out to facilitate radiolabeling of the protein, since mature pea 23K does not contain methionine. Like pre-GFP, this protein is subject to translocation reversal under these expression conditions but to a much lesser extent; we usually find that about 30–40% of the protein is correctly targeted into the thylakoid lumen. Some of the thylakoid-associated, mature size 23K is protected from proteolysis, suggestive of a luminal location (23). Fig. 4B shows fractionation tests to determine the location of the imported bands after pulse-chase assays ± nigericin, and as with the previous experiments, the data show that nigericin has no significant effect on the overall targeting process. In both the presence and absence of nigericin, imported pre-23K is found as both the intermediate and mature size forms in the stroma, and mature size protein is also present in the thylakoid fraction.

In higher plant leaves, the thylakoid proton motive force is almost entirely in the form of ΔpH under steady state conditions; the electrical potential, ΔΨ is negligible due to ion movements across the membrane (31). However, it has been suggested that the ΔΨ may actually contribute to the proton motive force under some circumstances (32), and this may explain, at least in part, the ability of the Tat system to function in the absence of a ΔpH in *Chlamydomonas*. To completely exclude the role of either component in Tat-dependent targeting of these substrates, we carried out a comparative test on the effects of selectively dissipating the ΔpH or ΔΨ alone with nigericin or valinomycin, respectively, or of eliminating both components using a combination of the two compounds. Valinomycin was used at a concentration of 2 μM,
which is a 2-fold excess compared with the concentrations used in previous studies (20, 21) to ensure that the ΔΨ was fully dissipated.

Fig. 5A shows pulse-chase assays in which protoplasts expressing pre-GFP were labeled for 1 h in the presence of nigericin, valinomycin, or both as indicated and then analyzed immediately (0 h) or after a 5-h chase as indicated. Cells expressing pre-GFPΔTPP were analyzed in parallel to provide a marker for iGFP. B, samples from the same control (Con), nigericin (Nig), valinomycin (Val), and nigericin + valinomycin (Nig+ Val) assays (5 h chase samples) were analyzed by autoradiography.

In vitro assays to study Tat function have invariably used higher plant chloroplasts and in this report we have sought to use the tobacco protoplast system to analyze the role of the ΔpH in Tat function in a higher plant. Barley leaves were also analyzed in the

\[ \Delta p\text{H-independent Operation of the Tat System in Tobacco Protoplasts} \]

substrates in transfected tobacco protoplasts resulted in “translocation reversal” within the Tat translocon to a remarkable extent. This may stem from the high level expression of substrates in this system, but this alone does not explain why translocation is aborted so frequently, since this phenomenon was not observed in several previous studies when saturation of the Tat pathway was achieved in vitro (e.g. Ref. 34). Alternatively, the stress associated with protoplast generation may lead to the production of factors that indirectly induce translocation reversal. It should, however, be emphasized that the protoplasts expressing the Tat substrates in this study are intact, the chloroplasts are equally intact, and photosynthetic efficiency is high, indicating that the cells as a whole are highly functional. Moreover, the same experimental system has been successfully used to study intracellular protein targeting by other pathways.

In vitro assay systems have been developed for the study of Tat-dependent protein transport in isolated chloroplasts, as expected from numerous previous studies on Tat substrates (e.g. (20)), but the barley 23-kDa protein was not formally identified as 23K, the levels of 23K in the stroma were not analyzed, and the kinetics of maturation were not addressed. These experiments are, technically, very difficult using intact leaves. The protoplast system, on the other hand, can be used to address these issues because radiolabeling and organelle isolation is relatively straightforward. Although the underlying causes of the observed translocation reversal observed recently are not understood, it is clear that the partial translocation of pre-GFP and the translocation of pre-23K to a protease-protected location is mediated in each case by the Tat pathway, and their maturation is carried out by TPP on the trans side of the thylakoid membrane (23).

In the present study, we have used sensitive assays to study the role of the ΔpH in this process, and the results again point to major differences between the in vitro and in vivo assays. While the transport of pre-GFP across the thylakoid membrane is entirely ΔpH-dependent in isolated chloroplasts, as expected from numerous previous studies on Tat substrates (e.g. (20)), dissipation of the ΔpH has no significant inhibitory effect on the maturation of either pre-GFP or pre-23K in transfected protoplasts. The data therefore demonstrate that the ΔpH is not required for the initial assembly of the Tat translocon in vivo or for partial translocation to the point where the signal peptide cleavage point is exposed on the trans side of the membrane. At this point, the GFP would be fully contained within the confines of the membrane bilayer, assuming that translocation occurs in a folded state. The data also raise the possibility that the ΔpH is not required for full translocation into the lumen; while the efficiency of pre-GFP translocation is very low because of the extensive translocation reversal, the pre-23K does appear to be transported into the lumen with higher efficiency, and its transport is not significantly affected by ΔpH removal. However, we are aware that 23K is transported with relatively low efficiency under these conditions and we cannot rule out the possibility that the ΔpH may stimulate translocation under conditions where translocation reversal is less prevalent. We conclude that key factors play a role in the energetics of Tat-dependent protein transport in vivo, and given the remarkable differences between the in vitro and in vivo targeting characteristics, a detailed understanding of these unknown factors is clearly essential for a full understanding of this system.
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