A simple method for isolating import-competent *Arabidopsis* chloroplasts

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Abstract We present a simple, rapid and low-cost method for isolating a high yield of *Arabidopsis* chloroplasts that can be used to study chloroplast protein import. Efficiency of chloroplast isolation was dependent upon the ratio between amount of plant tissue and the buffer volume, the size and speed of the homogenisation equipment, and the size of the homogenisation beaker. The import method proved useful when characterising different precursor proteins, developmental stages and import-defective mutants. Time-course experiments enabled the measurement of import rates in the linear range. Compared to protoplastation, this isolation method has significant time and cost savings (~80% and ~95%, respectively), and yields chloroplasts with a higher capacity to import proteins.

Key words: Chloroplast; Protein import; Precursor protein; *Arabidopsis thaliana*

1. Introduction

Most chloroplast proteins are encoded in the nucleus, translated in precursor form in the cytosol, and transported across the chloroplast envelope post-translationally [1,2]. Precursor proteins (preproteins) each have an amino-terminal targeting signal called the transit peptide that directs their translocation through a translocon complex in the envelope membranes, and is finally cleaved by a stromal processing peptidase to yield mature protein [3,4]. Imported proteins may subsequently be localised to different intrachloroplastic compartments [1].

The chloroplast protein import field has, during the last decade, undergone rapid progress concerning the discovery of translocon components [1,2]. Pea has been the model system of choice for studies of chloroplast protein import, but *Arabidopsis* is now emerging as an alternative model system [5–9]. Advanced genetic and molecular techniques and the complete genome sequence are enabling the import mechanism to be explored in greater detail, in vitro and in vivo. The exploitation of *Arabidopsis* in chloroplast import research, however, has been retarded by the inconsistency and low yield of *Arabidopsis* chloroplast isolation procedures. This was partly resolved by a method for isolating *Arabidopsis* chloroplasts from protoplasts [10]. However, because this method relies on protoplastation prior to chloroplast isolation, it is time-consuming and expensive. We present a simple, low-cost method that can be used to isolate large numbers of intact chloroplasts from *Arabidopsis* seedlings in ~1 h, and describe how the isolated chloroplasts can be used to study chloroplast protein import. The simplicity of the protocol, and the functional integrity of the isolated chloroplasts, indicate that the isolation method may prove useful in studies of many different aspects of chloroplast biology in addition to protein import.

2. Materials and methods

2.1. Plant growth conditions

Seeds of *Arabidopsis thaliana* (ecotype Columbia-0) were sterilised in 70% (v/v) ethanol, 0.05% (v/v) Triton X-100 for 5 min, followed by 10 min in 100% ethanol, and allowed to air-dry in a laminar flow hood. Seeds were sown on petri plates (9 cm in diameter; 100–120 seeds/plate) containing Murashige and Skoog salt and vitamin mixture (Sigma), 0.5% (w/v) sucrose, and 0.6% (w/v) bactoagar. Each plate was sealed with Leukopor tape (Beiersdorf), incubated at 4°C for 48 h to break seed dormancy, and thereafter grown at 20°C in 120 μmol/m^2/s white light with a long day cycle (16-h light/8-h dark) for exactly 10, 14 or 28 d in a tissue culture chamber (Percival).

2.2. Isolation of Arabidopsis chloroplasts

For each isolation procedure, 25–40 petri plates of 10-, 14- or 28-d-old plants were used; with 10-d-old plants, this is equivalent to ~2500–4000 individuals or ~7.5–12 g tissue. During the isolation procedure the plant material was kept at 4°C. Plants were homogenised for 3–4 s using a polytron (Kinematica PT20) with a small rotor (13-mm diameter, ~40% max speed) in 20 ml isolation buffer (0.3 M sorbitol, 5 mM $MgCl_2$, 5 mM EGTA, 5 mM EDTA, 20 mM HEPES/KOH, pH 8.0, 10 mM $NaHCO_3$) in a 50-ml beaker. The homogenate was filtered through a double layer of Miracloth (Calbiochem). The debris retained in the Miracloth was returned to the beaker with 20 ml fresh isolation buffer and the homogenisation was repeated. This homogenisation procedure was carried out five times in total. The combined homogenate was centrifuged at $1000\times g_{max}$ for 5 min (brake on) and the pellet was resuspended in ~500 μl isolation buffer.

The resuspended chloroplasts were loaded onto a two-step Percoll gradient or a linear Percoll gradient. Two-step gradients were prepared in 20-ml Corex tubes (DuPont), and linear gradients were prepared in 30-ml Nalgene tubes (Fisher). Two-step gradients consisted of a bottom layer (3 ml) comprising 2.55 ml Percoll solution (95% (w/v) Percoll, 3% (w/v) PEG 6000, 1% (w/v) Ficoll, 1% (w/v) BSA) plus 0.45 ml gradient mixture (25 mM HEPES–NaOH, pH 8.0, 10 mM EDTA, 5% (w/v) sorbitol) and a top layer (7 ml) comprising 2.94 ml Percoll solution plus 4.06 ml gradient mixture. Two-step gradients were centrifuged in a swing-out rotor at $1500\times g_{max}$ for 10 min (brake off). Linear gradients (26 ml) consisted of 13 ml Percoll, 13 ml 2× isolation buffer and 5 mg glutathione, and were pre-centrifuged in a fixed angle rotor at $43\,000\times g_{max}$ for 30 min (brake off). After adding the chloroplasts, linear gradients were centrifuged in a swing-out rotor at $7800\times g_{max}$ for 10 min (brake off).

The band that appeared between the phases when using two-step gradients, and the lower band that appeared when using linear gradients, contained intact chloroplasts. The upper band in each case contained broken chloroplasts. Broken chloroplasts were removed and discarded, and then the intact chloroplasts were recovered using

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a 1-ml Gilson pipette tip, cut at the end. Next, 30 ml HEPES–$MgSO_4$–sorbitol (HMS) buffer (50 mM HEPES, 3 mM $MgSO_4$, 0.3 M sorbitol), or, where specifically stated, 30 ml HEPES–sorbitol (HS) buffer (50 mM HEPES, 0.3 M sorbitol), was added to the chloroplasts and the tube was inverted carefully one time to wash off the Percoll. The chloroplasts were centrifuged in a swing-out rotor at $1000\times g_{max}$ for 5 min (brake on). The supernatant was decanted and discarded, and the pellet was resuspended in the residual HMS (or HS) buffer. Determination of the yield of chloroplasts was carried out using a haemocytometer, and the intactness of the chloroplasts was verified by phase-contrast microscopy (Zeiss) and transmission electron microscopy [11].

Isolation of chloroplasts from protoplasts was carried out as described previously [10], with the following minor changes. The growth conditions of the plants were as described above. The cellulase concentration was reduced two-fold to 2%; this lower cellulase concentration was found to be effective for isolating *Arabidopsis* chloroplasts for photosynthetic electron transport studies [12], and did not significantly affect chloroplast yield (Aronsson and Jarvis, unpublished observation). A two-step Percoll gradient was used in place of the linear Percoll gradient in some cases.

2.3. Import into Arabidopsis chloroplasts

Template DNA for the in vitro transcription/translation of preproteins was amplified by PCR from cDNA clones using M13 primers. Transcription/translation was performed using a wheat germ system (Promega) containing [^{35}S]-methionine and either T7 RNA polymerase (*Arabidopsis* pSS and pL11) or SP6 RNA polymerase (pea pSS) according to the manufacturer's instructions.

Import reactions were carried out in HS buffer [13], or HMS buffer containing 20 mM gluconic acid (potassium salt), 10 mM $NaHCO_3$ and 0.2% (w/v) bovine serum albumin [14]. Unless stated otherwise, each 150 μl import assay contained 10^7 chloroplasts (~20 μg chlorophyll when using wild-type) from 10-d-old plants, 5 mM MgATP, 10 mM methionine, and translation mixture not exceeding 10% of the total volume.

Import was performed in white light (100 μmol/m^2/s) at 25°C for 5, 10, 15 or 20 min exactly. When necessary, import reactions were divided in two for protease treatment: one half was treated with 100 μg/ml thermolysin on ice for 30 min, and the other half was left untreated. Thermolysin reactions, and all import reactions that were not subjected to thermolysin treatment, were stopped by adding an equal volume of ice-cold HS buffer containing 50 mM EDTA, followed by a short centrifugation burst (~4 s) in a microfuge. Pellets were resuspended in 2× denaturation buffer [15]. After heating at 95°C for 1.5 min, samples were resolved on 12.5% or 15% SDS–PAGE gels [15]; translation mixture equivalent to 10% of that added to each import reaction was run on each gel for quantification purposes. Gels were fixed in 7% (v/v) acetic acid, coated with NAMP100 fluorescent signal amplifier (Amersham), dried, and exposed to X-ray film or a phosphorimager screen. Quantification was performed using ImageQuant software (Molecular Dynamics).

To deplete endogenous ATP, chloroplasts were kept in the dark at room temperature for 10 min [9,16], or in the dark on ice in the presence of 6 μM nigericin (Sigma) for 10 min [17]. Small molecules, including ATP, were removed from the translation products by Sephadex G-25 filtration (Pharmacia). MgATP was added to import reactions, containing dark-adapted chloroplasts and ATP-depleted preprotein at different concentrations (50–5000 μM), or was omitted completely. Reactions were incubated in the dark at 25°C for 15 min, and then analysed as described above.

3. Results and discussion

3.1. Isolation of intact chloroplasts from Arabidopsis plants

To minimise the time needed for plant growth, and to allow the analysis of import-defective mutants during early development when their phenotypes are often more severe [5], we developed a chloroplast isolation protocol that can be used with 10-d-old *Arabidopsis* plants. To reduce the time and cost of the isolation procedure, we based our protocol on established methods that do not involve protoplastation [13,14]. During the development of the isolation method, we found

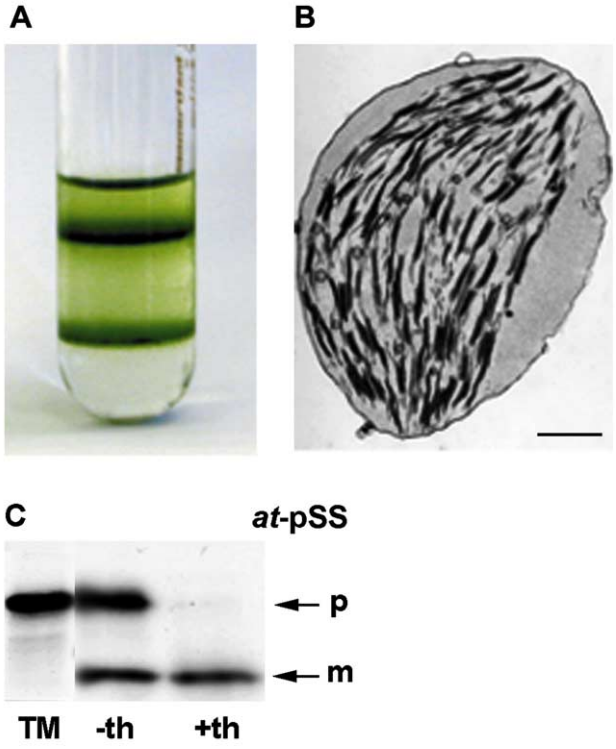

Fig. 1. Isolation of intact *Arabidopsis* chloroplasts. A: Typical two-step Percoll gradient showing the distribution of chloroplasts between two bands. The upper band contains broken chloroplasts, and the lower band contains intact chloroplasts. B: Electron micrograph of an intact chloroplast from the lower band of the Percoll gradient. Size bar indicates 1.0 μm. C: Import of *at*-pSS into intact chloroplasts. After 15 min import reactions, samples were either treated with thermolysin (+th) or left untreated (−th). TM, translation mixture; p, preprotein; m, mature.

the ratio between the amount of tissue and the volume of isolation buffer to be of great importance. The type of homogenisation equipment used was also found to be important, as was the size and speed of the polytron rotor, and the size of the homogenisation beaker. The isolation conditions presented here were found to be optimal. Increasing the size or speed of the polytron rotor, or using a kitchen blender instead of the polytron, resulted in a higher proportion of broken chloroplasts (data not shown). A key feature of the isolation procedure is the repetition of the homogenisation step, since this ensures a high yield of intact chloroplasts.

After homogenisation, intact chloroplasts were separated from damaged chloroplasts by Percoll density gradient centrifugation (two-step gradients were used for most experiments). A typical Percoll gradient showing the distribution of chloroplasts between two discrete bands is shown (Fig. 1A). Chloroplasts from the lower band were washed and estimated to be >85% intact by phase-contrast microscopy. The integrity of the isolated chloroplasts was also confirmed by transmission electron microscopy, and a typical micrograph is shown (Fig. 1B). A typical isolation procedure using ~2500 plants (~7.5 g tissue) yielded $>15\times10^7$ intact chloroplasts (~40 μg chlorophyll/g tissue), which was sufficient for at least 15 import assays each containing 10^7 chloroplasts; import using 2×10^7 chloroplasts per assay did not significantly increase import efficiency (data not shown). The yield of ~40 μg chlo-

rophyll/g tissue is approximately eight-fold higher than previously reported yields for direct isolation from *Arabidopsis* plants [7,10], but slightly less than the yield reported for isolation from protoplasts [10].

To assess the import competence of the chloroplasts, and to further confirm their integrity, an import assay using *Arabidopsis* Rubisco small subunit preprotein (*at*-pSS) was conducted (Fig. 1C). Radiolabelled pSS was imported with high efficiency (~10% of added preprotein was imported after 15 min), and the imported protein was found to be insensitive to exogenously applied thermolysin (Fig. 1C). These data demonstrate that the chloroplasts are highly import-competent and that their envelope membranes are intact.

3.2. Import using different preproteins

Several previous studies of *Arabidopsis* chloroplast protein import utilised heterologous import systems in which the preprotein and the chloroplasts were from different species [6,7,9,10]. To determine if the use of heterologous systems affects import efficiency, we compared the import efficiencies of *at*-pSS and pea pSS (*ps*-pSS) using chloroplasts from 10-d-old *Arabidopsis* plants (Fig. 2A). *ps*-pSS import was at least as efficient as *at*-pSS import, suggesting that, as with other species [18], there is no strong disadvantage in using a heterologous import system, at least in the case of pSS. Nevertheless, the use of homologous import systems is preferable, since doing so reduces the risk of obtaining spurious results. The *Arabidopsis* genome contains multiple genes for many import apparatus components [19], hinting at the existence of multiple import pathways in plastids, perhaps with different precursor recognition specificities [5,8]. In light of this remarkable complexity, it will be important to work with homologous import systems to ensure that the results obtained accurately reflect the in vivo situation.

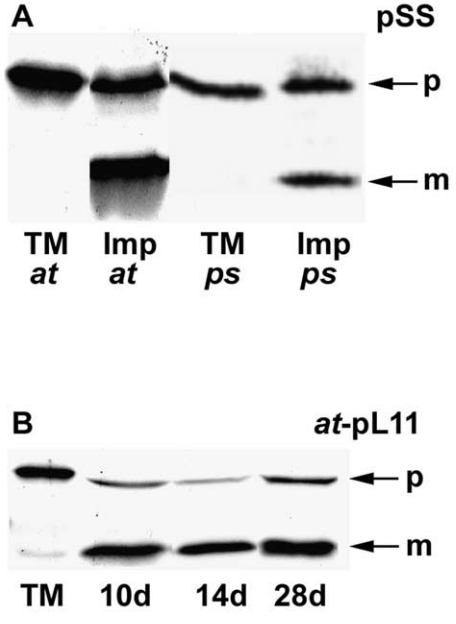

Fig. 2. Import using different preproteins and at different developmental stages. A: Comparison of homologous and heterologous import systems. *at*-pSS (lanes 1–2) and *ps*-pSS (lanes 3–4) were each imported (Imp) into *Arabidopsis* chloroplasts for 20 min. B: Import at different developmental stages. Chloroplasts isolated from 10-, 14- and 28-d-old plants were used in import assays with *at*-pL11; import was carried out for 15 min in each case.

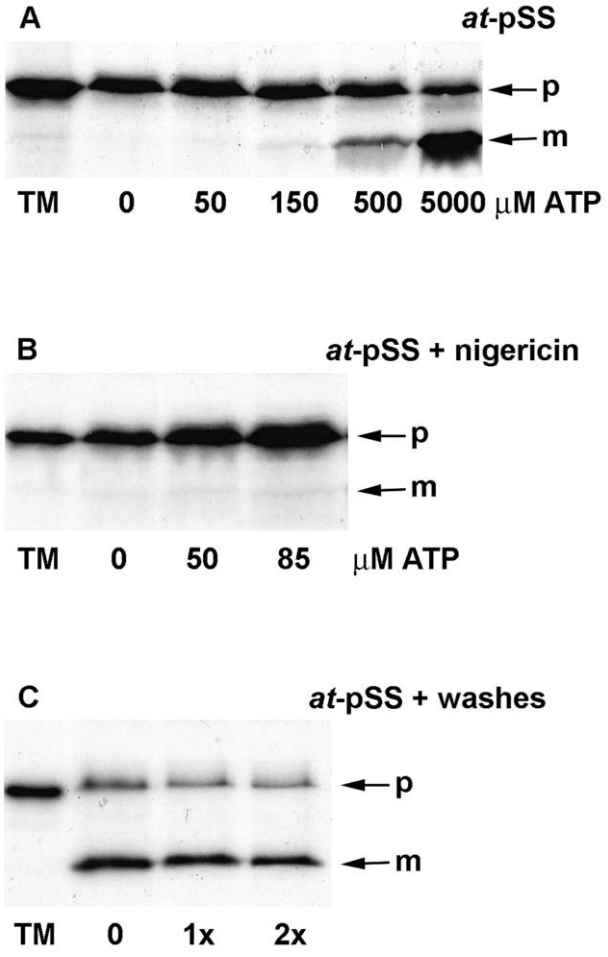

Fig. 3. Energetic requirements for binding and import. A: ATP-depleted chloroplasts and *at*-pSS were incubated together for 15 min in darkness. Reactions were carried out at different ATP concentrations as indicated. B: As A, except that the chloroplasts were depleted of ATP in the presence of nigericin. C: Bound preprotein cannot be removed by washing. Chloroplasts were incubated with *at*-pSS in the presence of 5 mM ATP in the light for 15 min, and were either analysed immediately (0) or washed once (1×) or twice (2×) in HS buffer prior to analysis.

In the past, import studies utilised pSS more than other preproteins since it is imported with high efficiency in vitro. However, the recently discovered complexity of the *Arabidopsis* import apparatus [19] means that it will be important to conduct future import studies using multiple precursors. We therefore investigated the competence of isolated *Arabidopsis* chloroplasts to import a second *Arabidopsis* preprotein: the 50S ribosomal subunit precursor, pL11 (Fig. 2B). This preprotein was selected since it is non-photosynthetic, whereas pSS is photosynthetic; comparisons of the import of functionally distinct proteins may be important, since photosynthetic and non-photosynthetic preproteins may follow different import pathways [8,20]. Import of pL11 was at least as efficient as pSS import (Fig. 2B, lane 2; ~10% of added pL11 was imported after 15 min), indicating that the method is useful for analysing precursors other than pSS. Several other *Arabidopsis* preproteins were also imported with high efficiency (Aronsson, Kubis and Jarvis, unpublished).

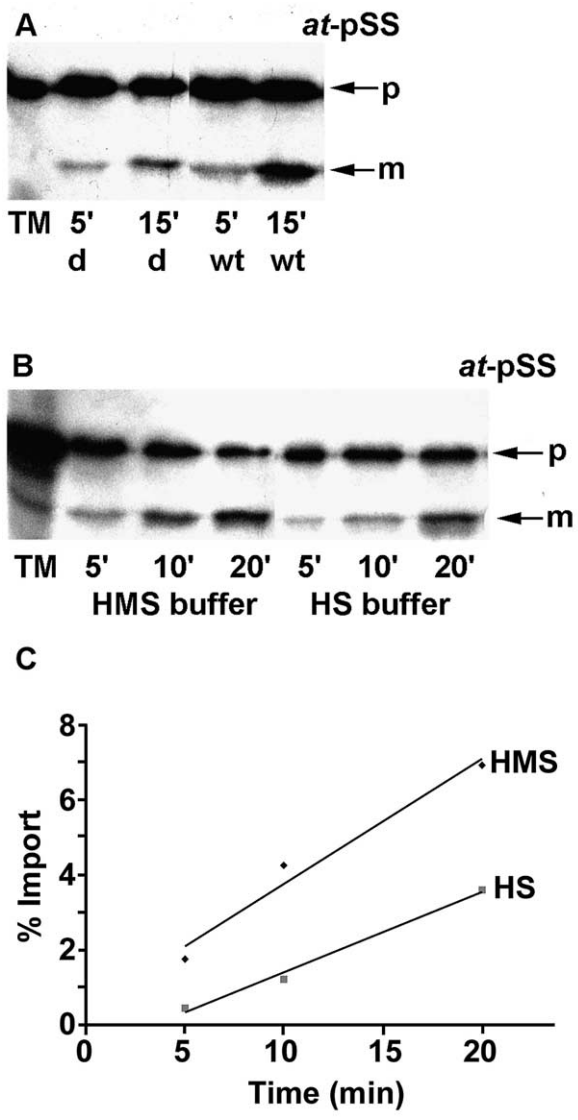

Fig. 4. Comparing import in different genotypes and using different import buffers. A: *at*-pSS was imported into chloroplasts from *dgd1* (d) and wild-type (wt) *Arabidopsis* plants for 5 and 15 min as indicated. B: *at*-pSS was imported into chloroplasts using HMS buffer or HS buffer for 5, 10 and 20 min as indicated. C: Radioactivity associated with each mature band in B was quantified and expressed as a percentage of the total pSS-associated radioactivity added to each reaction.

3.3. Import at different developmental stages

The isolation method was optimised for use with 10-d-old plants for reasons given earlier. Because it has been demonstrated that the import capabilities of pea and wheat chloroplasts can change significantly during development [21], and that the effects of *Arabidopsis* translocon mutations can change through development [5], it will be important to be able to study import at different developmental stages. To assess the suitability of the method for studying import at later stages, we isolated chloroplasts from 10-, 14- and 28-d-old plants and compared their import capabilities using pL11 (Fig. 2B). In each case, ~10% of the added pL11 was imported after 15 min, demonstrating that the protocol is indeed suitable for use with older plants. Interestingly, the import capabilities of chloroplasts from plants of different ages did not appear to differ significantly (Fig. 2B). A similar observation was made previously after comparing pSS import into chloroplasts isolated from 2-, 3-, 4- and 5-week-old *Arabidopsis* plants [10]. These results may indicate fundamental regulatory differences between the import systems of *Arabidopsis* and pea or wheat, or reflect the fact that whole plants were used to make the *Arabidopsis* import capacity comparisons, whereas developmentally different tissues were used in the pea and wheat experiments.

3.4. Energetic requirements for import

Protein import into pea chloroplasts can be divided into three distinct stages based on energetic requirements: energy-independent binding (ATP not required), import intermediate formation (< 100 μM ATP) and complete translocation (> 100 μM ATP) [16,22]. To assess the energetic requirements for protein import into *Arabidopsis* chloroplasts isolated using our method, import experiments containing different concentrations of ATP were conducted. As in pea, pSS bound to chloroplasts in the absence of ATP (Fig. 3A, lane 2), complete translocation only occurred at ATP concentrations exceeding 50 μM (Fig. 3A, lanes 4–6), and translocation was most efficient at mM ATP concentrations (Fig. 3A, lane 6).

However, import was observed to differ from pea import in two ways: (1) low ATP concentrations (50 μM) did not significantly stimulate preprotein binding (Fig. 3A, lane 3); (2) relatively high levels of preprotein remained bound to the chloroplast surface at all ATP concentrations (Fig. 3A). To determine if the former discrepancy was due to residual ATP within the chloroplasts, we repeated the experiment using chloroplasts depleted of ATP in the presence of the ionophore, nigericin [17]. This time, stimulated preprotein binding

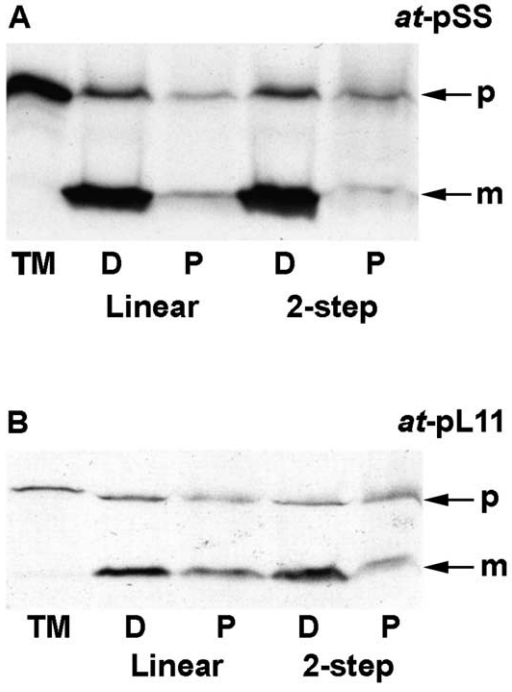

Fig. 5. Comparison of chloroplasts isolated using the direct and protoplast methods. *Arabidopsis* pSS (A) and pL11 (B) preproteins were imported into chloroplasts isolated from 14-d-old *Arabidopsis* plants by either the direct method (D) or the protoplast method (P) as indicated. Chloroplasts were purified using either a linear or a two-step Percoll gradient. Import was carried out for 15 min.

at low ATP concentrations was clearly observed (Fig. 3B, lanes 3–4), indicating that more stringent ATP-depletion conditions are required for *Arabidopsis* than pea [16]. High levels of preprotein binding have been observed previously [6,10], and this therefore appears to be a general feature of *Arabidopsis* chloroplasts. To assess the strength of association between the bound preprotein and the chloroplast surface, we attempted to remove the preprotein by persistent washing in HS buffer (Fig. 3C). Even after two washes, none of the bound preprotein could be removed, indicating that it is tightly bound to the chloroplast surface. High levels of preprotein binding may therefore reflect some specific property of the *Arabidopsis* import apparatus.

3.5. Studying import-defective mutants and quantifying import rates

The ability to characterise import in a range of different genotypes is an essential requirement for chloroplast import research using the *Arabidopsis* model system. We therefore tested our protocol using several different chloroplast biogenesis mutants, including *ppi1*, *dgd1* and *mgd1* [5,6,23], and found that it works efficiently in each case. Data generated using the mutants are intended for other papers, and so only data on the previously characterised mutant, *dgd1*, are shown here (Fig. 4A). The data are consistent with previous results [6], and confirm that *dgd1*, a mutant deficient in the plastidic galactolipid, digalactosyldiacylglycerol, is an import-defective mutant, clearly demonstrating the usefulness of the procedure for studying import defects in *Arabidopsis* mutants.

When characterising import-defective mutants, or when studying import at different developmental stages or under different environmental conditions, it may be necessary to make comparisons of import efficiency between samples. The best way to do this is to measure the import rate in each sample. Import rates decline during extended import reactions (data not shown), and so time-course experiments must be conducted during the first 20 min. We used time-course experiments to compare two different import buffers: HMS buffer [14] and HS buffer [13] (Fig. 4B). Quantification of the data demonstrates that import using HMS buffer is more efficient than import using HS buffer (Fig. 4C), and so HMS buffer was used for all our import experiments.

3.6. Comparison with chloroplasts isolated from protoplasts

The isolation of *Arabidopsis* chloroplasts from protoplasts has been described [12]. Recently, a modification of this protocol was used to isolate *Arabidopsis* chloroplasts for import studies (referred to as the protoplast method) [10]. Direct homogenisation methods have been used more commonly for chloroplast isolation, have been in use for many years [3,24], and have been shown to work well with several different species [20,21,25]. There are two major differences between the protoplast method and the direct homogenisation method described here (referred to as the direct method). First, the time between tissue harvesting and the beginning of the chloroplast isolation procedure is 4–5 h with the protoplast method [10]; with the direct method, chloroplast isolation starts immediately after tissue harvesting. Second, the use of the cell wall-degrading enzymes (cellulase and pectinase) for protoplastation adds an extra cost to each isolation, making the protoplast method ~20 times more expensive than the direct method. To determine if these time and cost disadvantages are compensated for by higher chloroplast yields or improved import-competence, we compared the two isolation procedures directly. To make the results directly comparable with those described previously [10], 14-d-old plants were used.

We used two different types of Percoll gradient to isolate intact chloroplasts from homogenates prepared using each method: a two-step gradient and a linear gradient. Using both types of gradient, we obtained a lower band containing intact chloroplasts with each method (data not shown). The different Percoll gradients used did not produce significantly different results. Chloroplast yield was found to be similar, although in some cases it was slightly higher using the protoplast method; in our hands, maximal yields using the protoplast method were 50–60 μg chlorophyll/g tissue. The import capabilities of chloroplasts from the direct and protoplast methods, isolated using linear and two-step gradients, were compared in import assays using pSS and pL11 (Fig. 5). The results demonstrate that the import of both preproteins is significantly more efficient with chloroplasts isolated using the direct method. In a previous study using the protoplast method, import efficiencies were also relatively low: only ~3% of the added pSS was imported after 30 min [9]. The very high import-competence of chloroplasts isolated using the direct method presumably reflects their integrity and normal functionality, indicating that the protocol may be useful for studying many different aspects of chloroplast biology in addition to protein import.

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