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Solubilization Method for Isolation of Photosynthetic Mega- and Super-complexes from Conifer Thylakoids

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[Abstract] Photosynthesis is the main process by which sunlight is harvested and converted into chemical energy and has been a focal point of fundamental research in plant biology for decades. In higher plants, the process takes place in the thylakoid membranes where the two photosystems (PSI and PSII) are located. In the past few decades, the evolution of biophysical and biochemical techniques allowed detailed studies of the thylakoid organization and the interaction between protein complexes and cofactors. These studies have mainly focused on model plants, such as *Arabidopsis*, pea, spinach, and tobacco, which are grown in climate chambers even though significant differences between indoor and outdoor growth conditions are present. In this manuscript, we present a new mild-solubilization procedure for use with "fragile" samples such as thylakoids from conifers growing outdoors. Here, the solubilization protocol is optimized with two detergents in two species, namely Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*). We have optimized the isolation and characterization of PSI and PSII multimeric mega- and super-complexes in a close-to-native condition by Blue-Native gel electrophoresis. Eventually, our protocol will not only help in the characterization of photosynthetic complexes from conifers but also in understanding winter adaptation.

Keywords: Blue-Native gel electrophoresis, Norway spruce, Scots pine, *Picea abies, Pinus sylvestris*, Photosystem I, Photosystem II, 2nd Dimension SDS-PAGE, Thylakoids

[Background] Being the basis of life on Earth, photosynthesis has become a major research focus from the mid-19th century (Hill, 1937 and 1939; Benson and Calvin, 1950; Benson *et al.*, 1950; Porter, 1950; Huzisige and Ke, 1993). Among several biochemical characterization methods, separation in polyacrylamide gel-based matrix of photosynthetic multi-subunit complexes, such as Photosystem I and II (PSI and PSII), was optimized from previous methods (Schägger and von Jagow, 1991; Kügler *et al.*, 1997; Järvi *et al.*, 2011; Haniewicz *et al.*, 2015; Farci *et al.*, 2017; Rantala *et al.*, 2017). Furthermore, the solubilization approach to extract those complexes evolved over the last decades, moving from "harsh" toward "gentle" procedures by using detergents with lower critical micelle concentration (CMC) and at lower concentrations. The same is true for all procedures aimed at extracting membrane protein complexes for structural and functional analyses. Yet, sample preparation and biochemical characterization remain challenging.

In almost all biochemical studies, the isolation of protein complexes is of fundamental importance for the success of any project. Protocols need to be robust and reproducible to minimize variations between

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extractions. A crucial factor for successful isolation and characterization is the employment of the best-suited detergent depending on the material used, considering the CMC, incubation time, and the temperature during solubilization but also the mixing procedure. It is an even more delicate task to obtain protocols that keep the isolated membrane protein complexes intact, in a close-to-native state. A protein complex in solution does not necessarily have a native and stable structure; additionally, a detergent useful for extraction can be unsuitable for purification procedures and subsequent studies. Thylakoid membranes are solubilized with different detergents, such as n-Dodecyl-β-D-Maltopyranoside (β-DDM), n-Dodecyl-α-D-Maltopyranoside (α-DDM), and digitonin, at an efficiency depending on the thylakoid region (grana or stoma lamellae) and species (Barera *et al.*, 2012; Haniewicz *et al.*, 2013 and 2015; Albanese *et al.*, 2016; Rantala *et al.*, 2017). Isolated photosynthetic complexes have been fully characterized in *Arabidopsis thaliana, Pisum sativum* L., and *Spinacia oleracea* L. (Albanese *et al.*, 2016; Rantala *et al.*, 2017; Wood *et al.*, 2018), and partially characterized in other species, such as *Nicotiana tabacum* L., *Physcomitrella patens*, *Picea abies* L. (Norway spruce), *Selaginella martensii*, *Oryza sativa* L., and *Zea mays* L. (D'Amici *et al.*, 2008; Romanowska *et al.*, 2008; Haniewicz *et al.*, 2013 and 2015; Ferroni *et al.*, 2014; Shen *et al.*, 2017; Iwai *et al.*, 2018; Grebe *et al.*, 2019; Kouřil *et al.*, 2020). Different detergent concentrations, mixtures of two detergents, and multi-step strategies (*e.g.*, differential solubilization) are also used to optimize solubilization (Wientjes *et al.*, 2013; Haniewicz *et al.*, 2015). These approaches gained detailed knowledge on oligomeric states, subunit composition, and dynamics in PSII. To achieve reproducibility, most of these studies were performed on chamber-grown plants, but they do not necessarily yield answers to how photosynthetic regulation takes place under natural conditions. Conversely, studies on tree species (*e.g.*, *P. abies*) growing in a challenging condition (like a boreal winter) provided a more complex picture of photosynthetic acclimation to environmental stress (Bag *et al.*, 2020; Grebe *et al.*, 2020; Chang *et al.*, 2021). For these species, several differences in composition and abundance in photosynthetic complexes were reported: the presence of triple phosphorylation of the novel LHCB1 variant, needed to achieve winter-adapted chloroplast structures (Bag *et al.*, 2020; Grebe *et al.*, 2020); higher-order mega complexes (Kouřil *et al.*, 2020), which are still a matter of debate in higher plants; and the absence of the PSI-NDH complex, mega-complexes, and the so-called M-LHCII complex (Bassi and Dainese, 1992; Nystedt *et al.*, 2013; Kouřil *et al.*, 2016). The latter is most likely an LHCII assembly containing both Lhcb3 and Lhcb6 that are missing in the Pinaceae family of conifers (Kouřil *et al.*, 2016; Grebe *et al.*, 2019) but present in *A. thaliana*.

Consequently, optimized biochemical procedures for the isolation and characterization of membrane complexes are needed to pursue such challenging studies, compare different species, and gain meaningful insights on structural/functional differences. In this manuscript, we use digitonin to apply and improve a mild-solubilization procedure for isolating thylakoid complexes from Norway spruce (*P. abies*) and Scots pine (*P. sylvestris*). The complexes are characterized by Blue-Native gel electrophoresis (BN-PAGE) and denaturing second dimension gel electrophoresis (2D SDS-PAGE). This newly optimized protocol will enable further characterization of PSI and PSII complexes by improving the extraction procedure in a close-to-native state, which, in these species, cannot be achieved by classic α-/β-DDM

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solubilization. Finally, the availability of this method opens paths for obtaining an in-depth understanding of the winter adaptation processes in evergreen pine trees.

Materials and Reagents

A. Consumables

1. Conical bottom microcentrifuge tubes, 1.5 ml
2. Conical bottom centrifuge tubes, 50 ml and 15 ml
3. Pipet tips (P1000, P200, and P10)

B. Plant material

P. abies and *P. sylvestris* L. plant materials collected from naturally grown trees in the Umeå University campus (63.8202° N, 20.3054° E)

C. Reagents

1. Invitrogen NativePAGE Bis-Tris (3-12% w/v gradient and 1.0 mm thickness, catalog number: BN1001BOX)
2. Precision Plus Protein Dual Color Standards (SDS-PAGE molecular marker; Bio-Rad, catalog number: 1610374)
3. SYPRO-ruby staining (ThermoFisher, catalog number: S12001)
4. APS (Ammonium persulfate, Sigma, catalog number: A3678)
5. Trizma (Sigma-Aldrich, catalog number: 93362)
6. Tricine (Sigma-Aldrich, catalog number: T0377)
7. Sodium Dodecyl Sulphate (SDS) (Sigma-Aldrich, catalog number: 436143)
8. Serva Coomassie Blue G (SERVA Electrophoresis, catalog number: 17524.02)
9. Bis-Tris (Sigma-Aldrich, catalog number: 14879)
10. 6-aminocaproic acid (Sigma-Aldrich, catalog number: A7824)
11. Glycerol (Sigma-Aldrich, catalog number: G5516)
12. miracloth (Merk Millipore)
13. Grinding buffer (B1) (see Recipes)
14. Shock buffer (B2) (see Recipes)
15. Storage buffer (B3) (see Recipes)
16. Aqueous acetone solution (80%) (see Recipes)
17. Washing buffer 1 (WB1) (see Recipes)
18. Washing buffer 2 (WB2) (see Recipes)
19. Washing buffer 3 (WB3) (see Recipes)
20. Solubilizing BTH buffer (SB) (see Recipes)
21. n-Dodecyl-α-D-Maltopyranoside (α-DDM) (see Recipes)
22. Digitonin (see Recipes)

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23. 100 mM α-DDM working solution (see Recipes)
24. 100 mM digitonin working solution (see Recipes)
25. Loading buffer (LB) (see Recipes)
26. Running buffer (RB) (see Recipes)
27. Soft-staining solution (see Recipes)
28. Acrylamide solution A (ThermoFischer) (see Recipes)
29. Acrylamide solution B (ThermoFischer) (see Recipes)
30. Cathode SDS buffer (CB) (see Recipes)
31. Anode SDS buffer (AB) (see Recipes)

Equipment

1. Blender (Multiblender Coline, Clas Ohlson)
2. Automatic pipettes (P1000, P200, and P10)
3. Heating block (95°C)
4. Cooled microcentrifuge (Eppendorf, model: 4530)
5. Microcentrifuge tube shaker (Orbit^{TM} Digital Microtube and Microplate Shaker)
6. Hoefer electrophoretic chamber (model: SE260 Mighty Small II)
7. Bio-Rad electrophoretic chamber (mini)
8. Bio-Rad mini-PAGE casting apparatus
9. Power Supply (Bio-Rad Power pack ultra)
10. pH meter (Bio-Rad)
11. UV-VIS spectrophotometer (Lumina, scanning range 150-800 nm)

Software

1. Chemidoc imaging system (Bio-Rad Image Lab version 6)

Procedure

A. Sample harvesting
   1. Mature needles were cut from branches with a pair of scissors, collected into a glass beaker, and kept on ice (T ≤ 0°C) in the dark.
   2. Needles were harvested on sunny days during June and July from south-facing branches of five different *P. sylvestris* and *P. abies* trees growing in the Umeå University campus (63.8202° N, 20.3054° E).
   3. Upon cutting, the samples were collected into a glass beaker (a separate icebox for each biological replicate was used) and placed in an icebox (35-40 cm deep; ¾ filled with ice and ¼ headspace).

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4. After collection of approximately 3-4 small branches (10-15 g of needles), the whole icebox was covered with a double layer of thick-black cloth to ensure darkness in the sunny summer days during the 2-5 min transportation time to the laboratory.
5. In the laboratory, samples were immediately subjected to isolation in the cold room (T ≤ 4°C) under dim green light. At no point from collecting branches until completion of isolation were samples moved from either icebox/cold room or darkness (see Figure S1 for sampling collection process and working setup).

B. Thylakoid isolation

Note: This step must be performed in a cold room.

(Buffers required - B1, B2, and B3; water and buffers were kept in the cold room overnight)

1. Samples were prepared according to Bag *et al.* (2020) with slight modifications to maximize protein complex isolation. Briefly, approximately 10-15 g (fresh weight) of needles were washed in ice-cold distilled water; then, 100-150 ml of ice-cold Grinding buffer (B1) was added (1:10 ratio of needles to buffer), and the mixture blended with a high-speed blender and pre-cooled at 4°C for 15 s in five repetitions, with a 30-s gap between each blending step [a high number of blending steps (5-6) at low blending time (15-20 s) and time lapse between each blending steps (25-40 s) are important for isolating intact higher-order complexes].
2. The blended needles were filtered through two layers of miracloth (Merk Millipore) and centrifuged (4,500 × *g* for 8 min, at 4°C). The obtained pellet was resuspended in 30-35 ml Shock buffer (B2) and centrifuged (4,500 × *g* for 8 min, at 4°C).
3. A subsequent resuspension, this time on Storage buffer (B3), was followed by a two-step centrifugation, first at low speed (200 × *g* for 2 min, at 4°C, discard pellet), then at higher speed (4,500 × *g* for 8 min, at 4°C).
4. The final obtained pellet was resuspended on 800 µl of B3 buffer, aliquoted in small fractions (50-100 µl), and either used immediately or frozen in liquid nitrogen and stored at -80°C for further use. In all steps, the pellet was resuspended with thin hair paintbrushes to prevent excessive mechanical shearing, which might damage the higher-order protein complexes.

C. Chlorophyll concentration determination

Considering the stoichiometric relationship between Chlorophyll and Chl-binding proteins, the sample concentration is expressed as milligrams of Chls in a milliliter of sample.

1. Chlorophyll concentration was calculated from 10 µl of samples extracted in 0.99 ml of 80% ice-cold acetone (1 ml final volume) by rigorous vortexing until no clumps could be seen (approximately 2 min; more time may be needed if the sample is very thick).
2. After vortexing, tubes were centrifuged at 8,000 × *g* for 5 min and measured according to Porra *et al.* (1989).

D. Sample preparation, electrophoresis, and isolation of protein complexes in native conditions

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Note: This step must be performed in a cold room.

Major optimization methods for conifer samples start from this part. From here onwards, steps are denoted chronologically using the same numbers as they appear on the graphical illustration (Figure 1). The volumes and concentrations of the samples, buffers, and detergents only focus on the optimal detergent concentrations that allow maximum separation. Samples, buffers, and detergents required for different tested detergent concentrations are provided in Tables 1 and 2.

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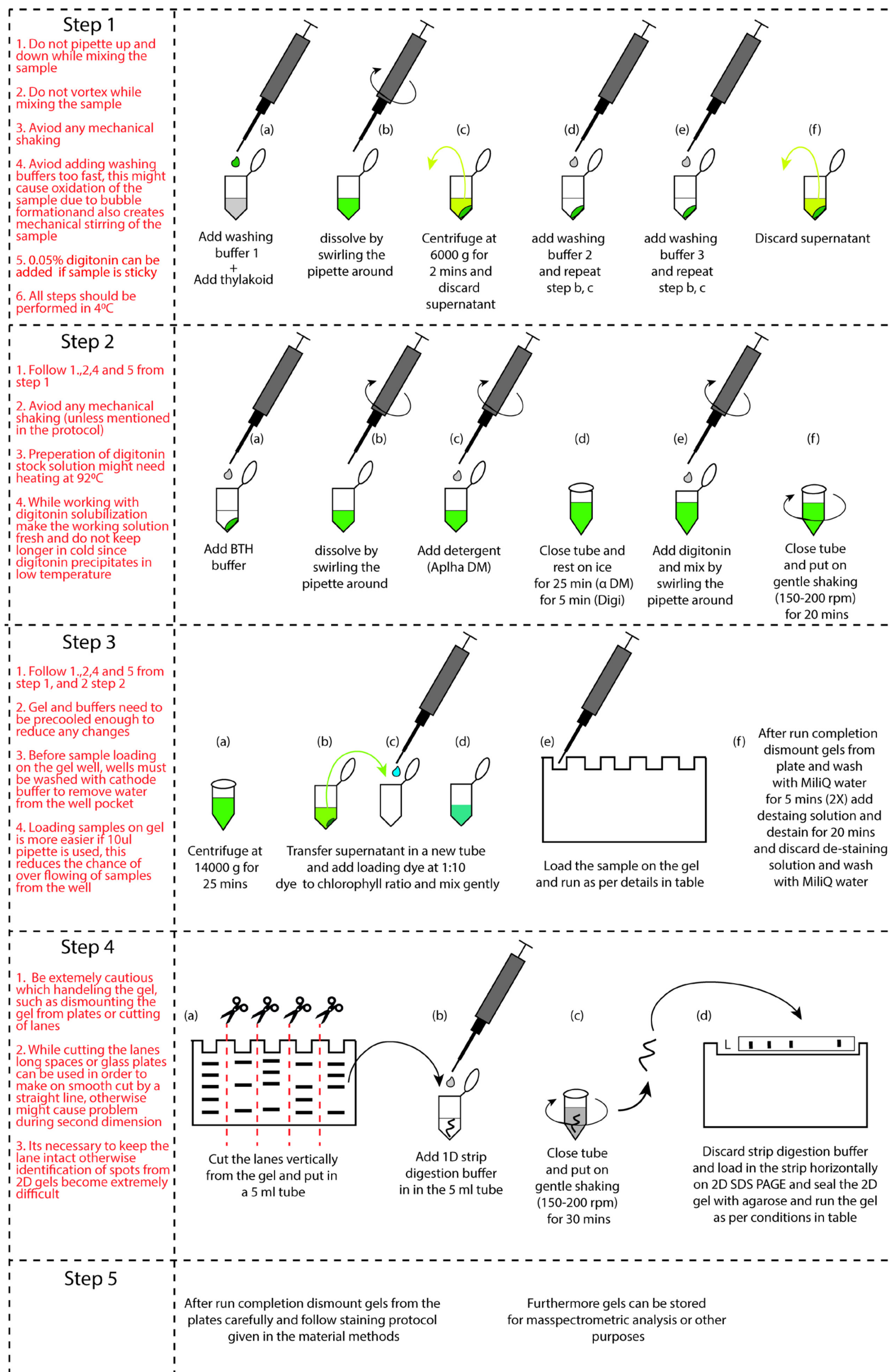

Figure 1. Graphical illustration of the solubilization, gel running, and band identification

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Table 1. Solubilization with increasing concentrations of α-DDM. The table shows the volumes of solubilizing buffer (SB) and volumes/concentrations of α-DDM used for obtaining the increasing detergent to chlorophyll ratios presented in Figure 2.

Specific volumes equal to 10 µg of chlorophyll were washed, pelleted down (Step 1), and solubilized (Step 2) with α-DDM	**SB**	**Detergent concentration**	**Detergent volume***	**Total sample volume**	**Final Chls concentration**
	17 µl	10 mM	2 µl	20 µl	0.5 µg/µl
	15 µl	20 mM	4 µl	20 µl	0.5 µg/µl
	13 µl	30 mM	6 µl	20 µl	0.5 µg/µl
	11 µl	40 mM	8 µl	20 µl	0.5 µg/µl
	9 µl	50 mM	10 µl	20 µl	0.5 µg/µl
	7 µl	60 mM	12 µl	20 µl	0.5 µg/µl
	5 µl	70 mM	14 µl	20 µl	0.5 µg/µl

*The volumes are referred to the 100 mM α-DDM working solution (see Recipes).

Table 2. Solubilization with increasing concentrations of digitonin. The table shows the volumes of SB and volumes/concentrations of digitonin used for obtaining the increasing detergent to chlorophyll ratios presented in Figure 3. Here, a two-steps solubilization was applied: first, 5 mM α-DDM, denoted as a constant +1 µl in the detergent volume column, were added 5 min prior to digitonin, which is added according to the desired concentration (changing volumes in the detergent volume column).

Specific volumes equal to 10 µg of chlorophyll were washed, pelleted down (Step 1), and solubilized (Step 2) with digitonin	**SB**	**Detergent concentration**	**Detergent volume***	**Total sample volume**	**Final Chls concentration**
	18 µl	4 mM	1 µl + 1 µl	20 µl	0.5 µg/µl
	17 µl	8 mM	2 µl + 1 µl	20 µl	0.5 µg/µl
	15 µl	16 mM	4 µl + 1 µl	20 µl	0.5 µg/µl
	13 µl	24 mM	6 µl + 1 µl	20 µl	0.5 µg/µl
	11 µl	32 mM	8 µl + 1 µl	20 µl	0.5 µg/µl
	9 µl	40 mM	10 µl + 1 µl	20 µl	0.5 µg/µl

*The volumes are referred to the 100 mM digitonin working stocking solution (see Recipes).

1. Washing thylakoid samples
   (Buffers required - WB1, WB2, and WB3)
   Considering the high tannin and polyphenolic contents in conifer needles, separation of native complexes needs several washing steps before solubilization; otherwise, the detergent activity

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becomes limited. Here, we used three different washing buffers before solubilization. First, 250 µl of WB1 was aliquoted in Eppendorf tubes and a certain volume of thylakoids corresponding to 10 µg of chlorophylls was suspended by mixing by swirling the pipette in the tube (vortexing or pipetting up and down is strictly prohibited since this can destroy protein complexes). After that, the sample was centrifuged (3,000 × *g* for 2 min, at 4°C) and the supernatant discarded. Then, the same process was repeated twice, once with WB2 and once with WB3.

2. Thylakoid solubilization
   (Buffers required - SB)
   For thylakoid solubilization, two different approaches are followed based on the detergent used.
   a. First, for α-DDM solubilization, we solubilized the washed thylakoid pellet in 8-9 µl of SB (depending on pellet size, the bigger the pellet, the smaller the volume of SB that should be added) to obtain 10 µl final volume of the resuspended washed thylakoids. In this way, the chlorophyll concentration of the sample becomes 1 mg/ml. After solubilization in SB, 100 mM of *n*-dodecyl-α-D- maltoside (α-DDM) is added at equal volume (obtaining a final chlorophyll concentration equal to 0.5 mg/ml and a detergent to chlorophyll ratio of 50:1) and solubilized on ice for 30 min at 4°C.
   b. The fractional solubilization process was applied as digitonin solubilization was reported to be unsuccessful in *Arabidopsis* protocols (Järvi *et al.*, 2011; Rantala *et al.*, 2017). First, thylakoids were solubilized in SB as before to a chlorophyll concentration of 1 mg/ml, then 1 µl of 100 mM *n*-dodecyl-α-D- maltoside (α-DDM) was added and the sample kept on ice for 5 min. Then, 6 µl of 100 mM digitonin was added and the final volume filled to 20 µl with SB (the final chlorophyll concentration was 0.5 mg/ml and the detergent to chlorophyll ratio 30:1). Gentle shaking was applied to tubes (max 200 rpm in Orbit™ Digital Microtube and Microplate Shake) and solubilization performed for 25 min at 4°C.
   c. Finally, both solubilized samples were centrifuged (14,000 × *g* for 25 min, at 4°C) and the obtained supernatant was used as for all analyses presented in this work.
3. BN-PAGE process
   a. Blue-Native Polyacrylamide Gel Electrophoresis
      (Buffer required - LB and RB)
      BN-PAGE was carried out using 3-12% w/v gradient and 1.0 mm thickness pre-casted 10 well gels. Before loading, samples were prepared at a ratio of 1:10 in loading buffer. A sample volume of 16 µl was loaded, which corresponds to a final concentration of 8 µg/ml Chls in each electrophoretic lane. Before loading the samples on the gel, the precast gels need to be installed on the electrophoretic chamber and the tank needs to be filled with running buffer and cooled down to 4°C. For our samples, we used a sub-zero water bath to keep the temperature of the electrophoretic system continuously below 2°C throughout the running period.
   b. Native electrophoretic conditions

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The run was performed on a Hoefer electrophoretic chamber at 65 V for 14 h (or overnight) at 4°C in the dark. The running buffer had the same composition for both anode and cathode, but before use, the cathode buffer was supplemented with loading buffer at a ratio of 1:1,000.

4. Post BN-PAGE processes

a. Soft staining and gel image acquisition

After the run, the gels were unmounted from the electrophoretic chambers and casting plates and washed in distilled water, fixed, and stained. BN-PAGEs were stained with the soft-staining solution (Figure 2) according to the procedure described by Farci *et al.* (2017). Gels were imaged on a white-light LED trans-illuminator using a digital camera (Canon EOS RP digital).

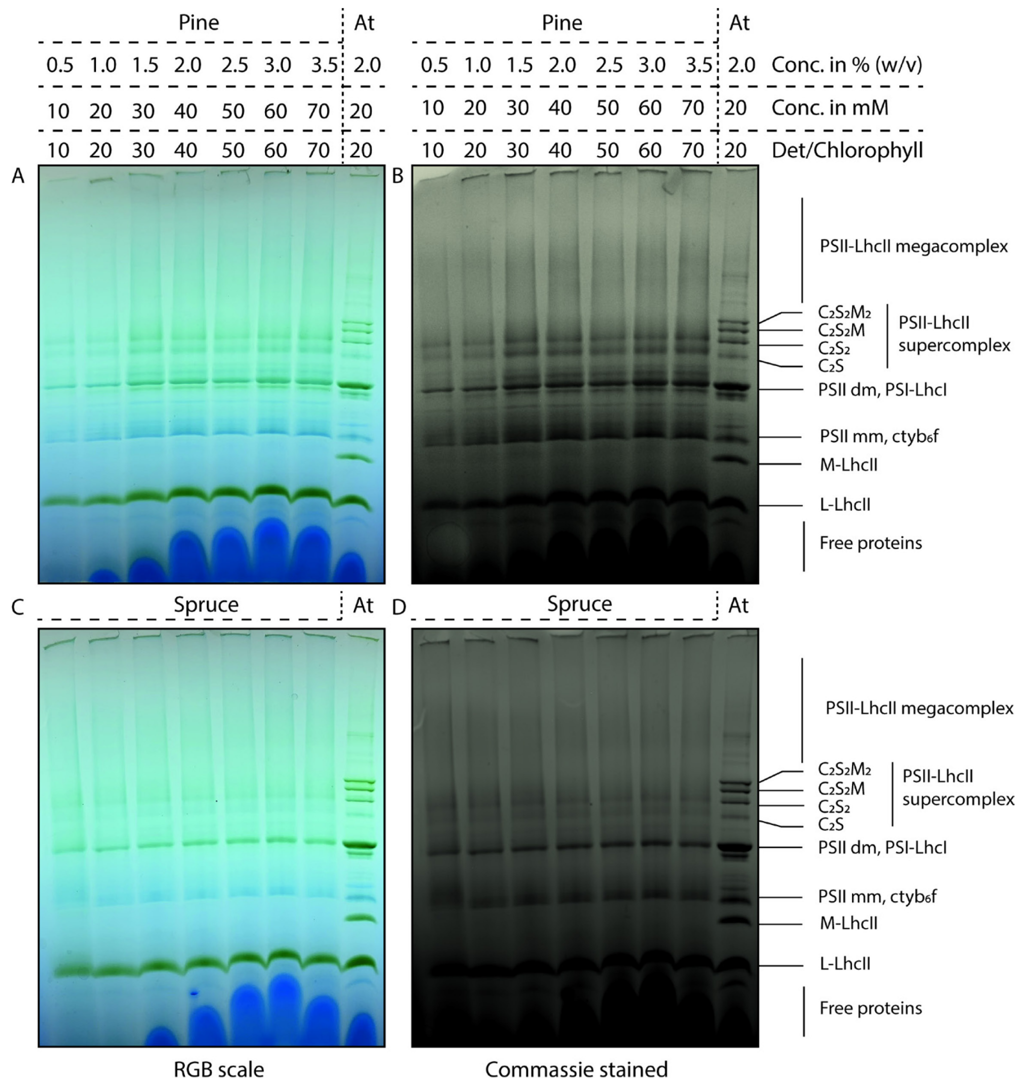

Figure 2. Solubilization of pine (A, B) and spruce (C, D) thylakoids with different concentrations of α-DDM. A and **C** are RGB images of soft-stained gels taken with a digital camera. **B** and **D** are Coomassie-stained images of the same gels.

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b. 2D Denaturing Polyacrylamide Gel Electrophoresis

For 2D separation, denaturing Sodium-Dodecyl-Sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Haniewicz *et al.* (2013). Briefly, the strips from the BN-PAGE were excised and denatured with Rotiload (Roth) at room temperature for 20 min. After denaturation, the strips were placed on top of a 10% denaturing SDS-PAGE (1.5 mm thickness) and sealed with 0.5% (w/v) agarose in cathode buffer. The molecular weight of the complexes isolated by 2D SDS-PAGE was estimated by plotting the retardation factor values (Rf, length of the band migration/length of the dye front) versus the log of the molecular weight of the molecular marker using a polynomial curve fit (second-order polynomial best-fit), according to the manufacturer's instructions. The obtained curve was used for calculating the apparent masses at the equivalent Rf of the 2D SDS-PAGE spots (data not shown). The masses were combined with western-blotting data for the identification of PSI and PSII subunits (see Step 5b).

c. Denaturing electrophoretic conditions

The run was performed on a Bio-Rad electrophoretic chamber and was carried out at 75 V for 3 h, at 4°C, in the dark. Freshly made cathode and anode running buffers were used.

5. Post 2D SDS-PAGE processes

a. Staining and gel image acquisition

After the run, the gels were unmounted from the electrophoretic chambers and casting plates and washed in distilled water, fixed, and stained. 2D SDS-PAGEs were stained with SYPRO-ruby staining (Figures 4 and 5) according to the manufacturer's protocol. Gels were imaged on a white-light LED trans-illuminator using a digital camera (Chemidoc imaging system).

b. Western blotting analysis

After 2D SDS-PAGE, proteins were transferred on PVDF membranes and further used for western blotting analysis and identification of PSI and PSII antibodies, as described in Bag *et al.* (2020).

Data analysis

A. Optimizing concentrations of detergents

Isolated native thylakoid membranes were solubilized as per the methods in two different detergents n-dodecyl-alpha-D-maltoside (α-DDM) and digitonin. The separation of thylakoid membrane complexes greatly depends on the optimal solubilization of samples (Haniewicz *et al.*, 2015), and the detergent to chlorophyll ratio may vary from species to species (Kouřil *et al.*, 2016 and 2020). For both detergents, we used a series of concentrations, as shown in Figure 2. For α-DDM, we started with the lowest concentration of detergent (0.5% w/vol =10 mM = 10:1 D:Chls) known to release complexes (to our knowledge) in other plant species (Shao *et al.*, 2011), and concentrations

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were increased up to 70 mM in 10 mM increments (Figure 2). With increasing detergent concentrations, the release of complexes increases and reaches the maximum at 50 mM. It is worth noting that previously it was reported that the use of 40 mM β-DDM releases $C_2S_2M_2$ as the largest complex, but here, 50 mM α-DDM released even larger complexes from the membrane (Figures 2 and 3), much larger than $C_2S_2M_2$ complexes found in *Arabidopsis*. Most likely, these bands could be similar to higher-order super-complexes previously identified in conifers (Kouřil *et al.*, 2020).

For digitonin solubilization, similar increasing detergent concentrations from 4 mM (0.4% w/vol = 4:1 D:Chls) have been used, but protocols like those used for other species (Järvi *et al.*, 2011) failed to release any complexes at all (Figure S2). We then supplemented digitonin with a very low concentration of α-DDM (5 mM) as this solubilization method is known to result in a better release of higher-order complexes (Wientjes *et al.*, 2013). Moreover, we obtained clearer results with a two-step solubilization (Figure 3), as explained in the methods and previously reported for other species where it was named "differential solubilization" (Haniewicz *et al.*, 2015). From different detergent concentrations, we chose 32 mM of digitonin to achieve maximum release of complexes when used in combination with 0.25 mM α-DDM in a two-steps solubilization process. By this, a total of six higher-order complexes (higher molecular weights than PSII dimer and PSI monomer) could be separated. The bands were named from 1-6 starting from the higher molecular weight (Figure 3E and 3F).

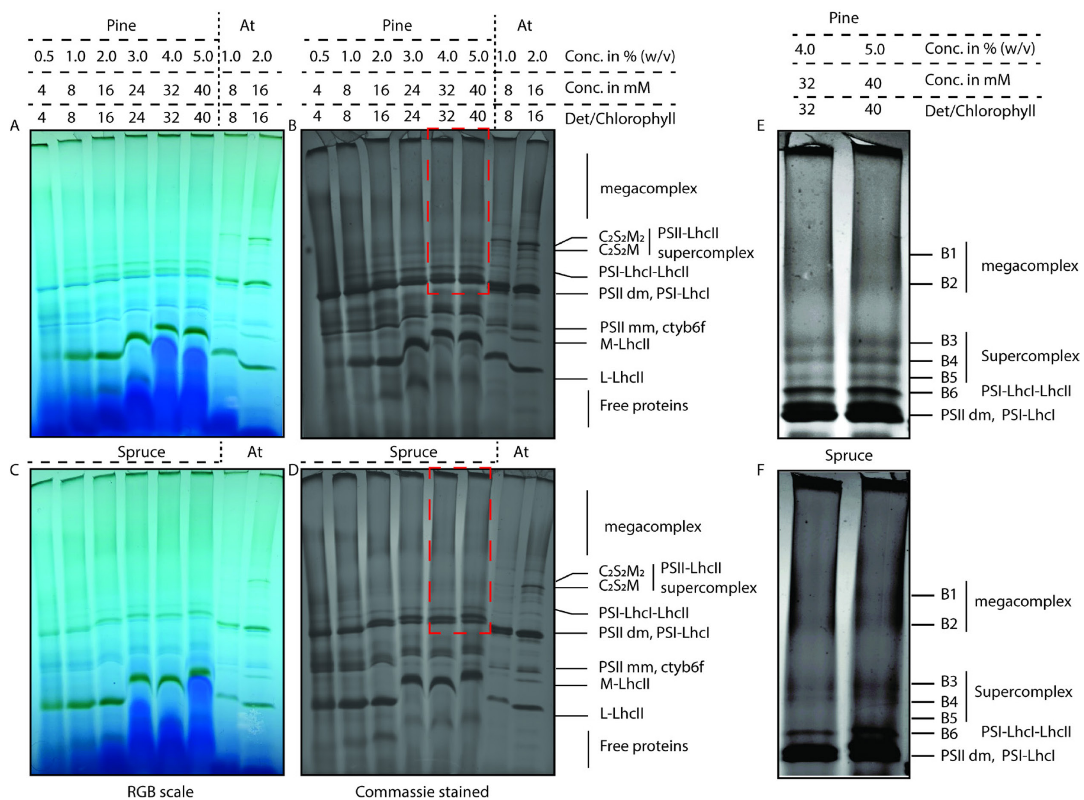

Figure 3. Solubilization of pine (A, B) and spruce (C, D) thylakoids with different concentrations of digitonin supplemented with 5 mM α-DDM. A and **C** are RGB images of soft-stained gels taken with a digital camera. **B** and **D** are Coomassie-stained images of the

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same gels. The red boxes marked in B and D are enlarged and depicted at high contrast in the insets E and F, respectively. These show six higher-order complexes (higher molecular weights than PSII dimer and PSI monomer) here named from B1 to B6 starting from the higher molecular weight.

B. Identification of complexes from 2D SDS-PAGE of 1D BN-PAGE strips

Identification of thylakoid membrane protein complexes separated on native gels have been done in *Arabidopsis* and Tobacco (Järvi *et al.*, 2011; Haniewicz *et al.*, 2015; Farci *et al.*, 2017). Recently, Grebe *et al.* (2019) identified proteins from native *P. abies* complexes released by β-DDM solubilization. We used the information from these maps to identify the protein composition of the complexes from pine (Figure 4C-ii, D-ii) and spruce (Figure 5C-ii, D-ii) and compared these with known *Arabidopsis* maps by running *Arabidopsis* thylakoids in parallel (Figure 4 and Figure 5C-i, D-i).

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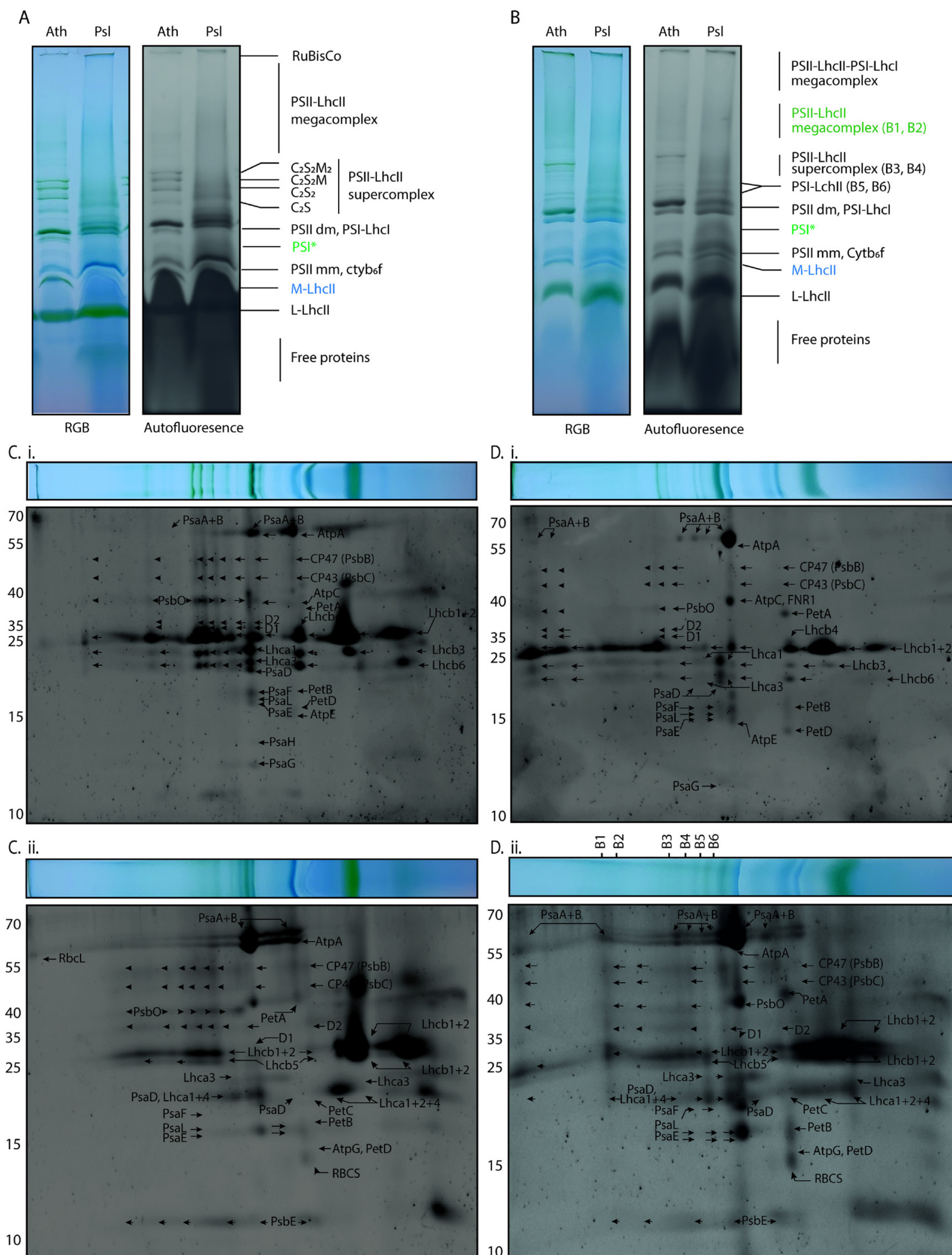

Figure 4. Identification of possible presence of thylakoid proteins by 2D SDS-PAGE of 1D BN-PAGE strips. Comparison of α-DDM (**A**) and digitonin solubilization (**B**) of pine (PsI) and *Arabidopsis thaliana* (Ath) thylakoid samples. 2D SDS-PAGE of 1D strips (**C**) cut from α-DDM solubilized *Arabidopsis* (**C-i**) and pine (**C-ii**); 2D SDS-PAGE of 1D strips (**D**) cut from digitonin solubilized *Arabidopsis* (**D-i**) and pine (**D-ii**).

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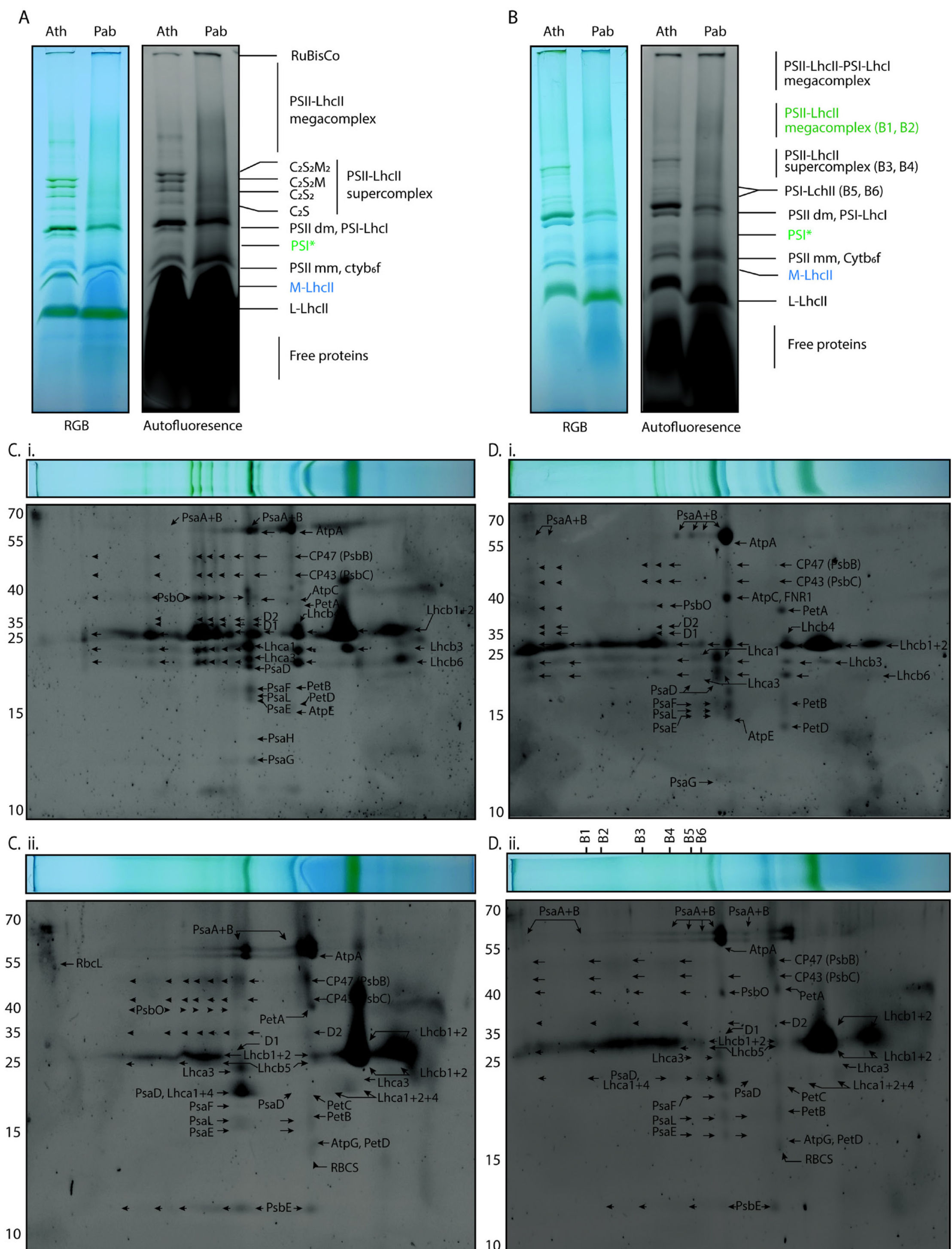

Figure 5. Identification of possible presence of thylakoid proteins by 2D SDS-PAGE of 1D BN-PAGE strips. Comparison of α-DDM (**A**) and digitonin solubilization (**B**) of spruce (Pab) and *Arabidopsis thaliana* (Ath) thylakoid samples. 2D SDS-PAGE of 1D strips (**C**) cut from α-DDM solubilized *Arabidopsis* (**C-i**) and spruce (**C-ii**); 2D SDS-PAGE of 1D strips (**D**) cut from digitonin solubilized *Arabidopsis* (**D-i**) and spruce (**D-ii**).

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The 2D SDS-PAGE of α-DDM solubilized BN strips (Figure 4C-ii and Figure 5C-ii) shows that both first and second bands contain mostly PSII proteins, *i.e.*, CP47, CP43, D1, D2, and LHCII (in comparison with band patterns from Grebe *et al.*, 2020). No spots or bands could be assigned to PSI proteins; thus, we conclude that these bands most likely comprised higher-order PSII complexes (either super- or mega- or oligomers of PSII).

Conversely, in 2D SDS-PAGE of digitonin solubilized BN strips, the first band (Figure 4D-ii and Figure 5D-ii) contains both PSI and PSII proteins, suggesting the presence of any of the three complexes, PSI-LHCI-PSII-LHCII (Rantala *et al.*, 2017), PSII-LHCII, and PSI-LHCI oligomer (Kouřil *et al.*, 2020). The second and third bands were mostly enriched in PSII subunits, while the fourth and fifth bands contain both PSI and PSII subunits. Finally, the last band was mainly represented by PSI core subunits, LHCI, and LHCII, but contained no PSII core subunits, suggesting this band to be either PSI-LHCI or PSI-LHCI-LHCII. This possibility of multiple complexes arises from the fact that, in conifers, there are unique higher-order complexes that are not present in so-called model plants such as *Arabidopsis*.

C. Future perspectives

The establishment of this protocol for thylakoid solubilization with non-ionic detergents facilitates further studies of higher-order complexes from conifers, for example, by mass spectroscopy or cryo-EM. This will enable a better understanding of the basis of photosynthetic adaptations in conifers, for example, during the boreal winter.

Recipes

Note: Stock/working solutions and buffers are prepared in Milli-Q water.

1. Grinding buffer **(B1)**
   50 mM HEPES-KOH pH 7.5
   330 mM sorbitol
   5 mM $MgCl_2$
   10% (w/v) PEG 6000
   10 mM NaF
   1 mM BSA
   0.65 mM Na-ascorbate
2. Shock buffer **(B2)**
   50 mM HEPES-KOH pH 7.5
   5 mM $MgCl_2$
   10 mM NaF
3. Storage buffer **(B3)**
   50 mM HEPES-KOH pH 7.5
   5 mM $MgCl_2$

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100 mM sorbitol
10 mM NaF

4. Aqueous acetone solution
   80% acetone
   20% water
5. Washing buffer 1 **(WB1)**
   50 mM Bis-Tris HCl (pH 7.0)
   4% sorbitol
   10 mM NaF
   2.5% Pefabloc
6. Washing buffer 2 **(WB2)**
   40 mM Bis-Tris HCl (pH 7.0)
   5% sorbitol
   10 mM NaF
   2.5% Pefabloc
7. Washing buffer 3 **(WB3)**
   35 mM Bis-Tris HCl (pH 7.0)
   6% sorbitol
   10 mM NaF
   2.5% Pefabloc
8. Solubilizing BTH buffer **(SB)**
   25 mM Bis-Tris HCl (pH 7.0)
   20% glycerol
   10 mM NaF
   2.5% Pefabloc
9. α-DDM
   200 mM stock solution in distilled water
10. Digitonin (200 mM stock solution in distilled water)
    Dissolving digitonin requires heating at 92-95°C for 10-12 min.
    If stock solution is frozen for further use, it needs similar heat treatment before it can be used again.
11. 100 mM α-DDM working solution
    25 mM Bis-Tris HCl (pH 7.0)
    20% glycerol
    10 mM NaF
    2.5% Pefabloc
    200 mM α-DDM
    This solution needs to be freshly made every time.
12. 100 mM digitonin working solution

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25 mM Bis-Tris HCl (pH 7.0)
20% glycerol
10 mM NaF
2.5% Pefabloc
200 mM digitonin
This solution needs to be freshly made every time.

13. Loading buffer **(LB)**
    5% serva Coomassie Blue G
    750 mM aminocaproic acid
    50 mM Bis-Tris HCl (pH 7.0)
    35% sucrose
14. Running buffer **(RB)**
    50 mM BisTris, pH 6.8
    50 mM Tricine
15. Soft-staining solution
    7% acetic acid
    10% ethanol
    83% distilled water
16. Acrylamide 40% (w/v) solution A (ready-to-use) (ThermoFischer)
17. Acrylamide 2% (w/v) solution B (ready-to-use) (ThermoFischer)
18. Cathode SDS buffer **(CB)**
    0.1 M Tris
    0.1 M Tricine
    0.1% (w/v) SDS
19. Anode SDS buffer **(AB)**
    0.2 M Tris, pH 8.9

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Competing interests

The authors declare no conflict or competing interests.

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