CHAPTER ONE

Analysis of the Effect of Elevated Cytokinin Content on the Photosynthetic Apparatus Using Blue Native PAGE

Anne Cortleven and Roland Valcke

Abstract Blue Native gel electrophoresis is a very powerful technique for the separation of highly hydrophobic membrane proteins and the isolation of native protein complexes. It offers an alternative for two-dimensional gel electrophoresis. To study the effects of cytokinins on the composition of the complexes in the photosynthetic apparatus, transgenic P*ssu-ipt* tobacco plants with increased cytokinin content are used and compared to wild-type plants. After isolation of intact chloroplasts of transgenic and wild-type plants, digitonin and dodecyl-β-maltoside (1%) are used to obtain the native conformation of the photosynthetic complexes. A separation in the first dimension under native conditions, Blue Native PAGE, is combined with SDS-PAGE for separation of the protein complexes into their subunits. This provides a better insight in the network of protein complexes of the photosynthetic apparatus and the physiological changes due to elevated cytokinin content.

Keywords Photosynthesis, membrane protein complexes, blue native PAGE, cytokinins

Introduction

Chloroplasts are highly specialized plant organelles, where photosynthesis takes place. They are important not only for photosynthesis, but also for other metabolic processes such as synthesis of specific lipids, amino acids, tetrapyrols and hormones. Chloroplasts are the most important target of cytokinins (Reski 1994). Cytokinins are plant hormones that play a multiple role in growth and development of the plant. They regulate light-mediated processes like de-etiolation and differentiation of chloroplasts by inducing the synthesis of chloroplast proteins and photosynthetic components (Chernyad'ev 2000).

To study the effect of cytokinins on the structure and the function of the photosynthetic apparatus, transgenic P*ssu-ipt* tobacco plants, containing the *ipt*-gene coding for isopentenyltransferase, a key enzyme in the biosynthesis of cytokinin, under control of the light-inducible P*ssu*-promoter, are used. This results in elevated endogenous cytokinin

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content. Previous studies showed that transgenic P*ssu-ipt* tobacco plants show a reduction in photosynthesis (Synková et al. 1999). To study the effects of elevated cytokinins on the structure of the photosynthetic apparatus, 2D-gel electrophoresis was used. This technique is not useful for the separation of native protein complexes or highly hydrophobic proteins (Santoni et al. 2000). The chloroplasts contain different highly hydrophobic protein complexes such as photosystem I and II, cytochrome b_6f complex and ATP-synthase. Therefore, Blue-native PAGE offers a good alternative to study the proteome of the chloroplasts and any structural changes that may occur due to elevated endogenous cytokinin content.

Materials and methods

Cultivation of plants. All plants were cultivated in a greenhouse. Wild-type plants, *Nicotiana tabacum* L. cv. Petit Havana SR1, were sown in potting soil (Universal potting soil, Agrofino, Agrofino Products N.V.). After 2 weeks, they were put on Grodan (Grodania A/S, Hedehusene, Denmark) saturated with half-strength Hoagland solution. The transgenic plants, containing the *ipt*-gene under control of the *Pisum sativum* ribulose-1,5-biphosphate carboxylase small subunit promoter sequence (*Pssu-ipt*), were obtained using the *Agrobacterium tumefaciens* system as described by Beinsberger et al. (1992). After transformation, the seeds were sown on Murashige-Skoog medium with kanamycin (100 mg/mL). Only kanamycin resistant seedlings (2–3 weeks old) were further cultivated under the same conditions as wild-type plants (temperature 18°C, humidity 60%). Additional illumination was provided 16 h a day with AgroSon T (400 W) and HTQ (400 W) lamps (photon flux density of 200 µmol quanta (m^{-2} s^{-1})).

Isolation of intact chloroplasts. Intact chloroplasts were isolated from 6-week-old wild-type plants and transgenic plants of comparable height, following a modified version of the method described by Bartlett et al. (1982). Leaves (50 g) were harvested and homogenized in 200 ml ice-cold grinding buffer (2 mM NaEDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 50 mM Hepes-KOH, pH 7.5, 0.33 M sorbitol, 5 mM sodium ascorbate) for three times 10 s, using a Braun MX-32 mixer. All subsequent steps were carried out at 4°C. The suspension was filtered through four layers miracloth and a fraction containing chloroplasts was sedimented by centrifugation at 1,400 g for 5 min. The green pellet was resuspended in grinding buffer (5 ml/50 g). Two milliliter of this suspension was loaded on a continuous 10–80% Percoll gradient (3% PEG 6000, 1% Ficoll, 1% BSA) and centrifuged for 20 min at 8,000 *g*. Two green bands are visible after centrifugation. The upper band contains the broken chloroplasts and the lower band the intact chloroplasts, which were collected with a syringe, washed with 5–10 volumes grinding buffer and centrifuged for 10 min. The intact chloroplasts are then resuspended in TMK-buffer (10 mM Tris pH 6.8, 10 mM $MnCl_2$, 20 mM KCl) and centrifuged for 10 min at 2,200 *g*. The pellet was dissolved in TMK buffer at a concentration of 1 mg chlorophyll/mL.

BN-PAGE/SDS-PAGE. BN-PAGE was performed according to Reisinger and Eichacker (2006). All steps of the sample preparation were performed on ice. Two different detergents (digitonin and dodecyl-β-maltoside) were used for solubilization of the membrane protein complexes. Before solubilization, the chloroplast suspension was centrifuged at 400 *g* during 3 min. The pelleted chloroplasts were then solubilized with 1% (w/v) dodecyl-β-maltoside (30 µl) or 2.5% (w/v) digitonin (50 µl) for 10 min. After solubilization, the samples were centrifuged for 10 min at 13,000 *g*. One microliter loading buffer (5% (w/v) Serva Blue G250, 750 mM ε–aminocaproic acid) was added to the supernatant and the mixture was loaded on a 5–15% acrylamide gradient gel (4% acrylamide stacking gel). Electrophoresis was performed in a Protean II electrophoresis system (Bio-Rad, USA) at 4°C applying a constant voltage of 250 V overnight. For SDS-PAGE, lanes of the first dimension were incubated in solubilization buffer (2% (w/v) SDS, 66 mM Na_2CO_3, 2% (w/v) β-mercapto-ethanol, 10% (w/v) glycerol, 0.5 M Tris/HCl pH 6.8) for 30 min at room temperature. Thereafter,

the lane was put on a second dimension gel (10% acrylamide, stacking gel: 4% acrylamide) and overlaid with agarose solution (25 mM Tris pH 8.8, 192 mM glycine, 0.1% SDS, 0.5% (w/v) agarose, 0.0002% bromophenolblue). Electrophoresis (1 h at 2 W/gel, followed by 1 h 4 W/gel and finally 4 h at 17 W/gel) was carried out in EttanDalt*six* system (Amersham Bioscience).

Staining and analysis. After electrophoresis, gels were fixed in fixation solution (45% (v/v) methanol, 5% (v/v) acetic acid) overnight. Blue Native gels were stained with Coomassie dye (50% (v/v) methanol, 7% (v/v) acetic acid, 0.025% (w/v) Coomassie Brilliant Blue G250) for 4 h and unstained overnight in methanol (50% v/v), acetic acid (7% v/v) after fixation, the second dimension gels were washed three times with 50% (v/v) ethanol for 20 min, incubated for 1 min in 0.02% (w/v) $NaS0_4$ and rinsed three times with water. Thereafter, the gels were incubated for 20 min in 0.2% (w/v) silvernitrate, rinsed three times with water and incubated in 0.04% formaldehyde, 6% (w/v) sodiumcarbonate until the spots were visible. This coloring reaction is stopped using 5% (v/v) acetic acid. After staining, the gels were scanned and analysed with Image Master Platina (Amersham Bioscience).

Results

Blue native PAGE of chloroplast protein complexes. Digitonin and dodecyl-β-maltoside proved to be very suitable detergents for the solubilization and stabilization of supercomplexes of chloroplasts (Heinemeyer et al. 2004; Kügler et al. 1997). To determine the optimal detergent-protein ratio for solubilization of the supercomplexes, intact chloroplasts were treated with different digitonin or dodecyl-β-maltoside concentrations and analysed by 1D Blue Native PAGE. Low concentration of detergent resulted in less sharp bands and reduced separation of the complexes. High concentrations of detergents resulted in micelle-formation. The best separation of the chloroplast complexes was obtained using 1% (w/w) dodecyl-β-maltoside and 2.5% (w/w) digitonin.

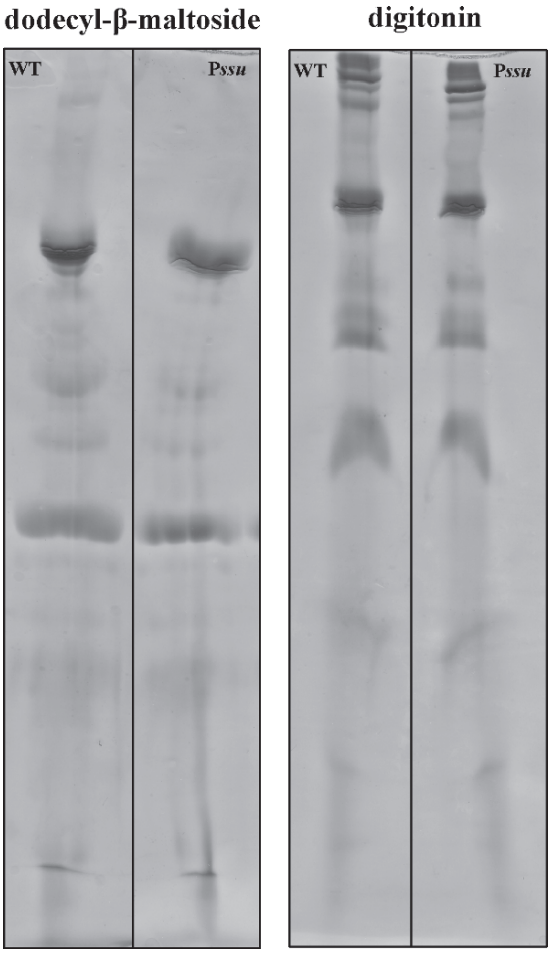

Fig. 1 Blue Native electrophoresis of intact chloroplasts of wild-type (WT) and transgenic (P*ssu-ipt)* tobacco plants after staining with Coomassie Brilliant Blue. Intact chloroplasts were solubilized in 1% (w/v) digitonin or 2.5% (w/v) dodecyl-β-maltoside. No differences in protein complexes are visible between WT and P*ssu-ipt* for dodecyl-β-maltoside as wel as for digitonin

Unfortunately, there were no qualitative differences in the supercomplex pattern between transgenic and wild-type (Fig. 1).

Analyses of the subunits of the protein complexes. To further investigate the protein complexes, Blue Native strips were subjected to SDS-PAGE in the second dimension. Under these conditions, protein-complexes dissociate into their subunits.

After staining and software analyses of these gels, no differences were found in subunit composition of the protein complexes (Fig. 2).

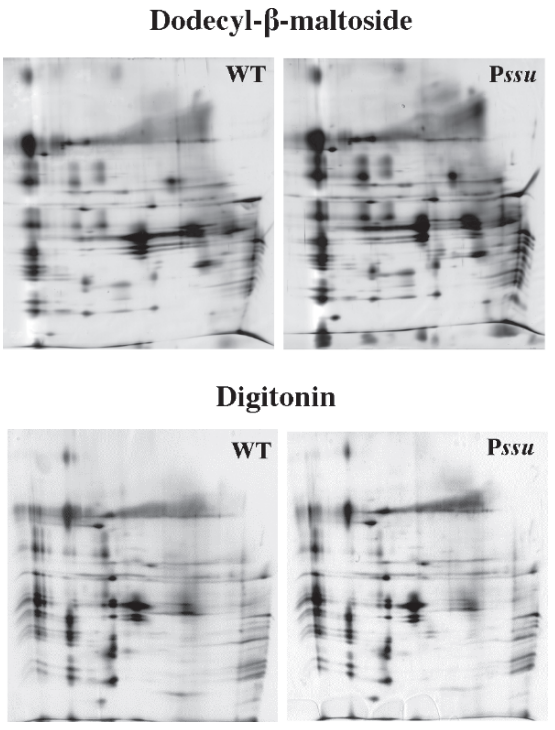

Fig. 2 Comparison of 2D BN/SDS PAGE (10% (w/v) polyacrylamide) between wild-type (WT) and transgenic (P*ssu-ipt*) tobacco plants for dodecyl-β-maltoside and digitonin. There are no differences in subunit composition between wild-type and transgenic plants

Discussion

Blue Native PAGE is an ideal technique to investigate the high hydrophobic protein complexes of the chloroplasts (Heinemeyer et al. 2004). Results of chlorophyll *a* fluorescence kinetics (unpublished data) suggested no effect of cytokinins on the transfer of excitons to the reaction centers, but a significant effect of cytokinins on the transfer of electrons once the photosynthetic apparatus is in the excited state. A possible hypothesis is that increased endogenous cytokinins levels induce structural alterations. This hypothesis could be consistent with the higher chlorophyll content of the transgenics (unpublished data). Using Blue Native PAGE, no qualitative differences in protein subunit composition could be observed between transgenic plants, with increased endogenous cytokinins content, and wild-type plants. A severe limitation of Blue Native PAGE is that it does not offer the possibility to search for quantitative differences. Further detailed 2D analysis (IEF/SDS-PAGE) will be necessary to look for quantitative differences.

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