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Effect of cytokinins on the expression of LHCP-genes

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Introduction

The light-harvesting chlorophyll a/b- binding proteins (LHCP) function as carrier proteins of antenna pigments and are located in the thylakoïd membrane of chloroplasts. LHCP's are present in both PSI and PSII. Encoded by a gene family, they consist of a large variety of polypeptides. The general structure of these polypeptides is shown in fig.1. The *Lhc*-genes are located on the nuclear DNA.

To get a better insight in the regulation of these genes, we use transgenic tobacco plants with an elevated cytokinin-level. Using a special construct (P*ssu-ipt*) with a light-inducible promotor (from *Pisum sativum*), the concentration of cytokinins in the plants can be changed. The influence of cytokinins on photosynthesis is already examined. The capacity of the electron transport chain is altered. The effects on the photosynthetic-capacity depend on the age of the leaves (Synková et al., 1997).

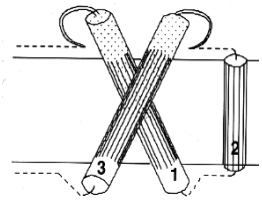

Fig. 1. (Jansson, 1994)

Generally, a small increase in cytokinin concentration has a positive effect on photosynthesis; at higher concentrations, photosynthesis decreases. It is still not clear how an elevated cytokinin concentration influences the photosynthetic proces or the structure of photosynthetic pigment-proteins. An *in vitro* application of cytokinin has no direct effect (no direct binding) on a component of the electrontransport chain (Valcke R., Pers. Com.).

Material and Methods

In the transgenic plants, an *ipt*-gene is inserted by means of the *Agrobacterium tumefaciens* transformation system. This gene codes for isopentenyl transferase, a key enzyme in cytokinin-biosynthesis. In the transgenic plants, the *ipt*-gene was coupled to the light-inducible P*ssu*-promotor (of *Pisum sativum*) of the small subunit of RubisCO (fig.2).

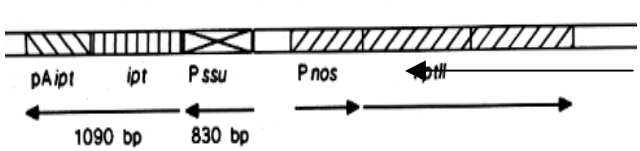

Fig.2. Chimere gene-construct *Pssu-ipt* (adapted from Beinsberger, 1991).

To show the presence of the *ipt*-gene in the transgenic plants, a PCR was performed (fig.3). Primer sequences are: + 5'-GCATATTATTCGCCACAAGTTACCC-3' and – 5'-GGCTAGCAAACAACATGGCATATC-3'.

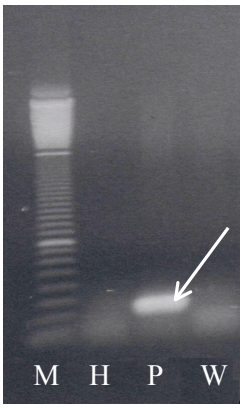

Fig.3. Result of PCR. Pssu-plants clearly have the *ipt*-gene (M: marker, H: H_2O, P: Pssu, W: WT).

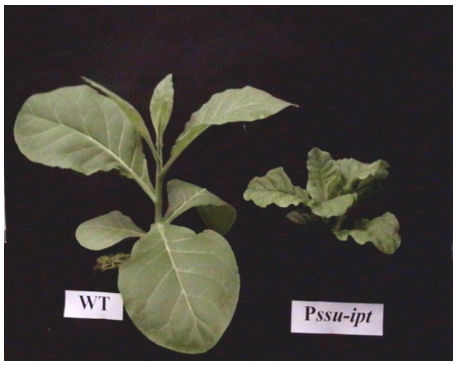

Fig.4. WT plant and Pssu-ipt plant

Plants (*Nicotiana tabacum* L. cv. Petit Havana SR1) are cultivated on grodan in a greenhouse. This is done for WT as well as for the transgenic plants (fig.4). Additional illumination is provided 16h a day with Agroson T (400W) and HTQ (400W) lamps (200µmol quanta m^{-2} s^{-1}).

Samples (leaf discs) are analysed by means of 1D-, 2D-electrophoresis and Western blotting. 1D–samples are prepared with an extraction buffer (0.33M sorbitol, 0.1M Tricine pH 7.8), after centrifugation, pellets are washed in buffer (5mM EDTA, 5mM Tricine pH 7.5). After homogenisation, chlorophyll content is measured (OD 652nm). After centrifugation (48.000g, 10 min.), pellets are solubilised in sample buffer (50mM Tris pH 6.8, 1mM EDTA, 2.5% SDS, 5% β-mercapto-ethanol) at 2mg chl/ml. For the separation, gradient gels are used (12%-18%).

2D-samples are prepared with an aceton-fenol extraction. IEF is performed with ImmobilineDry strips (Pharmacia) and SDS-Page with Excel Gels 8%-18% (Pharmacia). 1D-gels are stained with Coomassie-staining, 2D-gels with silverstaining (according to Blum et al., 1987).

Unstained gels are used for Western blotting. Antibodies were raised against Lhcb (generous gift from Dr. A. Radunz, Universität Bielefeld, FRG). Staining of the membranes was done by o-anisidine.

Results and discussion

Introduction and expression of the *ipt*-gene, encoding isopentenyl transferase, into plant genome under the control of a light-inducible promotor leads to an increased endogenous cytokinin level (Schmülling et al., 1989; Beinsberger et al., 1991). Transgenic plants exhibited a general cytokinin-syndrome, e.g. reduced rooth growth, reduced apical dominance, reduced leaf surface, reduced growth of the stem (Schmülling et al, 1989; Beinsberger et al., 1992). Also tobacco shoots constitutively expressing the *ipt*-gene display suppression of root formation (Schmülling et al, 1989). Cytokinins are known to be involved in development of chloroplasts (Parthier, 1979), so there is some indication that the photosynthetic apparatus can be affected. Plants with a slightly elevated cytokinin level show no changes in phenotype and/or photosynthesis (Šiffel et al, 1992). When the endogenous level exceeds a certain 'treshold', which is not easy to determine, changes in morphology, development and photosynthesis become significant. Disturbances in Chl synthesis, electron transport activity and water regime were also observed (Synková et al., 1999).

The light-harvesting chlorophyll protein complex of photosystem II (LHC-II) could be influenced by the elevated level of cytokinins. To examine this, we use a molecular approach. If we look at the polypeptide-level, is it possible to see differences between the transgenic and WT plants? By means of 1D-, 2D-electrophoresis and Western blotting, we try to compare the polypeptide pattern of both types of plants. This could give an indication of a cytokinin-effect in the transgenic plants.

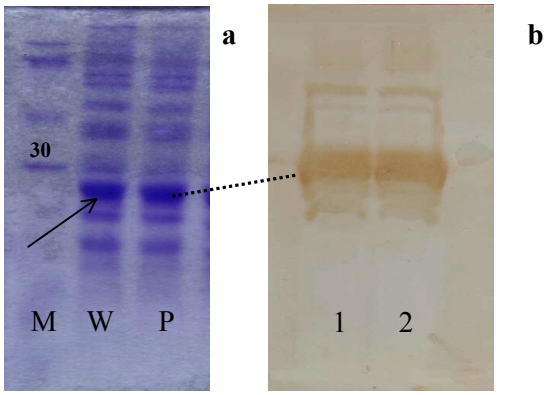

Fig.5. a. SDS-PAGE (12-18%) of WT thylakoid extract (W) and Pssu thylakoid extract (P); marker (M) / **b.** Immunoblot of thylakoid extract with Lhcb-antibodies (1: WT, 2: Pssu).

In fig.5a, the polypeptide-pattern of WT- and P*ssu*-plants are compared. The marker of 30kDa is indicated by 30 and the band of Lhcb is by an arrow. In the immunoblot (fig.5b), more than one band is colored. The antibodies are not specific for Lhcb. The patterns show no clear difference for the two plants compared.

The experimental conditions to perform the 2D-electrophoresis of the WT and P*ssu-ipt* proteins are identical. Equal amounts of protein in a final concentration of 5mg/ml are used. Silver staining of the 2D-gel gives a very complex pattern (fig.6a; polypeptide pattern of WT-plant extract). Equally runned 2D-gels are blotted on nitrocellulose membranes and treated with Lhcb-antibodies (fig.6b). Based on the assumption that the Lhcb-antibodies are rather specific (reaction in 28-30 kDa zone), the blot shows that the Lhcb-complex is composed of several polypeptides, characterized by different iso-electric points, but the same molecular weight. The patterns in the WT and the P*ssu-ipt* blots are qualitatively identical but the spots in the P*ssu-ipt* blot are much less intense compared to the WT. This indicates that in the transgenic P*ssu-ipt* plants, less Lhcb protein is present. The endogenous cytokinin level in the P*ssu-ipt* plants is much higher than in WT (Synková et al, 1999) and most probably exceeds a threshold value. This could result in reduced expression of several proteins, including the Lhcb-proteins. Another possible approach is that high endogenous amounts of cytokinins induce stress conditions. For instance, P*ssu-ipt* plants are characterized by a strongly reduced root-system. This implies a less efficient uptake of minerals and water which could result in mineral nutrition stress and water stress.

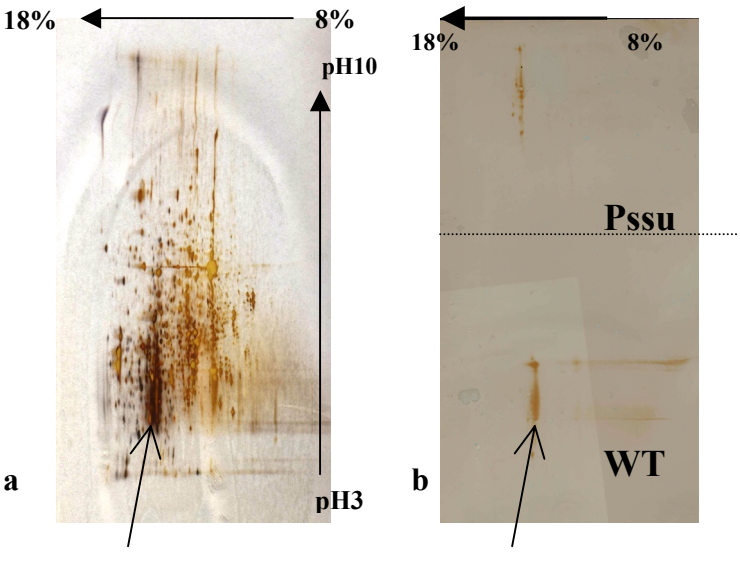

Fig 6a. 2D-polypeptide-pattern of WT- plant (pH 3-10, 8-18%). The region where LHC is located, is indicated by the arrow.
b. Immunoblot of WT and P*ssu-ipt* plant extract with Lhcb-antibodies. The strips which are used have a gradient from pH 4-9. Two strips are compared on one gel. Upper part: P*ssu*, lower part: WT plant. Antibodies against Lhcb show less protein in P*ssu*-plant. Equal amounts of total protein-sample are focused. Reduction of protein amount can be due to an elevated level of cytokinins.

Further investigation of the specific gene expression is needed to elucidate the effect of cytokinins on the genes of the LHC.

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