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Effect of cytokinins on the expression of the chloroplast *psbD-psbC* genes

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Introduction

Investigations of the role of cytokinins on the development of higher plants has been done extensively. In the past, effects of this plant hormone were determined by applying cytokinins exogenously to the plants. This method is accompanied by a lot of negative effects. First of all, high, non-physiological concentrations are applied and, secondly, most of the time leaf discs are used such that wound responses can not be avoided. Transgenic plants containing a gene which gives rise to an elevated level of cytokinins would provide a better system. The biosynthetic pathway of cytokinins in plants is poorly understood, but has been clearly demonstrated in the phytopathogenic bacterium *Agrobacterium tumefaciens* (Akiyoshi *et al.*, 1984; Barry *et al.*, 1984). Those bacteria contain a cytokinin biosynthetic *ipt*-gene coding for isopentenyl transferase, which is a key enzyme in the biosynthesis of cytokinin. Plants transformed with the *ipt*-gene under control of different promoters indeed lead to elevated cytokinin levels (Beinsberger *et al.*, 1991). The transgenic plants used in this work contain the *ipt*-gene under control of a light-inducible promoter. The role of cytokinins in the structure and function of the photosynthetic apparatus is still not clear. The aim of this work is to look at the effect of cytokinins on the expression of the chloroplast *psbD-psbC* genes. These genes are part of the *psbI-psbK-psbC-psbD*-operon in the chloroplast genome and code for two proteins of photosystem II. The *psbD*-gene codes for D2. The D2 and D1 (encoded by *psbA*) proteins are the key reaction center proteins of the photosystem II complex. The *psbC* gene codes for CP43, an integral membrane protein component of photosystem II. This chlorophyll protein is one of the interior transducers of excitation energy from the light-harvesting pigment proteins to the photochemical reaction center.

Materials and methods

Plants (*Nicotiana tabacum* L. cv. Petit Havana SR1) are cultivated in a greenhouse on grodan. This is done for wild types as well as for transgenic plants. The transgenic tobacco plants contain the *ipt*-gene under control of the light-inducible promoter of the small subunit of the RubisCO-enzyme of *Pisum sativum* (for the construct see figure 1). Additional illumination is provided 16h a day with AgroSon T (400 W) and HTQ (400 W) lamps (photon flux density of 200 µmol quanta $m^{-2}s^{-1}$). At harvest, samples are taken for DNA-isolation, gel-electrophoresis and Western blotting. The DNA of the plants is isolated using the DNeasy Plant Mini Kit from Qiagen. Primers against the *ipt*-gene are designed and the presence of the gene in the transgenic plants is controlled using PCR (primer sequences leading to a 194 bp PCR-product : upper primer 5'-GCATAT TATTCGCCACAAGTTACCC-3' and lower primer 5'-GGCTAGCAAACAACA TGGCATATC-3'). Sample preparation for 1D-electrophoresis is performed as follows. Leaf tissue is mixed in extraction buffer (0.33M

Sorbitol, 0.1M Tricine – pH 7.8) and filtered. After centrifugation (10 min. at 2000 g) the pellet is washed in resuspension buffer (5mM Tricine, 5mM EDTA – pH 7.5). The pellet is resuspended once more and after homogenisation (with a potter) part of the sample is used to determine the chlorophyll content (OD_{652nm}). The remaining sample is centrifuged (10 min. at 48000 g). The pellet is solubilised in sample buffer (50mM Tris, 1mM EDTA, 2.5% SDS, 5% β-mercapto-ethanol, 10% glycerol) until 2 mg chl/ml. 1D-electrophoresis is performed using an SDS-PAGE gradient gel (12% - 18%); 5 µl sample is loaded. After 1D-electrophoresis half of the gel is stained (0.25% Coomassie Brillant Blue, 7% HAc, 40% Methanol) and the other half is used for Western blotting. The gel is blotted on a nitrocellulose membrane during 1 hour. The membrane is blocked, washed and incubated overnight with antibodies against D2 (used in a 1/10.000 dilution). After conjugate incubation, the membrane is stained.

Results

The P*ssu-ipt* plants are morphological different from the wild type plants (WT). Figure 1 shows both types of plants, a wild type SR1 plant on the left and transgenic P*ssu-ipt* plant on the right. The P*ssu-ipt* plants have an extreme variability in their growth pattern and a highly reduced root system (Synková *et al.*, 1999). The leaves are more wrinkled than the ones of the wild types.

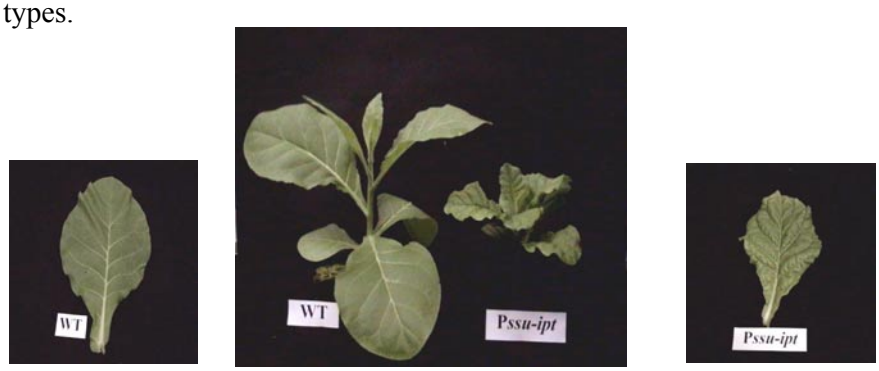

Fig. 1. Plant and leaf of wild type SR1 (left) and transgenic P*ssu-ipt* (right)

The construct of the transgenic P*ssu-ipt* plants is given in figure 2. After performing PCR with primers against the *ipt*-gene, we are able to prove the transgenic nature of the *ipt*-transformed plants. A result of such a PCR is given in figure 3. Lane 1 shows the marker (100 bp ladder). In lane 2 water is used instead of DNA (control). Lane 3 and 4 give the result of the PCR reaction when DNA of respectively a transgenic plant (P*ssu-ipt*) and a wild type is used. In lane 3 we clearly see a band near the 200 bp marker band corresponding to the PCR-product of 194bp. This clearly shows the presence of the *ipt*-gene in the DNA of the transgenic P*ssu-ipt* plants.

Fig. 3.
Result of PCR-reaction with primers against the *ipt*-gene:
Lane 1: 100bp ladder
Lane 2: Millipore water
Lane 3: DNA P*ssu-ipt*
Lane 4: DNA wild type

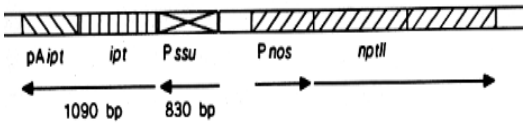

Fig. 2. Construct of ipt-gene under control of the light-inducible promoter (P*ssu-ipt*)

Figure 4 shows the protein pattern of the thylakoid membranes after 1D-electrophoresis on an SDS-PAGE gradient gel stained with CBB. The first lane is the marker (LMW, bands of 43 kDa and 30 kDa are indicated) and lane 2 and 3 contain samples of respectively wild type and P*ssu-ipt* plants. Since the proteins are clearly separated, the other side of the gel is appropriate for Western blotting. Figure 5 gives the result of a Western blot with antibodies against the D2 protein. The loaded samples all contain an equal chlorophyll content. If the samples from different plant types have an equal chlorophyll/protein ratio, it is expected that the same amount of protein is loaded on the gel when adding the same volume of sample. Taking this into account we are able to make some quantitative conclusions. Both lanes contain the same amount of protein, 5µl sample. The band corresponding with the D2 protein is less intense in the transgenic P*ssu-ipt* plant (lane 2) compared to the wild type plant (lane 1).

Fig. 4.
Result of SDS-PAGE stained with CBB
Lane 1: LMW marker
Lane 2: Wild Type
Lane 3: P*ssu-ipt*

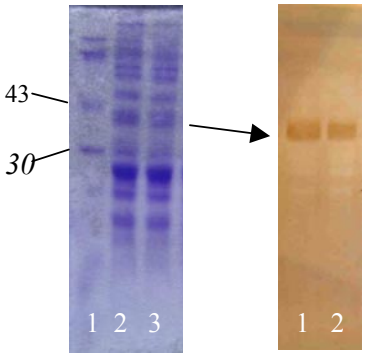

Fig. 5.
Western blot with antibodies against D2
Lane 1: Wild Type
Lane 2: P*ssu-ipt*

Discussion

Transgenic P*ssu-ipt* plants are morphological different from wild type plants. They have wrinkled leaves and a highly reduced root system (Synková *et al.*, 1999). It is generally accepted that a high auxin to cytokinin ratio is necessary to optimise rooting (Stenlid, 1982). In the P*ssu-ipt* plants this ratio is lower because of the elevated level of cytokinins (Synková *et al.*, 1999) and this leads to the disturbed root formation. With PCR techniques we are able to check the transgenic nature of plants and we see that the P*ssu-ipt* plants indeed contain the *ipt*-gene coding for isopentenyl transferase. To investigate protein patterns and expression of the D2 protein, 1D electrophoresis and Western blotting is performed. Proteins are clearly separated after 1D-electrophoresis and nice patterns are found. Western Blot with antibodies against the D2 protein shows a less intense band in the transgenic plants compared to the wild types. If both types of plants have the same chlorophyll/protein ratio, we can conclude that the expression of the D2 protein in the P*ssu-ipt* plants is lower compared to the wild types since the same amount of chlorophyll was loaded on the gel. This lower expression can be due to the higher cytokinin content, but can also be caused by stress. The plants get light 16h

a day leading to a high expression of the *ipt*-gene. This gives rise to a high cytokinin level which might exceed a certain threshold value. This can induce stress in the plants, causing a reduced expression of several proteins (McKersie *et al.,* 1994) including the D2 protein. Moreover the plants can suffer from other secondary effects of the elevated cytokinin level. The reduced root system makes it more difficult for plants to take up nutrients and water; resulting in mineral nutrition stress and water stress. Further experiments will give us more insight in what is really going on in the transgenic plants with an elevated level of cytokinins.

Acknowledgments

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