LIMBURGS UNIVERSITAIR CENTRUM

FACULTEIT WETENSCHAPPEN

Morphological characteristics and photosynthetic activity in *Nicotiana tabacum* L. cv. Petit Havana SR1 transformed with the *ipt*-gene from *Agrobacterium tumefaciens*.

Morfologische eigenschappen en fotosynthese-activiteit van *Nicotiana tabacum* L. cv. Petit Havana SR1 getransformeerd met het *ipt*-gen van *Agrobacterium tumefaciens*.

Proefschrift voorgelegd tot het behalen van de graad van
Doctor in de Biologie
aan het Limburgs Universitair Centrum

te verdedigen door

Karen VAN LOVEN

Promotoren:
Prof. dr. H. Clijsters
Prof. dr. R. Valcke

1995

Chapter 1: Introduction

The development of a plant is a complex and highly co-ordinated process which implicates growth and differentiation. It is regulated by endogenous mechanisms and responds adequately to external influences. It is inferred that the external factors can modulate an internal signal transduction, which is translated into a developmental program.

Phytohormones play a crucial role in controlling this process. Five major classes of phytohormones have been identified, to wit auxins, gibberellins, cytokinins, abscisic acid (ABA) and ethylene. Besides, other organic substances such as polyamines, oligosaccharides, glycoproteins and jasmonates have been shown to be involved in certain developmental processes (Altamura *et al.*, 1993; Bagni *et al.*, 1993; Parthier *et al.*, 1992; Tran Thanh Van *et al.*, 1985).

A certain extent of growth can be accomplished solely by water uptake. However, this is limited and very soon biosynthesis is required. Cell wall loosening is an important element in cell enlargement - this lays the foundation of the auxin mediated acid growth theory (Cleland, 1971), but the synthesis of new cell wall material and the accumulation of osmotic solutes are prerequisites for further growth (Brock and Cleland, 1990). Likewise, Pritchard *et al.* (1987, 1991) demonstrated that root growth is determined by cell wall extensibility and by turgor pressure, which was changed by alterations in the ionic composition and osmolarity. Besides, cell growth is limited and cell division takes place. In particular IAA and cytokinins are actively involved in this process (Cleland, 1987; Fosket and Short, 1973; Miller *et al.*, 1956; Rayle *et al.*, 1982).

In plants, biomass production is primarily the result of photosynthesis. However, no direct correlation with net leaf photosynthesis can be found (Bolhàr-Nordenkampf, 1987). On the other hand, dry matter production is related to the amount of absorbed irradiance (Loomis and Gerakis, 1975). Short term regulation of dry matter accumulation can be met at the level of carbon metabolism, directly via activation of enzymes of the Calvin cycle (Buchanan, 1991). Intermediates of the Calvin cycle dynamically regulate sucrose synthesis (Stitt *et al.*, 1988; Budde and Randall, 1990), and partitioning of carbohydrates in its turn affects photosynthesis (Martínez-Carrasco *et al.*, 1993). Bunce (1991) even suggested '*that availability of photosynthate may be a controlling factor in leaf structural growth and,*

consequently, in photosynthetic acclimation to light and temperature'.
Optimization of the excitation energy captation occurs via plant architecture and at a subcellular level, by ultrastructural and molecular adaptations (e.g., sun-shade acclimation, stoichiometric adjustments), that improve light interception (Critchley and Russell, 1993). Light, the driving force of photosynthesis, in itself is a major signal that induces photosynthetic adaptations; both light quality and quantity are involved in these processes.

1.1 Effects of phytohormones on plant architecture:

Plant anatomy is shown to be determined by the interaction of different phytohormones. The auxin-cytokinin balance is known to be critical in the control of apical dominance (for a review see Tamas, 1987; and Cline, 1994). In tissue culture, this ratio defines whether shoot or root induction is promoted. This capacity has been put to good use in plant regeneration. Stem elongation is affected by auxins and gibberellins; an influence of gibberellins on the biosynthesis of indole-3-acetic acid (IAA) has been proposed (Law and Davies, 1990), however, the insensitivity of stem elongation to auxin in gibberellin-deficient plants provided evidence against this hypothesis (see Klee and Estelle, 1991). Root growth is generally reduced by cytokinin or auxin treatment. However, Atzmon and van Staden (1993) demonstrated in *Pinus* seedlings that the nature of cytokinins is important, since zeatin was shown to be reductive and isopentenyladenine to affect positively lateral root growth. Leaf inclination is mainly species dependent and differs with leaf position along the stem, but in the *flacca* mutant of tomato plants auxin induced ethylene biosynthesis was believed to be the cause of an altered leaf orientation by inducing leaf epinasty (Tal *et al.*, 1979). Since these mutants are impaired in the ABA biosynthesis, it was concluded later that the ethylene increase might be a response to the water status of the plant, and a role for auxin was questioned (Neill *et al.*, 1986). The abscission of debladed petioles of cotton seedlings is another IAA stimulated ethylene effect. Morris (1993) showed that in this case synthesis of ACC, a precursor of ethylene, was induced by IAA.

Developmental processes, like germination and breaking of dormancy are known to be affected by changes of diverse phytohormones as a function of the developmental stage.
Sexual differentiation in *Mercurialis* flowers was demonstrated to be determined by different metabolic forms of cytokinins: *trans*-zeatin was abundant in female shoot

apices, whereas free nucleoside forms (isopentenyladenosine and zeatin riboside) were predominant in male structures (Louis *et al.*, 1990). This is in agreement with results obtained with Douglas-fir (Morris *et al.*, 1990). Moreover, in *Mercurialis* anther fertility is dependent on the ratio of *cis*- to *trans*-forms of the zeatin type cytokinins (Louis *et al.*, 1990).

A complete overview of the role of phytohormones on plant development is out of the scope of this report. The few examples cited just illustrate the importance of hormone interactions. In many experiments with exogenously applied phytohormones, these interactions have been overlooked and a direct relationship with the observed effect was assumed. Now it is clear that the more different types of endogenous phytohormones are identified, the more examples of phytohormone interactions can be found. New methods for quantitative analysis based on immunological and physicochemical techniques, like chromatographical methods combined with mass spectrometry, lend themselves to this purpose. In contrast with bioassays, frequently used in the past, these modern methods make a qualitative phytohormone analysis possible.

1.2 Effects of exogenously applied phytohormones on photosynthesis:

1.2.1 Abscisic acid:

The photosynthetic activity is known to be affected mainly by two types of phytohormones, ABA and cytokinins. The former was originally believed only to exert its influence on gas exchange by regulating the stomatal opening (Cummins *et al.*, 1971; Dubbe *et al.*, 1978). There is now supporting evidence for an effect at the level of the thylakoid membranes as well. In barley, grown in the presence of ABA (1 - 100μM), the granal chloroplast ultrastructure was disrupted and an increase in ß-centres of photosystem II (PSII-ß) relative to PSII-α was suggested (Maslenkova *et al.*, 1989). These ß-centres are found in stroma exposed thylakoid lamellae, they contain only part of the peripheral chlorophyll a/b light harvesting complex of PSII (LHCII) and are impaired in their plastoquinone-reducing properties (Melis, 1985; Melis, 1991). Moreover, an ABA-induced reduction in the total number of PSII centres is not excluded (Maslenkova *et al.*, 1989). ABA also inhibits the ribulose-1,5-bisphosphate carboxylase (RuBPCase) activity; this might be attributed to an effect on the electron transport, which in turn regulates the enzyme activity (Campbell and Ögren, 1990; Popova *et al.*, 1987; Ward and Bunce, 1987). On the other hand, protein synthesis is known to be affected as well (Rossi

and Iusem, 1994; Shriver and Mundi, 1990). Ivanov *et al.* (1992) noticed that *in vivo* ABA treatment (comparable to that applied by Maslenkova *et al.*, 1989) results in an enhanced accumulation of the LHCII complex, with a more pronounced grana stacking as a consequence. However, *in vitro* ABA treated thylakoid membranes were more negatively charged, possibly owing to the free, exposed carboxylic group from ABA molecules that are attached to specific binding sites at the chloroplast membranes (Kicheva and Ivanov, 1992). This could enhance the repulsive forces between the thylakoids and the grana stacking would be disrupted (Barber, 1982), which is in agreement with the results obtained on *in vivo* ABA treated plants by Maslenkova *et al.* (1989). These authors, however, found no influence of ABA application *in vitro*. These contradictory results might arise from differences in local ABA concentrations, which, induce opposite effects by the different molecular mechanisms proposed.

1.2.2 Cytokinins:

In contrast to ABA, Adedipe *et al.* (1971) reported no effects of cytokinins on the transpiration rate in dicotyledonous species. Other results, however, contradict this report: in *Digitalis* plantlets stomatal opening was stimulated and the water status of *Hevea* calli was negatively affected by increasing concentrations of exogenously applied cytokinins (Diettrich *et al.*, 1992; Etienne *et al.*, 1991). A cytokinin resistant mutant of *Nicotiana plumbaginifolia* was found to be wilty (Blonstein *et al.*, 1991a). This mutant had a low homeostatic ABA level and even failed to accumulate ABA under stress conditions. Therefore, it was hypothesized that cytokinin can regulate the ABA metabolism, and that wilting might not have been primarily related to the cytokinin insensitivity. Afterwards, Parry *et al.* (1991) have shown that the mutant was defective in the ABA metabolism and that cytokinin resistance should have appeared secondary.

Many effects of cytokinins on chloroplast development have been described (see Parthier, 1979). In particular, an interaction of cytokinins with chlorophyll synthesis has been noted. In greening experiments with excised or attached cucurbit cotyledons, depending on the duration of the cytokinin treatment prior to illumination, only the lag period in chlorophyll synthesis disappeared (short treatment, up to 6 h), or additionally the steady state rate of chlorophyll formation was increased (long treatment) (Dei, 1983, 1984; Cohen *et al.*, 1988). Moreover, the latter component required higher light fluence rates. Clearly there are two distinct phases in chlorophyll formation and they are differently affected by

cytokinins. The first phase, which corresponds to the lag period, was suggested to be limited by the activity of the enzyme NADPH:protochlorophyllide oxidoreductase (Dei, 1984). This enzyme catalyzes the photoconversion of protochlorophyllide into chlorophyllide. Hence, the accelerated chlorophyll synthesis was believed to result from a cytokinin induced accumulation of the enzyme. Up to now, the regulation of this enzyme is not understood. The lag phase in chlorophyll formation was also proposed to be related to the formation of δ-aminolevulinic acid (Cohen *et al.*, 1988; Lew and Tsuji, 1982). Interestingly, δ-aminolevulinic acid and chlorophyllide formation appear to be co-regulated (Söll, 1993). The nature of the second phase in chlorophyll formation is not known. The exact site where cytokinins might affect chlorophyll biosynthesis remains unclear.

In one chlorophyll mutant of maize, chlorophyll formation and normal development of the photosynthetic apparatus could be partially restored by treatment with benzyladenine (BA) and this was more effective when applied early during germination (Tonelli *et al.*, 1989). In this mutant the endogenous content of zeatin-riboside (ZR) was comparable to that in control seedlings and the concentration of dihydrozeatinriboside was even increased 1.5-fold. Therefore, the authors concluded that these results did not apply to a mutation in cytokinin metabolism, though that the mutant was defective in the cytokinin action mechanism. It is likely a 'sensitivity' mutant. Such mutant plants could provide interesting material to study the crucial steps in chlorophyll biosynthesis that are regulated by cytokinins.

Cytokinins are well-known to retard senescence. This has often been related to their sink enhancing effect (Fletcher *et al.*, 1970; Leopold and Kawase, 1964). In excised cotyledons, BA specifically accelerates hydrolysis of storage proteins (Leshem and Sussex, 1990). This could be important in promoting mobilization of metabolites towards developing (sink) tissues. However, outgrowth of these cotyledons preceeded the disappearance of the storage proteins.
During senescence, an overall protein degradation is observed. In detached leaves treated with kinetin, more protein is retained upon dark incubation in comparison to untreated leaves. Protein synthesis was stimulated only to a very limited extent, but protein degradation was strongly diminished (Lamattina *et al.*, 1987). Cytokinin treatment also reduces the increase in the chlorophyllase level (Sabater and Rodriguez, 1978).

These results clearly indicate that the mechanism of action of cytokinins on greening and on senescence is completely different, although both processes are characterized by a higher chlorophyll content.

Cytokinins can modulate gene expression. An effect at the transcriptional level was often suggested. Seyer and Lescure (1984) observed significant changes in the level of plastid mRNAs during the stationary phase of a tobacco cell culture, when grown in the presence or absence of kinetin. Among others, a positive control of cytokinin on the mRNA of the reaction centre of PSI was assumed. According to the present knowledge, this should be the mRNA corresponding to either of the two polypeptides of the PsaA/PsaB heterodimer.

Changes in mRNA levels of nuclear encoded genes have also been noticed. In the approach taken by Crowell and Amasino (1991) cytokinin-depletion induced changes in the mRNA pool were investigated in soybean cell culture. A cDNA library was constructed and this revealed one clone with a high degree of homology with the FeSOD gene from *Escherichia coli*. Moreover, the gene product of the relevant cDNA, after expression in *E. coli* cells, demonstrated FeSOD activity. Harding and Smigocki (1994) induced a cytokinin increase in *Nicotiana plumbaginifolia* plants, transformed with the isopentenyl transferase (*ipt*) gene from *Agrobacterium tumefaciens* (see below). Likewise, they isolated several cDNA clones among which two stress induced genes were found.

An enhanced accumulation of the mRNAs encoding the LHCP polypeptide and the small subunit (SSU) of the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) has been reported upon cytokinin application (Flores and Tobin, 1986; Lerbs *et al.*, 1984; Teyssendier de la Serve *et al.*, 1985). Flores and Tobin (1988) demonstrated that BA slightly stimulates the transcription of the LHCP-mRNA, and considerably influences its post-transcriptional regulation. A red light pulse, on the contrary, only affected the transcription. In etiolated watermelon colyledons a polypeptide with an apparent molecular weight of 32 kDa appeared after 6 h of exposure to BA (Longo *et al.*, 1986). It was speculated that this polypeptide corresponds to the precursor of LHCP and in the absence of any illumination even traces of the mature LHCP were found (Bracale *et al.*, 1988; Longo *et al.*, 1990). However, the polypeptide was not stabilized by integration into the thylakoid membrane. This was likely due to the lack of chlorophyll. In tobacco cell cultures grown under continuous light, cytokinin application led to a corresponding increase in LHCP and to a simultaneous chlorophyll accumulation (Axelos *et al.*, 1984). The accumulation rate was not affected, but in the cytokinin supplemented culture the increase started earlier, as if a lag phase was abolished. The authors formulated the hypothesis that in the absence of cytokinins, the mature LHCP-apoprotein is rapidly degraded as chlorophyll synthesis lags behind. It was recently confirmed that chlorophyll stabilizes the apoprotein of chlorophyll-protein-complexes and even

establishes a functional conformation (Ikegami and Katoh, 1991; Kim *et al.*, 1994; Mullet *et al.*, 1990). Kim and co-workers (1994) even suggest a reversed order of priority: since free chlorophyll is a potential photo-oxidant of the membranes, '*...it is reasonable for plastids to synthesize chlorophyll apoproteins in excess so that whenever chlorophyll is synthesized a chlorophyll apoprotein is always available*'. Hence, the presence of the mature LHCP apoprotein in etiolated cotyledons (Bracale *et al.*, 1988; Longo *et al.*, 1990) is meaningful.

Furthermore, Caers *et al.* (1985) noted that a heat induced decline in endogenous cytokinins in etiolated maize seedlings, followed by a greening period, significantly reduced the degree of grana stacking, and this effect was reversed by exogenous application of BA (Caers and Vendrig, 1986). A few reports present data showing a very limited stacking caused by cytokinin treatment in etiolated tissues (Bracale *et al.*, 1988; Chory *et al.*, 1994). This is directly related to the presence of LHCP (see before); a loose association of the apoprotein with the thylakoid membrane might already exist in cytokinin treated etioplasts (Longo *et al.*, 1990).

An effect of cytokinins on chloroplast DNA-replication has been reported (Kinoshita and Tsuji, 1984). Momotani *et al.* (1991) found that this phytohormone stimulates chloroplast division and advances this process in time. However, the effects were dependent on the developmental stage of the leaf. It was visible in young leaves only. In a later phase chloroplast division ceases anyhow; then enlargement of the organella is stimulated (Kinoshita and Tsuji, 1984).

Nuclear DNA accumulation is also enhanced, although later. This effect could be related to the stimulation of cell division by cytokinins. An increased number of replicon origins in the nuclear DNA has been demonstrated in response to BA treatment (Houssa *et al.*, 1990 and 1994). This might be essential in shortening the S phase of the cell cycle. This was supported by the recent nuclear co-localization of natural cytokinins with proteins that are assumedly involved in the onset of DNA-replication and cell proliferation (Ivanova *et al.*, 1994).

Momotani *et al.* (1991) suggested that RNA and protein accumulation preceeds DNA synthesis. Therefore, for rapid responses (other than the induction of cell division) transcriptional and post-transcriptional or translational regulation may be more susceptible to cytokinin modulation.

The photosynthetic activity in cytokinin treated tissues is enhanced: both the CO_2-assimilation and O_2-evolution rate, measured on leaf strips, are increased (Caers and Vendrig, 1986). The activities of RubisCO and other enzymes involved in CO_2-assimilation in C_4 plants were stimulated. However, Chernyad'ev *et al.* (1986) noted

that the activity of RubisCO expressed per protein remains constant, in contrast to the activity expressed per leaf area or per fresh weight. From these observations it was concluded that the specific activity of the enzyme remains unchanged, but that protein synthesis is enhanced, with a proportional increase of the RubisCO content. This was confirmed by Parthier *et al.* (1987). Even in etiolated cotyledons an increased accumulation of the enzyme has been detected (Ohya and Suzuki, 1991). As such, the results resemble those of LHCP: it appears that the light trigger was replaced by cytokinins. However, this hypothesis was questioned. In a two-factor analysis considering the effects of light and BA on the light stimulated development of the RuBPCase activity, Zimmermann *et al.* (1987) could distinguish at least two components. Only one of these appeared to be cytokinin dependent. This better fits the interpretation of Parthier *et al.* (1987), who stated that cytokinins do not trigger but merely stimulate the synthesis of RubisCO.

Under changing light conditions the quantity of RubisCO closely correlates with the mRNA level of its small subunit (SSU) (Prioul and Reyss, 1987). In contrast, the large subunit (LSU)-mRNA is hardly affected neither by the light fluence rate, nor by BA, and it is assumed to become equilibrated with the SSU polypeptide by post-transcriptional regulation (Bate *et al.*, 1991; Ohya and Suzuki, 1991). Van Oosten and Besford (1994) showed that the SSU-mRNA even responds to sucrose and glucose levels: after 24 h at high concentrations, the abundance of the transcript was reduced. Moreover, enzymes involved in sucrose metabolism are locally affected by BA application. Under conditions of enhanced sink demand in developing shoots, the sucrose phosphate synthase activity increased in the source leaf tissues (which corresponds to an enhanced sucrose mobilization), whereas in the young shoot sucrose synthase activity was promoted (Zieslin and Khayat, 1990). These effects were studied only after 1 to several days. It is unknown whether cytokinins rapidly modulate the amount of RubisCO through changes in the sugar levels. This mechanism might be more important as feedback control during the long-term development of a plant. So far, rapid changes (after 6 h) have only been correlated with the SSU-mRNA content.

Furthermore, the photosynthetic electron transport activity is affected. Chloroplasts isolated from kinetin-treated *Raphanus* cotyledons exhibited a higher Hill activity (Buschmann and Lichtenthaler, 1977). In contrast, Zerbe and Wild (1980) observed no effect of kinetin on the Hill activity, neither on the non-cyclic phosphorylation rate in *Sinapis* plants. During greening of cucumber cotyledons, kinetin stimulated the PSI dependent electron transport activity proportionally more

than the PSII mediated activity (Pedhadiya *et al.*, 1987). The authors suggested that the $NADP^+$-reducing system was affected. Besides, the intermediate electron carrier system, operating between PSII and PSI, also seemed to be promoted, which is in agreement with the finding that the plastoquinone and cytochrome f content (per leaf area or fresh weight) were increased (Buschmann and Lichtenthaler, 1977; Zerbe and Wild, 1980). Further, an enhanced P700 content per chlorophyll was recorded (Buschmann and Lichtenthaler, 1977).

During senescence, the electron transport rate declines: there is a loss in energy transfer from the antennae to the reaction centres, and the PSI mediated electron transport activity declines more rapidly than that of PSII (Le Pabic *et al.*, 1990). An impairment at the cytochrome b_6/f complex was suggested. After cytokinin treatment, the partial electron transport activities were preserved significantly better (Prasad *et al.*, 1988) and the loss of coupling between the electron transport and photophosphorylation was slightly reduced (Le Pabic *et al.*, 1990). However, in the latter report the electron transport activities rapidly diminished in contrast to the enhanced chlorophyll retention. This contradicted to the simultaneous chlorophyll accumulation and the establishment of the electron transport during greening, and was probably caused by a potassium deficiency (Le Pabic *et al.*, 1990).

A remarkable similarity between potassium and cytokinin induced effects on chloroplast activities has been reported. KCl treatment resulted in a stimulated chlorophyll and RubisCO synthesis, an enhanced electron transport rate, an increase in chloroplast size and the high protein content was retained much longer (Arnold and Fletcher, 1986; Le Pabic *et al.*, 1990; Ohya *et al.*, 1986). It is tempting to conclude - at least in part - a common pathway for the action mechanism of both potassium and cytokinins. However, for the potassium stimulated RubisCO accumulation, light was still indispensable (Ohya *et al.*, 1986) and in the report of Arnold and Fletcher (1986) the lag phase in chlorophyll accumulation was not found to be eliminated. Besides, the effect of potassium was drastically inhibited by cycloheximide but hardly by actinomycin-D, whereas BA-stimulated chlorophyll production could be blocked by actinomycin-D and both translation inhibitors chloramphenicol and cycloheximide. Therefore, different regulation mechanisms were concluded: cytoplasmic translation was of major importance for the potassium stimulated greening and BA regulation essentially took place at the transcriptional level (Arnold and Fletcher, 1986).

Despite the homology in effects, the action of cytokinins has a number of unique features that distinguishes it from the controlling mechanisms influenced by e.g.

potassium or light.

1.2.3 Indole-3-acetic acid and gibberellins:

A substantial amount (30 - 40%) of the intracellular IAA pool has been localized in the chloroplast (Sandberg *et al.*, 1990). Consequently, a physiological role on chloroplast metabolism could be expected. However, reports of IAA effects on chloroplast activities are scarce. In isolated chloroplasts, IAA stimulates CO_2-fixation and phosphorylation, although the latter effect was not always reproducible (Robinson *et al.*, 1978; Tamas *et al.*, 1974). In intact plants IAA may also stimulate photosynthesis by promoting stomatal opening (see Mansfield, 1987).

Gibberellins were also suggested to stimulate photosynthesis. Applied to roots, the leaf carbon assimilation rate was promoted (Arteca and Dong, 1981). However, foliar treatment with GA_3 caused contradictory results. In some studies the photosynthetic rate was increased, in others it was not affected. It was suggested that the increases, if any, were due to a stimulated leaf expansion. However, this conclusion contradicts to the results of Arteca and Dong (1981), who found an enhanced activity expressed on a leaf area basis. Prasad *et al.* (1988) observed that gibberellic acid (GA_3) was not very effective in inhibiting senescence. GA_3 was less efficient than the cytokinin BA in accelerating the greening process (Pedhadiya *et al.*, 1987). Zimmermann *et al.* (1987) suggested that GA_3 and BA do not act independently, but e.g., GA_3 could decrease the BA uptake or BA action.

1.3 Studying the influence of endogenous cytokinin levels:

Since effects of exogenously applied cytokinins on photosynthesis are manifold (see 1.2.2), the question arises whether natural endogenous cytokinins regulate the normal development of the photosynthetic apparatus.

Changing the endogenous hormonal balance by exogenous supply includes uncontrolled factors concerning hormone uptake, distribution and metabolism. Interactions with the metabolism of different classes of phytohormones were illustrated above (see 1.1): the influence of IAA on ACC-synthase, a potential effect of gibberellins on auxin biosynthesis and the ABA-cytokinin interrelation can be recalled. Vaňková *et al.* (1992) demonstrated a transient effect of auxin on the free base and riboside forms of zeatin and isopentenyladenine. The reverse (cytokinins affecting the auxin content) had been shown already before (see references in

Vaňková *et al.*, 1992). In *ipt* transformed *Nicotiana* and potato calli, with an increased endogenous cytokinin content (see below), auxin autonomous growth has been reported and the IAA level was shown to be slightly increased (Binns *et al.*, 1987; Ondřej *et al.*, 1990; Smigocki and Owens, 1988; Van Onckelen *et al.*, 1988). Naturally occuring cytokinins as well as synthetic ones, like BA and kinetin, are both metabolized after exogenous application: riboside- and nucleotide-forms, and glucosylated products can be recovered in treated tissues (for a review see Letham and Palni, 1983). Besides, addition of BA to an *in vitro* cultivation medium leads to a decline in endogenous cytokinin level in tobacco and potato plantlets (Pospíšilová *et al.*, 1993). Due to these complex metabolic interrelations, it is inconceivable how to control the endogenous hormonal balance by exogenous application.

One could propose to determine the endogenous cytokinins and try to relate these with the development of the photosynthetic apparatus. This could be realized when distinct phases in chloroplast development can be separated *in vivo*. A separation in time might be achieved during greening, a spatial separation is present in grass-like leaves that develop by intercalary meristematic growth. Methods for quantitative analysis of phytohormones have been substantially improved over the last two decades: the spectrum of different metabolic forms has been refined and the sensitivity up-graded (for a review see Hedden, 1993). With immunological techniques *in situ* localization is possible. In other words, the biochemical and biophysical technology is now well advanced to support this approach. Nevertheless, it would be limited to those situations where the different stages of chloroplast development are clearly separated in time or space, and it would be difficult to distinguish between cause and effect.

Alternatively, a molecular-genetic approach can open new perspectives. There is, however, a lack of understanding in cytokinin synthesis and metabolism (see Letham and Palni, 1983; Binns, 1994). In analogy to the biosynthetic pathway induced by the plant pathogenic bacterium *Agrobacterium tumefaciens*, it is assumed that in plants the initial reaction in the *de novo* synthesis is the addition of isopentenyl pyrophosphate to the N^6 position of 5'-adenosine monophosphate (5'-AMP), to yield isopentenyl-5'-AMP (iPeAMP). In *A. tumefaciens*, this reaction is catalyzed by the enzyme isopentenyl transferase (IPT).
Up to now the search for a homologue of the *ipt*-gene from *A. tumefaciens* in the plant genome has not been successful. Nevertheless, in extracts of cytokinin

autonomous tobacco callus tissue, a similar enzyme activity has been shown (Chen, 1982). Only partial purification of the enzyme was achieved, and no complete identification was obtained. Moreover, it could not be reproduced. Therefore, there was some doubt whether the callus tissue utilized in this study was really an untransformed, habituated strain. After feeding immature *Zea mays* kernels with tritium labelled 5'-AMP and isopentenyl pyrophosphate, the radioactive labelling could be found in isopentenyladenosine (iPA). Even though the intermediate product iPeAMP was not clearly recovered, these results also support the view that a mechanistically similar reaction as the IPT catalyzed one, naturally occurs in plants (Blackwell and Horgan, 1994).

Some enzymes, required for the metabolic interconversion between different cytokinin forms, have been partially characterized. A zeatin O-glucosyl and O-xylosyl transferase in *Phaseolus* is well documented (see Binns, 1994). The identification of the corresponding gene sequences may be expected in the near future. Enzymes catalyzing the catabolic, oxidative cleavage of the N^6 side-chain of cytokinins have been found in many species (for a review see Hare and van Staden, 1994). Quite some differences in apparent molecular mass and pH-optimum appeared, even within the same plant species. It was suggested that different isoenzymes might exist. Up to now, one putative cytokinin oxidase gene has been isolated from a cDNA expression library from *Zea mays* seedlings (Burch and Horgan, 1992). Subsequent reports concerning cloning and further identification have not come to my attention, yet. Obviously, a break-through in unraveling the cytokinin metabolism to the molecular level can be expected.

Besides, there is a lack of cytokinin deficiency mutants. Since cytokinins are involved in almost any crucial step of plant development, these mutations might be lethal. A sideshoot-less mutant of Craigella tomato seemed a good candidate for a cytokinin deficient mutant. It was shown, however, that cytokinin biosynthesis in the root tips was not affected, but the mutant produced tissue specific alterations in the metabolism (Sossountzov *et al.*, 1988). In the moss *Physcomitrella patens* cytokinin overaccumulating mutants have been isolated (Futers *et al.*, 1986; Wang *et al.*, 1981). They were characterized by the overproduction of sterile gametophores, but the genes that are involved have not been identified. Recently, in mutagenized *Arabidopsis thaliana* seedlings, one mutant (*amp1*) has come across with increased cytokinin concentrations. It was selected by its altered morphology (number of cotyledons and rosette leaves, and increased leaf growth) (Chaudhury *et al.*, 1993). Further characterization still has to be realized. The recessive *rms-2* mutation in *Pisum sativum* plants also produces a typical phenotype (among others

a reduced stem length and a smaller root system). Although no information is still available, neither on the cytokinin levels, nor on enzyme activities (e.g. ß-glucosidases, cytokinin oxidases), Beveridge *et al.* (1994) suggested that the mutation might involve a block in the catabolism of active cytokinins. Nevertheless, any conclusion about a cytokinin induced physiological effect in these mutants is premature.

A number of cytokinin resistant mutants has been isolated (Ashton *et al.*, 1979; Blonstein *et al.*, 1991a; Su and Howell, 1992), suggested to be defective in the cytokinin response pathway. However, in *Nicotiana plumbaginifolia*, it concerned an ABA deficient mutant (see 1.2.2; Parry *et al.*, 1991). In *Physcomitrella* the mutant phenotype could mostly be reversed by auxins (Ashton *et al.*, 1979). These facts underscore the importance of the selection criteria applied (Blonstein *et al.*, 1991b). When cytokinin resistant mutants are selected on media containing high (toxic) overdoses of cytokinins, generally stress-resistant plants are isolated. Moreover, the interrelations between the different phytohormones frequently result in multiple resistances for a single mutation. Two *det* mutants (*det1* and *det2*) from *Arabidopsis* (Chory *et al.*, 1991 and 1994) are also likely cytokinin response mutants. They were not selected by growth on high cytokinin levels, but were retained because of some of their physiological properties (induction of de-etiolation under dark conditions) that had been related to cytokinin activity. Different subsets of light-regulated responses appear to be affected by the 2 *det* alleles. In contrast to other - unspecific - cytokinin response mutants, these are (still) more promising for the study of the action mechanism of cytokinins in this physiological response.

As mentioned above, the plant pathogen *A. tumefaciens* contains the *ipt*-gene that codes for the enzyme catalyzing the initial step of cytokinin biosynthesis. The gene has been localized on an extrachromosomal "Tumor-inducing" (Ti) plasmid, within a defined domain surrounded by border sequences: the T-DNA. Depending on the bacterial strain it contains two T-DNA regions (octopine type) or only one (nopaline strains). In both cases, the T-DNA is entirely transferred to the plant cell upon infection, and is covalently incorporated into the nuclear plant genome. The processes involved in infection, T-DNA transfer and integration into the plant DNA have been reviewed by Zambryski (1992). It has been shown that 3 genes localized on the T-DNA are responsible for tumorigenesis: the genes 1, 2 and 4. The genes 1 and 2 encode for the enzymes tryptophan-mono-oxygenase and indole-acetamide hydrolase, respectively. In sequence, these enzymes catalyze the synthesis of IAA

brought about by the *ipt*-gene and those arising from the experimental set-up.

Secondly, the photosynthetic activity will be studied. The attention will mainly be focused on the "light reactions". The photosynthetic electron transport and photophosphorylation will be analyzed by measuring partial reactions *in vitro* and with non-invasive methods (*in vivo* fluorescence and absorbance measurements).

The results will be discussed in relation to data of the endogenous cytokinin content.

1.5 General outline of the thesis:

The following chapter describes the plant material, cultivation conditions and the general methods applied.

The effect of cytokinins in the *in vitro* assay for the electron transport was tested (chapter 3). In chapter 4, an analysis of the effects of heat treatment on the photosynthetic activity is reported. This was by way of preparation of the experimental work with the transgenic plants containing the heat shock promoter - *ipt*-gene construction.

In chapter 5 and 6 results are described, obtained with the *ipt*-transgenic plant material with the heat shock or the light responsive promoters, respectively.

In the chapters 3 to 6 the presentation of the results is immediately followed by the discussion. Chapter 7 contains concluding remarks.

The results from chapter 4 have been published already in a preliminary form in:

Van Loven, K. and Valcke, R. (1992) The effect of heat treatment on the capacity of the electron transport: interaction of light and structural organization. In: Regulation of Chloroplast Biogenesis. (Argyroudi-Akoyunoglou, J.H., ed.) Plenum Press, New York. Pp. 451-455.

The morphometric section of chapter 5 has been published in:

Van Loven, K., Beinsberger, S.E.I., Valcke, R.L.M., Van Onckelen, H.A. and Clijsters, H.M.M. (1993) Morphometric analysis of the growth of P*hsp70-ipt* transgenic tobacco plants. Journal of Experimental Botany 44: 1671-1678.

Chapter 2: Materials and Methods

2.1 Plant material:

2.1.1 Transgenic constructions:

Transgenic Tobacco plants (*Nicotiana tabacum* L. *cv.* Petit Havana SR1 (Maliga *et al.*,1973)) containing a supplementary *ipt*-gene were generated by means of the *Agrobacterium tumefaciens* transformation system. Two different genetic constructions are employed in this study. In the first one the *ipt*-gene is coupled to a *hsp70* promoter (Schmülling et al., 1989). The heat inducible promoter of the *hsp70*-gene from *Drosophila melanogaster* (Spena et al., 1985) has been isolated and positioned upstream of the entire coding region of the *ipt*-gene. The latter was a *Bal*31- derived fragment of the *ipt*-gene from the T-DNA of the Ti plasmid pTi15955, including 16 bp of the untranslated leader region and 373 bp of the 3'-flanking sequences. A kanamycin-resistance gene (*npt*II = neomycin phosphotransferase II gene of transposon Tn5) with the P*nos* promoter sequence and pA*ocs* (polyadenylation sequence) was inserted in the vector (fig. 2.1).

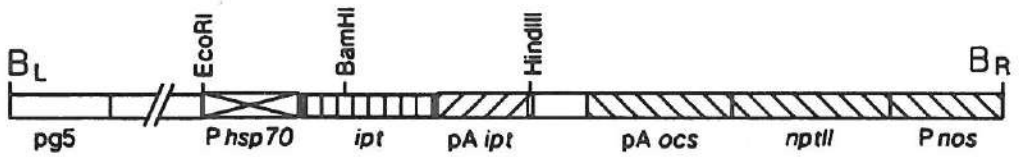


Figure 2.1: Scheme of the vector containing the chimeric construction P*hsp70-ipt* (after Schmülling *et al.*, 1989).

After transfer to *E. coli*, this vector was mobilized to *A. tumefaciens* strain GV3101.

Transformation of tobacco occured by cocultivation of leaf protoplasts from *N. tabacum* L. cv. Petit Havana SR1 with *A. Tumefaciens* GV3101 containing the plasmid construction described above. Kanamycin resistant transformants were selected. Subsequently calli were grown, shoot formation was induced by BA

(0.5 mg/l) on solid MS-medium (Murashige and Skoog, 1962). These shoots could be rooted on hormone-free medium. The regenerated plants yielded seeds. After self-fertilization homozygous plants were obtained. The progeny of these transgenic plants is used here. They are further defined as HSIPT, an abbreviation of P*hsp70-ipt*.

The second construction consists of the *ipt*-gene connected to the promoter sequence of the *rbcS*-gene (P*ssu*) of *Pisum sativum* (Herrera-Estrella *et al.*, 1984). This light inducible promoter was cloned as a 830 bp *Bgl*II/*Bam*HI fragment into the pGV831 vector, containing the chimeric P*nos-npt*II selection gene (Deblaere *et al.*, 1985). The resulting recombined vector (pGV894) was mobilized to *A. tumefaciens* and cointegrated into its Ti plasmid pGV2260 (pGV2260::pGV894 = pGV2488) (fig 2.2).

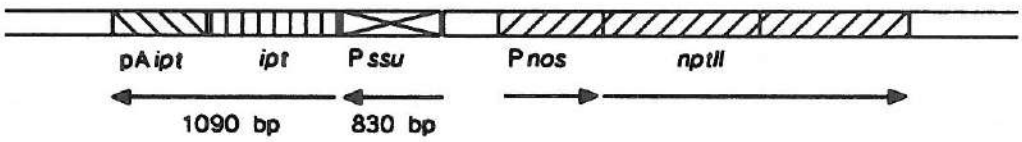


Figure 2.2: Scheme of the chimeric construction P*ssu-ipt* (adapted from Beinsberger, 1993).

Tobacco plants were transformed by the leaf disc transformation procedure (Simpson *et al.*, 1985) in cocultivation with the *A. tumefaciens* strain containing the pGV2488 plasmid described above. Callus development was promoted and shoot formation was induced by changing the IAA/BA ratio in the LS-medium (Linsmaier and Skoog, based on MS-medium, with addition of sucrose 3%, thiamine 10 mg/l, pyridoxine 1 mg/l, nicotinic acid 1 mg/l, myo-inositol 200 mg/l, agar 0.8%, pH 5.9) from 1/0.1 to 0.1/1 (mg/l). However, these shoots failed to root upon addition of different concentrations of NAA/BA. To proceed to the development of expanding leaf tissue (most interesting for photosynthetic analysis) the shoots were grafted on a wild type stalk with root base (Beinsberger *et al.*, 1992a). The progeny of these grafts segregated into kanamycin resistant and - sensitive seedlings. The latter also lacked the *ipt*-gene, indicating the heterozygous nature of the grafts. When the kanamycin resistant seedlings were transferred to soil, they developed further to mature plants with a small root

system. These plants are called P*ssu-ipt* plants throughout this work. The kanamycin sensitive seedlings developed, in the absence of kanamycin, to plants indistinguishable from wild type plants, and they are marked as Km^S plants.
The grafts of a P*ssu-ipt* transgenic shoot on a wild type stalk are named 'P*ssu-ipt* graft'. Non-transformed plants of the cv. Petit Havana SR1 are in this work defined as wild type plants. They are also named 'SR1-plants'.

2.1.2 Cultivation conditions:

2.1.2.1 P*ssu-ipt* transgenic shoots: multiplication and grafting.

Transgenic shoots of the P*ssu-ipt* strain which were used for grafting, were isolated from callus tissue and multiplied on LS-medium (see above) containing NAA (1 mg/l) and BA (0.1 mg/l). Newly formed grafts were kept under high humidity by covering them with a plastic bag. When the grafts had started growing, the bag was removed and grafts were grown under the same *'in vivo'* conditions as mentioned below (see 2.1.2.4).

2.1.2.2 *In vitro* pre-cultivation of seedlings:

SR1, HSIPT, P*ssu-ipt* and Km^S seeds were germinated and pre-cultivated *in vitro* after surface sterilization. This procedure consisted in washing the seeds for one minute with ethanol 70%, followed by 10 min with hypochlorite 30% containing a few droplets Tween-20; finally the seeds were washed three times consecutively with sterile water. Hereafter the seeds were sown on LS-medium with 1% agar. For the transgenic HSIPT seeds this medium was supplemented with kanamycin sulphate (50 mg/l). Seeds from the P*ssu-ipt* grafts were sown on a kanamycin free medium since transgenic and Km^S seedlings could be distinguished in the absence of kanamycin on a morphological basis (Beinsberger, 1993); this allowed the Km^S seedlings to grow up. From June 1992 on the *in vitro* growth medium (LS) was replaced by half strength Hoagland solution with 1.5% agar. Changing the medium did not influence the development of the seedlings.
The *in vitro* growth occured under low irradiance (20 - 50 μmol quanta $m^{-2}s^{-1}$) at a 16h day / 8h night cycle at 20°C. After 4 - 6 weeks of *in vitro* cultivation the seedlings were transferred to soil (nutrient composition NPK 7-7-10; pH 5.5 - 6.5) in polyethylene pots (diameter 18 cm).

2.1.2.3 *In vivo* pre-cultivation of seedlings:

Since the HSIPT strain is homozygous in the chimeric gene, selection on kanamycin sulphate has become superfluous. *In vitro* pre-cultivation can be omitted. HSIPT and the corresponding control SR1 seedlings were sown in soil (see above) in a commercial sowing tray that keeps the germination conditions humid. After three weeks the seedlings were planted in polyethylene pots containing the same soil.

Although the P*ssu-ipt* and Km^s seedlings could be distiguished on a kanamycin-free medium, they were never sown directly in soil, since it was easier to identify them in *in vitro* culture.

2.1.2.4 *In vivo* growth conditions:

Originally, plants were grown in a growth chamber at a day (12h - 25°C)/night (12h - 20°C) regime (relative humidity: 65%; illumination: c. 150 μmol quanta $m^{-2}s^{-1}$ fluorescent light (Sylvania Cool White supplemented with Phillips Philinea lamps; spectrum see fig. 2.3).

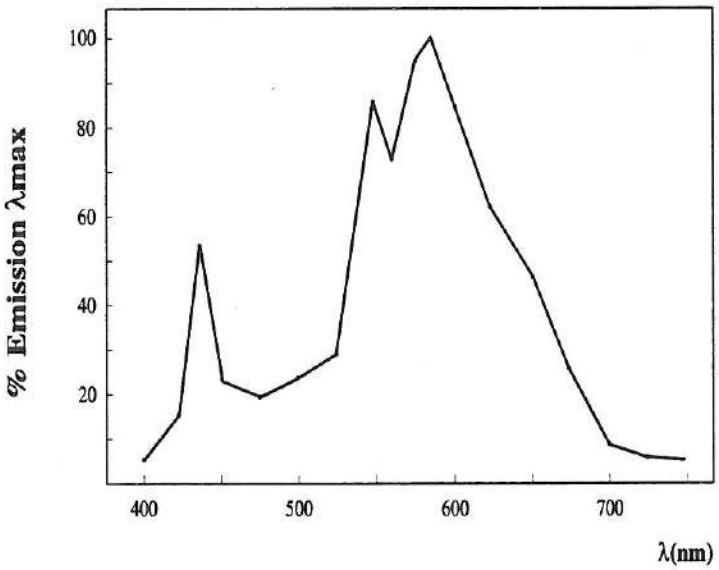


Figure 2.3: Emission spectrum of the Sylvania Cool White fluorescent lamps supplemented with Phillips Philinea lamps, recorded in the growth chamber.

In late summer 1993 the plant culture was transferred to a greenhouse, and from October 1993 on all plants were cultivated there under the following conditions: 25°C day / 18°C night, relative humidity 60%, additional illumination was provided 12h / day with AgroSon T (400W) and HTQ (400W) lamps (photon flux density c. 200 μmol quanta $m^{-2}s^{-1}$; spectrum see fig. 2.4).

Wild type and HSIPT plants were watered with tap water and once a week supplemented with half-strength Hoagland solution. The P*ssu-ipt* grafts and -plants daily received half strength Hoagland solution.

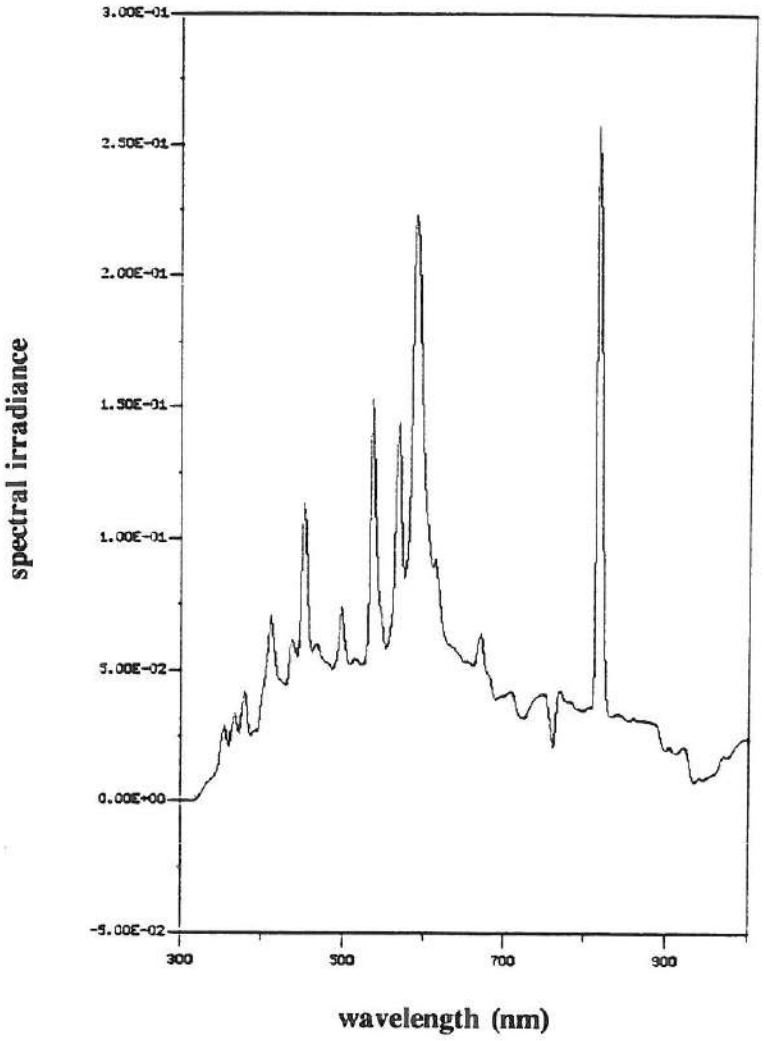


Figure 2.4: Emission spectrum of the AgroSon T and HTQ lamps, as recorded in the greenhouse.

2.1.2.5 Heat treatment:

Ten days after transfer of *in vitro* pre-cultivated seedlings (HSIPT and SR1, respectively) to soil, they were comparable in size with *in vivo* pre-cultivated seedlings three days after transfer to the larger pots (see chapter 5 - length of the stem of the different series at the start of the experiment). At that moment heat treatment was started. This treatment consisted of a daily exposure to 40°C in the morning, analogous to the treatment performed by Schmülling et al (1989) on the transgenic callus tissue. The plants were transferred from the growth chamber to a heat shock chamber with relatively low irradiance (50 μmol quanta m^{-2} s^{-1}; Phillips TLD 18W/33 fluorescent lamps). There, temperature was raised from 25°C to 40°C within 15 min. This was maintained for 1 h. Temperature was decreased to 25°C again in 15 to 20 min, after which the plants were replaced into the growth chamber. It was maintained up to 8 weeks. Consequently, the weeks during this experimental growth period were numbered from one to eight, according to the period of treatment.

In chapter 4 also results are presented with a heat treatment given at high irradiance (c. 2000 μmol quanta $m^{-2}s^{-1}$; Phillips HNF 206 lamp). The other conditions were kept constant.

2.1.3 Morphological analysis:

Quantifiable growth parameters, such as total stem length, the number of internodes, the length of the successive internodes (numbered acropetally) and the diameter at the middle of the internodes, and fresh weight of the axillary shoots were determined.

From wild type and HSIPT plants, with or without heat treatment, cross-sections of the stem were made for light microscopy. After fixation in FAA (formaldehyde 40%:ethanol:acetic acid:water 4:47.5:43.5:5 v/v/v/v) the tissues were preserved in ethanol 70%. Woody samples were softened 24h in H_2O:glycerol:ethanol (1:1:1 v/v/v) and washed with ethanol 50% and ethanol 70%, prior to embedding in paraffin. Sections (5 - 10 μm) were made with a rotary microtome (Jung) and stained with fast green FCF and safranine (see Appendix). Observations were made with the Polyvar microscope (Reichert-Jung).

Further analysis was descriptive, supported by photographs.

2.2 Cytokinin analysis:

2.2.1 Method used for the HSIPT and corresponding control (SR1) plants and for P*ssu-ipt* grafts:

This method is similar to that given in Van Loven *et al.* (1993). The main steps are enumerated, here.

Samples were frozen in liquid nitrogen and stored at -20°C until analysis. For extraction of the cytokinins, the tissues were homogenized in methanol (9 ml g^{-1} fresh weight). At this first step, a standard amount of *trans*-[^{3}H]-zeatin riboside dialcohol was added. After incubation (minimal 1h at -20°C) and centrifugation (15 min at 28000g), the supernatant was purified by passage through Seppack C18 cartridges. The eluate was dried and redissolved first in methanol, and a second time in ethanol:TBS-buffer (10 mM Tris-HCl, pH 7.4; 0.14 M NaCl; 0.1% NaN_3) (1:7 v/v). The percentage of recovery after purification was determined by analysis of the radioactivity (dpm ^{3}H).

The quantification was done by radioimmunoassay (RIA) according to Weiler (1980). The antibodies that were used (anti-ZR and anti-iPA), were kindly provided by Drs. P. Redig (laboratory of plant biochemistry, UIA). These were purified from egg yolks of immunized hens (Jensenius *et al.*, 1981).
The original cytokinin concentration was calculated against a calibration curve (between 0.5 and 100 pmol) after logit-transformation (Rodbard, 1974) and expressed as pmol ZR- or iPA-equivalents g^{-1} fresh weight (for further details, see Beinsberger, 1993).
The cytokinin analysis in P*ssu-ipt* grafts was carried out by Dr. S. Beinsberger.

2.2.2 Method used for the P*ssu-ipt* plants:

These plants have been analysed by Dr. G. Kudoyarova. The method is described in detail in (Kudoyarova *et al.*, in preparation).

Essentially, the method is as follows. Samples were homogenized in methanol as in the previous method (see 2.2.1), but without addition of the tritium-labelled internal standard. To estimate the recovery, the homogenized samples were divided in two parts, to one of which an unlabelled standard amount of cytokinins was added. The increase in immunoreactivity in the final quantification was a measure for the recovery.

The homogenate was incubated over-night at -20°C, filtrated, and passed through a Bond-Elut RP-C18 column. The aqueous phase of the eluate was applied on a DEAE-Sephadex column (formate form), which retains the cytokinin nucleotides. The eluate containing the free base and riboside forms, was further rinsed and concentrated on a Bond-Elut RP-C18 cartridge. This was eluted with methanol 80% and the sample was dried. The cytokinin nucleotides were eluted from the DEAE-Sephadex column with NH_4formate (1 M), converted to nucleosides after incubation with alkaline phosphatase and extracted with n-butanol. The latter was evaporated.

For further purification and separation the samples were chromatographed by thin layer chromatography (TLC) (silica gel 60 F-254 plates, Merck; eluent: n-butanol:NH_4OH (14 M): H_2O, 6:1:2 v/v/v) (Van der Krieken *et al.*, 1991). The TLC-zones were eluted with phosphate buffer (0.1 M - pH 7.4) by thorough sonication (30 min). The silica gel was precipitated by centrifugation.

The quantification was carried out by enzyme-linked immunosorbent assay (ELISA), making use of kits for cytokinin determination (Vicont, Ufa 450073, box 7512, Russia) (Kudoyarova *et al.*, 1990).

2.3 Photosynthetic parameters:

2.3.1 Pigment dosage:

For a determination of the total chlorophyll content in thylakoid suspensions (see 2.3.2.1), a thylakoid sample was diluted (1:100) in acetone 80% (v/v). This solution was centrifuged (5 min at 3000g) and the absorbance of the supernatant was measured at 652 nm. The chlorophyll concentration was calculated according to Bruinsma (1963) with the following equation:

$$\text{chlorophyll a+b (mg ml}^{-1}\text{ suspension)} = (A652/36)\cdot 100.$$

In leaf discs, chlorophyll a, chlorophyll b and the total concentration of carotenoids was determined by a method adapted from Lichtenthaler and Wellburn (1983). A leaf disc was ground in a Potter homogenizer in acetone 80% (v/v) with a pinch of $CaCO_3$. The suspension was centrifuged (5 min at

3000g) and the absorbance of the supernatant was measured at 470, 646, 663 and 710 nm. The value of A710 was subtracted from the other absorbances to correct for impurities, giving the values A'470, A'646 and A'663, respectively. The pigment concentrations were calculated with the following equations:

$$\text{chlorophyll a } (\mu g / ml) = 12.21 \cdot A'663 - 2.81 \cdot A'646 = Ca,$$

$$\text{chlorophyll b } (\mu g / ml) = 20.13 \cdot A'646 - 5.03 \cdot A'663 = Cb \text{ and}$$

$$\text{carotenoids } (\mu g / ml) = (1000 \cdot A'470 - 3.27 \cdot Ca - 104 \cdot Cb) / 229.$$

After multiplication with the volume of supernatant, this yielded the corresponding pigment concentrations (μg) in the leaf disc.

2.3.2 The capacity of the photosynthetic electron transport chain:

The electron transport activity was measured with an oxygen electrode, making use of artificial electron donors, electron acceptors and inhibitors. This activity, determined on isolated thylakoid membranes under specific artificial, generally not limiting conditions, is further called the capacity of the electron transport chain.

2.3.2.1 Leaf sampling and isolation of thylakoid membranes:

As a control expanding leaves (leaf 7 - 11) from 3 - 4.5 weeks old SR1 plants, were used unless otherwise indicated.

From the transgenic HSIPT plants the largest leaves from 0 to 6 weeks heat treated plants were sampled and compared with wild type plants from the same age, subjected to the same treatment. Non-heated plants (HSIPT and SR1) of the same age were assayed likewise.

In the P*ssu-ipt* grafts almost fully expanded leaves from non-flowering branches were used. These leaves were much smaller than those from wild type plants and 2 to 4 leaves were collected for one extraction.

Since the growth rate of the P*ssu-ipt* plants was much slower and varied rather strongly in comparison to SR1 plants, the age of the plants was determined according to their size (see chapter 6). The largest, not completely expanded leaves were collected. This was always done before flower induction had

occured.
The development of Km^S plants, segregating from the P*ssu-ipt* plants, was similar to that of the wild type and equivalent leaves were harvested.

Due to the large amounts of starch in the transgenic P*ssu-ipt* grafts and -plants (Beinsberger, 1993; Winters, 1994), it was impossible to overcome disruption of the plastids during isolation. Therefore, no intact chloroplasts were used in this study, but crude thylakoid membrane suspensions were prepared.

The general isolation procedure for thylakoid membranes is as follows. The recipients and solutions used were ice cooled. The mid-vein of the leaves was stripped, the blades were sliced and blended in isolation medium (sorbitol 0.35M; $MnCl_2$ 1mM; $MgCl_2$ 1mM; EDTA 2mM; K_2HPO_4 0.5mM; Hepes (KOH - pH8) 0.05M; BSA 0.4% w/v). This suspension was filtered through 1 layer of miracloth and 1 layer of nylon (80 mesh). The filtrate was centrifuged for 5 min at 3000g. The pellet was washed once and after centrifugation (5 min, 3000g) resuspended with a paint brush and further homogenized with a Potter homogenizer in the resuspension medium (sorbitol 0.33M; EDTA 2mM; $MgCl_2$ 1mM; $MnCl_2$ 1mM; Tricine (KOH - pH7.5) 0.05M) to a final concentration of about 1 mg Chl/ml. This extract was immediately stored in a refrigerator. Any modification to this general extraction procedure will be mentioned on the appropriate place in the text.

2.3.2.2 a) Polarographic determination of the electron transport capacity:

The capacity of partial reactions of the electron transport chain was measured with a liquid phase Clark type oxygen electrode (Hansatech Ltd.). The thylakoid suspension was diluted 20 times in the assay medium (1 ml reaction volume), containing K_2HPO_4 2mM; NaCl 2mM; $MgCl_2$ 5mM; NaN_3 0.25mM; and Tricine (KOH - pH 7.8) 0.01M. Several redox-active chemicals were added, in combination with certain inhibitors. The components, the concentrations used and the presumed interaction sites in the electron transport chain are summarized in table 2.1.
The measurement was performed at 25°C and the cuvette was illuminated either with a slide projector lamp (c. 1240 μmol quanta PAR $m^{-2}s^{-1}$; cooling the light by passage through a water bath) or with a LS2-light source (Hansatech) (1500 μmol quanta PAR $m^{-2}s^{-1}$) guided by optic fiber when a cuvette with black painted water jacket was used. In both cases light was saturating.

2.3.2.2 b) Spectrophotometric measurement of the electron transport capacity:

The activity of the electron transport mediated by the PSII reaction centre without the oxygen evolving complex, was measured on a split beam spectrophotometer (SLM Aminco DW2000) with DCPIP (0.1mM) as electron acceptor and DPC (0.67mM) as electron donor (see table 2.1). The thylakoid membranes were incubated on ice for 15 min with NH_2OH prior to the measurement in order to inhibit the oxygen evolving complex (Kretschmann *et al.*, 1991; Mei and Yocum, 1991). After the inhibitory treatment, the thylakoid suspension was diluted to a final concentration of 20 μg Chl/ml in the assay medium (KCl 20mM; $MgCl_2$ 5mM; Tricine (KOH - pH 6.5) 10mM). The absorbance change at 590 nm was assayed upon switching on actinic illumination (SLM Aminco Tungsten - Iodide lamp; Calflex C and Schott RG645 filtered the light that was cooled by passage through a water bath), perpendicular to the measuring beam. The cuvette was thermostatted at 25°C. An extinction coefficient ϵ = 22000 (Dawson *et al.*, 1986) was used for calculating DCPIP reduction.

Table 2.1: Partial electron transport reactions measured in the presence of the redox components and inhibitors mentioned. The presumed interaction sites (1-10) are indicated on the scheme opposite to this table.

e⁻donor[i]	e⁻acceptor[i]	inhibitor	presumed e⁻donation and acceptor side; + eventually inhibition site	references
H_2O	MV (0.25mM)	(DBMIB)[j]	1 → 10	a,b,c
H_2O	FeCy (6.25mM)	-	1 → 6,9	a
H_2O	SiMo (0.1mM)	DCMU (2μM)	1 → 4; + 5	d,e
H_2O	DAD/FeCy (0.5mM/1.2mM)	-	1 → 6	a
H_2O	DCPIP/FeCy (0.125mM/1.2mM)	-	1 → 6,9	a
H_2O	PD/FeCy (0.5mM/1.2mM)	-	1 → 6	a
DPC (0.67mM)	DCPIP (0.1mM)	NH_2OH	3 → 6,9; + 2[k]	a,g
DAD/red[h] (0.5mM)	MV (0.25mM)	DCMU (50μM)	7 → 10; + 5	a,e
DCPIP/red[h] (0.125mM)	MV (0.25mM)	DCMU (50μM)	8,9 → 10; + 5	a,e
TMPD/red[h] (1.25μM)	MV (50μM)	DCMU (33μM)	7,8 → 10; + 5	a,e

$$\underset{1}{H_2O} \rightarrow \underset{2}{o.e.c.} \rightarrow \underset{3}{P680} \rightarrow \underset{4}{Q_A} \underset{5}{Q_B} \rightarrow \underset{6}{PQ} \rightarrow \underset{7}{(Fe\text{-}S)}\ \underset{8}{b_6f} \rightarrow \underset{9}{PC} \rightarrow P700 \rightarrow A_0A_1 \rightarrow$$

$$F_X \rightarrow \underset{10}{F_{A,B}} \rightarrow Fd \rightarrow NADP^+$$

(a) Izawa, 1980; (b) Böger and Kunert, 1978; (c) Fujii *et al.*, 1990; (d) Schansker and van Rensen, 1993; (e) Izawa, 1977; (f) Trebst, 1980; (g) Kretschmann *et al.*, 1991; Allen and Holmes, 1986 - thylakoid suspensions (1 mg chlorophyll/ml) were pre-incubated during 15 min in darkness on ice with 1mM NH_2OH, pH 7; (h) DAD was reduced by addition of 4mg $NaBH_4$/ml stock solution (20mM), after 1 min 10μl HCl (concentrated) was added as in Allen and Holmes (1986), this was done on ice; DCPIP and TMPD were prepared as double concentrated stock solutions (5mM resp. 0.125M) and reduced by addition of equal amount of sodium ascorbate (22.7mM) solution.
(i) MV, FeCy, TMPD and NH_4Cl were dissolved in deionized water; DCMU, DBMIB, DPC, DCPIP and PD stock solutions were prepared in ethanol (resp. 20%, 96%, 96%, 10%, 75%); DAD was dissolved in ethanol:ethylene glycol (1:1) and SiMo in DMSO:water (1:1) mixtures.
(j) DBMIB was optionally added after the uncoupling of reaction 1 → 10 by addition of 10mM NH_4Cl (see chapter 6); other inhibitors were added before the presumable reaction was recorded, though this did not exclude sequential recording e.g. first reaction 1 → 10 was measured, then DCMU (site 5 inhibitor) was added, followed by DAD/red (reaction 7 → 10).
(k) measured spectrophotometrically (see 2.3.2.2 b).

2.3.2.3 Photophosphorylation:

ATP production was assayed by comparing a 1 min illuminated and a dark treated sample. ATP was quantified by detection of the luminescence from luciferin by the firefly luciferase catalyzed reaction (McElroy and DeLuca, 1981; ATP monitoring kit 1243-102 - LKB). ADP was determined after chemical conversion to ATP using pyruvate kinase.

2.3.2.3.1 Sample preparation:

A 10 μl sample from freshly prepared thylakoid suspension (1 mg Chl/ml) was diluted 20 times in a microcentrifuge tube in an adapted resuspension medium (KCl 25mM; $MgCl_2$ 6mM; ADP 1mM; NaH_2PO_4 1mM; MV 0.1mM; Hepes (KOH - pH 8.0) 10mM). The microcentrifuge tube was placed in the cuvette of an oxygen electrode maintained at 25°C and illuminated during 1 min with cooled light from a slide projector lamp (saturating light intensity). Before switching off the light 200 μl TCA-solution (trichloroacetic acid 10% (w/v); EDTA 4mM) was added to the extract in order to block ATP converting reactions. The sample was centrifuged for 4 min at 15800g (Eppendorf centrifuge 5415C). The supernatant was transferred to a glass tube and small quantities of KOH (4N) were added until pH 7.75 was reached. This solution was diluted 5-fold in Tris-EDTA (Tris (acetic acid - pH 7.75) 0.1M; EDTA 0.1M) before carrying out the assay. In parallel a non-illuminated sample was prepared.

2.3.2.3.2 Luminescence measurement:

In a polystyrene cuvet 150 μl Tris-EDTA and 50 μl ATP monitoring reagent (LKB - Wallac 1243-200) were mixed to yield the basic luminescence level (measured at constant temperature (25°C) with LKB - Wallac Luminometer 1250). The luminescence signal was registered upon addition of 5 - 50 μl sample; finally an internal standard (5 μl ATP $5 \cdot 10^{-5}$M, dissolved in millipore water) was added. For ADP determination, additionally pyruvate kinase (10 μl 800 units/ml) and phospho-enol-pyruvate (10 μl 0.1M) were injected and the reaction was followed until the maximum output signal was obtained; thereafter the internal standard was added. With this method we obtained a good linear response within a range of 10^{-10} - 10^{-6}M ATP (final concentration).

2.3.3 Oxygen evolving capacity in leaf discs:

The photosynthetic activity *in vivo* was assayed by means of a leaf disc electrode (LDE, Hansatech Ltd.; Delieu and Walker, 1983). With this apparatus oxygen evolution (polarographic method) and fluorescence can be measured simultaneously. The following measuring routine was adapted: a leaf disc of 10 cm^2 was mounted and a wake up procedure was applied (5 min light, 50% of full intensity of the LED light source, *i.e.* 520 μmol quanta $m^{-2}s^{-1}$ - 3 min darkness). Although plants were not dark adapted prior to the measurement, it was proven that by this brief light induction the photosynthetic carbon assimilation reactions were initiated (Walker, 1981). CO_2 supply was provided by a capillary matting humidified in $KHCO_3$ (1M - pH 9) solution and by flushing the leaf chamber with CO_2 enriched air between every stage of the measuring procedure which was divided into 3 parts.

In the first part oxygen evolution and chlorophyll fluorescence kinetics were recorded under conditions perturbing steady state photosynthesis. After 1 min dark recording the leaf disc was illuminated for 5 min (45% of the maximum intensity of LEDs, *i.e.* 470 μmol quanta $m^{-2}s^{-1}$). Again 1 min darkness was followed by a 5 min re-illumination at higher light intensity (90% of full LED intensity, *i.e.* 960 μmol quanta $m^{-2}s^{-1}$). After this recording a 5 min dark interval preceeded the second act.

In this second part oxygen evolution was measured at increasing light intensities. This measurement was conducted by computer (Leafdisc Program Version R1.00 - Hansatech) changing the output of the LED source from 0 to about 1000 μmol $m^{-2}s^{-1}$ PAR in 20 steps of 45 seconds. The rate of oxygen evolution as a function of photon flux density (PFD) was calculated.

To evaluate the low intensity range more accurately, a second computerized recording of the oxygen evolution was performed with decreasing intensity of actinic illumination in 16 autosteps of 45 seconds each, from c. 150 to 0 μmol quanta $m^{-2}s^{-1}$. This is the third and final part of the measuring procedure. The measurement was performed at 20°C. Immediately afterwards fresh weight of the leaf disc was determined and the sample was frozen for pigment dosage (method adapted from Lichtenthaler and Wellburn (1983), see 2.3.1).

2.3.4 Chlorophyll a fluorescence characteristics of leaves:

2.3.4.1 LDE - slow kinetics at room temperature on leaf discs:

A first impression of the kinetic behaviour of chlorophyll fluorescence was obtained in the simultaneous measurement of the oxygen evolution and fluorescence of leaf discs, as mentioned in the previous section (see 2.3.3). This represents slow induction kinetics (minute scale - Kautsky effect) under non steady state conditions. In normal leaves, like those from wild type plants, the light regime applied induces a dampening oscillation of the fluorescence light, with an antiparallel oscillation pattern in the oxygen evolution (see results, fig. 6.7 a-b). The factors that influence the quenching of fluorescence were analyzed more in detail by means of a modulated (PAM) fluorometer.

2.3.4.2 Pulse Amplitude Modulation Fluorometer - quenching analysis:

2.3.4.2.1 Background of the quenching analysis:

The chlorophyll fluorescence emission evolves at room temperature a time dependent course upon dark - light transition in leaves, which is known as the Kautsky effect (Schreiber, 1983). After an initial, transient fluorescence maximum (the P-phase in the 'OIDPSMT' Kautsky sequence) the signal is quenched. This quenching has various origins; these are usually classified as photochemical (q_P) and non photochemical (q_N) quenching.

The photochemical quenching depends on the reductive state of Q_A, the primary electron acceptor of PSII (Schreiber *et al.*, 1986). As Q_A becomes reoxidized it is available again as oxidant for the reaction centre of PSII. This enables the primary photochemical charge separation in the reaction centre; in that way the fluorescence emission is quenched.

The underlying mechanism of the non photochemical quenching (q_N) is more complex (Krause and Weis, 1984). It is the sum of several factors, all causing a decline in fluorescence. Under physiological conditions the major component is the energized state quenching (q_E) (Horton, 1983; Quick and Horton, 1984). Coupled photosynthetic electron transport leads to a build up of a proton gradient and brings the thylakoid membrane in a so-called high-energy state. This presumably induces secondary changes in the thylakoid membrane which would explain the difference in time between acidification of the thylakoid lumen and the increase in q_E (Bolhàr-Nordenkampf *et al.*, 1989; Krause and Weis, 1984; Schreiber *et al.*, 1986). It results in a higher radiationless, thermal

de-excitation. The exact mechanism is still a matter of discussion (for a review see Krause and Weis, 1991 and Horton *et al.*, 1991).
A second component of q_N is the photoinhibitory quenching (q_I) which is correlated to conditions of excessive radiation. High light intensities, in combination with other experimental conditions, are responsible for this nearly irreversible quenching. Conditions favouring state 1 - state 2 transitions, by phosphorylation of the LHCII, account for the factor q_T. Whether this comprises the event of spillover of excitation energy from PSII to PSI under physiological conditions is not clear. Quenching can be generated by Mg^{+2} depletion. This results in a destacking of the grana, which also might increase the probability of spillover; again the physiological relevance of this quenching factor can be questionned. Other specific conditional quenching circumstances, e.g. involved in the generation of pigment radicals, can be recognized.

The distinction between q_P and q_N can be made by means of the light doubling effect (Bradbury and Baker, 1981). It is based on the difference in response time: q_P corresponds to the fast phases of fluorescence induction (complete reduction of Q_A - and thus suppression of q_P - can be achieved by a saturating light intensity within a fraction of seconds), whereas q_N changes are relatively slow (a short light flash will not be reflected by a change in q_N).

With the development of the pulse amplitude modulation fluorometer ('PAM' fluorometer) this distinction can be performed easily, since this technique allows the use of changing light regimes throughout the measurement. Short (millisecond range), saturating light pulses superimposed on a relatively low actinic illumination can be separated from the fluorescence signal. Deconvolution then yields information about photochemical activity in PSII and about energetic conditions of the photosynthetic apparatus. However, Havaux *et al.* (1991) presented evidence against the strict distinction between q_P and q_N. Therefore the results should be interpreted with care.

2.3.4.2.2 Instrumentation and measuring procedure:

A leaf disc was placed on a matting humidified with $KHCO_3$ solution (1M - pH 9) for CO_2 supply. A branched optic fiber, which guided the different light beams to and the fluorescence signal from the leaf, was mounted on the upper surface of the leaf. This connection was covered by a black cloth, to avoid light penetration from the surroundings to the leaf. The PAM chlorophyll fluorometer (H. Walz, Effeltrich, Germany) was used, in combination with the KL1500 electronic and KL1500-FL103 light sources (Walz). The modulated

measuring light (c. 0.35 μmol quanta $m^{-2}s^{-1}$), provided by the PAM 101 control unit, was checked not to cause fluorescence induction.

Analogous to the measuring procedure with the LDE (see 2.3.3) a wake up illumination was introduced: 5 min of actinic illumination, followed by 3 min of darkness. The F_o and F_M (by a saturating flash light of 800 ms) were determined. Because the plant material was not in a full dark adapted state, F_M is underestimated (Öquist and Chow, 1992). Inaccuracy can also be expected in the determination of F_o. In dark adapted leaves the F_o level exceeds that in leaves adapted to strong actinic light (Bilger and Schreiber, 1986), due to a fluorescence contribution from inactive centres of PSII (Guenther *et al.*, 1990). Havaux and co-authors (1991) proposed therefore another method to measure F_o in light adapted leaves. However, we persisted with the wake up sequence, to analyse the fluorescence course of leaf discs under comparable conditions as in the initial recordings with the LDE (see 2.3.4.1).
Following the registration of F_o and F_M, actinic illumination (150 W m^{-2}) and a superimposed flash light (800 ms saturating flashes (1320-1800 W m^{-2}) at a flash frequency of 0.1 Hz) was given during 5 min. Data acquisition and manipulation were handled by the dataprocessor DA100 (Walz).

2.3.5 Redox kinetics of P700, the reaction centre of PSI, in intact leaves:

2.3.5.1 Principle:

As pointed out before, the oxidation state of PSII can be monitored by analysis of the chlorophyll fluorescence signal. Namely, q_P describes the quenching of fluorescence provoked by the photochemical charge separation at the reaction centre and thus $(1-q_P)$ reflects the relative extent of Q_A reduction (Schreiber *et al.*, 1986).
At room temperature PSI is only a very weak fluorescence emittor (emission maximum at about 730 nm). However, redox changes of P700 can be detected by absorbance changes centred around 700 nm and also at 820 nm. P700 in the oxidized state has an increased absorbance at 820 nm as compared to the reduced state. This increase in absorbance is also accomplished by the oxidation of other chlorophyll molecules and by pheophytin reduction (for a review, see Harbinson and Woodward, 1987). Yet, because of the difference in half-times of the reduction kinetics and because of the accumulation of the oxidized $P700^+$ under continuous illumination, which does not happen with $P680^+$, the absorbance

change at 820 nm provides a good method to measure the redox state of P700.

Complete oxidation of P700 can be accomplished by far red illumination (>715 nm). When in this state a single or a multiple turnover flash illumination is given, P700 is transiently reduced by the activity of PSII and subsequently re-oxidized by the far red background illumination. By comparison of the complementary area between the redox-curve after that multiple vs. single turnover flash and the stationary level of $P700^+$ (MT-area and ST-area, respectively), the functional pool size of intersystem electrons per PSI reaction centres can be estimated (Schreiber *et al.*, 1988; Asada *et al.*, 1992).
When actinic light is provided, a steady state level of $P700^+$/P700 will be established. After turning out the actinic light, $P700^+$ is transiently reduced by the pool of electrons that was accumulated during the actinic illumination and that can be donated to $P700^+$. Thereafter, the far red background illumination completely re-oxidizes the P700-pool again. This functional pool size of electrons is reflected in the complementary area between the redox-curve after cessation of the actinic illumination and the stationary level of $P700^+$ (AL-area). From the comparison of the AL- and MT-area, Asada *et al.* (1992) have concluded that stromal components contribute to the AL-area. Thus, the equation

(AL-area - MT-area) / ST-area

represents the pool size of electrons from the stromal donors per P700.
Because at the time of this experiment, there was no ST-flash lamp to our disposal, it was not possible to estimate the number of reaction centres of PSI by this technique in the intact leaves. Besides, leaf structure and chlorophyll content was highly variable in the different plants and grafts (see results, chapter 6). For this reason differences in concentration of P700 per unit of leaf area could be expected. By making the ratio of the AL- and MT-areas this problem was overcome. A measuring procedure was established in which the AL-area and the MT-area were determined.

2.3.5.2 Instrumentation and measuring procedure for intact leaves:

An intact leaf, still attached to the plant, was enclosed into the leaf clip fitting to the MFMS fluorometer (Hansatech Ltd.). This leaf clip has an open bottom, through which CO_2 supply was ensured by means of a dark painted

matting humidified with a $KHCO_3$ solution (1M - pH 9). The leaf was dark adapted for 20 min in this position prior to the measurement. During this period the pulsed measuring light (820 nm; 0.065 Wm^{-2}) was but present.
After this period the measurement was started. First, far red light was switched on (102-FR LED - emission maximum at 735 nm). After 2 min - by this time P700 was completely oxidized - actinic illumination was given (KL1500 electronic - Walz or MFMS LED 9306 - Hansatech Ltd., providing different light intensities - see results chapter 6) for 3 min. Upon turning off the actinic light, P700 was re-oxidized by the far red background illumination. When full re-oxidation was achieved a multiple turnover flash (XMT 103 - Walz; 50 ms saturating light intensity) was given. When the quick re-oxidation was completed, far red illumination was switched off, and the original absorbance level was established again within a few minutes.

The complementary areas between the re-oxidation curves and the stationary level of $P700^+$ after the reduction either by actinic illumination (AL-area) or by flash illumination (MT-area) were determined as by Asada and co-workers (1992) (see fig. 2.5).

The fiberoptic system was always placed towards the upper surface of the leaf. Due to the black coloured matting, reducing the reflection of the measuring light, the P700 signal was mainly from the palisade parenchyma chloroplasts.

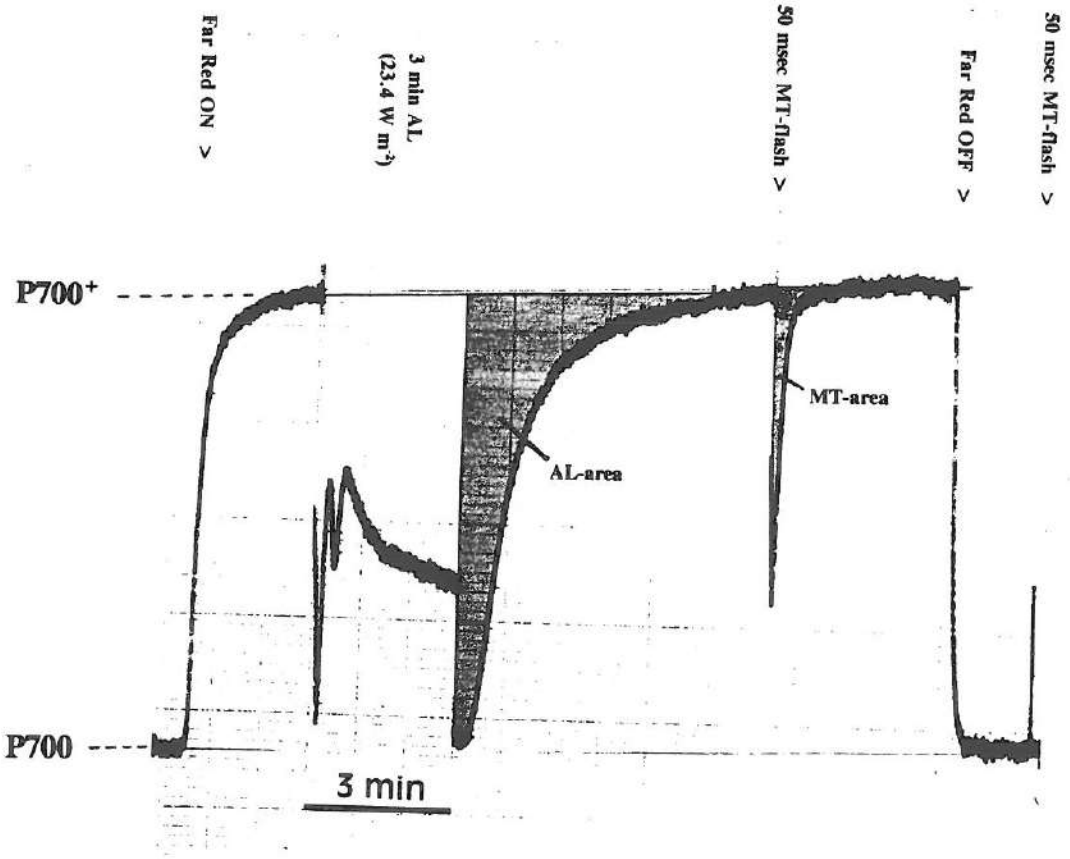


Figure 2.5: Example of a recording of the absorbance signal of P700 (arbitrary units). The different illuminations provided are marked on the figure. The AL- and MT-area are the shaded areas indicated. For further details, see text (2.3.5.2).

Chapter 3: Control experiment on the *in vitro* application of cytokinins and IAA on the determination of the photosynthetic electron transport capacity.

Exogenous application of kinetin and IAA to seedlings or greening cotyledons (Behera and Choudhury, 1990; Buschmann and Lichtenthaler, 1977; Pedhadiya *et al.*, 1987) was shown to stimulate partial electron transport reactions, among which the Hill activity, PSI activity and possibly the $NADP^+$-reducing system. On the other hand, Le Pabic and co-workers (1988) have demonstrated an inhibitory effect of some synthetic cytokinins on PSII activity. In 1990 Le Pabic *et al.* showed that upon BA treatment of detached cotyledons, PSI and PSII activity on a chlorophyll basis, was lower than in the control; this was presumably due to a change in the antenna-reaction centre relation.

As pointed out in Materials and Methods the electron transfer capacity of thylakoid membranes was determined in a liquid phase oxygen electrode, on thylakoid suspensions. As it is our aim to work with transgenic plants overproducing cytokinins, it might be expected that crude thylakoid suspensions are enriched in cytokinins. This implies that increased concentrations of natural phytohormones could be present during the measurement in the cuvette of the oxygen electrode.

To rule out any possible direct interference of cytokinins in the determination of the electron transport capacity, thylakoid suspensions from wild type plants were prepared and were incubated with different phytohormones before measurement. The influence of the natural occuring zeatin (Z) and zeatin riboside (ZR) and the synthetic N-benzyl-6-aminopurine (BA) and kinetin was tested over a wide concentration range. Because in the transgenic tissues the increased cytokinin content was accompanied by a rise of the indole-3-acetic acid (IAA) level (Van Onckelen *et al.*, 1988; Ondřej *et al.*, 1990; Beinsberger, 1993), the effect of IAA addition to the thylakoid suspension was examined as well.
The incubation period was relatively short (up to 30 min). Direct, and thus prompt effects on the electron transport system would be discerned.

3.1 Experimental approach:

Crude thylakoid suspensions from wild type plants were prepared as described in Materials and Methods (2.3.2.1), although without washing, *i.e.* after the first centrifugation the membranes were immediately resuspended in the resuspension medium to the final concentration of 1.0 ± 0.2 mg chlorophyll/ml.

Stock solutions ($2 \cdot 10^{-3}$M) of Z, ZR and IAA were made in resuspension medium, BA was either dissolved in HCl (10^{-3}N) or in DMSO (1% v/v) and kinetin was also dissolved in DMSO (1% v/v). All hormone stocks were diluted in resuspension medium.

An equal volume of hormone solution was added to the thylakoid membrane suspension in a range from $2 \cdot 10^{-11}$M to $2 \cdot 10^{-3}$M. In table 3.1 the resulting hormone/chlorophyll ratio is calculated.

Table 3.1: final hormone/chlorophyll ratio after addition of different concentrations of hormone solution to isolated thylakoid suspensions (1 mg chlorophyll/ml).

concentration (M) of hormone solution	hormone/chlorophyll (mol/mg)
$2 \cdot 10^{-3}$	$2 \cdot 10^{-6}$
$2 \cdot 10^{-4}$	$2 \cdot 10^{-7}$
$2 \cdot 10^{-5}$	$2 \cdot 10^{-8}$
$2 \cdot 10^{-7}$	$2 \cdot 10^{-10}$
$2 \cdot 10^{-9}$	$2 \cdot 10^{-12}$
$2 \cdot 10^{-11}$	$2 \cdot 10^{-14}$

Since in wild type plants a chlorophyll concentration of about 1 mg/g fresh weight is recorded (cf. chapter 6), addition of hormone solutions of $2 \cdot 10^{-9}$M to $2 \cdot 10^{-7}$M to the isolated thylakoid suspensions corresponds to a physiological concentration range *in vivo* (cf. chapter 5; Beinsberger, 1993).

This 'hormone treated' sample was used for measurement of the capacity of the electron transport of the whole chain and of partial reactions (see table 2.1).

First, the effect of the incubation period, up to 30 min, was tested with high concentrations of BA and with a high and a low concentration of kinetin. Thereafter, a series of concentrations of the different cytokinins and of IAA was assayed, in accordance with table 3.1. In that case the incubation period was always within half an hour.

3.2 Results:

Figure 3.1 shows the influence of the synthetic cytokinins kinetin and BA on the capacity of the electron transport from H_2O over both photosystem I and II to MV (reaction 1 → 10; table 2.1) as a function of the incubation period. The results are presented as percentage of the capacity of untreated samples.

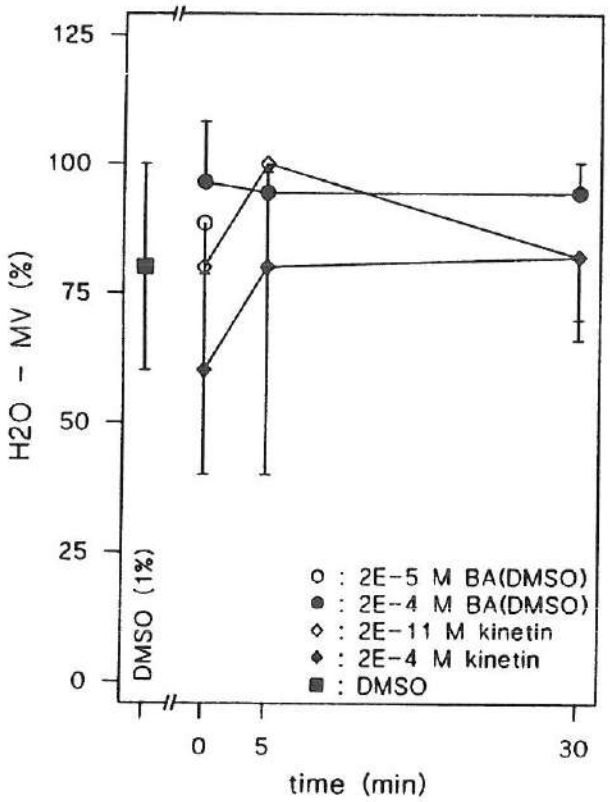


Figure 3.1: Effect of the addition of BA or kinetin on the electron transport capacity mediated by PSII and PSI (H_2O → MV) as a function of the incubation period. The data (mean ± standard error) are expressed as percentage of the activity of a non-incubated thylakoid suspension.

Kinetin was tested at the highest and lowest concentrations used in the dose-response experiment (see figs. 3.3-4 and 3.6). The apparent initial reduction in the whole chain electron transport (fig. 3.1) disappeared within 5 min. After half an hour of incubation (on ice, in darkness), taking the standard error into account, no differences had appeared. The effect of BA was pursued at high concentrations ($2 \cdot 10^{-5}$ - $2 \cdot 10^{-4}$M, dissolved in DMSO), but again no stimulation or inhibition occured over a period of 30 min.

On the partial reations with DAD as electron acceptor from PSII (fig. 3.2-a; reaction 1 → 6; table 2.1) or with $DADH_2$ as electron donor to PSI (fig. 3.2-b; reaction 7 → 10; table 2.1) BA and kinetin did not affect the electron transport rate within the incubation period.

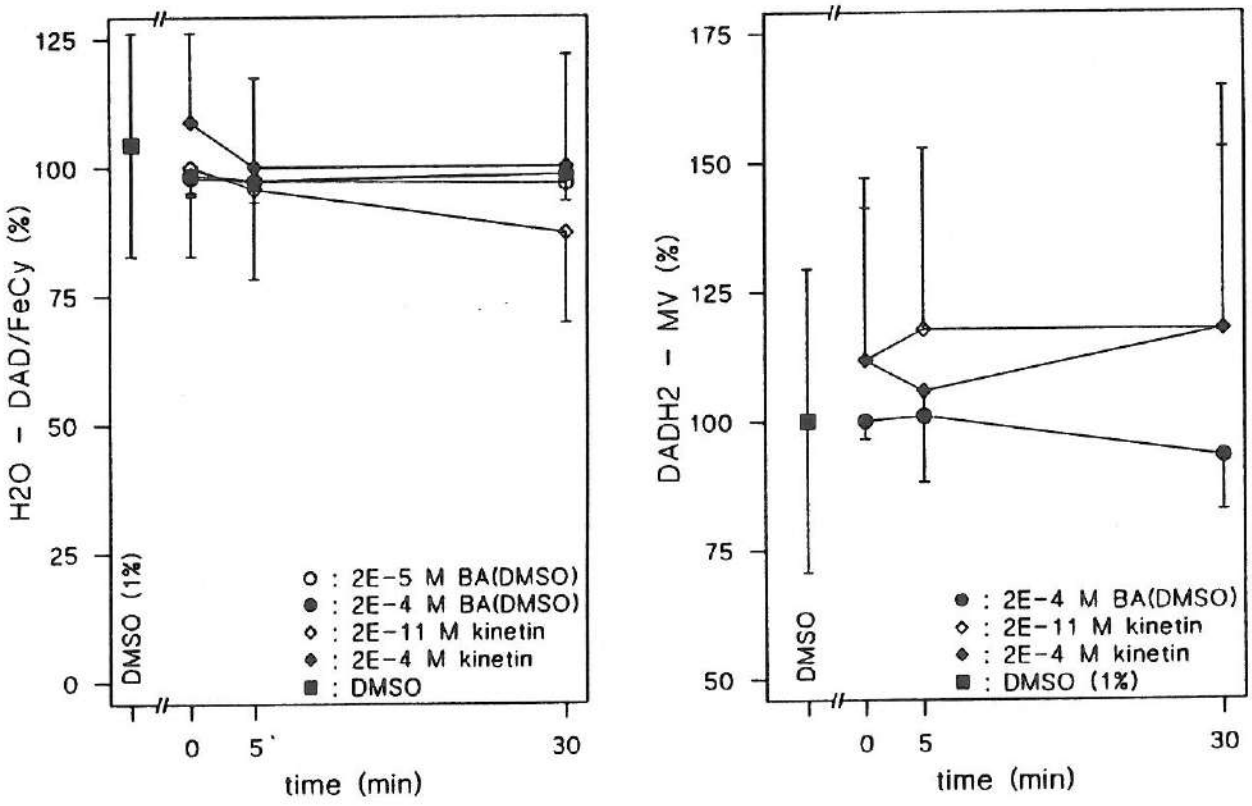


Figure 3.2: Effect of the addition of BA or kinetin on the electron transport capacity **a)** mediated by PSII (H_2O → DAD/ferricyanide) and **b)** mediated by PSI ($DADH_2$ → MV), as a function of the incubation period.

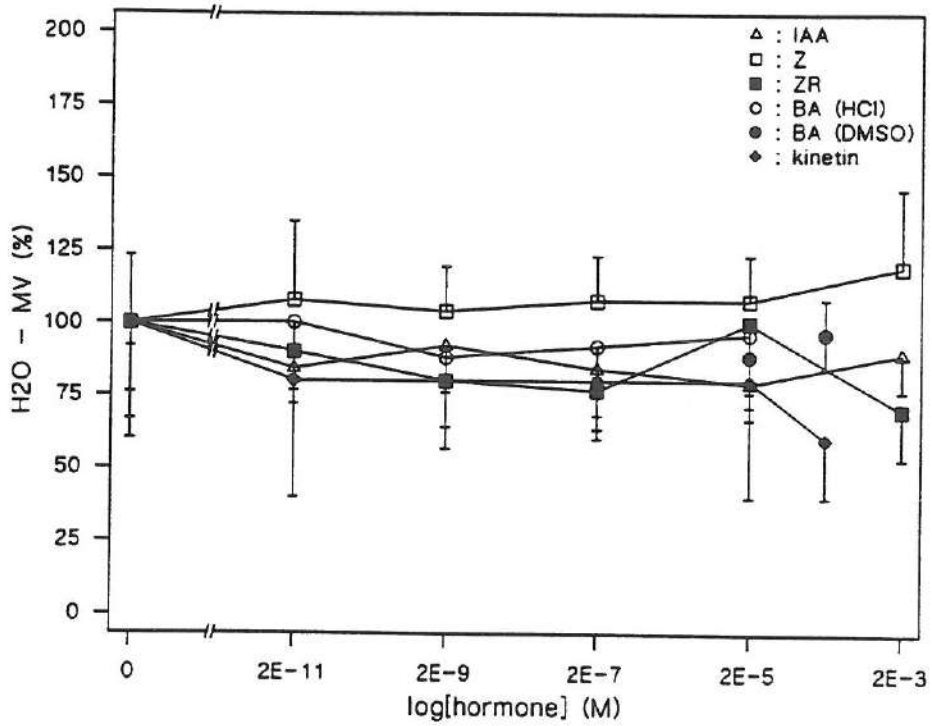


Figure 3.3: Effect of the addition of cytokinins or IAA on the electron transport capacity mediated by PSII and PSI ($H_2O \rightarrow MV$) as a function of the concentration of phytohormones applied. The data are expressed as percentage of the activity in a non-incubated thylakoid suspension.

In figure 3.3 the capacity of the whole chain electron transport (from H_2O to MV, *i.e.* reaction 1 → 10; table 2.1) is depicted as a function of the concentration of the hormones applied. Reckoning the standard error, no significant changes occured upon addition of any of the phytohormones used.

When focusing on the partial reactions mediated by photosystem II (figs. 3.4-5) with DAD or DCPIP as electron acceptors (reactions 1 → 6,9; table 2.1), or by photosystem I (figs. 3.6-7), making use of $DADH_2$ or $DCPIPH_2$ as electron donors (reactions 7,8 → 10; table 2.1), no distinctive effect of hormone treatment could be observed, either. In particular the higher activity of the reaction H_2O → DCPIP/FeCy (fig. 3.5) upon ZR addition should be qualified, noting the large standard error and the drop in the curve at $2 \cdot 10^{-5}$M. Moreover, no stimulation was observed in the reaction H_2O → DAD/FeCy, which spans a comparable partial electron transfer reaction.

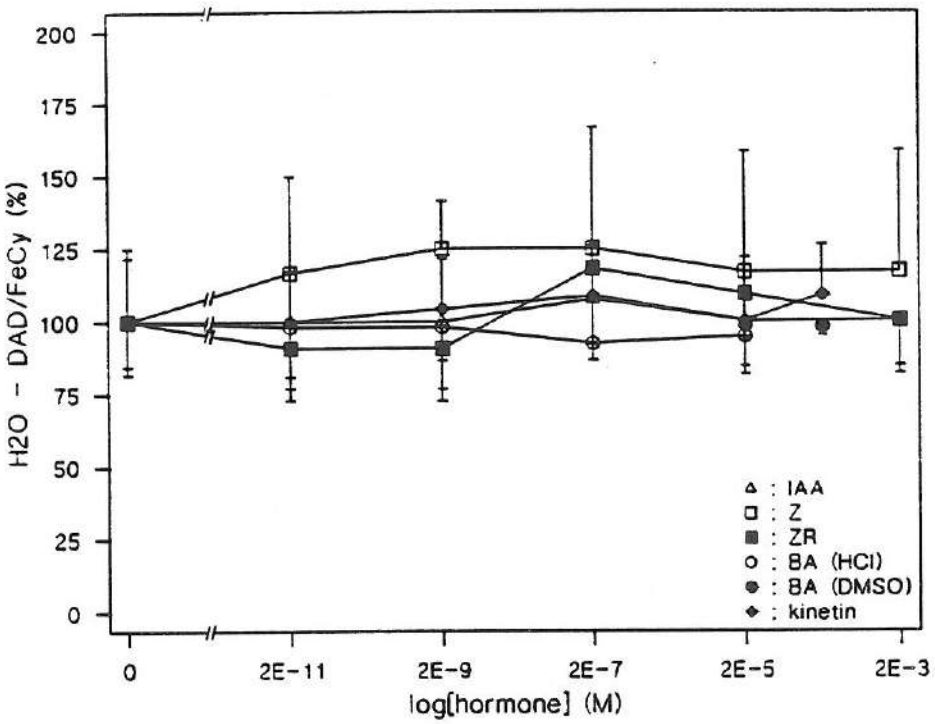


Figure 3.4: Effect of the addition of cytokinins or IAA on the electron transport capacity mediated by PSII ($H_2O \rightarrow$ DAD/ferricyanide) as a function of the concentration of phytohormones applied.

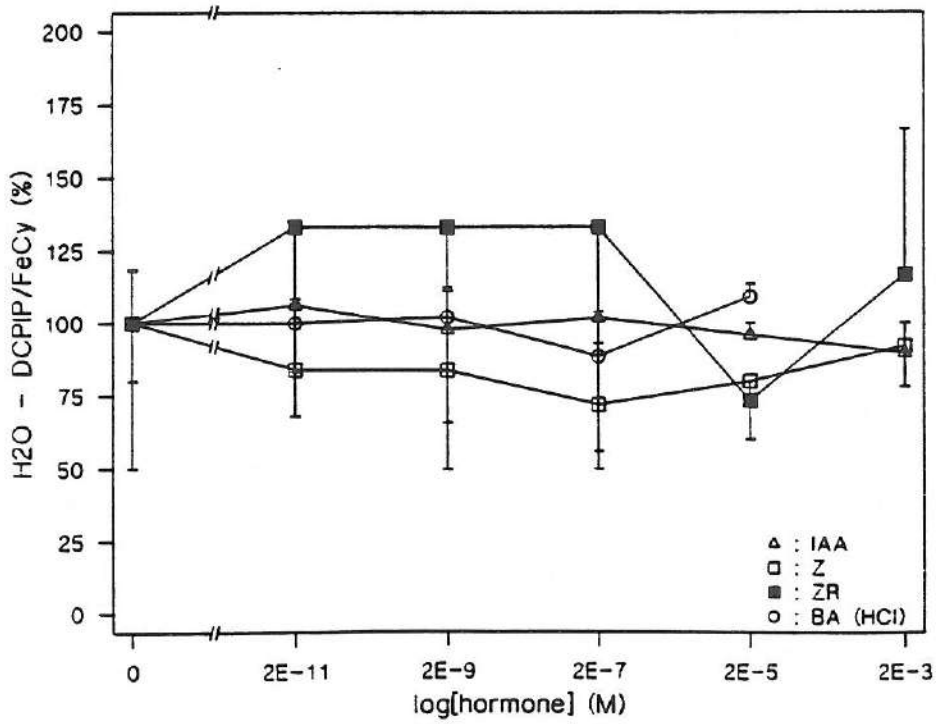


Figure 3.5: Similar to figure 3.4, but with DCPIP/ferricyanide as artificial electron acceptor.

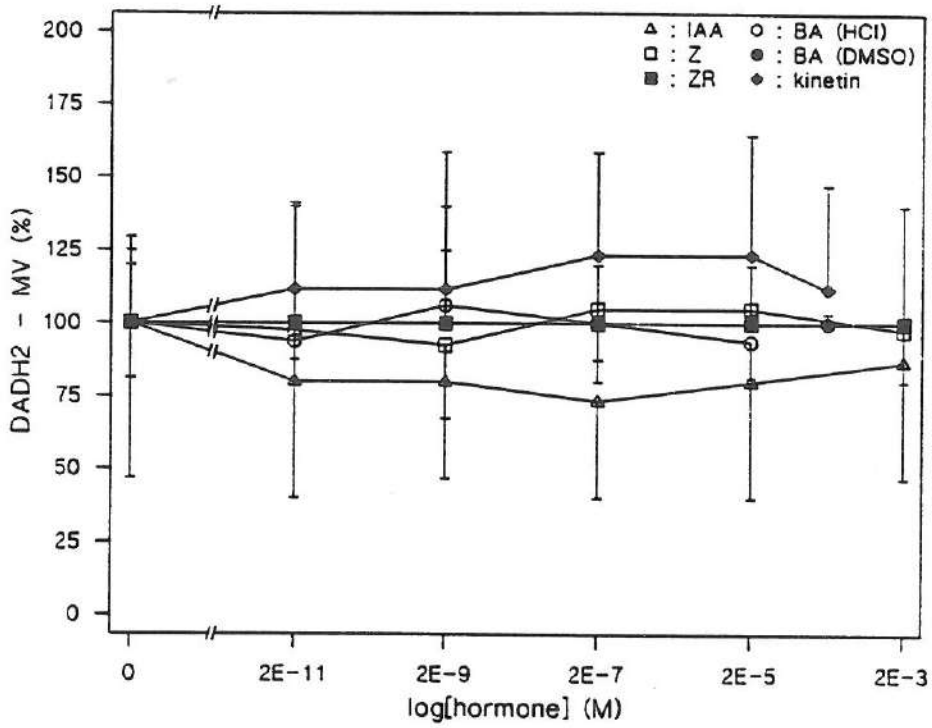


Figure 3.6: Effect of the addition of cytokinins or IAA on the electron transport capacity mediated by PSI ($DADH_2 \rightarrow MV$) as a function of the concentration of phytohormones applied.

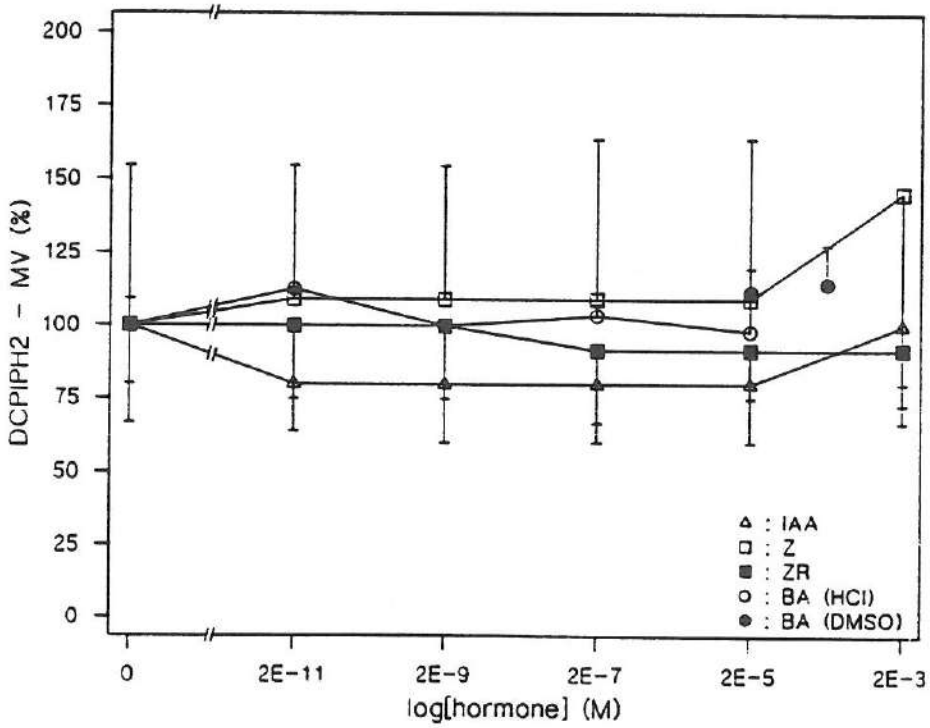


Figure 3.7: Similar to figure 3.6, but with $DCPIPH_2$ as artificial electron donor.

When BA was dissolved in HCl, at $2 \cdot 10^{-3}$M it caused inhibition of the PSII mediated electron transport (data not shown); this was purely due to acidification of the medium. Therefore the higher concentrations of BA ($2 \cdot 10^{-5}$ - $2 \cdot 10^{-4}$M) were tested with a stock ($2 \cdot 10^{-3}$M) made up in DMSO 1% (see also figs. 3.1 and 3.2 a-b). Addition of 1% DMSO itself had no effect, neither on the electron transport rate of the whole chain (fig. 3.1), nor on the partial reactions mediated by PSII or by PSI (fig. 3.2 a-b). Even at the high concentrations no effect of BA was noticed on any of the electron transport reactions measured.

3.3 Discussion:

Neither the natural and synthetic cytokinins that were studied, nor IAA did affect the capacity of the photosynthetic electron transport over a broad concentration range up to 30 min after application. These results confirm the data of Buschmann and Lichtenthaler (1977), who found that incubation of chloroplasts up to 30 min with 0.5mM IAA or kinetin, did not change the Hill activity e.g. by means of uncoupling of photophosphorylation.

These results are in contrast to the competitive inhibition of adenine derivatives for the atrazine binding site, recorded by Le Pabic *et al.* (1988). They determined that the interaction of BA and DCMU was a non competitive one. Several other alkyl and acyl adenine derivatives were active at the same site, inducing from 50 to 100% inhibition of the PSII activity. The interaction of the adenine derivatives with the herbicides atrazine and DCMU indicates that they act at the level of the 32kDa polypeptide of PSII, blocking the electron transfer from Q_A to Q_B. However, they found that the stronger inhibiting adenine derivatives usually had weaker cytokinin properties, as determined in a bioassay. Moreover, BA which exhibited good anti-senescence (cytokinin) action at $6 \cdot 10^{-6}$M, had to be used at millimolar concentrations to reduce the electron transport by about 50%. This was an order of magnitude higher than the concentration used here, and it was far exceeding the physiological range.

Effects of BA at the quinone pool like in the plant alternative cyanide-resistant respiratory chain (Chauveau *et al.*, 1983), could not be concluded from our results.

It can be concluded that no direct interference from the phytohormones on the *in vitro* measurement of the electron transport rate appears within a large concentration range. Accordingly, in the transgenic plant material with increased endogenous cytokinin content, an enhancement of the hormone concentration in the assay, e.g. by contamination of the thylakoid suspension with a cytokinin enriched microsomal fraction, will have no influence. Therefore, any difference measured in the electron transport capacity of the transgenic plants may be interpreted as a physiological effect.

Chapter 4: Effect of heat treatment on the photosynthetic electron transport.

The *ipt*-gene, the gene of interest in this study, was introduced in tobacco plants by way of two different genetic vector contructions (see 2.1.1). In the first one the *ipt*-gene was controlled by the *hsp70* heat shock promoter, in the second it was coupled to the light inducible *ssu*-promoter. In the former construction heat treatment is required to induce the expression of the *ipt*-gene and consequently to elevate the cytokinin biosynthesis. However, heat treatment itself evokes a number of physiological changes in the plant, such as induction of heat shock proteins (for a review see Vierling, 1991), increased sensitivity to photoinhibitory damage (Schuster *et al.*, 1989), changes in the lipid composition of thylakoid membranes (Guillot-Salomon *et al.*, 1991; Ivanova *et al.*, 1993; Süss and Yordanov, 1986) and inhibition of whole leaf photosynthesis (Berry and Björkman, 1980). There is not only a thermal effect on the biochemical reactions of CO_2-fixation (Weis, 1981), but the PSII-activity is highly vulnerable to heat damage. Oxygen evolution and electron transport around PSII in particular have been recognized as most heat sensitive processes (Berry and Björkman, 1980; Santarius and Weis, 1988). Light has a key position in this effect. The interaction with light can either be protective or injurious, depending on its intensity (Havaux and Strasser, 1990; Kislyuk, 1979; Schreiber and Berry, 1977). Heat stress is also supposed to cause dissociation of the LHCII-complex (Armond *et al.*, 1980; Gounaris *et al.*, 1984).

To define the effects of heating on the photosynthetic electron transport, the following experiments were all realized on wild type (SR1) plants. Heat treatment was first applied to isolated thylakoid suspensions, devoid of cellular protection or repair mechanisms based on protein synthesis. Subsequently the influence of heat treatment on detached leaves was measured. In this case the leaf was submerged in a waterbath to assure a rapid and accurate temperature control. Finally heat treatment was evaluated *in planta*. Intact plants were subjected to a single heat treatment, similar to that used to induce the *hsp70-ipt* gene expression in calli (Schmülling *et al.*, 1989; see 2.1.2.5).

The electron transport mediated by PSII was evaluated under all these heat treatments. In the heated thylakoid suspensions the electron transport capacity of PSI and of the whole chain (PSI + II) was also measured. In intact plants, besides the capacity of the electron transport (*in vitro*), oxygen evolution of leaf discs was

evaluated. The influence of light was considered by applying various intensities of white light during the treatment. The photon flux densities will be mentioned at the respective experiments.

4.1 The electron transport capacity of isolated thylakoid membranes after heat treatment:

4.1.1 Experimental approach:

Thylakoid membrane suspensions were isolated from leaves of wild type plants in a shortened procedure. In contrast to heat treatment of isolated thylakoid membranes, the treatment on intact leaf and whole plant level preceeded the isolation procedure. In order to exclude rapid reorganization after the treatment, of e.g. the lipid phase of the membranes, washing and centrifugation steps in the isolation procedure were omitted. Accordingly, leaves were homogenized in a minimal volume of isolation medium (see 2.3.2.1) and filtered through miracloth and nylon. A chlorophyll concentration of 0.5 $\pm$ 0.1 mg/ml was obtained on average.
This membrane suspension was immediately poured into a darkened test-tube (wrapped in tinfoil) and stored in a refrigerator (4°C) or placed for 0 - 60 min in a waterbath at 25, 30, 35, 40 and 50°C, respectively. Alternatively a non-darkened, wide (diameter about 2.5 cm) test-tube was used. As such the suspension was exposed to low light intensities at the working table (approximately 5 μmoles $m^{-2}s^{-1}$) or the suspension was illuminated with 50 μmoles $m^{-2}s^{-1}$ cooled light from a slide projector lamp (the light intensity was adjusted with neutral density filters). In the latter case also a glass beaker was used, in which a thin layer (c. 3 mm) of suspension was poured. The average chlorophyll concentration used here was only 0.23 $\pm$ 0.06 mg/ml. As such the sample was illuminated more homogenously. For the experiment at 4°C the beaker was placed on top of ice, at 25°C it was placed in a thermostatted waterbath.

The whole chain electron transport (from water to MV) and the activity of PSI, with either reduced $DADH_2$ or $DCPIPH_2$ as electron donor and MV as electron acceptor (see 2.3.2.2a; table 2.1), were measured. The oxygen evolving PSII-activity was measured with DAD and ferricyanide as artificial electron acceptors. The measurements were done in a thermostatted oxygen electrode at 25°C.

In a subsequent experiment, the oxygen evolving complex was inhibited by hydroxylamine and the electron transport capacity of the reaction center of PSII was measured spectrophotometrically. Herefore, the chlorophyll concentration of the

thylakoid suspension was adjusted to 1 mg/ml prior to the heat treatment, thus one centrifugation (5 min, 3000g) was added after which the membranes were resuspended to the final concentration in the isolation medium. Immediately after the respective heat treatment under light or dark conditions, the sample was incubated with the inhibitor NH_2OH during 15 min (in darkness, on ice). The electron transport from DPC to DCPIP was measured as explained in Materials and Methods (see 2.3.2.2b).

The data were statistically compared with a one way analysis of variance, combined with the Fisher's LSD test ($\alpha = 0.05$; two tailed).

4.1.2 Results:

The electron transport capacity from water to MV (whole chain electron transfer) vs. time of incubation of the thylakoids at different temperatures in darkness is presented in figure 4.1. It is obvious that low temperature incubation (4°C) did not cause any change in capacity within one hour. At 25 - 30°C a moderate inhibition (30 - 40% after 1 h) was attained. At higher temperatures (40 to 50°C) the loss of activity proceeded much faster and much more dramatic. At 50°C a complete inhibition was even obtained within 30 min.

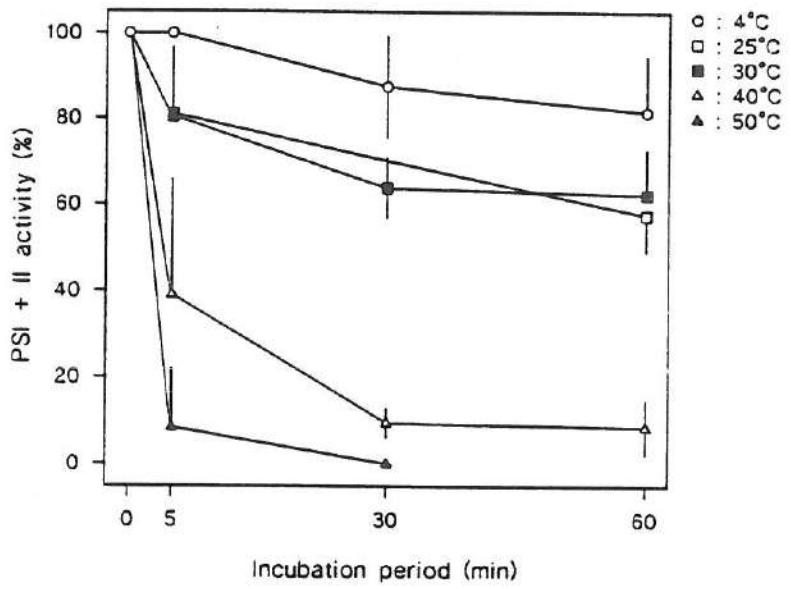


Figure 4.1: The capacity of the photosynthetic electron transport over PSI and PSII (water → MV) as a function of the incubation period of the thylakoid suspension at different temperatures in the dark. The data are represented as percentage of the original activity, before incubation (mean ± standard error).

This does not agree with the temperature response of the electron transport capacity mediated by PSI alone. Heat stress had an inhibitory effect on the activity of PSI when measured with $DADH_2$ as electron donor. In darkness a small inhibition was observed from 25°C on (fig. 4.2-a). At 40°C the reduction in PSI activity became more pronounced. After 1 h the activity had diminshed by 40%. At 50°C the decline in activity was already statistically significant after 30 min (30% loss), in comparison to the sample incubated at 4°C. The loss in PSI activity by heat treatment never reached the level observed for the whole chain electron transfer. The inactivation process was slower and based on these results no clear distinction between the different temperatures could be made.

With $DCPIPH_2$ the changes in PSI activity by heat were completely different (fig. 4.2-b). At low temperatures (4 - 25°C) the activity of PSI was hardly affected, while at 40°C the oxygen uptake was markedly enhanced. At 50°C a significant and maximal increase was established within a few minutes and it was maintained over the period observed.

With a weak illumination (5 μmol quanta $m^{-2}s^{-1}$) the activity measured with $DADH_2$ was slightly stabilized during incubation at 25°C (fig. 4.3-a). At 40 and 50°C the inhibition was a little enhanced by light in comparison to the dark incubated samples (fig. 4.3-a vs. fig. 4.2-a).

Similarly, when measured with $DCPIPH_2$ (fig. 4.3-b) a mild stimulation in activity by low light was found at 25°C. At 50°C, after a strong initial rise, the activity slowly decreased throughout time.

Thus, the changes that were registered in PSI activity upon heat incubation of thylakoid suspensions were strongly dependent on the artificial electron donor that was employed. A low photon flux density during the incubation had a moderate effect, either positive or negative depending on the incubation temperature. These facts will be discussed further (see 4.6 Discussion).

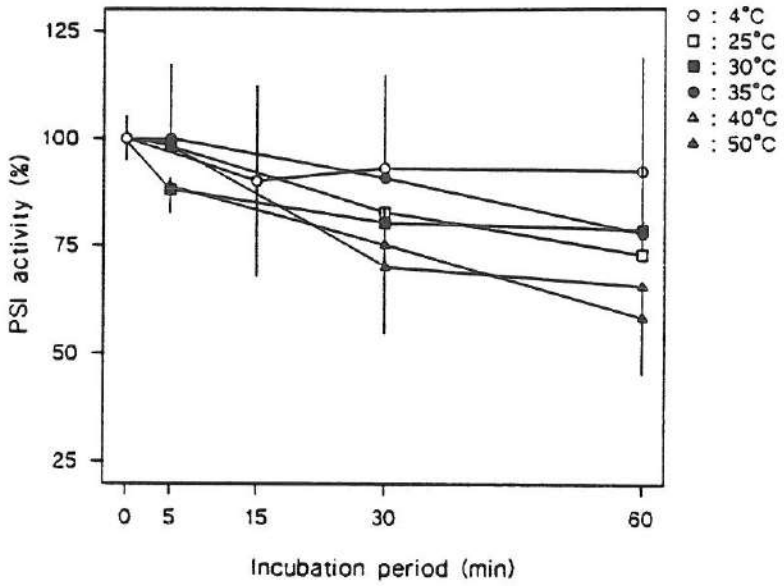


Figure 4.2-a: Evolution of the electron transport capacity of PSI ($DADH_2 \rightarrow MV$) of thylakoid membranes incubated in darkness at different temperatures as a function of the incubation period.

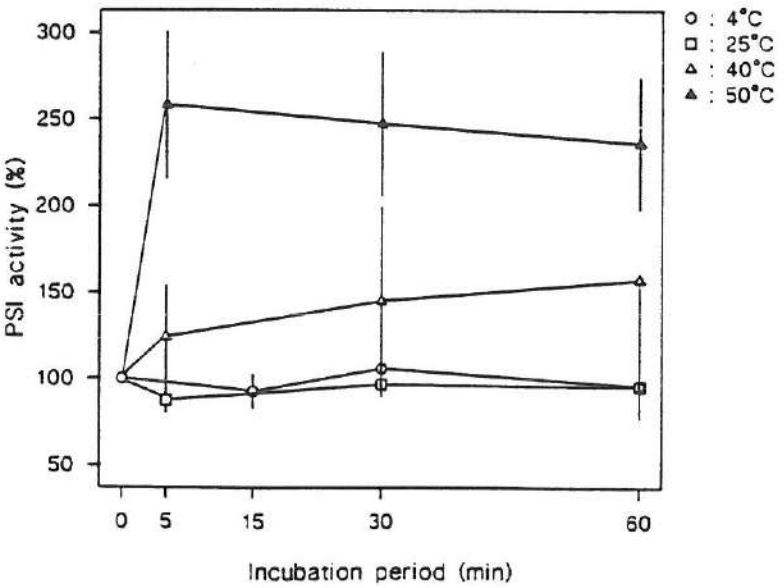


Figure 4.2-b: The same as in figure 4.2-a, but with $DCPIPH_2$ as electron donor.

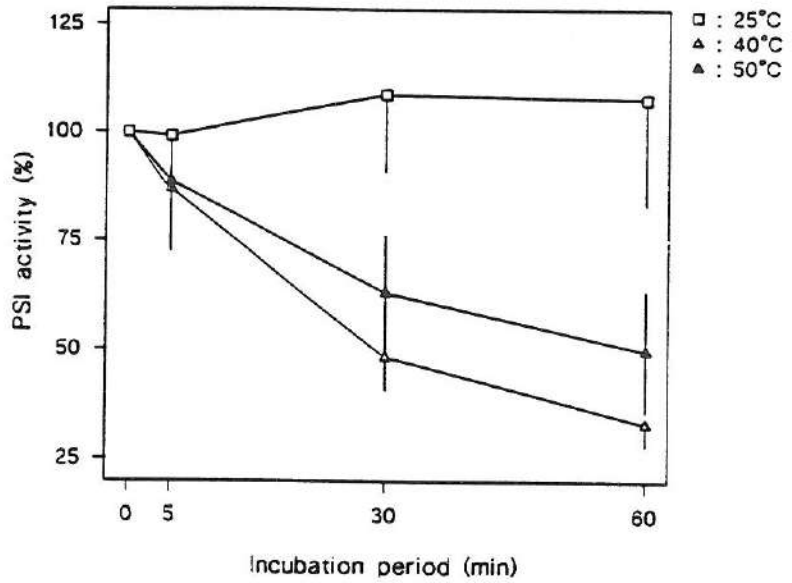


Figure 4.3-a: The same as in figure 4.2-a, but with thylakoid suspensions incubated at a low photon flux density (5 μmol quanta $m^{-2}s^{-1}$).

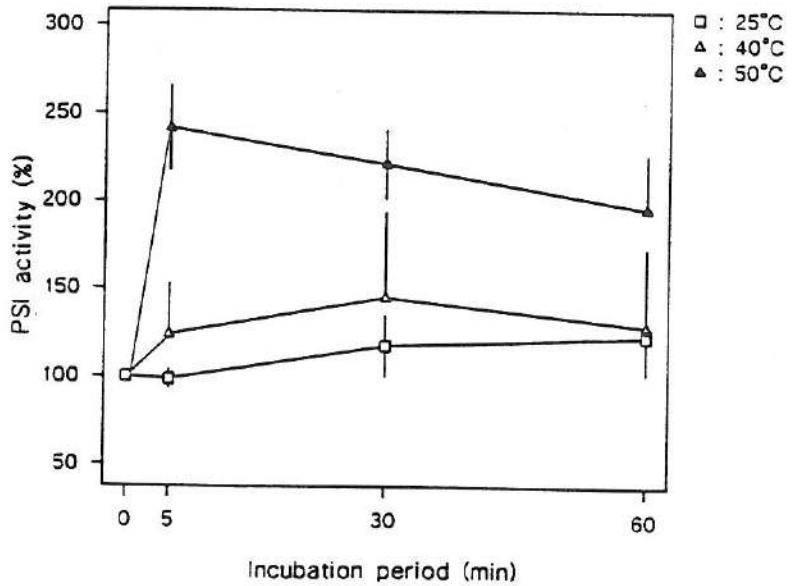


Figure 4.3-b: The same as in figure 4.2-b, but with thylakoids incubated at a low photon flux density (5 μmol quanta $m^{-2}s^{-1}$). Compare also with figure 4.3-a; but the electron donor used here, was $DCPIPH_2$.

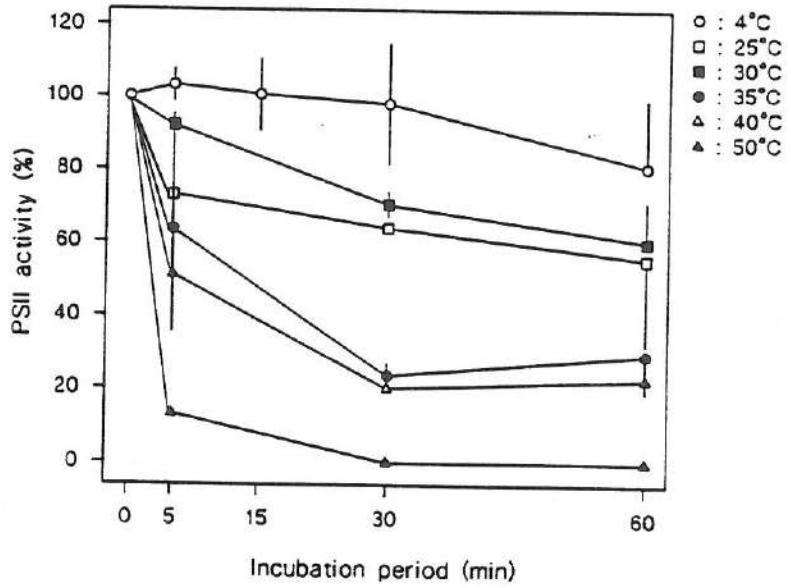


Figure 4.4: The electron transport capacity of PSII (water → DAD/ferricyanide) of thylakoid membranes incubated in darkness at different temperatures as a function of the incubation period.

In figure 4.4 the capacity of the PSII mediated electron transport (from water to DAD/ferricyanide) can be seen after incubation of the thylakoid suspension in darkness at the temperatures indicated. The response of PSII reflects that of the whole chain electron transport (see fig. 4.1) fairly well. A detailed analysis of the data of PSII activity (fig. 4.4) revealed the distinction between 4 different patterns of temperature response. The first one was represented by the curve at 4°C: the oxygen evolution hardly changed during incubation of the thylakoid sample in darkness; only after 1 h a small but significant decrease was observed.

The second pattern was defined by the curves at 25 and 30°C. After 5 min the activity was inhibited to 90 or 70% of the initial value. The decline continued steadily, leading to a significant reduction (40%) in PSII activity after 1 h, compared to the same period of incubation at 4°C.

A third pattern of evolution was observed at 35 - 40°C. The activity decreased more rapidly: after 5 min it was already significantly different from the value measured at 4°C and after 30 min dark incubation only about 20% of the original activity was left. The activity did not decrease any further in the following half hour.

The fourth pattern was here represented by the incubation at 50°C. This

temperature caused an instantaneous drop in PSII activity. After 30 min no remaining activity was measured.
These four patterns that were distinguished as a function of temperature suggest that at least three temperature dependent effects were responsible for the inactivation of PSII observed upon heat treatment in the dark, to wit one for the transition from the pattern at 4°C to that at 25 - 30°C, a second for the transition further to the 35 - 40°C pattern and a third one for the shift to the 50°C inactivation pattern. The putative biochemical nature of the changes that may account for these transitions will be resumed in the discussion (see 4.6 Discussion).

When the incubation at 25°C did not take place in complete darkness, but in a test-tube under weak illumination (5 μmol quanta $m^{-2}s^{-1}$; fig. 4.5) oxygen evolution remained constant throughout the 1 h incubation period. In contrast, at 40°C the inhibition was enhanced by the weak light, leading to a complete inhibition after 30 min. At a photon flux density of 50 μmol quanta $m^{-2}s^{-1}$ the same course was observed (incubation in test-tube - fig. 4.6): no reduction in activity at 25°C and 100% inhibition at 40°C within 30 min.
In an open beaker however a significant inhibition was measured after 30 min incubation at 25°C. This difference was likely an effect of oxygen. In the latter case the interface air-thylakoid suspension was much larger. Oxygen has been shown to take part in the conversion from reversibly inactivated PSII reaction centres to non functional ones (Schnettger *et al.*, 1992; Aro *et al.*, 1993). Under conditions of acceptor side photoinhibition overreduction of the primary electron acceptor Q_A increases the probability for the formation of the triplet state of P680 by a recombination of $P680^+Pheo^-$. This unstable state can be transmitted to oxygen with the formation of the highly reactive singlet oxygen (Barber and De Las Rivas, 1993), which can further lead to membrane damage by lipid peroxidation (Takahama and Nishimura, 1975). At 4°C in a beaker, the weak light also caused a significant reduction of the activity after 1 h, supporting the hypothesis of an oxygen effect. However, Šetlík *et al.* (1990) and Kirilovsky *et al.* (1994) have shown that also complete anaerobic conditions induce an additional fast process of photoinactivation.

The PSII electron transport activity was further analyzed when the oxygen evolving complex (o.e.c) was inhibited with NH_2OH. Figures 4.7-a and -b show that incubation at 25°C did not cause any change in the electron transport from DPC to DCPIP over a period of 1 h, neither in the dark, nor under weak light (5 μmol quanta $m^{-2}s^{-1}$). This indicates that the light induced changes in the electron transfer

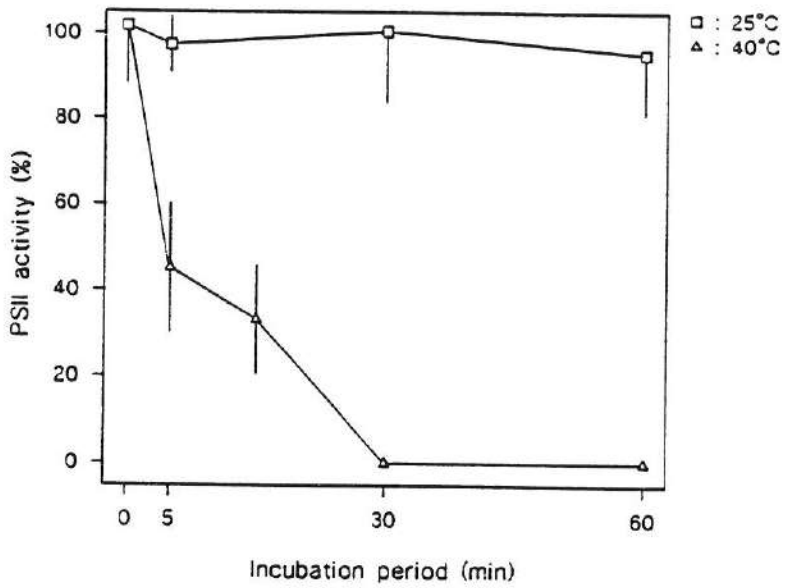


Figure 4.5: The effect of a low photon flux density (5 μmol quanta $m^{-2}s^{-1}$) during the incubation of thylakoid suspensions at 25°C and 40°C on the electron transport capacity of PSII (water → DAD/ ferricyanide).

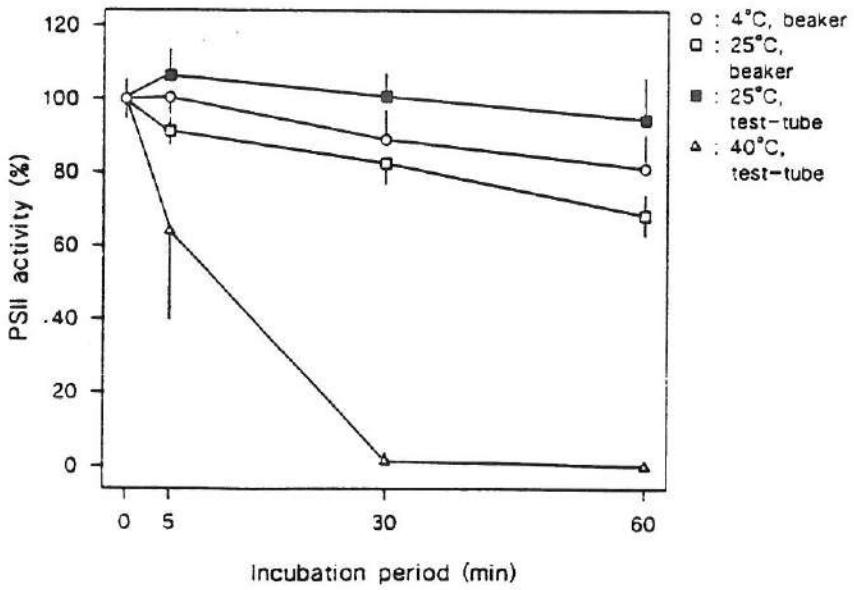


Figure 4.6: The same as in figure 4.5, but with an incubation photon flux density of 50 μmol quanta $m^{-2}s^{-1}$. The incubation at 4°C and 25°C (open symbol) took place in a beaker and at 25°C (closed symbol) and 40°C in a test-tube.

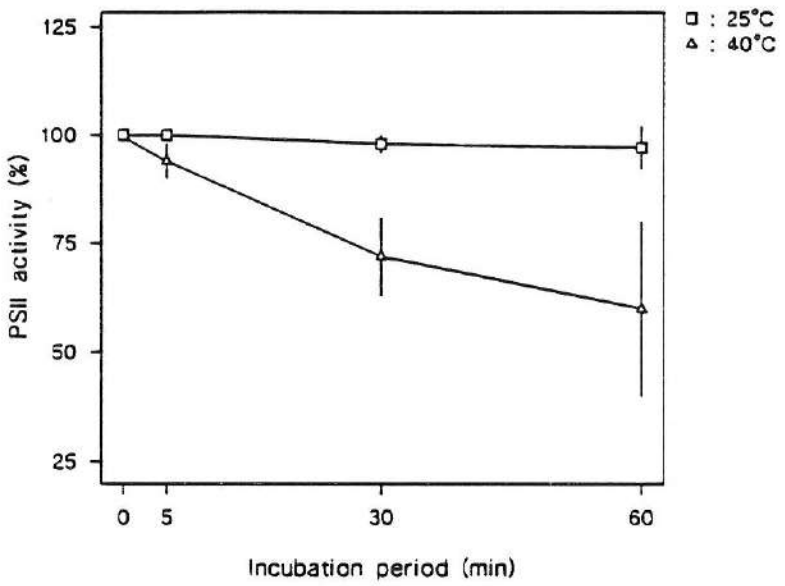


Figure 4.7-a: The effect of dark incubation of thylakoids at 25 °C and 40 °C on the PSII activity, measured spectrophotometrically (DPC → DCPIP).

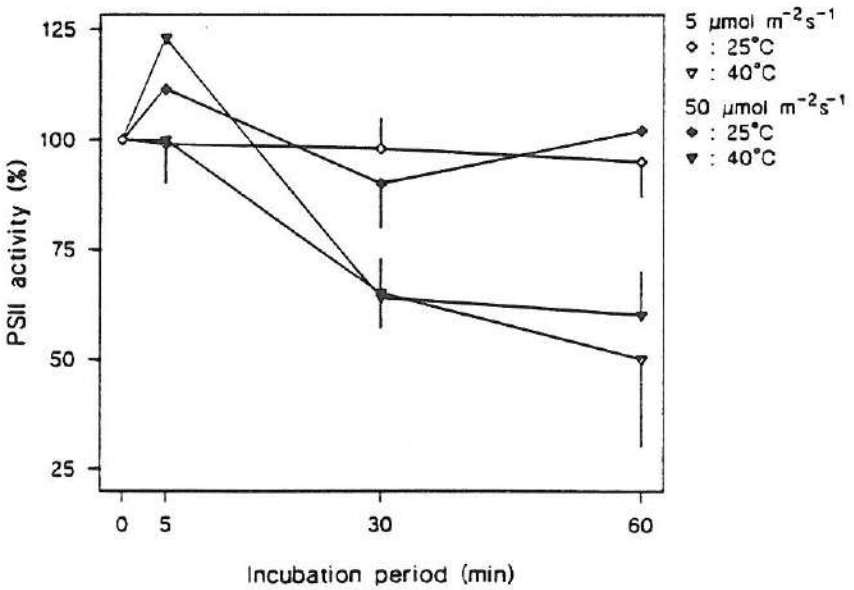


Figure 4.7-b: The same as in figure 4.7-a, but the incubation of the thylakoid samples was performed under weak irradiance of 5 μmol quanta $m^{-2}s^{-1}$ (open symbols) or 50 μmol quanta $m^{-2}s^{-1}$ (closed symbols).

reaction from water to DAD/ferricyanide (fig. 4.3 vs. fig. 4.2) are localized at the o.e.c. In darkness the oxygen evolving capacity was partially lost, apparently by inactivation of the o.e.c., which was overcome with a photon flux density of 5 μmol quanta $m^{-2}s^{-1}$. This is in agreement with the finding that light is required for the reactivation of the o.e.c. upon Mn-depletion. Photo-oxidation of Mn^{2+} to $Mn^{\geq 3+}$ appears to be necessary for its ligation into an active water oxidizing centre (Tamura and Cheniae, 1988). Yamashita *et al.* (1990 a and b) reported that an electron transport reaction from the added reductant dithiothreitol under aerobic conditions maintained by the weak light stimulates the Mn incorporation. They suggested that the photo-oxidation/reduction cycle of Mn^{2+}/Mn^{3+} mediated this electron transport. Ono and Inoue (1991) hypothesized that under weak light a positive charge accumulates at the donor side of PSII, first on the tyrosine-160 of D1 (*i.e.* the secondary donor Z) and subsequently on a histidine residue. This can oxidize exogenous Mn^{2+} after which assembly of the Mn-cluster can take place. The oxidized form of histidine is though unstable, and degradation or inactivation will occur unless it is rereduced quickly. In this respect the positive intervention of dithiotreitol may be considered. At a photon flux density of 50 μmol quanta $m^{-2}s^{-1}$ in a wide test-tube the PSII reaction centre activity was not affected neither (fig. 4.7-b). We did not perform the experiment in an open beaker to test the hypothesis of the enhanced acceptor side inhibition due to the excess of oxygen.

At 40°C there was a significant inhibition of the PSII reaction centre activity, but it was only a fraction of the inhibition observed with an active o.e.c. and it only started after more than 5 min. Comparing figure 4.7-a with figure 4.4, this shows that the fast component of the inhibition during the first 5 min at 40°C can be attributed to changes at the o.e.c. The longer term effect could be the result of inhibition towards the acceptor side of PSII. The effects at the acceptor side were not light dependent (fig. 4.7-a vs. fig. 4.7-b). This implies that at 40°C the acceleration of the degradation of the PSII-activity ($H_2O \rightarrow$ DAD/ferricyanide) by light should be localized at the water oxidizing donor side of PSII. Up to now, this light effect can not be explained.

It can be summarized that the inhibition of the PSII activity at 25°C in darkness is due to damage at the donor side, more in particular at the o.e.c. Low irradiance overcomes this process. The PSII complex is stabilized by weak light, as long as the acceptor side impairment is not enhanced, e.g. by high oxygen concentration or by increased temperature.

4.2 Influence of the presence of a PSII electron acceptor during heat treatment:

A distinction between acceptor-side and donor-side limiting conditions for electron transport is being made at present. Both situations have been discerned leading to photoinhibitory damage (Aro *et al.*, 1993; Barber and Andersson, 1992). The results already described (see 4.1.2) point out a rapid impairment of the oxygen evolving donor side during incubation. Enhancement of this donor side depletion is expected to accelerate the inhibition of the PSII electron transfer reactions. To test this hypothesis oxidizing conditions were created by addition of electron acceptors during the incubation.

4.2.1 Experimental approach:

Thylakoid membranes were isolated by the short procedure (see 4.1.1; chlorophyll concentration of 0.26 $\pm$ 0.05 mg/ml) and incubated at 25°C , either or not in the presence of the electron acceptor couple DAD (1mM) - ferricyanide (24mM). After incubation, a sample was diluted 20-fold in the assay medium. As such, the final concentration of the artificial electron acceptor corresponded with that in previous experiments (cf. table 2.1) and the oxygen evolving capacity could be measured instantaneously. Light (50 μmol quanta $m^{-2}s^{-1}$) and dark incubation conditions were compared over a period of 30 min.

4.2.2 Results:

At 25°C in the absence of artificial electron acceptors, the dark accelerated inhibition of PSII mediated oxygen evolution was not repeated (compare fig. 4.8 (closed circle) with fig. 4.4 (open square)). Hence, we could not consolidate the light stabilizing effect. It is even though clear that the oxidizing conditions are destructive (fig. 4.8). In darkness the oxygen evolving capacity was highly reduced by the presence of the electron acceptors. Dim light reinforced the breakdown. This might be an effect induced by the light driven photosynthetic electron transport. However, bleaching of chlorophyll brought about by ferricyanide can also be part of an underlying mechanism (Bendall *et al.*, 1971).

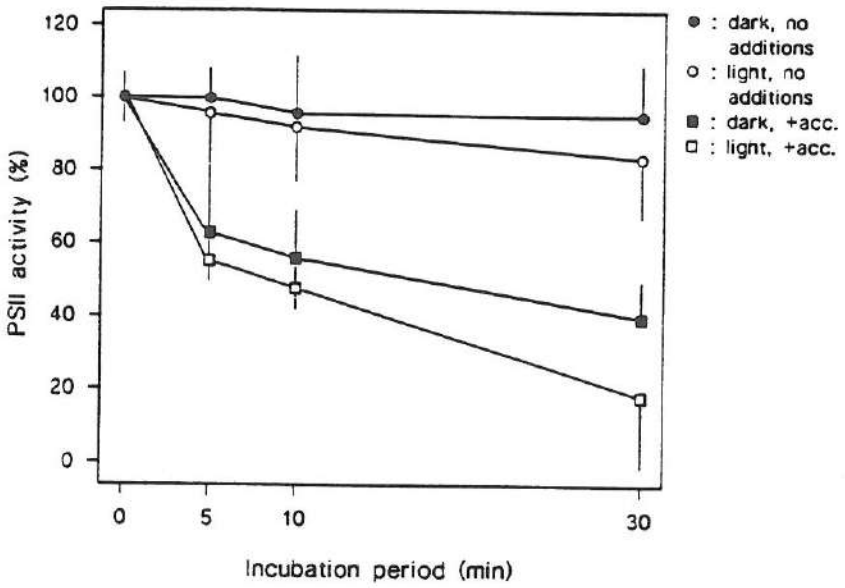


Figure 4.8: The effect of the addition of the electron acceptors DAD and ferricyanide during light (50 μmoles $m^{-2}s^{-1}$; open symbols) or dark (closed symbols) incubation of thylakoid membranes at 25°C, on the electron transport capacity of PSII (water → DAD/ferricyanide).

4.3 The electron transport capacity of heat treated intact leaves:

The effects of heat shock on the PSII activity in intact tissues and in isolated chloroplasts show some similarity (Guillot-Salomon *et al.*, 1991; Santarius, 1980), although heat tolerance *in vivo* is higher than *in vitro* (Krause and Santarius, 1975; Santarius and Müller, 1979). The same heat treatment is therefore expected to cause less inactivation in leaves than in isolated chloroplasts. This was controlled in the following experiments.

4.3.1 Experimental approach:

For an efficient control of the incubation temperature, detached leaves were submerged in water of 25°C and 40°C, respectively, at various photon flux densities. White light was provided by a slide projector equiped with neutral density filters. After an incubation period of 5, 30 or 60 min, thylakoids were isolated from

the illuminated leaf area by the short procedure described above (see 4.1.1). Because of the small leaf area used in each isolation, the chlorophyll content was only 0.19 ± 0.07 mg/ml. Therefore the membrane suspension was diluted 10-fold (in stead of 20-fold) in the assay for the PSII activity. This was measured polarographically with DAD and ferricyanide as electron acceptors (see 2.3.2.2a; table 2.1).

4.3.2 Results:

Incubation of detached leaves at 25°C hardly affected the oxygen evolving capacity at the photon flux densities applied in previous experiments (0 - 50 μmol quanta $m^{-2}s^{-1}$) (fig. 4.9 a-c). At higher irradiance, light appeared to exert a stabilizing effect. At 1500 μmol quanta $m^{-2}s^{-1}$, however, this stabilization vanished and light inhibition was observed. This is accentuated by using the logaritmic scale for the X-axis (irradiance) in the figures 4.9 (a-c).

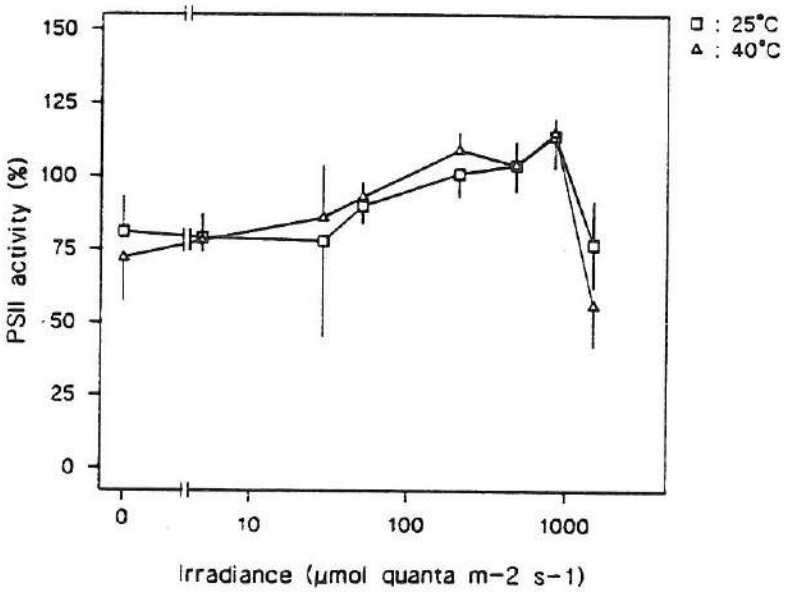


Figure 4.9-a: The electron transport capacity of PSII (water → DAD/ferricyanide) in leaves incubated for 5 min at 25°C or 40°C at various photon flux densities, ranging from 0 to 1500 μmol quanta $m^{-2}s^{-1}$. The electron transport rate measured on a freshly prepared thylakoid suspension of a non-incubated leaf equals 100%.

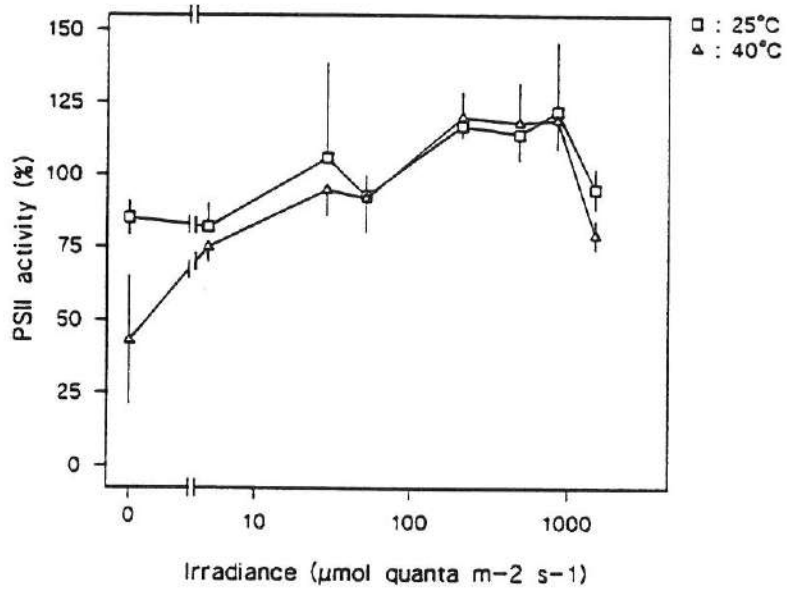


Figure 4.9-b: The same as in figure 4.9-a, after 30 min of incubation.

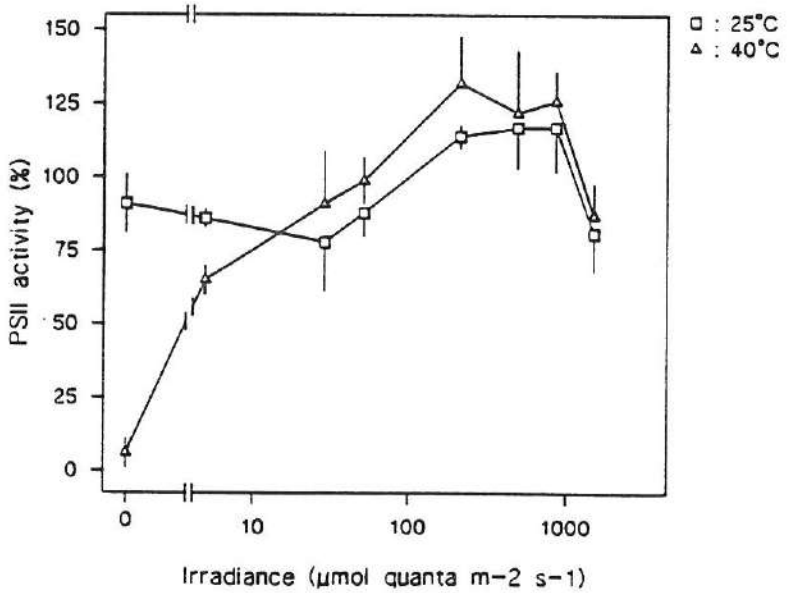


Figure 4.9-c: The same as in figure 4.9-a, after 1 h of incubation.

At 40°C a strong inhibition was observed in darkness (zero-point in fig. 4.9-b and c) as a function of incubation time; it resulted in a more pronounced loss of the oxygen evolving capacity than for thylakoids (see fig. 4.4). Low light fluence rates were found to be stabilizing at 40°C as well. This effect was originally seen at 25°C in isolated thylakoids. It is now confirmed in detached leaves at 25°C and at 40°C. The reason can be different since in leaves the D1 repair cycle must be considered as well (Mattoo *et al.*, 1984; Ohad *et al.*, 1984; Adir *et al.*, 1990). This process requires protein synthesis, which is disconnected in chloroplast subfractions.

Applied at 40°C, the highest photon flux density seemed to induce a slightly more pronounced photoinhibition than at 25°C. The reduction in oxygen evolving capacity depended on the duration of light exposure: short exposure (fig. 4.9-a) was more inhibitory than illumination during 1 h (fig. 4.9-c).

4.4 The electron transport capacity of PSII after heat treatment of intact plants:

4.4.1 Experimental approach:

The impact of heat treatment, as ultimately applied to the transgenic HSIPT plants (see Chapter 5), was evaluated. Intact plants were placed in a temperature controlled chamber in which the air temperature was raised from 25°C to 40°C in 15 min (see 2.1.2.5). After 30 and 60 min of exposure to 40°C or after the same period at control temperature (25°C), thylakoid suspensions were rapidly isolated (see 4.1.1) and PSII activity was determined (see 2.3.2.2a; table 2.1). The average chlorophyll concentration of the thylakoid samples was 0.4 $\pm$ 0.2 mg/ml
During the heat treatment three different light conditions were tested: darkness, 50 μmol quanta $m^{-2}s^{-1}$ and c. 2000 μmol quanta $m^{-2}s^{-1}$.

4.4.2 Results:

Under the light conditions applied heat treatment of intact plants did not alter the electron transport capacity of PSII. In contrast to the loss of oxygen evolving capacity observed in detached leaves (see figs. 4-9 a-c), exposure to 40°C, neither in darkness (fig. 4.10-a) nor in high light (fig. 4.10-b - closed symbols), resulted in any effect.

The conditions for treatment of detached and attached leaves are rather different. Submersion of the leaves brings them under conditions of low oxygen partial pressure (p_{O_2}). An inhibitory effect on PSII of this condition cannot be

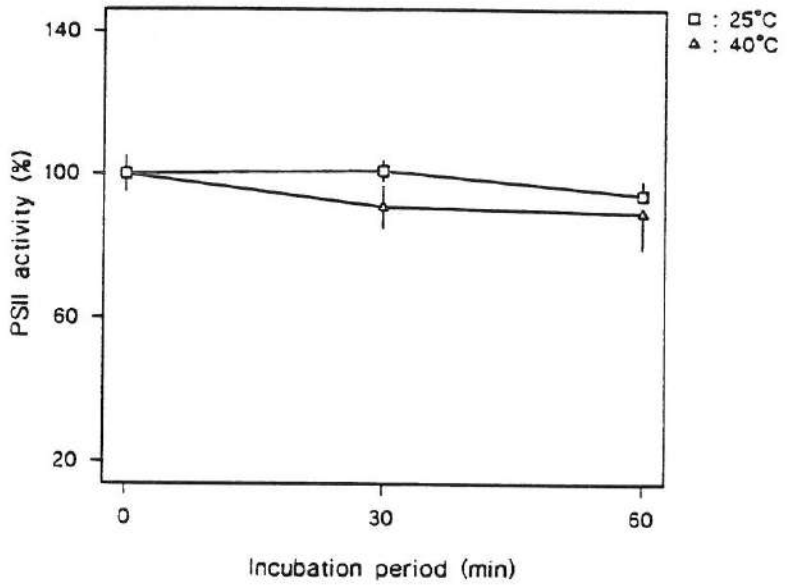


Figure 4.10-a: The effect on the electron transport capacity of PSII (water → DAD/ferricyanide) after treatment of intact plants in a heat shock chamber at 25°C or at 40°C in darkness.

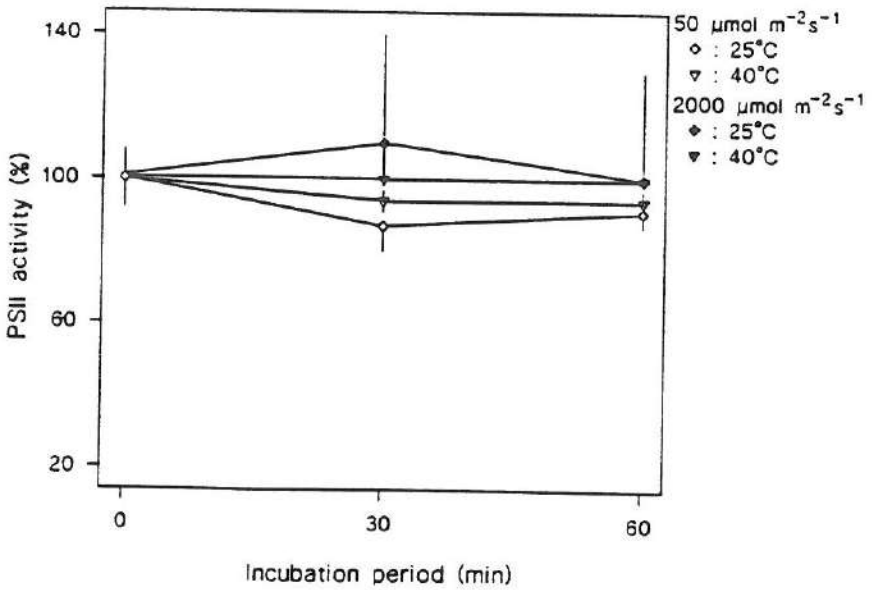


Figure 4.10-b: The effect on the electron transport capacity of PSII (water → DAD/ferricyanide) of light incubation (50 μmol quanta $m^{-2}s^{-1}$ = open symbols; 2000 μmol quanta $m^{-2}s^{-1}$ = closed symbols) of intact plants in a heat shock chamber at 25°C or at 40°C.

excluded, since an additional fast phase ($t_{1/2}$ of 4 - 12 min) of photoinactivation has been characterized under complete anaerobiosis *in vitro* (on isolated thylakoids) (Šetlík *et al.*, 1990; Kirilovsky *et al.*, 1994). It has been ascribed to the formation of a stabilized reduced state of Q_A, *i.e.* Q_A^-, and its protonated form. This phase is absent in the presence of oxygen, but oxygen and its radical forms may enhance another slow photoinactivation process, or may induce a separate pathway of photoinactivation.
The way of heat transmission is also different for the two treatments. Submerged in a waterbath, temperature is closely controlled. Intact plants are exposed to heated air. By this way, heat transmission to the leaf interior is much slower: under weak irradiance the leaf surface temperature did not exceed 35°C for an air temperature of 40°C (see chapter 5). The temperature rise may have been too low to induce a noticeable inactivation of the PSII mediated electron transport *in vivo*.

4.5 Photosynthetic activity of leaf discs after heat treatment of the intact plant:

So far, *in vitro* partial reactions of the electron transport chain have been measured. In this section, the influence of heat treatment on photosynthesis *in vivo* will be evaluated in terms of oxygen evolution on intact leaf tissues.

4.5.1 Experimental approach:

The heat treatment was the same as in 4.4.1. Immediately after the heat treatment (15 min warming up, 1 h 40°C) at a photon flux density of 50 μmol quanta $m^{-2}s^{-1}$, the plants were placed back in the growth chamber. Leaf discs (10 cm^2) were collected before and immediately after the treatment, and further at various intervals during recovery of the plants in the growth chamber. For comparison, plants that were not removed from the growth chamber, were sampled at corresponding times.
Oxygen evolution was measured with a leaf disc electrode at 20°C (see 2.3.3).

4.5.2 Data collected:

The results were obtained by measuring light response curves as explained in Materials and Methods (see 2.3.3). Several characteristics of these curves, schematically depicted in figure 4.11 were calculated according to Walker (1990).

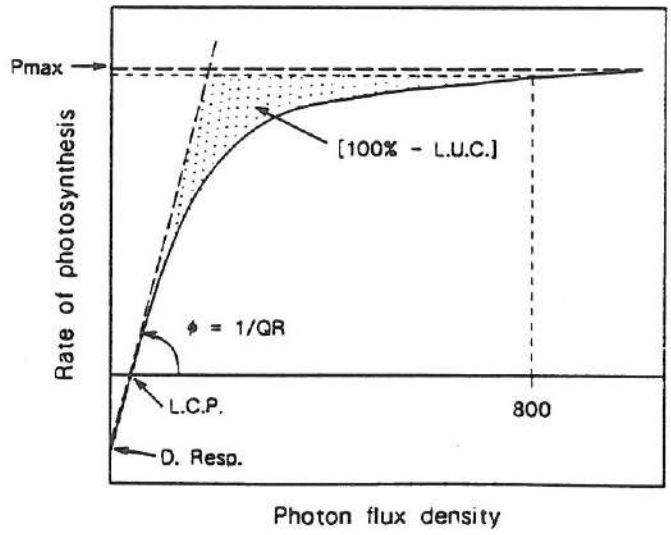


Figure 4.11: Schematic representation of the light response curve (D.Resp. = dark respiration; L.C.P. = light compensation point; L.U.C. = light utilization capacity; QR = quantum requirement; ϕ = quantum yield).

1) The quantum requirement (QR) is the reciprocal of the quantum yield (Φ), which corresponds to the initial slope of the light response curve. The apparent quantum requirement, not corrected for the actual absorbtance of the leaf, was estimated from the low intensity range measurement (the third part of the whole measuring sequence, see 2.3.3).

2) Pmax is the photosynthetic rate at light saturation. It was determined as the maximal rate of oxygen evolution in the curve spanning the wide photon flux density range (part two of the described procedure).

3) Dark respiration (D. Resp.) and the light compensation point (L.C.P.) are the intercept of the Y-axis (rate of photosynthesis) and of the X-axis (photon flux density), respectively. Photorespiration was suppressed in these measurements by providing the leaves with a sufficiently high CO_2-supply.

4) The light utilization capacity (L.U.C.) is defined as the area under the light response curve as a percentage of the area bounded by the initial slope (quantum yield) and a horizontal ceiling corresponding to the photosynthetic rate at 800 μmol quanta $m^{-2}s^{-1}$.

5) The convexity coefficient (Θ) is derived from the equation describing a non-rectangular hyperbola (Terashima and Takenaka, 1986; Ögren, 1993):

$$\Theta P^2 - (\Phi \cdot I + Pmax) \cdot P + \Phi \cdot I \cdot Pmax = 0$$

$$\text{and thus, } \Theta = ((\Phi \cdot I + Pmax) \cdot P - \Phi \cdot I \cdot Pmax) / P^2 ,$$

where Φ is the quantum yield and P the rate of photosynthesis at irradiance I.

A model of Farquhar and co-workers resolves the photosynthetic CO_2 assimilation being limited at low light by the rate of electron transport, at high light by the capacity of RubisCO; at the transition between these two limitations photosynthetic efficiency is determined by Θ (Ögren, 1993). This coefficient is considered to depend to a certain extent on the leaf structure and to the corresponding light gradient within the leaf. A gradient in Pmax (related to the concentration of RubisCO in the leaf) is supposed to develop in accordance to this light gradient (Terashima and Saeki, 1985). The more mismatch between these two gradients, the lower Θ. Changes in the convexity coefficient should be reflected in the light utilization capacity. This capacity, however, includes the level of dark respiration (Walker, 1989) and incorporates a more complex regulation mechanism in response to several factors e.g. stress.

4.5.3 Results:

In table 4.1 these photosynthetic characteristics are presented as measured during the day. The apparent quantum requirement (QR) was rather high in the control plants. It was still comparable with the values recorded by Björkman and Demmig (1987), based on the incident light and not corrected for the leaf absorptance. QR did not change markedly throughout the day in the plants staying in the growth chamber. The maximal photosynthetic activity, Pmax, significantly reduced during the day. Dark respiration and the L.C.P. were unaffected by daytime. There was a minor increase in L.U.C., although significant, in parallel to the decrease in Pmax. The convexity of the curve (Θ) showed the same trend, but this increase was not significant. The shape of the light compensation curves was rather constant. Nevertheless, the decrease in Pmax in particular, demonstrates that it is important when comparing data from the photosynthetic activity *in vivo*, to do this at corresponding times. Otherwise, intrinsic day rhythms may have implications for the interpretation.

After heat shock at 40°C under low light conditions, Pmax decreased and recovered afterwards (table 4.2). Later on, a decline during the day was not evident (in contrast with an earlier report of Valcke and Van Loven, 1992). No effect on QR, dark respiration or L.C.P. were registered. The day response of the L.U.C. and Θ was completely absent. With the exception of Pmax, the features of the light response curves after heat treatment were still in accordance with the control at corresponding times.

Table 4.1: Evolution during the day, of the parameters from the light response curve of plants in the growth chamber at 25°C. Data that are marked with the same character, are not significantly different (α = 0.05; ANOVA and Fisher's LSD test; mean ± standard error).
QR = apparent quantum requirement; Pmax = maximal photosynthetic rate (μmol O_2 $m^{-2}s^{-1}$); D.Resp. = dark respiration (μmol O_2 consumption $m^{-2}s^{-1}$); L.C.P. = light compensation point (μmol quanta $m^{-2}s^{-1}$); L.U.C. = light utilization capacity (%); Θ = convexity coefficient.

time	QR	Pmax	D.Resp.	L.C.P.	L.U.C.	Θ
9.45 AM	10.9 ± 0.9 a	14 ± 2 b	1.9 ± 0.3 a	20 ± 3 a	91 ± 1 a	0.66 ± 0.08 a
11 AM	11 ± 2 a	13 ± 2 b	1.7 ± 0.3 a	19 ± 3 a	92 ± 2 ab	0.7 ± 0.1 a
12 AM	11.2 ± 1.0 a	12 ± 2 b	1.8 ± 0.3 a	20 ± 3 a	93 ± 3 b	0.7 ± 0.2 a
1 PM	11 ± 1 a	12 ± 2 ab	1.8 ± 0.3 a	20 ± 3 a	93 ± 2 b	0.7 ± 0.1 a
3 PM	12 ± 1 a	11 ± 1 a	1.8 ± 0.5 a	21 ± 6 a	93 ± 2 b	0.75 ± 0.04 a

Table 4.2: Photosynthetic characteristics of plants placed in the heat shock chamber at a photon flux density of 50 μmol quanta $m^{-2}s^{-1}$, warming up from 9.45 AM untill 10 AM, followed by 1 h at 40°C. Subsequent recovery in the growth chamber was recorded. Abbreviations see table 4.1. Data that are not significantly different from those in table 4.1 at corresponding times are marked with the letter 'a'.

time	QR	Pmax	D.Resp.	L.C.P.	L.U.C.	Θ
11 AM	10.9 ± 0.4 a	10.9 ± 0.8 b	1.9 ± 0.2 a	20 ± 2 a	94 ± 2 a	0.71 ± 0.07 a
12 AM	11.2 ± 0.5 a	12 ± 1 a	1.9 ± 0.1 a	21 ± 2 a	94 ± 3 a	0.73 ± 0.10 a
1 PM	11.1 ± 0.7 a	11 ± 2 a	2.0 ± 0.3 a	22 ± 4 a	94 ± 3 a	0.71 ± 0.09 a
3 PM	11.4 ± 0.8 a	11 ± 2 a	1.9 ± 0.5 a	21 ± 6 a	92 ± 2 a	0.7 ± 0.1 a
11 AM(*)	10.8 ± 0.4 a	12 ± 4 a	2.0 ± 0.2 a	22 ± 3 a	93 ± 3 a	0.71 ± 0.06 a

(*) after 24 h recovery in the growth chamber.

4.6 Discussion:

PSII is generally considered to be a most sensitive complex, particularly susceptible to heat stress. This was confirmed by the experiments described. However, also pronounced effects of heat on the PSI activity were shown.

Lajkó *et al.* (1991) have demonstrated spectrophotometrically that the PSI mediated photo-oxidation of $DCPIPH_2$ is not heat sensitive. Therefore, they concluded that the PSI activity *sensu stricto* is not affected by heat shock. Nevertheless, in this study, heat treatment appeared to be either inhibitory (figs. 4.2-a and 4.3-a) or stimulatory (figs. 4.2-b and 4.3-b) for PSI (measured polarographically), depending on the reductant utilized. This is consistent with several other reports (Thomas *et al.*, 1986; Boucher *et al.*, 1990; Boucher and Carpentier, 1993). Different interpretations have been put forward to explain this increased oxygen uptake. The heat induced dissociation of the LHCII complex from the core complex of PSII and membrane alterations, such as increases in lateral mobility and destacking-like phenomena, have been argued to enlarge the cross-section of PSI absorption and to enhance spillover (Velitchkova *et al.*, 1989; Ivanov and Velitchkova, 1990). This hypothesis is supported by the increases in F_o and in the ratio F_{735}/F_{685}, as these authors reported. However, it contradicted with the results of Boucher *et al.* (1990), showing that heat treatment of PSI submembrane preparations results in the same stimulation of oxygen uptake. The latter authors confirmed the results of Thomas *et al.* (1986), demonstrating that the affinity of the cytochrome b_6/f complex for DCPIP increases upon heat treatment and considered this as the site for heat stimulation of the PSI activity. A conformational modification of the lipid phase at the vicinity of the cytochrome b_6/f complex or a change at the cytochrome b_6/f complex itself could increase the accessibility of $DCPIPH_2$ to existing or to new oxidation sites. The absence of stimulation by TMPD (Thomas *et al.*, 1986) could be explained by a difference in electron donation site, assuming that only $DCPIPH_2$ gets access to the newly exposed oxidation sites which preceed the site for TMPD.

Więckowski and Fiedor (1990) showed that NADPH enhanced the oxygen uptake after heat treatment of chloroplasts. They suggested that this process is possibly catalyzed by the ferredoxin-NADP reductase (FNR), as a reverse electron flow from NADPH. In heat treated leaf discs oxygen uptake has also been observed. Havaux *et al.* (1987) attributed this to the Mehler reaction in which oxygen is reduced as terminal acceptor of the electron transfer chain. They argued that an internal electron donor is implied. As the Mehler reaction with O_2 is promoted

under conditions leading to a high NADPH/$NADP^+$-ratio, NADPH might be regarded as this electron source, fitting in with the postulated 'ferredoxin-$NADP^+$ reductase catalyzed' hypothesis.
On the other hand Lajkó *et al.* (1991) noticed that the reduction of NADPH is diminished by high temperature. A gradual release of the membrane bound FNR was considered to be responsible for this effect. Nevertheless, Więckowski and Fiedor (1990) showed that the FNR-activity is highly heat resistant.

Lajkó and co-authors (1991) first postulated the role of SOD. Oxygen is a product of the reaction catalyzed by SOD (reaction 3, see below). The observed enhancement of the oxygen uptake can thus be caused either by an increased Mehler reaction with MV (reaction 2), or by a diminished O_2-production in the SOD-catalyzed reaction (reaction 3). Lajkó *et al.* (1991) showed spectrophotometrically that the redox-activity of PSI is not affected. Accordingly, they held a loss in SOD activity responsible for the net increase in oxygen uptake. It was suggested to result from the dissociation of the enzyme from the thylakoid membrane.

$$2\ MV^{2+} + 2\ e^- \rightarrow 2\ MV^+ \qquad (1)$$
[reduction of MV by photosynthetic electron transport]

$$2\ MV^+ + 2\ O_2 \rightarrow 2\ MV^{2+} + 2\ O_2^- \qquad (2)$$
[Mehler reaction]

$$2\ O_2^- + 2\ H^+ \rightarrow H_2O_2 + O_2 \qquad (3)$$
[SOD catalyzed]

Alternatively, Boucher and Carpentier (1993) proposed that superoxide can be directly reduced by electron donors like $DCPIPH_2$ and duroquinol or eventually by ascorbate (reactions 4 and 5).

$$2\ O_2^- + DH_2 + 2\ H^+ \rightarrow D + 2\ H_2O_2 \qquad (4)$$
[DH_2 = electron donor, like $DCPIPH_2$ or duroquinol]

$$2\ O_2^- + AscH^- + 3\ H^+ \rightarrow Asc + 2\ H_2O_2 \qquad (5)$$
[Asc = ascorbate]

These reactions do not involve any oxygen production, and the substrate for the SOD-catalyzed reaction is diminished. TMPD is not a suitable candidate for the

reaction (4), since its oxidation is a monovalent reaction without proton release (Izawa, 1980). As such, this electron donor is unable to reduce O_2^-. This would explain for the difference in response to heat treatment as has been recorded with $DCPIPH_2$ or TMPD, respectively, as PSI electron donor.
However, $DADH_2$ is a two electron - two proton donor, just like $DCPIPH_2$, and the same response would be expected. Moreover, heat inactivation of SOD should also affect the measurements with TMPD, namely net oxygen consumption would be increased. This does not agree with the observations.

If, on the other hand, SOD would be heat stable, the following hypothesis could be formulated. Under normal temperature conditions, with TMPD the reactions 1, 2 and 3 are executed. TMPD was reduced by the addition of ascorbate (see Materials and Methods, table 2.1); therefore, reaction 5 must be considered as well. After heat treatment the activity measured with TMPD is not changed. Hence, these reactions (1, 2, 3 and 5) should be heat resistant. With $DCPIPH_2$ reaction 4 must be incorporated. Making the assumption that at higher temperature, the accessibility of $DCPIPH_2$ to the membrane plane increases, the contribution of reaction 4 might be enhanced. This reaction competes with SOD for the substrate superoxide. Accordingly, oxygen release by SOD is reduced and the net result is an enhanced oxygen uptake after heat treatment. In the case of $DADH_2$, the reaction of ascorbate with superoxide (reaction 5) can be disregarded, because DAD was reduced with $NaBH_4$ (see Materials and Methods, table 2.1). Because of the more lipophilic nature of $DADH_2$, one could assumed that reaction 4 is equally important before and after heat treatment. Consequently, O_2 production by the SOD reaction is not further reduced by an enhancement of the competing reactions after heat treatment.
Several reports make mention of a chloroplast associated Cu/Zn-type SOD (Burke and Oliver, 1992; Kanematsi and Asada, 1990), but after separation by gel electrophoresis it is not found to be heat stable. On the other hand, in *Nicotiana plumbaginifolia* Tsang *et al.* (1991) showed that the mRNA level of the cytosolic Cu/Zn-SOD increased rapidly during a heat shock treatment. In these plants, however, no chloroplastic Cu/Zn-SOD has been recovered. In stead, a stromal Fe-SOD was found, but its transcript level was not consistently affected by heat shock. The activity of a Mn-SOD, occuring in the mitochondria, is temperature insensitive within a range from 10 to 45°C (Burke and Oliver, 1992). Hayakawa *et al.* (1985) had found indications for the existence of a thylakoid bound Mn-SOD, but this has been questioned. Other, non-specific components could have reacted with superoxide (Palma *et al.*, 1986), or impurities from mitochondrial or microbody

(glyoxysomes, peroxisomes) fractions might have provided the Mn-SOD (Sandalio and Del Río, 1987). The knowledge of the chloroplastic SODs is still too contradictory and incomplete to support or reject the hypothesis presented above. This hypothesis better suits to explain the different heat responses recorded with the different artificial reductants, however, our data do not contain the necessary information to test its validity. Besides, the light stimulated stability of the activity at 25°C, and the light induced inhibition (measured with $DADH_2$) or diminished stimulation (with $DCPIPH_2$) at high temperatures is not explained yet, by any of the models.

Nevertheless, it can be concluded that, in contrast to PSII (see below), the reaction centre of PSI is not likely to be a primary target for heat induced damage. The contribution of the intermediate electron transfer system, linking PSII and PSI, is probably more important in the heat induced alterations that were recorded.

In further experiments the attention was focused on PSII. The decrease in antenna-size upon heat treatment, due to disconnection of the LHCII (see above), is not of great influence in the results here, since the measurements were always performed under saturating light conditions. Limitations in excitation energy transfer from the antenna to the reaction centres is than omitted.

The results obtained with isolated thylakoids, suggest three temperature transitions leading to the inactivation of PSII. The first transition between 4°C and 25°C is related to the destabilization of the o.e.c. Light prevents this process, presuming that the effects of this first transition are localized at the manganese cluster of the o.e.c. (see 4.1.2). Hence, dark incubation of thylakoid membranes at 25°C causes donor side impairment. Addition of an artificial electron acceptor, enhancing the donor side limitations (fig. 4.7 a-b), amplified the inactivation in darkness and extended it to low light conditions. Under such conditions the PQ pool becomes predominantly oxidized. Gong and Ohad (1991) proposed that a high PQ/PQH_2 ratio accelerates the degradation of the PSII core protein D1. This is related to 'irreversible' damage to PSII. Recovery from this state is only possible by resynthesis of D1. This might explain for the failure of the light stabilization in the presence of the artificial electron acceptor.

In an attempt to preserve PSII for the highly oxidized state, we applied dithiothreitol in order to create reducing conditions. But after this, the PSII activity was not measurable anymore with the polarographic method. Instead of oxygen production, its consumption was recorded (data not shown), due to an additional Mehler reaction (Allen, 1977). However, Barber (1992) did show indeed on a Western blotting that

in the presence of both an electron donor (Mn^{2+}) and acceptor (silicomolybdate), the photoinduced degradation of D1 was more or less prevented: breakdown products of the polypeptide were hardly detected.

Temperature increase to 40°C leads to a second temperature transition. This involves a rapid effect at the o.e.c. and a slower effect at the PSII core or towards the acceptor side.

Drawing a parallel, during recovery from photoinhibition two phases have been recognized: one related to the reactivation of the donor side of PSII centres and another involving synthesis of D1 and reassembly into functional PSII units (van Wijk and van Hasselt, 1993). Jegerschöld *et al.* (1990) have shown that after inactivation of the o.e.c. by Cl^- depletion the D1 protein was more sensitive to light dependent degradation. Similarly, heat shock induced inactivation of the oxygen evolution is enhanced by Cl^- depletion (Critchley and Chopra, 1988). These data support the idea that damage at D1 should be enhanced at 40°C. Taking into account the light dependence of the turnover of D1 (Mattoo *et al.*, 1984) one would expect that the light enhanced loss in oxygen evolving activity at 40°C (compare open triangles in figs. 4.5 and 4.6 vs. fig. 4.4) should be reflected in the reaction centre activity (fig. 4.7-b), since repair is excluded in the thylakoid suspensions. However, this is not the case: the inhibition of the reaction centre activity was equal in darkness and under low light conditions (compare open and closed triangles in figs. 4.7 a-b). The reason for it is not clear.

Furthermore, the reaction centre activity was less inhibited than the PSII mediated electron transport measured with DAD/ferricyanide under any condition. Bukhov *et al.* (1990) showed that the redox state of Q_A is shifted after brief heating of chloroplasts. This could additionally affect the electron transport activity.

The sequence of events is probably dependent on the experimental conditions; it is not the aim of this study to resolve this sequence. Although it is clear that the transition to 40°C does induce irreversible damage to the reaction centre of PSII.

Further increase of the temperature to 50°C can result in modifications of the lipid matrix of the membranes, inducing alterations in the lipid-protein interactions. The membrane permeability to ions increases. This leads to swelling and unstacking of the granal thylakoids (Guillot-Salomon *et al.*, 1991). After 3 min at 50°C, Cramer *et al.* (1981) observed in chloroplasts a shift in the midpoint potential of cytochrome b-559. This is probably a secondary effect due to changes at the protein environment of PSII. Thompson *et al.* (1989) investigated the protein denaturation as a function of temperature. They noticed, by differential scanning calorimetry, that several chlorophyll binding proteins degrade at peak temperatures of 54 and

59.5°C. These temperatures are lower in isolated core fractions of PSII than in the whole PSII membranes. This accentuates once more that the membrane environment affects the susceptibility to heat stress of the photosynthetic complexes.

In intact leaf tissues, a higher level of organization, the response to heat shock treatment was different to that observed in isolated photosynthetic complexes. Komenda *et al.* (1992) showed that in the cyanobacterium *Synechococcus*, at growth temperature *in vivo*, predominantly acceptor side inhibition occurs under high irradiance. As far as these results can be transposed to leaves, they could explain for the inhibition observed at high light intensities in figures 4.9 a-c. On the other hand, these authors quoted that at e.g. high temperatures donor side inhibition can be involved, resulting in a fast inactivation even at low light intensity. This might explain the difference in PSII activity between 25°C and 40°C in the low light intensity range of the curves (figs. 4.9 a-c).
Havaux and Strasser (1990) analyzed the chlorophyll fluorescence in more detail under heat stress in combination with light. They concluded that the quenching of fluorescence after heat treatment in darkness resembles that of a high energy state, and suggested that conformational changes arise, which increase the probability for dissipation of the excitation energy as heat. Under continuous background illumination the $P680^{+}Pheo^{-}$ state is stabilized. Under these conditions the damage to the reaction centre by heat stress is assumed to be limited.

The slight time-healing effect we observed under high irradiance was quite remarkable (figs. 4.9 a-c). It might correspond to a progressive adaptation to the high temperature, as was observed also by Havaux and Strasser (1990). These authors demonstrated that the initial decline of F_M was followed by a slow increase when the heat treatment was given under light conditions. However, they used a light fluence rate of 45 W m^{-2} and in our experiments the time effect occured at 1500 μmol quanta $m^{-2}s^{-1}$ (about 600 W m^{-2}).

In intact plants, dark and high light intensity inactivation of the PSII electron transport capacity was completely absent. At this organization level, more complex stabilization and heat avoiding processes are involved. Moreover, to explain the difference in response between detached leaves and whole plants, the differences in the mode of application of the heat treatment must be considered (see 4.4.2).

From the data in tables 4.1 and 4.2 it can be concluded that heat treatment of wild type plants has only a limited effect on the photosynthetic activity *in vivo*. Pmax is reduced but recovers soon afterwards. The quantum requirement remains

unchanged, in contrast to observations by Havaux and Strasser (1990). They noticed a decline in Pmax and in quantum yield after heat treatment in darkness. Here, however, *in vivo* heat treatment was executed under a low light intensity

Consequently, the heat treatment that will be applied for inducing the gene expression in the HSIPT plants is not negative on its own for the photosynthetic activity of the plant. Since Pmax changes as a function of time during the day (see table 4.1), the repeated heat treatment of intact plants was further consistently applied at the same moment of the day (see Chapter 5).

Chapter 5: Morphology, hormonal content and photosynthetic activity of P*hsp70-ipt* transgenic plants.

As was shown in the previous chapter, a single heat treatment by raising the temperature from 25°C to 40°C, does hardly affect the photosynthetic activity of intact plants. Only a minor, transient effect on Pmax could be demonstrated. Therefore, chimeric gene constructions with a heat inducible promoter are attractive tools to investigate the effect of the coding gene introduced. The expression of the foreign gene can than be controlled in the plants by applying heat shock treatments. This approach has been used by several laboratories and different heat-shock promoter sequences are now in circulation. In this study the *hsp70* promoter from *Drosophila* is utilized (Spena *et al.*, 1985).

A problem encountered in many of the chimeric heat-shock constructions was the leaky expression of the gene, *i.e.* the expression without specific heat shock induction. In *hsp70-ipt* transgenic tobacco tissues (this was the starting material for the clone used in this study) Schmülling *et al.* (1989) only found a low level of uninduced gene expression. With a *hsp70-iaaM* construction, containing the same promoter, Kares *et al.* (1990) noticed uninduced accumulation of indole-3-acetamide in the plants at control temperature. This is a result of the *iaaM*-gene product activity. In 2 types of *hsp70-ipt* transgenic *Nicotiana* plants, Medford *et al.* (1989) and Smigocki (1991) showed altered phenotypes related to cytokinin accumulation at control temperature. The latter author had to deal with this problem only when the plants were grown *in vitro*.

To evaluate the importance of leakiness in our plant material and to study the effect of heat treatment itself, transgenic and non-transformed plants are compared in this chapter. First, a detailed morphological analysis of wild type and 'HSIPT' (P*hsp70-ipt*) transgenic tobacco plants, either subjected or not to heat treatment, is presented. To study the effect of growth conditions on the expression of the *ipt*-gene, the plants were pre-cultivated *in vitro* or they were immediately sown in soil. Subsequently, photosynthesis in these plants will be examined. The effects will be discussed as a function of the hormonal content in the respective tissues.

5.1 Experimental approach:

The growth conditions are described in Materials and Methods (Chapter 2). After a pre-cultivation of 6 weeks *in vitro* or 3 weeks *in vivo*, the plants were repotted in soil. The daily heat application was started 10 or 3 days later (see 2.1.2.5); only half of each series of plants (transgenic and wild type, pre-cultivated *in vitro* and *in vivo*) was treated. Before heat treatment, the soil was well irrigated in order to prevent water stress. At the day of sampling, no heat treatment was given anymore. As such, long-term and/or permanent modifications brought about by the treatment were envisaged.

Hormone analysis and morphometric measurements were performed on the same plants, and thus the sampling was destructive. For each hormone sample the tissues of 6 plants (9 for the first week) were pooled.

Measurements of the photosynthetic activity were performed in a separate experiment, for which the plants were pre-cultivated *in vitro*. Their phenotype was similar to that of the plants utilized for morphometry and hormone analysis.

5.2 Results:

5.2.1 Morphology:

In figure 5.1 (a-c) the general appearance of the SR1 and HSIPT plants, cultivated under different conditions (pre-cultured *in vitro* or *in vivo*; with or without heat treatment) is shown. Different aspects are discussed hereafter.

5.2.1.1 Stem elongation:

Heat treatment of the transgenic plants resulted in stunted growth compared to the untreated ones. In non-transgenic plants heat treatment caused a similar reduction in length (see fig. 5.1 b-c). This effect was clearly visible after 4 weeks of treatment, irrespective of the pre-cultivation conditions (see fig. 5.2-a and -b). *In vitro* and in soil pre-grown plants showed significant responses to heat treatment. *In vitro* pre-cultivation, however, caused a reduction in length growth in the 4 types of plants (wild type or transgenic; control or heat treated). This *in vitro* effect became highly significant only when the plants were in a late elongation phase (after 4 weeks; fig. 5.1-a). Comparing *in vitro* (fig. 5.2-b) and soil (fig. 5.2-a) pre-cultivation, for the 4 plant types the calculated probability was maximally $P = 0.0055$. During the first and second week of the experiment, no significant effect of *in vitro* pre-cultivation was observed. At this early stage the effect of heat shock was still not expressed.

Figure 5.1: Phenotype of the plants after an experimental growth period of 4 weeks: **a)** from left to right: SR1-C plant, pre-cultivated in soil, SR1-C plant, pre-cultivated *in vitro*, and HSIPT-C plant, pre-cultivated *in vitro*; **b)** HSIPT-C (left) and HSIPT+HS (right) plant, both pre-cultivated *in vitro*; **c)** SR1-C (left) and SR1+HS (right) plant, both pre-cultivated in soil; **d)** flower of a SR1-C plant; **e)** flower of a HSIPT-C plant.

The transformation itself did not affect stem length: under identical experimental conditions HSIPT and wild type plants were not statistically different. Only after 2 weeks the HSIPT-C series was significantly longer for plants grown in soil, but after 4 weeks this difference had disappeared.
These data show that there is no effect of the presence and the activation of the *ipt*-gene on the overall stem elongation growth in this HSIPT transgenic line.

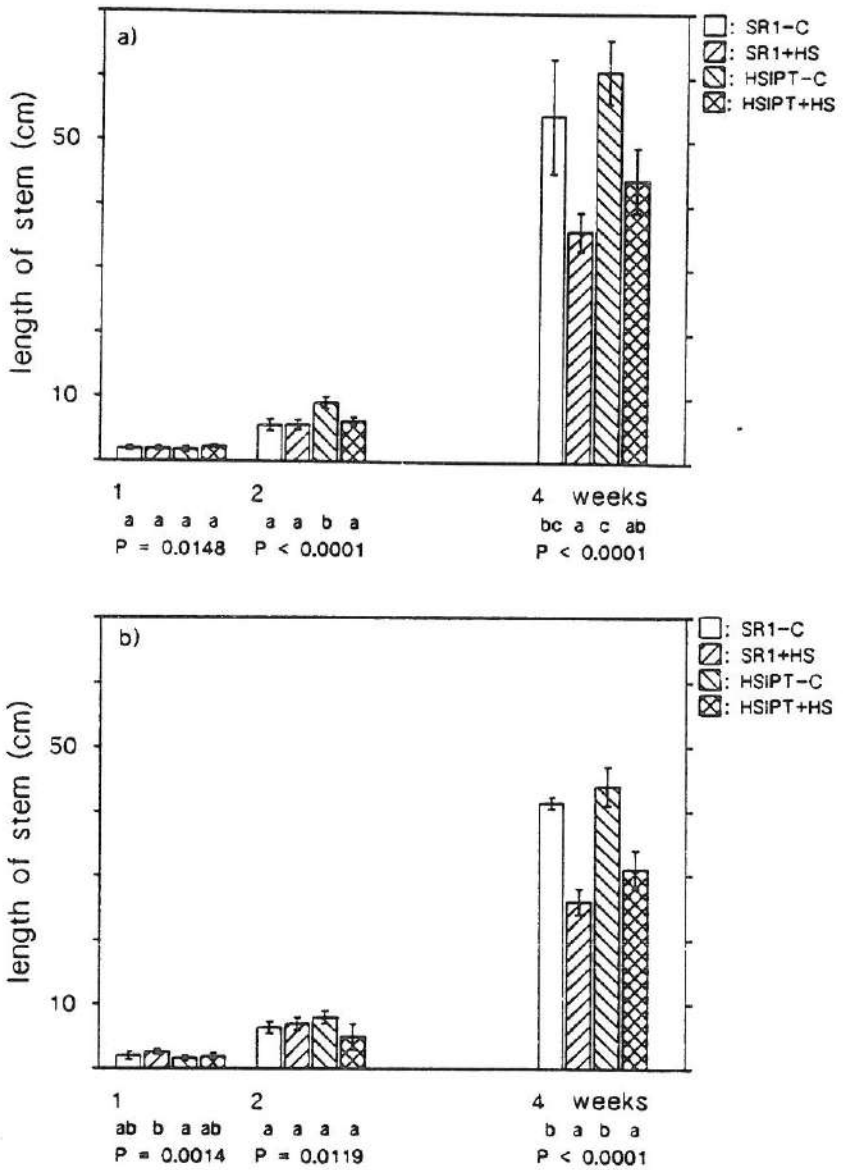


Figure 5.2: Total length of the stem of SR1 and HSIPT plants after an experimental growth period of 1, 2 or 4 weeks, with (+HS) or without (-C) heat treatment. The plants were pre-cultivated **(a)** in soil or **(b)** *in vitro*. Mean ± stand.error; data marked with the same character below the figure are not significantly different (α = 0.01; ANOVA and multiple range test of Scheffé).

5.2.1.2 Internode growth:

The number of stem internodes was not altered by the transformation or by the experimental growth conditions. In all series approximately 17 stem internodes were counted.

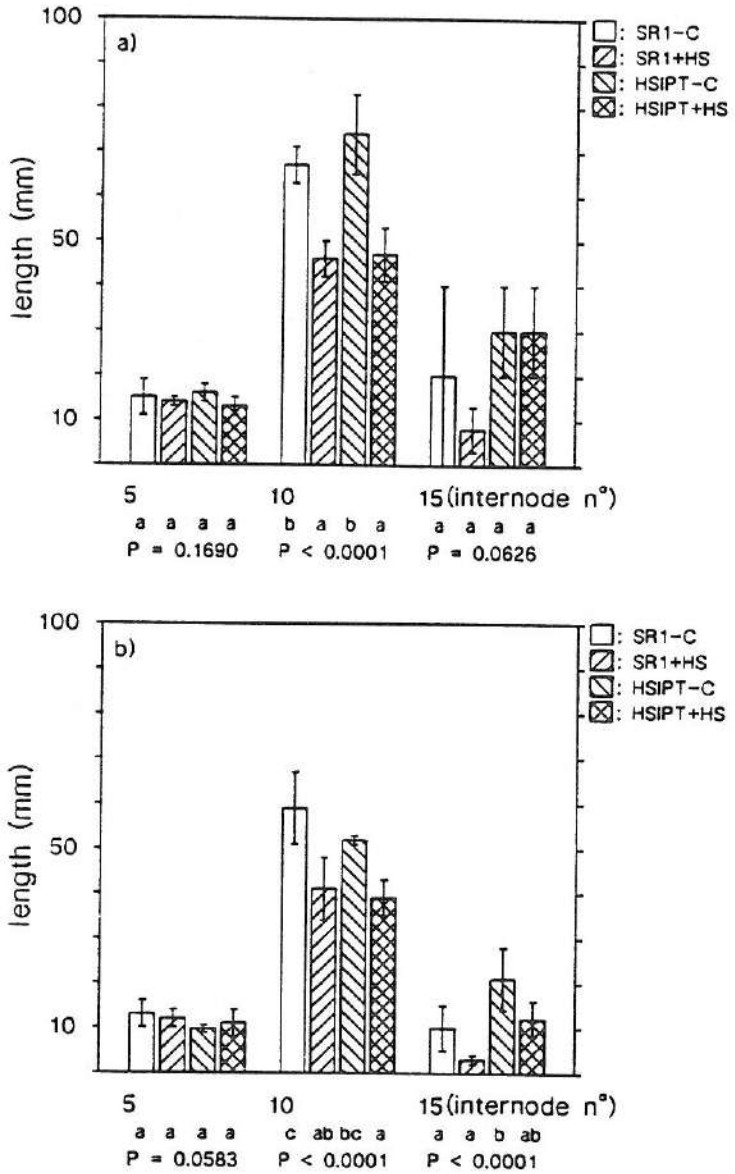


Figure 5.3: Length of the fifth, tenth and fifteenth internodes of SR1 and HSIPT plants after an experimental growth period of 4 weeks, with (+HS) or without (-C) heat treatment. The plants were pre-cultivated **(a)** in soil or **(b)** *in vitro*. Mean ± stand.error as in fig. 5.2.

The effects on the total stem length after 4 weeks of experimental growth were further examined in 3 zones of the stem: a basal (fifth), middle (tenth) and apical (fifteenth) internode. The diameter of these internodes was measured as well.

At the basal part of the stem no difference in internode length was observed between transgenic and wild type (HSIPT vs. SR1). Heat treatment did not result in length reduction of the 5th internode (fig. 5.3 a and b). *In vitro* pre-cultivation conditions reduced the elongation of the 5th internode in the HSIPT-C plants ($P = 0.0002$).
The diameter became smaller after heat treatment of the *in vitro* pre-cultivated plants (compare +HS vs. -C in fig. 5.4-b), although this reduction was not significant. The stem thickening observed in the HSIPT+HS plants from soil pre-culture was not significant either (fig. 5.4-a). The effect of *in vitro* background on the diameter of the 5th internode varied: in both heat treated plant series the diameter had decreased (SR1+HS, $P = 0.0014$; HSIPT+HS, $P = 0.0024$), in the HSIPT-C series it had increased ($P = 0.0182$) and in the SR1-C plants there was no change.
The development of the 5th internode appears to be already predestinated before the start of the experiment, since no pronounced or unambiguous effect of heat shock, transformation or *in vitro* pre-cultivation was observed.

The length of the 10th internode reflected the total stem length. Heat treatment as well as *in vitro* growth conditions caused a significant reduction in internode length in all cases. No effect of the transformation could be registered. In the plants that originated from the *in vitro* pre-culture, the 10th internode in the HSIPT-series was shorter than in the SR1-plants, but this difference was not significant.
In wild type plants heat shock reduced the diameter of internode 10. On the contrary, in the transgenic plants stem thickening was promoted by this treatment. Compared to the respective untreated plant series, these effects were not significant. However, this opposite response in wild type and transgenic plants resulted in a significant difference after heat treatment (SR1+HS vs. HSIPT+HS; fig. 5.4-a and -b). This effect was further examined on cross sections for light microscopy. Although during the preparation of the sections the cortex parenchyma was frequently ruptured, still, figure 5.5 indicates that in this cell layer no differences between the 4 plant series were produced. Also in the vascular tissue, no distinct changes were observed; however, it should be mentioned that the stem of the transgenic HSIPT plants appeared more lignified after several weeks of heat

treatment. The most pronounced differences were found in the pith parenchyma. The number of cells on a radius of a transverse stem section was equal in the 4 series of plants (all pre-cultivated in soil), but the cell dimensions had changed. In SR1 plants the diameter of these parenchyma cells was notably smaller after heat treatment (average diameter about 13.2 μm vs. 14.6 μm for SR1-C), in the HSIPT plants it was enlarged (17.0 μm vs. 15.1 μm for HSIPT-C). Thus, differences in cell expansion of the pith parenchyma were responsible for the alterations in stem diameter.

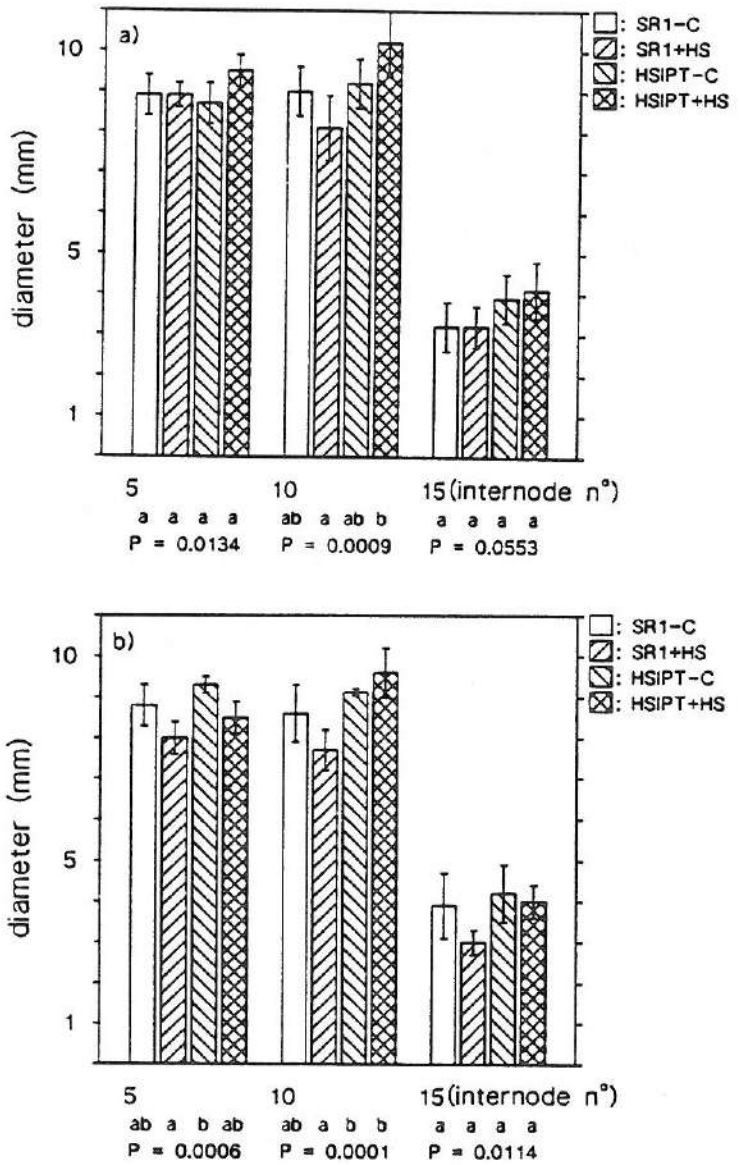


Figure 5.4: Diameter of the fifth, tenth and fifteenth internodes of the same plants as in figure 5.3. The plants were pre-cultivated **(a)** in soil or **(b)** *in vitro*. Mean ± stand.error as in fig. 5.2.

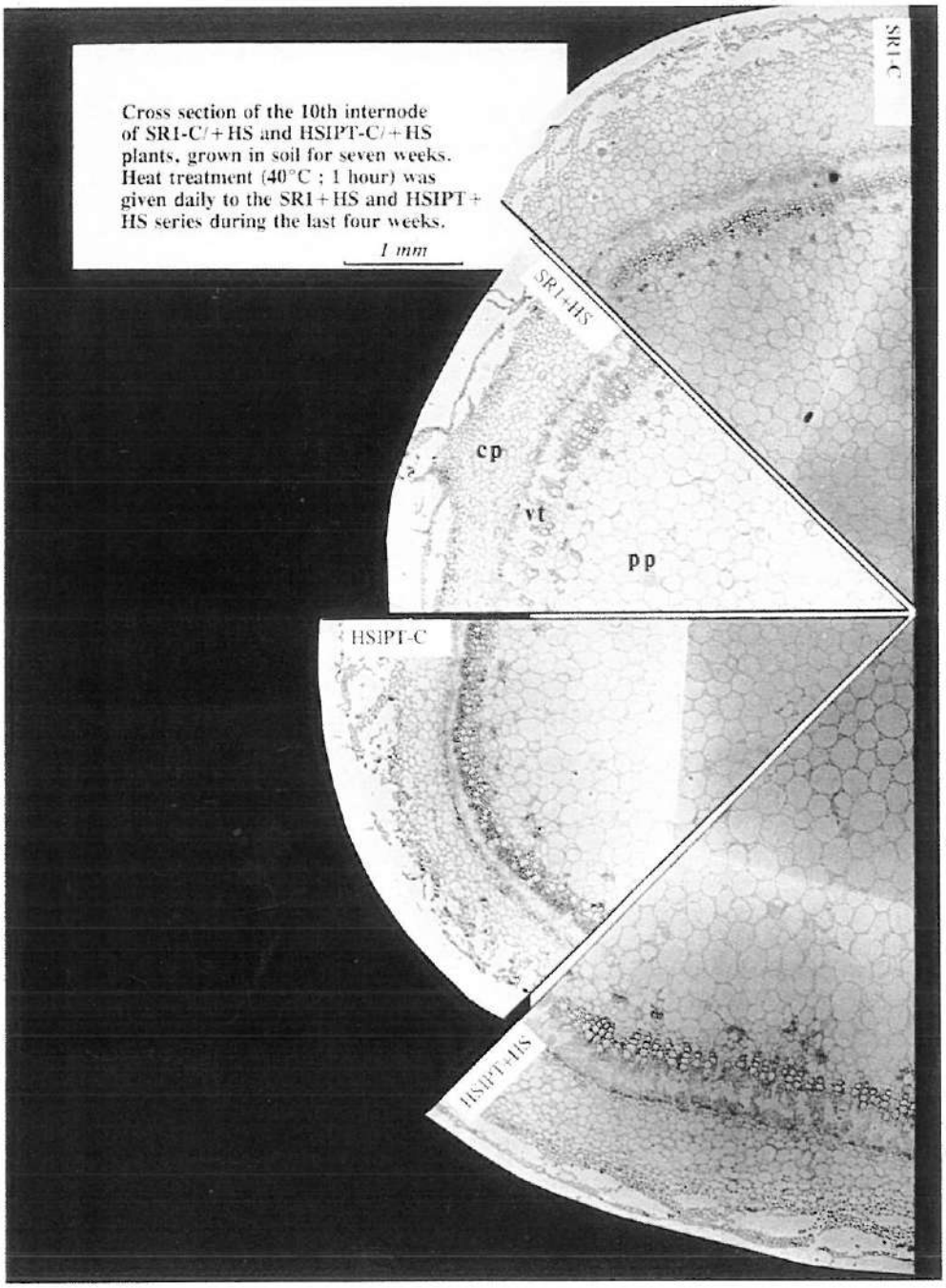


Figure 5.5: Cross-sections of the tenth internode of SR1 and HSIPT plants after an experimental growth period of 4 weeks, with our without heat treatment. The plants were pre-cultivated in soil.
Size bar = 1 mm. (cp = cortex parenchyma, pp = pith parenchyma, vt = vascular tissue)

The apical part of the stem showed a rather large variation in internode length, particularly for plants from the soil pre-culture. In this period (at week 4) flower development took place. In the mean while a substantial elongation of the apical stem zone still came about. Flower development was not exactly synchronized and this might be the cause of the large standard error in figure 5.3-a.
The inhibitory effect of heat shock, as observed on internode 10, was also present in the 15th internode of *in vitro* pre-cultivated plants. But, this effect was not significant. There was yet a significant difference in length of internode 15 between SR1-C and HSIPT-C (see fig. 5.3-b). In the latter case a retardation in flower development may have occured, which would lead to an extended elongation period. The diameter of internode 15 was reduced by heat shock in both SR1 and HSIPT plants, but only for *in vitro* pre-cultivated plants (fig. 5.4-b), and even this difference was not significant. The *in vitro* pre-cultivation itself did not pronouncedly affect the thickness of internode 15, a lately initiated stem internode.

5.2.1.3 Development of the axillary shoots and the inflorescence:

The fresh weight of the axillary shoots was measured. Since the weight of a single axillary shoot is very low, all shoots from 6 plants (per series) were collected and pooled. The bars in figure 5.6 represent the calculated average weight of a pooled sample; hence, these data were not analyzed statistically. Nevertheless, well founded conclusions can be made, because no noticeable differences between plants within each sample were found but the differences between the samples were pronounced.

In the plants pre-cultivated in soil, only the HSIPT+HS series displayed an enhanced bud release (fig. 5.6-a). In particular the middle part of the stem was affected. The heat treatment (SR1+HS vs. SR1-C) or transformation itself (HSIPT-C vs. SR1-C) did not affect the outgrowth of the axillary shoots. In these 3 plant series (SR1-C, SR1+HS and HSIPT-C) buds only grew out when the plants had started flowering. These axillary shoots did not develop to the same extent as for the HSIPT+HS plants at their flowering stage.
After pre-cultivation *in vitro* a similar increase in fresh weight of the axillary shoots was seen in the HSIPT+HS plants (fig. 5.6-b). Without repetitive heat treatments there was some growth of the axillary shoots in the transgenic plants, but not to the same extent as in the HSIPT+HS plants.
In the HSIPT+HS plants the inflorescences were highly reduced and often flower buds were aborted. The flower morphology was not affected (see fig. 5.1 d-e).

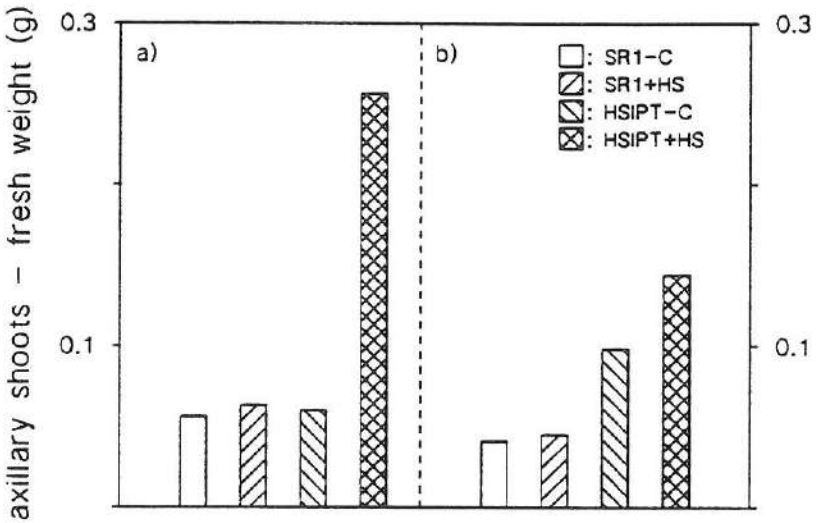


Figure 5.6: Fresh weight of the axillary shoots of SR1 and HSIPT plants after an experimental growth period of 4 weeks, with (+HS) or without (-C) heat treatment. The plants were pre-cultivated **(a)** in soil or **(b)** *in vitro*.

5.2.1.4 Root development:

The development of the root system was not evaluated quantitatively, since the plants were grown in soil during the experiment. It was impossible to remove the soil without damaging the roots. Instead, it can be estimated qualitatively. Therefore, photographs (fig. 5.7) were taken after 8 weeks of heat treatment. A reduction of the root system can be seen in the HSIPT+HS plants, pre-cultivated *in vitro*. At the same time, untreated and wild type plants had a well developed root system and no difference between these series were demonstrated.

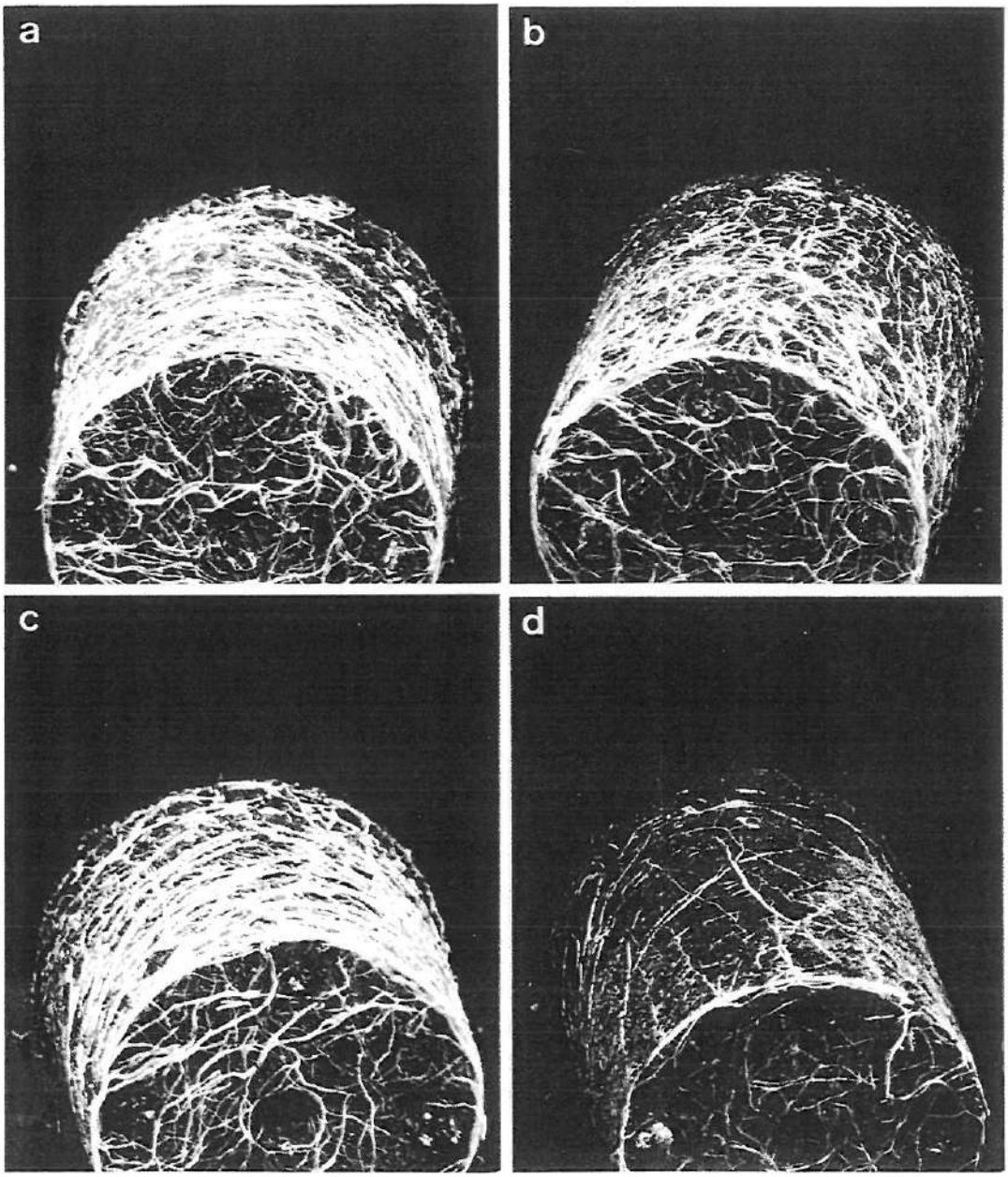


Figure 5.7: Root system after an experimental growth period of 8 weeks of **(a)** SR1-C, **(b)** SR1+HS, **(c)** HSIPT-C and **(d)** HSIPT+HS plants. All plants were pre-cultivated *in vitro*.

5.2.2 Hormone analysis:

The cytokinin concentration was determined by radio immuno assays (see 2. 2.1)

In the stems of young plants and in young stem tissues of older plants, the cytokinin concentration was higher than in older stem segments (table 5.1). No distinct pattern of distribution among the different plant series comes forward after the first week. In the upper half of the stem after 2 weeks and in the 10th internode after 4 weeks a similar evolution was seen in plants pre-cultivated in soil. In these tissues heat treatment caused a reduction in the level of ZR- and iPA-equivalents in wild type plants. In the transgenic plants, on the contrary, the cytokinin content was increased by heat shock.

This respons was partly repeated in the *in vitro* pre-cultivated plants. After 2 weeks the heat treatment also reduced the cytokinin concentration in the upper half of the stem in wild type plants and increased the concentration of iPA-equivalents in the HSIPT plants. The ZR-content remained constant in the transgenic plants. After 4 weeks in the SR1 plants from *in vitro* culture, no further decrease appeared, on the contrary the cytokinin concentration was doubled by heat treatment in internode 10. But in the transgenic plants the increase was somewhat more pronounced. In the apical part of the stem after 4 weeks, again high endogenous levels of cytokinins were present and the respons to heat treatment was more variable.

From the data of SR1-C and HSIPT-C plants, it cannot be concluded that uninduced expression of the *ipt*-gene occured in these tissues, since the cytokinin content was equal and in some cases even lower in the transgenic plants. However, these results should be interpreted with care, because they consist of single determinations.

In leaves the cytokinin content was measured during the first 2 weeks only (table 5.2). After 2 weeks leaf 5 had expanded substantially; therefore, the sample was divided in two parts: the mid vein and the rest of the leaf blade.

In leaves also the concentration was the highest in young tissues (leaf 10) and decreased with age (compare leaf 5 at week 1 and 2), in particular in the wild type plants. The effects produced by heat treatment were not unambiguous. Besides there were no morphological alterations e.g. in leaf shape or visible changes in leaf pigmentation.

Table 5.1: Cytokinin content in pooled samples of stem tissues from SR1 and HSIPT plants after an experimental growth period of 1, 2 or 4 weeks, with or without heat treatment. The plants were pre-cultivated in soil or *in vitro*. The data are expressed in pmol ZR- and iPA-equivalents g^{-1} fresh weight. (nd = not determined)

pmol ZR-eq. g^{-1}fr.weight		SR1-C	SR1+HS	HSIPT-C	HSIPT+HS
in soil	- week 1	119	107	210	153
	- week 2				
	lower half	235	159	91	88
	upper half	145	78	28	89
	- week 4				
	internode 10	45	10	20	88
	intern.15 - top	62	196	66	85
in vitro	- week 1	149	129	144	143
	- week 2				
	lower half	71	85	70	67
	upper half	66	25	55	58
	- week 4				
	internode 10	21	47	37	97
	intern.15 - top	86	161	107	198
pmol iPA-eq. g^{-1}fr.weight					
in soil	- week 1	23	22	31	26
	- week 2				
	lower half	nd	15	14	13
	upper half	43	24	23	27
	- week 4				
	internode 10	6	3	4	9
	intern.15 - top	15	18	74	18
in vitro	- week 1	nd	27	31	31
	- week 2				
	lower half	10	7	6	10
	upper half	20	10	10	30
	- week 4				
	internode 10	3	5	7	17
	intern.15 - top	30	48	13	39

Table 5.2: Cytokinin content in pooled samples of leaf tissues from SR1 and HSIPT plants after an experimental growth period of 1, 2 or 4 weeks, with or without heat treatment. The plants were pre-cultivated in soil or *in vitro*. The data are expressed in pmol ZR- and iPA-equivalents g^{-1} fresh weight. (nd = not determined)

pmol ZR-eq. g^{-1}fr.weight		SR1-C	SR1+HS	HSIPT-C	HSIPT+HS
in soil	- week 1				
	leaf 5, in total	56	64	29	21
	- week 2				
	leaf 5, mid vein	nd	10	12	8
	leaf 5, rest of leaf blade	nd	15	16	17
	leaf 10, in total	167	115	57	71
in vitro	- week 1				
	leaf 5, in total	38	35	12	18
	- week 2				
	leaf 5, mid vein	11	9	8	7
	leaf 5, rest of leaf blade	14	15	11	9
	leaf 10, in total	194	238	52	128
pmol iPA-eq. g^{-1}fr.weight					
in soil	- week 1				
	leaf 5, in total	14	9	8	8
	- week 2				
	leaf 5, mid vein	nd	2	2	1
	leaf 5, rest of leaf blade	nd	3	3	2
	leaf 10, in total	61	40	13	24
in vitro	- week 1				
	leaf 5, in total	6	4	3	6
	- week 2				
	leaf 5, mid vein	2	2	2	2
	leaf 5, rest of leaf blade	2	2	1	2
	leaf 10, in total	nd	56	10	37

As expected, in the axillary shoot, with young, deviding cells, the cytokinin concentration was high (table 5.3). On a fresh weight basis, the ZR- and iPA-content was markedly lower in the HSIPT+HS plants grown in soil than in the 3 other plant series, that were resembling among themselves. In contrast, in the *in vitro* pre-grown plants, the heat treatment increased the ZR-content in SR1 and HSIPT plants (table 5.3). The iPA-content decreased by heat treatment in wild type, but increased in HSIPT plants.

However, when the data were recalculated and expressed as pmol ZR- or iPA-equivalents in the axillary shoots per plant instead of per fresh weight of the examined tissue, in the soil pre-cultivated plants the ZR-content only was increased in the HSIPT+HS plants. In the HSIPT+HS plants from *in vitro* pre-culture ZR- and iPA-concentrations on a plant basis were increased in comparison with the other plant series.

Table 5.3: Cytokinin content in pooled samples of axillary shoots from SR1 and HSIPT plants after an experimental growth period of 4 weeks, with or without heat treatment. The plants were pre-cultivated in soil or *in vitro*. The data are expressed as pmol ZR- and iPA-equivalents g^{-1} fresh weight or as pmol ZR- and iPA-equivalents per plant.

	SR1-C	SR1+HS	HSIPT-C	HSIPT+HS
pmol ZR-eq. g^{-1}fr.weight				
in soil	305	249	257	98
in vitro	112	273	142	271
pmol iPA-eq. g^{-1}fr.weight				
in soil	97	95	95	20
in vitro	92	43	25	54
pmol ZR-eq. $plant^{-1}$				
in soil	17.0	15.6	15.4	25.1
in vitro	4.6	12.3	13.9	39.0
pmol iPA-eq. $plant^{-1}$				
in soil	5.4	6.0	5.7	5.1
in vitro	3.8	1.9	2.5	7.8

5.2.3 Photosynthetic electron transport capacity:

The capacity of the photosynthetic electron transport was measured in wild type and transgenic HSIPT plants, both pre-cultivated *in vitro*, after different periods of heat treatment. The data were compared with those of untreated plants of the same age. The most expanded leaf was selected for thylakoid isolation (see 2.3.2.1). This was about leaf 6 during the first 2 weeks and shifted to more apical leaves later on. From week 4 on leaf 10 was used. During the last 2 weeks this leaf gradually got older.

Figure 5.8 presents the electron transport capacity mediated by PSI and PSII, measured with the artificial electron acceptor MV. The variability of the results was quite large during the first weeks, but decreased as the leaves further developed. The electron transport capacity decreased with ageing. No remarkable differences between the 4 series of plants were noticed. This is also true for the PSI and PSII activities separately. The trend of the electron transport capacity to decline as function of leaf age is less pronounced for the PSII activity (fig. 5.9) than for the PSI mediated electron transport (fig. 5.10). No effect could be attributed to heat treatment, neither in the wild type, nor in the transgenic plants. Also the transformation itself had no influence (HSIPT vs. SR1).

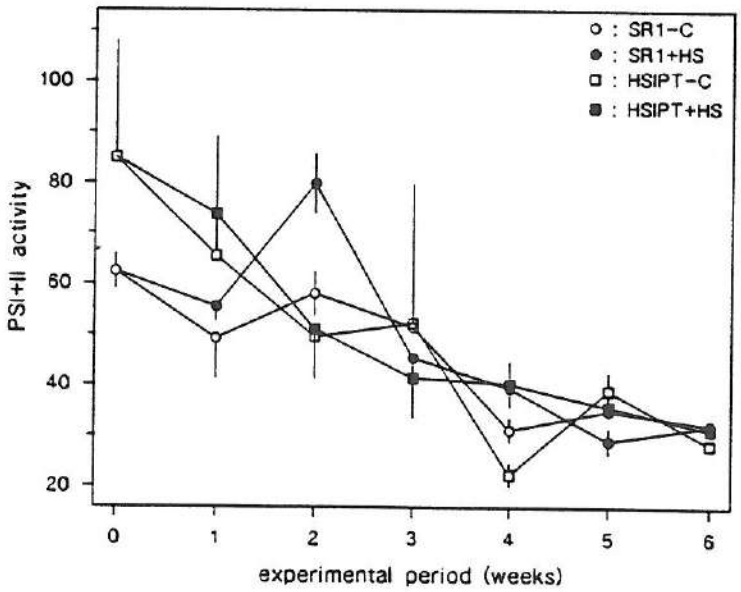


Figure 5.8: Photosynthetic electron transport capacity mediated by PSI and II ($H_2O \rightarrow MV$), measured on a thylakoid suspension prepared from the largest leaf of SR1 and HSIPT plants, with (+HS) or without (-C) heat treatment (μmol O_2 evolution (mg Chl)$^{-1}$h^{-1}).

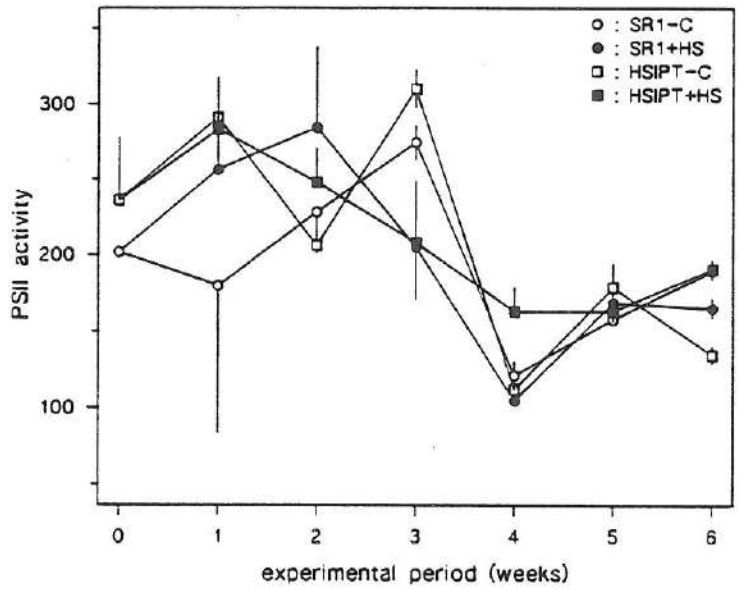


Figure 5.9: Photosynthetic electron transport capacity of PSII ($H_2O \rightarrow$ DAD/ferricyanide) of the largest leaf of SR1 and HSIPT plants, with (+HS) or without (-C) heat treatment (μmol O_2 production (mg Chl)$^{-1}$ h^{-1}).

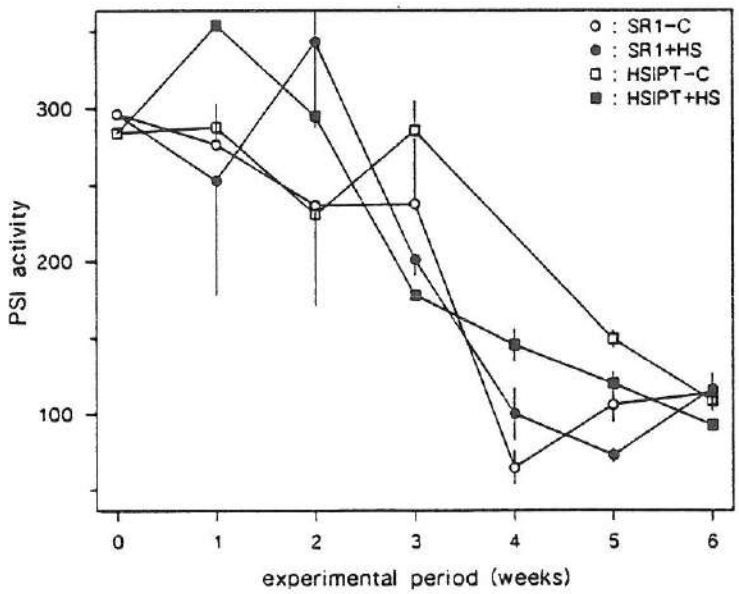


Figure 5.10: Photosynthetic electron transport capacity of PSI ($DADH_2 \rightarrow$ MV) of the largest leaf of SR1 and HSIPT plants, with (+HS) or without (-C) heat treatment (μmol O_2 evolution (mg Chl)$^{-1}$ h^{-1}).

5.3 Discussion:

The experimental set-up allows to distinguish between the effects actually provoked by the activation of the *ipt*-gene expression, by comparing HSIPT-C and HSIPT+HS plants, and changes induced by other factors. Comparison of wild type SR1 and transgenic HSIPT plants, both cultivated at control temperature, yields information about the effect of the genetic transformation itself or can give indication of uninduced expression of the *ipt*-gene, while comparison of SR1-C and SR1+HS plants specifies the effects of heat treatment. Finally, the influence of differences in early cultivation conditions can be distinguished since plants were either grown immediately in soil or pre-cultivated *in vitro* on a selective medium (see 2.1.2.2-3).

A predominant feature was the reduction in elongation growth (fig. 5.2 a-b). This was mainly induced by heat treatment. Besides, the *in vitro* pre-cultivation had an overall inhibitory effect on stem elongation. The effects on total stem elongation were reflected in particular in the central part of the stem (internode 10; fig. 5.3). An attempt has been made to distinguish between the contribution of cell division and cell expansion to the altered stem elongation. Therefore, longitudinal stem sections were made, but they failed to give a decisive answer because the tissues were frequently distorted during embedding for light microscopy. This was due to the large difference in elasticity of the individual tissue layers in the lignifying stem. Consequently, the number of valid, independent observations was too small to be conclusive.

Stunted growth is often reported as an effect of the expression of the *ipt*-gene (Ainley *et al.*, 1993; Medford *et al.*, 1989; Ondřej *et al.*, 1991). In *rol*C-transgenic plants reduced length growth was also related to an increase in the level of free cytokinins (Schmülling *et al.*, 1993). Exogenous application of cytokinins was shown to reduce the apical dominance (Li and Bangerth, 1992); concomitant the lateral shoot growth overtakes the growth activity of the apical shoot, which should result in a diminished length of the major stem. However, in our experiment no effect of the *ipt*-gene expression on stem length could be measured. Namely, the magnitude of inhibition of the elongation growth is equal in transgenic and wild type plants. This failure could be explained assuming that a maximal reduction of length growth that can be induced at a particular developmental stage, was already realized by the heat treatments. As such the effect of the *ipt*-gene expression would be masked. Noteworthy in this respect is the enhanced inhibition of the elongation of the 10th internode in the HSIPT-C plants by *in vitro* pre-cultivation, in comparison with the SR1-C plants (fig. 5.3-b). This observation will be called to mind further on.

The thickness of the stem is also affected by heat treatment, especially at the 10th internode (fig. 5.4). Moreover, after 4 weeks stem diameter appears to correlate with the cytokinin content in the plants from soil pre-culture (table 5.1). In wild type plants heat treatment caused a reduction of the stem diameter, coinciding with a decrease in the content of ZR- and iPA-equivalents. Itai and Benzioni (1974) reported a decrease in cytokinins in xylem exudate after a short exposure of the root system to heat. Leaves responded to the same fashion. Caers *et al.* (1985) confirmed that heat treatment of maize roots causes a decline in the endogenous cytokinin concentration in green leaves. In the transgenic HSIPT plants heat treatment led to an increased cytokinin level by the activation of the *ipt*-gene. This stimulated stem thickening. The hormonal changes that attend this differential response are relatively small. However, Hocart *et al.* (1992) and Ainley *et al.* (1993) found likewise an increase in stem diameter in *ipt*-transgenic plants corresponding with a limited cytokinin increase. Thickening is shown to be the result of increased cell expansion of the pith parenchyma (fig. 5.5). Additionally, Ainley *et al.* (1993) observed cell expansion in the stem cortex and of the secondary xylem in their HSIPT transformants. In PSAUR-*ipt* transgenic plants characterized by a weak expression, cortical parenchyma and epidermis cells had expanded, and these are the tissues where the SAUR-promoter was expressed (Li *et al.*, 1992). In these plants, the vascular system of the stem had also increased. However, in P*chs-ipt* plants stem thickening was brought about by promotion of cell division and cell enlargement of the pith tissue (Hocart *et al.*, 1992). On the contrary, Medford *et al.* (1989) reported a reduction in stem diameter in both transgenic P*hsp70-ipt* tobacco and *Arabidopsis*, if the latter was cultivated in short days. This correlated with a reduction of xylem proliferation. In these plants the cytokinin level was much more elevated after heat induction. Yet, the stem diameter was reduced to the same extent in transgenic plants maintained at the control temperature. In their HSIPT-C plants the cytokinin content in the leaves was three- to sevenfold higher for ZR and its monophosphate, respectively, than in the wild type, indicating some leakage of the gene expression. But, this limited increase in cytokinins resulted in a similar decline in xylem tissue. This is remarkable, the more so as cytokinins are believed to play a role in the induction of xylem differentiation (Aloni, 1982). However, since the cytokinin requirement is limited to the initiation phase, a localized and/or temporal decline in the hormone signal or in the sensitivity in the HSIPT plants could have induced a reduction in the vascular system. This interpretation is hypothetical, since Medford *et al.* (1989) did not measure kinetics of the hormone content and only leaves were analyzed.

Another effect of the *ipt*-gene expression is the sprouting of the axillary buds. It was predominant in the central stem region, where the buds grew out to heavy branches. After 6 weeks of treatment in the HSIPT+HS plants the fresh weight of the axillary shoots at node 10 exceeded a 100-fold increase in comparison with the other plant series (data not shown). This effect was reported in most of the other *ipt*-transgenic plants also. When cytokinin content in the axillary shoots was expressed on a plant basis, there was a correlation between sprouting and the cytokinin content (table 5.3). On a fresh weight basis the correlation was inverse in the plants pre-cultivated in soil. This can be explained by the fact that the entire shoots were analyzed, whereas the cytokinin concentration was probably mainly increased in the actively dividing, meristematic cells. In the buds from the HSIPT+HS series the fresh weight had increased already (fig. 5.6), reducing the relative part of the meristematic tissue. As such a dilution effect by the increase in fresh weight was introduced. This can be overcome by expressing the results per axillary shoot or per plant (because here all the individual shoots were pooled together per plant series).

Procházka and Jacobs (1984) showed that cytokinins are rapidly mobilized to axillary buds that are induced to grow out. Li *et al.* (1992) demonstrated that local cytokinin accumulation enhances the sink strength. Hence, nutrients are translocated towards these tissues, which could support their growth.
Apical dominance is related to a basipetal auxin gradient along the stem. Release of the lateral shoots should involve a rearrangement of this gradient. Cytokinins may take part in this process. Li and Bangerth (1992) suggested that an increase in cytokinins stimulates IAA export from the buds, which would redefine their dominance relationships within the plant. On the other hand Panizza *et al.* (1993) emphasized the regulatory role of ethylene in benzyladenine stimulated budding. At this moment the regulation of the apical dominance is still far from being understood. Some hypotheses interrelate diverse factors but, causal modelling is precocious, yet (Cline, 1994). The *ipt* stimulated axillary bud development confirmes again the cytokinin involvement.

In the plants pre-cultivated *in vitro* the fresh weight of the axillary shoots increased also without thermal induction in the transgenic plants (HSIPT-C; fig. 5.6-b). This result, however, did not coincide with a cytokinin increase. Smart *et al.* (1991) and Smigocki (1991) also showed that in the HSIPT-C plants the axillary shoot development was intermediate to that in wild type and in HSIPT+HS plants; likewise their transgenic plants were pre-cultivated *in vitro* prior to transfer to soil. Heat shock genes are not expressed upon a transitory temperature rise only, as could

be misjudged from their name. Other stress conditions have also been shown to induce the *hsp70*-gene family (Heikkila *et al.*, 1984). *In vitro* culture produces unfavourable, stressful conditions for growth, to which plants exhibit a short term acclimation (Pospíšilová *et al.*, 1988; Tichá *et al.*, 1988). Dr. Synková showed that upon transfer from *in vitro* to soil culture the fluorescence characteristics of SR1 plants improved: the photochemical efficiency of PSII (F_V/F_M) as well as a 'vitality index', calculated as $(F_M - F_S)/F_S$ (Lichtenthaler and Rinderle, 1988), increased (personal communication). This supports the idea of *in vitro* stress. Consequently, during the early development in the *in vitro* pre-cultivated transgenic plants, the *hsp70* promoter may have been activated and the *ipt*-gene transcribed. The appearance of a transient cytokinin peak is assumed, which might induce morphological changes. In a later stage, during proliferation these changes would come to expression in e.g. a limited axillary shoot growth. Hence, this effect does not indicate leakage of the gene expression *sensu stricto* because this is defined as uninduced gene expression while here the induction was achieved by another stress factor than heat.

The moderate reduction in length of the 10th internode in the HSIPT-C plants from *in vitro* pre-culture might be explained in this way as well. The *in vitro* stress could have induced *ipt*-gene expression, which might have changed the stem development and this becomes only visible after complete expansion. This would support the interpretation by other research groups of a cytokinin evoked growth retardation (see before).

Root development is reduced in the heat induced HSIPT plants. This is consistent with the *ipt* exerted root growth inhibition demonstrated in other HSIPT transformed plants (Medford *et al.*, 1989; Smigocki, 1991). It would result from a reduction in the root elongation zone. In *ipt*-transformed plants with the constitutive 35S-promoter root formation was even completely inhibited (Smigocki and Owens, 1989). The increase in cytokinin content resulted in a higher shoot-to-root ratio: lateral branches emerged and root growth was reduced. This is in agreement with the bushy morphology of P*ssu-ipt* transgenic tobacco grafts, made from shoots that were unable to develop roots (Beinsberger *et al.*, 1992a). In contrast, two transformed potato lines with the *ipt*-gene under control of its own promoter, displayed stimulated root growth (Ooms *et al.*, 1991). In other clones root development was not affected. These authors propose a hypothesis based on an altered sensitivity to cytokinins. They assume that meristematic tissues, leaves or both can produce an enhanced sensitivity after transformation. This organ specificity

could be due to the position of the *ipt*-gene after integration into the nuclear plant genome. However, this hypothesis still does not explain why these authors found a stimulation in root development. In *rol*C-transgenic potato plants root development was also enhanced (Schmülling *et al.*, 1993). This gene encodes for a cytokinin-ß-glucosidase (Estruch *et al.*, 1991a), which catalyzes the release of free cytokinins from inactive N-glucosides; as a consequence the active cytokinin pool is increased. The difference in response for rooting might not just be explained in relation to the cytokinin concentration, but the interaction with other hormones and the tissue sensitivity may be determinant (Trewavas, 1991). Furthermore, Pospíšilová *et al.* (1993) showed that tobacco plants were more sensitive to exogenous cytokinins than potato. Species specific differences might contribute as well.

In several *ipt*-transformed plants leaf size was reduced and eventually the shape was altered: the length-to-width ratio was often reduced and the lamina was wrinkled (Ainley *et al.*, 1993; Beinsberger, 1993; Li *et al.*, 1992; Medford *et al.*, 1989). Exogenous application of cytokinins to leaves was shown to reduce the leaf surface area (Bosselaers, 1983). On the other hand enhanced cotyledon expansion has often been used as bioassay for cytokinins (Esashi and Leopold, 1969). This shows clearly that cytokinins affect leaf morphology.
None of these changes was observed in our transgenic plants. The absence of effects might correspond to an organ specific enhancement of the sensitivity to cytokinins (Ooms *et al.*, 1991); accordingly, the *Phsp70-ipt* plants should display a lower sensitivity in leaves in comparison with meristems and stem tissues. Moreover, the cytokinin content was not increased in the leaves of the transformed plants subjected to heat treatment (table 5.2). This treatment was apparently not very effective in increasing the leaf temperature. After 1 h in the heat shock chamber at 40°C, leaf surface temperature had only increased to about 32°C. Plants dispose of mechanisms to avoid the actual leaf temperature to rise. When heat stress is not accompanied by water stress, evaporative cooling can significantly reduce leaf temperature compared to the air temperature (Campbell *et al.*, 1990), and as mentioned before, water stress was avoided in our experiment. If radiative heat transfer is limited, heating is additionally hindered by the boundary layer effect. Local heat treatment by contact of the leaf with a temperature controlled block of plexiglas, as applied by Havaux and Strasser (1990), is more effective. Smart *et al.* (1991) also imposed local heat shocks by clamping a small, tightly temperature controlled chamber onto the leaf. This was shown to be efficient: in the heated leaf area of the HSIPT transgenic plants the cytokinin content was clearly elevated. After

repetitive treatment senescence was delayed in this part of the leaf. However, this method of treatment shows also distinct limitations. Only a small area can be treated at once. Besides, the surrounding, non-heated tissue cannot simply be considered as control since its phytohormone content also slightly alters after the treatment, probably due to diffusion, active transport and metabolism (Smart *et al.*, 1991).

Taking into consideration that the heat treatment applied does not affect leaf morphology, nor leaf cytokinin content (table 5.2), it is not surprising that no effect of the treatment can be observed when the photosynthetic electron transport capacity of wild type and transgenic leaves is compared (fig. 5.8-10). Based on fluorescence characteristics, Šiffel *et al.* (1992) found in *ipt*-transformed potato plants with elevated cytokinin content, that the reaction centre complexes of PSI and PSII were not affected. They demonstrated only a difference in the electrophoretic mobility of the light harvesting complex.
On the other hand, in SR1 and HSIPT plants, 4 weeks of heat treatment were shown to affect the ultrastructure of the chloroplasts: the thylakoids are swollen (Vandenborn, 1990; H. Synková, personal communication). It is thus quite remarkable that no effect of this repetitive treatment on the electron transport capacity appears. The swelling was more pronounced in the transgenic plants, in particular in the older leaves. Moreover, non-treated HSIPT plants had thicker thylakoids than SR1-C plants. This swollen aspect was also noticed in P*ssu-ipt* transgenic grafts and seedlings, in which the leaf cytokinin content is increased (Beinsberger, 1993). Accordingly, thylakoid swelling could be an effect of *ipt*-gene expression. It can be questioned whether in the HSIPT transformants the cytokinin level never had changed during leaf ontogeny. To solve this question a more refined determination of the hormonal kinetics and of the spectrum of the different cytokinins is required.

In conclusion, these HSIPT transgenic plants show distinct morphological changes upon heat treatment: reduced elongation growth, increased stem diameter, axillary sprouting and a reduction in root growth. The latter three effects could be attributed to the expression of the *ipt*-gene. Stem elongation was likewise diminished by heat treatment in the non-transgenic plants.
A point in favour of this genetic construction is the well controlled gene expression: when grown in soil, without heat induction the plant morphology was indistiguishable from wild type plants. A disadvantage is that gene expression fails in leaves, the major photosynthetic organ. Therefore, these plants are not a good model to study

the role of cytokinins on photosynthesis. A more effective heat treatment of the leaves might overcome this problem. However, Ooms *et al.* (1991) showed that a variation of organ sensitivity can occur in different clones of potato plants transformed with one and the same vector. We therefore decided not to improve the inductive heat shock treatment, but to proceed with another transgenic line of *Nicotiana tabacum* with high levels of *ipt*-gene expression specifically in leaves, viz. P*ssu-ipt* transformants.

Chapter 6: Morphology, hormonal content and photosynthetic characteristics of P*ssu-ipt* transgenic tobacco.

In the previous chapter the results were discussed, obtained with the P*hsp70-ipt* transgenic tobacco. The effects of the *ipt*-gene expression, however, were merely localized in non-photosynthesizing organs. The level of gene transcription was not analyzed in the different organs; therefore it could not be concluded whether the *ipt*-gene was transcribed or not in the leaves under the experimental conditions. The levels of iPA and ZR-equivalents, products of the isopentenyl-transferase initiated biosynthetic pathway, however, were not increased in leaves.

In this chapter the results are presented of transgenic tobacco, where the *ipt*-gene was coupled to the promoter of the *rbcS*-gene, coding for the small subunit (SSU) of RubisCO. This promoter, also named *ssu*-promoter, is light responsive and is predominantly expressed in leaves (Fladung *et al.*, 1993; Jefferson *et al.*, 1987). Therefore, this transgenic plant material is more appropriate to investigate the effect of the introduced *ipt*-gene on photosynthesis.

Since light triggers the expression of the *ipt*-gene in the P*ssu-ipt* transgenic construction, the cultivation of photosynthetic competent plants cannot be separated from the induction of the *ipt*-gene expression. In these conditions the question rises how to define a suitable control. Moreover, the developmental pattern of the transgenic plant material was completely altered. In contrast to the HSIPT plants, no corresponding leaf, regarding position on the stem, leaf size or age, could be determined in comparison to wild type plants (see 6.1).
In this study, the results of photosynthetic characteristics of transgenic plants were always compared to those from wild type plants at a specified developmental stage, namely after 3 to 4.5 weeks of growth in large pots in soil. This is before flower bud development has started.

The plant material used was derived from transgenic tobacco calli. Shoot induction was easily obtained in these calli. But, as already explained in Materials and Methods (see 2.1.1), every attempt to induce root formation had failed (Beinsberger *et al.*, 1992a). Therefore, the transgenic shoots were grafted on non-transformed stalks. This was a successful method. The grafts developed into a large bushy crown on a wild type stem.

These grafts appeared to be heterozygous in the chimeric gene construction, since the F1 progeny segregated into kanamycin-resistant and -sensitive plants. The kanamycin-resistance factor (*npt*II-gene) was closely linked to the chimeric *ipt*-gene. This implies that the absence of the resistance factor is associated with the absence of the *ipt*-gene. Conversely, in the kanamycin-resistant (Km^R) progeny the *ipt*-gene was present (Beinsberger, 1993). Seeds from the grafts were sown *in vitro* on a kanamycin free medium, in order to keep the kanamycin-sensitive (Km^S) seedlings viable. After a few weeks a clear phenotypical distinction between Km^R and Km^S seedlings could be made, even in the absence of the selective marker itself. The Km^S seedlings developed normally and when they were transferred to soil after 4 to 5 weeks of *in vitro* pre-cultivation, their development was indistinguishable from wild type (SR1) plants. In the Km^R seedlings, true P*ssu-ipt* transgenic ones, the radicula and two small, dark green cotyledons appeared after germination, but the seedlings did not further develop *in vitro*. After 4 to 5 weeks they were also transferred to soil and the growth of these plants, called 'P*ssu-ipt* plants', was followed.

Analogous to chapter 5, first the morphological characteristics will be presented, followed by the hormonal analysis and by the photosynthetic properties. The morphology and hormonal content of the P*ssu-ipt* grafts has been described by Beinsberger (1993) and only the major features will be repeated.

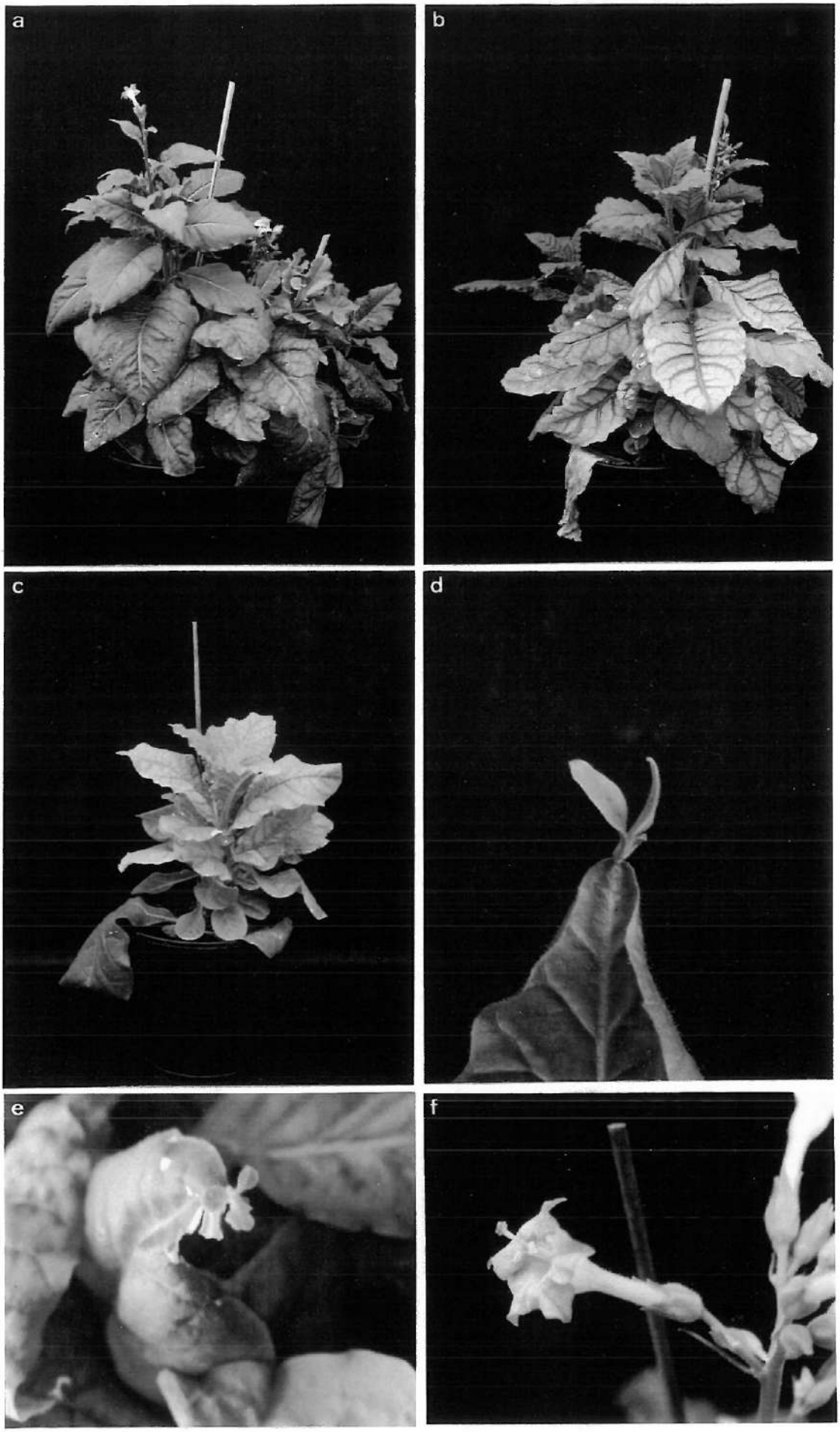
6.1 Results:

6.1.1 Morphology:

6.1.1.1 P*ssu-ipt* grafts:

No morphometric study was realized on grafted P*ssu-ipt* transgenic shoots. A description of the phenotype, referring to figure 6.1, is more appropriate here.

Figure 6.1: (see opposite page) Phenotype of the P*ssu-ipt* grafts: **a)** old, dark green graft; **b)** old, pale graft; **c)** young graft; **d** and **e)** leaves with viviparous shoots on the leaf tip; **f)** flowers, mind the over-elongated pistil.


a
b
c
d
e
f

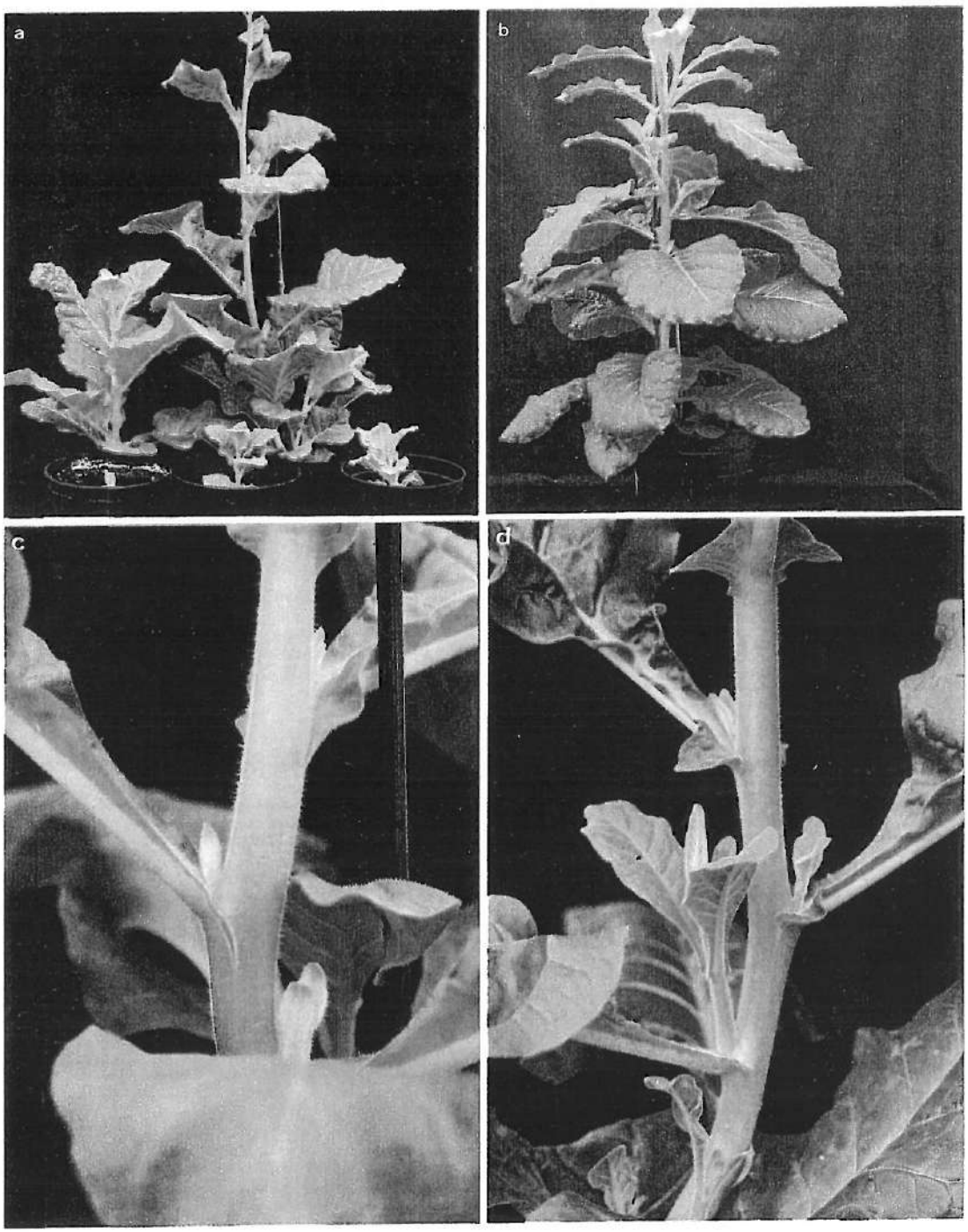
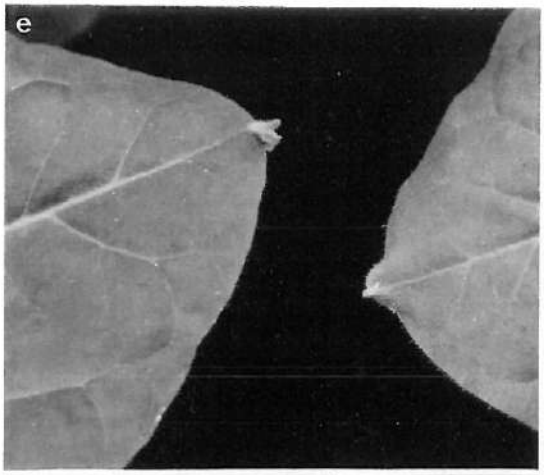


Figure 6.2: Phenotype of the P*ssu-ipt* plants: **a)** a collection of P*ssu-ipt* plants, all of the same age (5 weeks *in vitro* culture and 11 weeks in soil); **b)** old, green P*ssu-ipt* plant; **c** and **d)** sprouting axillary shoots;



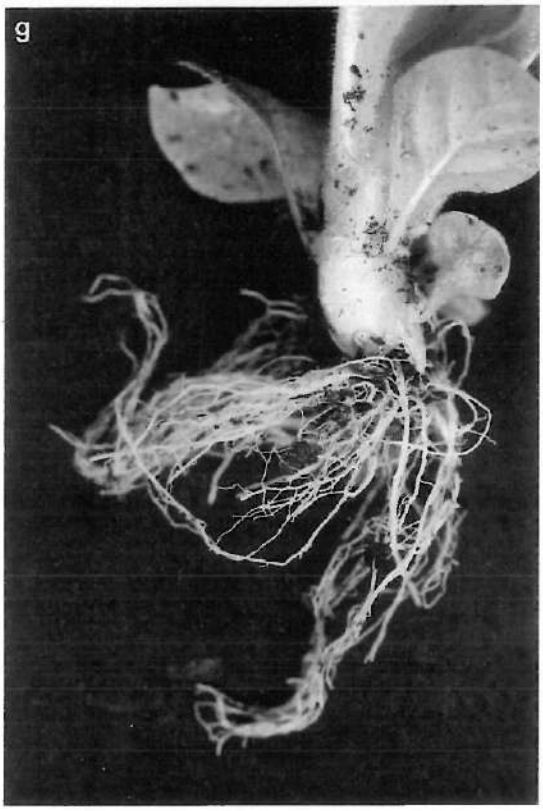
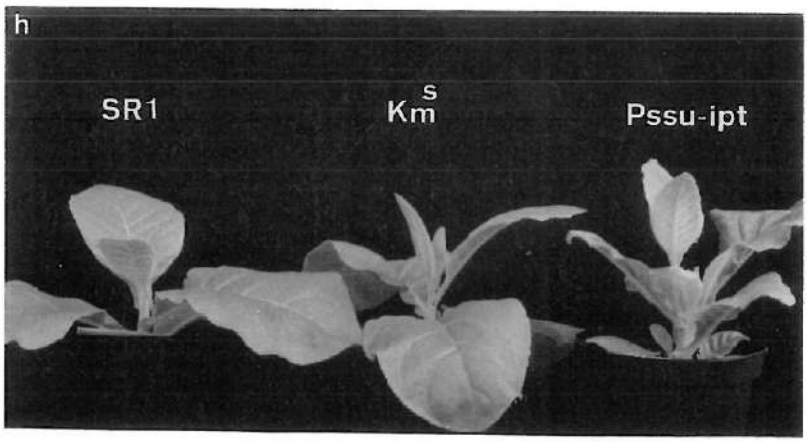


Figure 6.2-continued: **e)** viviparous shoots on leaf tips; **f)** flowers with over-elongated pistil; **g)** highly reduced root system; **h)** SR1, Km^S plants of 4 weeks in soil, and P*ssu-ipt* plants of comparable size, grown for 8 weeks in soil.



On the grafted shoots many of the axillary buds grew out. Together with the short internodes this resulted in a typical bushy appearance (fig. 6.1 a-c). Apical dominance was drastically reduced.
The leaves were small, with a wrinkled lamina. Often viviparous shoots were formed on the leaf tip (fig. 6.1-d). They could also appear on the mid vein in the middle of the leaf, but this was rather exceptional. In young grafts the leaves were light green (fig. 6.1-c). As the grafts got older, the leaves turned either intensely green, or they were bleached between the veins (see fig. 6.1-a,b; for chlorophyll content: see below, table 6.2).
Flowers frequently aborted in an early stage; if not, they showed an over-elongation of the pistil (fig. 6.1-f). Consequently, self pollination was excluded and manual intervention was required. The yield of seeds was lower than in wild type plants. The grafts developed slowly and it was remarkable that complete leaf senescence holded off for a long period (several months).

6.1.1.2 P*ssu-ipt* plants:

A very striking characteristic of these plants was the extreme variability in their growth pattern. Figure 6.2-a shows a collection of plants, all pre-cultivated *in vitro* for 5 weeks and subsequently grown in soil for 11 weeks. Extreme differences in development could be found: some plants had hardly grown after transfer to soil, others were fully grown and were flowering already. After 13 weeks of growth in soil, the stem length of 107 plants has been measured. The date were divided into classes at an interval size of 5 cm. The frequency of each length class is represented in a histogram in figure 6.3. Obviously, the frequency distribution is not unimodal. In this sample two major groups could be distinguished: one containing very small plants, the other consisting of individuals with a stem length centered around 80 - 85 cm. The growth capacity of the first group of plants was very poor. Some of them did not grow at all after transfer to soil; these seedlings died soon after the length measurement was done (13 weeks in soil), which still represents an extremely long period of survival under growth arrest. The second group consisted of slowly growing plants, in comparison with SR1 (wild type) plants, but they did reach maturity. In between these two distinct groups, the distribution pattern is less clear. It corresponds to the large variation in stage of development as was shown in figure 6.2-a.

The maximal stem length of full grown P*ssu-ipt* plants even exceeded that of the wild type. SR1 plants that had completed elongation growth, had a stem length of 91 $\pm$ 5 cm. The transgenic plants often reached a length of more than

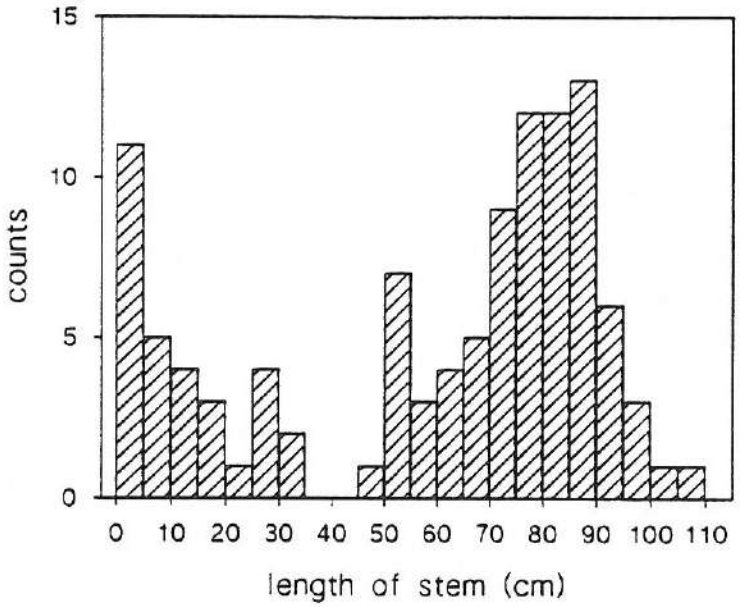


Figure 6.3: Frequency histogram of the stem length of P*ssu-ipt* plants pre-cultivated *in vitro* (5 weeks), and subsequently grown for 13 weeks in soil. (Interval size of the classes: 5 cm; n = 107)

1 meter (see highest length classes in figure 6.3). However, sometimes the elongation of the main stem was not maximal and growth was taken over by one or more side branches.

Budding of the axillary shoots was rather variable: in some plants primarily the basal axillary shoots, in others more apical axillary shoots developed. The extent of this shoot development was also variable, but in all cases sprouting was more pronounced than in wild type plants (fig. 6.2-c,d). In P*ssu-ipt* plants with an average stem length of 60 $\pm$ 10 cm (n = 9), the combined fresh weight of all axillary buds and shoots per plant was 10 $\pm$ 8 g, in comparison to a value of 0.056 g in SR1 plants of 54 $\pm$ 9 cm in height (plants pre-cultivated in soil and grown in large pots for 4 weeks, see previous chapter). However, there is an important difference in bud development between P*ssu-ipt* plants and grafts. In the latter ones, almost all axillary buds immediately grew out, and second to even third order buds emerged. In the P*ssu-ipt* plants the buds were swollen already in an early stage, nevertheless the outgrowth was mostly delayed until flower development had started.

In the P*ssu-ipt* plants the stem diameter at the tenth internode was increased, viz. 12 ± 1 mm in plants of 60 ± 10 cm in height. Internode 10 was situated below the middle of the stem. The basal internodes were shorter than in SR1 plants, and the number of internodes had increased to 22 ± 1. In P*ssu-ipt* transgenic plants of a comparable size as SR1 plants, the stem was composed of a higher number of shorter internodes. A similar observation was reported on PSAUR-*ipt* transgenic tobacco (Li *et al.*, 1994).
The diameter at the middle of the stem of the P*ssu-ipt* plants amounted to 9.1 ± 0.5 mm. This is comparable to the diameter of internode 10 in wild type plants, which is located at the middle of the stem (see previous chapter).

Since the amount of internodes increased, the number of leaves was also elevated. The size of the central leaves was comparable to that of wild type plants or even larger, but their appearance was more like that of the leaves of the P*ssu-ipt* grafts: leaves were wrinkled, viviparous shoots appeared on the leaf tip (fig. 6.2-e) and plants with a dark green leaf pigmentation as well as with bleaching leaves could be found.

In some transgenic plants the inflorescence was reduced; many flower buds did not develop and were aborted. In other plants the inflorescence was rather normal. In all cases, the structure of the flowers was comparable to that of the grafts: the pistil was more or less over-elongated (fig. 6.2-f).

Root and shoot development were not proportional. In figure 6.2-g the highly reduced root system of a mature plant is shown. There was a lag period in the root growth of at least one week after transfer of the seedlings to soil. The length of this lag period and the subsequent rate of root growth might be the cause of the variability in shoot development. Different levels of gene expression are not excluded. At this moment, we have no idea about possible variation in the number of *ipt*-gene copies in these plants. Besides, methylation of the *ssu*-promoter might have occured to different degrees. This gene silencing process has been observed in transformed plants carrying T-DNA genes. Methylation of these T-DNA genes was related to phenotypic variation (Amasino *et al.*, 1984; John and Amasino, 1989).

6.1.2 Cytokinin content:

The endogenous cytokinin content in P*ssu-ipt* grafts has been determined by means of radioimmunoassays (see 2.2.1) and the results have been discussed elsewhere (Beinsberger *et al.*, 1992a and b; Beinsberger, 1993). In summary, these results show that at every moment in the development of the grafts, the ZR content was more abundant in older than in the younger leaves. As the development of the green grafts proceeded, the leaf ZR concentration decreased. The concentration of iPA-equivalents was the highest in the younger tissues, but was still below the value of ZR. It was concluded that in these grafts the expression of the *ipt*-gene was permanent, only controlled by light and not influenced by the developmental stage. In young viviparous shoots the cytokinin content was extremely high, in particular the level of iPA-equivalents. As these shoots developed, the cytokinin content decreased. This enormous cytokinin accumulation is probably responsible for the de-differentiation of leaf cells into meristematic cells (Estruch *et al.*, 1991b).

In the P*ssu-ipt* plants, at one developmental stage a more detailed cytokinin analysis was performed by means of enzym-immunoassays, after a chromatographical separation of different metabolic forms (see 2.2.2). Young plants were chosen and they were compared with SR1 plants of the same stature. Five consecutive leaves were analyzed. These were numbered in a basipetal order: leaf position 1 was the most apical one, leaf position 5 the most basal.
In these P*ssu-ipt* plants the total amount of Z, ZR and the nucleotide forms was higher than in wild type plants (personally communicated by G. Kudoyarova; see table 6.1). In the apical, young leaves the level of the free base, zeatin, was much higher than in older leaves, and it was substantially higher than in SR1 plants. In the ZR content a similar gradient appeared, not differing between transgenic and wild type plants. An opposite gradient was found for the Z-nucleotides: they accumulated predominantly in the basal leaves of the transgenic P*ssu-ipt* plants. In the SR1 plants, the nucleotide concentration remained low in all the leaves, except in the most apical one (leaf position 1).

Table 6.1: Concentration (ng Z-equivalents / g fresh weight) of zeatin, zeatin riboside, nucleotides and the sum of the three (Σ) in a young P*ssu-ipt* plant in comparison with a SR1 plant. Five successive leaves were analyzed. Leaf position 1 corresponds with the most apical, position 5 with the most basal one.

	Leaf position	zeatin	zeatin riboside	zeatin nucleotides	Σ
SR1:	# 1	177	260	25	462
	# 2	45	24	2.8	71.8
	# 3	1.8	7	4	12.8
	# 4	2.5	4	2	8.5
	# 5	4.5	6	3	13.5
P*ssu-ipt* plant:	# 1	600	159	16	775
	# 2	63	54	7	124
	# 3	7.3	8.4	24	39.7
	# 4	7.5	10	72	89.5
	# 5	10	20	315	345

6.1.3 Photosynthetic characteristics:

It is inherent to the study of the photosynthetic characteristics of P*ssu-ipt* grafts and plants that there is no exact control, *i.e.* a transformed plant that develops in the light without expression of the supplementary *ipt*-gene. Due to the light sensitivity of the *ssu*-promoter it is impossible indeed to obtain photosynthetically active P*ssu-ipt* grafts or plants without induction of the *ipt*-gene.

To evaluate the effect of grafting, wild type shoots were grafted on wild type stems. These 'SR1 grafts' exhibited the same morphological characteristics as the SR1 plants and the capacity of the photosynthetic electron transport was not different (data not shown).

Therefore, the transformed grafts and plants were both compared with wild type plants. To take into account the heterogeneity in growth rate of P*ssu-ipt* grafts and plants, they were subdivided in two classes, according to their size. P*ssu-ipt* grafts with branches up to approximately 15 cm were called 'young'. Above that they were considered as old, and a distinction between dark green and pale grafts was being made. If the level of pigmentation is not specified intense green grafts were used.

The P*ssu-ipt* plants were determined as young up to maximally 20 cm in stem length. Larger plants were named old and in accordance with the grafts dark green and pale plants were distinguished. The absolute age of these plants was quite variable, although, in practice P*ssu-ipt* plants were selected for a moderate growth rate, in order to reduce heterogeneity.

Wild type (SR1) and Km^s plants were always used between 3 and 4.5 weeks of growth in large pots in soil.

In figure 6.2-h a SR1, a Km^s and a young P*ssu-ipt* plant are shown side by side. The former two were grown in the large containers in soil for 4 weeks, the latter one for 8 weeks.

In general, the leaves in the middle of the stem, resp. on the branches of the grafts, were sampled, unless otherwise specified. Flowering shoots were excluded for this sampling.

Data were statistically analyzed with a one way analysis of variance, combined with the Fischer's LSD test ($\alpha = 0.01$; two tailed).

6.1.3.1 Chlorophyll and carotenoid content:

Table 6.2 presents the pigment concentration of the plant types previously defined. The pigments were extracted from leaf discs of 10 cm^2 in acetone 80%. Some measurements of the chlorophyll a/b ratio and of total chlorophyll/carotenoids ratio were additionally performed on thylakoid suspensions, diluted 100-fold in acetone 80%. The concentrations were calculated according to Lichtenthaler and Wellburn (1983) (see Materials and Methods, 2.3.1).

Table 6.2: Pigment concentrations (μg / g fresh weight) and ratios of chlorophyll a/b and chlorophyll/carotenoid in leaves of P*ssu-ipt* transgenic grafts, resp. plants and in SR1 plants.
Data marked with the same character, are not significantly different (α = 0.01; ANOVA and Fisher's LSD test; mean $\pm$ standard error). (Chl = chlorophyll; Carot. = carotenoids)

	Chl a	Chl b	Chl a/b	Carot.	Chl/Carot.
SR1	900 $\pm$ 100 bc	220 $\pm$ 40 ab	4.0 $\pm$ 0.3 bc	190 $\pm$ 30 b	6.1 $\pm$ 0.8 a
Graft - young	600 $\pm$ 200 ab	150 $\pm$ 40 a	4.1 $\pm$ 0.4 c	140 $\pm$ 30 a	5.4 $\pm$ 0.5 a
- old - green	1300 $\pm$ 400 d	400 $\pm$ 100 c	3.7 $\pm$ 0.4 b	230 $\pm$ 70 c	7 $\pm$ 1 b
- old - pale	500 $\pm$ 200 a	170 $\pm$ 60 ab	2.8 $\pm$ 0.3 a	80 $\pm$ 20 a	8 $\pm$ 2 b
P*ssu-ipt* plant - green	1100 $\pm$ 300 cd	300 $\pm$ 100 bc	4.0 $\pm$ 0.5 bc	210 $\pm$ 40 bc	6 $\pm$ 1 a

Young grafts were characterized by a lower chlorophyll content in comparison to wild type plants. The chlorophyll a/b ratio was not changed. The carotenoid content was also lower, but not to the same extent as the chlorophyll concentration. This can be seen in the slightly reduced ratio of total chlorophyll/ carotenoid content.

In dark green, older grafts the chlorophyll concentration was significantly increased. The chlorophyll a/b ratio was slightly, but in comparison to wild type plants not significantly, decreased. The carotenoid content was also higher, but not to the same extent as for the chlorophylls. Accordingly, the chlorophyll/

carotenoid ratio was significantly increased. In young and old, green grafts the pigmentation evolved in opposite directions in comparison to SR1 plants.
Old, pale grafts had a low chlorophyll content, like in young grafts, but, the chlorophyll a/b ratio had shifted towards chlorophyll b. This is indicative for a lower content of photosynthetic reaction centres relative to antenna pigments. The total carotenoid content had greatly diminished, so that it even resulted in a very high chlorophyll/carotenoid ratio, comparable to that of dark green grafts. This is expected to have consequences on the dissipation of excess exitation energy, in which carotenoids play an important role (Demmig *et al.*, 1987; Demmig-Adams, 1990; Horton *et al.*, 1991).
In dark green P*ssu-ipt* plants the pigment concentration was only moderately increased as compared to SR1 plants. Although these plants had an intense green appearance, the chlorophyll and carotenoid content on a fresh weight basis was intermediate to that in wild type plants and in dark green grafts.

6.1.3.2 The electron transport capacity:

This was determined *in vitro* on isolated thylakoid membrane suspensions. The membrane isolation was performed as described in Materials and Methods (see 2.3.2.1). The various partial electron transfer reactions measured, are summarized in table 2.1.

The capacity of the electron transport mediated by the two photosystems, PSI and II, was measured with either methylviologen (MV) or ferricyanide as terminal electron acceptor. Nearly the whole electron transport chain is involved in this measurement. In table 6.3 the results of the reactions ($H_2O \rightarrow MV$) and ($H_2O \rightarrow FeCy$) are presented proportional to the electron transport rate measured on thylakoid suspensions of wild type plants (= 100%).

The electron transport capacity of Km^s and SR1 plants was similar. In the transgenic plant material the electron transport was reduced. In the P*ssu-ipt* grafts the reduction amounts to c. 70%. The reduction was less pronounced in the green P*ssu-ipt* plants (30 - 50%, depending on the artificial electron acceptor). In pale P*ssu-ipt* plants the electron trasport rate was more comparable with that in grafts. However, when P*ssu-ipt* plants and grafts with a corresponding level of pigmentation are compared, the electron transport capacity was still lower in the grafts.

After addition of NH_4Cl, the maximal, uncoupled electron transport rate was registered. In table 6.3 the strength of coupling, *i.e.* the uncoupled electron transport rate expressed as percentage of the rate before uncoupling, can be seen

in the 2nd and 4th data column. These numbers are not refering to SR1 as 100% but to the 'coupled' rate of the respective plant material. The effect of the uncoupler was stronger in SR1 and Km^S plants than in the transgenic material, irrespective of the electron acceptor used. The ability to build up a proton gradient across the thylakoid membrane appears to be lower in the P*ssu-ipt* transgenic tissues (see below).

Table 6.3: Capacity of the photosynthetic electron transport mediated by PSI and II, expressed as percentage of the capacity in SR1 plants. The effect of uncoupling, by addition of NH_4Cl, is expressed as percentage of the coupled rate (% uncoupled/coupled rate). Data marked with the same character, are not significantly different. (nd = not determined)

		$H_2O \rightarrow MV$	$H_2O \rightarrow MV$ + NH_4Cl	$H_2O \rightarrow FeCy$	$H_2O \rightarrow FeCy$ + NH_4Cl
SR1		100 ± 20 d	240 ± 30 b	100 ± 9 c	270 ± 40 b
Km^S		86 ± 6 d	260 ± 60 b	90 ± 10 c	290 ± 30 b
Graft	- old - green	30 ± 20 a	150 ± 30 a	39 ± 6 a	180 ± 70 a
	- old - pale	20 ± 20 a	170 ± 20 a	29 ± 5 a	140 ± 30 a
P*ssu-ipt* plant	- young	50 ± 10 bc	180 ± 20 a	62 ± 7 b	180 ± 10 a
	- old - green	50 ± 20 b	nd	70 ± 10 b	nd
	- old - pale	33 ± 9 ab	nd	50 ± 10 b	nd

The electron transport mediated by each photosystem separately was further analyzed by measurement of the partial reactions of the electron transport chain with several electron donor and acceptor combinations.

For the measurements of the electron transport capacity around PSII, water was always the initial electron donor (see table 6.4). The o.e.c. capacity was not separately investigated e.g. by comparing the NH_2OH inhibited and non-inhibited electron transport rate.

Table 6.4: Capacity of the electron transport mediated by PSII, expressed as percentage of the capacity in SR1 plants. Data marked with the same character, are not significantly different. (nd = not determined)

		$H_2O \rightarrow$ PD/FeCy	$H_2O \rightarrow$ DAD/FeCy	$H_2O \rightarrow$ SiMo
SR1		100 ± 30 b	100 ± 8 c	100 ± 20 c
Km^s		nd	110 ± 20 d	nd
Graft	- old - green	100 ± 30 b	70 ± 10 b	80 ± 20 b
	- old - pale	nd	60 ± 7 b	nd
P*ssu-ipt* plant	- young	nd	32 ± 6 a	nd
	- old - green	90 ± 30 b	nd	46 ± 3 a
	- old - pale	30 ± 10 a	40 ± 30 a	30 ± 10 a

With diaminodurene (DAD) as electron acceptor (ferricyanide was added to reoxidize the electron acceptor), a moderate reduction of the electron transport rate can be noted in the grafts. In the P*ssu-ipt* plants the reduction was stronger. p-Phenylenediamine (PD) is an electron acceptor very similar to DAD, although a little more lipophilic. Consequently, PD has a better access to the lipid membrane phase where the interaction with the quinone pool takes place (Izawa,

1980). The reduction in electron transport rate measured with DAD in green grafts was no longer noticable with PD. This could indicate that in the transgenic grafts the accessibility of the artificial acceptors to the electron carriers is altered, without any serious change in the activity of the reaction centre of PSII.
Also in old, green P*ssu-ipt* plants the capacity measured with PD was comparable with that in SR1 plants. But in the pale transgenic plants still a considerable reduction was found. In dark green P*ssu-ipt* plants the activity with DAD was not measured. Hence, from the large difference in activity with PD between green and pale P*ssu-ipt* plants, it can not be concluded yet whether in the green specimens the accessibility to the membrane embedded carriers had changed, whereas in the pale ones additionally the reaction centre activity was altered.

The PSII mediated activity was also measured with silicomolybdate (SiMo). This rate better represents the electron transport of the PSII complex, since SiMo is capable to accept electrons from the primary quinone electron acceptor (Q_A) of PSII. For this measurement, the thylakoid suspension was diluted in the assay medium (pH 7.8) in a darkened oxygen electrode. DCMU (2 μM) was added, and the dark signal was recorded during 1 min. SiMo (0.1 mM) was added and only after a short equilibration in darkness (approximately 30 sec), saturating light was given. At pH 7.6 the interaction between SiMo and DCMU is apparently competitive, and notwithstanding the higher affinity of PSII for DCMU at this alkaline pH (Schansker and van Rensen, 1993), the respective concentrations still provide fast displacement of DCMU for SiMo. Therefore, the dark equilibration is indispensable. In our experimental set-up it might have been not sufficiently long for obtaining the maximal displacement, but the length of the dark period was equal in all measurements. The initial rate upon illumination was recorded.

The electron transport activity in old, green grafts was only moderately reduced, in agreement with the results obtained with PD and DAD. In old, pale P*ssu-ipt* plants a strong reduction in PSII activity was noted, suggesting that the lower electron transport rate found with DAD or with PD is primarily evoked by PSII reaction centre limitations and not by thylakoid membrane alterations. In green P*ssu-ipt* plants also the electron transport rate measured with SiMo was low, suggesting that the electron transport capacity through PSII reaction centres is diminished. However, the large discrepancy between PD- and SiMo-mediated PSII electron transport measurements is not understood.

PSI-mediated electron transport was measured in the presence of DCMU, with MV as terminal electron acceptor and with different electron donors: reduced DAD, DCPIP or TMPD (table 6.5).

The activity in Km^S plants was, with the different electron donors, nearly the same as in wild type plants.

With $DADH_2$ as electron donor, the PSI-mediated electron transport was 60 to even 80% reduced in P*ssu-ipt* plants and grafts, respectively. With $DCPIPH_2$, the reduction was less pronounced. These two electron donors differ in their potential electron donation sites. $DADH_2$ is assumed to donate electrons at or near the oxidizing side of the cytochrome f-region. $DCPIPH_2$ is a cytochrome f/plastocyanin region electron donor. Additionally, DCPIP may have access to P700 directly in its phenolate anion form ($DCPIPH^-$; Hauska *et al.*, 1975). Because in the presence of a proton gradient $DCPIPH^-$ is expelled from the thylakoid lumen, this electron donation is dependent on membrane integrity (Hauska *et al.*, 1975; Izawa, 1980). In the P*ssu-ipt* grafts and plants, coupling was reduced (see before); this could signify that the membrane integrity is weakened. Accordingly, the direct reduction of P700 could be substantial, and the measurement with $DCPIPH_2$ in the transgenic grafts and plants would give a closer approximation of the PSI reaction centre activity. The results in table 6.5 indicate that the activity of the reaction centre is maximally reduced by 20 - 30% and 50 - 60% in P*ssu-ipt* plants and grafts, respectively. This suggests that the larger reduction measured with $DADH_2$ is not situated at the reaction centre of PSI, but rather at the cytochrome b_6/f complex.

TMPD has electron donor properties very similar to $DADH_2$. The lipophilic nature and the redoxpotential allow electron donation near the cytochrome f-region. The main difference is that TMPD is a one electron donor, while $DADH_2$ exchanges at the same time protons and electrons. Accordingly, the reaction with TMPD is not coupled to photophosphorylation, in contrast to that with $DADH_2$ (Hauska, 1977).

The activity with TMPD confirms the reduction of PSI activity measured in grafts with $DADH_2$. In green P*ssu-ipt* plants the TMPD-activity is decreased to the same extent as in young or pale P*ssu-ipt* plants, measured with $DADH_2$. In contrast, in pale transgenic plants the reaction (TMPD → MV) is only a little less than in SR1 plants, and corresponds to the electron transport rate measured with $DCPIPH_2$. The limitations in the electron transport chain preceding P700, the reaction centre of PSI, (as registered with $DADH_2$) seem to be abolished with TMPD in the pale P*ssu-ipt* plants. This suggests that the establishment of a

proton gradient is the controlling factor.
In the green grafts the reaction with TMPD was not essentially improved in comparison to the reaction with $DADH_2$. In these grafts the effect of uncoupling was the lowest (see table 6.3; reaction $H_2O \rightarrow MV$). Besides, there is further evidence that in the grafts the build-up of the pH gradient is disturbed (see 6.1.3.6). This supports the idea that indeed the reduction in the electron transport reaction ($DADH_2 \rightarrow MV$) in the pale transgenic plants is related to the back-pressure of a pH gradient; on the other hand a stronger reduction in the PSI reaction centre activity may be concluded in the green grafts.

Table 6.5: Capacity of the electron transport mediated by PSI, expressed as percentage of the capacity in SR1 plants. Photosystem II was inhibited by DCMU. Data marked with the same character, are not significantly different. (nd = not determined)

		$DADH_2 \rightarrow MV$	$DCPIPH_2 \rightarrow MV$	$TMPD \rightarrow MV$
SR1		100 ± 10 d	100 ± 8 d	100 ± 20 c
Km^S		80 ± 10 c	97 ± 8 cd	nd
Graft	- old - green	20 ± 10 a	50 ± 10 a	30 ± 20 a
	- old - pale	30 ± 20 ab	43 ± 5 a	nd
Pssu-ipt plant	- young	40 ± 6 b	70 ± 20 b	nd
	- old - green	nd	nd	42 ± 7 a
	- old - pale	39 ± 1 ab	80 ± 10 bc	80 ± 20 b

6.1.3.3 The effect of the inhibitor DBMIB:

In order to obtain more insight in the functioning of the cytochrome b_6/f complex, the influence of DBMIB (= 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone) was tested. This was realized by addition of DBMIB during *in vitro* measurements of the whole chain electron transport ($H_2O \rightarrow MV$) on isolated chloroplasts (cf. results in 6.1.3.2).

On the one hand this inhibitor is known to interfere at the oxidation site for plastoquinol (the Q_Z-site; Jones and Whitmarsh, 1987), blocking the electron donation to the (Fe-S) centre of the cytochrome b_6/f complex. A putative second binding site with a lower affinity, by which DBMIB inhibits the electron flow from the (Fe-S) centre to cytochrome f has been suggested (Jones and Whitmarsh, 1988; fig. 6.4). On the other hand the molecule has distinct redox properties (Rich *et al.*, 1991). As a benzoquinone derivative, a differential distribution across the thylakoid membrane can be expected as a proton gradient develops (Hauska *et al.*, 1975). Besides, Rich *et al.* (1991) have shown that cytochrome f reduction by $DBMIBH_2$ via the Q_Z site or via the plastocyanin binding site is pH dependent. A proper control of the pH at the site of action of DBMIB is desirable. Therefore, NH_4Cl was added before the addition of DBMIB, to unload the proton gradient established by the coupled electron transport. Thereby differences in the coupling status between thylakoid suspensions from wild type or transgenic plants or grafts were eliminated.

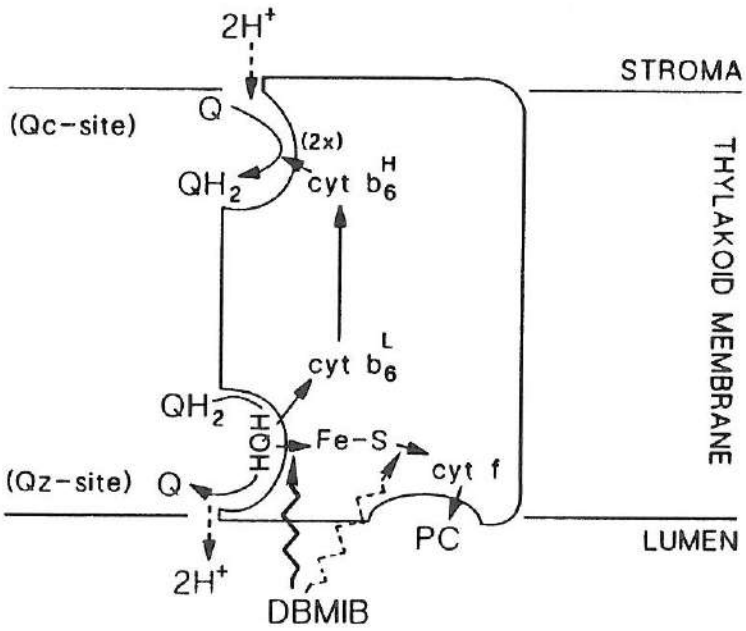


Figure 6.4: Schematic representation of the cytochrome b_6/f complex, with indication of the proposed inhibition sites of DBMIB.

The assay medium for the polarographic measurements was essentially the same as given in Materials and Methods (see 2.3.2.2a), but the pH was adjusted either to 8.0 by Hepes-KOH (20 mM) or to pH 6.0 by MES-KOH (20 mM). The thylakoid isolation was identical to that described in Materials and Methods (see 2.3.2.1). The chlorophyll content in the cuvette was always 50 μg/ml, to respect the chlorophyll/inhibitor ratio.

The redox characteristics of DBMIB also make that it can function as electron acceptor. At high concentrations, DBMIB acts as PSII electron acceptor in a reaction that supports phosphorylation (Izawa, 1980). Therefore, a concentration range from 0.1 to 10 μM (final concentration in the cuvette) was tested.

The figures 6.5-a and -b demonstrate the influence of DBMIB applied in the light to the uncoupled electron transport reaction ($H_2O \rightarrow MV$), measured at pH 8.0 (fig. 6.5-a) or at pH 6.0 (fig. 6.5-b). The rate of electron transport after inhibition is expressed as a percentage of the uncoupled electron transport rate and plotted against the concentration of DBMIB. Wild type plants, old, green P*ssu-ipt* grafts and green P*ssu-ipt* plants were used. The uninhibited rate is fixed at 100% in the 3 plant series.

At the more alkaline pH the net inhibitory effect of DBMIB was stronger. This becomes manifest when comparing the data of wild type plants at pH 8.0 vs. pH 6.0. However, in the presence of duroquinone, DBMIB was shown to be rapidly reduced, resulting in a weaker inhibition (Rich *et al.*, 1991). It can be argued that in the reduced form the PSII electron acceptor properties of $DBMIBH_2$ are minimal. At pH 6.0 the proportion of the oxidized form may have been relatively larger. Accordingly, DBMIB may intercept electrons from the electron transfer chain, and thus the reduction of MV^{2+} is less. This should also result in a lower oxygen consumption, due to the diminished auto-oxidation reaction of MV^{+}. This is expected to occur at the highest concentration of DBMIB only. However, this does not agree with our results: at any concentration of DBMIB, the net inhibition recorded was stronger at pH 8.0 than at pH 6.0.

It should be mentioned that at more acidic pH the uninhibited electron transport rate was lower (only 27% of the rate measured at pH 8.0). It has been shown that acidification to a pH value below 5.5 causes the inactivation of PSII (Krieger and Weis, 1992). Furthermore, the surface charge density of the thylakoid membrane is pH-dependent. This might affect the interaction with the artificial electron transport mediators, like DBMIB. These elements should be

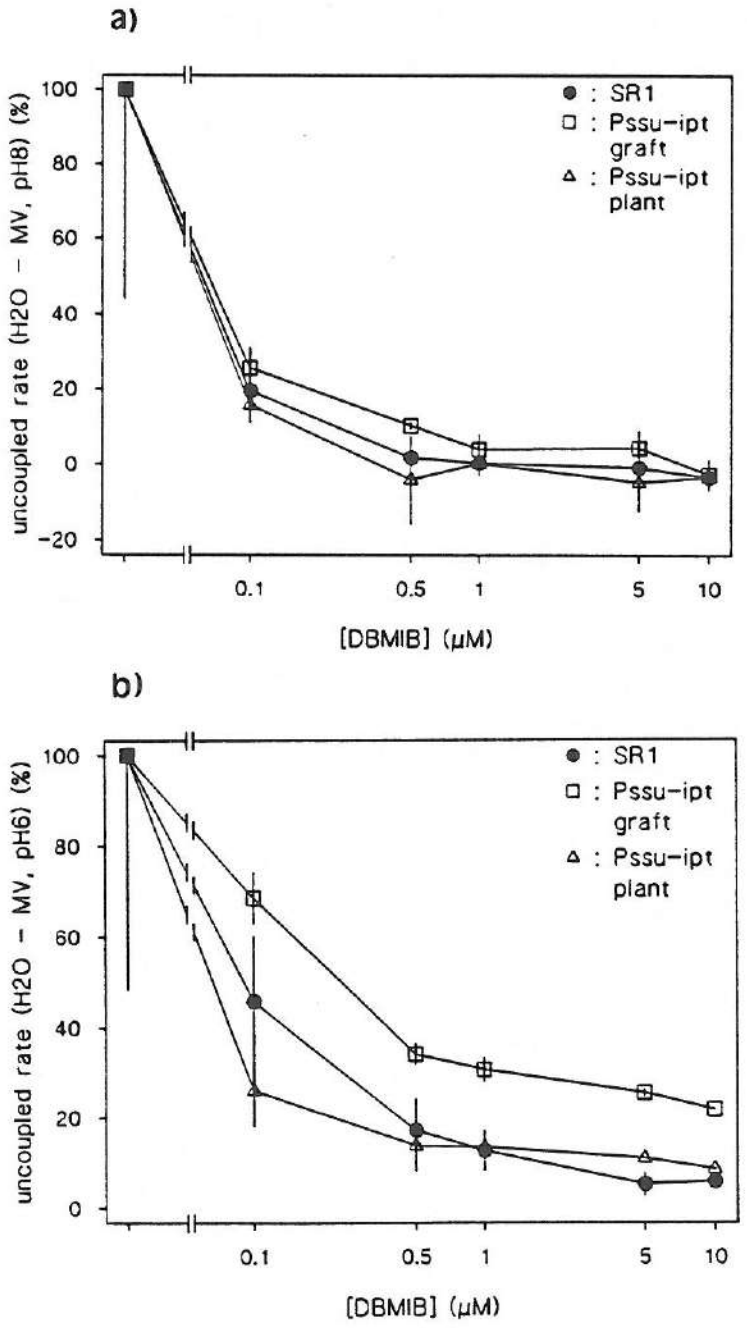


Figure 6.5: Effect of DBMIB on the uncoupled electron transport capacity ($H_2O \rightarrow MV$), measured at pH 8.0 **(a)** or pH 6.0 **(b)** in SR1 plants, green P*ssu-ipt* grafts and green P*ssu-ipt* plants. The results are presented as the DBMIB-inhibited rate, expressed as a percentage of the uncoupled rate before inhibition vs. the concentration of DBMIB.

incorporated when explaining the differences in inhibition by DBMIB between both pH values.

Furhter, a comparison between the transgenic and wild type plant material was made. At either pH, at the lowest DBMIB concentration (0.1 μM) no significant differences between P*ssu-ipt* grafts, P*ssu-ipt* plants or SR1 plants were found. At higher concentrations the effect of DBMIB was less pronounced in the transgenic grafts. At pH 8.0 this effect was not significant, but at pH 6.0 after addition of 1 to 5 μM DBMIB, its inhibitory effect on the electron transport was significantly lower in grafts than in wild type plants. In the P*ssu-ipt* plants, however, even at high DBMIB concentrations there was no significant difference with SR1 plants. These results, although preliminar, point to alterations of the cytochrome b_6/f complex in the grafts only.

6.1.3.4 Photophosphorylation:

The ATP/ADP ratio was comparatively determined in thylakoid samples illuminated for 1 min, or kept in the dark (see 2.3.2.3). In figure 6.6 these ratios are presented on a logaritmic scale. Before illumination, in the dark adapted samples, no significant difference could be shown between SR1, Km^S and P*ssu-ipt* transgenic samples. In green P*ssu-ipt* grafts this ratio appeared to be higher in the dark adapted sample, although, the standard error on these results was large.

After 1 min of illumination with actinic light, the ATP/ADP ratio had increased in the thylakoid samples of SR1, Km^S and P*ssu-ipt* plants. Photophosphorylation had clearly taken place in these thylakoid suspensions. In contrast, in the P*ssu-ipt* grafts the ATP/ADP ratio hardly changed upon illumination. Consequently, this ratio was significantly lower than in wild type plants. No significant difference could be found between the P*ssu-ipt* grafts and Km^S and P*ssu-ipt* plants, due to the variation within each plant type. Nevertheless, figure 6.6 clearly shows that in the samples of Km^S and P*ssu-ipt* plants photophosphorylation took place, whereas in those from the P*ssu-ipt* grafts no such activity could be demonstrated. These data do not indicate at which step ATP-synthesis is blocked in the grafts, but previous results suggest that the build up of a proton motive force may be limiting (see the effect of uncoupling, table 6.3). Further evidence will be collected from the analysis of the fluorescence quenching (see 6.1.3.6).

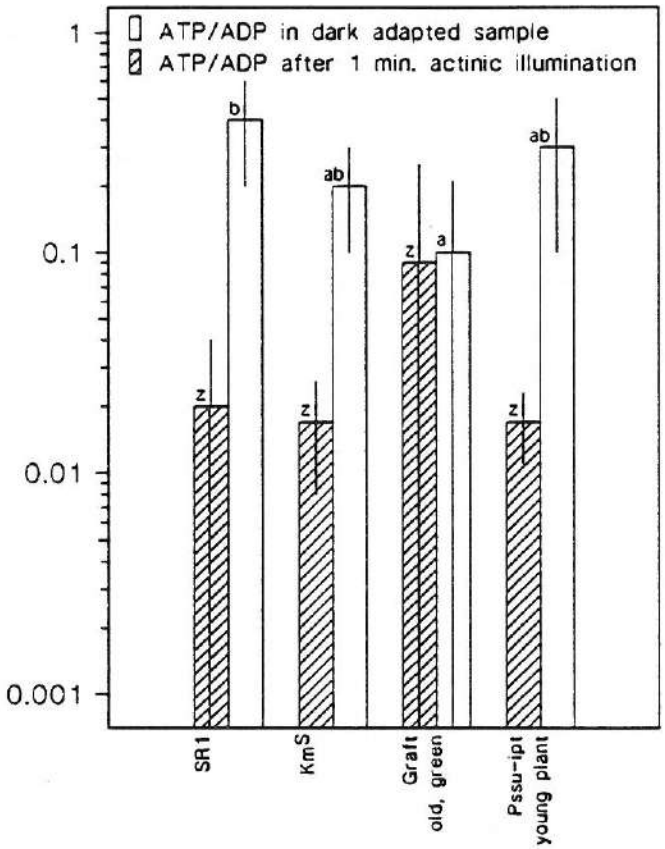


Figure 6.6: ATP/ADP ratio of wild type (SR1), Km^S and young P*ssu-ipt* plants, and P*ssu-ipt* grafts in a dark adapted sample and after 1 min of actinic illumination. The results of the dark adapted samples were statistically compared among one another and similarly for the illuminated samples. Data marked with the same character are not significantly different ($\alpha = 0.01$).

6.1.3.5 Photosynthetic activity, measured with the leaf disc electrode:

Light response curves were drawn up after measurement of the fluorescence induction kinetics in the three step procedure as described in Materials and Methods (see 2.3.3).

The fluorescence induction was measured at a slow time scale. Under these conditions, precise analysis of the initial phases of the induction sequence (OIDP-phases; Schreiber, 1983) is not possible, because this phenomenon is over within a second range. However, the dark-light transition procedure applied was more informative in the subsequent decay to a steady state through a number of successive M-peaks. This oscillatory decay was prominent in leaf discs from wild type plants. The oscillation was also discernible in the rate of oxygen evolution (see fig. 6.7 a). This oscillation was anti-parallel to the one in the fluorescence trace, with a little phase shift: a maximum in the fluorescence yield anticipated a minimum in O_2-evolution. This is in agreement with earlier reports (Walker, 1981; Walker *et al.*, 1983). However, in P*ssu-ipt* plants the oscillations were often diminished and in old P*ssu-ipt* grafts they were completely absent, as demonstrated in figure 6.7 b.

Different parameters from the light response curves were calculated as before (see 4.5.2). For these measurements green grafts and green, middle-aged P*ssu-ipt* plants (stem length of approximately 25 cm) were used.

The apparent quantum requirement (QR), dark respiration (D.Resp.) and the light compensation point (L.C.P.) were not significantly different in wild type and transgenic grafts and plants (see table 6.6). This means that the limitations imposed in the low light intensity region were similar for the different plants and grafts. In contrast, at light saturation, the maximal photosynthetic rate (Pmax) was significantly altered. In young grafts, Pmax was increased in comparison to SR1 plants. In the distal (*i.e.* the youngest) leaves of old grafts, Pmax was lower than in wild type plants. This effect was not significant. In old (the more proximal) leaves of the P*ssu-ipt* grafts and in P*ssu-ipt* plants Pmax was intermediate to that in wild type plants and in young grafts. Expressed on a chlorophyll basis (see table 6.7), the value of Pmax in the old leaves of grafts and in the leaves of the P*ssu-ipt* plants is more similar to that in SR1 plants. Table 6.7 shows the corresponding chlorophyll content and the chlorophyll a/b ratio of the leaf discs that were assayed. These data, on an area basis, are in agreement with those in table 6.2, showing a reduction in chlorophyll concentration in the young grafts. In the green P*ssu-ipt* plants and in the older leaves of the old grafts the total chlorophyll content was increased. Also the chlorophyll a/b ratio

corresponds to that in table 6.2. Looking at Pmax on a chlorophyll basis, an inverse relationship with the chlorophyll content becomes evident (see discussion). The light utilization capacity (L.U.C.) and the convexity coefficient (Θ), both calculated parameters giving an indication of the sharpness of the transition between the low and high light intensities, were both higher in wild type than in transgenic plants and grafts (see table 6.6).

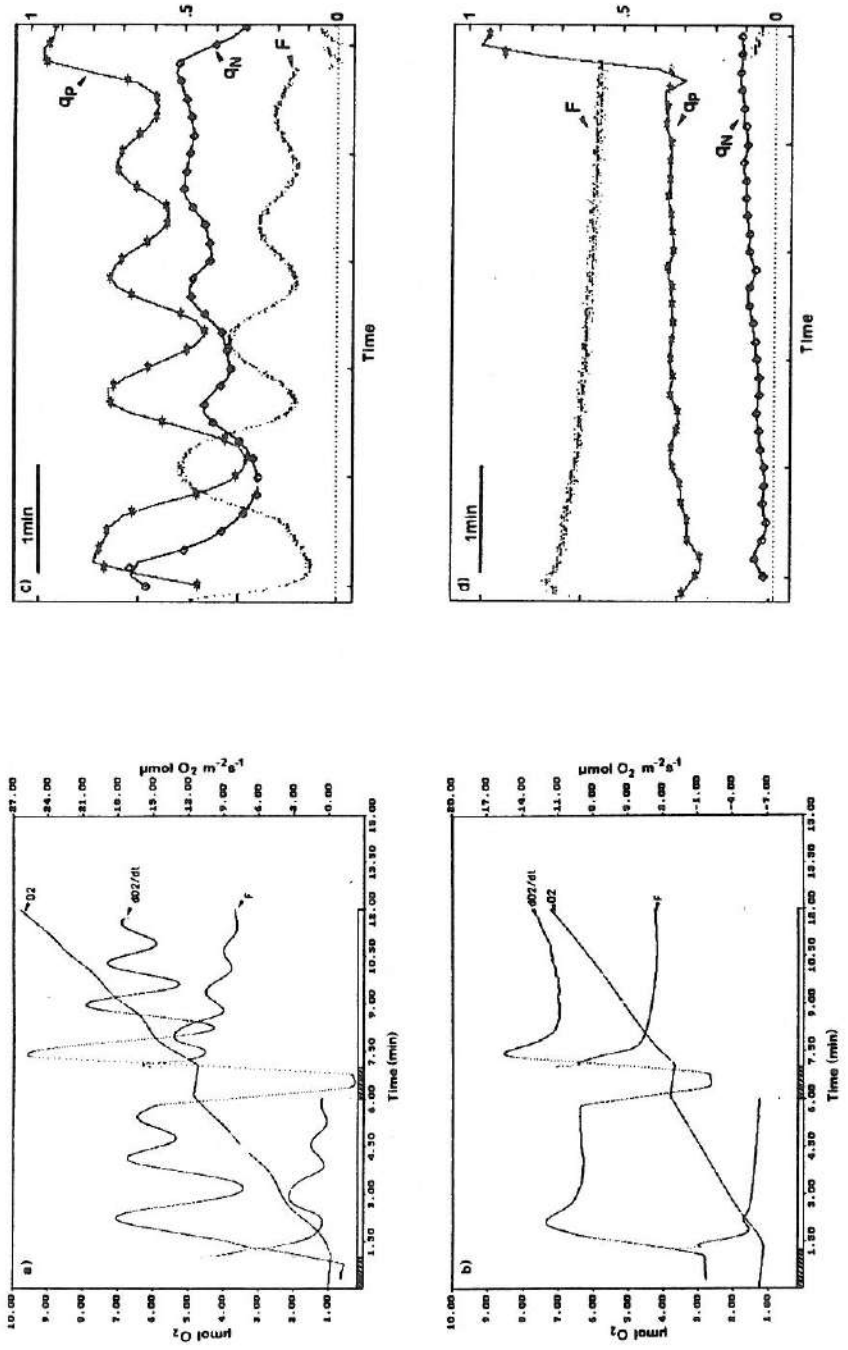


Figure 6.7: Left: Example of the course of chlorophyll fluorescence (F; arbitrary units) and the oxygen evolution (O_2; μmol O_2) measured simultaneously, after a wake up procedure, on a leaf disc **a)** from a young SR1 plant and **b)** from an old, green P*ssu-ipt* graft. The calculated rate of oxygen evolution (dO_2/dt; μmol O_2 $m^{-2}s^{-1}$) is also shown. The dark-light cycle during the measurement is indicated by the bar (hatched: dark; open bar: light) at the bottom of the respective figures. For further details, see Materials and Methods (2.3.3).
Right: Course of the chlorophyll fluorescence (F; dotted trace; arbitrary units) and of the quenching parameters q_P (*; right Y-scale) and q_N (◇; right Y-scale) upon a dark-light transition, following a wake up procedure, as described in Materials and Methods (2.3.4.2.2). The measurement was performed on a leaf disc **c)** from a SR1 plant and **d)** from an old, green P*ssu-ipt* graft.

Table 6.6: Parameters from the light response curve of P*ssu-ipt* transgenic grafts or respective plants and wild type plants, measured at the end of a photoperiod. Data marked with the same character, are not significantly different. (QR = apparent quantum requirement; Pmax = maximal photosynthetic rate (μmol O_2 m^{-2} s^{-1}); D.Resp. = dark respiration (μmol O_2 consumption m^{-2} s^{-1}); L.C.P. = light compensation point (μmol quanta m^{-2} s^{-1}); L.U.C. = light utilization capacity (%); Θ = convexity coefficient)

	QR	Pmax	D.Resp.	L.C.P.	L.U.C.	Θ
SR1	11 ± 1 a	11 ± 2 a	1.9 ± 0.4 a	23 ± 5 a	93 ± 2 b	0.75 ± 0.08 b
Graft - young	12.2 ± 0.6 a	15 ± 4 b	1.9 ± 0.3 a	24 ± 3 a	89 ± 1 a	0.6 ± 0.1 a
- old -young leaf	10 ± 1 a	8 ± 4 a	2.0 ± 0.2 a	21 ± 1 a	91 ± 3 ab	0.6 ± 0.2 a
- old -old leaf	11 ± 1 a	13 ± 2 ab	1.78 ± 0.07 a	21 ± 2 a	89 ± 2 a	0.5 ± 0.1 a
P*ssu-ipt* plant - green, middle-aged	11 ± 1 a	12 ± 2 ab	2.2 ± 0.3 a	28 ± 6 a	89 ± 1 a	0.52 ± 0.07 a

Table 6.7: Maximal photosynthetic rate expressed on a chlorophyll base (μmoles O_2 mg(Chla+b)$^{-1}$ h^{-1}) and the respective chlorophyll content (mg m^{-2}) and chlorophyll a/b ratio; data corresponding with table 6.6. Data marked with the same character, are not significantly different.

	Pmax	Chl (a+b)	Chl a/b
SR1	200 ± 40 b	210 ± 20 ab	4.1 ± 0.2 a
Graft - young	300 ± 40 c	180 ± 50 a	4.1 ± 0.3 a
- old -young leaf	130 ± 50 a	240 ± 10 bc	3.9 ± 0.2 a
- old -old leaf	160 ± 20 ab	300 ± 50 c	3.8 ± 0.6 a
P*ssu-ipt* plant - green, middle-aged	200 ± 60 ab	230 ± 50 bc	4.2 ± 0.2 a

6.1.3.6 Photochemical yield and fluorescence quenching parameters:

The chlorophyll a fluorescence was measured at room temperature with a Pulse Amplitude Modulation fluorometer in a saturating flash mode, which allows the analysis of the quenching parameters (see 2.3.4.2). A wake up procedure consisting of 5 min of actinic light, followed by a 3 min dark period, was given prior to the measurement. As such, a similar dampening oscillation was induced as had been shown in the coupled measurement of chlorophyll fluorescence and oxygen evolution (fig. 6.7 a-b). For comparison, in figure 6.7 c-d the fluorescence kinetics, with the calculated values of q_P and q_N of a SR1 plant and a P*ssu-ipt* graft are shown.

Since F_o and F_M were determined immediately after the wake up, the values are only approximate. These values have been used for calculation of the following parameters: Φ_p^o, Φ_p, q_P and q_N.

The fluorescence quenching parameters q_P (photochemical quenching) and q_N (non-photochemical quenching) were calculated automatically with the data processor DA100. There, the assumption is made that F_o remains constant throughout the measurement. No q_o quenching is considered. Consequently, q_P and q_N were calculated after every saturating flash, without corresponding determination of the F_o-level immediately after the flash illumination.

The photochemical and non-photochemical quenching are defined as:

$$q_P = (F_M'-F) / (F_M'-F_o') \text{ and}$$

$$q_N = 1 - [(F_v'/F_o') / (F_v/F_o)] ,$$

with F_M' and F_o' being the fluorescence intensities with all PSII reaction centres closed, respectively open, in a light adapted state; F the fluorescence intensity just before the saturating flash; $F_v = (F_M-F_o)$, *i.e.* the maximal variable fluorescence with F_M and F_o the maximal (reaction centres closed) and minimal (reaction centres open) fluorescence intensity in the dark adapted state, and corresponding $F_v' = (F_M'-F_o')$ in a light adapted state (van Kooten and Snel, 1990).

With the assumption made that $F_o = F_o'$, q_P and q_N can be calculated as:

$$q_P = (F_M'-F) / (F_M'-F_o) \text{ and}$$

$$q_N = (F_M-F_M') / (F_M-F_o) .$$

In table 6.8 the near steady state values of q_P and q_N are given. In young P*ssu-ipt* grafts only, q_P was significantly increased. These grafts were also characterized by a high rate of photosynthesis at light saturation (Pmax; see 6.1.3.5). There was no difference in q_P between the SR1 plants, the old grafts (green and pale) and the old, green P*ssu-ipt* plants.

The non-photochemical quenching component (q_N) was strongly reduced in all P*ssu-ipt* grafts. This suggests that under continuous actinic illumination, the membrane energization is reduced in comparison with wild type plants, although, these results must be interpreted with care, because of the inaccuracy of F_M. The more F_M is underestimated, the lower the value of q_N. Since F_M was measured by a saturating light flash, q_P is definitely eliminated (Bilger and Schreiber, 1986; Bradbury and Baker, 1981). At the end of the wake up period (after 3 min in darkness), q_N-relaxation was probably not completed. The remaining q_N could have interfered in the F_M determination. A lower calculated value of q_N in the subsequent light period, as is observed in the grafts, can also be explained by assuming a slower decay of q_N in the dark, resulting in a lower apparent F_M. However, the lower coupling status, demonstrated before (see table 6.3), gives evidential support for a reduced membrane energization.

Table 6.8: Characteristics of chlorophyll a fluorescence, at room temperature, of P*ssu-ipt* transgenic grafts or resp. plants and wild type plants. Data marked with the same character, are not significantly different. (q_P, photochemical quenching $= (F_M' - F) / (F_M' - F_o)$; q_N, non-photochemical quenching $= (F_M - F_M') / (F_M - F_o)$; Φ_p°, maximal quantum yield for photochemistry in PSII $= F_v / F_M$; Φ_p, actual quantum yield $= (F_M - F_S) / F_M$)

		q_P	q_N	Φ_p°	Φ_p
SR1		0.5 ± 0.2 a	0.6 ± 0.1 b	0.74 ± 0.02 a	0.59 ± 0.04 bc
Graft	- young	0.8 ± 0.1 b	0.16 ± 0.03 a	0.80 ± 0.02 c	0.70 ± 0.09 c
	- old - green	0.4 ± 0.2 a	0.13 ± 0.06 a	0.74 ± 0.03 a	0.4 ± 0.1 a
	- old - pale	0.6 ± 0.2 a	0.14 ± 0.05 a	0.76 ± 0.02 ab	0.5 ± 0.1 ab
P*ssu-ipt* old, green plant		0.5 ± 0.2 a	0.4 ± 0.3 b	0.78 ± 0.02 bc	0.5 ± 0.2 abc

In the old P*ssu-ipt* plants q_N was only slightly reduced, compared to wild type plants. Neither coupling status, nor photophosphorylation have been measured in comparatively old P*ssu-ipt* plants. However, in young transgenic plants, ATP-synthesis was active and the coupling was intermediate to that in wild type plants and P*ssu-ipt* grafts (see fig. 6.6 and table 6.3). This supports the hypothesis that in the transgenic plants thylakoid membrane energization was only moderately diminished, in contrast to the more drastic effects in grafts.

The maximal quantum yield or maximal photochemical efficiency of PSII (Φ_p°) is defined for the dark adapted state as:

$$\Phi_p^\circ = F_v / F_M$$

with F_v being the maximal variable fluorescence ($= F_M - F_o$) and F_M the maximal fluorescence intensity under conditions where all PSII reaction centres are open. The data presented in table 6.8 were calculated with the approximate F_M and F_o values that were determined after the wake up. When in SR1 plants the wake up was omitted, than Φ_p° was a little enhanced (data not shown). This indicates that indeed F_M was underestimated. Consequently, it must be kept in mind that Φ_p° was also slightly underestimated.

In young grafts Φ_p° was increased in comparison to older grafts and to SR1 plants. Also in P*ssu-ipt* plants Φ_p° was higher than in green, old grafts and in wild type plants in particular. In old grafts Φ_p° is not different from the value in SR1 plants. This indicates that in the transgenic plant material *in vivo* the photochemical charge separation in the reaction centre of PSII is certainly not limiting when the reaction centres are open. Refering to the results of the electron transport capacity of PSII measured *in vitro* (see 6.1.3.2), in old, green grafts a moderate reduction in reaction centre activity was concluded and in old, green P*ssu-ipt* plants the results were contradictory, pleading either for a low or for a high inhibition at the PSII reaction centres (see table 6.4; cf. the reactions measured with SiMo and with PD). The *in vivo* results, presented here, provide additional evidence for a good preservation of the PSII reaction centre activity, both in grafts and in green P*ssu-ipt* plants.

Under steady state conditions, the actual quantum yield, Φ_p, was calculated here as:

$$\Phi_p = (F_M - F_S) / F_M$$

with F_M the maximal fluorescence intensity under dark conditions and F_S the fluorescence intensity at steady state photosynthesis. It is self-evident that also this parameter is underestimated.

In young grafts this value was increased, although not significantly; in old grafts, on the contrary, it was lowered, in particular in the dark green ones. The green P*ssu-ipt* plants take an intermediate position between the old, green grafts and SR1 plants. This pattern corresponds to the differences found for q_P. High values of q_P reduce F_S, and consequently Φ_P increases. Thus, under steady state conditions in old, green grafts the actual photochemical efficiency of PSII appears to be limited by the redox state of Q_A. As the maximal quantum yield is not reduced, this leads to the conclusion that the limitation generated in the light is imposed on the acceptor side of PSII.

6.1.3.7 Redox kinetics of P700:

The redox state of P700, the reaction centre of PSI, can be estimated from the absorbance change at 820 nm. As described in Materials and Methods (see 2.3.5) the redox kinetics of P700 following after a flash illumination or after a period of continuous actinic illumination, give information on the functional size of intersystem electron pools or stromal electron pools, respectively. Here, the kinetics upon a 50 msec multiple turnover (MT) flash and a 3 min actinic illumination were traced. During the actinic illumination the magnitude of P700 oxidation showed a complex oscillatory behaviour, dampening towards a stationary level, as could be expected from the oscillations in PSII photochemistry (an example is shown in figure 2.5).

The MT-area and AL-area were quantified after the MT-flash and actinic illumination respectively, as depicted in figure 2.5 (see 2.3.5). The ratio of the areas (AL-MT)/MT was calculated. This relation corresponds to the ratio of the functional pool size of stromal electrons to that of intersystem electrons.

In figure 6.8 this ratio is presented vs. the fluence rate that was used for the 3 min actinic illumination. With the lowest light fluence rates, negative values were obtained. This means that during the 3 min illumination the

functional intersystem pool was not even fully reduced, as it was attained with the 50 msec flash (Schreiber *et al.*, 1988; Schreiber *et al.*, 1989). With increasing irradiance, the ratio (AL-MT)/MT rapidly rose to a plateau level. In wild type plants its maximal value was on average 6. In young P*ssu-ipt* grafts, in old leaves of old, green grafts and in green, middle-aged P*ssu-ipt* plants the average value ranged between 5 and 6. In young leaves of old, green grafts, the (AL-MT)/MT ratio followed the same course as in the previously mentioned plant material under low irradiance. At higher light intensities, 55 and 96 W m^{-2}, the ratio decreased to 2. Only at very low irradiance levels (1.7 and 1.95 W m^{-2}) the (AL-MT)/MT value of SR1 leaves was statistically different from that in young grafts. The physiological significance of this can be questioned.

More relevant for discussion was the fact that the absolute maximal value of (AL-MT)/MT, registered here in tobacco, was very high. This is in contradiction with results of Asada *et al.* (1992): in several C_3 plants they recorded values for the ratio of the areas AL/MT in the range of 2 to 3, and accordingly the ratio (AL-MT)/MT is still one unit lower. In the C_4 species maize, a much higher value was found: with an actinic light intensity above 65 W m^{-2}, the AL/MT-area ratio was estimated to be about 10 (Asada *et al.*, 1993).
Under anaerobiosis, the ratio AL/MT could be increased in C_3 plants. Under these conditions, the MT-area is not affected, but the AL-area is increased, possibly as a result of a cyclic electron flow through PSI. This might give an explanation for the high (AL-MT)/MT values, found in this study. To supply the assayed leaves with CO_2, a matting moistened with a 1 M carbonate/bicarbonate solution (pH 9) was placed under the leaf. This provides conditions for CO_2-saturating photosynthesis by increasing the CO_2-concentration relative to O_2 (Öquist and Chow, 1992). A high CO_2-concentration stimulates carbon assimilation, until ATP becomes limiting. As 1,3-diphosphoglycerate is no longer synthesized, 3-phosphoglyceraldehyde cannot be formed and the NADPH consumption declines (Walker *et al.*, 1983). With the accumulation of NADPH, linear electron flow is temporary decreased, in favour of the cyclic pathways (Laisk *et al.*, 1991; Weis, *et al.*, 1990), until the ATP/NADPH ratio is re-balanced. Therefore, under the experimental conditions as were described in Materials and Methods (see 2.3.5.2), a strong accumulation of NADPH or reduced intermediates of cyclic electron transfer in the stroma is suggested. This appears to be of the same order of magnitude in the wild type plants and the transgenic plants and grafts.

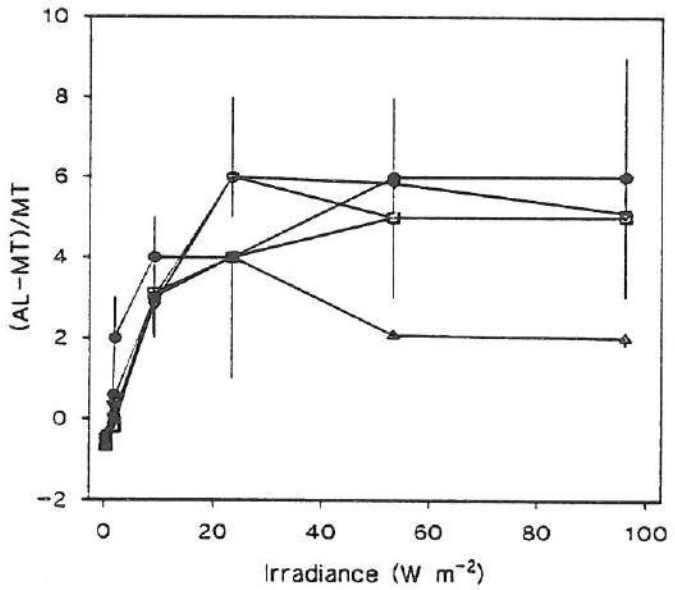


Figure 6.8: The ratio of the areas (AL-MT)/MT determined from the re-oxidation kinetics of P700 after 3 min actinic illumination (AL) and after a 50 msec multiple turnover flash (MT) vs. the actinic light intensity (W m^{-2}), in SR1 plants, in green, old P*ssu-ipt* grafts (young and old leaves) and in green, middle-aged P*ssu-ipt* plants. (mean ± standard error)

6.2 Discussion:

6.2.1 Morphology and cytokinin content:

Considering the morphology of the P*ssu-ipt* transgenic plant material, a comparison with the HSIPT plants is necessary. Several changes in the phenotype of the HSIPT plants that were attributed to the expression of the *ipt*-gene, appear more pronounced in the P*ssu-ipt* strain.

The apical dominance was much more reduced. In the P*ssu-ipt* grafts in particular, the axillary shoot development was promoted, resulting in the bushy phenotype. In the progeny of the grafts, *i.e.* the P*ssu-ipt* plants, this effect was strongly reduced. Only late in the development the axillary shoots grew out.
In the P*ssu-ipt* plants stem elongation was much retarded and the internode length was reduced. This could be considered as stunted growth. However, the elongation growth persisted much longer and finally the total stem length could even exceed that of wild type plants. Consequently, flower development was delayed, like in *rol*A transformed tobacco (Dehio *et al.*, 1993). It was recently shown that in the latter plants the basipetal auxin gradient was attenuated and that in the apical stem region a transient cytokinin accumulation had occured (Prinsen, 1993a).

Stem thickening was one of the most typical features of the *ipt*-gene expression in the HSIPT plants. However, in the P*ssu-ipt* transgenic strain problems were encountered in making an adequate comparison. In the grafts, the absence of one main stem was counterbalanced by an abundance of smaller branches. Therefore, it was impossible to make a comparative measurement with a SR1-stem. When comparing the diameter of corresponding internodes in P*ssu-ipt* and wild type plants, stem thickening was obvious for the former. However, when the comparison with wild type plants was made at the same height, this effect disappeared, due to the increased number of shorter internodes. Besides, the extreme variability in growth rate made the comparison more difficult.

Rooting, which was completely inhibited in the P*ssu-ipt* shoots that were used for grafting, was no longer excluded in the P*ssu-ipt* seedlings. Still, it was highly reduced as compared to the wild type. As a consequence, the shoot/root ratio was out of balance, even more than in the HSIPT plants after repetitive induction of the *ipt*-gene. This probably corresponds to the level of *ipt*-gene expression. Transcriptional regulation mechanisms, such as DNA methylation, are likely involved (see 6.1.1.2).

Specifically in the P*ssu-ipt* transformed tobacco, effects of the *ipt*-gene expression were perceptible in leaves. Wrinkling of the lamina has been mentioned before only in one other *ipt*-transformed tobacco strain (Ainley *et al.*, 1993). This effect was also found as one of the characteristics of the 'hairy root syndrome', in plants transformed with the *rol*-genes from *Agrobacterium rhizogenes* (Spena *et al.*, 1987; Tepfer, 1984). In particular the *rol*A-gene was able to provoke leaf wrinkling (Dehio *et al.*, 1993; Schmülling *et al.*, 1988 and 1993). Beside the influence of *rol*A-gene expression on the auxin distribution and on cytokinin kinetics (Prinsen, 1993a) it was shown that a normal phenotype could be partially restored by application of GA_3 (Schmülling *et al.*, 1993). In *rol*B transgenic plants leaf necrosis frequently occured. In a tetracycline-dependent expression system the *rol*B phenotype was more pronounced than under constitutive expression and wrinkled leaves developed (Röder *et al.*, 1994). The *rol*B-gene encodes for an enzyme with indoxyl-ß-glucoside hydrolase activity and this activity correlates with an increased IAA content (Estruch *et al.*, 1991b). *In vitro rol*B-glucosidases were shown to perform cytokinin-O-glucoside hydrolysis as well (Prinsen, 1993b). Complex changes in the auxin/cytokinin ratio, and probably more generally in the total hormonal balance during the early leaf ontogeny is supposed to be the cause of wrinkling by disproportional cell division and expansion of parenchyma cells with respect to vascular differentiation (Chriqui *et al.*, 1990).

Adventitious shoots, here also called viviparous shoots, were typically produced on leaf tips of P*ssu-ipt* grafts and plants. The cytokinin concentration in these shoots was very high, especially in a young stage. Supposedly, vascular transport can lead to an enormous accumulation of cytokinins at the leaf tip, where viviparous shoots arise from vascular parenchyma cells (Estruch *et al.*, 1991b).

From a morphological point of view, P*ssu-ipt* plants in general appear to take an intermediate position between wild type plants and P*ssu-ipt* grafts. Šiffel and co-workers (1992) mentioned that in the second generation the cytokinin increase was less pronounced than in the original plants, directly regenerated after transformation. A normalization in the morphology of *ipt*-transformed potato tissues is also described under continuous *in vitro* subcultivation (Čatský *et al.*, 1993a). Yusibov *et al.* (1991) have shown that, together with a lesser accumulation of cytokinins, the *ipt*-mRNA was down-regulated in regenerated

transgenic plants in comparison to the original calli and the primary regenerated shoots. Also, after transfer to soil culture, transcriptional regulation of *ipt*-transformed plants has been observed (Dymock *et al.*, 1991).
A homeostatic regulation of the active phytohormone pool is essential for the eventual physiological effect. It is generally assumed that the free cytokinin bases are the active products. Nucleotides may be associated with cytokinin transport and the different glucoside-forms have been suggested to be temporal storage forms or deactivation products (see Letham and Palni, 1983). In the P*ssu-ipt* grafts only ZR- and iPA-equivalents have been measured. In the P*ssu-ipt* plants, free zeatin, ZR and the nucleotides were distinguished. The glycosylated cytokinin pool was not analyzed, and no estimation of the interconversion reactions was made. Therefore, the actual active cytokinin pool cannot be evaluated from the present results.
Still, in comparison to SR1 plants, the level of cytokinins was higher in leaves from P*ssu-ipt* plants and even higher in grafts. This is an interesting starting-point to evaluate the impact of cytokinins on the photosynthetic apparatus.

6.2.2 Photosynthetic characteristics:

In vitro measurements demonstrated that the photosynthetic electron transport and the photophosphorylation capacity in Km^S plants was not essentially different from that in SR1 plants. With Southern blot analysis it has been proven that these Km^S plants do not contain the *ipt*-gene (Beinsberger, 1993). This can be explained by considering these plants as the non-transgenic progeny from heterozygous P*ssu-ipt* grafts. In this study, *ipt*-transformed plant material is examined in comparison to wild type plants. Accordingly, the Km^S plants were not further considered.

Cytokinins have been recognized to accelerate chlorophyll accumulation during the de-etiolation process (Arnold and Fletcher, 1986; Caers and Vendrig, 1986; Dei, 1984). Recently, additional evidence for a role of cytokinins in de-etiolation was given by the *det*-mutation in *Arabidopsis* (Chory *et al.*, 1994). Cytokinins are also actively involved in the delay of senescence (van Staden *et al.*, 1988; Grossmann *et al.*, 1991). Therefore, an effect on the chlorophyll content could be expected.

In young P*ssu-ipt* grafts the chlorophyll and carotenoid content per leaf fresh weight was reduced. Likewise, in *rol*C-transgenic plants leaf pigmentation

was reduced (Spena *et al.*, 1989). In these plants the level of free cytokinins was moderately increased, due to the *rol*C encoded cytokinin-glucoside hydrolase activity (Estruch *et al.*, 1991a). In the young P*ssu-ipt* grafts the cytokinin concentration was also increased (Beinsberger, 1993); in particular very high concentrations of ZR have been recorded.

In comparison to shade plants, sun type plants are characterized by a decline in pigmentation per fresh weight, but by an increase in chlorophyll content on a leaf area basis, an increased chlorophyll a/b ratio, enhanced photosynthetic activity and large starch granules in the chloroplasts, with reduced grana stacking (Lichtenthaler *et al.*, 1981). It has been demonstrated before that the formation of sun type chloroplasts can be mimiced by the exogenous application of cytokinins (Lichtenthaler and Buschmann, 1978). In the P*ssu-ipt* grafts, large amounts of starch have been found (Beinsberger, 1993) and the oxygen evolution in leaf discs from young grafts is significantly enhanced (table 6.6 and 6.7). In that respect young P*ssu-ipt* grafts apparently develop sun type leaves. However, in young P*ssu-ipt* grafts the chlorophyll content on a leaf area basis was somewhat decreased (table 6.7), which contradicts to the sun-type acclimation.

In older P*ssu-ipt* grafts and in P*ssu-ipt* plants the chlorophyll content could be increased significantly. Zerbe and Wild (1980) have demonstrated that kinetin treatment of *Sinapis* plants also results in a higher pigment concentration, with an increase in chlorophyll a in particular, thus increasing the chlorophyll a/b ratio. On the contrary, in the intense green P*ssu-ipt* grafts, the chlorophyll a/b ratio was reduced, indicating an accumulation of the LHCP-complex relative to the photosynthetic reaction centres (Čatský *et al.*, 1993b; Šiffel *et al.*, 1992). This supports the results obtained by exogenous application of cytokinins, by which an enhanced accumulation of LHCP-mRNA and of the polypeptide was accomplished (Axelos *et al.*, 1984; Longo *et al.*, 1990; Teyssendier de la Serve *et al.*, 1985). Flores and Tobin (1988) already suggested that BA exerts an influence at the transcriptional and post-transcriptional regulation.

Pale P*ssu-ipt* grafts were also produced. The occurence of both extremes (dark green vs. pale) was quite unexpected. However, different responses in chlorophyll accumulation in *ipt*-transformed plants have been reported: no change (Ondřej *et al.*, 1991; Šiffel *et al.*, 1992), a decline (Čatský *et al.*, 1993a; Ondřej *et al.*, 1990) or an increase (Li *et al.*, 1992; Smigocki, 1991). Ainley *et al.* (1993) noticed that after a heat induction of their HSIPT plants the leaves were either completely chlorotic, or greener with chlorotic margins. Ondřej *et al.* (1990) suggested that the chlorophyll decline occurs when the cytokinin

concentration exceeds an optimal value. This was confirmed by *in vitro* cultivation of plantlets on a concentration range of BA: upon addition of 0.01 μg/l BA to the cultivation medium for tobacco several photosynthetic parameters raised to a maximum; at higher concentrations they decreased again (Pospíšilová *et al.*, 1993). Beside different levels of *ipt*-gene expression, the reports cited above emphasize the presence of regulating mechanisms that control the pool of active cytokinins within the plant. This regulation might be different among individual P*ssu-ipt* grafts and plants.

In vitro measurements of the capacity of the whole chain electron transfer show a reduction for the P*ssu-ipt* plants which was even stronger in the grafts (table 6.3). For PSI and PSII separately, at first glance P*ssu-ipt* grafts and plants appear to respond in opposite way: in the grafts damage seems to have occured predominantly at PSI and in the P*ssu-ipt* plants mainly at PSII (tables 6.4 and 6.5).

A more profound comparison with different electron acceptors and donors leads to the conclusion that in green P*ssu-ipt* grafts the electron transport capacity of PSII is hardly affected (see 6.1.3.2); this is confirmed by the high value of Φ_p° (see table 6.8). The capacity of PSI is more reduced.
Changes in chlorophyll a and b content indicate an enhanced accumulation of LHCP in grafts (see before). Since the electron transport rate was calculated on a chlorophyll basis, the reduction in reaction centre activity might have been overrated. If this extra LHCP corresponds mainly to LHCII (connected with PSII under state 1-conditions), the incident exitation energy directed towards PSII would be benefited. This could explain the differential reduction in PSII vs. PSI activity. Alternatively, a change in the photosystem stoichiometry could be assumed. However, by growing peas under conditions of light enriched in the red wavelength range or by supplementary far red light pulses (preferentially absorbed by PSI), the PSII/PSI ratio could be elevated (Chow *et al.*, 1990a and 1990b). Under saturating white light conditions Pmax was increased in these plants, especially when expressed on a chlorophyll base. This is not in agreement with our results (see old grafts, table 6.7).

An inhibitory effect at the vicinity of the cytochrome b_6/f complex is suggested, based on the difference with $DADH_2$ and $DCPIPH_2$ (table 6.5); this result is supported by the reduced inhibitory effect of DBMIB (see 6.1.3.3). Other studies on *ipt*-transformed plants also pointed out that the reaction centres were not the primary target, although the net photosynthetic rate was reduced

(Ondřej *et al.*, 1990; Šiffel *et al.*, 1988 and 1992). Analogous to our conclusion, Čatský *et al.* (1993a) suggested that in a clone with a high cytokinin accumulation some change was induced between PSII and PSI, rather than at the reaction centres themselves. In contrast, exogenous application of kinetin during greening was shown to promote plastoquinone and cytochrome f accumulation (Buschmann and Lichtenthaler, 1977; Zerbe and Wild, 1980). This difference (inhibition vs. enhanced accumulation) might be related to differences in the endogenous cytokinin concentrations. This result, however, underscores the key role of the intersystem carriers in regulating the activity of the electron transport.

Sabat *et al.* (1989) studied the effect of leaf ageing on the photosynthetic electron transport. They concluded that ageing resulted in an enhanced inhibition by DBMIB. Furthermore, they noted that the activity with TMPD and $DCPIPH_2$ was increased. In that respect, comparison of P*ssu-ipt* grafts and SR1 plants would suggest that the grafts are physiologically younger, which consolidates the proposed anti-senescence role of cytokinins. Taking into account the occurence of pale grafts and the complexity of the phenomenon of senescence, this conclusion might be over-hasty.

Although in green P*ssu-ipt* plants the SiMo-mediated electron transport was highly reduced, the results with PD (table 6.4) as well as the *in vivo* determination of Φ_p^o on leaf discs (table 6.8) indicate that the PSII reaction centre activity is hardly affected. On the other hand, in pale P*ssu-ipt* plants the PSII reaction centre was most probably inhibited.
Photosystem I was moderately affected in young and pale P*ssu-ipt* plants; comparison of measurements with the electron donors $DCPIPH_2$ and $DADH_2$ shows that the higher inhibition recorded with $DADH_2$ could not be attributed to a limitation in PSI reaction centre activity itself, but rather at the cytochrome b_6/f complex (see 6.1.3.2). Based on the results with TMPD, in green P*ssu-ipt* plants PSI activity was more reduced.
In the P*ssu-ipt* (green and pale) grafts also a difference was found between the reaction mediated by $DADH_2$ or by $DCPIPH_2$. This points again to an effect located at the intersystem chain. Hence, the alterations in the partial reaction of the photosynthetic electron transport chain in P*ssu-ipt* grafts and plants are quite similar.

In young grafts q_P was increased. This corresponds to the higher Φ_p and the elevated Pmax. In a weak expressing *ipt*-transformed tobacco the carbon

assimilation rate was likewise increased (Čatský *et al.*, 1993b). Moreover, Pospíšilová *et al.* (1993) showed that at a low concentration BA stimulates q_P. However, at higher concentrations of BA, q_P was lower than in untreated plants. In old, green grafts no limitation is expected from the reaction centre of PSII (cf. Φ_p^o), but rather further down the electron transport chain. For this reason, an enhanced accumulation of reduced plastoquinone can be expected, which may increase the back pressure on Q_A oxidation. This would reduce q_P. Assuming that q_N does not significantly increase in the mean while (the membrane energization in the grafts is very low), the fluorescence yield is expected to rise, F_S will be higher and consequently Φ_p will drop. This suggested sequence would derive benefit from an unimpairment of the cytochrome b_6/f complex.

In the P*ssu-ipt* grafts the build up of a proton motive force is reduced (see effect of uncoupling, table 6.3) and as a consequence, the photophosphorylation capacity is low (see 6.1.3.4). This is corroborated by the low value of q_N (table 6.8). Reminding the inaccuracy in the determination of F_M, an alternative hypothesis has been formulated to explain the decline in q_N (see 6.1.3.6). If the assumption of a slower decay of q_N in grafts is correct, the error in F_M determination would become more important and thus Φ_p^o should be upgraded relatively more in the grafts. It would even reinforce the promotion in Φ_p^o in the young and old, pale grafts in comparison to wild type plants. This is very unlikely to be true, since the *in vitro* PSII capacity was not stimulated at all. This again validates the decline in q_N for real.

After dark-light transition, a typical oscillatory behaviour in the chlorophyll fluorescence induction kinetics was reported in SR1 plants. It was completely absent in the P*ssu-ipt* grafts, as were the oscillations in the rate of oxygen evolution (fig. 6.7 a-d).

The transient M-peaks in the kinetics of the chlorophyll fluorescence are associated with the carbon assimilation cycle (Walker, 1981). The ATP/ADP ratio in particular, is assumed to be involved in the regulation to establish the steady state level. This ratio directly modulates the equilibrium concentrations of several intermediates of the Calvin cycle. A high carbon assimilation rate, producing high levels of triose phosphates, involves a high rate of ATP-consumption. When ATP becomes limiting, photophosphorylation is favoured. This causes a discharge of the proton gradient, abolishing the membrane energization component of the non photochemical quenching (Sivak *et al.*, 1985; Sivak and Walker, 1986). High electron transport rates are resumed, as can be

observed in the high oxygen evolution rate, increasing the photochemical fluorescence quenching.
Stitt and Grosse (1988) demonstrated the dependence of photosynthesis on sucrose synthesis, with the release of phosphate. Recycling of phosphate links the cytosolic metabolism to chloroplastic activities (Stitt *et al.*, 1988). This supports the central position of phosphate (Laisk and Walker, 1986; Sivak and Walker, 1986).

On the other hand, Laisk *et al.* (1991) emphasized that the regulation can be exerted either by the phosphorylation potential or by NADPH-production. When NADPH accumulates, electron transport is driven into alternative, often cyclic, pathways. These still support ATP-synthesis, which favours the ATP/NADPH ratio. Subsequently, the ATP enhanced carbon assimilation can result in a NADPH depletion, via the D-glyceraldehyde-3-phosphate formation (catalyzed by glyceraldehyde-phosphate dehydrogenase ($NADP^+$)). As such, the reducing and the phosphorylating potential can keep one another in balance. Further, in the report of Laisk *et al.* (1991) calculated values of phosphate appear to be higher at the minima than at maxima in photosynthesis, which contradicts to the idea of phosphate being the only limiting factor. However, it has already been shown that the photophosphorylation in green P*ssu-ipt* grafts is limited (see 6.1.3.3). This has probably affected the regulatory mechanism that adjusts photosynthesis after disturbance of the steady state, eliminating the oscillations.

In the light response curves no substantial differences in the low light intensity region could be observed. This initial part of the curve is proportional to the leaf absorptance and to the quantum yield (Terashima and Takenaka, 1990). Although Φ_p was shown to be changed, the apparent quantum requirement, the light compensation point and dark respiration were not significantly different in the P*ssu-ipt* grafts or plants (table 6.6). Other reports on *ipt*-transformed tobacco and potato plantlets indicated though a modulation in the respiratory activity (Čatský *et al.*, 1993 a and b), consistent with effects produced by exogenous application of BA in a cell culture of *Beta vulgaris* (Vaňková *et al.*, 1991).
The alterations at saturating light intensity have already been mentioned when discussing the chlorophyll content. Apparently, there was a correlation between the chlorophyll concentration and Pmax. In broad outline this could be interpreted in analogy to the sun vs. shade acclimation, some characteristics, however, did not fit into this view. At light saturation, photosynthesis is mainly

restricted by the carbon assimilation rate (Farquhar *et al.*, 1980; Terashima and Takenaka, 1990). The carboxylase and oxygenase activity of RubisCO have been determined and they were shown to be reduced (per unit of protein) in old leaves of P*ssu-ipt* grafts and in green P*ssu-ipt* plants (Winters, 1994; R. Valcke, personal communication). The results corresponded to a relative decline of the large subunit (LSU) of the enzyme while the amount of the the small subunit (SSU) remained constant. In younger grafts, the opposite was observed: the LSU was more abundant relative to the constant level of SSU and the carboxylase activity was increased. The activity of RubisCO itself appears to determine Pmax. The changes in LSU suggest that the amount of the active RubisCO enzyme was regulated. The mechanism of this regulation is still obscure.

A transition zone links the region of low and high light intensity in the light response curve. The sharper this transition (the higher the values of L.U.C. or Θ), the more advantageous the adjustment of the photosynthetic apparatus to the light gradient within the leaf. This transition was more blunted in the P*ssu-ipt* grafts and plants. Several reasons can be put forward. (1) For young grafts, the appearance of more sun-type-like chloroplasts was suggested. If the chloroplasts of the spongy parenchyma cells also shift to this sun acclimation, the light harvesting antenna would be reduced, more like in palisade parenchyma chloroplasts, and the cross section area of light absorbance at the deeper levels in the leaves would decline. In old grafts, where the accumulation of LHCP is promoted (more shade-type chloroplasts) the incident light deeper in the leaf would be dimmed. In both cases, the chloroplast architecture would not be properly adapted to the intra-leaf light gradient (Terashima and Takenaka, 1990). (2) Differences in leaf structure have been observed on transversal sections for light microscopy: leaves from P*ssu-ipt* plants are thicker than corresponding leaves in wild type plants, but the number of cell layers is not changed (S. Veselov, personal communication). This might have altered the light absorbtance in the different cell layers of the leaf, due to differences in light scattering. (3) Leaf inclination towards the stem might have differed. The measurements were done on leaf discs, with the actinic light source perpendicular to the adaxial leaf surface. During these measurements, the leaf would thus experience a light gradient, different from the gradient to which it was adapted. All these elements would lead to a decline in convexity of the light response curves.

From the kinetic measurements of the redox-state of P700 no estimation of the amount of P700 could be made (no single turnover flash illumination was provided; see Materials and Methods, 2.2.4). Moreover, the output signal from the fluorometer equipment (Hansatech MFMS fluorometer) was first amplified by an adjustable electronic back-off/amplifier before it was sent to the chart recorder. The absolute absorbance at 820nm (A820) was not registered. Therefore, determination of the normalized absorbance change $\Delta A820/A820$ could not be recorded, thus also by that means no estimation of the P700 content was made. After all, the large differences in chlorophyll content among the different leaves would have interfered.

Yet, the kinetic measurements, as presented in 6.1.3.7, give information about the influence of stromal components on the photosynthetic electron transport. As potential stromal electron donors, peripheral ferredoxin, pyridine nucleotides and other metabolites, such as triose phosphates have been proposed. Asada *et al.* (1993) have come to the conclusion that triose phosphates are good candidates to form the stromal electron reservoir; NADPH probably functions as a mediator to render the electrons back into the electron transport chain. As was discussed earlier (see 6.1.3.7), under conditions that favour a cyclic electron flow, the electron pool giving rise to the AL-area likely consisted of the intersystem pool plus electrons accumulated in NADPH and in reduced intermediates of a cyclic electron transfer chain through PSI. This pool was shown to be equal in P*ssu-ipt* transgenic plant material and in SR1 plants.

This supports the idea that the absence of oscillations in chlorophyll fluorescence and in the oxygen evolution in the P*ssu-ipt* grafts upon a dark-light transition was caused by the incapability in photophosphorylation (see 6.1.3.4), rather than due to an effect on the regulation of the reducing potential.
This effect was accompanied by a strong accumulation of starch in the chloroplasts. In contrast, a starchless mutant produced oscillations with an unusual long period (Peterson and Hanson, 1991). These authors deduced that this is underlain by a limited availability of phosphate. Cytoplasmic phosphate depletion can also be induced experimentally in many C_3 species by feeding mannose. This results in a reduced photosynthetic rate, but starch formation is stimulated by allosteric activation (due to a high stromal 3-phosphoglyceric acid/phospate ratio) of the enzyme ADPglucose pyrophosphorylase, which plays an important role in starch synthesis (Edwards and Walker, 1983).
Based on these results it might be hypothesized that the sink strength enhancing

effect of cytokinins (Leshem and Sussex, 1990; Muñoz *et al.*, 1990) is accomplished by influencing the phosphorylation activity in the leaves. The question about the mechanism of action of cytokinins on this activity, however, is open.

In conclusion, genetic transformation of tobacco with the P*ssu-ipt* construction affects the morphology as well as the photosynthetic activity. The *ssu*-promoter is responsible for a leaf accompanied expression and the induction of the *ipt*-gene expression already starts from the moment that the cotyledons are unfolded and exposed to light. The difference in tissue specificity and the more stringent induction in comparison to the HSIPT plants is presumably the cause of the stronger morphological changes.

In isolated chloroplasts, partial reactions of the electron transport chain are differentially influenced. The activity of the reaction centre of PSII is hardly affected; the PSI activity was more reduced, especially in the P*ssu-ipt* plants; the intersystem electron transport chain is negatively influenced, particularly in the P*ssu-ipt* grafts. The site that is affected, is suggested to be the cytochrome b_6/f complex. Changes in the physico-chemical properties of the thylakoid membrane may also be involved. This could provide an explanation for the difference in accessibility of DAD relative to PD in green grafts (see 6.1.3.2).

In vivo, the photosynthetic regulation was in the first instance limited by the phosphorylation capacity. The photosynthetic activity was further determined by cytokinin induced changes at the chloroplast ultrastructure (Beinsberger, 1993) which resembled a sun-type acclimation in young P*ssu-ipt* grafts and a shade-type acclimation in old grafts and in P*ssu-ipt* plants. However, the pigmentation of the leaves on an area basis did not change correspondingly. Modifications of the leaf structure may not be overlooked.

The opposite responses in young and old P*ssu-ipt* grafts support the notion that the development of the photosynthetic apparatus is stimulated by cytokinins up to a maximal concentration. Above that threshold value, the system becomes inefficient, due to the growing disproportion in the energy converting reactions.

Chapter 7: Concluding remarks

In this study two different *ipt*-transformed tobacco strains have been examined. In the first one, the P*hsp70-ipt* strain, clear morphological effects have been described. Heat treatment was shown to induce effects of the *ipt*-gene expression in axillary buds, stem tissues and roots, but not in leaves. Although the chloroplast ultrastructure was affected (thylakoid swelling was observed), the electron transport capacity was not changed. A difference in tissue sensitivity may be the cause, although it was shown that the temperature rise might have been too limited in the leaves to activate the transcription of the P*hsp70-ipt*-gene. Besides, transport and metabolic interconversion of the cytokinins have not been considered.

In the second, P*ssu-ipt* transgenic strain, grafted shoots and their progeny, *i.e.* rooted 'P*ssu-ipt* plants', were studied. Some typical morphological effects (diminished rooting and reduced apical dominance) were even more pronounced than in the HSIPT plants. Moreover, effects of the *ipt*-gene expression on leaves were present. Under control of the *ssu*-promoter, the *ipt*-expression was stronger (higher levels of endogenous cytokinins were found) and the expression was specific for leaves.

Most striking was the high degree of variability in growth of the P*ssu-ipt* plants. This added to the problem to define a basis for comparison. The absolute age of the P*ssu-ipt* plants and grafts could not be used. Here, we applied a visual classification, based on plant size and leaf pigmentation level. This appeared to be physiologically relevant, since Pmax was inversely related to the chlorophyll concentration and in young grafts the photosynthetic activity was enhanced in contrast to the reduced activity in old grafts.

The results indicate that in the *ipt*-transformed tissues the reaction centres of PSI and PSII are not primarily affected. Rather, the acceptor side of PSII and the intersystem carriers, linking PSII and PSI, were suggested to be impaired. The photophosphorylation capacity is reduced, particularly in old grafts. The build up of a proton motive force is disturbed in these grafts. Membrane alterations are likely involved. This would also explain the differences in electron transport capacity mediated by PSII as measured with DAD or PD as electron acceptors (see 6.1.3.2).

In contrast, during the cytokinin induced delay in senescence, coupling is less reduced (Le Pabic, 1990). Furthermore, during senescence a loss in the functional cytochrome b_6/f content has been reported (Le Pabic *et al.*, 1990, and the references therein). Therefore, it could be imagined that cytokinins positively

affect this intersystem complex during retardation of senescence. Likewise, it has been shown that cytokinins stimulate the accumulation of plastoquinone and cytochrome f during greening (Buschmann and Lichtenthaler, 1977; Zerbe and Wild, 1980). These results apparently are in contradiction with our hypothesis of an inhibition of the intersystem electron transport in the P*ssu-ipt* plants and grafts, in particular. However, there probably exists an optimal endogenous cytokinin concentration for thylakoid membrane functioning. The direction of the cytokinin effect (stimulation or inhibition) might be related to the magnitude of the endogenous cytokinin increase.

When comparing young and old grafts, two different responses appear. In young grafts the chlorophyll content was lowered and the photosynthetic activity *in vivo* was increased, in agreement with the report of Čatský *et al.* (1993b) on a weak expressing *ipt*-transformed tobacco line. In old grafts, the photosynthetic activity was lowered and the chlorophyll content was either increased or highly reduced. Exogenous application of BA has revealed an optimal concentration, below which BA addition is stimulatory for several photosynthetic parameters and beyond that level, the reverse effect is seen (Pospíšilová *et al.*, 1993). To that respect, the suggested continuous accumulation of cytokinins in the P*ssu-ipt* grafts (Beinsberger, 1993) is an interesting fact. This would imply that the young grafts are situated below the cytokinin optimum and the old grafts have already surpassed it. Moreover, gene regulation mechanisms and metabolic activities essentially determine the plant response. Differences among P*ssu-ipt* plants and with respect to the grafts might be situated at that level. Further analysis of this P*ssu-ipt* strain could reveal interesting information concerning molecular and biochemical regulation.

Only a limited set of cytokinins was analyzed, and no other phytohormones were considered. It cannot be claimed that the effects registered are directly resulting from a rise in total cytokinin content. It can be argued that these are phenotypical and physiological effects, resulting from the introduction and the expression of the *ipt*-gene, which starts a biosynthetic pathway for cytokinins. The mechanism by which the alterations are produced was not the topic of this investigation.

A refinement of the control of the level of gene expression and the incorporation of other phytohormones are desirable for the further analysis of the effects that have been located in this study.

Appendix: Staining of stem sections for light microscopy.

dyes: - safranin O (Gurr) made up as a 3% solution (w/v) in ethanol 50%,
- fast green FCF (Gurr) made up as a 1% solution (w/v) in ethanol 96%.
Both solutions were filtrated.

Procedure: The mounted sections (5 - 10 μm) were stained by passing them through the solutions enumerated consecutively in the following table.

Solution	time
xylene	10 min
xylene	10 min
ethanol 96%	10 min
ethanol 85%	5 min
ethanol 75%	5 min
ethanol 50%	5 min
safranin	30 min
water	rinsed in running tap water until the water remains colorless
ethanol 96%	dipped up and down three times
fast green FCF	20 sec
iso-propanol	dipped up and down two to three times
ethanol - absolute	maximally 30 sec
ethanol - absolute	maximally 30 sec
xylene	10 min
xylene	10 min

In the whole procedure denatured ethanol was used except for the final dehydration (as indicated: ethanol - absolute).

References:

Adedipe, N. O., Hunt, L. A. and Fletcher, R. A. (1971) Effects of benzyladenine on photosynthesis, growth and senescence of the bean plant. *Physiol. Plant.* **25:** 151-153.

Adir, N., Shochat, S. and Ohad, I. (1990) Light-dependent D1 protein synthesis and translocation is regulated by reaction center II. *J. Biol. Chem.* **265:** 12563-12568.

Ainley, W. M., McNeil, K. J., Hill, J. W., Lingle, W. L., Simpson, R. B., Brenner M.L., Nagao, R. T. and Key, J. L. (1993) Regulatable endogenous production of cytokinins up to 'toxic' levels in transgenic plants and plant tissues. *Plant Mol. Biol.* **22:** 13-23.

Akiyoshi, D. E., Klee, H., Amasino, R. M., Nester, E. W. and Gordon, M. P. (1984) T-DNA of *Agrobacterium tumefaciens* encodes an enzyme of cytokinin biosynthesis. *Proc. Natl. Acad. Sci. USA* **81:** 5994-5998.

Allen, J. F. (1977) Superoxide and photosynthetic reduction of oxygen. *In:* Superoxide and superoxide dismutases. (Michelson, A. M., McCord, J. M., Fridovich, I., eds.) Acad. Press, pp. 417-436.

Allen, J. F. and Holmes, N. G. (1986) Electron transport and redox titration. *In:* Photosynthesis - energy transduction. A practical approach. (Hipkins, M. F., Baker, N. R., eds.) IRL Press, Oxford, pp. 103-141.

Aloni, R. (1982) Role of cytokinin in differentiation of secondary xylem fibers. *Plant Physiol.* **70:** 1631-1633.

Altamura, M. M., Torrigiani, P., Falasca, G., Rossini, P. and Bagni, N. (1993) Morpho-functional gradients in superficial and deep tissues along tobacco stem: polyamine levels, biosynthesis and oxidation, and organogenesis *in vitro*. *J. Plant Physiol.* **142:** 543-551.

Amasino, R. M., Powell, A. L. T. and Gordon, M. P. (1984) Changes in T-DNA methylation and expression are associated with phenotype variation and plant regeneration in a crown gall line. *Mol. Gen. Genet.* **197:** 437-446.

Armond, P. A., Björkman, O. and Staehelin, L. A. (1980) Dissociation of supramolecular complexes in chloroplast membranes. A manifestation of heat damage to the photosynthetic apparatus. *Biochim. Biophys. Acta* **601:** 433-442.

Arnold, V. and Fletcher, R. A. (1986) Stimulation of chlorophyll synthesis by benzyladenine and potassium in excised and intact cucumber cotyledons. *Physiol. Plant.* **68:** 169-174.

Aro, E.-M., Virgin, I. and Andersson, B. (1993) Photoinhibition of photosystem II. Inactivation, protein damage and turnover. *Biochim. Biophys. Acta* **1143:** 113-134.

Arteca, R. N. and Dong, C.-N. (1981) Increased photosynthetic rates following gibberellic acid treatment to the roots of tomato plants. *Photosynth. Res.* **2:** 243-249.

Asada, K., Heber, U. and Schreiber, U. (1993) Electron flow to the intersystem chain from stromal components and cyclic electron flow in maize chloroplasts, as detected in intact leaves by monitoring redox change of P700 and chlorophyll fluorescence. *Plant Cell Physiol.* **34:** 39-50.

Asada, K., Heber, U. and Schreiber, U. (1992) Pool size of electrons that can be donated to $P700^+$, as determined in intact leaves: donation to $P700^+$ from stromal components via the intersystem chain. *Plant Cell Physiol.* **33:** 927-932.

Ashton, N. W., Grimsley, N. H. and Cove, D. J. (1979) Analysis of gametophytic development in the moss *Physcomitrella patens* using auxin- and cytokinin-resistant mutants. *Planta* **144:** 427-435.

Atzmon, N. and van Staden, J. (1993) The effect of zeatin and *iso*-pentenyladenine on root growth of *Pinus pinea* L. *J. Plant Physiol.* **141:** 366-369.

Axelos, M., Barbet, J. and Péaud-Lenoël, C. (1984) Influence of cytokinins on the biosynthesis of light-harvesting chlorophyll a/b proteins in tobacco cell suspensions: detection by radioimmunological assay. *Plant Sci. Lett.* **33:** 201-212.

Bagni, N., Altamura, M. M., Biondi, S., Mengoli, M. and Torrigiani, P. (1993) Polyamines and morphogenesis in normal and transgenic plant cultures. *In:* Morphogenesis in plants - Molecular approaches. (Roubelakis-Angelakis, K. A., Tran Thanh Van, K., eds.) Plenum Press, New York, pp. 89-111.

Barber, J. (1982) Influence of surface charges on thylakoid structure and function. *Ann. Rev. Plant Physiol.* **33:** 261-295.

Barber, J. (1992) The isolated photosystem two reaction centre reveals details of the molecular processes of photoinhibition. (Review) *Photosynthetica* **27:** 63-80.

Barber, J. and Andersson, B. (1992) Too much of a good thing: light can be bad for photosynthesis. *Trends Biochem. Sci.* **17:** 61-66.

Barber, J. and De Las Rivas, J. (1993) A functional model for the role of cytochrome b-559 in the protection against donor and acceptor side photoinhibition. *Proc. Natl. Acad. Sci. USA* **90:** 10942-10946.

Barry, G. F., Rogers, S. G., Fraley, R. T. and Brand, L. (1984) Identification of a cloned cytokinin biosynthetic gene. *Proc. Natl. Acad. Sci. USA* **81:** 4776-4780.

Bate, N. J., Rothstein, S. J. and Thompson, J. E. (1991) Expression of nuclear and chloroplast photosynthesis-specific genes during leaf senescence. *J. Exp. Bot.* **42:** 801-811.

Behera, L. M. and Choudhury, N. K. (1990) Effect of organ excision and kinetin treatment on chlorophyll content and DCPIP photoreduction activity of chloroplasts of pumpkin cotyledons. *J. Plant Physiol.* **137:** 53-57.

Beinsberger, S. (1993) Fysiologische gevolgen van de introductie van het *Agrobacterium tumefaciens* T-DNA 'cytokinine' gen 4 (*ipt*) op de ontwikkeling van *Nicotiana tabacum* L. cv. Petit Havana SR1. Ph.D.Thesis, Universitaire Instelling Antwerpen, 187 p.

Beinsberger, S. E., Clijsters, H. M., Valcke, R. L. and Van Onckelen, H. A. (1992a) Morphological characteristics and phytohormone content of *ipt*-transgenic tobacco. *In:* Progress in plant growth regulation. (Karssen, C. M., van Loon, L. C., Vreugdenhil, D., eds.) Kluwer Acad. Publ., the Netherlands, pp. 738-745.

Beinsberger, S. E. I., Valcke, R. L. M., Clijsters, H. M. M., De Greef, J. A. and Van Onckelen,

H. A. (1992b) Effects of enhanced cytokinin levels in *ipt* transgenic tobacco. *In:* Physiology and biochemistry of cytokinins in plants. (Kamínek, M., Mok, D. W. S., Zažímalová, E., eds.) SPB Acad. Publ. bv, The Hague, pp. 77- 82.

Bendall, D. S., Davenport, H. E. and Hill, R. (1971) Cytochrome components in chloroplasts of the higher plants. *In:* Methods Enzymol. (San Pietro, A., ed.) Acad. Press, Vol. 23 A, pp. 327-344.

Berry, J. and Björkman, O. (1980) Photosynthetic response and adaptation to temperature in higher plants. *Ann. Rev. Plant Physiol.* **31:** 491-543.

Beveridge, C. A., Ross, J. J. and Murfet, I. C. (1994) Branching mutant *rms-2* in *Pisum sativum*. - Grafting studies and endogenous indole-3-acetic acid levels. *Plant Physiol.* **104:** 953-959.

Bilger, W. and Schreiber, U. (1986) Energy-dependent quenching of dark-level chlorophyll fluorescence in intact leaves. *Photosynth. Res.* **10:** 303-308.

Binns, A. N. (1994) Cytokinin accumulation and action: biochemical, genetic, and molecular approaches. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **45:** 173-196.

Binns, A. N., Labriola, J. and Black, R. C. (1987) Initiation of auxin autonomy in *Nicotiana glutinosa* cells by the cytokinin- biosynthesis gene from *Agrobacterium tumefaciens*. *Planta* **171:** 539-548.

Björkman, O. and Demmig, B. (1987) Photon yield of O2 evolution and chlorophyll fluorescence characteristics at 77K among vascular plants of diverse origins. *Planta* **170:** 489-504.

Blackwell, J. R. and Horgan, R. (1994) Cytokinin biosynthesis by extracts of *Zea mays*. *Phytochemistry* **35:** 339-342.

Blonstein, A. D., Parry, A. D., Horgan, R. and King, P. J. (1991a) A cytokinin-resistant mutant of *Nicotiana plumbaginifolia* is wilty. *Planta* **183:** 244-250.

Blonstein, A. D., Stirnberg, P. and King, P. J. (1991b) Mutants of *Nicotiana plumbaginifolia* with specific resistance to auxin. *Mol. Gen. Genet.* **228:** 361-371.

Bolhàr-Nordenkampf, H. R. (1987) Shoot morphology and leaf anatomy in relation to photosynthesis. *In:* Techniques in bioproductivity and photosynthesis. 2nd ed. (Coombs, J., Hall, D. O., Long, S. P., Scurlock, J. M. O., eds.) Pergamon Press, Oxford, pp. 107-117.

Bolhàr-Nordenkampf, H. R., Long, S. P., Baker, N. R., Öquist, G., Schreiber, U. and Lechner, E. G. (1989) Chlorophyll fluorescence as a probe of the photosynthetic competence of leaves in the field: a review of current instrumentation. *Functional Ecol.* **3:** 497-514.

Bosselaers, J. P. (1983) Cytokinin effects on leaf architecture in *Phaseolus vulgaris* L. *J. Exp. Bot.* **34:** 1007-1017.

Boucher, N. and Carpentier, R. (1993) Heat stress stimulation of oxygen uptake by photosystem I involves the reduction of superoxide radicals by specific electron donors. *Photosynth. Res.* **35:** 213-218.

Boucher, N., Harnois, J. and Carpentier, R. (1990) Heat-stress stimulation of electron flow in a photosystem I submembrane fraction. *Biochem. Cell Biol.* **68:** 999-1004.

Böger, P. and Kunert, K. J. (1978) Phytotoxic action of paraquat on the photosynthetic apparatus. *Z. Naturforsch.* **33 c:** 688-694.

Bracale, M., Longo, G. P., Rossi, G. and Longo, C. P. (1988) Early changes in morphology and polypeptide pattern of plastids from watermelon cotyledons induced by benzyladenine or light are very similar. *Physiol. Plant.* **72:** 94-100.

Bradbury, M. and Baker, N. R. (1981) Analysis of the slow phases of the *in vivo* chlorophyll fluorescence induction curve. Changes in the redox state of photosystem II electron acceptors and fluorescence emission from photosystem I and II. *Biochim. Biophys. Acta* **63:** 542-551.

Brock, T. G. and Cleland, R. E. (1990) Biophysical basis of growth promotion in primary leaves of *Phaseolus vulgaris* L. by hormones versus light. *Planta* **182:** 427-431.

Bruinsma, J. (1963) The quantitative analysis of chlorophylls a and b in plant extracts. *Photochem. Photobiol.* **2:** 241-250.

Buchanan, B. B. (1991) Regulation of CO_2 assimilation in oxygenic photosynthesis: the ferredoxin/thioredoxin system. - Perspective on its discovery, present status, and future development. *Arch. Biochem. Biophys.* **288:** 1-9.

Buchmann, I., Marner, F.-J., Schröder, G., Waffenschmidt, S. and Schröder, J. (1985) Tumour genes in plants: T-DNA encoded cytokinin biosynthesis. *EMBO J.* **4:** 853-859.

Budde, R. J. A. and Randall, D. D. (1990) Light as a signal influencing the phosphorylation status of plant proteins. *Plant Physiol.* **94:** 1501-1504.

Bukhov, N. G., Sabat, S. C. and Mohanty, P. (1990) Analysis of chlorophyll a fluorescence changes in weak light in heat treated *Amaranthus* chloroplasts. *Photosynth. Res.* **23:** 81-87.

Bunce, J. A. (1991) Control of the acclimation of photosynthesis to light and temperature in relation to partitioning of photosynthate in developing soybean leaves. *J. Exp. Bot.* **42:** 853-859.

Burch, L. R. and Horgan, R. (1992) Cytokinin oxidase and the degradative metabolism of cytokinins. *In:* Physiology and biochemistry of cytokinins in plants. (Kamínek, M., Mok, D.W.S., Zažímalová, E., eds.) SPB Acad. Publ. bv, The Hague, pp. 29-32.

Burke, J. J. and Oliver, M. J. (1992) Differential temperature sensitivity of pea superoxide dismutases. *Plant Physiol.* **100:** 1595-1598.

Buschmann, C. and Lichtenthaler, H. K. (1977) Hill-activity and P700 concentration of chloroplasts isolated from radish seedlings treated with -indoleacetic acid, kinetin or gibberellic acid. *Z. Naturforsch.* **32 c:** 798-802.

Caers, M., Rüdelsheim, P., Van Onckelen, H. and Horemans, S. (1985) Effect of heat stress on photosynthetic activity and chloroplast ultrastructure in correlation with endogenous cytokinin concentration in maize seedlings. *Plant Cell Pysiol.* **26:** 47-52.

Caers, M. and Vendrig, J. C. (1986) Benzyladenine effects on the development of the photosynthetic apparatus in *Zea mays*: studies on photosynthetic activity, enzymes and (etio)chloroplast ultrastructure. *Physiol. Plant.* **66:** 685-691.

Campbell, W. J., Allen, L. H. and Bowes, G. (1990) Response of soybean canopy photosynthesis

to CO_2 concentration, light, and temperature. *J. Exp. Bot.* **41**: 427-433.

Campbell, W. J. and Ögren, W. L. (1990) Electron transport through photosystem I stimulates light activation of ribulose bisphosphate carboxylase/oxygenase (RubisCO) by Rubisco-activase. *Plant Physiol.* **94**: 479-484.

Čatský, J., Pospíšilová, J., Macháčková, I., Synková, H., Wilhelmová, N. and Šesták, Z. (1993a) High level of endogenous cytokinins in transgenic potato plantlets limits photosynthesis. *Biol. Plant.* **35**: 191-198.

Čatský, J., Pospíšilová, J., Macháčková I., Wilhelmová, N. and Šesták, Z. (1993b) Photosynthesis and water relations in transgenic tobacco plants with T-DNA carrying gene 4 for cytokinin synthesis. *Biol. Plant.* **35**: 393-399.

Chaudhury, A. M., Letham, S., Craig, S. and Dennis, E. S. (1993) *amp1* - a mutant with high cytokinin levels and altered embryonic pattern, faster vegetative growth, constitutive photomorphogenesis, and precocious flowering. *Plant J.* **4**: 907- 916.

Chauveau, M., Dizengremel, P. and Roussaux, J. (1983) Interaction of benzylaminopurine with electron transport in plant mitochondria during malate oxidation. *Plant Physiol.* **73**: 945-948.

Chen, C.-M. (1982) Cytokinin biosynthesis in cell free systems. *In:* Plant growth substances. (Wareing, P. F., ed.) Acad. Press, New York, pp. 155-164.

Chernyad'ev, I. I., Chvojka, L., Friedrich, A., Volfová, A. and Březinová, A. (1986) Photosynthesis in sugar beet leaves under the influence of cytokinins and triazine herbicide. *Photosynthetica* **20**: 196-203.

Chory, J., Nagpal, P. and Peto, C. A. (1991) Phenotypic and genetic analysis of *det2*, a new mutant that affects light- regulated seedling development in *Arabidopsis*. *Plant Cell* **3**: 445-459.

Chory, J., Reinecke, D., Sim, S., Washburn, T. and Brenner, M. (1994) A role for cytokinins in de-etiolation in *Arabidopsis*. - *det* mutants have an altered response to cytokinins. *Plant Physiol.* **104**: 339-347.

Chow, W. S., Goodchild, D. J., Miller, C. and Anderson, J. M. (1990a) The influence of high levels of brief or prolonged supplementary far-red illumination during growth on the photosynthetic characteristics, composition and morphology of *Pisum sativum* chloroplasts. *Plant Cell Environm.* **13**: 135-145.

Chow, W. S., Melis, A. and Anderson, J. M. (1990b) Adjustment of photosystem stoichiometry in chloroplasts improve the quantum efficiency of photosynthesis. *Proc. Natl. Acad. Sci. USA* **87**: 7502-7506.

Chriqui, D., Tepfer, M., Prinsen, E. and Van Onckelen, H. (1990) Composantes morphogénétiques et hormonales du phénotype hairy root chez *Nicotiana tabacum* cv. Xanthi. *Bull. Soc. Bot. Fr., Actual. Bot.* **137**: 79-91.

Cleland, R. E. (1987) Auxin and cell elongation. *In:* Plant hormones and their role in plant growth and development. (Davies, P. J., ed.) Martinus Nijhoff Publ., Dordrecht, pp. 132-148.

Cleland, R. E. (1971) Cell wall extension. *Ann. Rev. Plant Physiol.* **22**: 197-222.

Cline, M. G. (1994) The role of hormones in apical dominance. New approaches to an old problem in plant development. (Minireview) *Physiol. Plant.* **90:** 230-237.

Cohen, L., Arzee, T. and Zilberstein, A. (1988) Mimicry by cytokinin of phytochrome-regulated inhibition of chloroplast development in etiolated cucumber cotyledons. *Physiol. Plant.* **72:** 57-64.

Cramer, W. A., Whitmarsh, J. and Low, P. S. (1981) Differential scanning calorimetry of chloroplast membranes: identification of an endothermic transition associated with the water-splitting complex of photosystem II. *Biochemistry* **20:** 157- 162.

Critchley, C. and Chopra, R. K. (1988) Protection of photosynthetic O_2 evolution against heat inactivation: the role of chloride, pH and coupling status. *Photosynth. Res.* **15:** 143- 152.

Critchley, C. and Russell, A. W. (1993) Maximizing light interception: the role of chloroplast disposition in cells and thylakoid membrane architecture. *In:* Photosynthesis - Photoreactions to plant productivity. (Abrol, Y. P., Mohanty, P., Govindjee, eds.) Oxford & IBH Publishing, New Dehli, pp. 257-276.

Crowell, D. N. and Amasino, R. M. (1991) Induction of specific mRNAs in cultured soybean cells during cytokinin or auxin starvation. *Plant Physiol.* **95:** 711-715.

Cummins, W. R., Kende, H. and Raschke, K. (1971) Specificity and reversibility of the rapid stomatal response to abscisic acid. *Planta* **99:** 347-351.

Dawson, R. M. C., Elliot, D. C., Elliot, W. H. and Jones, K. M. (1986) Data for biochemical research. 3rd ed. Oxford Sci. Publ., New York, 580 p.

Deblaere, R., Bytebier, B., De Greve, H., Deboeck, F., Schell, J., Van Montagu, M. and Leemans, J. (1985) Efficient octopine Ti plasmid-derived vectors for *Agrobacterium*-mediated gene transfer to plants. *Nucl. Acids Res.* **13:** 4777-4787.

Dehio, C., Grossmann, K., Schell, J. and Schmülling, T. (1993) Phenotype and hormonal status of transgenic tobacco plants overexpressing the *rol*A gene of *Agrobacterium rhizogenes* T-DNA. *Plant Mol. Biol.* **23:** 1199-1210.

Dei, M. (1983) Benzyladenine-induced stimulation of chlorophyll formation in attached cotyledons of etiolated cucumber seedlings. *Plant Sci. Lett.* **30:** 251-257.

Dei, M. (1984) Benzyladenine-induced stimulation of two components of chlorophyll formation in etiolated cucumber cotyledons. *Physiol. Plant.* **62:** 521-526.

Delieu, T. J. and Walker, D. A. (1983) Simultaneous measurement of oxygen evolution and chlorophyll fluorescence from leaf pieces. *Plant Physiol.* **73:** 534-541.

Demmig B., Winter, K., Krüger, A. and Czygan, F.-C. (1987) Photoinhibition and zeaxanthin formation in intact leaves. - A possible role of the xanthophyll cycle in the dissipation of excess light energy. *Plant Physiol.* **84:** 218-224.

Demmig-Adams, B. (1990) Carotenoids and photoprotection in plants: A role for the xanthophyll zeaxanthin. *Biochim. Biophys. Acta* **1020:** 1-24.

Diettrich, B., Merinat, H. and Luckner, M. (1992) Reduction of water loss during *ex vitro*

acclimation of micropropagated *Digitalis lanata* clone plants. *Biochem. Physiol. Pflanz.* **188:** 23-31.

Dubbe, D. R., Farquhar, C. D. and Raschke, K. (1978) Effect of abscisic acid on the gain of feedback loop involving carbon dioxide and stomata. *Plant Physiol.* **62:** 413-417.

Dymock, D., Risiott, R., de Pater, S., Lancaster, J., Tillson, P. and Ooms, G. (1991) Regulation of *Agrobacterium tumefaciens* T-*cyt* gene expression in leaves of transgenic potato (*Solanum tuberosum* L. cv. Désirée) is strongly influenced by plant culture conditions. *Plant Mol. Biol.* **17:** 711-725.

Edwards, G. and Walker, D. A. (1983) The regulation of photosynthetic carbon assimilation. *In:* C3,C4: mechanisms, and cellular and environmental regulation, of photosynthesis. Blackwell Scientific Publications, Oxford, pp. 247-274.

Esashi, Y. and Leopold, A. C. (1969) Cotyledon expansion as a bioassay for cytokinins. *Plant Physiol.* **44:** 618-620.

Estruch, J. J., Chriqui, D., Grossmann, K., Schell, J. and Spena, A. (1991a) The plant oncogene *rol*C is responsible for the release of cytokinins from glucoside conjugates. *EMBO J.* **10:** 2889-2895.

Estruch, J. J., Prinsen, E., Van Onckelen, H., Schell, J. and Spena, A. (1991b) Viviparous leaves produced by somatic activation of an inactive cytokinin-synthesizing gene. *Science* **254:** 1364-1367.

Etienne, H., Berger, A. and Carron, M. P. (1991) Water status of callus from *Hevea brasiliensis* during induction of somatic embryogenesis. *Physiol. Plant.* **82:** 213-218.

Farquhar, C. D., von Caemmerer, S. and Berry, J. A. (1980) A biochemical model of photosynthetic CO_2 assimilation in leaves of C_3 species. *Planta* **149:** 78-90.

Fladung, M., Ballvora, A. and Schmülling, T. (1993) Constitutive or light-regulated expression of the *rol*C gene in transgenic potato plants has different effects on yield attributes and tuber carbohydrate composition. *Plant Mol. Biol.* **23:** 749-757.

Fletcher, R. A., Hofstra, G. and Adedipe, N. O. (1970) Effects of benzyladenine on bean leaf senescence and the translocation of ^{14}C-assimilates. *Physiol. Plant.* **23:** 1144-1148.

Flores, S. and Tobin, E. M. (1986) Benzyladenine modulation of the expression of two genes for nuclear-encoded chloroplast proteins in *Lemna gibba*: apparent post-transcriptional regulation. *Planta* **168:** 340-349.

Flores, S. and Tobin, E. (1988) Cytokinin modulation of LHCP mRNA levels: the involvement of post-transcriptional regulation. *Plant Mol. Biol.* **11:** 409-415.

Fosket, D. E. and Short, K. C. (1973) The role of cytokinins in the regulation of growth, DNA synthesis and cell proliferation in cultured soybean tissues (*Glycine max* var. Biloxi.). *Physiol. Plant.* **28:** 14-23.

Fujii, T., Yokoyama, E., Inoue, K. and Sahurai, H. (1990) The sites of electron donation of photosystem I to methyl viologen. *Biochim. Biophys. Acta* **1015:** 41-48.

Futers, T. S., Wang, T. L. and Cove, D. J. (1986) Characterization of a temperature-sensitive gametophore over- producing mutant of the moss *Physcomitrella patens*. *Mol. Gen. Genet.* **203:** 529-532.

Gong, H. and Ohad, I. (1991) The PQ/PQH_2 ratio and occupancy of photosystem II-Q_B site by plastoquinone control the degradation of D1 protein during photoinhibition *in vivo*. *J. Biol. Chem.* **266:** 21293-21299.

Gounaris, K., Brain, A. P. R., Quinn, P. J. and Williams, W. P. (1984) Structural reorganisation of chloroplast thylakoid membranes in response to heat stress. *Biochim. Biophys. Acta* **776:** 198-208.

Grossmann, K., Kwiatkowski, J. and Häuser, C. (1991) Phytohormonal changes in greening and senescing intact cotyledons of oilseed rape and pumpkin: influence of the growth retardant BAS 111 .. W. *Physiol. Plant.* **83:** 544-550.

Guenther, J. E., Nemson, J. A. and Melis, A. (1990) Development of PSII in dark grown *Chlamydomonas reinhardtii*. A light- dependent conversion of $PSII_\beta$, Q_B-non reducing centers to $PSII_\alpha$, Q_B-reducing form. *Photosynth. Res.* **24:** 35-46.

Guillot-Salomon, T., Bahl, J., Ben-Rais, L., Alpha, M.-J., Cantrel, C. and Dubacq, J. P. (1991) Heat stress and changes of lipid and carotenoid composition in spinach, a temperate plant, and jojoba, a desert plant. *Plant Physiol. Biochem.* **29:** 667-679.

Harbinson, J. and Woodward, F. I. (1987) The use of light-induced absorbance changes at 820 nm to monitor the oxidation state of P-700 in leaves. *Plant, Cell Environm.* **10:** 131-140.

Harding, S. A. and Smigocki, A. C. (1994) Cytokinins modulate stress response genes in isopentenyl transferase-transformed *Nicotiana plumbaginifolia* plants. *Physiol. Plant.* **90:** 327-333.

Hare, P. D. and van Staden, J. (1994) Cytokinin oxidase: biochemical features and physiological significance. (minireview) *Physiol. Plant.* **91:** 128-136.

Hauska, G. (1977) Artificial acceptors and donors. *In:* Encycl. Plant Physiol. (Trebst, A., Avron, M., eds.) Springer-Verlag, Berlin, Vol. 5, pp. 253-265.

Hauska, G., Oettmeier, W., Reimer, S. and Trebst, A. (1975) Shuttles of artificial electron donors for photosystem I across the thylakoid membrane. *Z. Naturforsch.* **30c:** 37-45.

Havaux, M., Canaani, O. and Malkin, S. (1987) Oxygen uptake by tobacco leaves after heat shock. *Plant, Cell Environm.* **10:** 677- 683.

Havaux, M. and Strasser, R. J. (1990) Protection of photosystem II by light in heat-stressed pea leaves. *Z. Naturforsch.* **45 c:** 1133-1141.

Havaux, M., Strasser, R. J. and Greppin, H. (1991) A theoretical and experimental analysis of the qP and qN coefficients of chlorophyll fluorescence quenching and their relation to photochemical and nonphotochemical events. *Photosynth. Res.* **27:** 41-55.

Hayakawa, T., Kanematsu, S. and Asada, K. (1985) Purification and characterization of thylakoid-bound Mn-superoxide dismutase in spinach chloroplasts. *Planta* **166:** 111-116.

Hedden, P. (1993) Modern methods for the quantitative analysis of plant hormones. *Ann. Rev. Plant*

Physiol. Plant Mol. Biol. **44**: 107-129.

Heikkila, J. J., Papp, J. E. T., Schultz, G. A. and Bewley, J. D. (1984) Induction of heat shock protein messenger RNA in maize mesocotyl by water stress, abscisic acid and wounding. *Plant Physiol.* **76:** 270-274.

Herrera-Estrella, L., Van de Broeck, G., Maenhaut, R., Van Montagu, M., Schell, J., Timko, M. and Cashmore, A. (1984) Light- inducible and chloroplast-associated expression of a chimaeric gene introduced into *Nicotiana tabacum* using a Ti plasmid vector. *Nature* **310:** 115-120.

Hocart, C. H., Letham, D. S., Wang, J., Cornish, E. and Parker, C. W. (1992) Control of cytokinin levels by inhibitors of metabolism, symbiosis and genetic manipulation. *In:* Progress in plant growth regulation. (Karssen, C. M., van Loon, L. C., Vreugdenhil, D., eds.) Kluwer Acad. Publ., the Netherlands, pp. 607-616.

Horton, P. (1983) Relations between electron transport and carbon assimilation: simultaneous measurement of chlorophyll fluorescence, transthylakoid pH gradient and O2 evolution in isolated chloroplasts. *Proc. Roy. Soc. Lond. B* **217:** 405-416.

Horton, P., Ruban, A. V., Rees, D., Pascal, A. A., Noctor, G. and Young, A. J. (1991) Control of the light-harvesting function of chloroplast membranes by aggregation of the LHCII chlorophyll- protein complex. *FEBS Lett.* **292:** 1-4.

Houssa, C., Bernier, G., Pieltain, A., Kinet, J.-M. and Jacqmard, A. (1994) Activation of latent DNA-replication origins: a universal effect of cytokinins. *Planta* **193:** 247-250.

Houssa, C., Jacqmard, A. and Bernier, G. (1990) Activation of replicon origins as a possible target for cytokinins in shoot meristems of *Sinapis*. *Planta* **181:** 324-326.

Ikegami, I. and Katoh, S. (1991) Antenna chlorophyll a has a structural role in stabilizing the functional conformation of P700-chlorophyll-protein complexes. *Biochim. Biophys. Acta* **1059:** 275-280.

Itai, C. and Benzioni, A. (1974) Regulation of plant response to high temperature. *In:* Mechanisms of regulation of plant growth. (Bieleski, R. L., Ferguson, A. R., Cresswell, M. M., eds.) Roy. Soc. New Zealand, Wellington, Bulletin 12, pp. 477-482.

Ivanov, A. G., Kicheva, M. I., Christov, A. M. and Popova, L. P. (1992) Effects of abscisic acid treatment on the thermostability of photosynthetic apparatus in barley chloroplasts. *Plant Physiol.* **98:** 1228-1232.

Ivanov, A. and Velitchkova, M. (1990) Heat-induced changes in the efficiency of P700 photo-oxidation in pea chloroplast membranes. *J. Photochem. Photobiol. B (Biology)* **4:** 307-320.

Ivanova, A. P., Yordanov, I. T., Stefanov, K. L., Seizova, K. A. and Popov, S. S. (1993) Effect of heat stress on the lipid and fatty acid composition of the thylakoid membranes in two cultivars of pea acclimated to elevated temperatures. *Photosynthetica* **29:** 131-137.

Ivanova, M. I., Todorov, I. T., Atanassova, L., Dewitte, W. and Van Onckelen, H. A. (1994) Co-localization of cytokinins with proteins related to cell proliferation in developing somatic embryos of *Dactylis glomerata* L. *J. Exp. Bot.* **45:** 1009-1017.

Izawa, S. (1977) Inhibitors of electron transport. *In:* Encycl. Plant Physiol. (Trebst, A., Avron, M., eds.) Springer-Verlag, Berlin, Vol. 5, pp. 266-282.

Izawa, S. (1980) Acceptors and donors for chloroplast electron transport. *In:* Methods Enzymol. (San Pietro, A., ed.) Acad. Press, Vol. 69 c, pp. 413-434.

Jefferson, R. A., Kavanagh, T. A. and Bevan, M. W. (1987) GUS fusions: ß glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **6:** 3901-3907.

Jegerschöld, C., Virgin, I. and Styring, S. (1990) Light- dependent degradation of the D1 protein in photosystem II is accelerated after inhibition of the water splitting reaction. *Biochemistry* **29:** 6179-6186.

Jensenius, I. C., Andersen, I., Hau, J., Crone, M. and Koch, C. (1981) Methods for the purification of yolk IgG. *J. Immunol. Meth.* **46:** 63-68.

John, M. C. and Amasino, R. M. (1989) Extensive changes in DNA methylation patterns accompany activation of a silent T-DNA *ipt-* gene in *Agrobacterium tumefaciens* transformed plant cells. *Mol. Cell. Biol.* **9:** 4298-4303.

Jones, R. W. and Whitmarsh, J. (1987) Inhibition of electron transfer in the cytochrome *b/f* complex by NQNO and DBMIB: evidence for two quinone binding sites. *In:* Progress in photosynthesis research (Biggens, J., ed.) Martinus Nijhoff Publ., Dordrecht, Vol. II, 7, pp. 441-444.

Jones, R. W. and Whitmarsh, J. (1988) Inhibition of electron transfer and the electrogenic reaction in the cytochrome *b/f* complex by 2-n-nonyl-4-hydroxyquinoline N-oxide (NQNO) and 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB). *Biochim. Biophys. Acta* **933:** 258-268.

Kanematsi, S. and Asada, K. (1990) Characteristic amino acid sequences of chloroplast and cytosol isozymes of CuZn-superoxide dismutase in spinach, rice and horsetail. *Plant Cell Physiol.* **31:** 99-112.

Kares, C., Prinsen, E., Van Onckelen, H. and Otten, L. (1990) IAA synthesis and root induction with *iaa* genes under heat shock promoter control. *Plant Mol. Biol.* **15:** 225-236.

Kicheva, M. I. and Ivanov, A. G. (1992) A comparative analysis of the effects of *in-vivo* and *in-vitro* abscisic-acid treatment on the surface electrical properties of barley chloroplast membranes. *Planta* **188:** 232-237.

Kim, J., Eichacker, L. A., Rüdiger, W. and Mullet, J. E. (1994) Chlorophyll regulates accumulation of the plastid-encoded chlorophyll proteins P700 and D1 by increasing apoprotein stability. *Plant Physiol.* **104:** 907-916.

Kinoshita, I. and Tsuji, H. (1984) Benzyladenine-induced increase in DNA content per chloroplast in intact bean leaves. *Plant Physiol.* **76:** 575-578.

Kirilovsky, D., Rutherford, A. W. and Etienne, A.-L. (1994) Influence of DCMU and ferricyanide on photodamage in photosystem II. *Biochemistry* **33:** 3087-3095.

Kislyuk, I. M. (1979) Protecting and injurious effects of light on photosynthetic apparatus during and after heat treatment of leaves. *Photosynthetica* **13:** 386-391.

Klee, H. and Estelle, M. (1991) Molecular genetic approaches to plant hormone biology. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **42:** 529-551.

Klee, H., Horsch, R. and Rogers, S. (1987) *Agrobacterium*-mediated plant transformation and its further applications to plant biology. *Ann. Rev. Plant Physiol.* **38:** 467-486.

Komenda, J., Masojídek, J., Prášil, O. and Boček, J. (1992) Two mechanisms of photosystem 2 photoinactivation: Do they exist *in vivo*? *Photosynthetica* **27:** 99-108.

Krause, G. H. and Santarius, K. A. (1975) Relative thermostability of the chloroplast envelope. *Planta* **127:** 285- 299.

Krause, G. H. and Weis, E. (1984) Chlorophyll fluorescence as a tool in plant physiology. II. Interpretation of fluorescence signals. (review) *Photosynth. Res.* **5:** 139-157.

Krause, G. H. and Weis, E. (1991) Chlorophyll fluorescence and photosynthesis: the basics. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **42:** 313-349.

Kretschmann, H., Pauly, S. and Witt, H. T. (1991) Evidence for a chemical reaction of hydroxylamine with the photosynthetic water splitting enzyme S in the dark.- Possible states of manganese and water in the S cycle. *Biochim. Biophys. Acta* **1059:** 208-214.

Krieger, A. and Weis, E. (1992) Energy-dependent quenching of chlorophyll a fluorescence: the involvement of proton-calcium exchange at photosystem 2. *Photosynthetica* **27:** 89-98.

Kudoyarova, G. R., Veselov, S. Yu, Karavaiko, N. N., Gyuli-Zade, V. Z., Cheredova, E. P., Mustafina, A. R., Moshkov, I. E. and Kulaeva, O. N. (1990) Immunoenzyme test system for assaying cytokinins. *Plant Physiol. (russian)* **37:** 193-199.

Kudoyarova, G. R., Veselov, S. Yu, Van Onckelen, H. and Valcke, R. Distribution of cytokinins in P*ssu-ipt* transformed tobacco plants. *(in preparation)*

Laisk, A., Siebke, K., Gerst, U., Eichelmann, H., Oja, V. and Heber, U. (1991) Oscillations in photosynthesis are initiated and supported by imbalances in the supply of ATP and NADPH to the Calvin cycle. *Planta* **185:** 554-562.

Laisk, A. and Walker, D. A. (1986) Control of phosphate turnover as a rate-limiting factor and possible cause of oscillations in photosynthesis: a mathematical model. *Proc. Roy. Soc. Lond. B* **227:** 281-302.

Lajkó, F., Kadioglu, A. and Garab, G. (1991) Involvement of superoxide dismutase in heat-induced stimulation of photosystem I-mediated oxygen uptake. *Biochem. Biophys. Res. Comm.* **174:** 696- 700.

Lamattina, L., Anchoverri, V., Conde, R. D. and Pont Lezica, R. (1987) Quantification of the kinetin effect on protein synthesis and degradation in senescing wheat leaves. *Plant Physiol.* **83:** 497-499.

Law, D. M. and Davies, P. J. (1990) Comparative indole-3-acetic acid levels in the slender pea and other pea phenotypes. *Plant Physiol.* **93:** 1539-1543.

Le Pabic, C., Hoffelt, M. and Roussaux, J. (1988) Adenine derivatives as inhibitors of photosystem II: relation with cytokinin activity. *Plant Cell Physiol.* **29:** 289-294.

Le Pabic, C., Hoffelt, M. and Roussaux, J. (1990) Photosynthetic electron transport in plastids in detached cucumber cotyledons treated with 6-benzylaminopurine and potassium. *Plant Cell Physiol.* **31:** 333-339.

Leopold, A. C. and Karvase, M. (1964) Benzyladenine effects on bean leaf growth and senescence. *Am. J. Bot.* **51:** 294-298.

Lerbs, S., Lerbs, W., Klyachko, N. L., Romanko, E. G., Kulaeva, O. N., Wollgiehn, R. and Parthier, B. (1984) Gene expression in cytokinin- and light-mediated plastogenesis of *Cucurbita* cotyledons: ribulose-1,5-bisphosphate carboxylase/oxygenase. *Planta* **162:** 289-298.

Leshem, B. and Sussex, I. M. (1990) Exogenous cytokinin acts independently to regulate growth and storage protein metabolism in cultured melon embryos. *J. Plant Physiol.* **137:** 238-240.

Letham, D. S. and Palni, L. M. S. (1983) The biosynthesis and metabolism of cytokinins. *Ann. Rev. Plant Physiol.* **34:** 163-197.

Lew, R. and Tsuji, H. (1982) Effect of benzyladenine treatment duration on δ-aminolevulinic acid accumulation in the dark, chlorophyll lag phase abolition, and long-term chlorophyll production in excised cotyledons of dark-grown cucumber seedlings. *Plant Physiol.* **69:** 663-667.

Li, C. J. and Bangerth, F. (1992) The possible role of cytokinins, ethylene and indoleacetic acid in apical dominance. *In:* Progress in plant growth regulation. (Karssen, C. M., van Loon, L. C., Vreugdenhil, D., eds.) Kluwer Acad. Publ., the Netherlands, pp. 431-436.

Li, Y., Hagen, G. and Guilfoyle, J. (1992) Altered morphology in transgenic tobacco plants that overproduce cytokinins in specific tissues and organs. *Developm. Biol.* **153:** 386-395.

Li, Y., Shi, X., Strabala, T. J., Hagen, G. and Guilfoyle, T. J. (1994) Transgenic tobacco plants that overproduce cytokinins show increased tolerance to exogenous auxin and auxin transport inhibitors. *Plant Science* **100:** 9-14.

Lichtenthaler, H. K. and Buschmann, C. (1978) Control of chloroplast development by red light, blue light and phytohormones. *In:* Chloroplast development - Developments in plant biology. (Akoyunoglou, G., Argyroudi-Akoyunoglou, J. H., eds.) Elsevier, Amsterdam, Vol. 2, pp. 801-816.

Lichtenthaler, H. K., Buschmann, C., Döll, M., Fietz, H.-J., Bach, T., Kozel, U., Meier, D. and Rahmsdorf, U. (1981) Photosynthetic activity, chloroplast ultrastructure, and leaf characteristics of high-light and low-light plants and of sun and shade leaves. *Photosynth. Res.* **2:** 115-141.

Lichtenthaler, H. K. and Rinderle, U. (1988) The role of chlorophyll fluorescence in the detection of stress conditions in plants. *In:* Critical reviews in analytical chemistry. CRC Press Inc., Florida, Vol. 19 (Suppl. 1), pp. S29-S85.

Lichtenthaler, H. K. and Wellburn, A. R. (1983) Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. *Biochem. Soc. Trans.* **11:** 591-592.

Linsmaier, E. M. and Skoog, F. (1965) Organic growth factor requirements of tobacco tissue cultures. *Physiol. Plant.* **18:** 100-127.

Longo, G. P., Bracale, M., Rossi, G. and Longo, C. P. (1986) Effect of benzyladenine on the polypeptide pattern of plastids in excised watermelon cotyledons. *Physiol. Plant.* **68:** 678-684.

Longo, G. P. M., Bracale, M., Rossi, G. and Longo, C. P. (1990) Benzyladenine induces the appearance of LHCP-mRNA and of the relevant protein in dark-grown excised watermelon cotyledons. *Plant Mol. Biol.* **14:** 569-573.

Loomis, R. S. and Gerakis, P. A. (1975) Productivity of agricultural ecosystems. *In:* Photosynthesis and productivity in different environments. (Cooper, J. P., ed.) Cambridge Univ. Press, Cambridge, pp. 145-172.

Louis, J. P., Augur, C. and Teller, G. (1990) Cytokinins and differentiation processes in *Mercurialis annua*. - Genetic regulation, relations with auxins, indoleacetic acid oxidases, and sexual expression patterns. *Plant Physiol.* **94:** 1535-1541.

Maliga, P., Sz.-Breznovits, A. and Marton, L. (1973) Streptomycin-resistant plants from callus culture of haploid tobacco. *Nature* **244:** 29-30.

Mansfield, T. A. (1987) Hormones as regulators of water balance. *In:* Plant hormones and their role in plant growth and development. (Davies, P. J., ed.) Martinus Nijhoff Publ., Dordrecht, pp. 411-430.

Martínez-Carrasco, R., Cervantes, E., Pérez, P., Morcuende, R. and Martín del Molino, I. M. (1993) Effect of sink size on photosynthesis and carbohydrate content of leaves of three spring wheat varieties. *Physiol. Plant.* **89:** 453-459.

Maslenkova, L. T., Zanev, Y. and Popova, L. P. (1989) Effect of abscisic acid on the photosynthetic oxygen evolution in barley chloroplasts. *Photosynth. Res.* **21:** 45-50.

Mattoo, A. K., Hoffman-Falk, H., Marder, J. B. and Edelman, M. (1984) Regulation of protein metabolism: coupling of photosynthetic electron transport to *in vivo* degradation of the rapidly metabolized 32-kilodalton protein of the chloroplast membranes. *Proc. Natl Acad. Sci. USA* **81:** 1380-1384.

McElroy, W. D. and DeLuca, M. (1981) The chemistry and applications of firefly luminescence. *In:* Bioluminescence and chemiluminescence - Basic chemistry and analytical applications. (DeLuca, M. A., McElroy, W. D., eds.) Acad. Press, pp. 179-186.

Medford, J. I., Horgan, R., El-Sawi, Z. and Klee, H. J. (1989) Alterations of endogenous cytokinins in transgenic plants using a chimeric isopentenyl transferase gene. *Plant Cell* **1:** 403-413.

Mei, R. and Yocum, C. F. (1991) Calcium retards NH_2OH inhibition of O_2 evolution activity by stabilization of Mn(2+) binding to photosystem II. *Biochemistry* **30:** 7836-7842.

Melis, A. (1985) Functional properties of photosystem II_β in spinach chloroplasts. *Biochim. Biophys. Acta* **808:** 334-342.

Melis, A. (1991) Dynamics of photosynthetic membrane composition and function. (review) *Biochim. Biophys. Acta* **1058:** 87-106.

Miller, C. O., Skoog, F., Okomura, F. S., von Saltza, M. H. and Strong, F. M. (1956) Isolation, structure and synthesis of kinetin, a substance promoting cell division. *J. Amer. Chem. Soc.*

78: 1345-1350.

Momotani, E., Aoki, K. and Tsuji, H. (1991) Effect of benzyladenine on diurnal changes in DNA content per chloroplast and chloroplast replication in intact bean leaves. *J. Exp. Bot.* **42:** 1287-1293.

Morris, D. A. (1993) The role of auxin in the apical regulation of leaf abscission in cotton (*Gossypium hirsutum* L.). *J. Exp. Bot.* **44:** 807-814.

Morris, J. W., Doumas, P., Morris, R. O. and Zaerr, J. B. (1990) Cytokinins in vegetative and reproductive buds of *Pseudotsuga menziesii*. *Plant Physiol.* **93:** 67-71.

Mullet, J. E., Klein, P. G. and Klein, R. R. (1990) Chlorophyll regulates accumulation of the plastid-encoded chlorophyll apoproteins CP43 and D1 by increasing apoprotein stability. *Proc. Natl. Acad. Sci. USA* **87:** 4038-4042.

Muñoz, J. L., Martin, L., Nicolas, G. and Villalobos, N. (1990) Influence of endogenous cytokinins on reserve mobilization in cotyledons of *Cicer arietinum* L.: reproduction of endogenous levels of total cytokinins, zeatin, zeatin riboside and their corresponding glucosides. *Plant Physiol.* **93:** 1011-1016.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15:** 473-497.

Neill, S. J., McGaw, B. A. and Horgan, R. (1986) Ethylene and 1- aminocyclopropane-1-carboxylic acid production in *flacca*, a wilty tomato mutant, subjected to water deficiency and pretreatment with abscisic acid. *J. Exp. Bot.* **37:** 535-541.

Ohad, I., Kyle, D. J. and Arntzen, C. J. (1984) Membrane protein damage and repair: removal and replacement of inactivated 32- kilodalton polypeptides in chloroplast membranes. *J. Cell Biol.* **99:** 481-485.

Ohya, T., Kojima, S., Naito, K. and Suzuki, H. (1986) Differential effects of benzyladenine and potassium on ribulose-1,5-bisphosphate carboxylase synthesis in detached cucumber cotyledons. *J. Plant Physiol.* **125:** 115-121.

Ohya, T. and Suzuki, H. (1991) The effects of benzyladenine on the accumulation of messenger RNAs that encode the large and small subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase and light-harvesting chlorophyll a/b protein in excised cucumber cotyledons. (short communication) *Plant Cell Physiol.* **32:** 577- 580.

Ondřej, M., Bavrina, T. V., Dudko, N., Hrouda, M., Krekule, J., Lozhnikova, V. N., Macháčková, I., Seidlová, F. and Vlasák, J. (1991) Transgenic tobacco plants with T-DNA phytohormone synthesis genes. *Biol. Plant.* **33:** 40-48.

Ondřej, M., Macháčková, I., Čatský, J., Eder, J., Hrouda, M., Pospíšilová, J. and Synková, H. (1990) Potato transformation by T-DNA cytokinin synthesis gene. *Biol. Plant.* **32:** 401-406.

Ondřej, M., Vlasák, J., Hrouda, M., Macháčková, I. and Kostřica, P. (1992) Regeneration of plants carrying *Agrobacterium tumefaciens* T-DNA gene for cytokinin synthesis. *In:* Physiology and biochemistry of cytokinins in plants. (Kamínek, M., Mok, D. W. S., Zažímalová, E., eds.) SPB Acad. Publ. bv, The Hague, pp. 111-113.

Ono, T.-A. and Inoue, Y. (1991) A possible role of redox-active histidine in the photoligation of manganese into a photosynthetic O_2-evolving enzyme. *Biochemistry* **30:** 6183-6188.

Ooms, G., Risiott, R., Kendall, A., Keys, A., Lawlor, D., Smith, S., Turner, J. and Young, A. (1991) Phenotypic changes in T-*cyt*- transformed potato plants are consistent with enhanced sensitivity of specific cell types to normal regulation by root- derived cytokinin. *Plant Mol. Biol.* **17:** 727-743.

Ögren, E. (1993) Convexity of the photosynthetic light-response curve in relation to intensity and direction of light during growth. *Plant Physiol.* **101:** 1013-1019.

Öquist, G. and Chow, W. S. (1992) On the relationship between the quantum yield of photosystem II electron transport, as determined by chlorophyll fluorescence and the quantum yield of CO_2- dependent O_2-evolution. *Photosynth. Res.* **33:** 51-62.

Palma, J. M., Sandalio, L. M. and Del Río, L. A. (1986) Manganese superoxide dismutase and higher plant chloroplasts: a reappraisal of a controverted cellular localization. *J. Plant Physiol.* **125:** 427-439.

Panizza, M., Mensuali-Sodi, A. and Tognoni, F. (1993) Role of ethylene in axillary shoot proliferation of Lavandin. - Interaction with benzyladenine and polyamines. *J. Exp. Bot.* **44:** 387-394.

Parry, A. D., Blonstein, A. D., Babiano, M. J., King, P. J. and Horgan, R. (1991) Abscisic-acid metabolism in a wilty mutant of *Nicotiana plumbaginifolia*. *Planta* **183:** 237-243.

Parthier, B. (1979) The role of phytohormones (cytokinins) in chloroplast development. (review) *Biochem. Physiol. Pflanzen* **174:** 173-214.

Parthier, B., Brückner, C., Dathe, W., Hause, B., Herrmann, G., Knöfel, H.-D., Kramell, H.-M., Kramell, R., Lehmann, J., Miersch, O., Reinbothe, St, Sembdner, G., Wasternack, C. and Zur Nieden, U. (1992) Jasmonates: metabolism, biological activities, and modes of action in senescence and stress responses. *In:* Progress in plant growth regulation. (Karssen, C. M., van Loon, L. C., Vreugdenhil, D., eds.) Kluwer Acad. Publ., the Netherlands, pp. 276-285.

Parthier, B., Lehmann, J., Lerbs, S., Lerbs, W., Weidhase, R. A. and Wollgiehn, R. (1987) Hormone and light actions in the differentiation program of chloroplasts. *In:* Molecular biology of plant growth control. Alan R. Liss Inc., pp. 391-400.

Pedhadiya, M. D., Vaishnav, P. P. and Singh, Y. D. (1987) Development of photosynthetic electron transport reactions under the influence of phytohormones and nitrate nutrition in greening cucumber cotyledons. *Photosynth. Res.* **13:** 159-165.

Peterson, R. B. and Hanson, K. R. (1991) Changes in photochemical and fluorescence yields in leaf tissue from normal and starchless *Nicotiana sylvestris* with increasing irradiance. *Plant Sci.* **76:** 143-151.

Popova, L. P., Tsonev, D. T. and Vaklinova, S. G. (1987) A possible role for abscisic acid in regulation of photosynthetic and photorespiratory carbon metabolism in barley leaves. *Plant Physiol.* **83:** 820-824.

Pospíšilová, J., Čatský, J., Synková, H., Macháčková, I. and Solárová, J. (1993) Gas exchange and

in vivo chlorophyll fluorescence in potato and tobacco plantlets *in vitro* as affected by various concentrations of 6-benzylaminopurine. *Photosynthetica* **29:** 1-12.

Pospíšilová, J., Solárová, J., Čatský, J., Ondřej, M. and Opatrný, Z. (1988) The photosynthetic characteristics during the micropropagation of tobacco and potato plants. *Photosynthetica* **22:** 205-213.

Prasad, P., Chanda, S. V., Vaishnav, P. P. and Singh, Y. D. (1988) Hormonal effects on the components of photosynthetic electron transport in *Dolichos falcatus* L. leaf discs incubated in the dark. *Photosynthetica* **22:** 554-561.

Prinsen, E. (1993a) *Agrobacterium rhizogenes* TL and *rol*A transgenic *Nicotiana tabacum* cv. Xanthi plants: endogenous indole-3-acetic acid, cytokinins and abscisic acid. *In:* Phytohormones in plant-bacterium interactions. Ph.D.Thesis, Universitaire Instelling Antwerpen, pp. 107-118.

Prinsen, E. (1993b) Expression of the *rol*B gene in *Nicotiana tabacum* SR1 anthers correlates with a reduction in indoxyl- glucoside content and an increase in free and bound IAA content. *In:* Phytohormones in plant-bacterium interactions. Ph.D.Thesis, Universitaire Instelling Antwerpen, pp. 119-131.

Prioul, J.-L. and Reyss, A. (1987) Acclimation of ribulose bisphosphate carboxylase and mRNAs to changing irradiance in adult tobacco leaves. *Plant Physiol.* **84:** 1238-1243.

Pritchard, J., Tomos, A. D. and Wyn Jones, R. G. (1987) Control of wheat root elongation growth. I. Effect of ions on growth rate. Wall rheology and cell water relations *J. Exp. Bot.* **38:** 948-959.

Pritchard, J., Wyn Jones, R. G. and Tomos, A. D. (1991) Turgor, growth and rheological gradients of wheat roots following osmotic stress. *J. Exp. Bot.* **42:** 1043-1049.

Procházka, S. and Jacobs, W. P. (1984) Transport of benzyladenine and gibberellin A1 from roots in relation to the dominance between the axillary buds of pea (*Pisum sativum* L.) cotyledons. *Plant Physiol.* **76:** 224-227.

Quick, W. P. and Horton, P. (1984) Studies on the induction of chlorophyll fluorescence quenching by redox state and transthylakoid pH gradient. *Proc. Roy. Soc. Lond. B* **220:** 371- 382.

Rayle, D. L., Ross, C. W. and Robinson, N. (1982) Estimation of osmotic parameters accompanying zeatin-induced growth of detached cucumber cotyledons. *Plant Physiol.* **70:** 1634-1636.

Rich, P. R., Madgwick, S. A. and Moss, D. A. (1991) The interactions of duroquinol, DBMIB and NQNO with the chloroplast cytochrome *bf* complex. *Biochim. Biophys. Acta* **1058:** 312-328.

Robinson, S. P., Wiskich, J. T. and Paleg, L. G. (1978) Effects of indoleacetic acid on CO_2 fixation, electron transport and phosphorylation in isolated chloroplasts. *Aust. J. Plant Physiol.* **5:** 425-431.

Rodbard, D. (1974) Statistical quality control and routine data processing for radioimmunoassays and immunoradiometric assays. *Clin. Chem.* **20:** 1255-1270.

Rossi, M. and Iusem, N. D. (1994) Tomato (*Lycopersicon esculentum*) genomic clone homologous to a gene encoding an abscisic acid-induced protein. *Plant Physiol.* **104:** 1073-1074.

Röder, F. T., Schmülling, T. and Gatz, C. (1994) Efficiency of the tetracycline-dependent gene expression system: complete suppression and efficient induction of the *rolB* phenotype in transgenic plants. *Mol. Gen. Genet.* **243:** 32-38.

Sabat, S. C., Grover, A. and Mohanty, P. (1989) Senescence induced alteration in the electron transport in wheat leaf chloroplasts. *J. Photochem. Photobiol., B: Biology* **3:** 175-183.

Sabater, B. and Rodriguez, M. T. (1978) Control of chlorophyll degradation in detached leaves of barley and oat through effect of kinetin on chlorophyllase levels. *Physiol. Plant.* **43:** 274-276.

Sandalio, L. M. and Del Río, L. A. (1987) Localization of superoxide dismutase in glyoxysomes from *Citrullus vulgaris*. Functional implications in cellular metabolism. *J. Plant Physiol.* **127:** 395-409.

Sandberg, G., Gardeström, P., Sitbon, F. and Olsson, O. (1990) Presence of indole-3-acetic acid in chloroplasts of *Nicotiana tabacum* and *Pinus sylvestris*. *Planta* **180:** 562-568.

Santarius, K. A. (1980) Membrane lipids in heat injury of spinach chloroplasts. *Physiol. Plant.* **49:** 1-6.

Santarius, K. A. and Müller, M. (1979) Investigations in heat resistance of spinach leaves. *Planta* **146:** 529-538.

Santarius, K. A. and Weis, E. (1988) Heat stress and membranes. *In:* Plant membranes. - Structure, assembly and function. (Harwood, J. L., Walton, T. J., eds.) The Biochem. Soc., London, pp. 97-112.

Schansker, G. and van Rensen, J. J. S. (1993) Characterization of the complex interaction between the electron acceptor silicomolybdate and photosystem II. *Photosynth. Res.* **37:** 165- 175.

Schmülling, T., Beinsberger, S., De Greef, J., Schell, J., Van Onckelen, H. and Spena, A. (1989) Construction of a heat- inducible chimaeric gene to increase the cytokinin content in transgenic plant tissue. *FEBS Lett.* **249:** 401-406.

Schmülling, T., Fladung, M., Grossmann, K. and Schell, J. (1993) Hormonal content and sensitivity of transgenic tobacco and potato plants expressing single *rol* genes of *Agrobacterium rhizogenes* T- DNA. *Plant J.* **3:** 371-382.

Schmülling, T., Schell, J. and Spena, A. (1988) Single genes from *Agrobacterium rhizogenes* influence plant development. *EMBO J.* **7:** 2621-2629.

Schmülling, T., Schell, J. and Spena, A. (1992) Construction of cytokinin overproducing mutants in higher plants. *In:* Physiology and Biochemistry of cytokinins in plants. (Kamínek, M., Mok, D. W. S., Zažímalová, E., eds.) SPB Acad. Publ. bv, The Hague, pp. 87-94.

Schnettger, B., Santore, U. J., Critchley, C., Leitsch, J. and Krause, G. H. (1992) Light-induced turnover of the D1 protein of the photosystem II reaction centre and photoinhibition *in vivo*. *In:* Research in photosynthesis. (Murata, N., ed.) Kluwer Acad. Publ., the Netherlands, Vol. IV, pp. 423-426.

Schreiber, U. (1983) Chlorophyll fluorescence yield changes as a tool in plant physiology. I. The measuring system. (Technical review) *Photosynth. Res.* **4:** 361-373.

Schreiber, U. and Berry, J. A. (1977) Heat induced changes of chlorophyll fluorescence in intact leaves correlated with damage to the photosynthetic apparatus. *Planta* **136:** 233-238.

Schreiber, U., Klughammer, C. and Neubauer, C. (1988) Measuring P700 absorbance change around 830 nm with a new type of pulse modulation system. *Z. Naturforsch.* **43c:** 686-698.

Schreiber, U., Neubauer, C. and Klughammer, C. (1989) Devices and methods for room-temperature fluorescence analysis. *Phil. Trans. Roy. Soc. Lond. B* **323:** 241-251.

Schreiber, U., Schliwa, U. and Bilger, W. (1986) Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. *Photosynth. Res.* **10:** 51-62.

Schuster, G., Shochat, S., Adir, N. and Ohad, I. (1989) Inactivation of photosystem II and turnover of the D1-protein by light and heat stresses. *In:* Techniques and new developments in photosynthetic research. (Barber, J., Malkin, R., eds.) Plenum Press, New York, pp. 499-510.

Šetlík, I., Allakhverdiev, S. I., Nedbal, L., Šetlíková, E. and Klimov, V. V. (1990) Three types of photosystem II photoinactivation. 1. Damaging processes on the acceptor side. *Photosynth. Res.* **23:** 39-48.

Seyer, P. and Lescure, A. M. (1984) Evidence for changes in plastid mRNA populations during cytokinin-induced chloroplast differentiation in tobacco cell suspensions. *Plant Sci. Lett.* **36:** 59-66.

Shriver, K. and Mundi, J. (1990) Gene expression in response to abscisic acid and osmotic stress. *Plant Cell* **2:** 503-512.

Šiffel, P., Durchan, M., Květoň, J., Ondřej, M. and Šesták, Z. (1988) Photosynthesis in regenerants of tobacco transformed by plasmids of *Agrobacterium*. 2. Fluorescence emission spectra and age induced changes in slow fluorescence induction. *Photosynthetica* **22:** 214-220.

Šiffel, P., Šindelková, E., Durchan, M. and Zajícová, M. (1992) Photosynthetic characteristics of *Solanum tuberosum* L. plants transformed by *Agrobacterium* strains. 1. Pigment apparatus. *Photosynthetica* **27:** 441-447.

Simpson, J., Timko, M. P., Cashmore, A. R., Schell, J., Van Montagu, M. and Herrera-Estrella, L. (1985) Light-inducible and tissue-specific expression of a chimaeric gene under control of the 5' flanking sequence of a pea chlorophyll a/b-binding protein gene. *EMBO J.* **4:** 2723-2729.

Sivak, M. N., Heber, U. and Walker, D. A. (1985) Chlorophyll a fluorescence and light-scattering kinetics displayed by leaves during induction of photosynthesis. *Planta* **163:** 419-423.

Sivak, M. N. and Walker, D. A. (1986) Photosynthesis *in vivo* can be limited by phosphate supply. *New Phytol.* **102:** 499-512.

Smart, C. M., Scofield, S. R., Bevan, M. W. and Dyer T. (1991) Delayed leaf senescence in tobacco plants transformed with *tmr*, a gene for cytokinin production in *Agrobacterium*. *Plant Cell* **3:** 647-656.

Smigocki, A. C. (1991) Cytokinin content and tissue distribution in plants transformed by a

reconstructed isopentenyl transferase gene. *Plant Mol. Biol.* **16:** 105-115.

Smigocki, A. C. and Owens, L. D. (1988) Cytokinin gene fused with a strong promoter enhances shoot organogenesis and zeatin levels in transformed plant cells. *Proc. Natl. Acad. Sci. USA* **85:** 5131-5135.

Smigocki, A. C. and Owens, L. D. (1989) Cytokinin-to-auxin ratios and morphology of shoots and tissues transformed by a chimeric isopentenyl transferase gene. *Plant Physiol.* **91:** 808-811.

Sossountzov, L., Maldiney, R., Sotta, B., Sabbagh, I., Habricot, Y., Bonnet, M. and Miginiac, E. (1988) Immunocytochemical localization of cytokinins in Craigella tomato and a sideshootless mutant. *Planta* **175:** 291-304.

Söll, D. (1993) Chlorophyll biosynthesis and protein synthesis. Handout of July 1 from the course Plant Biochemistry 1993, June 30 - July 18, University of Wisconsin - Madison.

Spena, A., Aalen, R. B. and Schulze, S. (1989) Cell-autonomous behaviour of the *rol*C gene of *Agrobacterium rhizogenes* during leaf development: a visual assay for transposon excision in transgenic plants. *Plant Cell* **1:** 1157-1164.

Spena, A., Estruch, J. J., Aalen, R. D., Prinsen, E., Parets- Soler, A., Nacken, W., Sommer, H., Chriqui, D., Grossmann, K., Van Onckelen, H. and Schell, J. (1992) Transgenic plants and transgenic plant mosaics for the expression of pathogen derived genes able to affect phytohormone activity. *In:* Progress in plant growth regulation. (Karssen, C. M., van Loon, L. C., Vreugdenhil, D., eds.) Kluwer Acad. Publ., the Netherlands, pp. 724-730.

Spena, A., Hain, R., Ziervogel, U., Saedler, H. and Schell, J. (1985) Construction of a heat-inducible gene for plants. Demonstration of heat-inducible activity of the *Drosophila* hsp70- promoter in plants. *EMBO J.* **4:** 2739-2743.

Spena, A., Schmülling, T., Koncz, C. and Schell, J. (1987) Independent and synergistic activities of the *rol*A, B and C loci in stimulating abnormal growth in plants. *EMBO J.* **6:** 3891-3899.

Stitt, M. and Grosse, H. (1988) Interactions between sucrose synthesis and CO_2 fixation. I. Secondary kinetics during photosynthetic induction are related to a delayed activation of sucrose synthesis. *J. Plant Physiol.* **133:** 129-137.

Stitt, M., Grosse, H. and Woo, K.-C. (1988) Interactions between sucrose synthesis and CO_2 fixation. II. Alterations of fructose 2,6-bisphosphate during photosynthetic oscillations. *J. Plant Physiol.* **133:** 138-143.

Su, W. and Howell, S. H. (1992) A single genetic locus, *ckr1*, defines *Arabidopsis* mutants in which root growth is resistant to low concentrations of cytokinin. *Plant Physiol.* **99:** 1569-1574.

Süss, K.-H. and Yordanov, I. T. (1986) Biosynthetic cause of *in vivo* acquired thermotolerance of photosynthetic light reactions and metabolic responses of chloroplasts to heat stress. *Plant Physiol.* **81:** 192-199.

Takahama, U. and Nishimura, M. (1975) Formation of singlet molecular oxygen in illuminated chloroplasts. Effects on photoinactivation and lipid peroxidation. *Plant Cell Physiol.* **16:** 737-748.

Tal, M., Imber, D., Erez, A. and Epstein, E. (1979) Abnormal stomatal behaviour and hormonal

imbalance in *flacca*, a wilty mutant of tomato. V. Effect of abscissic acid on indoleacetic acid metabolism and ethylene evolution. *Plant Physiol.* **63:** 1044- 1048.

Tamas, I. A. (1987) Hormonal regulation of apical dominance. *In:* Plant hormones and their role in plant growth and development. (Davies, P. J., ed.) Martinus Nijhoff Publ., Dordrecht, pp. 393-410.

Tamas, I. A., Schwartz, J. W., Hagin, J. W. and Simmonds, R. (1974) Hormonal control of photosynthesis in isolated chloroplasts. *In:* Mechanisms of regulation of plant growth. (Beileski, R. L., Ferguson, A. R., Cresswell, M. M., eds.) Roy. Soc. New Zealand, Wellington, pp. 261-268.

Tamura, N. and Cheniae, G. M. (1988) Photoactivation of the water oxidizing complex: the mechanism and general consequences to photosystem 2. *In:* Light-energy transduction in photosynthesis: higher plant and bacterial models. (Stevens, S. E. ,Jr, Bryant, D. A., eds.) pp. 227-242.

Tepfer, D. A. (1984) Transformation of several species of higher plants by *Agrobacterium rhizogenes*. *Mol. Gen. Genet.* **206:** 17-23.

Terashima, I. and Saeki, T. (1985) A new model for leaf photosynthesis incorporating the gradients of light environment and of photosynthetic properties of chloroplasts within a leaf. *Ann. Bot.* **56:** 489-499.

Terashima, I. and Takenaka, A. (1986) Organization of photosynthetic system of dorsiventral leaves as adapted to the irradiation from the adaxial side. *In:* Biological control of photosynthesis. (Marcelle, R., Clijsters, H., Van Poucke, M., eds.) Martinus Nijhoff Publ., Dordrecht, pp. 219-230.

Terashima, I. and Takenaka, A. (1990) Factors determining light response characteristics of leaf photosynthesis. *In:* Current research in photosynthesis. (Baltscheffsky, M., ed.) Kluwer Acad. Publ., the Netherlands, Vol. IV, pp. 299-306.

Teyssendier de la Serve, B., Axelos, M. and Péaud-Lenoël, C. (1985) Cytokinins modulate the expression of genes encoding the protein of the light-harvesting chlorophyll a/b complex. *Plant Mol. Biol.* **5:** 155-163.

Thomas, P. G., Quinn, P. J. and Williams, W. P. (1986) The origin of photosystem I-mediated electron transport stimulation in heat-stressed chloroplasts. *Planta* **167:** 133-139.

Thompson, L. K., Blaylock, R., Sturtevant, J. M. and Brudvig, G. W. (1989) Molecular basis of the heat denaturation of photosystem II. *Biochemistry* **28:** 6686-6695.

Tichá I., Ondřej, M., Hrouda, M., Čatský, J., Zima, J., Pospíšilová, J. and Solárová, J. (1988) Photosynthesis in regenerants of tobacco transformed by plasmids of *Agrobacterium*. 1. Morphology, anatomy and gas exchange of transformants. *Photosynthetica* **22:** 350-363.

Tonelli, C., Salvi, C., Gavazzi, G., Marziani Longo, G. P., Rossi, G. and Longo, C. (1989) Benzyladenine partially restores the normal phenotype in a chlorophyll mutant of maize. *J. Plant Physiol.* **133:** 713-718.

Tran Thanh Van, K., Toubart, P., Cousson, A., Darvill, A. G., Gollin, D. J., Chelf, P. and Albersheim, P. (1985) Manipulation of the morphogenetic pathways of tobacco explants by

oligosaccharins. *Nature* **314:** 615-617.

Trebst, A. (1980) Inhibitors in electron flow: tools for the functional and structural localization of carriers and energy conservation sites. *In:* Methods Enzymol. (San Pietro, A., ed.) Acad. Press, Vol. 69 c, pp. 675-715.

Trewavas, A. (1991) How do plant growth substances work? II. *Plant Cell Environm.* **14:** 1-12.

Tsang, E. W. T., Bowler, C., Hérouart, D., Van Camp, W., Villaroel, R., Genetello, C., Van Montagu, M. and Inzé, D. (1991) Differential regulation of superoxide dismutases in plants exposed to environmental stress. *Plant Cell* **3:** 783-792.

Valcke, R. and Van Loven, K. (1992) Does a heat-shock treatment affect photosynthesis in *Nicotiana tabacum*? *In:* Regulation of chloroplast biogenesis. (Argyroudi-Akoyunoglou, J. H., ed.) Plenum Press, New York, pp. 457-462.

Van der Krieken, W. M., Van Eldik, G., Nijtmans, L., Croes, A. F. and Wullems, G. J. (1991) Endogenous cytokinin in flower bud forming explants of tobacco. *J. Exp. Bot.* **42:** 77-80.

van Kooten, O. and Snel, J. F. H. (1990) The use of chlorophyll fluorescence nomenclature in plant stress physiology. *Photosynth. Res.* **25:** 147-150.

Van Loven, K., Beinsberger, S. E. I., Valcke, R. L. M., Van Onckelen, H. A. and Clijsters, H. M. M. (1993) Morphometric analysis of the growth of *Phsp70-ipt* transgenic tobacco plants. *J. Exp. Bot.* **44:** 1671-1678.

Van Onckelen, H., Rüdelsheim, P., Beinsberger, S., Inzé, D., Van Montagu, M. and De Greef, J. (1988) Mutual interaction of plant specific and T-DNA controlled IAA metabolism in transformed tobacco tissue. *In:* Physiology and biochemistry of auxins in plants. (Kutáček, M., Bandurski, R. S., Krekule, J., eds.) SPB Acad. Publ., The Hague, pp. 379-387.

Van Oosten J.-J. and Besford, R. T. (1994) Sugar feeding mimics effect of acclimation to high CO_2. - Rapid down regulation of RuBisCO small subunit transcripts but not of the large subunit transcripts. *J. Plant Physiol.* **143:** 306-312.

van Staden, J., Cook, E. L. and Noodén, L. D. (1988) Cytokinins and senescence. *In:* Senescence and aging in plants. (Noodén, L. D., Leopold, A. C., eds.) Acad. Press, San Diego, pp. 281-328.

van Wijk, K. J. and van Hasselt, P. R. (1993) Kinetic resolution of different recovery phases of photoinhibited photosystem II in cold-acclimated and non-acclimated spinach leaves. *Physiol. Plant.* **87:** 187-198.

Vandenborn, R. (1990) Morfologische studie van de rol van endogene cytokinine op de biogenese van het fotosynthese apparaat. Thesis, Universitaire Instelling Antwerpen, 51 p.

Vaňková, R., Gaudinová, A., Kamínek, M. and Eder, J. (1992) The effect of interaction of synthetic cytokinin and auxin on production of natural cytokinins by immobilized tobacco cells. *In:* Physiology and biochemistry of cytokinins in plants. (Kamínek, M., Mok, D. W. S., Zažímalová, E., eds.) SPB Acad. Publ. bv, The Hague, pp. 47-51.

Vaňková, R., Hsiao, K.-C., Bornman, C. H. and Gaudinová, A. (1991) Effect of synthetic cytokinins on levels of endogenous cytokinins and respiration patterns of *Beta vulgaris* cells in

suspension. *J. Plant Growth Regul.* **10:** 197-199.

Velitchkova, M., Ivanov, A. and Petkova, R. (1989) Mechanism of heat induced stimulation of PSI activity in pea chloroplasts. *In:* Techniques and new developments in photosynthesis research. (Barber, J., Malkin, R., eds.) Plenum Publ. Corp., pp. 587-590.

Vierling, E. (1991) The roles of heat shock proteins in plants. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **42:** 579-620.

Walker, D. A. (1981) Secondary fluorescence kinetics of spinach leaves in relation to the onset of photosynthetic carbon assimilation. *Planta* **153:** 273-278.

Walker, D. A. (1989) Automated measurement of leaf photosynthetic O_2 evolution as a function of photon flux density. *Phil. Trans. Roy. Soc. Lond. B* **323:** 313-326.

Walker, D. (1990) *In:* The use of the oxygen electrode and fluorescence probes in simple measurements of photosynthesis. 2nd ed. Oxygraphics Ltd. (Publ.).

Walker, D. A., Sivak, M. N., Prinsley, R. and Cheesbrough, J. K. (1983) Simultaneous measurement of oscillations in oxygen evolution and chlorophyll a fluorescence in leaf pieces. *Plant Physiol.* **73:** 542-549.

Wang, T. L., Buetelmann, P. and Cove, D. J. (1981) Cytokinin biosynthesis in mutants of the moss *Physcomitrella patens*. *Plant Physiol.* **68:** 739-744.

Ward, D. A. and Bunce, J. A. (1987) Abscissic acid simultaneously decreases carboxylation efficiency and quantum yield in attached soybean leaves. *J. Exp. Bot.* **38:** 1182-1192.

Weiler, E. W. (1980) Radioimmunoassay for *trans*-zeatine and related cytokinins. *Planta* **149:** 155-162.

Weis, E. (1981) Reversible heat inactivation of the Calvin cycle: a possible mechanism of the temperature regulation of photosynthesis. *Planta* **151:** 33-39.

Weis, E., Lechtenberg, D. and Krieger, A. (1990) Physiological control of primary photochemical energy conversion in higher plants. *In:* Current research in photosynthesis. (Baltscheffsky, M., ed.) Kluwer Acad. Publ., the Netherlands, Vol. IV, pp. 307-312.

Więckowski, S. and Fiedor, L. (1990) Oxygen uptake by heat- pretreated chloroplasts isolated from cucumber cotyledons at the early stages of greening. *J. Plant Physiol.* **136:** 696-700.

Winters, M. (1994) Studie van metabolieten en enzymen van het fotosynthese-apparaat. Vergelijking tussen wild type en transgene tabaksplanten. thesis KIHL, Diepenbeek, 103 p.

Yamashita, T., Kobayashi, T., Morita, Y. and Shoji, T. (1990a) The roles of weak light, oxygen and dithiothreitol in the photoreactivation of the oxygen evolving center of tris-washed and 2,6-dichlorophenol indophenol-treated chloroplasts. *Photosynth. Res.* **26:** 1-8.

Yamashita, T., Kobayashi, T., Morita, Y. and Shoji, T. (1990b) Weak light dependent coupling of the photoreduction of oxygen and the photoreactivation of oxygen evolving center. *In:* Current Res. Photosynth. (Baltscheffsky, M., ed.) Kluwer Acad. Publ., Vol. I, pp. 913-916.

Yusibov, V. M., Il, P. C., Andrianov, V. M. and Piruzian, E. S. (1991) Phenotypically normal

transgenic T-*cyt* tobacco plants as a model for the investigation of plant gene expression in response to phytohormonal stress. *Plant. Mol. Biol.* **17:** 825-836.

Zambryski, P. C. (1992) Chronicles from the *Agrobacterium*-plant cell DNA transfer story. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **43:** 465-490.

Zerbe, R. and Wild, A. (1980) The effect of kinetin on the photosynthetic apparatus of *Sinapis alba*. *Photosynth. Res.* **1:** 53-64.

Zieslin, N. and Khayat, E. (1990) Effects of alternating night temperature and cytokinin application on the activity of enzymes of sucrose metabolism in leaves, young shoots and petals of roses. *Plant Cell Physiol.* **31:** 845-849.

Zimmermann, K.-H., Chudy, M., Preusser, E. and Göring, H. (1987) Relationships between light and hormone action on ribulose-1,5- bisphosphate carboxylase in cucumber cotyledons. *Biochem. Physiol. Pflanzen* **182:** 407-415.

Acknowledgement - dankwoord:

Een doctoraat voorbereiden doe je niet alleen. Het werk dat in dit proefschrift wordt voorgesteld, is tot stand gekomen dank zij de kritische en practische ondersteuning van velen en ik zou allen daarvoor hartelijk willen danken.

Prof. Dr. H. Clijsters, u als promotor, en ik hebben bij de voorbereiding van dit proefschrift heel wat stof tot discussie gehad. U leerde daarbij mijn gedachtengang lezen, en ik leerde die wetenschappelijk te formuleren. Dit was een leerrijke ervaring voor mij. Dank u wel voor de tijd die u daar telkens voor vrijmaakte.

Prof. Dr. R. Valcke, eveneens promotor, u heeft me ingewijd in een bijzonder boeiend domein van de wetenschap. Steeds kon ik bij u terecht om nieuwe ideeën te toetsen. Ik ben blij dat ik hier een leerproces heb kunnen en mogen doorlopen om als jonge onderzoeker zelf ook het roer in handen te nemen. Dank u wel, dat u me behoed heeft voor averij. Ook dank voor de vele aansporingen en de steun die ik heb ervaren.

Prof. Dr. H. Van Onckelen, in het labo biochemie (UIA) heb ik op een indrukwekkende wijze kennis gemaakt met het phytohormonen-onderzoek. Langs de zijde van Dr. S. Beinsberger heb ik enkele doseringen kunnen uitvoeren. De grote technische bekwaamheid en de aangename contacten met de vele mensen in het labo doen me steeds graag terugkeren. Langs deze weg wil ik u ook danken voor de efficiënte en kritische besprekingen.

Prof. Dr. R. Strasser, I want to express my appreciation for your constructive criticism. Thank you very much for providing me with a theoretical background on chlorophyll fluorescence and also thank you for your encouragements.

Susy, zoals reeds gezegd, jij hebt me bijzonder goed opgevangen voor de hormoondoseringen. Ik heb me vaak opgetrokken aan jou enorm doorzettingsvermogen. Ik ben blij dat ik je heb kunnen navolgen tot op dit stadium, het voorleggen van dit proefschrift.

Important contributions have been made by Dr. G. Kudoyarova and Dr. H. Synková. Guzel, you and your husband Stas have done an enormous job with the cytokinin analysis of the P*ssu-ipt* plants. Our discussions were sometimes intense, but have certainly given me a deeper understanding of the problems.
Side by side with Helena, we have tried to unravel fragments of the photosynthetic electron transport chain. It was a challenge, but together we have managed to do a great deal of work. Helena, in these periods you were like a mentor to me, but let us keep these memoirs for later.
Naďa, the contact with you has been brief up to now, but I realize that you have given already impulses for further work.
Beside science, the personal contact with all of you has been enriching.

Seeds from the transgenic P*hsp70-ipt* plants were kindly provided by Dr. T. Schmülling and Dr. A. Spena (Max Planck Institut für Züchtungsforschung, 5000 Köln 30,

FRG). Dr. S. Beinsberger stelde de P*ssu-ipt* transgene lijn ter beschikking.

Prof. Dr. H. Callaert wil ik danken voor zijn verhelderende suggesties bij de statistische analyse.

Wat de technische ondersteuning betreft, mocht ik beroep doen op zeer bekwame mensen. Katleen, jij was een grote aanwinst binnen het FKFO-project en hebt dit werk mee mogelijk gemaakt. Voor jou precisie en je sterke inzet heb ik bewondering. Bijna alle planten werden door jou opgekweekt en tot in de eindfase kon ik bij je langslopen, met onder andere een bundel correctiewerk. Daarvoor zeg ik: dank je wel. Greet, vele handen maken het werk lichter, dank je wel voor die van jou. Verder mocht ik putten uit de ervaring van Brigitte (ook buiten de practica heb ik veel van je geleerd), Nelia, en de FTO-biologie-mensen. Jan en Jef, in het bijzonder voor al jullie werk aan de serre en heat-shock kasten: bedankt! Jo, als bureau-genote heb je vaak klankbord gespeeld, en dat heb je prima gedaan. Voor computer- en electronische snufjes kon ik terecht bij Danny, Koen en Kristof. Danny, ook van harte bedankt voor je hulp bij het maken van een aantal figuren.
Verder werd mijn werkomgeving kleurrijk aangevuld met collega's dierkunde en biochemie, en passeerden nog enkele niet vermelde collega's plantkunde de revue. 's Avonds stond Sali me steeds op een fijne wijze te hulp. Jullie hebben mee de "ups and downs" beleefd, en droegen bij tot een sfeer waarin ik graag vertoefde.

Voor al het overige ... waren daar familie en vele vrienden. Zij toonden een aangename interesse, en kwamen in de laatste maanden zelfs met een 'Karen-AID actie' opzetten. Hiermee hebben zij een drukke periode opgevrolijkt.

Een bijzondere plaats werd ingenomen door mijn ouders. Ma en pa, te allen tijde kon ik bij jullie terecht, voor een luisterend oor en voor twee paar helpende handen. Jullie creativiteit is zelfs tot in Diepenbeek gekend en jullie geloof in mij heeft me voortgestuwd. Dank je wel voor al hetgene dat hier niet te noemen is.

Voor een aantal buitenlandse activiteiten werden mij kredieten verschaft door het Limburgs Universitair Centrum[1] (LUC) en het Nationaal Fonds voor Wetenschappelijk Onderzoek[2] (NFWO):

- Advanced Course on Techniques in Photosynthesis, R. Hill Institute, University of Sheffield, UK, 1990[1].
- International Meeting on the Regulation of Chloroplast Biogenesis, Kreta, 1991[2].
- Course Plant Biochemistry - 1993, University of Wisconsin, USA, 1993[1].
- 9th FESPP Congress, Brno, Czech Rep., 1994[1].

Verder ontving ik financiële steun van achtereenvolgens het Instituut tot aanmoediging van het Wetenschappelijk Onderzoek in Nijverheid en Landbouw (IWONL), LUC en NFWO.

Tenslotte, wil ik mijn vroegere leraar biologie, Leo Michiels, gelijk geven toen hij - nu bijna 10 jaar geleden - zei dat fotosynthese in werkelijkheid veel complexer is, en dat er nog véél onderzoek naar dient te gebeuren ... Leo, dit was een stimulans waar ik nu nog jaren mee verder kan.

Karen.