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Anti-sense RNA efficiently inhibits formation of the 10 kd polypeptide of photosystem II in transgenic potato plants: analysis of the role of the 10 kd protein

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A chimeric gene encoding an anti-sense RNA of the 10 kd protein of the water-splitting apparatus of photosystem II of higher plants under the control of the CaMV 35S promoter was introduced into potato using *Agrobacterium* based vectors. The expression of the anti-sense RNA led to a significant reduction of the amounts of the 10 kd protein and RNA in a number of trangenic plants. In three out of 36 plants tested, the level of the 10 kd protein was only up to 1-3% compared with the wild-type control. The drastic reduction of the 10 kd protein did not influence the accumulation of other photosystem II associated polypeptides at both the RNA and protein level. Furthermore no phenotypic differences were observed between potato plants expressing wild-type and drastically reduced levels of the 10 kd protein with respect to growth rate, habitus or ultrastructure of the chloroplasts. Measurements of the relaxation of the flash-induced enhancement in the fluorescence quantum yield as determined in intact leaves and the rates and characteristic oscillation pattern of O_2 evolution as determined in isolated thylakoid samples however, show that the elimination of the 10 kd protein on the one hand retards reoxidation of Q_A- and on the other hand introduces a general disorder into the PSII complex.

Key words: anti-sense RNA/photosystem II/10 kd protein/transgenic plants

Introduction

Photosynthesis represents a process of fundamental importance with respect to the development and maintenance of all organisms. Durig recent years the combined application of both molecular biology and biochemical/biophysical methods has considerably improved both the understanding of mechanisms underlying the process of photosynthesis and structural features of the complex involved (Glazer and Melis, 1987; Andréasson and Vänngard, 1988; Rochaix and Erickson, 1988). Probably the best understood photosynthetically active complex is the photosynthetic reaction centre of the purple bacterium *Rhodopseudomonas viridis* where X-ray crystallographic analysis has led to a complete unravelling of its structure (Deisenhofer *et al.*, 1984, 1985).

It is obvious that the results of these experiments are of ultimate importance also for the understanding of the structure of the reaction centre of photosystem II of higher plants. In contrast to the reaction centre of purple bacteria, however, photosystem II is capable of light-induced oxidation of water, thus being the major source of all oxygen found on earth. In higher plants, photosystem II is formed by a large number of different polypeptides the majority of which can be assigned to three functional domains, the light-harvesting system, the photochemical reaction centre and the water-splitting activity. Some of these polypeptides are encoded by the plastid genome and others are the products of nuclear genes (Herrmann *et al.*, 1985; Rochaix and Erickson, 1988).

While the location of the functional redox groups of the photosystem II reaction centre within a heterodimer of polypeptides D1 and D2 is now widely accepted (Trebst, 1986; Deisenhofer and Michel, 1989), the structural basis of the water-splitting complex still needs to be unravelled (Renger, 1987; Homann, 1988; Brudvig *et al.*, 1989; Rutherford, 1989). Four polypeptides of 33, 23, 16 and 10 kd located in the thylakoid lumen are closely associated with this activity (Jansson *et al.*, 1979; Åkerlund *et al.*, 1982; Kuwabara and Murata, 1982; Yamamoto *et al.*, 1983; Ljungberg *et al.*, 1986; Miyao and Murata, 1989). However, none of them carries the catalytic manganese cluster, they are rather of structural and regulatory importance for the functional integrity of the water-splitting complex (Homann, 1988; Brudvig *et al.*, 1989; Rutherford, 1989). With regard to the central role these polypeptides play in the process of oxygen evolution, a more complete understanding of their function would be highly desirable. In the past, reconstitution experiments (Åkerlund *et al.*, 1982; Ono and Inoue, 1984) and mutational analysis (Mayfield *et al.*, 1987a,b) have mainly been used to exploit the contribution of the different polypeptides with respect to oxygen evolution. These approaches have led to the conception that the 33 kd polypeptide is necessary to preserve the manganese in its active site (Ohio and Inoue, 1983, 1984); it is thus an obligatory requirement for photosystem II activity *in vivo* (Mayfield *et al.*, 1987a). On the other side, the 23 kd polypeptide is to some extent dispensable. A *Chlamydomonas* mutant lacking this protein still maintains the capacity of light-induced oxygen evolution, although at very reduced levels (Mayfield *et al.*, 1987b). As inferred from biochemical analysis, the function of the 23 kd protein is mainly concerned with the binding of Ca^{2+} and chloride, two essential cofactors for photosystem II activity (for a review: Homann, 1988, Rutherford, 1989).

In contrast to what is known about the roles of the 33 kd and 23 kd polypeptides, only limited information is available concerning the roles of the 16 and 10 kd proteins. No mutants have been isolated yet to elucidate their functions

in vivo. Ljungberg and coworkers (Ljungberg *et al.*, 1984, 1986) have obtained some evidence indicating that the 10 kd protein serves a structural role in the water-splitting complex by providing binding sites for the 23 kd polypeptides. However, no clear-cut conclusions could be drawn from these reconstitution experiments.

In order to gain more insight into the function of the 10 kd protein and to circumvent the problems inherent in *in vitro* experiments we decided to use an *in vivo* approach using reversed genetics with the aim of suppressing the expression of the 10 kd protein in intact plants. To this end we introduced chimeric genes into transgenic potato plants expressing an anti-sense RNA with respect to the 10 kd protein.

Here we describe the results of the analysis of potato plants expressing the wild-type level of the 10 kd protein in comparison with transgenic potato displaying only 1–3% of the wild-type level with respect to various physiological, biochemical and biophysical parameters.

Results

Construction of the 10 kd anti-sense gene and its integration into the potato genome

A full length cDNA clone of the 10 kd transcript (Eckes *et al.*, 1985) was fused in reverse orientation to the strong promoter of the CaMV 35S transcript (position −526 to +4 relative to the transcription start site), followed by the polyadenylation signal of the octopine synthase gene derived from the T-DNA of the octopine plasmid pTiACH 5 (Figure 1).

This construct was inserted into the binary vector Bin 19 (Bevan,1984) and *Agrobacteria* transformed with this construct were used for transforming potato plants via the leaf disc technique (Rocha-Sosa *et al.*, 1989). Regenerated plants were tested via DNA blot hybridization experiments for the correct integration of the anti-sense gene (data not shown) and only those plants containing non-rearranged copies of the construct were used in further experiments.

Some transgenic plants contain a dramatically reduced level of the 10 kd protein

Thirty-six transgenic potato plants containing the anti-sense construct were grown in the greenhouse. In between these plants no differences concerning growth rates and habitus were observed (see below). In order to determine whether or not any of these transgenic plants would be affected with respect to the expression level of the 10 kd protein, all 36 plants were screened by Western blot analysis. Within the population analysed the amount of the 10 kd protein varied by a factor of ~100. In six out of the 36 plants tested the amount of this protein was below the detection limit imposed by this kind of analysis (data not shown).

The reduction of the 10 kd protein is seemingly unrelated to the expression level of the anti-sense RNA

Twelve plants displaying drastic differences with respect to the amount of 10 kd protein present were chosen for a more detailed analysis at both RNA and protein levels. In order to estimate the amount of transcript encoding the 10 kd protein at the steady state RNA level, total RNA extracted from leaves was probed with a strand specific anti-sense RNA probe generated by the SP6/T7 transcription system (Figure 2A) in a RNA blot experiment.

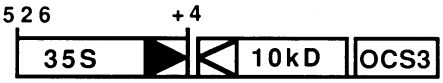

Fig. 1. Structure of the chimeric gene expressing an anti-sense RNA of the 10 kd protein encoding cDNA. The promoter of the CaMV 35S RNA (position −526 to +4 relative to the transcription start site) was fused in front of a full length cDNA clone of the 10 kd a transcript (Eckes *et al.*, 1985) and provided with the poly A site of the octopine synthase gene of the Ti-plasmid pTiACH 5. The 5′ to 3′ direction of the promoter is indicated by a filled triangle, the (original) 5′ to 3′ direction of the cDNA clone is indicated by an open triangle.

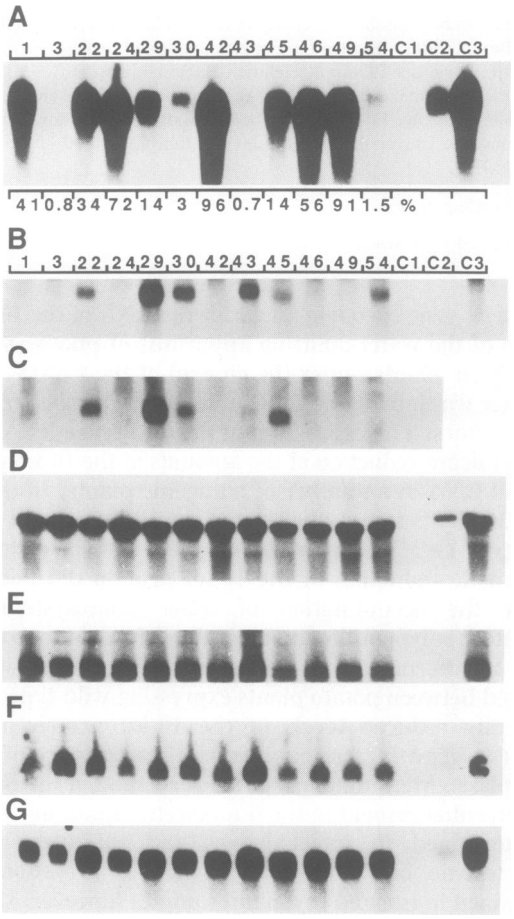

Fig. 2. Northern analysis of total RNA isolated from leaves (A, B, D–G) or roots (C) of different transgenic and control potato plants. After isolation total RNA (50 μg each except lanes C1, C2, C3) was subjected to gel electrophoretic separation using formaldehyde gels (cf. Materials and methods), blotted on nylon membranes and subjected to hybridization using different molecular probes. The following probes were used: **A:** an RNA probe encoding the anti-sense RNA of the 10 kd protein; **B,C:** an RNA probe encoding the sense RNA of the 10 kd protein; **D:** a nick-translated DNA probe encoding the plastid gene psbA; **E:** a nick-translated DNA probe encoding the plastid gene psbB; **F:** a nick-translated cDNA probe encoding the nuclear 33 kd polypeptide; **G:** a nick-translated cDNA probe encoding the nuclear small subunit of the ribulose bisphosphate carboxylase gene. The numbers 1, 3, 22, 24, 29, 30, 42, 43, 45, 46, 49, 54 on top of panel A identify different independently transformed potato plants harbouring the construct encoding the anti-sense RNA to the 10 kd protein (cf. Figure 1). Lanes C1, C2 and C3 contain 0.5, 5 and 50 μg of total RNA isolated from leaves of a nontransformed control potato plant. The numbers given below the lanes in panel A indicate the relative amount of the 10 kd transcript in percentage as compared with the level observed in a wild-type plant.

The relative transcript levels as determined by scanning the autoradiographs with a laser scanner are given in Figure 2A. Plant numbers 3, 30, 43 and 54 contain the lowest amount of 10 kd encoding RNA amounting to ~1–5% of the wild-type level whereas the level of this RNA in the plant nos 42 and 49 does not significantly differ from that found in wild-type plants (cf Figure 2A).

The same blots were also used to determine the corresponding amount of the anti-sense RNA transcribed from the transferred chimeric gene by using sense RNA as probe (Figure 2B). In addition, as the estimation of the amount of anti-sense RNA present in leaves could be misleading due to the presence of sense RNA total root RNA which is devoid of sense RNA was included in this analysis (Figure 2C). In some transformants the relative amount of detectable anti-sense RNA was similar in leaves and roots (Figure 2B and C: plant numbers 1, 22, 29,30), whereas in others the relative amount varies between leaves and roots (Figures 2B and C: plant numbers 43,45,54). It is important to note however that there is no obvious correlation between the amount of anti-sense RNA detectable and the suppression of the 10 kd transcript levels (compare Figures 2A-C).

Level of steady state RNA of other nuclear and plastid genes involved in photosynthesis

As outlined in the Introduction, photosystem II is composed of a large number of polypeptides encoded either by the nuclear or the plastid genome. The expression of both sets of genes therefore has to be co-ordinated by signals exchanged between the chloroplasts and the nucleus. Within this respect one might expect that the reduction of the 10 kd protein and its transcript levels would have an influence on the accumulation of either other nuclear and plastid encoded photosystem II transcripts or on the transcript levels of other nuclear genes encoding chloroplast-located proteins such as the genes encoding the small subunit of the ribulose bisphosphate carboxylase. The plastid genes *psbA* (Figure 2D), *psbB* (Figure 2E), as well as a cDNA from the nuclear encoded 33 kd polypeptide (which is a component of the oxygen-evolving complex of photosystem II) (Figure 2F) and a cDNA encoding the small subunit of the ribulose bisphosphate carboxylase (Figure 2G) were used as probes. (Note that in Figure 2E, only the band representing the mature RNA of the *psbB* hybridizing transcripts with a size of 1800 nucleotides is shown for this comparison).

A comparison of the transcript levels of these genes in different transgenic potato plants and wild-type plants indicates that they are seemingly not influenced by the expression level of the 10 kd protein (compare Figures 2A–G).

Steady state level of photosystem II polypeptides

In order to answer the question of whether the 10 kd polypeptide is an essential structural component involved in the assembly and/or stabilization of other photosystem II polypeptides the amount of steady state protein of some photosynthetic polypeptides was analysed in transgenic potato plants harbouring the 10 kd anti-sense contruct. To this end extracts from young and old leaves were analysed by Western blot experiments. As shown in Figure 3A, the level of the 10 kd protein closely follows the steady state RNA levels (compare Figures 2A and 3A). This result was obtained by using protein extracts from both young leaves

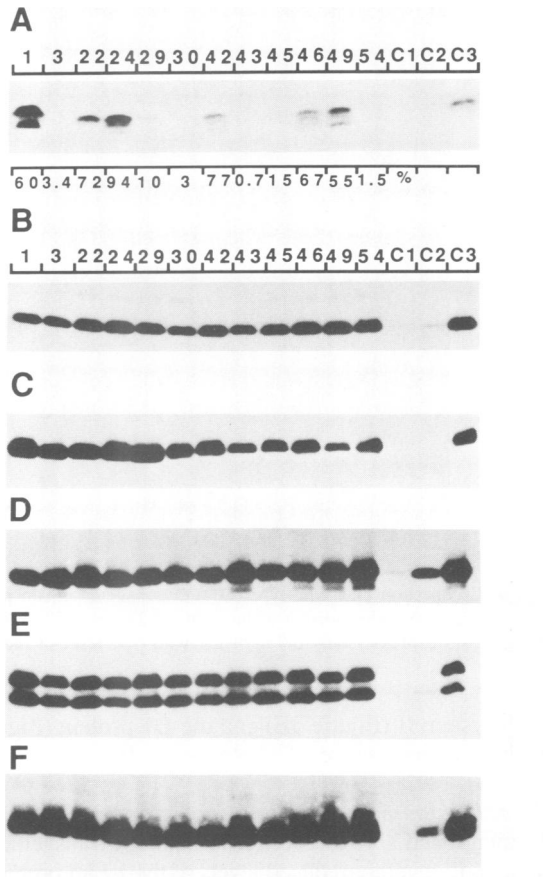

Fig. 3. Western-type analysis of total protein isolated from leaves of different transgenic and control potato plants. After isolation total protein (50 μg each except lanes C1, C2 and C3) was subjected to PAGE under denaturing conditions, transferred to nitrocellulose membranes and subsequently analysed for the presence of different proteins using antibodies against the following proteins: **A:** 10 kd protein of the water-splitting apparatus of photosystem II; **B:** 16 kd protein of the oxygen-evolving complex of photosystem II; **C:** 23 kd of the oxygen-evolving complex of photosystem II; **D:** 33 kd protein of the oxygen-evolving complex of photosystem II; **E:** 22 kd protein associated with photosystem II; **F:** D1 protein of the reaction centre of photosystem II. The numbering given above panels A and B identifies independent transgenic potato plants harbouring the construct encoding the anti-sense RNA to the 10 kd protein (cf. Figure 1). Lanes C1, C2 and C3 contain 0.5, 5 and 25 μg total protein isolated from leaves of a nontransformed control potato plant. The numbers given below the lanes in panel A indicate the relative amount of the 10 kd protein in percentage as compared with the level present in wild-type plants. In the case of the 10 kd and the 22 kd proteins, each two protein bands immunologically reacting with the respective antibodies are seen in the Western blot. For the 22 kd protein the higher molecular weight protein is a nonspecific protein band not related to the 22 kd protein. For the 10 kd protein, where a monospecific antibody raised against a fusion protein produced in *Escherichia coli* (Stockhaus, 1989) was used, the different bands recognized most likely represent proteolytic degradation products.

and older fully expanded leaves (cf. Materials and methods) thus excluding a possible accumulation of the 10 kd protein in older leaves (data not shown). The steady state protein levels of the 16 kd (Figure 3B), 23kd (Figure 3C) and 33 kd (Figure 3D) polypeptides which have been demonstrated to be components of the oxygen-evolving complex of photosystem II, are not affected by the suppression of the amount of the 10 kd polypeptide. The same result was obtained for a 22 kd protein which is associated with

Fig. 4. Comparison of a wild-type (D) and transgenic potato plant (no. 43) when grown under normal white light conditions (150 W/m^2). Plant no. 43 expresses only 0.7% of the wild-type level of the 10 kd protein.

photosystem II (Figure 3E) and the D1 protein (Figure 3F) of the reaction centre of photosystem II.

Growth rates and morphological aspects

As mentioned above several independent transgenic plants containing severely reduced levels of the 10 kd protein show similar growth rates to the plants expressing wild-type levels of this protein.

In plant 43 (Figure 4) the amount of the 10 kd protein reaches only 0.7% of the wild-type level. Obviously however, no morphological difference between plant 43 and plants expressing wild-type levels of the 10 kd protein (Figure 4D) can be observed. This holds true for all transgenic plants analysed which contain reduced levels of the 10 kd protein (cf. Figure 5A).

In order to test whether under low light conditions plants containing a reduced level of the 10 kd protein would show a phenotype different from the wild-type, a set of plants was grown under limiting light conditions (15 W/m^2). Under these growth conditions the plants are characterized by elongated stems and small rudimentary leaves. Again no significant changes with respect to growth rate were observed between plants containing different amounts of the 10 kd protein (cf. Figure 5B).

Finally the ultrastructure of the chloroplasts of wild-type plants and plants showing a reduced level of the 10 kd protein was analysed. To this end plants were grown under two different light regimes, i.e. under normal white light (150 W/m^2) as well as under limiting light conditions (15 W/m^2). The rationale behind this experiment was that the depletion of the 10 kd protein could possibly lead to alterations of the chloroplastid ultrastructure such as the loss of the thylakoid stacking regions.

Figure 6 shows the electron micrographs of chloroplasts of a 10 kd protein depleted plant (Figure 6A: plant 43) and of a wild-type plant (Figure 6B) grown under normal white light (150 W/m^2) as well as of a 10 kd protein depleted plant (Figure 6C: plant 43) and a wild-type plant (Figure 6D) grown under limiting light (15 W/m^2).

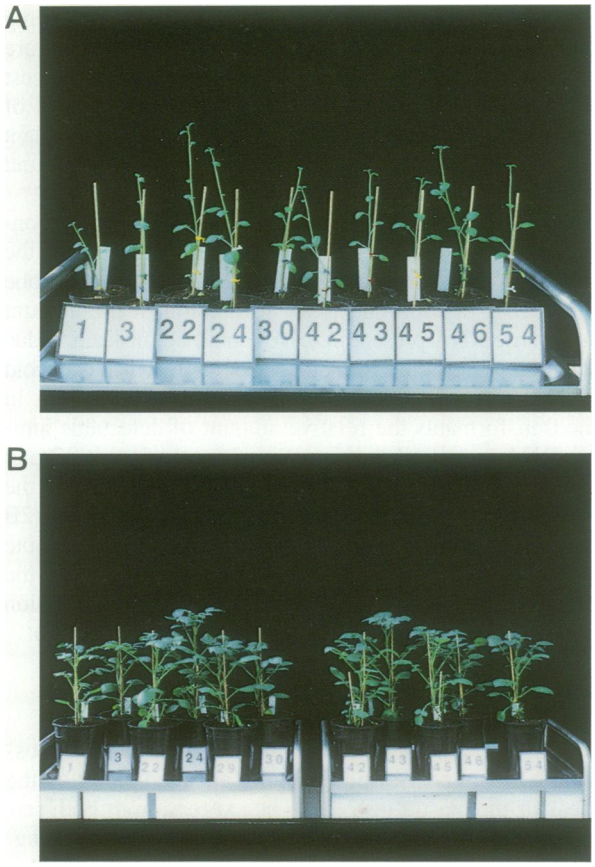

Fig. 5. Comparison of different transgenic potato plants varying widely in the residual level of the 10 kd protein (cf. Figure 3A) growing under low-light (A) and normal-light (B) conditions. Low light means 15 W/m^2, normal light means 150 W/m^2. Numbers identify independent transgenic potato plants harbouring the 10 kd anti-sense construct.

Chloroplasts of all plants grown under normal light conditions clearly display starch granules and grana stacks irrespective of the level of the 10 kd protein (Figure 6A and B). The ultrastructure of both plastid types of the plants is also very similar when grown under low-light conditions. In this case the plastids are characterized by the presence of a large number of grana regions (Figure 6C and D).

Functional analysis of the photosystem II

In order to analyse possible effects of the nuclear encoded 10 kd protein on the functional integrity of the PS II, two different kinds of experiments were performed: (i) the relaxation of the flash-induced enhancement in the fluorescence quantum yield, as determined in intact leaves; and (ii) the rates and characteristic oscillation pattern of O_2 evolution, as determined in an isolated thylakoid sample.

Figure 7 shows traces of transient fluorescence quantum yield changes induced by a train of two laser flashes in the dark-shaped leaves of control (dotted curve) and a 10 kd depleted plant (no. 3; full curve). The data reveal significantly slower relaxation kinetics in leaves of the transgenic plant lacking the 10 kd protein. This was observed in several independent experiments using leaves of different sizes and using independent vegetatively propagated plants of the same original transformant (data not shown). It is well established that the decay of the flash-induced fluorescence quantum yield reflects via a nonlinear relationship (Joliot and

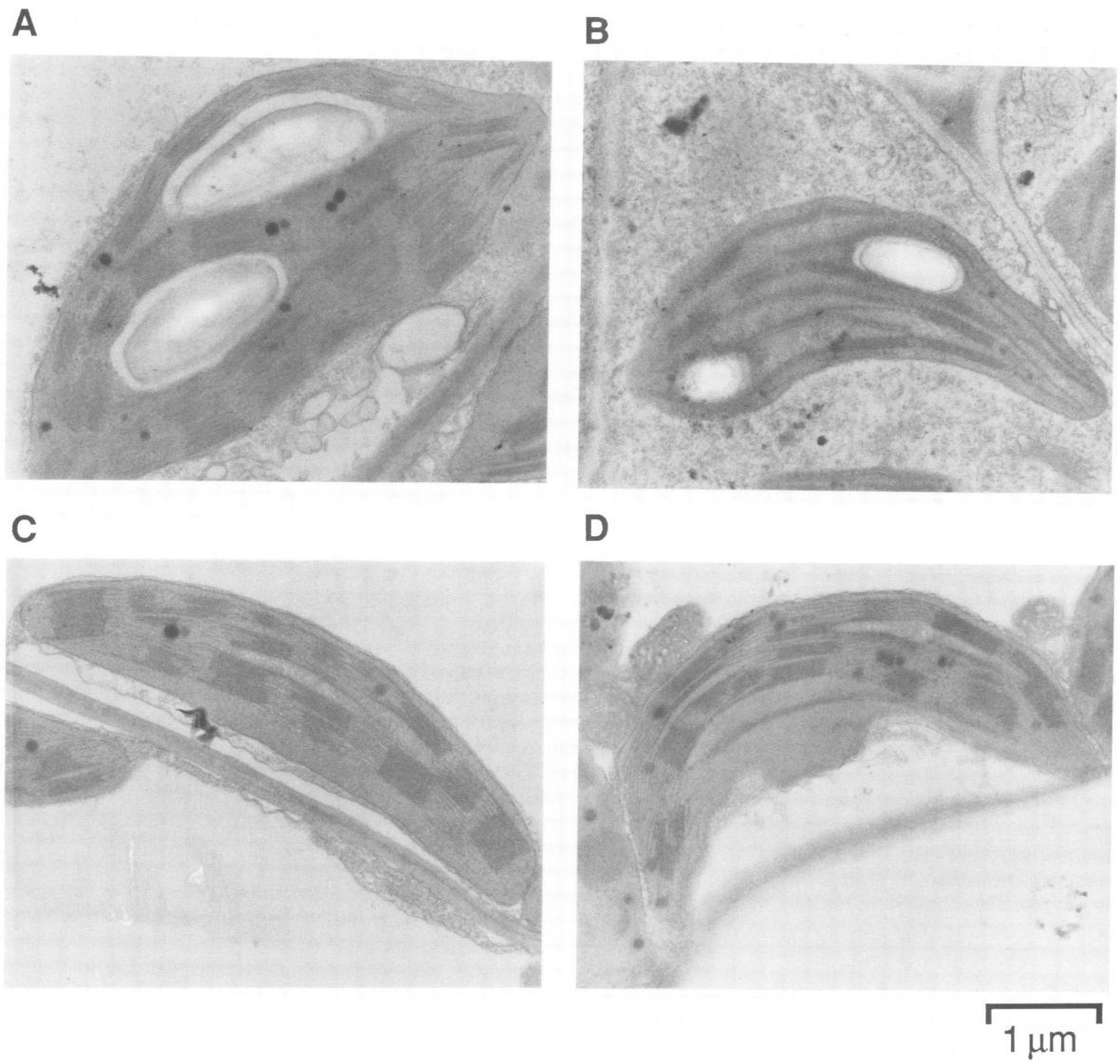

Fig. 6. Electron micrograph of chloroplasts of a wild-type (**B** and **D**) and transgenic potato plants containing only 0.7% of the wild-type level of the 10 kd protein (plant no. 43, panels **A** and **C**). Plants were kept either under low light (15 W/m^2; panels A and B) or normal white light (150 W/m^2; panels C and D) conditions.

Joliot, 1964) the reoxidation of Q_A- by Q_B and Q_B- (Robinson and Crofts, 1983). Therefore the results of Figure 7 clearly indicate that the elimination of the nuclear encoded 10 kd protein retards the the reoxidation of Q_A-.

With regard to the oxygen evolution measurements, the wild-type thylakoid samples exhibited maximum rates at 300−350 µmol O_2/mg Chl h in the presence of phenyl-*p*-benzoquinone (0.2 mM) and $K_3Fe(CN)_6$ (2mM) as the electron acceptor and 2mM NH_4Cl as uncoupler. Omission of the phenyl-*p*-benzoquinone led to much slower rates (~100 µmol O_2/mg Chl h).

In comparison with the wild-type under identical assay conditions the rates of O_2 evolution for 10 kd depleted transgenic plants (i.e. plants nos 3 and 43) were generally lower and showed a different pH dependence. At alkaline pH (>7.5), the initial rates were only 30−40% of those observed in the wild-type while at neutral and acid pH (down to 5.5) the differences were much less, the initial rates being 80% of the wild-type. The results are summarized in Table I.

As the steady state measurements are often complicated by acceptor limitations we also determined the O_2 yield induced by a train of short flashes in dark-adapted thylakoids in the absence of an exogenous electron acceptor. A typical period four oscillation pattern is observed which reflects the dioxygen formation via a sequence of four univalent redox steps at the catalytic site of water oxidation (for a recent review see Renger, 1987). As depicted in Figure 8, the overall oscillation pattern is not markedly disturbed in the thylakoid samples isolated from the 10 kd depleted transgenic plant. However, a close inspection of the data reveals that a higher damping occurs in this plant (i.e. transformant no. 3) compared with the wild-type. This phenomenon is very reproducible and was observed in different preparations of the same transformant as well as in preparations of a different transformant (plant no. 43; data not shown). Thus the lack of the 10 kd protein must also have some influence on the reaction pattern of the photosynthetic water cleavage.

Discussion

The photosynthetic apparatus of higher plants represents a very complex structure. It is composed of numerous different

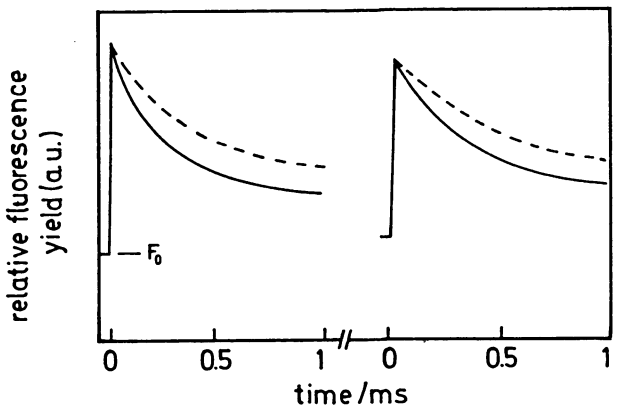

Fig. 7. Transient changes of fluorescence yield induced by a train of two laser flashes in intact leaves of wild-type (dotted curve) and 10 kd protein lacking transgenic potato plants. Dark time between the laser flashes: 1 s.

Table I. Altered pH dependence in the steady state rates of O_2 evolution of a 10 kd depleted transgenic potato plant (no. 3) in comparison with the wild-type.

pH	Q_2 Evolution initial rate (−10 kd)/initial rate (+10 kd)
5.5	0.79
6.3	0.76
7.0	0.76
7.5	0.65
8.0	0.42

These data are based on three to five measurements from two different preparations of transformant no. 3. The error limit of the rates was +10%. Similar rates were obtained for transformant no. 43 (data not shown).

proteins which are encoded by two different genomes, i.e. the plastome and the nuclear genome, which obviously asks for a tight interaction between the expression of both these genomes. Furthermore it has to react to a variety of environmental and developmental changes.

In order to understand the contribution and the role of the different polypeptides contained within this complex, one approach which has been used extensively in the past is the isolation of different structures depleted of one or more proteins and their *in vitro* analysis by biochemical and biophysical methods. This approach has its limitations and needs to be complemented by constructing *in vivo* complexes which are devoid of or at least heavily depleted by one and/or more polypeptides.

One way to overcome this problem is to screen for mutants. Though this approach has been successfully used especially in the case of the algae *Chlamydomonas*, it is not so easily feasible with higher plants especially when the mutant phenotype is unknown which is true for most mutations in any of the photosynthetic proteins. We therefore decided to use the anti-sense RNA approach to try to suppress the expression of a well-defined gene encoding the 10 kd protein of the water-splitting apparatus of photosystem II.

Expression of anti-sense RNA leads to efficient suppression of the 10 kd mRNA and protein amounts

In order to express the anti-sense RNA the full length cDNA was fused to the promoter of the 35S RNA of CaMV. This

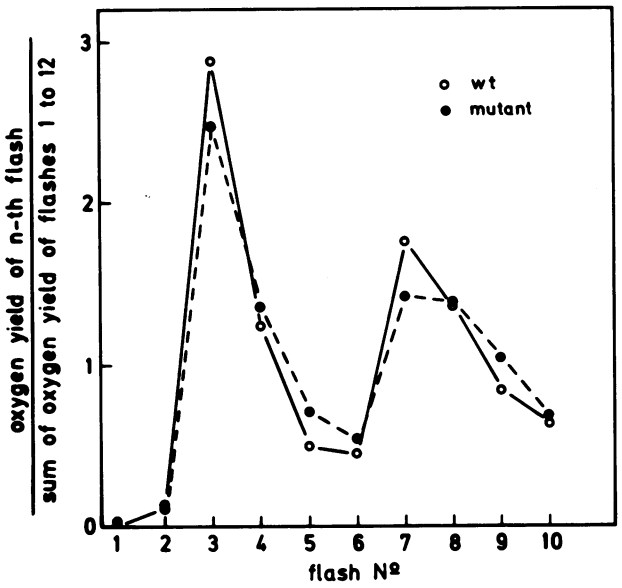

Fig. 8. Normalized oxygen yield as a function of flash number in dark-adapted thylakoids from wild-type (wt) and transgenic mutant potato plants. The data are normalized to the integral oxygen evolution due to flashes 1−12 of the train. Experimental details as described in Materials and methods.

promoter was chosen for two reasons. (i) As the 35S CaMV promoter is expressed in a variety of tissues and cells and is not restricted to photosynthetically active cells like the promoter of the 10 kd protein gene (Stockhaus *et al.*, 1989b), anti-sense RNA is already accumulating in the tissue before the promoter of the 10 kd gene is activated and sense RNA is produced. (ii) Due to the activity of the 35S promoter in root tissue, the level of anti-sense RNA can be determined in roots without possible interference due to the presence of sense RNA as in leaf tissue.

As described in Results, the expression of the 10 kd protein was suppressed to different levels in independent transgenic potato plants; in some transformants the steady state mRNA level reached only ~1% of the wild-type level. Thus the expression of anti-sense RNA in transgenic potato plants reduces the expression of the 10 kd protein in a most effective manner. An important notion with respect to the mechanism by which anti-sense RNA induced inhibition works is the finding that there is no obvious correlation between the steady state level of the anti-sense RNA and the 10 kd gene steady state transcript levels.

The mechanism of the inhibitory effect of anti-sense RNA on the expression of the target gene is not understood. In principle anti-sense RNA could act at the level of transcription, processing and transport of the RNA out of the nucleus, at the level of translation or could generally affect RNA stability. Irrespective of the mechanism, if the anti-sense RNA interacts with the sense RNA, a bimolecular reaction has to be postulated. This should result in a concentration dependence of the observed inhibiting effect.

Concerning transgenic plants, anti-sense RNA expression has been used in few cases to inhibit the expression of an endogenous gene. Unfortunately the level of anti-sense RNA was not in all cases determined and compared with the sense RNA. A clear correlation between the inhibitory effect of the anti-sense RNA and its transcript levels has only been described by Delauney and colleagues (Delauney *et al.*,

1988) thus favouring the idea that the RNA is destabilized by a mechanism most likely involving heteroduplex formation. In the case of inhibition of the expression of the chalcone synthase gene by anti-sense RNA however, Van der Krol *et al.* (1988) described a rather complex pattern indicating a complex mechanism. Thus, different pigmentation patterns were observed in flowers of independent transgenic plants irrespective of the fact that these plants expressed the same steady state levels of chalcone synthase anti-sense RNA. On the other hand, cases were described where very low steady state levels of anti-sense RNA showed a pronounced effect on flower pigmentation (Van der Krol *et al.*, 1988). The data described in Results provide evidence for a more complex mechanism of anti-sense RNA inhibition. Although the fact that there is no clear correlation between the level of anti-sense RNA and suppression of the 10 kd gene expression does not exclude mechanisms involving heteroduplex formation between sense and anti-sense RNA, this assumption is not sufficient to explain these results. In order to explain our data, parameters have to be incorporated into a model. One possibility is that the integration site of the anti-sense construct with respect to the location of the target gene is of importance. If the inhibition of expression occurs very early after transcription of the sense RNA one might envision that different integration sites of the anti-sense RNA could lead to different local concentrations of the anti-sense RNA at the position of the target gene if the movement of the RNA within the nucleus is not diffusion-controlled, thus resulting in different efficiencies of suppression of the sense RNA.

Accumulation of chloroplastidic and nuclear encoded transcripts of genes involved in photosynthesis is unaffected by reduction of the 10 kd RNA and protein

Both photosystems I and II are composed of a large number of polypeptides encoded either in the nucleus or in the plastids thus requiring a tight coordination of gene expression. Recently, evidence for direct as well as indirect regulatory interactions between the nucleus and the plastids was obtained in different laboratories (reviewed in Taylor, 1989). Thus it has been shown that the state of chloroplast development drastically affects the expression of nuclear genes encoding chloroplastid proteins and it has been suggested that a plastid derived factor is involved in the transcriptional control of the expression of a number of genes encoding chloroplastidic polypeptides (Bradbeer *et al.*, 1979; Mayfield and Taylor, 1984; Oelmüller and Mohr, 1986; Batschauer *et al.*, 1986; Stockhaus *et al.*, 1989a). One possible mechanism would be a feedback-type regulation of certain transcripts and/or polypeptides involved in the photosynthetic process.

As shown in Figure 2 the steady state transcript levels of several plastid and nuclear genes analysed are not influenced significantly by the reduction of the 10 kd mRNA. This indicates that the transcription of these genes is not coupled to the expression of the 10 kd gene via a feedback control mechanism.

Effects of the 10 kd polypeptide depletion

In order to understand the role of the 10 kd protein, several experimental lines were followed. On the one hand plants showing a residual level of only 1% of the 10 kd protein were compared with wild-type plants with respect to the composition of other polypeptides belonging to photosystem II. This is especially important as one assumption based on reconstitution experiments is that the 10 kd protein might be necessary for the binding of the 23 kd polypeptide (Ljungberg *et al.*, 1986) which has been shown to play a regulatory role in oxygen evolution (Miyao and Murata, 1989). The analysis of the accumulation of some of the photosystem II polypeptides including the 23 kd shows that they are unaffected by the depletion of the 10 kd polypeptide. In a second line of experiments macroscopic parameters such as habitus, growth rates and assimilation capacity were determined for both wild-type plants and plants depleted in the 10 kd protein. As described in Results no obvious changes were observed. This indicates that the photosynthetic capacity of the transgenic plants depleted in the 10 kd protein is not significantly affected under the growth conditions used. Additional analysis performed at the microscopic level again revealed no change in the ultrastructural characteristics of the chloroplasts.

In a third type of experiment the functional integrity of the PSII complex of the wild-type and of plants depleted in the 10 kd protein was analysed by measurements of light-induced transient changes of the fluorescence quantum yield and of oxygen evolution. The fluorescence measurements clearly indicate that the lack of the 10 kd protein significantly retards the reoxidation of Q_A- by Q_B and Q_B-. As these functional redox groups are embedded in the heterodimer consisting of polypeptides D1 and D2 (for a review see Trebst, 1986) the results of this study provide evidence for an allosteric influence on the kinetics of acceptor side reactions as discussed previously (Renger *et al.*, 1981). The mechanistic implications of these findings will be analysed in more detail in a forthcoming paper.

The light saturated rates of oxygen evolution revealed a lower activity in thylakoids isolated from the transgenic plants lacking the 10 kd protein compared with those of the control. The extent of this diminishing effect greatly varied with pH. Therefore the 10 kd could act as a pH-dependent stabilizing polypeptide. A mechanically interesting phenomenon is the higher damping of the period four oscillation of oxygen yield in dark-adpated thylakoids from plants lacking the 10 kd protein.

An increase of the probability of misses could conceivably be due to a shift of the redox equilibrium comprising the couple Q_A/Q_A- and the plastoquinone bound to the Q_B site of polypeptide D1 in a way that leads to slightly modified properties of the Q_B site. This idea is supported by the fluorescence measurements described in this study but additional effects due to destabilizations of the donor site cannot be excluded.

Alternatively a general disorder introduced into the PSII complex by the lack of the 10 kd protein may slightly modify the properties throughout the PSII reaction sequence, without a particular target site of action. Detailed biophysical studies on the reduction kinetics of Q_A, the herbicide binding and the fluorescence induction curves are under progress to clarify this point.

In other organisms such as for example cyanobacteria or the green alga *Chlamydomonas reinhardtii*, photosystem II mutants are available for analysis of the assembly of photosystem II *in vivo* (Pakrasi *et al.*, 1989; Rochaix and Erickson, 1988; Kuchka *et al.*, 1989). Thus it has been

shown for *Chlamydomonas* that a deficiency of the nuclear gene product OEE1 (oxygene evolving enhancer) affects the stability of the photosytem II core, whereas the deficiency of the polypeptide OEE2 did not have any effect on the accumulation of the other photosystem II polypeptides Mayfield *et al.*, 1987a/b).

By using the anti-sense approach we have extended this kind of structural analysis of photosystem II to higher plants. This powerful approach will be very useful for the functional analysis of a number of different polypeptides of photosystem II which, like the 10 kd protein, are seemingly unique to photosystem II of higher plants.

Materials and methods

Material

DNA probes for the various nuclear and plastid genes were isolated from *Flaveria trinearvia* (M.Höfer and P.Westhoff, unpublished results). Antisera used are described in Oswald *et al.* (1990)

Recombinant DNA techniques

Standard procedures were used for recombinant DNA work (Maniatis *et al.*, 1982).

Transformation of potato plants and tissue techniques

The chimeric genes were inserted in the vector BIN19 (Bevan, 1984) and introduced into the *Agrobacterium tumefaciens* strain pGV2260 (Deblaere *et al.*, 1985) by direct transformation according to Höfgen and Willmitzer (1988). The transformation and regeneration of *Solanum tuberosum* cv. Desiree plants was performed as described (Rocha-Sosa *et al.*, 1989).

Growth conditions and analysis of transgenic plants

Regenerated plants used for the biochemical, molecular and ultrastructural analyses were grown in a greenhouse under controlled temperature and light conditons (16 h light, temperature: 19°C; 8h dark, 10°C). Plants with a height of 60 cm were used for harvesting material. Young leaves means the fifth to seventh leaf (counting from top to bottom of the plant); old leaves means completely expanded, however, not yet senescent leaves of the plant.

Isolation of DNA and RNA from transgenic plants and their subsequent analysis by blot hybridizations were performed as described (Eckes *et al.*, 1986; Sanchez-Serrano *et al.*, 1987). The plant material was routinely harvested in the middle of the light period, i.e. at ~2 p.m.

Preparation of mesophyll protein fractions and Western blotting

Leaf tissue was homogenized in ice-cold buffer A (330 mM sorbitol, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 2 mM EGTA, 5 mM dithiothreitol, 2 mM diethyldithiocarbamic acid, 50 mM Tris pH 7.5). The membrane fraction was collected by centrifugation and washed with buffer A. The membrane fraction was resuspended in buffer B (10% sucrose, 20 mM Na carbonate, 50 mM dithiothreitol, 1 mM PMSF, 2 mM diethyldithiocarbamate) and sonicated twice for 15s with a Branson sonicator. The protein concentrations of cell extracts were determined as described by Bradford (1976). Protein gel electrophoresis was performed according to Schägger and von Jagow (1987). Separated proteins were electrophoretically transferred to nitrocellulose membranes (PH79: Schleicher & Schüll) using a semi-dry blotting device (Pharmacia LKB, Freiburg). Immunodetection with antibodies, incubation with ^{125}I-labelled protein A (Amersham Buchler) and fluorography was performed as described by Westhoff *et al.*, (1985).

Fluorescence measurements

The flash-induced changes of fluorescence yield were measured as described in Renger *et al.*, (1988) using leaves as probing material.

Thylakoid isolation and O_2 measurements

Potato leaf material was homogenized in extraction buffer (330 mM sorbitol, 5 mM $MgCl_2$, 5 mM Na isoascorbate, 50 mM HEPES pH 7.5) with a Waring Blendor, passed through Miracloth and the chloroplasts were collected by centrifugation of 1 min at 1500 *g*. The chloroplasts were resuspended in lysis buffer (5 mM $MgCl_2$, 50 mM HEPES pH 7.5). The thylakoids were collected by centrifugation at 6000 *g* for 5 min and washed (330 mM sorbitol, 5 mM $MgCl_2$, 50 mM HEPES pH 7.5). The chlorophyll concentration was determined spectrophotometrically according to Arnon (1949). The rates of oxygen evolution were determined with a Clark-type electrode in a cuvette illuminated with white light of saturating intensity. The suspension contained: thylakoids (5 μg/ml Chl) 5 mM NaCl, 2mM NH_4Cl MES/NaOH and 2 mM $K_3Fe(CN)_6$ in the absence or presence of 1 mM phenyl-p-benzoquinone (Ph-p-BQ) as electron acceptor. The oxygen yield per flash induced by a train of saturating Xenon flashes (FWHM 10 μs) in dark-shaped samples was determined with a Joliot-type electrode as described in Hanssum *et al.*, (1985). These experiments were performed at pH 7.6 (50 mM Tricine/NaOH) in the presence of 5 mM $MgCl_2$ and 20 mM NaCl without electron acceptor.

Ultrastructure of the chloroplasts

Small leaf samples were embedded in Poly/Bed 812 Embedding Media (Polysciences, Inc.) as described by the supplier. Ultrathin sections were inspected by electron microscopy.

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