The Manganese-stabilizing Protein Is Required for Photosystem II Assembly/Stability and Photoautotrophy in Higher Plants*

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Interfering RNA was used to suppress the expression of two genes that encode the manganese-stabilizing protein of photosystem II in Arabidopsis thaliana, MSP-1 (encoded by psbO-1, At5g66570), and MSP-2 (encoded by psbO-2, At3g50820). A phenotypic series of transgenic plants was recovered that expressed high, intermediate, and low amounts of these two manganese-stabilizing proteins. Chlorophyll fluorescence induction and decay analyses were performed. Decreasing amounts of expressed protein led to the progressive loss of variable fluorescence and a marked decrease in the fluorescence quantum yield ($F_v/F_m$) in both the absence and the presence of dichloromethylurea. This result indicated that the amount of functional photosystem II reaction centers was compromised in the plants that exhibited intermediate and low amounts of the manganese-stabilizing proteins. An analysis of the decay of the variable fluorescence in the presence of dichlorophenyldimethylurea indicated that charge recombination between QA− and the S2 state of the oxygen-evolving complex was seriously retarded in the plants that expressed low amounts of the manganese-stabilizing proteins. This may have indicated a stabilization of the S2 state in the absence of the extrinsic component. Immunological analysis of the photosystem II protein complement indicated that significant losses of the CP47, CP43, and D1 proteins occurred upon the loss of the manganese-stabilizing proteins. This indicated that these extrinsic proteins were required for photosystem II core assembly/stability. Additionally, although the quantity of the 24-kDa extrinsic protein was only modestly affected by the loss of the manganese-stabilizing proteins, the 17-kDa extrinsic protein dramatically decreased. The control proteins ribulose bisphosphate carboxylase and cytochrome f were not affected by the loss of the manganese-stabilizing proteins; the photosystem I PsaB protein, however, was significantly reduced in the low expressing transgenic plants. Finally, it was determined that the transgenic plants that expressed low amounts of the manganese-stabilizing proteins could not grow photoautotrophically.

In higher plants and cyanobacteria, at least six intrinsic proteins appear to be required for oxygen evolution by photosystem II (PS II).1–3. These are CP47, CP43, the D1 and D2 proteins, and the $\alpha$ and $\beta$ subunits of cytochrome b$_{597}$. Inertional inactivation or deletion of the genes for these components results in the complete loss of oxygen evolution activity. Additionally, a number of low molecular mass, intrinsic membrane protein components are associated with PS II (4–6), although the functions of many of these proteins remain obscure. Although PS II complexes containing only these intrinsic components can evolve oxygen, they do so at low rates (about 25–40% of control), are extremely susceptible to photoactivation, and require high, non-physiological levels of calcium and chloride for maximal activity (1, 3).

In higher plants, three extrinsic proteins, with apparent molecular masses of 33, 24, and 17 kDa, are required for high rates of oxygen evolution at physiological inorganic cofactor concentrations (7). The 33-kDa component has been termed the manganese-stabilizing protein (MSP) due to its stabilization of the manganese cluster during exposure to low chloride concentrations or to exogenous reductants. The 24- and 17-kDa proteins appear to modulate the calcium and chloride requirements for efficient oxygen evolution. These three extrinsic components interact with intrinsic membrane proteins and possibly with each other to yield fully functional oxygen-evolving complexes.

The MSP is encoded by the psbO gene. The mature MSP protein is highly conserved (7), particularly among higher plant species. In the cyanobacterium Synechocystis 6803, mutants in which the psbO gene was deleted exhibited photoautotrophic growth, and their PS II reaction centers were present in near normal amounts (~75%) (2, 25). In Chlamydomonas, however, a mutant that did not accumulate the MSP was also deficient in the assembly of the PS II reaction centers and could not grow photoautotrophically (9).

Arabidopsis contains two genes for the MSP, psbO-1 (At5g66570) and psbO-2 (At3g50820), which encode MSP-1 and MSP-2, respectively (10, 11). Both of these genes are normally expressed, with about 70% of the MSP complement being MSP-1. A point mutation in the psbO-1 gene was recovered that led to the introduction of a premature stop codon and the loss of the MSP-1 protein (12). This mutant exhibited slow growth, aberrant fluorescence induction characteristics, and an increased expression of MSP-2.

RNAi is a post-transcriptional gene-silencing process in which double-stranded RNA induces the degradation of homologous mRNA sequences (13). RNAi has been successfully applied as a powerful gene-silencing tool in a variety of organisms, including Caenorhabditis elegans and Drosophila

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1 The abbreviations used are: PS, photosystem; chl, chlorophyll; DCMU, dichlorophenyldimethylurea; LiDS, lithium dodecyl sulfate; MES, 2-[N-morpholino]ethanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)methyl]glycine; MSP, manganese-stabilizing protein; MS medium, Murashige and Skoog medium; RNAi, interfering RNA; LHC, light-harvesting chlorophyll.

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**RESULTS AND DISCUSSION**

**Screening and PCR Confirmation of Transgenic Plants**—Seeds from wild type and from plants transformed with the RNAi-1 construct were distributed on agar plates containing either MS medium or MS medium supplemented with 50 mg of kanamycin/liter. Although wild type seeds could not germinate successfully on medium containing kanamycin, a few kanamycin-resistant seedlings were recovered from plants transformed with the RNAi construct. If, however, seeds from the treated plants were sown onto agar plates containing MS medium + kanamycin + sucrose, many more kanamycin-resistant seedlings were recovered (data not shown). This indicated that many of the kanamycin-resistant plants required sucrose for successful germination and growth. The presence of the RNAi construct in the 35 kanamycin-resistant plants that germinated and grew on the sucrose-containing medium was confirmed by PCR amplification of the cauliflower mosaic virus promoter region. All of the plants that exhibited kanamycin resistance also exhibited the 1.0-kbp cauliflower mosaic virus S promoter region, which was absent in the wild type plants (data not shown).

**Immunological Characterization of Thylakoid Proteins**—For a more in-depth analysis of the protein complement of the thylakoid membranes, chloroplasts were isolated from wild type and three mutant plant lines that expressed different levels of the MSPs. These lines, designated RNAi-1A, RNAi-1B, and RNAi-1C, expressed high, intermediate, and low amounts of the MSPs, respectively. Leaves were ground in a glass homogenizer with a chloroplast isolation buffer (100 mM sucrose, 200 mM NaCl, 5 mM MgCl₂, 50 mM sodium potassium phosphate buffer, pH 7.4.), the homogenate was then passed through two layers of Miracloth (Calbiochem), and the chloroplasts were pelleted by centrifugation at 6,000 x g for 5 min. The chloroplasts were then resuspended in a small amount of 300 mM sucrose, 15 mM NaCl, 10 mM MgCl₂, and 50 mM MES-NaOH, pH 6.0, and the chlorophyll concentration was determined by the method of Arnon (20). LiDS-PAGE was performed on a 12.5–20% gradient gel with 3 μg of chlorophyll loaded/lane. Western blotting, blocking, and probing with primary and secondary antibodies were as described above. For detection of the immobilized antibodies, a chemiluminescent substrate (SuperSignal West Pico chemiluminescent substrate, Pierce) was used, and the blots were exposed to x-ray film. After development, the x-ray films were scanned with a UMax PowerLook III scanner at 300-dpi resolution and an 8-bit color depth.

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in this screen). These results are consistent with the results obtained in other RNAi studies that target other proteins. In almost all instances, different RNAi-containing plant lines exhibit different degrees of suppression of the protein targets (21, 22), often allowing the isolation of a graded phenotypic series of mutants deficient in the targeted component.

Plants That Express Low Levels of the MSPs Cannot Grow Photoautotrophically—RNAi-1-containing plants that were germinated on MS medium + kanamycin + sucrose were transplanted onto MS medium + kanamycin. Prior to transplanting, all of the plants appeared green and healthy. After transplanting, the RNAi-1-U plant, which expressed low amounts of the MSPs, yellowed and died, whereas the RNAi-1-S plant, which expressed intermediate amounts of MSPs, grew more slowly than wild type and was a paler shade of green (Fig. 2). Similar results were obtained if intermediate and low expressing plants were transplanted onto a soilless potting mixture. All of the plants that expressed low amounts of the MSPs died (data not shown) when not supplied with sucrose. These results indicated that the MSPs were required for photoautotrophic growth in Arabidopsis.

In the cyanobacterium Synechocystis 6803, deletion mutants that lack the single MSP found in this organism grow photoautotrophically under the normal calcium and chloride conditions of BG-11 medium (2, 23). Under calcium- or chloride-depleted conditions, however, these strains cannot grow photoautotrophically (23, 24). In the green alga Chlamydomonas reinhardtii, however, the loss of the single MSP found in this organism in the FdA44 mutant led to a loss of photoautotrophy (9). Our results indicate that the higher plant requirement for MSP, as expected, is similar to that observed for Chlamydomonas.

Low Levels of MSP Expression Lead to Decreased Levels of Functional PS II—To assess the effect of the loss of the expression of MSPs on the functional competence of PS II, chlorophyll fluorescence induction and decay were examined in wild type and the transgenic plants. Fig. 3 illustrates fluorescence induction and decay curves for wild type and for the transgenic plants RNAi-1A, RNAi-1B, and RNAi-1C either in the absence (Fig. 3A) or in the presence (Fig. 3B) of DCMU. These fluorescence curves were normalized to the Fo fluorescence level and were offset for clarity. Fluorescence traces were performed either in the absence (A) or in the presence (B) of 40 μM DCMU. Up arrows indicate the onset of strong actinic illumination, whereas down arrows indicate the termination of strong actinic illumination. ■, wild type; ●, RNAi-1A; ▲, RNAi-1B; ▼, RNAi-1C.

The rate constant (k−1) for the fluorescence decay in the absence of DCMU also increased through the phenotypic series. In the presence of DCMU, the fluorescence decay from Fo was dominated by charge recombination between QA− and the S2 state of the oxygen-evolving complex. The increased rate constants observed for the RNAi-1B and RNAi-1C indicated that this charge recombination was markedly slowed in these plants. This may have indicated an increased stability of the S2 state. It should be noted that we consider the fluorescence decay time of the RNAi-1C plant as only approximate. The very small Fo− observed in this plant made the estimation of its decay time difficult and problematic. Nevertheless, multiple fluorescence decay measurements on this plant and others with the
low expressing phenotype) yielded consistently slow apparent rates of fluorescence decay.

In *Synechocystis* 6803, a similar slowing of the decay of variable fluorescence was observed in mutants lacking the MSP (25). These authors observed a 2–3-fold increase in the rate constants for fluorescence decay. No similar MSP (25). These authors observed a 2–3-fold increase in the rate constants for fluorescence decay. No similar

| Plant type | F_0 | F_m | F_x | F_x/F_m | Fluorescence decay
|-------------|-----|-----|-----|--------|------------------|
| + DCMU      |     |     |     |        |                  |
| Wild type   | 0.54 | 1.91 | 1.37 | 0.72 | ND
| RNAi-1A     | 0.34 | 1.41 | 1.07 | 0.76 | ND
| RNAi-1B     | 0.99 | 1.28 | 0.29 | 0.23 | ND
| RNAi-1C     | 0.67 | 0.80 | 0.13 | 0.16 | ND
| − DCMU      |     |     |     |        |                  |
| Wild type   | 0.87 | 1.58 | 0.70 | 0.44 | 0.89 ± 0.04
| RNAi-1A     | 0.77 | 1.40 | 0.66 | 0.47 | 0.82 ± 0.03
| RNAi-1B     | 1.73 | 2.09 | 0.35 | 0.17 | 1.46 ± 0.08
| RNAi-1C     | 0.85 | 0.88 | 0.03 | 0.04 | 0.96 ± 1.89

* Fit to a single exponential decay curve.
* Errors for this fluorescence data were ± 10%.
* ND, not determined.

![Fig. 4. Immunological analysis of the protein complement of the thylakoid membranes of wild type and transgenic plants containing high, intermediate, and low amounts of the MSPs.](image)

**Table 1**

Fluorescence characteristics of wild type and RNAi-1-containing Arabidopsis

*RNAi Suppression of MSP Expression*

**Lowered Expression of the MSPs Leads to Loss of Other PS II Components**—To determine whether decreased expression of the MSPs led to a loss of other PS II components, immunological analysis using chemiluminescent detection was performed. It should be noted that chemiluminescent detection is significantly more sensitive than the colorimetric reagents used during screening (Fig. 1) of the RNAi-1 transgenic plants. The relative amounts of some selected PS II and control proteins present were analyzed in chloroplast preparations of wild type *Arabidopsis* and the transgenic plants in the phenotypic series (Fig. 4). Three proteins that are present in the intrinsic core of PS II, CP47, CP43, and D1 were examined. The amount of CP47, CP43, and the D1 protein all decreased in the phenotypic series, with RNAi-1A being the least affected and RNAi-1C being the most affected. LHC II, the light-harvesting chlorophyll a/b protein antenna of PS II, was also examined. No change in the amount of LHC II present was observed in the RNAi-1-containing plants. These results indicated that the loss of the MSPs led to a decrease in the accumulation of PS II reaction center components and that the expression of LHC II was not related to the presence or absence of PS II. In the *Chlamydomonas* FuD44 mutant, the loss of the MSP led to a concomitant loss of other PS II core polypeptides including CP47, CP43, D1, and D2 (9). The amount of LHC II, however, was not affected. The results obtained for these proteins in *Chlamydomonas* were essentially identical to those that we observed in the higher plant system. It should be noted that in the *Chlamydomonas* study, the complete absence of the MSP led to nearly the full loss of the examined PS II core components. The effects of intermediate levels of the MSP could not be examined in that system. In our study, we demonstrated that even at intermediate MSP expression levels (i.e. in the RNAi-1B plant), the amounts of a number of the examined core PS II components, including CP43 and CP47, were substantially reduced. This may have indicated a rather tight coupling of the expression of these components to the expression level of the MSPs that was not detected in the *Chlamydomonas* system. Alternatively, decreased levels of expression of the MSPs may have led to enhanced rates of degradation of the PS II core components, which then led to decreases in their steady state levels. Interestingly, Hashimoto *et al.* (27) demonstrated that there was a large pool of unassembled PS II extrinsic proteins comprising 20–50% of the total extrinsic proteins present in the thylakoid lumen and that these proteins were competent in binding to the photosystem. They suggested that this unbound pool participated in the maintenance of homeostasis with respect to turnover and assembly of PS II. Our observation that a lower level of the MSPs in the RNAi-1B mutant led to the loss of PS II reaction centers supported the hypothesis of Hashimoto *et al.* (27).

In addition to the intrinsic PS II core components, the expression of the extrinsic 24- and 17-kDa extrinsic proteins was also assessed. In wild type and the RNAi-1A and RNAi-1B transgenic plants, two immunoreactive bands were observed that bound the anti-24-kDa antibody. The nature of the minor
band is unclear at this time, although it may represent a migrational variant of the 24-kDa protein. This 24-kDa component was only modestly affected by the loss of the MSPs. Only in the RNAi-1C plant was a reduction in the amount of the 24-kDa protein evident, with the major immunoreactive band being reduced in intensity and the minor band disappearing. A markedly different pattern was observed for the 17-kDa protein. Chloroplasts from the RNAi-1B plant exhibited a modest reduction of the 17-kDa protein, whereas the RNAi-1C plant exhibited a complete loss of this extrinsic component.

Although a similar result was obtained in Chlamydomonas for the 24-kDa protein, the 17-kDa result was quite different (9). In Chlamydomonas, the complete loss of MSP did not lead to any observable change in the expression of the 17-kDa protein. It is unclear at this time whether the loss of the 17-kDa protein in Arabidopsis was due to decreased synthesis or increased degradation of this component. It should be noted, however, that large pools of unassembled mature 17-kDa protein can exist in the thylakoid lumen without being degraded either in the presence (27) or in the absence (8, 28, 29) of assembled and functional PS II reaction centers. Consequently, the loss of the 17-kDa component must have been the direct result of the loss of expression of the MSPs and not a consequence of the reduced levels of PS II observed as a result of decreased expression of the MSPs. This unexpected result indicated that decreased expression of the MSPs in Arabidopsis led to a loss of PS II reaction centers and decreased amounts of the extrinsic proteins associated with the oxygen-evolving complex, with the 17-kDa protein being dramatically affected.

In addition to these PS II proteins, three control proteins were also examined. As expected, the amounts of the large subunit of ribulose bisphosphate carboxylase and cytochrome f, a component of the cytochrome b of complex, were not affected by decreased expression of the MSPs. Interestingly, the amount of the PsaB protein, a core component of photosystem I, was markedly decreased in the RNAi-1C plant. It should be noted that this could not be the result of a general loss of thylakoid membranes since such a situation would also lead to a loss of the cytochrome b complex. Similar results were obtained for the nuclear mutant hcf136, which is deficient in the HCF136 protein and does not assemble PS II reaction centers (18). The loss of PS II reaction centers apparently led to a loss in the accumulation of PS I but not the cytochrome b complex. The mechanism for this phenomenon has not been established and is being investigated.

Conclusions—The use of RNAi methodology has proved a useful tool in probing the effects of a reduction of the MSPs in Arabidopsis. Decreased amounts of the MSPs led to a loss of photoautotrophy, decreased variable fluorescence yield, and a loss of PS II reaction center components. Although overall the effects were similar to those observed in the Chlamydomonas system (9), our results seem to have indicated a tight coupling of the amount of the MSPs that accumulated with the amount of PS II reaction centers assembled. Additionally, in contrast to the Chlamydomonas system, the accumulation of the 17-kDa protein seemed to be very dependent on the amount of the MSPs present.

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