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Research Notes

Changes in the Activity and the Polypeptide Composition of the Oxygen-Evolving Complex in Photosystem II of Tobacco Leaves Infected with Cucumber Mosaic Virus Strain Y

Hideki Takahashi and Yoshio Ehara

Faculty of Agriculture, Tohoku University, 1-1, Tsutsumidori Amamiyamachi, Aoba-ku, Sendai 981, Japan
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The amount of 22- and 23-kDa polypeptides in a 23-kDa protein family, which plays a regulatory role in photosynthetic oxygen evolution, decreased significantly during the progress of appearance of chlorotic spots in cucumber mosaic virus strain Y (CMV[Y])-inoculated tobacco (*Nicotiana tabacum* 'Ky57') leaves (Plant Mol. Biol. 16:689-698, 1991). The present study was conducted to determine if the amount of other polypeptides of the oxygen-evolving complex and the oxygen-evolving activity also decreased in CMV(Y)-inoculated tobacco leaves. The amount of a 33-kDa polypeptide, which is essential to oxygen evolution, did not decrease in CMV(Y)-inoculated leaves showing early symptoms, although the amount of 22- and 23-kDa polypeptides began to decrease. However, comparative analysis of electron transport in thylakoid membranes indicated that the oxygen-evolving activity in CMV(Y)-inoculated tobacco leaves was only partly reduced, compared with the activity in CMV(O)-inoculated tobacco leaves which did not show clear symptoms. Partial inhibition of the oxygen-evolving activity, by a differential decrease in the amount of polypeptides of the oxygen-evolving complex, seems to be associated with the primary molecular process of symptom expression in CMV(Y)-inoculated tobacco leaves.

Plants systemically infected with viruses often show various severities of chlorosis or mosaic symptoms. The mechanism(s) of symptom expression is not understood at the molecular level. However, over the years, there have been many studies to associate virus symptom expression with chloroplast function (Goodman *et al.* 1986; Fraser 1987). Recently, Beachy and co-workers indicated that the photosynthetic electron transport activity of photosystem II (PSII) in chloroplasts was inhibited in tobacco and spinach infected with an aggressive tobacco mosaic virus (TMV) strain, whereas no reduction in PSII was observed in plants infected with a mild strain (Reinero and Beachy 1989; Hodgson *et al.* 1989). However, Van Kooten *et al.* (1990) suggested that symptom expression did not appear to be related to the influence of TMV on PSII activity. Therefore, a consistent model of the linkage of symptom expression with chloroplast function at the molecular level has not been established.

To understand the molecular basis of symptom expression in virus-infected plants, we have compared proteins extracted from tobacco (*Nicotiana tabacum* L. 'Ky57') leaves inoculated with either of two strains of cucumber mosaic virus (CMV[Y] or CMV[O]) or mock-inoculated, using two-dimensional (2-D) polyacrylamide gel electrophoresis. CMV(Y) produces severe chlorotic spots, and CMV(O) does not show clear symptoms in inoculated tobacco leaves, although both strains multiply to a similar extent (Takahashi and Ehara 1988). CMV(Y) contains a satellite RNA, but CMV(O) does not (Takanami *et al.* 1977). However, the satellite RNA does not cause chlorotic spot symptoms in CMV(Y)-inoculated tobacco leaves (H. Takahashi *et al.*, unpublished results). Recently, we indicated that the oxygen-evolving complex of PSII in tobacco chloroplasts contains a 23-kDa protein family, containing 22-, 23-, and 24-kDa polypeptides, which corresponds to the 23-kDa protein of the oxygen-evolving complex in spinach chloroplasts. The amount of two polypeptides, 22- and 23-kDa, in the 23-kDa protein family, decreased significantly with the appearance of early chlorotic spots in CMV(Y)-inoculated tobacco leaves but did not decrease in mock- or CMV(O)-inoculated leaves. The amount of 24-kDa polypeptide in the 23-kDa protein family does not decrease until severe chlorotic spot symptoms appear in CMV(Y)-inoculated leaves (Takahashi *et al.* 1991).

In the process of photosynthesis, electrons, stripped from water to produce oxygen, are transferred into the PSII reaction center and then transported through the electron transport chain to photosystem I (PSI) (Fig. 1). Oxygen evolution in PSII plays an essential role in photosynthesis. The oxygen-evolving complex is composed of three polypeptides of 33-, 23-, and 18-kDa in spinach (Murata and Miyao 1987). The 23- and 18-kDa polypeptides play a regulatory role in photosynthetic oxygen evolution, whereas the 33-kDa polypeptide is essential (see review by Yamamoto 1989). To understand the molecular process of symptom expression, it is important to study the relationship between the composition of polypeptides in the oxygen-evolving complex and the inhibition of oxygen-evolving activity in CMV(Y)-inoculated tobacco leaves.

Address correspondence to H. Takahashi.

The conditions of plant growth and virus inoculation were described previously (Takahashi *et al.* 1991). The time-course of appearance of chlorotic spots in CMV(Y)-inoculated tobacco leaves is shown as the change of chlorophyll content in the inoculated leaves (Fig. 2B). In CMV(Y)-inoculated leaves, early chlorotic spot symptoms usually appeared between 3 and 4 days after inoculation and then developed to severe chlorotic spot symptoms between 5 and 6 days after inoculation. We have studied the patterns of decrease in the amount of the 18- and 33-kDa polypeptides, in addition to those of the 23-kDa protein family containing 22-, 23-, and 24-kDa polypeptides, during the progress of symptom expression.

The identity of the 33-kDa polypeptide spot (g) in 2-D gels (Fig. 2A) was confirmed by determining the N-terminal amino acid sequence. The N-terminal amino acid sequence of the 33-kDa polypeptide was determined to be EGVPKXL with a gas-phase protein sequence analyzer (ABI 473A). The N-terminal amino acid sequence of the 33-kDa protein of the oxygen-evolving complex in spinach was EGGPKRL (Tyagi *et al.* 1987). The time-course of decrease in the amount of 33-kDa polypeptide in CMV(Y)-inoculated leaves was analyzed by measuring the intensity of the polypeptide spots in 2-D gels in a Shimazu CS-9000 densitometer. The procedure of 2-D gel electrophoresis was described previously (Takahashi *et al.* 1991). As shown in Figure 2B, the 33-kDa polypeptide showed a similar pattern of decrease to the 24-kDa polypeptide during the appearance of chlorotic spots in CMV(Y)-inoculated leaves. The amount of 33-kDa polypeptide did not decrease 4 days after inoculation when early chlorotic spot symptoms had appeared, but it began to decrease 6 days after inoculation, when severe chlorotic spot symptoms appeared. Although the 33-kDa polypeptide resolved into three spots by 2-D gel electrophoresis using Ampholine (LKB), the N-terminal amino acid sequences of the three polypeptide spots were closely related to that of the 33-kDa polypeptide of the oxygen-evolving complex in spinach (data not shown).

We also determined the N-terminal amino acid sequences of most of the 18-kDa polypeptide spots in the 2-D gel. None were homologous to the N-terminal amino acid sequence of the 18-kDa protein of the oxygen-evolving complex of spinach (data not shown). The amino acid sequence of the 18-kDa polypeptide of the oxygen-evolving complex may not be highly conserved among higher plants. We compared the pattern of polypeptide spots in 2-D gels between mock-, CMV(O)-, and CMV(Y)-inoculated tobacco leaves during the progress of symptom expression. The amount of all 18-kDa polypeptides did not decrease significantly in CMV(Y)-inoculated tobacco leaves (data not shown).

In general, a decrease in the amount of the 23-kDa polypeptide induces a reduction in the oxygen-evolving activity of spinach. However, because polypeptides of the 23-kDa protein family in the oxygen-evolving complex of tobacco are differentially down-regulated during symptom expression, and the amount of the 33-kDa polypeptide, which is essential to oxygen evolution, does not decrease in CMV(Y)-inoculated tobacco leaves showing early chlorotic spot symptoms, it is not certain that the oxygen-evolving activity of PSII decreases significantly during the progress of symptom expression in CMV(Y)-inoculated tobacco leaves. Then, we measured the electron transport activity of thylakoid membranes that were prepared 4 days after inoculation from mock- or CMV(O)-inoculated tobacco leaves not showing clear symptom and from CMV(Y)-inoculated tobacco leaves showing early chlorotic spot symptoms.

Tobacco leaf homogenized in 50 mM sodium-potassium phosphate (pH 6.9) containing 300 mM sucrose and 50 mM NaCl, was centrifuged at 10,000 rpm for 10 min at 4° C. The pellet fraction was gently resuspended in the same buffer, or in 80 mM Tris-HCl (pH 8.0) containing 300 mM sucrose and 50 mM NaCl, and incubated for 20 min at 4° C. Tris treatment specifically inhibits oxygen evolution (Yamashita and Butler 1968: Fig. 1, Table 1). After centrifugation again, the thylakoid membrane pellet was gently resuspended in 25 mM MES-NaOH (pH 6.5) containing 300 mM sucrose, 10 mM NaCl, and 0.05% bovine serum albumin (Buffer A), and the electron transport activity was measured (Table 1). The thylakoid membrane electron transport assay from water to 2,6-dichlorophenol indophenol (DCIP), as an artificial electron acceptor, indicated that electron transport activity in CMV(Y)-inoculated leaves was reduced to about 33% of that in mock-inoculated leaves. In CMV(O)-inoculated leaves, it

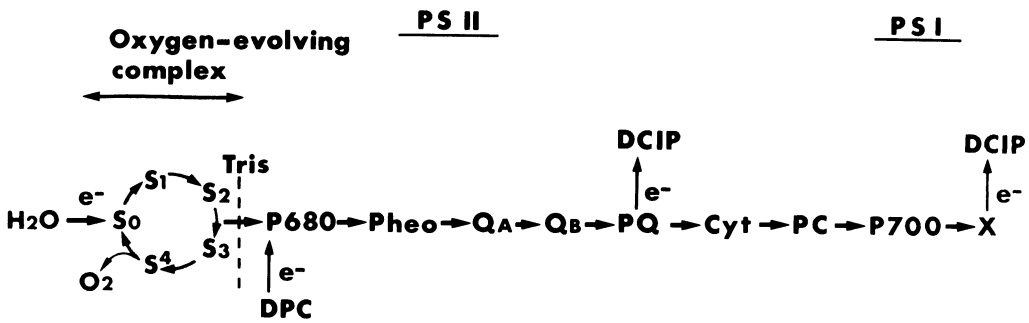

Fig. 1. Photosystem electron transport. Electrons removed from water to produce oxygen, can be transferred to DCIP via photosystem II (PSII) reaction center chlorophyll (P680), pheophytin (Pheo), plastoquinone molecules Q_A, Q_B and PQ), cytochrome b/f (Cyt), plastocyanin (PC), and photosystem I (PSI) reaction center chlorophyll (P700) and Fe-S center (X). Other electron transport components are not shown here. S-cycle model of oxygen evolution (Kok *et al.* 1970) is shown as S_0-S_4. Tris-treatment specifically inhibits oxygen evolution. 2,6-Dichlorophenol indophenol (DCIP) and 1,5-diphenylcarbazide (DPC) are used as artificial electron acceptor and donor, respectively. Ca^{2+} is capable of partially restoring the oxygen-evolving activity in PSII particles depleted of 24- and 18-kDa polypeptides.

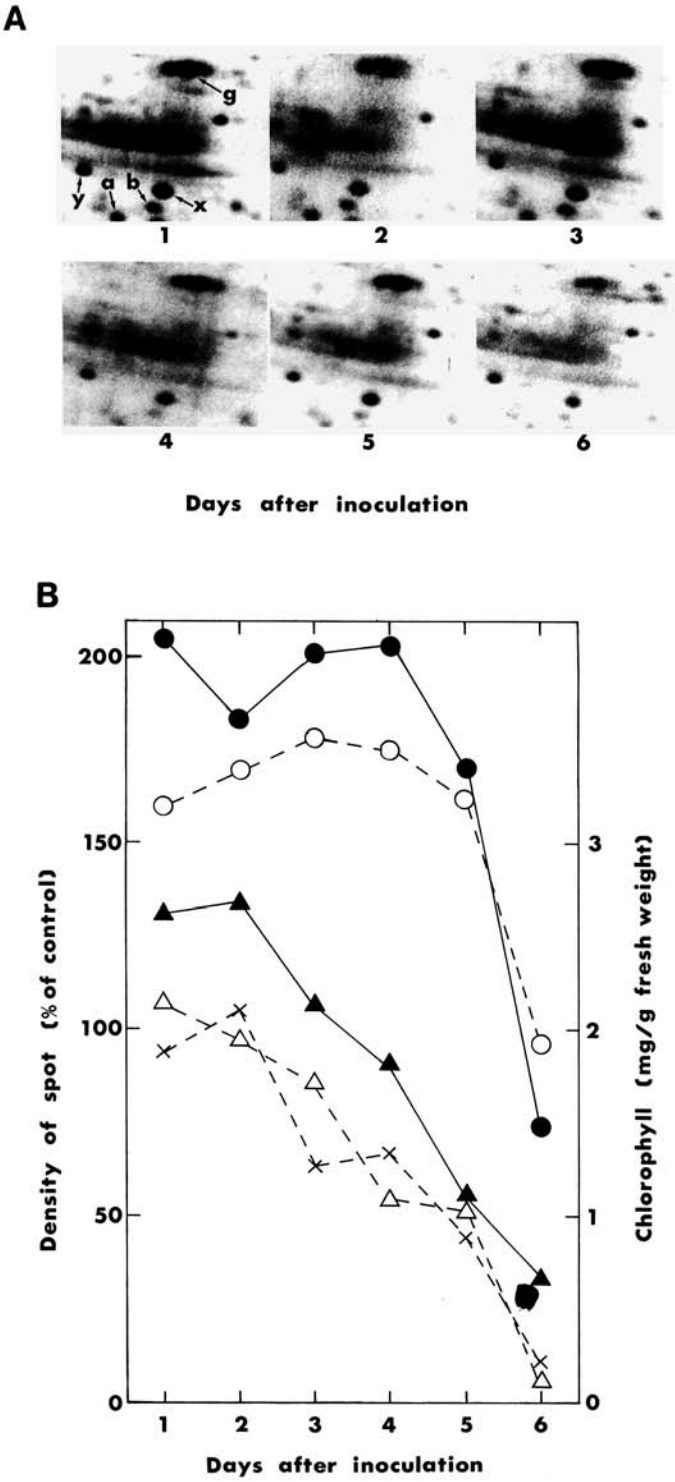

Fig. 2. Time-course of decreases in the amounts of 22-, 23-, 24-, and 33-kDa polypeptides and chlorophyll content with the progress of symptom expression in CMV(Y)-inoculated tobacco leaves. Data for 22-, 23-, and 24-kDa polypeptides are taken from Takahashi *et al.* (1991). **A,** 22- (a), 23- (b), 24- (x), and 33-kDa (g) polypeptides were analyzed by 2-D gel electrophoresis. *y* indicates the spot taken as a control standard for measuring decreases in the amounts of 22-, 23-, 24-, and 33-kDa polypeptides. **B,** The relative intensities of 22-kDa (X-----X), 23-kDa (△-----△), 24-kDa (○-----○), and 33-kDa (●——●) species are shown as the percentage of a standard spot (labeled *y*) that did not change in intensity during symptom expression. Chlorophyll content (▲——▲) was measured by the method of Arnon (1949).

was reduced only to 83% of the mock value. On the other hand, the electron transport assay from 1,5-diphenylcarbazide (DPC, an artificial electron donor that bypasses the oxygen evolution system) to DCIP in Tris-washed thylakoid membranes, indicated that the electron transport activity in CMV(O)- and CMV(Y)-inoculated tobacco leaves was reduced by only 10 and 23%, respectively. These results suggest that inhibition of the oxygen-evolving activity causes the reduction of photosynthetic electron transport in CMV(Y)-inoculated tobacco leaves that show early chlorotic spot symptoms. The small decrease in electron transport activity from DPC to DCIP in CMV(Y)-inoculated leaves, compared with that in CMV(O)-inoculated leaves, may be caused by the degradation of chlorophyll as an adjunct in the inactivation of a light-harvesting chlorophyll-protein complex.

Although the oxygen-evolving activity is partly reduced by removal of the 24- and 18-kDa polypeptides from PSII particles in spinach, addition of Ca^{2+} to assay mixtures depleted of the 24- and 18-kDa polypeptides reconstituted the oxygen-evolving activity (Murata and Miyao 1987). The purified thylakoid membrane pellet was gently suspended in Buffer A containing 5 mM $CaCl_2$, incubated at 4° C for 20 min, and the electron transport activity from water to DCIP was measured. The electron transport activity in CMV(Y)-inoculated leaves could be partly restored by Ca^{2+} ions (Table 1).

These results indicate that the partial reduction in oxygen-evolving activity in CMV(Y)-inoculated tobacco leaves showing early chlorotic spot symptoms is not caused

Table 1. Activities of photosynthetic electron transport reaction in mock-, cucumber mosaic virus (strain O) CMV[O]- and CMV(Y)-inoculated tobacco leaves

	Electron transport activity[a]				
	DCIP Reduction (μM/hr·μg protein ±SE.)			DCIP Reduction (% of mock-inoculation)	
	mock	CMV(O)	CMV(Y)	CMV(O)	CMV(Y)
$H_2O \longrightarrow$ DCIP[(1)]	84 ± 8	70 ± 12	28 ± 7	83.3	33.3
$H_2O \xrightarrow{\text{Tris}}$ DCIP[(1)]	11 ± 1	13 ± 6	1 ± 1	...	...
$H_2O \xrightarrow{\text{Tris}}$ DCIP[(2)]	189 ± 21	171 ± 9	146 ± 15	90.5	77.2
$H_2O \xrightarrow{\text{DPC}}$ DCIP[(3)] + $CaCl_2$	161 ± 17	126 ± 7	79 ± 9	78.3	49.1

[a] Electron transport activity was measured as follows: The amounts of thylakoid membrane for measuring the electron transport activity are expressed as protein content, because the chlorophyll concentration of tobacco leaf is changed by CMV infection. Thylakoid membrane proteins were quantified by the method of Bradford (1976). The experiments were repeated five times. (1) The activity of electron transport from H_2O to DCIP was measured photometrically by following the change in absorbance at 585 nm at a light intensity of 660 W/m^2 in a reaction mixture containing 25 mM MES-NaOH (pH 6.5), 300 mM sucrose, 10 mM NaCl, 0.05% BSA, 0.06 mM DCIP, and thylakoid membrane fraction equivalent to 20 μg of protein. The extinction coefficient of DCIP at pH 6.5 was calculated as 20.7 $mM^{-1} \cdot cm^{-1}$ (Armstrong 1964). (2) When the activity of electron transport from DPC to DCIP was measured, 1 mM DPC was added to the reaction mixture. (3) When the activity of electron transport from H_2O to DCIP was measured in the presence of Ca^{2+}, 5 mM $CaCl_2$ was added to the reaction mixture.

by a decrease in the amount of all polypeptides in the oxygen-evolving complex, but only by loss of the 22- and 23-kDa polypeptides in that complex. Decrease in the amount of 24- and 33-kDa polypeptides of the oxygen-evolving complex seems to coincide with more severe chlorotic spot symptoms 6 days after CMV(Y)-inoculation.

Symptom expression is due to complex interactions between host and virus and involves many processes at the molecular, cellular, and tissue levels. Other factors, apart from the 22- and 23-kDa polypeptides of the oxygen-evolving complex, are likely to be associated with symptom expression because inhibition of electron transport activity occurred in TMV-infected tobacco leaves that remained symptomless (Van Kooten *et al.* 1991). A single reaction cannot be identified as the sole cause of symptom expression. However, the present results indicate that inhibition of the oxygen-evolving activity in PSII is not a secondary consequence of symptom expression but is associated with the primary molecular process of symptom expression.

In CMV(Y)-inoculated tobacco leaves, it is interesting to note the differential down-regulation of the 23-kDa protein family and 33-kDa polypeptides of the oxygen-evolving complex in PSII during the progress of symptom expression. Although there are reports showing differential expression of 33- and 23-kDa polypeptides in a mutant of *Chlamydomonas reinhardtii* and in norflurazon-treated maize seedlings (Mayfield *et al.* 1987; Burgess and Taylor 1987), the regulatory mechanism between the 33- and 23-kDa polypeptides was unknown. On the other hand, symptom expression in virus-inoculated tobacco leaves is related to an amino acid change in the coat proteins of TMV or AlMV (Dawson *et al.* 1988; Neeleman *et al.* 1991).

To understand the molecular basis of the appearance of chlorotic spots in CMV(Y)-inoculated leaves, we must understand the mechanism of the differential down-regulation of the oxygen-evolving complex polypeptides following reduction of the oxygen-evolving activity and study the influence of coat protein on this down-regulation in CMV(Y)-inoculated tobacco leaves.

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