1 Artificial induction of a plant virus protein in transgenic tobacco provides a synchronous
2 system for analyzing the process of leaf chlorosis

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20 Abstract

21 The underlying molecular mechanism of chlorosis, a typical symptom of plant viral diseases,
22 remains poorly understood. To establish an experimental system to determine the molecular
23 changes during chlorosis, especially in the early phase, we generated transgenic tobacco
24 plants expressing *Cauliflower mosaic virus* Transactivator/viroplasmin (Tav) under the control
25 of a chemically inducible promoter. Induction of Tav resulted in visible chlorosis in ten days,
26 a statistically significant decrease in chlorophyll content in two days, decreased expression of
27 chloroplast protein genes, and abnormal thylakoid stacks, indicating that this system
28 reproduces the common features of chlorosis in virus-infected plants.

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30 Keywords: *Cauliflower mosaic virus*, chloroplast, synchronous system, thylakoid,
31 transactivator/viroplasmin

32

1. Introduction

One of the common features in plant virus diseases is chlorosis or yellowing, which results from the reduction in photosynthetic pigments such as chlorophyll. Because photosynthesis plays a major role in plant production, understanding the mechanism underlying chlorosis is important in avoiding crop damage caused by virus infection. Although the process of chlorosis has been extensively studied, the precise mechanism of this phenomenon remains to be clearly described. Recent studies have shown that the bright yellow symptoms in tobacco plants infected with *Cucumber mosaic virus* (CMV) harboring Y-satellite RNA (Y-sat) is attributed to silencing mediated by Y-sat-derived siRNA of magnesium protoporphyrin chelatase subunit I (ChlI) involved in chlorophyll biosynthesis [1, 2]. Another study has shown that siRNA derived from *Peach latent mosaic viroid* (PLMVd) directs the silencing of chloroplast heat-shock protein 90 (cHSP90), and consequently causes severe chlorosis or albinism [3]. Although these studies showed the involvement of RNA silencing in chlorotic symptom development, this pathway is unlikely to be a common mechanism for chlorosis induced by different viruses.

A possible common mechanism for chlorosis is the delayed or impaired resistance response. Delayed and reduced expression of pathogenesis-related (PR) proteins have been observed in plants infected with compatible viruses, although the dramatic induction of PR protein expression has been known as a representative molecular change during resistance response [4 - 6]. CMV mutants with different amino acids at a particular site (129th residue) of coat protein (CP) induce various types of symptoms in the same host plants ranging from vein chlorosis, pale green mosaic, white mosaic, systemic necrosis, and vein necrosis, to local necrotic spots which resemble the resistant response [7]. Given a single host component has a role in the perception of different CMV CP mutants, the difference in symptoms could be attributed to a difference in the efficiency of CP perception, which would lead to varying responses from a resistant response to different severity of symptoms. This notion supports

the idea that different severity of chlorosis responses share a common pathway and reflect the difference in pathogen perception.

Transcriptomic and proteomic studies have been conducted to understand the mechanism underlying symptom development [5, 8 - 15], but there is limitation resulting from the nature of materials. Because virus infection spreads gradually cell-to-cell within an inoculated leaf, the infected leaves are a mixture of cells at different stages of virus infection. This difference in stages of virus infection makes it difficult to analyze temporal changes in the process of symptom expression and to identify a cause-effect relationship among different molecular events observed during symptom expression. To solve this problem, we established a system using transgenic tobacco plants expressing *Cauliflower mosaic virus* (CaMV) multifunctional protein, transactivator/viroplasmin (Tav) [16], under the control of a chemically inducible promoter [17]. These transgenic lines reproducibly showed virus symptom-like chlorosis, as reported for those constitutively expressing Tav [18 - 20], but only after induction of Tav. Characterization of the transgenic plants supported the idea that they would provide a good system for analyzing early events as well as temporal changes in the process of virally induced chlorosis.

2. Materials and Methods

2.1 Construction and plant transformation

The *Tav* gene was amplified using primers Sal-TavF and Tav-BlnR (Supplementary Table S1), GXL DNA polymerase (Takara Bio, Ohtsu, Japan), and pFastWt [21] as a template. The amplified fragment was cloned, sequenced, and introduced into pTA7002 [17], resulting in pGVG-Tav. *Nicotiana tabacum* SR1 was aseptically grown on a twice-diluted MS medium free of any vitamins or plant hormones but with 0.8% agar (half-MS). Tobacco was transformed using *Agrobacterium tumefaciens* GV3101 harboring pGVG-Tav as described previously [22]. The transformants were selected on a medium containing 30 µg/mL

hygromycin B (Hyg) and confirmed for the transgene by PCR (Supplementary method).

2.2 Growth of transgenic tobacco and induction of Tav expression

Transgenic tobacco seeds were sown on half-MS containing 30 µg/mL Hyg, and the Hyg-resistant seedlings were transferred to Jiffy-7 (4.2 cm in diameter before swelling; Jiffy Products International, Kristiansand, Norway) supplemented with a small amount of soil one week later. The plants were further grown for up to six weeks for experiments, and watered once a week each with tap water and 1000-times diluted Hyponex 6-10-5 (Hyponex Japan, Osaka, Japan). Water was replaced with a solution containing 50 µM dexamethasone (Dex; Nacalai tesque, Kyoto, Japan) to induce the *Tav* transgene three or four weeks after the transfer to Jiffy-7. Six plants were kept in 18-cm square plastic trays, which received 50 mL Dex solution every other day. The expression of the *Tav* protein was examined by western blotting using an antiserum raised against *Tav* (see Supplementary information). Some seeds of the transgenic lines of the T2 generation were sown onto half-MS containing 1 µM Dex, because higher concentration of Dex inhibited the germination of tobacco seeds in both control and transgenic plants.

2.3 Determination of chlorophyll content

About 100 mg of leaf tissue was ground to a fine powder in a centrifuge tube using a bead-beater (Microsmash; Tomy, Tokyo, Japan) with two 5-mm stainless-steel beads. Pigments were extracted in 10 mL/g leaf tissue of 99.5% methanol. Absorbance at 652.0, 665.2, and 750 nm was measured using a spectrophotometer (UV-1800; Shimadzu, Kyoto, Japan). Total chlorophyll content (a + b) was calculated as described [23].

2.4 Gene expression analysis

About 100 mg of leaf tissue was ground to a fine powder as mentioned above, and

total RNA was extracted essentially as described [24] using Sepasol-RNA 1 Super G (Nacalai tesque) with a slight modification to the manufacturer's protocol. After phenol/chloroform extraction, the aqueous phase was once extracted with chloroform/isoamylalcohol (24:1) to improve RNA purity. About 10 µg of RNA preparation was treated with the recombinant DNase I (Takara Bio) in the presence of recombinant ribonuclease inhibitor (Takara Bio) in a 50 µL reaction mixture. The DNase-treated RNA was purified using Sepasol-RNA 1 Super G. The cDNA was synthesized from the RNA by PrimeScript reverse transcriptase (Takara Bio) using random hexamer primers. Semi-quantitative PCR was performed to see the expression levels of some chloroplast protein genes using the primers shown in Table S1 and reaction conditions described in Supplementary methods. The analyses were performed in duplicate samples from independent plants. The amounts of PCR products were quantified using Image Lab software (ver. 5; BioRad, Hercules, CA) and the ratios to day 0 samples of the mean values of duplicates are shown as the relative expression levels.

2.5 Electron microscopy

Ultrastructural change in chlorotic leaf cells was examined by comparing Dex-treated and untreated transgenic lines under electron microscopy as described [7] with a slight modification. Leaf tissues were cut into 2 × 5 mm pieces and fixed overnight at 4°C with 4% glutaraldehyde in 60 mM HEPES, pH 7.0, containing 125 mM sucrose. After post-fixation in Dalton's buffered 1% osmium tetroxide for 2 h at 4°C and dehydration, the tissues were embedded in Spurr resin (Nisshin EM, Tokyo, Japan). Ultra-thin sections (100–120 nm) were stained with 2% uranyl acetate and Sato's lead aqueous solution (1% lead nitrate, 1% lead acetate, 1% lead citrate, 2% sodium citrate, 0.18 N NaOH), and examined using a Hitachi 7500 electron microscope (Hitachi, Tokyo, Japan) at 80 kV.

3. Results

3.1 Induction of the *Tav* transgene results in symptom-like phenotype

Agrobacterium-mediated transformation brought 18 Hyg-resistant shoots, and 17 independent fertile transgenic tobacco lines harboring the *Tav* transgene (Supplementary Fig. S1). Hyg-selected T1 seedlings (five weeks old) from each transgenic line were treated with 20 mg/L Dex as described before and monitored for visible changes. All the transgenic lines showed chlorosis and growth suppression, albeit with variations in severity, at 7 days post-treatment (dpt; Fig. 1C, D, E, and F). Non-transformed control plants (SR1) as well as control transformants, which harbor the *L³* tobamovirus resistance gene [25] introduced into the same vector showed no visible chlorosis after Dex treatment (Fig. 1A and B). The expression of the *Tav* protein in the Dex-treated transgenic plants was examined by western blotting, revealing that the *Tav* protein was detectable as early as 1 dpt, reached a maximum at 3 dpt, and thereafter decreased (Fig. 2). Transgenic lines 3 and 5 showed higher levels of *Tav* expression than line 12. These results suggest that the induced expression of *Tav* can give rise to a symptom-like phenotype (SLP) consisting of chlorosis and growth suppression, which were observed in transgenic tobacco constitutively expressing *Tav* [18 - 20]. When the expression of *Tav* transgene was examined by RT-PCR, low levels of expression was observed in all transgenic lines in the absence of Dex treatment (Fig. 2B, C and D, lower panels), suggesting that the accumulation of *Tav* protein to a level above a threshold is required for the induction of SLP. As reported in the transgenic plants constitutively expressing *Tav*, some lines showed a mosaic-like patterned phenotype while others showed uniform chlorosis after Dex treatment (Fig. 1G and H).

SLP was also observed in transgenic lines grown on a Dex-containing medium (Fig. 3). When seeds were sown on Dex-containing medium, cotyledons of transgenic lines showed chlorosis (Fig. 3D, F and H), whereas they grew normally on the medium without Dex (Fig. 3C, E and G). Non-transformed control plants grew similarly on both medium with and

without Dex (Fig. 3A and B), suggesting that chlorosis observed in transgenic lines on Dex-containing medium should be attributed to the expression of the *Tav* protein.

3.2 Pathogenic-like change can be detected early after Dex treatment

The characterization of early events preceding chlorosis rather than changes found in chlorotic tissue, would lead to understanding the molecular mechanism underlying chlorosis. Because visual examination distinguished SLP as late as 7 dpt, we measured chlorophyll content to evaluate the progress of chlorosis at earlier time points. In all three lines, a statistically significant decrease in chlorophyll content of Dex-treated plants was observed at 2 dpt and thereafter (Fig. 4B, C, and D), although no difference was observed between Dex-treated and untreated control plants (Fig. 4A). The decrease in chlorophyll content within the first 2 days was slower in line 12, which expressed lower levels of *Tav* after induction than lines 3 and 5, indicating that the expression levels of *Tav* affect the progression of chlorosis. These results suggest that signaling towards chlorosis is activated within a day or two after induction of the *Tav* transgene.

3.3 Downregulation of chloroplast protein genes in transgenic plants with Tav-induced SLP

The downregulation of chloroplast- and photosynthesis-related genes is a common feature in virus infected plants [5, 6, 10, 26]. To test if this is also the case in *Tav*-induced SLP, we tested the expression of a few nuclear genes encoding chloroplast proteins: a small subunit of ribulose biphosphate carboxylase/oxygenase (RSSU), light harvesting chlorophyll a/b binding protein (LHC1), and magnesium protoporphyrin chelatase subunit I (ChlI). The expression levels of the elongation factor 1 α (EF1 α) gene were not affected by Dex treatment in both the transgenic lines and control plants (Fig. 5A). In contrast, the decreased expression levels of RSSU, LHC1, and ChlI were clearly observed by 7 dpt (Fig. 5B, C, and D) in transgenic plants but not in control SR1 plants, supporting the idea that *Tav*-induced

SLP shares the downregulation of chloroplast protein genes with the virus disease. The downregulation of these genes was observed at earlier time points in lines 3 and 5, which showed a more rapid decrease in chlorophyll content than line 12 (Fig. 4B, C, and D), indicating again that chlorosis progression differs within transgenic lines.

3.4 Upregulation of PR1a expression in transgenic plants during Tav-induced SLP expression

The expression of pathogenesis-related protein 1a (PR1a) was reported in transgenic tobacco constitutively expressing Tav [20]. In contrast, Tav reportedly suppresses the PR1a gene activation in transgenic *Arabidopsis* and transiently transformed *Nicotiana benthamiana* [27, 28]. Consistently with the former report, the upregulation of PR1a gene expression was found after the Dex-induced expression of Tav (Fig. 6). Although the expression levels of PR1a gene were not affected by Dex-treatment in control SR1 plants, transgenic line showed high levels of PR1a expression at 1 dpt and thereafter. Two independent transgenic lines, line 3 and line 12, showed slightly different temporal expression patterns of PR1a gene. The former showed dramatic decrease in PR1a expression at 7 dpt, whereas the decrease in PR1a expression at 7 dpt was moderate in the latter.

3.5 Ultrastructural changes in chloroplasts of transgenic plants with Tav-induced SLP

In virus and viroid infected plants, ultrastructural changes in chloroplasts have been reported in several studies [7, 29 - 31]. To characterize Tav-induced SLP, the chloroplast ultrastructure was compared in Dex-treated and untreated transgenic lines. No abnormality was found in chloroplasts in the control SR1 plants regardless of Dex treatment (Fig. 7A and B). In contrast to Dex-untreated transgenic plants showing almost normal chloroplast structure (Fig. 7E and F), severe alteration in thylakoid ultrastructure highlighted by the lack of granum stacks was observed in Dex-treated, chlorotic transgenic plants (Fig. 7C and D). The impaired grana structure has commonly been observed in plants infected with viruses and

viroids [7, 30, 31]. A hyper-accumulation of starch grains (HAS), which was also observed in virus infected cells [7, 29], was observed in the transgenic plants, even in the untreated transgenic plants (Fig. 7E, F, G, and H). The HAS in untreated plants may have been induced by the leaky expression of Tav in the absence of Dex, because the thylakoid structure was slightly affected in untreated transgenic plants (Fig. 7G and H).

Tav is known to be the major component of CaMV viroplasm or virus inclusion bodies [16]. Tav inclusion bodies similar to the CaMV viroplasm were found in cytoplasm of transgenic *Arabidopsis* constitutively expressing Tav [32]. However, we have not observed any viroplasm-like structure in the chlorotic Tav transgenic tobacco, most likely because the Tav protein levels decreased from 3 to 7 dpt. Because Tav has been shown to be a nucleocytoplasmic shuttling protein [33], it is unlikely that Tav invades chloroplasts and affects their structure.

4. Discussion

In plant virus studies, analysis of temporal changes in molecular levels is difficult because of the asynchronous nature of virus infection in plant tissues. Using protoplasts enabled the establishment of synchronous replication systems, in which temporal regulation of virus gene expression and replication has widely been studied (for example, ref. [34]). Nevertheless, the study of host responses to viruses is still difficult because the cues from viruses are given in an asynchronous manner in the infected plant tissue, and protoplast systems are not long-lasting enough to observe late responses including chlorosis. To establish a synchronous chlorosis system suitable for analyzing both early and late events during the progression of chlorosis, we employed a viral protein, CaMV Tav, which was reported to induce SLP in transgenic tobacco and *Arabidopsis* expressing the protein constitutively [18 - 20, 32]. A widely-used, chemically inducible promoter enabled the artificial induction of Tav-mediated SLP (Fig. 1 and 3).

There are two types of transgenic tobacco lines harboring inducible Tav transgene: one includes those represented by lines 3 and 5 showing mosaic-like chlorosis after Dex treatment and the other includes those represented by line 12 showing uniform chlorosis after Dex treatment (Fig. 1G and H). It is presently unknown what causes such a difference, but the expression levels of Tav after Dex treatment may affect the progression and pattern of chlorosis. Line 12 showed lesser accumulation of Tav protein than lines 3 and 5 when treated with Dex, whereas all the untreated transgenic lines showed low levels of Tav mRNA expression (Fig. 2). In concert with the difference in the expression levels, line 12 showed slower progression of chlorosis after Dex treatment than lines 3 and 5 as manifested by changes in chlorophyll content and chloroplast protein gene expression (Fig. 4 and 5). Although the mosaic pattern in virus-infected tissues is reportedly determined by the balance of the spread of virus and anti-viral RNA silencing [35], there could be an additional factor leading to mosaic-patterned chlorosis.

Studies have shown that Tav affects plant gene expression not only in transgenic Arabidopsis and tobacco plants, but also in plants transiently expressing Tav [20, 27, 28, 36]. Our expression analysis revealed the downregulation of chloroplast protein genes, which is a common feature in plants with chlorosis [5, 6, 10, 26]. In addition, we observed that PR1a expression was upregulated after Dex -induced Tav expression. Although it is consistent with previous results in transgenic tobacco constitutively expressing Tav [20], it is contradictory to the results from transgenic Arabidopsis and transiently transformed tobacco plants [27, 28, 36]. A possible explanation for this contradiction is that Tav suppresses salicylic acid-dependent pathway in host and transiently transformed non-host plants, but activates it in transgenic non-host tobacco plants. Involvement of ethylene pathway in Tav-dependent SLP was suggested in transgenic Arabidopsis [37]. Further studies are needed to understand the involvement of defense pathways in SLP expression in Tav transgenic tobacco plants.

A decrease in chlorophyll content was observed within 2 days of Dex treatment,

indicating that tobacco cells are committed to chlorosis soon after the accumulation of the Tav protein. In contrast, visible chlorosis was evident only after 7 days of Dex treatment, indicating that the progression of chlorosis to visible levels takes longer. These results collectively suggest that the analysis of symptomatically virus infected plant tissues is not suitable for studying the molecular mechanism underlying the commitment to chlorosis, although such studies have shown the molecular events in the execution phase of chlorosis [5, 6, 10, 26] It is not feasible to distinguish virus infected and non-infected cells in virus infected plant tissue, or to identify cells that will turn yellow in the near future. That is, it is not possible to analyze cells in the commitment phase of chlorosis. Dex treatment under the indicated condition reproducibly induced chlorosis in transgenic tobacco. Therefore, the inducible Tav transgenic plants provide a suitable system to elucidate the early molecular events in the commitment phase that precedes the execution of chlorosis.

The experimental system presented here would enable identifying genes upregulated or downregulated early in the process of chlorosis by common transcriptome analysis. The downregulation of chloroplast protein gene expression, which is likely to be an execution phase event, was detected as early as 2 dpt. The changes in gene expression in the commitment phase may take place within a day or two and therefore, transcriptome analysis should be performed at earlier time points. Some changes in protein levels would precede the transcriptomic changes, for example, the physical interaction of some host proteins with Tav, the degradation of Tav or some host proteins or both, and post-translational modifications such as phosphorylation, ubiquitination, or nitrosylation of Tav or some host proteins or both. In addition to such analytical studies, the present system is also useful for testing gene function using virus-induced gene silencing (VIGS), because VIGS in tobacco plants is possible using the *Apple latent spherical virus* vector [38]. Furthermore, the experimental system would also be useful for analyzing the process of chloroplast damage, because the chlorophyll decrease was not rapid and therefore, chloroplasts at different stages of damage

would be available using the system. In conclusion, the experimental system using transgenic tobacco with the inducible *Tav* transgene would pave the way to a better understanding of the molecular mechanism underlying chlorosis. It would be an open question whether or not the present experimental system can be regarded as a general model for virally induced chlorosis. This question could only be answered by testing the outcome from the present system in several virus-infected plants, because this is the first synchronous system in which the mechanism for virally induced chlorosis can reliably be analyzed.

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Figure legends

Fig. 1. Chlorosis and growth suppression in Dex-treated transgenic tobacco plants. *Nicotiana tabacum* cv. SR1, a non-transformed control (A), a control transformant with the same vector containing the *L*³ tobamovirus resistance gene (B), transgenic lines 2 (C), 3 (D and G), 5 (E), and 12 (F and H) harboring the inducible *Tav* transgene at 5 weeks old received water (Dex[−]) or a solution containing 20 mg/L Dex (Dex⁺), and were photographed at 7 days post-treatment.

Fig. 2. Immunoblot and RT-PCR analysis of *Tav* expression in Dex-treated and untreated plants. Non-transformed control (A) and transgenic lines 3 (B), 5 (C), and 12 (D) at 5 weeks old were treated as in Fig. 1. Samples were taken before (D0) the initial Dex treatment, and 1 day (D1), 2 days (D2), 3 days (D3), 5 days (D5), and 7 days (D7) after the initial Dex treatment. + and −, Dex-treated and untreated plants, respectively. The positions and molecular weights of standard proteins are shown on the left (upper panels) and the arrows indicate the 524bp specific PCR products amplified from the transcripts of *Tav* transgene (lower panels).

Fig. 3. Symptom-like phenotype of transgenic plants grown on a medium containing Dex. Seeds of non-transformed control (A and B) and transgenic lines 3 (C and D), 5 (E and F), and 12 (G and H) were sown onto half-MS medium (A, C, E, and G) or medium containing 1 μM Dex (B, D, F, and H). Bars indicate 3 mm.

Fig. 4. Decrease in chlorophyll content in Dex-treated transgenic plants. Non-transformed control (A) and transgenic lines 3 (B), 5 (C), and 12 (D) at 5 weeks old were treated as in Fig. 1. Samples were taken before the initial Dex treatment, and 1, 2, 3, 5, and 7 days after the initial Dex treatment. Vertical axis shows chlorophyll content (μg/mg fresh weight of leaf

tissue). Means $\pm$ standard deviation (error bar) of three biological samples from independent plants of Dex-treated (squares) and untreated (circles) plants are shown. Single and double asterisks indicate statistically significant difference with $p < 0.05$ and $p < 0.01$, respectively, in Student's T test.

Fig. 5. Downregulation of chloroplast protein gene expression in Dex-treated transgenic plants. Non-transformed control (SR1) and transgenic lines 3, 5, and 12 at 5 weeks old were treated as in Fig. 1. Samples were taken before the initial Dex treatment (0), and 1, 2, 3, 5, and 7 days after the initial Dex treatment. The expression of EF1 α (A) RSSU (B), LHC1 (C), and ChlI (D) mRNA were analyzed by RT-PCR (see supplementary Fig. S2 for gel photographs and methods). Relative expression to day 0 samples are shown. White and black bars denote Dex-treated and untreated plants, respectively.

Fig. 6. Upregulation of PR1a expression in transgenic plants during Tav-induced SLP expression. Non-transformed control (SR1) and transgenic lines 3 (L3), and 12 (L12) at 5 weeks old were treated as in Fig. 1. Samples were taken before (D0) the initial Dex treatment, and 1 day (D1), 2 days (D2), 3 days (D3), 5 days (D5), and 7 days (D7) after the initial Dex treatment. + and –, Dex-treated and untreated plants, respectively. The expression of EF1 α (not shown) and PR1a mRNA were analyzed by RT-PCR. Electrophoretic gel images (A) were analyzed and the relative expression levels to day 0 samples at different time points are shown (B). White and black bars denote Dex-treated and untreated plants, respectively.

Fig. 7. Ultrastructural change in chloroplasts of Dex-treated transgenic plants. Non-transformed control (SR1; A and B) and transgenic line 12 (L12; C, D, E, F, G, and H) were treated as in Fig. 1. Chloroplasts of untreated (A, E, F, G, and H) and Dex-treated (B, C, and D) plants were observed under an electron microscope. Arrowheads indicate affected

481 thylakoid membranes in untreated plants. D, F, and H show magnification of indicated area in
482 C, E, and G, respectively, to highlight the structural changes in granum stacks. Bars indicate 1
483 μm .

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