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学位論文全文に代わる要約
Extended Summary in Lieu of Dissertation

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学位論文題目：
Title of Dissertation

Comparative analysis of mechanisms underlying the chlorosis development in transgenic tobacco expressing different chlorosis triggers
(異なる退緑黄化発症因子を発現する遺伝子組換えタバコにおける退緑黄化発症機構の比較解析)

学位論文要約：
Dissertation Summary

One of the major factors responsible for crop losses is the infection by virus and viroid. Epidemics on crop plants due to virus infection possess a serious threat to global food security. Leaf chlorosis which represents the plants' impaired morphology and physiology, is the most common symptom that occurs during the plant-virus interaction and, eventually leads to significant yield reduction. As strict intracellular pathogens, they cannot be controlled chemically and prophylactic measures consist mainly of the destruction of infected plants and excessive pesticide applications to limit the population of vector organisms. A powerful alternative frequently employed in agriculture relies on the use of crop genetic resistances, the approach that depends on mechanisms governing plant-virus interactions. Therefore, understanding the mechanisms of chloroplast dysfunction would lead us to the establishment of counter-measures against crop loss.

Although the molecular events during chlorosis have been extensively documented, the precise mechanism for the reduced chloroplast activities had remained to be elucidated until recent pioneering studies, which have shown the involvement of RNA silencing of chloroplast protein genes in the development of chlorosis by sub-viral RNAs. Two groups have independently shown that the bright yellow symptoms in tobacco plants infected with *cucumber mosaic virus* (CMV) harboring Y-satellite RNA (Y-sat) is attributed to the RNA silencing of magnesium protoporphyrin chelatase I subunit (CHLI) involved in chlorophyll biosynthesis mediated by the Y- sat-derived small interfering RNA (siRNA). Another study has shown that siRNA derived from *peach latent mosaic viroid* (PLMVd) directs the RNA silencing of chloroplast heat-shock protein 90 (HSP90C) and consequently causes severe chlorosis or albinism in peach trees, which is the natural host of the viroid. A recent study identified an additional pathogenic determinant of yellow mosaic in peach, PLMVd-sRNA40 that guides cleavage of the mRNA encoding a thylakoid translocase subunit required for chloroplast development.

To investigate the molecular mechanisms of chlorosis induced by the RNA silencing of chloroplast proteins, previously two experimental systems were generated in our laboratory (Figure 11), by using the chemically inducible promoter to drive RNAs to induce RNA silencing of CHLI and HSP90C in transgenic tobacco plants.

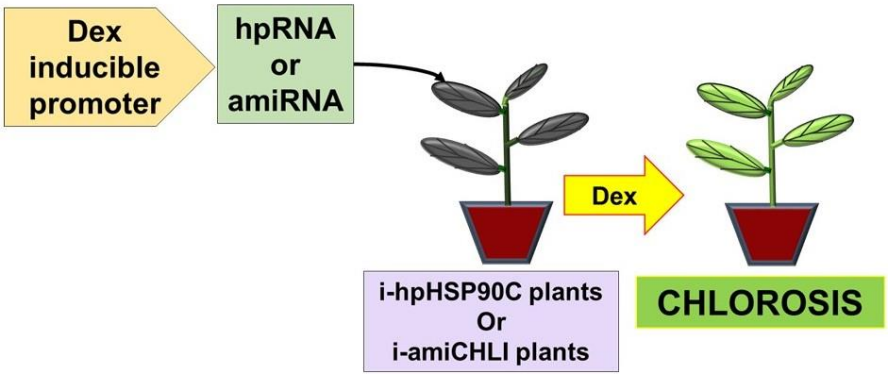

Figure 1. A schematic of the development of inducible-silencing systems for CHLI and HSP90C in tobacco.

The potential of these inducible transgenic systems for analyzing those plant cells that have committed to developing but not exhibited chlorosis, make them more advantageous over the conventional experimental systems with the virus- or viroid-infected plants. By exploiting the benefits of these systems, in this current study, an RNA-seq method was implemented to explore the early molecular changes, which lead to chlorosis development. Unlike previous studies analyzing the transcriptome changes in virus- and viroid-infected plants, this study successfully identified transcriptome changes that precede the detectable chlorosis.

Below, the flowchart shows the typical work-flow which is followed in an RNA-seq analysis.

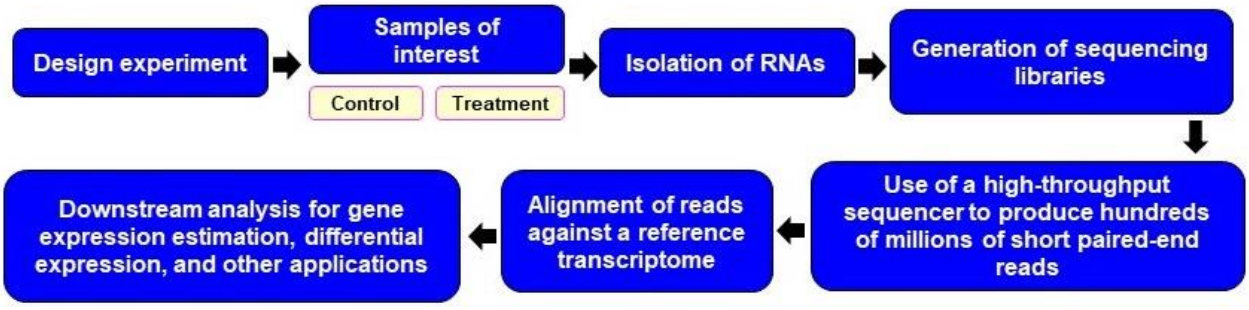

Figure 2. Typical method for data generation in an RNA-seq analysis.

Transcriptomics is the study of the transcriptome using high-throughput methods, such as DNA microarrays and next-generation sequencing technologies called RNA-Seq. Comparison of transcriptomes allows the identification of genes that are differentially expressed in distinct cell populations, or in response to different treatments. In a typical RNA-seq analysis, following a careful design of the experiment, RNAs are isolated from the samples of interest. Then a sequencing library is generated and, by utilizing high-throughput sequencer

hundreds of millions of short paired-end reads are produced. After the alignment of reads against a reference transcriptome, finally, downstream analysis for gene expression estimation and differential expression analysis is done.

Now, in the first part of the study. Three-week-old i-hpHSP90C transgenic line H-4 and non-transformant SR1 were Dex- or control-treated, and RNA was extracted at 24 h post-Dex-treatment. The RNA sequencing gave 23 M reads/sample on average from the 12 samples, 80–90% of which were mapped to the tobacco reference transcriptome (For details - Islam et al., 2020, https://www.mdpi.com/1422-0067/21/12/4202#supplementary).

The overall similarity within samples was evaluated by a histogram from DESeq2 analysis. The result displayed that HD2 and HD3 samples showed clear differences from the control samples including the non-transformed SR1 samples regardless of the treatment (Figure 3).

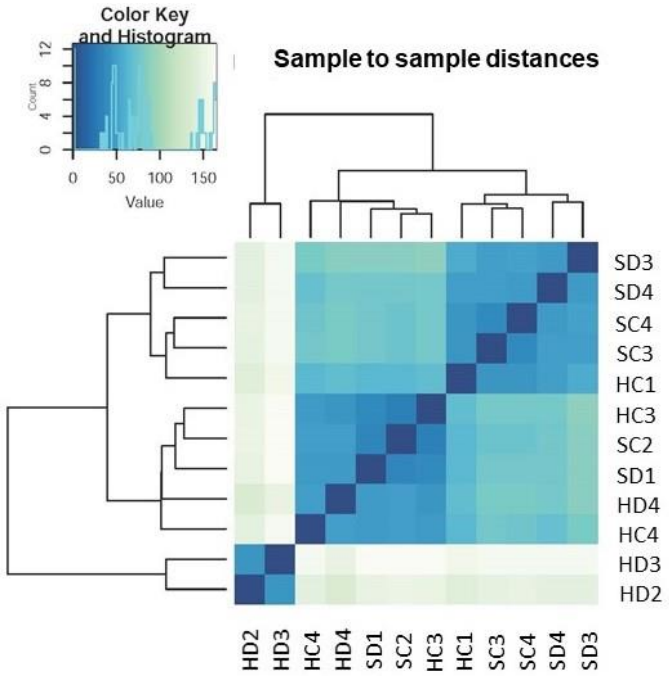

Figure 3. Histogram from the RNA-seq data showing the sample to sample distance in i-hpHSP90C plants.

In contrast, one of the Dex-treated i-hpHSP90C plants (HD4) clustered together with the control samples (Figure 3). The higher normalized HSP90C transcript counts in HD4 samples indicated inadequate HSP90C silencing in this sample. This suggests that the transcriptomic changes are observed only after a significant HSP90C downregulation. To test the hypothesis, the expression levels of HSP90C and representative nuclear genes that show clear up- and downregulation found in the RNA-seq analysis was compared by qRT-PCR. As a representative of upregulated genes the systemic acquired resistance deficient 1 (SARD1) and, to

represent downregulated genes, a light-harvesting chlorophyll a/b-binding protein (LHC a/b), was selected. The gene expression levels of SARD1 and LHC a/b correlated with the HSP90C expression negatively and positively, respectively. The SR1 plant did not respond to Dex treatment in the expression of the genes examined. These results support the hypothesis above; therefore, the HD4 sample was omitted from the differential expression analysis.

The gene expression of Dex-treated H-4 samples (HD2 and HD3 samples) were compared using DESeq2 with all three groups, untreated line H-4, Dex-treated SR1, and untreated SR1. Differentially expressed genes (DEGs) were selected from the DESeq2 data based on the combined criteria of log2(FC) values below –1 or above 1 and adjusted p-values less than 0.05. Out of the differentially expressed mRNAs, 4896 upregulated mRNAs had annotation with 2746 different AGI codes comprising 61.4% of DEGs with AGI annotation common in three comparisons, and 6307 downregulated mRNAs had 3490 (59.0%) AGI codes. The AGI codes associated with the up- and downregulated mRNAs were used for the GO enrichment analysis. The enriched GO terms associated with the upregulated genes include innate immune response, response to wounding, response to oxidative stress and, response to phytohormones. In addition, biosynthetic genes of SA and JA were also upregulated. The results indicated that the reduced supply of HSP90C to chloroplast elicits defense response involving some phytohormone pathways. Moreover, genes involved in the response to ER stress were remarkably upregulated after the impaired supply of HSP90C. This observation could be attributed to the non-specific silencing of ER-localizing HSP90 family protein by the HSP90C hairpin RNA (hpRNA). Therefore, the expression levels of six HSP90 family proteins were examined and, the true target of hpRNA-mediated silencing, HSP90C or HSP90.5, was significantly downregulated. The expression levels of cytosolic HSP90.1 and HSP90.4, nucleo-cytosolic HSP90.2, and mitochondrial HSP90.6 were unaffected, while, ER-localized HSP90.7 were significantly upregulated in HD2 and HD3 samples. These findings indicate a potential interplay between the protein quality control system of ER with that in plastid. However, activation of SA or immune response could also contribute in the upregulation of the ER-localizing HSP90 and other ER protein quality control components.

The enriched GO terms under the downregulated genes include photosynthesis, pigment metabolic process, and plastid organization, which is in agreement with pivotal roles of HS90C in chloroplast biogenesis. Moreover, some primary metabolic process like carbohydrate metabolic process, lipid metabolic process, cellular amino acid metabolic process and, some cellular process genes annotated with cell cycle, cell wall organization, and, cellular homeostasis were also downregulated, which is steady with the enrichment of cell-death-related

genes.

The significant upregulation of the cell death pathway leads to the detection of cell death in i-hpHSP90C lines. Since lower (older) leaves displayed more clear chlorosis than the upper (younger) leaves the cell death in those leaves were examined separately. The trypan blue staining followed by electrolyte leakage assay were used for the detection of cell death in this transgenic line. Below a schematic model in Figure 4 illustrates the procedure which was followed for the determination of cell death.

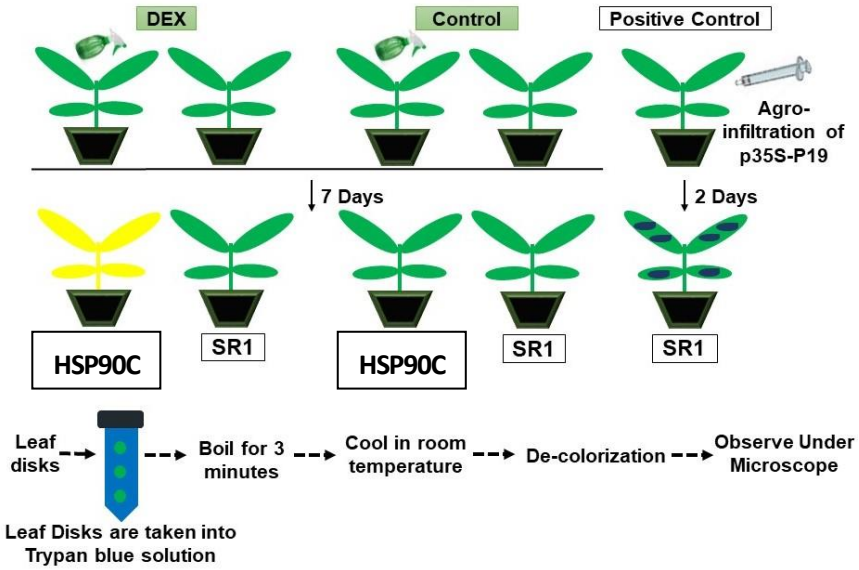

Figure 4. A schematic model is showing the method of cell death assay (For more details - Islam et al., 2020; https://www.mdpi.com/1422-0067/21/12/4202/htm).

From the trypan blue staining data more intense staining in older leaves of Dex-treated H-4 and H-6 plants were found compared the younger upper leaves, although to a lesser degree than the positive control. For the positive control plants, *tomato bushy stunt virus* (TMSV) P19 protein was transiently expressed for two days. The staining of lower leaves from Dex-treated H-4 and H-6 plants was uneven, suggesting the cell death induction in those leaves was sporadic within the leaf tissue. Electrolyte leakage assay confirmed the cell death in lower leaves but not in upper leaves. Microscopic observation of trypan-blue-stained leaf tissue indicated that dead cells in lower leaves of Dex-treated H-4 and H-6 plants were barely shrunken and, the upper leaves exhibited small patches of dead cells, suggesting the sporadic and age-dependent natures of cell death in Dex-treated H-4 and H-6 plants. A small fraction of cells in lower but not upper leaves of untreated H-6 showed blue staining, suggesting that the hpRNA to HSP90C had been expressed in a leaky fashion in a small number of cells in the untreated H-6 line and that the cell death observed is age-dependent. Dex-treated and untreated control plants (SR1) and untreated H-4 did not show any signs of cell death. These results collectively suggest that the

death of cells, in which HSP90C supply is impaired, is stochastically initiated and proceeds somewhat slowly, although the RNA-seq suggests that it is a plant-type hypersensitive response.

The upregulation of genes involved in the response to oxidative stress suggests the production of reactive oxygen species (ROS) in Dex-treated H-4 and H-6 plants. Because ROS is well studied as a signal mediator and an executor of plant cell death, 3,3'-Diaminobenzidine (DAB) staining were used to detect H_2O_2 as a representative of ROS. A schematic model in Figure 5 illustrates the DAB staining method.

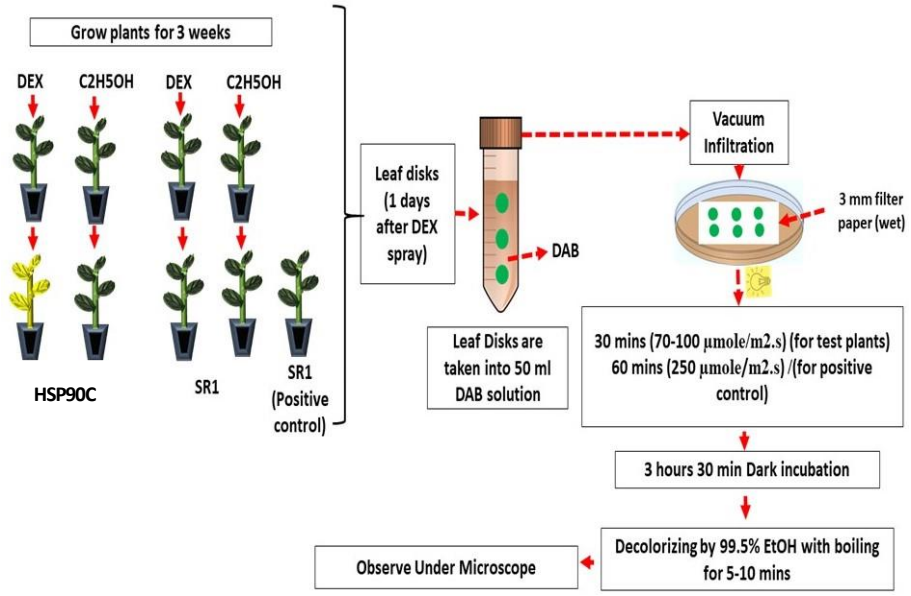

Figure 5. A schematic model is showing the method of DAB staining (For more details - Islam et al., 2020; https://www.mdpi.com/1422-0067/21/12/4202/htm).

Positive control with intense light resulted in the accumulation of brownish DAB precipitate in chloroplasts. Intense DAB staining of chloroplasts was barely visible in Dex-treated and untreated control plants (SR1) and in untreated H-4 and H-6 plants. Evident DAB staining of chloroplasts was observed in leaves of Dex-treated H-4 and H-6 plants at 1-dpt, but it was not observed when cell death was observed at 7-dpt (data not shown). The results suggest that, upon the loss of sufficient levels of HSP90C, chloroplasts produce ROS, triggering the cell death response. Although the overall DAB pigmentation was more intense in lower leaves, especially in H-4, pigmentation of each chloroplast was comparable between upper and lower leaves. The observation suggests that the extents of cell death correlate with the number of cells producing ROS but not with the magnitude of ROS production.

In the second part of my study, an RNA-seq approach was used to investigate the variation in gene expression early after the induction of CHLI silencing. Similar to i-hpHSP90C plants, three-week-old i-amiCHLI

transgenic line C-1 and non-transformed tobacco (SR1) were treated with a dexamethasone (Dex) solution or a control solution, and RNA was extracted at 24 h post-treatment. The RNA sequencing gave 23 M reads/sample on average from the 12 samples, about 85% of which were mapped to the tobacco reference transcriptome. The overall similarity within samples was evaluated by a histogram from DESeq2 analysis (Figure 6).

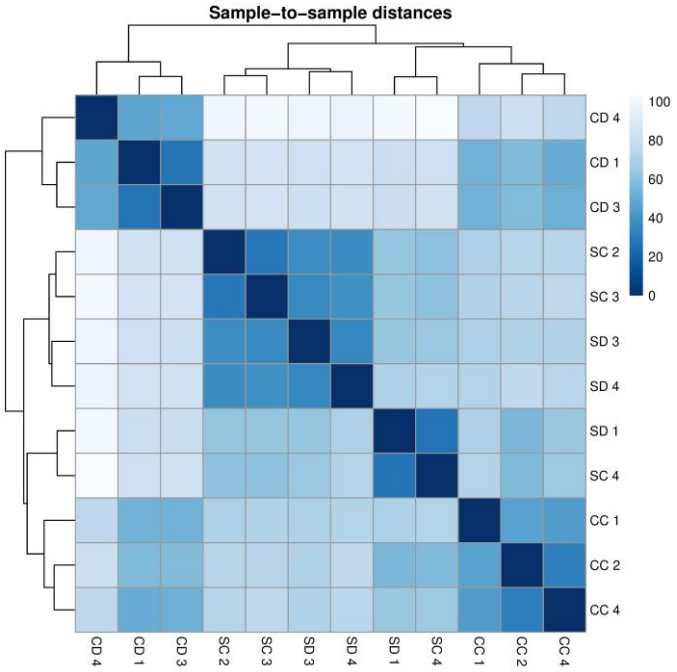

Figure 6. Histogram from the RNA-seq data showing the sample to sample distance in i-amiCHLI plants.

The result showed that all three Dex-treated C-1 samples showed clear differences from the control samples, including the non-transformed SR1 samples. However, control-treated C-1 plants have also shown an apparent difference from the non-transformed SR1 plants (Figure 6). This difference between non-transformant SR1 and control-treated i-amiCHLI plants could be due to a slight downregulation of the CHLI gene in the control-treated plants. To verify the reduced CHLI expression in the control-treated C-1 plants, the expression levels of CHLI and representative genes for up-/down-regulated DEGs, SARD1 and LHC a/b, respectively, were compared by qRT-PCR. Unlike the data from RNA-seq analysis, the control-treated C-1 plants showed comparable expression levels of CHLI, SARD1, and LHC a/b to SR1 plants, while Dex-treatment induced reproducible CHLI silencing accompanied by the up- and down-regulation of SARD1 and LHC a/b, respectively. The SR1 plants did not respond to Dex-treatment in the expression of the genes examined. These results indicate that the different transcriptome profiles of control-treated C-1 plants from those in SR1 plants cannot be attributed to the difference in the temporal CHLI expression levels.

The gene expression of Dex-treated i-amiCHLI plants (group CD; CD1, CD3 and CD4) was compared

in a pairwise manner using DESeq2 with all three groups, control-treated line C-1 (group CC; CC1, CC2 and CC4), Dex-treated SR1 (group SD; SD1, SD3 and SD4), and control-treated SR1 (group SC; SC2, SC3 and SC4), which have never shown chlorosis in our repeated experiments. DEGs were picked up from the DESeq2 data based on the combined criteria of log2(FC) values below -1 or above 1, and the adjusted p-values lesser than 0.05. The analyses of differentially expressed mRNA transcripts annotated with the Arabidopsis AGI codes in three different comparisons above, CD vs CC, CD vs SD, and CD vs SC, identified 1481, 2088, and 1425 up-regulated genes, respectively, and 1046, 2237, and 937 down-regulated genes, respectively. Because the control-treated C-1 plants have shown some difference in the overall expression pattern from both control SR1 plants (group SC and SD) and Dex-treated C-1 plants (group CD), it was considered that the use of commonly up- or down-regulated genes in 3 comparisons for gene ontology (GO) analysis was inadequate. It is possible that genes essential for chlorosis development would be up- or down-regulated in both CD and CC groups. Therefore, in GO enrichment analysis 1428 (45.9%) and 980 (31.8%) genes were used, which were commonly up- and down-regulated in at least two comparisons, respectively.

In addition to DESeq2, I have used the Short Time-series Expression Miner (STEM) program, for the analysis of four groups of RNA-seq data. Although STEM was designed to analyze RNA-seq data from time-course samples, it was considered that it would be useful for the analysis of RNA-seq data in the present study, because it can cluster genes based on the expression profiles. The relative normalized count data from four plant/treatment groups, SC, SD, CC, and CD, of 15,498 genes annotated with Arabidopsis AGI codes, were subjected to the analysis. The STEM clustering algorithm grouped all the genes into 50 different profiles, and among them, significantly enriched profiles (p-values lesser than 0.05) were highlighted with different color for each cluster of profiles. The 5 profiles for the up-regulated genes in Dex-treated C-1 plants (profile 18, 40, 42, 29 and, 30), contained total 3568 up-regulated DEGs. Profiles 27, 9, 11, 26, 34, 23 and, 31 which were selected for the downregulated genes, contained a total of 3582 downregulated DEGs. It is notable that 276 genes in the profile 27 were up-regulated in CC group but downregulated in CD as compared to SR1 plants (SC and SD). The STEM analysis facilitated the selection of more DEGs than those was found in the pairwise analyses, in which some important genes in chlorosis development could have been masked by the unexpectedly affected transcriptome of control-treated C-1 plants.

I performed GO enrichment analysis with the 1428 up- and 982 down-regulated DEGs from the pairwise analysis and 3635 up- and 3583 down-regulated DEGs from STEM analysis. GO terms enriched by DEGs detected by two different methods were compared, indicating that about half of the GO terms are common

to the both. The GO terms enriched by the up-regulated DEGs comprise innate immune response, defense response, response to wounding, response to hormones [salicylic acid (SA), jasmonic acid (JA), and abscisic acid (ABA)], and plant-type hypersensitive response were present. Moreover, SA biosynthetic genes were also found to be up-regulated. These results suggest that the reduction of CHLI levels in chloroplast induces defense response, including the activation of some phytohormone pathways.

The GO terms (biological process) enriched by the downregulated genes include photosynthesis, photoinhibition, pigment metabolic process, and plastid organization. Several GO terms that correspond to some primary metabolism genes were also downregulated. These GO terms include glycine metabolic process, carbohydrate metabolic process, lipid metabolic process, and vitamin metabolic process. Some cellular process genes annotated with the GO terms of cellular homeostasis, chaperone-mediated protein folding, and cellular component organization were downregulated, which is consistent with the upregulation of cell death-related genes. Also, genes involved in cellular response to chemical stress, response to oxidative stress, and response to heat were downregulated.

Interestingly, similar to i-hpHSP90C plants, the genes under the GO term of cell death, was significantly upregulated in i-amiCHLI plants. This finding encouraged us to detect cell death in i-amiCHLI lines. Trypan blue staining and electrolyte leakage assay was used to detect the cell death as shown in Figure 4. Micrographs of the Trypan blue-stained leaf tissue proved cell death in lower leaves of the Dex-treated C-1 and C-2 plants, albeit with the lesser extent and less shrunken morphology, compared to those in the positive control. These observations suggest that the cell death events in Dex-treated i-amiCHLI plants are sporadic and slower than that of hypersensitive response, although the genes annotated with GO term of plant-type hypersensitive response were upregulated therein. The upper leaves of Dex-treated C-1 plants showed cell death of a lesser extent and those of Dex-treated C-2 plants did not show any staining, which was similar to the cases in control-treated i-amiCHLI and non-transformant SR1 plants. These observations suggest that the cell death observed in this study is an age-dependent event. Electrolyte leakage assay supported the notion above: an age-dependent, sporadic, and slow cell death.

GO enrichment analysis in i-amiCHLI plants, failed to detect upregulation of genes related to ROS response but rather some of them were downregulated. However, a subset of ROS response-related genes were significantly up-regulated in one of the Dex-treated C-1 plants. Also, the other two Dex-treated C-1 plants were differentially clustered from control-treated C-1 plants and SR1 plants in the clustering analysis of ROS response-related gene expression of all the plants. These results prompted the detection of the H_2O_2 production as

a representative of ROS. In the positive control samples, the accumulation of brownish precipitate in chloroplasts was found by DAB staining. Intense DAB staining of chloroplasts was barely detected in Dex- and control-treated SR1 plants, and control-treated C-1 and C-2 plants. Clear DAB staining of chloroplasts was detected in leaves of Dex-treated C-1 and C-2 plants at 1-dpt, unlike the samples from 7-dpt when cell death was observed (data not shown). These results suggest that the silencing of CHLI induces ROS production in the chloroplasts.

To find out the common features in the chlorosis development in the two inducible-silencing system of i-hpHSP90C and i-amiCHLI, I compared the results from GO enrichment analyses with up- and down-regulated genes. I have found 105 and 179 common GO terms enriched in the analyses with up- and down-regulated genes, respectively, suggesting the involvement of common pathways in the chlorosis development in two chlorosis model systems. The results suggest that similar pathways including the defense responses accompanying cell death have roles in chlorosis development in two chlorosis models with different chlorosis triggers. To confirm the specific nature of those up- and down-regulated genes, I further analyzed them by clustering. The results showed that the CD4 sample exhibited similar expression patterns to HD2 and HD3, which are analyzed as the chlorosis-developing samples in i-hpHSP90C line. The findings of this comparative analysis support the idea that chlorosis development in two systems shares molecular mechanisms. In addition to those common GO terms, we also found the GO terms "ERAD (endoplasmic reticulum-associated degradation) pathways" and "Response to ER (endoplasmic reticulum) stress" were specifically up-regulated in the HSP90C system. To test the hypothesis that the silencing of CHLI leads downregulation of HSP90C, which drives transcriptome changes leading to chlorosis development, I examined the expression levels of six HSP90 family proteins in the RNA-seq data. In contrast to HD2 and HD3 samples, which showed downregulation of the silencing target HSP90C (HSP90.5) and upregulation of ER-localizing HSP90.7, CD group plants showed marginal decrease of HSP90C expression but no significant change in HSP90.7 expressions. Instead, CC and CD group plants showed reduced expression of cytoplasmic HSP90.1. However, it remains unknown whether or not such differences in HSP90 family proteins reflect the difference of molecular mechanisms underlying the chlorosis development. Further studies are necessary to elucidate the involvement of the ER-localizing and cytoplasmic HSP90 proteins in chlorosis development.

The summary of the molecular events discussed above that lead to the development of chlorosis after the induced silencing of the HSP90C and/or CHLI gene are shown in Figure 7.

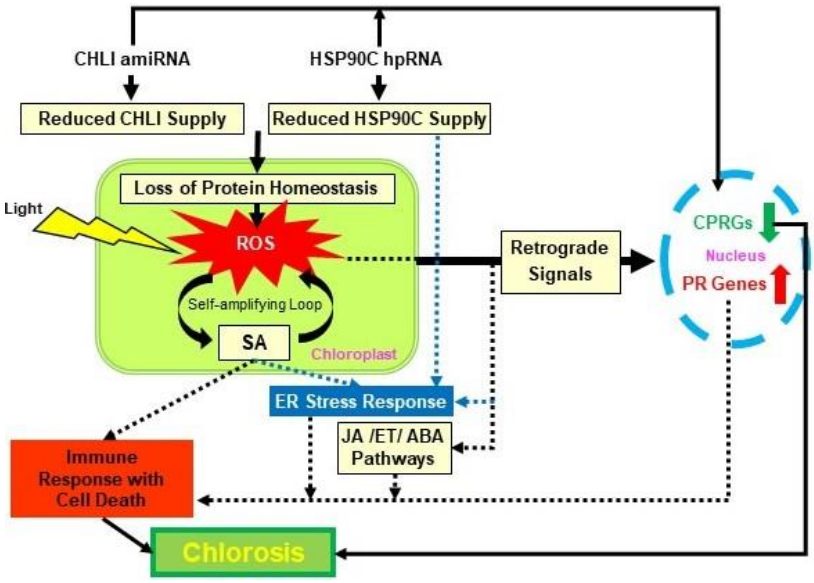

Figure 7. A schematic model for the molecular mechanism of chlorosis following the inducible silencing of the HSP90C and/or CHLI gene.

Inducible silencing of the HSP90C or CHLI gene would cause a reduction of HSP90C or CHLI levels in the chloroplast, which would cause the loss of protein homeostasis in the chloroplast. The disruption of protein homeostasis would lead chloroplasts to produce ROS in a light-dependent manner. The effects of ROS and SA would be enhanced by a self-amplifying loop. ROS would serve as the chloroplast retrograde signal and activate other retrograde signaling pathways, leading to the upregulation of pathogenesis-related (PR) genes and the downregulation of CPRGs. Reduced supply of HSP90C in chloroplast could induce the activation of the ER stress response, which was absent in the case of CHLI silencing. However, the upregulation of JA/ET/ABA pathway genes was common in both i-hpHSP90C and i-amiCHLI plants, likely through SA, ROS, or other signaling pathways. ROS, SA, the activation of PR genes, and other stress responses including JA/ET/ABA pathways could stimulate the cell death pathway. The CPRGs downregulation would be the primary cause of chlorosis, and ROS-mediated triggering of HR-like cell death in the chlorotic tissues could have a role in the development of chlorosis. Solid line arrows indicate the steps with experimental supports, whereas broken line arrows are hypothetical. Unique upregulation of the ER stress-responsive gene after HSP90C silencing was indicated with a blue color arrow.

(注)　要約の文量は，学位論文の文量の約１０分の１として下さい。図表や写真を含めても構いません。
(Note)　The Summary should be about 10％ of the entire dissertation and may include illustrations