Tetrapyrrole Metabolism Is Involved in Lesion Formation, Cell Death, in the Arabidopsis lesion initiation 1 Mutant

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The Arabidopsis lesion initiation 1 (len1) mutant develops lesions on leaves without pathogen attack. The len1 plants display lesion formation as they grow under short-day conditions (SD), but not under long-day conditions (LD). This study was conducted to examine how lesion formation, viz., cell death, in len1 plants occurs under SD. I present genetic and physiological data to show that tetrapyrrole metabolism is necessary for lesion formation in len1 plants. Lesion formation was suppressed in the len1lin2 double mutant under SD. lesion initiation 2 (lin2) is another lesion mimic mutant with a defect in tetrapyrrole biosynthesis. Suppression of lesion formation in len1 plants was also observed when they were crossed with the mutants that had defects in other steps in tetrapyrrole metabolism. Suppression was correlated with reduced chlorophyll (Chl) levels in the double mutants. Furthermore, I found that dark-to-light transition caused a bleached phenotype in len1 plants, as in the case of antisense ACD1 (acd, accelerated cell death) plants. ACD1 encodes pheophorbide a oxygenase (PaO), which is involved in Chl catabolism in Arabidopsis. These results suggest that tetrapyrrole metabolism, especially Chl breakdown, might be involved in lesion formation in len1 plants.

Key words: Arabidopsis; lesion; chlorophyll; tetrapyrrole

The biosynthetic pathway of chlorophylls (Chls) (Fig. 1A) begins with the condensation of eight molecules of 5-aminolevulinic acid (ALA) to uroporphyrinogen III, the first cyclic tetrapyrrole. Uroporphyrinogen III is converted to coproporphyrinogen III and then to protoporphyrin IX (Proto), which is the branch-point intermediate to Chls and hemes. Chelation of magnesium into Proto results in the formation of Mg-protoporphyrin IX (MgProto), which is converted to MgP monomethyl ester by a methyl transferase.1,2) Angiosperms include two chlorophyll species, Chl a and Chl b. Chl b is converted to Chl a, and only Chl a enters the Chl catabolic pathway.2,3) First the phytol chain of Chl a is removed from its porphyrin ring to produce chlorophyllide a by chlorophyllase. Secondly, the Mg2+ of chlorophyllide is removed by Mg-dechelatase to generate a green pigment, pheophorbide a. Thirdly, the macrocyclic ring of pheophorbide a is cleaved by pheophorbide a oxygenase (PaO) to generate a red pigment, the red chlorophyll catabolite (RCC). RCC is further catabolized to primary fluorescent Chl catabolites (pFCCs), FCCs, and nonfluorescent Chl catabolites (NCCs) (Fig. 1B).2,3)

Porphyrin compounds, the precursors and breakdown products of both Chls and hemes, are extremely phototoxic; thus, their synthesis and degradation are highly compartmentalized and regulated.2) Chls can absorb light and donate active electrons. In the absence of productive outlets for these active electrons, they can be donated to other compounds, including molecular oxygen, forming free radicals that can be toxic and can act as cellular signals. Accumulation of porphyrin compounds, caused by inactivation of enzymes in the porphyrin metabolic pathway, can cause cell death in animals and plants.4) For example, the Les22 mutant of maize accumulates uroporphyrin III and forms light dependent cell death lesions on its leaves.5) It has also been suggested that prophyrin compound is the cause of lesion initiation in the lin2 mutant.6) LIN2 encodes coproporphyrinogen III oxidase, a key enzyme in the biosynthetic pathway of Chls and hemes, a tetrapyrrole pathway, in Arabidopsis.6) The phenotypes of porphyrin accumulation in plants appear to be similar to the induction of defenses in pathogen resistance, including cell death and defense gene transcription through reactive oxygen species (ROS) production. This is termed a lesion mimic phenotype.7) Lesion mimic mutants have been isolated in many species, including the accelerated cell death (acd) and lesions simulating disease (lsd) series of Arabidopsis mutants.7) acd1 and acd2 have been found to develop a light-dependent lesion mimic phenotype and to be deficient in PaO and
red chlorophyll catabolite reductase (RCCR) respectively.\textsuperscript{8–11} It has been suggested that the phenotypes of the mutants are caused by an accumulation of phototoxic pheophorbide $a$ for $acd1$ and RCC for $acd2$ plants.\textsuperscript{8–11} In some lesion mimic mutants, the initiation or propagation of lesions is regulated by light conditions. For example, $lsd1$ and $lsd3$ mutants are suppressed under short-day conditions (SD), but $lsd2$ and, to a lesser extent, $lsd5$ are suppressed under long-day conditions (LD).\textsuperscript{12} The effects of light indicate that some lesion mimic phenotypes might require light energy, light signaling, or active chloroplast metabolism.

Porphyrin metabolism is also involved in plastid-to-nucleus signaling. Five mutants have been identified (genome uncoupled: $gun1$–$5$) that express nuclear-encoded photosynthetic genes in the absence of proper chloroplast development.\textsuperscript{13} To date, four of the $GUN$ loci, $GUN2$–$5$, have been cloned.\textsuperscript{14} The $gun2$ and $gun3$ mutants are alleles of $long$ hypocotyl1 ($hy1$) and $hy2$ respectively.\textsuperscript{14} $HY1/GUN2$ encodes heme oxygenase, and $HY2/GUN3$ encodes phytochromobilin synthase.\textsuperscript{15,16} These enzymes are required for the synthesis of phytochromobilin, the chromophore of phytochrome. The $GUN5$ gene encodes the H-subunit of Mg-chelatase (ChlH).\textsuperscript{17} Mg-chelatase catalyses the first reaction in the Chl branch of tetrapyrrole biosynthesis, inserting Mg$^{2+}$ into the protoporphyrin ring. Mg-chelatase is composed of three subunits, which are referred to as ChlH, ChlD, and ChlI. $CS$ encodes ChlI.\textsuperscript{17,18} Even though $cs$ plants have less Mg-chelatase activity than $gun5$ plants, $cs$ plants do not exhibit a $gun$ phenotype.\textsuperscript{17} $GUN4$ has recently been cloned, and encodes a novel chloroplast protein that binds both Proto and MgProto, and activates Mg-chelatase \textit{in vitro}.\textsuperscript{19} Thus, the $GUN2$–$5$ proteins are essential for normal tetrapyrrole metabolism.

A previous report from my laboratory indicated that the leaves of $len1$ plants developed cell death associated with lesion formation as they grew under SD, but that cell death was suppressed under LD.\textsuperscript{20} As $LEN1$ encodes a chloroplast chaperonin 60 (Cpn60$\beta$), loss of function of chloroplasts is probably the cause of lesion formation, \textit{viz.}, cell death, in $len1$ plants.\textsuperscript{20} It remains to be determined, however, why lesion formation in $len1$ plants is suppressed under LD. A genetic analysis has been conducted to identify the mechanisms of lesion formation, cell death, in $len1$ plants under SD.

**Materials and Methods**

\textit{Plant materials.} \textit{Arabidopsis thaliana} (ecotype Co-
ly.6,20) The genotypes of enhancer/promoter trap lines, as described previously, were calculated using the equations of Moran,21) and normalized for each line.

**Results and Discussion**

**Chlorophyll measurements.** Chls were extracted from approximately 0.05 g of leaves with N,N-dimethylformamide at 12 h at 4 °C in complete darkness. The extract was subjected to spectrophotometric measurements at 603, 647, and 664 nm. Specific chlorophyll content was calculated using the equations of Moran,21) and normalized to the total fresh weight for each sample.

**Determination of plant size.** The diameters of plants grown under SD were measured. Five plants were measured for each line.

**Light-dark-light transitions of plants.** Plants were grown under continuous illumination for 3 weeks, incubated under darkness for 8 d, transferred again into light conditions, and maintained for 7 d under constant illumination.

**Results and Discussion**

**Depletion of salicylic acid does not affect lesion formation in len1 plants**

As observed with several lesion mimic mutants, the gene encoding pathogenesis-related (PR)-1 protein was expressed in the leaves of len1 plants under SD.12) To assess the epistatic relationship between salicylic acid (SA) accumulation and PR-1 expression or lesion formation, transgenic Arabidopsis, which was unable to accumulate SA due to expression of the salicylate hydroxylase (nahG) gene, was used in a cross with len1 plants. Lesioned F2 progeny were evaluated for expression of nahG and PR-1 by RNA blot analysis. Expression of PR-1 was substantially reduced in nahG expressing len1 plants (len1nahG) (data not shown), but the phenotype of len1nahG plants was the same as that of len1 single mutants (data not shown). These results suggest the lesion formation in len1 plants is determined prior to or independently of accumulation of SA, but that accumulation of SA is required for expression of the PR-1 gene.

**LIN2 is required for the len1 mutant phenotype**

Another lesion mimic mutant, lin2, isolated in my laboratory, develops cell death under LD, but only very slightly under SD.6) During complementation analysis with len1 and lin2 plants, it was proved that lesion formation in len1lin2 double mutants was suppressed under SD (Fig. 2A, B). This suggests that LIN2 is required for lesion formation in len1 plants under SD. LIN2 encodes coproporphyrinogen III oxidase, a key enzyme in the biosynthetic pathway of Chls and hemes, a tetrapyrrole pathway, in Arabidopsis.5) lin2 has also been found to be one of the gun mutants.19) Communication between the chloroplast and the nucleus was disrupted in the lin2 mutant. Consequently, lin2 might cause suppression of lesions in len1 plants under SD by either of two mechanisms. In the first mechanism, a LIN2-dependent functional tetrapyrrole pathway is required for lesion formation in len1 plants. In the second, the LIN2-dependent plastid-to-nucleus signal transduction pathway is involved in lesion formation in len1 plants. To explore these two possible mechanisms further, a genetic approach was used. Double mutants of len1 with hy1/gun2, hy2/gun3, and cs, which had defects in the ability to function in the tetrapyrrole pathway (Fig. 1), were generated and characterized. These double mutants were grown under SD, and the lesion phenotypes were examined (Fig. 2C). hy1, hy2, and cs plants did not show any lesion formation under these conditions (Fig. 2C). len1hy2-101, -103, -104, and -105 plants showed lesion formation on their leaves, as len1 plants did (Fig. 2C and data not shown), whereas len1cs and len1hy2-100 plants did not (Fig. 2C). This result suggests that CS and HY1/GUN2, but not HY2/GUN3, might be required for lesion formation in len1 plants under SD. Since cs is not a gun mutant, it appears that, instead of a LIN2-dependent plastid-to-nucleus signal transduction pathway, a LIN2-dependent functional tetrapyrrole pathway is required for lesion formation in len1 plants.

**Growth inhibition of len1 plants**

During growth, len1 plants exhibit two major visible stress reactions, lesion formation, viz., cell death, and growth inhibition. As seen in Fig. 2A, len1 plants are much smaller than wild-type plants.20) To examine whether the LIN2-dependent tetrapyrrole pathway affects growth, I measured the plant sizes of the double mutants described above (Fig. 3). The size of len1hy2-101 plants was much smaller than that of hy2-101 plants. len1cs and len1hy2-100 almost equaled cs and hy2-100
in size. Thus the plants, which had initiated lesion formation, showed growth inhibition, and the extent of inhibition appeared to be correlated with that of lesion formation. This response resembles a stress tolerance strategy used by plants that are exposed to drought, heat, light, or cold stress. Under these adverse environmental conditions, plants may pass into a state of minimal metabolic activity that persists until the stress is relieved. In fact, the growth inhibition of len1 plants was reversible. After the plants were returned to LD, they resumed normal growth (data not shown). Thus it appears likely that plants that show cell death in the leaves are exposed to heavy stress and their growth is inhibited.

Relationship between Chl levels and lesion formation in double mutants

len1cs and len1hy1-100 plants, which did not initiate lesion formation, showed a pale green phenotype (Fig. 2C). Hence, Chl (Chl a and Chl b) levels in various mutants were analyzed to determine the relationship between Chl levels and lesion formation (Fig. 4). cs and hy1-100 plants contained Chls at lower levels than did wild-type plants. hy2-101 plants contained Chls at slightly lower levels than did wild-type plants. The Chl content in len1 plants was somewhat greater than that in wild-type plants. The contents in len1cs and len1hy1-100 plants, which formed no lesions, were less than half those in the len1 plants. len1hy2-101 plants, which formed lesions, contained Chls at higher levels than did other double mutants without lesions (Fig. 4). len1hy2-103, -104, and -105 plants, which also showed lesion formation, contained Chls at the same levels as len1hy2-101 plants (data not shown). Thus

**Fig. 2.** Phenotypic Analysis of Double Mutants.
A. Wild-type (wt), len1, lin2, and len1lin2 plants were grown under SD for 8 weeks. B. Detached leaves of these plants from (A). C, hy2-101, len1hy2-101, cs, len1cs, hy1-100, and len1hy1-100 plants were grown under SD for 8 weeks. The photo shows the detached leaves of the plants.

**Fig. 3.** Plant Sizes of Double Mutants.
Wild-Type (wt), cs, hy1-100, hy2-101, len1, len1cs, len1hy1-100, and len1hy2-101 plants were grown under SD for 8 weeks and their sizes were measured. Average diameter of wt and mutant plants with standard deviations. n = 5.

**Fig. 5.** Effects of Light–Dark–Light Transitions on Wild-Type (wt) and len1 Plants.
Plants were grown under continuous illumination for 3 weeks, incubated under darkness for 8 d, transferred again into light conditions, and maintained for 7 d under continuous illumination. The photo shows the detached leaves of the plants.
higher Chl levels might be required for lesion formation in len1 plants under SD.

**Dark-to-light transition caused a bleached phenotype in len1 plants**

As mentioned above, len1 plants display lesion formation as they grow under SD, but lesion formation is suppressed under LD. A question arises as to why deletion of a Cpn60β gene leads to cell death in len1 plants under SD but not under LD. The quality of light is equal as between SD and LD. The difference between the two is the quantity, the length, of the light/dark time. The dark period under SD (15 h) is longer than that under LD (8 h). It is well-known that degradation of Chl is induced when plants are kept under darkness. The breakdown products of Chls are extremely phototoxic. The products can absorb light and donate active electrons. In the absence of productive outlets for these active electrons, they can be donated to other compounds, including molecular oxygen, forming free radicals that can be toxic and can act as cellular signals. Tanaka et al. produced transgenic Arabidopsis plants in which antisense RNA for ACD1 was constitutively expressed. When the antisense ACD1 (AsACD1) plants were transferred to darkness, their leaves were waved and appeared to be dehydrated. And when the plants were returned to illumination, the leaves of AsACD1 turned white and dried after 24 h to 72 h of illumination. This phenotype resembles that of an acd2 mutant that has a defect in the RCCR. Light-dependent bleaching of leaves has commonly been observed in mutants that exhibit defective tetrapyrole metabolism. Thus the phenotype of AsACD1 suggests that the plants accumulated porphyrin compounds due to a defect in Chl breakdown during dark treatment. In fact, the AsACD1 plants accumulated pheophorbide a during dark incubation. These results suggest that accumulation of Chl breakdown products, caused by inactivation of enzymes in the Chl catabolic pathway, can cause cell death after dark-to-light transition through its photo-sensitizing properties in Arabidopsis.

As shown in Fig. 4, len1hy2-101 plants, which showed lesion formation, contained Chls at higher levels than did other double mutants without lesion formation. This suggests that the Chl flow of lesion forming plants, which can enter the Chl catabolic pathway, is higher than that of other plants without lesions under SD. Thus, if the chlorophyll breakdown process is perturbed in len1 plants, as in the case of AsACD1 plants, greater Chl flow leads to greater accumulation of porphyrin compounds, which can lead to cell death after dark-to-light transition through its photo-sensitizing properties. Hence, I hypothesized that the lesion formation induced in len1 plants under SD is due to perturbation of Chl catabolism, as in AsACD1 and acd2 plants. To test this hypothesis, experiments with light-dark-light transitions were conducted (Fig. 5). Wild-type and len1 plants were grown under continuous illumination for 3 weeks. No lesion formation was observed in either (data not shown). Then I kept the plants under darkness for 8 d. Again, no differences were observed in the phenotype between the two plant lines (data not shown). Finally, the plants were illuminated again for 7 d. Only the len1 plants showed a bleached phenotype on their leaves (Fig. 5). These results suggest that Chl breakdown is induced in both wild-type and len1 plants in a similar way in darkness, but that the Chl breakdown process is perturbed in len1 plants as in AsACD1 plants. Since LEN1 encodes a chloroplast Cpn60β, some protein(s) involved in Chl breakdown,
such as ACD1 and ACD2, might not work well in len1 chloroplasts. Thus the Chl breakdown products might be accumulated in len1 plants during the dark period. The Chl breakdown products absorb light and donate active electrons, which leads to the production of ROS. The ROS might be a cause of lesion formation, cell death, in len1 plants. In fact, len1 plants accumulated H$_2$O$_2$ in the region developing cell death under SD.\(^{20}\) Taken together, the answer to the above question appears to lie in the photosensitive nature of Chl breakdown products, which might accumulate in len1 plants under SD (long nights), but not under LD (short nights).

In contrast to AsACD1 plants, the leaves of len1 plants did not dry after illumination (Fig. 5). This suggests that the defect in Chl breakdown in len1 plants is not as strong as that in AsACD1 plants. Future studies, including metabolic profiling and proteome analysis of len1 chloroplasts, are required to identify the molecular mechanisms involved in cell death in len1 plants.

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### References


