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4. After synthesis of mitochondrially encoded proteins in the presence of [^{35}S]methionine for 20 min at 30°C, mitochondria were reisolated and washed twice with 1 ml of cold SHKCl buffer [0.6 M sorbitol, 80 mM KCl, 50 mM Hepes-KOH (pH 7.2)] to remove nonincorporated [^{35}S]methionine. Mitochondria were then carefully resuspended in translation buffer at a concentration of 1.5 mg/ml. For proteolysis, samples of 30 or 100 μl (for gel filtration analysis) were further incubated at 37°C. At the time points indicated, samples were immediately centrifuged at 4°C for 4 min at 13,200*g*. Radioactivity recovered in the supernatant was determined after the addition of scintillation fluid (Ultima Gold Canberra Packard) (1 ml) and vigorous vortexing. Mitochondrial pellets were resuspended in SHKCl buffer (30 μl) and precipitated with TCA [12.5% (w/v)] for 15 min at −80°C. After centrifugation for 15 min at 36,000*g*, TCA-soluble material was recovered and radioactivity determined as above. TCA-insoluble material was resuspended in 30 μl of LiDS sample buffer and processed for counting as above. To monitor the integrity of mitochondrial membranes, we routinely analyzed duplicate samples before TCA precipitation by SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblotting with antisera directed against the matrix protein Mge1 and the intermembrane space protein cytochrome b_2.
5. Recovered supernatants (100 μl; ~700,000 cpm) were subjected to gel filtration on a Sephadex Peptide column (7.5/300; Pharmacia) that was equilibrated with 40% (v/v) acetonitrile in 0.1% (v/v) TFA. Fractionation was carried out at room temperature at a flow rate of 0.3 ml/min. Fractions (0.3 ml) were collected and counted after the addition of scintillation fluid (1 ml). More than 95% of the radioactivity loaded was recovered from the column in each case. Rat gastrin (2126 daltons), substance P (1348 daltons), penta-glycine (303 daltons), methionine (149 daltons), and diglycine (132 daltons) were used for calibration.
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15. For generation of Mdl1- and Mdl2-specific antisera, the synthetic peptides CDEEGKGGVIDLD (corresponding to amino acid residues 677 to 688 of Mdl1) and CDDIEKSVEHLLKD (corresponding the amino acid residues 794 to 806 of Mdl2) were coupled with maleimide-activated carrier protein (Imject, Pierce) to keyhole limpet hemocyanin or ovalbumin, respectively, and used for generation of antibodies in rabbits.
16. Mitochondrial membranes (0.5 mg) were solubilized in 0.2% (v/v) Triton X-100, 20 mM tris/HCl (pH 7.5), 80 mM KCl, 4 mM Mg-acetate, and 1 mM ATP at a concentration of 5 mg/ml and fractionated by a Superose 12 gel filtration column equilibrated with the same buffer. Eluate fractions were analyzed by SDS-PAGE and immunoblotting with Mdl1- and Mdl2-specific antisera. Apoferritin (11.75 ml; 443 kD), alcohol dehydrogenase (13.5 ml; 150 kD), and carboanhydrase (16.25 ml; 29 kD) were used for calibration.
17. *MDL1* and *MDL2* were deleted in W303 wild-type cells by polymerase chain reaction–targeted homologous recombination with the heterologous auxotrophic markers *HIS3MX6* and *KanMX4* (*41*). *Δmdl1Δmdl2* and *Δyme1Δmdl1* strains were obtained by mating the corresponding single-mutant strains, sporulation, and isolation of haploid progenies.
18. Statistical significance was determined with the Student's unpaired *t* test, assuming unequal variances. In each case, mitochondria isolated from mutant strains were directly compared with the wild type.
19. For overexpression, Mdl1 was subcloned into the centromeric plasmid pYX113 (Novagen) and expressed from a galactose-inducible promoter in *Δmdl1* cells. Western blot analysis revealed a ~20-fold overexpression of Mdl1 in *Δmdl1* cells relative to wild-type cells.
20. Site-directed mutagenesis was carried out with appropriate oligonucleotides and the Quick-Change-Kit (Stratagene). Wild-type and mutant variants of Mdl1 were expressed in *Δmdl1* cells from a CEN-based plasmid under the control of the *ADH1* promoter.
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27 October 2000; accepted 6 February 2001

Arabidopsis NPL1: A Phototropin Homolog Controlling the Chloroplast High-Light Avoidance Response

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Chloroplasts relocate their positions in a cell in response to the intensity of incident light, moving to the side wall of the cell to avoid strong light, but gathering at the front face under weak light to maximize light interception. Here, *Arabidopsis thaliana* mutants defective in the avoidance response were isolated, and the mutated gene was identified as *NPL1* (NPH-like 1), a homolog of *NPH1* (nonphototropic hypocotyl 1), a blue light receptor used in phototropism. Hence, NPL1 is likely a blue light receptor regulating the avoidance response under strong light.

Plants need light not only for photosynthesis but also for precise regulation of their development. To compete successfully and effectively under varying environmental conditions, plants sense the surrounding light conditions, including wavelength, intensity, direction, duration, and in some instances even the plane of polarization. This information is used to regulate their development or prepare for forthcoming seasonal changes. Phytochromes and cryptochromes are the photoreceptors thought to mediate the multitude of responses involved (*1*).

To maximize photosynthesis, plants orient their growth and leaf angle to maximize light interception (a process known as phototropism), open their stomata in the presence of light to facilitate gas exchange, and relocate their chloroplasts within the cell. Plants use blue light in these responses, both to sense light direction and light intensity. However, a blue light receptor for increasing the

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efficiency of photosynthesis through the above three phenomena is known only for phototropism, where phototropin (NPH1) functions under weak light conditions (*2, 3*). Phototropin mediates phototropism of hypocotyls and roots, but to date has not been shown to mediate any other blue light responses. Although light-induced chloroplast movement is a phenomena well known since the 19th century (*4*), a blue light receptor that regulates this movement has not been identified. Phytochrome is known to be a photoreceptor for red light–induced chloroplast movement in the green alga *Mougeotia scalaris* (*5*) and the fern *Adiantum capillus-veneris* (*6*), but these are rare cases.

To identify the blue light receptor that regulates chloroplast movement in *A. thaliana*, a simple method for detecting chloroplast avoidance movements was developed (Fig. 1A) (*7*), and used to screen about 100,000 ethylmethane sulfonate (EMS)–mutagenized F_2 seeds and 15,000 T-DNA–tagged lines. More than 10 mutants, named *cav* (defective in chloroplast avoidance movements) were obtained. Four of the mutants, *cav1-1* to *cav1-4*, were found to belong to a single complementation group. All mutants were single, nuclear, and recessive.

Chromosome mapping of the *CAV1* gene placed it between the ILL2 and PLC1a markers at 113 centimorgans on chromosome V (Fig. 2A) (*8*). This region includes the recently described *NPL1* gene, which encodes a protein similar in sequence to phototropin (*9*), a blue light receptor for phototropic responses (*2, 3*). Thus, the NPL1 protein is a strong candidate to be encoded by *CAV1*. After screening a library of T-DNA–tagged lines, we obtained one line with an insertion in *NPL1* (*10*), which lacked the avoidance movement of chloroplasts. We designated this mutant *cav1-5*, because it was allelic to the previously isolated *cav1* mutants. The mutation sites of all the *cav1* mutants were located within the *NPL1* gene (Fig. 2B), confirming that *CAV1* and *NPL1* are the same. In *cav1-2*, the conserved threonine residue at 727 in subdomain VIII for substrate recognition of serine/threonine kinases region (*11*) was mutated to isoleucine, indicating that a signal for the chloroplast avoidance movement might be generated by phosphorylation. Accumulation of *NPL1* mRNA was not detected in *cav1-5* by reverse transcription polymerase chain reaction (RT-PCR), suggesting that *cav1-5* is a null mutant of *NPL1*.

The movement of an individual chloroplast in a mesophyll cell at the top layer of the palisade tissue was also examined, using a recording system to monitor chloroplast movement every 1 min under a microbeam irradiator (*12*). A part of the cell surface was irradiated with a small beam of weak or strong blue light (20 μm in diameter) (Fig. 1, B and C) (*13*). Chloroplasts in *cav1* mutant

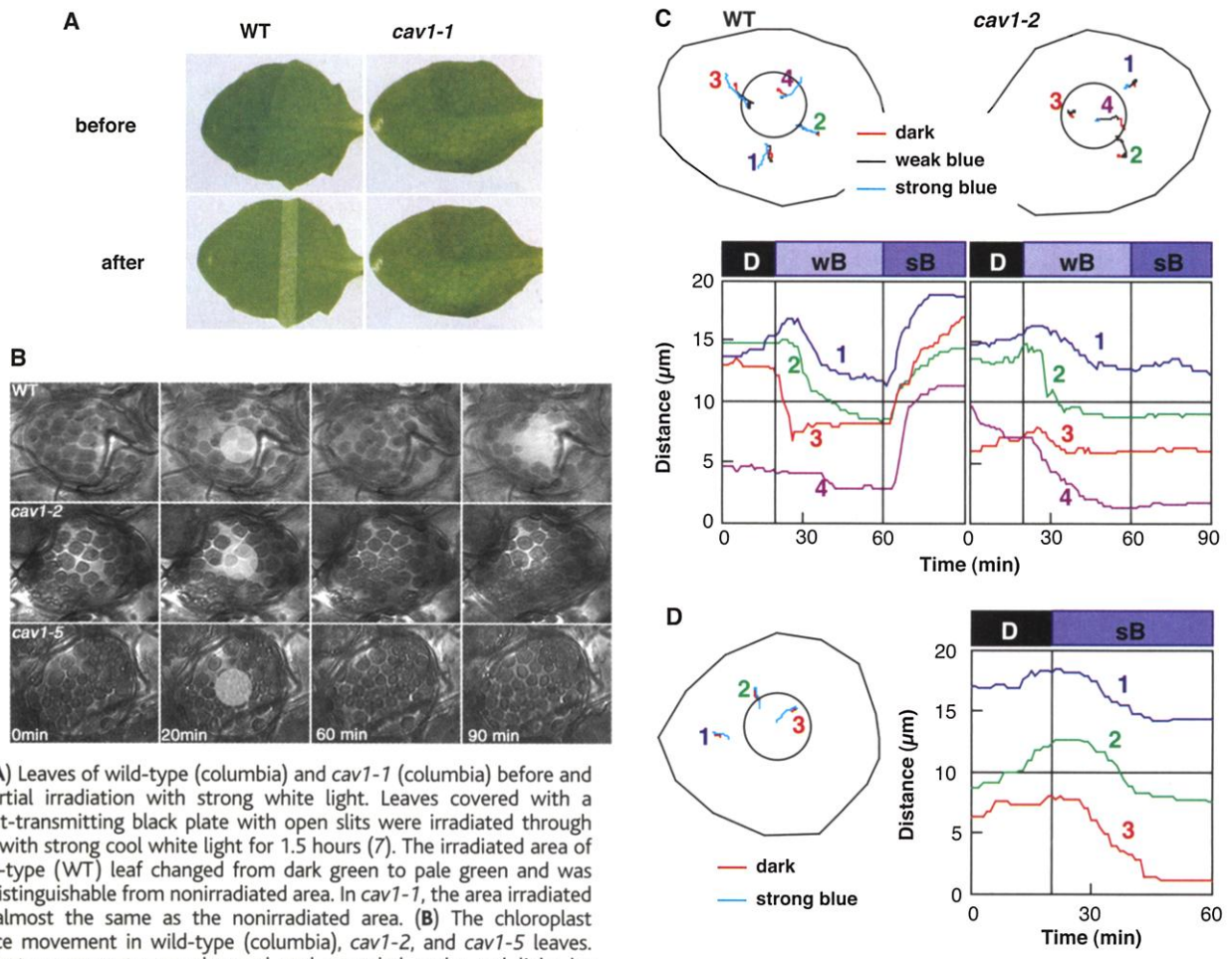

Fig. 1. (**A**) Leaves of wild-type (columbia) and *cav1-1* (columbia) before and after partial irradiation with strong white light. Leaves covered with a non–light-transmitting black plate with open slits were irradiated through the slits with strong cool white light for 1.5 hours (*7*). The irradiated area of the wild-type (WT) leaf changed from dark green to pale green and was clearly distinguishable from nonirradiated area. In *cav1-1*, the area irradiated looked almost the same as the nonirradiated area. (**B**) The chloroplast avoidance movement in wild-type (columbia), *cav1-2*, and *cav1-5* leaves. Chloroplast movement was observed and recorded under red light by methods described in (*12*). The cells were partially irradiated with a microbeam (20 μm in diameter shown as "white holes" in the photographs taken at 20 min) of weak blue light (2 μmol m^{-2} s^{-1}) from 20 to 60 min and with strong blue light (40 μmol m^{-2} s^{-1}) at the same position from 60 min to the end of experiments. (**C**) The movement of each chloroplast was traced during the experiment (upper panel) and the distance between the beam center and each chloroplast recorded (lower panel). These data were obtained using the same cells as shown in (B). Chloroplasts in both wild-type and *cav1-5* accumulated in the spot of weak blue light (wB: 2 μmol m^{-2} s^{-1}). When the beam was changed to strong blue light (sB: 40 μmol m^{-2} s^{-1}), chloroplasts in a wild-type cell moved away from the irradiated area but chloroplasts in the *cav1-5* did not. (**D**) A part of a cell of dark-adapted *cav1-1* was irradiated with a microbeam of strong blue light (40 μmol m^{-2} s^{-1}, 20 μm in diameter) from 20 to 60 min. Chloroplasts showed accumulation movement without stopping at the edge of the microbeam and entered into the irradiated area. Other conditions are the same as in (C).

cells moved toward the area irradiated with weak blue light (2 μmol m^{-2} s^{-1}) as did chloroplasts in wild-type cells. Unexpectedly, however, chloroplasts in these mutant cells also accumulated in the area irradiated with strong blue light (more than 40 μmol m^{-2} s^{-1}) (Fig. 1D); chloroplasts in a wild-type cell did not enter into the area irradiated with strong light (*12*). These results show that a blue light receptor for the accumulation movement is present in the irradiated area of the mutant cells and that the strong blue light irradiation itself can generate a signal for accumulation response, at least when the avoidance response is inactivated. In a wild-type cell, a signal for the accumulation response might also be generated under strong blue light. However, because no chloroplast was found to enter into the microbeam-irradiated area, the signal for avoidance is probably stronger than that for accumulation within the microbeam-irradiated area.

The *cav1* plants are defective in chloroplast avoidance movement but, to date, are normal in other blue light–induced physiological responses examined, such as phototropism, the chloroplast accumulation movement, inhibition of hypocotyl elongation, and the timing of flowering (*14*). The level of *NPL1* mRNA accumulated in wild-type plants was higher in leaves than in stems and flowers, and was very low in roots (Fig. 3A). These results are perhaps not surprising given that chloroplast movement occurs mostly in leaves. When etiolated seedlings of wild-type plants were irradiated with red, blue (10 μmol m^{-2} s^{-1}, respectively), or white light (30 μmol m^{-2} s^{-1}) for 4 hours, the level of accumulation of *NPL1* mRNA increased compared to a dark control (Fig. 3B). The blue light on the level of *NPL1* mRNA was tested in more detail by varying the fluence rate of blue light. It was found that the accumulation level more than doubled at the higher fluences (Fig. 3C) (*15*). Similarly, the mRNA level of the *OsNPH1b* gene, a *NPL1* homolog in rice, increased in etiolated leaves after light irradiation (*16*).

The NPL1 protein and phototropin are highly similar in their amino acid sequences (Fig. 2C) (*9*). The NPL1 protein has two highly conserved LOV (light, oxygen, and voltage) domains, to which FMN (flavin mononucleotide), a chromophore for blue light perception, was found to bind in phototropin from oat and phytochrome 3 from the fern *A. capillus-veneris* (*17*). Because NPL1 LOV domains are known to absorb blue light (*18*), NPL1 is likely to be the fourth blue light receptor found in *A. thaliana*. Physiological experiments using polarized blue light suggest that the photoreceptors for the avoidance response in *A. thaliana* are localized on or close to the plasma membrane with their dipole moment arranged parallel to the membrane (*12*), as is the case with lower plants (*6*). Together with the fact that phototropin is located on the plasma membrane (*19*), NPL1 may also function at the plasma membrane as a blue light photoreceptor.

The action spectra for chloroplast photorelocation movements under weak and strong light conditions are very similar, both having peaks at 450 and 480 nm in the water plant *Lemna trisulca* and the moss *Funaria hygrometrica* (*20*). It has been suggested that photoreceptors for both weak and strong light responses are the same. Because the accumulation movement occurs in the *cav1* mutants under strong blue light and because NPL1 protein could be the photoreceptor for the avoidance movement, there must exist a different blue light receptor for the accumulation movement. We obtained more than 10 *npl1* mutants for the avoidance movement but did not find any candidates for photoreceptor mutants for the accumulation response. A mutant defective in the photoreceptor for the accumulation movement might be lethal or the photoreceptors may be genetically redundant and necessary for this response under weak light conditions.

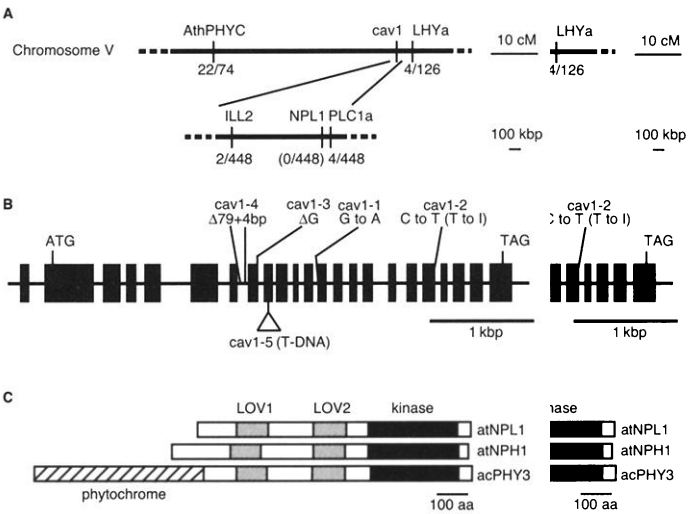

Fig. 2. *cav1* cloning and ture. (**A**) *cav1* map on chr The *cav1-1* mutation wa to be located between AthPHYC and LHYa by cl fied polymorphic seque (*8*), and then between ILL where the *NPL1* gene i combination rates for when crossed between lumbia) and WT (La shown underneath and *NPL1* appearance is als parenthesis. (**B**) Struct *NPL1* gene and positic mutants. The locatior (AGT) and stop (TAG) indicated. Twenty-three es) and 22 intron (line were determined by co genomic sequence with sequence. Mutations or *cav1-1*, *cav1-2*, *cav1-3*, *cav1-4*, and *cav1-5* are as follows: base 3121 from the start codon, the last nucleotide in intron 11 was changed from G to A in *cav1-1*; base 4425 was changed from C to G, changing threonine (T) to isoleucine (I) in *cav1-2*; a deletion of G at base 2408 caused a frame shift in *cav1-3*; and deletion of 79 base pairs (bp) from 2192-2270 and addition of 4 bp in *cav1-4*; and insertion of T-DNA into exon 9 in *cav1-5*. (**C**) Structural features of some plant proteins that have two LOV domains. Shown are *A. thaliana* NPL1 (atNPL1) (*9*), phototropin (atNPH1) (*2*), and *Adiantum capillus-veneris* phytochrome 3 (acPHY3) (*21*). LOV, kinase, and phytochrome domains are shown as meshed, black, and slashed boxes, respectively.

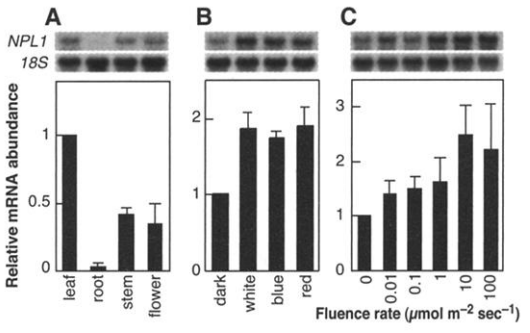

Fig. 3. *NPL1* gene expression in *Arabidopsis* wild-type (Landsberg *erecta*). RNA gel blot hybridization was performed with the ^{32}P-radiolabeled *NPL1* cDNA and 18*S* ribosomal DNA as probes against 5 μg of total RNA. The graphs show the relative abundance of *NPL1* mRNA measured by quantitative RT-PCR. The value was calculated relative to that of the leaf tissue sample in (A) or the sample mock irradiated for 4 hours in (B and C) versus the 18*S* ribosomal RNA signal. (**A**) *NPL1* gene expression in different tissues of adult plants grown on soil under continuous white light at 21°C for 4 to 5 weeks. Tissues were at an early flowering stage. (**B**) *NPL1* gene expression in 4-day-old etiolated seedlings after irradiation for 4 hours under various light conditions. (**C**) *NPL1* gene expression in 4-day-old etiolated seedlings after 4 hours exposure to blue light at the various intensity as described (*22*).

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7. Leaves of 2-week-old plants mutagenized by EMS (Lehle seeds, TX) or T-DNA insertion (Arabidopsis Biological Resource Center, OH) in *A. thaliana* were removed and placed adaxial side up on the surface of

an agar plate solidified with 1% agar. These leaves were covered by a black plate with open slits approximately 1 mm in width arranged in parallel and irradiated through these slits with strong cool white light (~30 W m^{-2}) for 1.5 hours using a metal halide lamp. The area of wild-type leaves irradiated through the slit became pale green, while the covered area remained dark green (Fig. 1A). The light-induced change in the green color is a consequence of the movement of chloroplasts to the side walls of cells. Thus, the color change will not be observed in mutant leaves lacking the avoidance response. Mutants *cav1-1* to *cav1-3* from EMS-treated seeds and *cav1-4* from T-DNA–tagged lines were screened by this method.

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13. Monochromatic blue light was obtained through an interference filter (Vacuum Optics of Japan, Tokyo) that has a transmission peak at 452.4 nm and a half band-width of 23.3 nm.
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15. Preparation of the seedlings and RNA gel blotting for the analysis of the *NPL1* gene expression was done as described (*22*). For the analysis using various light qualities, white light was provided by a white fluorescent lamp (model FL20SSW/18; National, Tokyo) at the fluence rate of 30 μmol m^{-2} s^{-1}. Blue and red light were provided by a light-emitting diode (LED), blue light lamps, and red light lamps (LED-mB; LED-mR; Eyela, Tokyo) with a maximum wavelength of 470 nm (30 nm half band-width) and of 660 nm (20 nm half band-width) at the fluence rate of 10 μmol m^{-2} s^{-1}, respectively. Quantitative RT-PCR was done by using ABI PRISM 7700 sequence detection system (PE Applied Biosystems, Foster City, CA) with TaqMan EZ RT-PCR Kit (PE Applied Biosystems). RNA samples (100 ng and 1 ng) treated by deoxyribonuclease I were used for the quantitative RT-PCR of *NPL1* mRNA and 18*S* rRNA, respectively. Nucleotide sequences of PCR primers for the *NPL1* gene were 5′-GAGACGCAATTAGAGATCAGAGG-3′ and 5′-CCGATGAAGTATTGAAGCTCTC-3′. Sequence of the probe to quantify the RT-PCR product was a 5′-CCACTTGCAACCTATGCGTGATCAGAAG-3′. This probe had a FAM (6-carbosyfluorescein) as a 5′-reporter dye and a TAMRA (6-carbosy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine) as a 3′-quencher dye. RT-PCR primer and the probe for 18*S* rRNA were used of TaqMan Ribosomal RNA Control Reagents (PE Applied Biosystems).
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23. We are grateful to W. R. Briggs and J. M. Christie of Carnegie Institution of Washington (Stanford, CA) and J. A. Banks of Purdue University (West Lafayette, IN) for critical reading of the manuscript. This work was partly supported by PRESTO, Japan Science and Technology Corporation, Japan, to T.K. and by grants from PROBRAIN (Program for Promotion of Basic Research Activities for Innovative Biosciences), Mitsubishi Foundation, Grant-in-Aid for International Scientific Research (Joint Research, no. 10044214) and for Scientific Research (B, no. 09440270) from the Ministry of Education, Sports, Science and Technology (MEXT) of Japan to M.W. Also supported by a grant from the program Grants-in-Aid for Scientific Research on Priority Areas (no. 10182101); a fund from the Joint Studies Program for Advanced Studies from MEXT, the Human Frontier Science Program, the Mitsubishi Foundation to K. Okada; and by funds from the Japan Society for the Promotion of Science to T.S. as a research fellow.

29 September 2000; accepted 18 January 2001

Defensive Function of Herbivore-Induced Plant Volatile Emissions in Nature

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Herbivore attack is known to increase the emission of volatiles, which attract predators to herbivore-damaged plants in the laboratory and agricultural systems. We quantified volatile emissions from *Nicotiana attenuata* plants growing in natural populations during attack by three species of leaf-feeding herbivores and mimicked the release of five commonly emitted volatiles individually. Three compounds (*cis*-3-hexen-1-ol, linalool, and *cis*-α-bergamotene) increased egg predation rates by a generalist predator; linalool and the complete blend decreased lepidopteran oviposition rates. As a consequence, a plant could reduce the number of herbivores by more than 90% by releasing volatiles. These results confirm that indirect defenses can operate in nature.

Plants defend themselves against herbivores with chemical and physical defenses that directly influence herbivore performance and indirectly through traits that attract the natural enemies of herbivores (*1*–*3*). One such indirect defense, the release of volatile organic compounds (VOCs) specifically after herbivory, is known to attract parasitoids and predators to actively feeding larvae in the laboratory (*4*, *5*), and evidence from agricultural systems suggests a role for herbivore-induced VOCs in increasing predation pressure (*6*–*8*). However, conclusive evidence has been lacking, and it is not even known whether plants growing in natural populations increase VOC emissions after herbivore attack. VOCs might be able to function as indirect defenses only in simplified agroecosystems, in which a single natural enemy species of a herbivore can act as an important biocontrol agent on an agricultural plant (*9*). In contrast, in natural systems, herbivore mortality is more commonly mediated by a suite of generalist enemies (*10*). Moreover, both the qualitative and quantitative characteristics of herbivore-induced plumes of VOCs are known to vary among plant genotypes (*11*–*13*); the genetic variation commonly found in natural populations may undermine the reliability of VOCs as a signal for natural enemies because prior exposure is often needed to associate plant VOCs with the occurrence of a feeding herbivore (*5*, *14*, *15*). Herbivore-induced plant VOCs may also influence herbivore host-location behavior, potentially increasing herbivore attack on plants releasing VOCs (*1*, *2*).

To evaluate the role of herbivore-induced VOCs in nature, we characterized the VOCs released from *Nicotiana attenuata* Torr. ex Wats (Solanaceae) plants growing in a native population (*16*–*18*) in the Great Basin desert of southwest Utah, which were under continuous attack by three numerically dominant folivores: the caterpillars of *Manduca quinquemaculata* (Lepidoptera, Sphingidae), the leaf bug *Dicyphus minimus* (Heteroptera, Miridae), and the flea beetle *Epitrix hirtipennis* (Coleoptera, Chrysomelidae) (*19*). We used an open-flow trapping design (*13*) to collect VOCs individually from 32 plants growing in a natural population that each had one leaf attacked by one of the three herbivore species or remained undamaged (*13*, *20*) (Fig. 1, A and B). All plants were growing in a 150-m^2 portion of the population (*18*) and were sampled simultaneously for 7 hours. Gas chromatography–mass spectrometry (GC-MS) analysis (*21*) of the trapped VOCs revealed that all three herbivore species elicited increases in the same suite of VOCs, although the odor profiles were not identical (Fig. 1B). The pattern and amount of herbivore-induced VOCs trapped from *N. attenuata* growing in the field were very similar to those found in laboratory studies with plants attacked by *Manduca sexta* larvae (*13*).

The emitted VOCs common to all three herbivores are derived from three biosynthetic pathways. Green leaf volatiles (*cis*-3-hexene-1-ol, *cis*-3-hexenyl acetate, and *cis*-3-hexenyl butyrate; 1, 3, and 6, respectively, in Fig. 1) derived from the octadecanoid path-

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