hypokalemic periodic paralysis, we have found a recombinant between this disorder and the sodium channel gene (*22*), suggesting that separate loci may exist.

The ultimate proof of a defect in the sodium channel gene in HYPP will require definition of the molecular lesion. This is the next step toward understanding of the structure and function of the sodium channel, establishment of molecular diagnostic procedures in HYPP, and realization of an animal model of the disorder.

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16. PCR primers were constructed with GC clamps and restriction enzyme sites according to the general design previously described (T. S. Khurana, E. P. Hoffman, L. M. Kunkel, *J. Biol. Chem.*, in press). The primer sequences are as follows: Na2 forward, 5′-ggggGGATCCggagatgaacaacctacagatt-3′; Na2 reverse, 5′-gggAAGCTTcatgaagacaatgaaggtctc-3′; Na3 forward, 5′-ggggGGATCCgatgtgctatgagacctgggag-3′; Na3 reverse, 5′-gggAAGCTTcacctcctgcttcctctt-3′. Single-stranded cDNA was produced by reverse transcription of poly(dT)-primed total RNA from human skeletal muscle with the use of avian reverse transcriptase. Complementary DNA (50 ng) was mixed with 1 μg of each primer, and amplified during 30 cycles of the following conditions: 94°C 1 min for denaturing, 40°C 2 min for annealing, and 72°C 3 min for elongation. The PCR product of the size expected from the rat sequence was excised from a 1.0% low-melting point agarose gel, purified, digested with Bam HI and Hind III, and subcloned into a similarly digested plasmid vector (Bluescript). Sequence analysis of the plasmid inserts was done by dideoxy sequencing with T7 and T3 primers (Sequenase protocol, U.S. Biochemicals).
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Salicylic Acid: A Likely Endogenous Signal in the Resistance Response of Tobacco to Viral Infection

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Some cultivars of tobacco are resistant to tobacco mosaic virus (TMV) and synthesize pathogenesis-related (PR) proteins upon infection. In a search for the signal or signals that induce resistance or PR genes, it was found that the endogenous salicylic acid levels in resistant, but not susceptible, cultivars increased at least 20-fold in infected leaves and 5-fold in uninfected leaves after TMV inoculation. Induction of PR1 genes paralleled the rise in salicylic acid levels. Since earlier work has demonstrated that treatment with exogenous salicylic acid induces PR genes and resistance, these findings suggest that salicylic acid functions as the natural transduction signal.

LIVING ORGANISMS HAVE EVOLVED A complex array of biochemical pathways that enable them to recognize and respond to signals from the environment. These pathways include receptor organs, hormones, second messengers, and enzymatic modifications. At present, little is known about the signal transduction pathways that are activated during a plant's response to attack by a pathogen, although this knowledge is central to our understanding of disease susceptibility and resistance. A common form of plant resistance is the restriction of pathogen proliferation to a small zone surrounding the site of infection. In many cases, this restriction is accompanied by localized death (necrosis) of host tissues. Together, pathogen restriction and local tissue necrosis characterize the hypersensitive response (HR). In addition to local defense responses, many plants respond to infection by activating defenses in uninfected parts of the plant. As a result, the entire plant is more resistant to a secondary infection. This systemic acquired resistance (SAR) can persist for several weeks or more (*1*) and often confers cross-resistance to unrelated pathogens (*2*).

The interaction between tobacco (*Nicotiana tabacum* Linn.) and TMV has been used extensively as a model for the study of plant disease and resistance. In general, infection of tobacco leaves with TMV results in one of two distinct responses. TMV-infected tobacco cultivars that have the dominant N gene are resistant and display both HR and SAR (*1*). In contrast, tobacco cultivars that lack the N gene (nn genotype) are susceptible to TMV. The virus replicates and spreads rapidly throughout these plants, causing stunting and the appearance of mosaic patterns of chlorosis on the youngest leaves.

Resistant (NN genotype), but not susceptible (nn), cultivars produce several new proteins in response to TMV infection, including five distinct families of proteins referred to as pathogenesis-related (PR) proteins (PR1 through PR5) (*3, 4*). PR proteins are found in many plants, and different subsets can be induced by various viral, viroid, fungal, and bacterial pathogens and by certain environmental and chemical stresses (*5*). The defense-related enzymatic activities of some of these PR proteins (*6, 7*) and the correlation between PR gene expression and resistance suggest that these

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proteins are involved in SAR and HR (*8*). However, the recent demonstration that transgenic tobacco plants constitutively expressing a single introduced PR gene did not exhibit enhanced resistance implies that multiple factors may be necessary for resistance (*9, 10*).

Application of exogenous salicylic acid or its derivative acetyl salicylic acid is particularly effective in inducing PR genes in many plants, including tobacco (*11, 12*). Application of salicylic acid also results in increased resistance of the treated areas to TMV and to some other viruses (*13*) and fungi (*14*).

To determine whether endogenous salicylic acid is involved in activation of PR genes, the closely related tobacco cultivars Xanthi (nn) and Xanthi nc (NN) [in which the N gene has been crossed to Xanthi (nn) from *Nicotiana glutinosa*] were mock- or TMV-inoculated, and the endogenous levels of salicylic acid and PR1 gene expression were monitored. Salicylic acid did not exceed the basal levels (~0.01 μg per gram of fresh weight) in mock-inoculated Xanthi nc (NN) (Fig. 1A) or in TMV- or mock-inoculated Xanthi (nn) tobacco, which is susceptible to this virus. In Xanthi nc (NN), little, if any, salicylic acid was detected in TMV-infected leaves at 0, 6, 12, or 18 hours after inoculation. Salicylic acid levels began to rise at 24 to 36 hours and increased 20-fold or more over basal levels by 42 to 48 hours. Salicylic acid levels remained high for the remainder of the experiment (Fig. 1A).

Induction of PR1 gene expression paralleled increased salicylic acid levels. Little PR1 mRNA was seen in mock- or TMV-inoculated Xanthi (nn) plants or in the mock-inoculated leaves of resistant Xanthi nc (NN) tobacco (*8*). In contrast, in TMV-infected leaves of the resistant Xanthi nc (NN) plants, PR1 mRNA levels were detectable at 24 to 36 hours and were maintained at high levels throughout the remainder of the experiment (Fig. 1B). Thus, not only does treatment with exogenous salicylic acid induce PR1 gene expression (Fig. 1C), but also the endogenous levels of salicylic acid and expression of PR1 genes rise in parallel in TMV-resistant plants undergoing the HR.

In resistant plants, there is tissue damage associated with lesion formation during the HR. However, no endogenous salicylic acid was detected when leaves of Xanthi nc (NN) plants were severely abraded, sliced with a razor blade, or injured with Dry Ice chips. These observations indicate that the salicylic acid induction that follows TMV infection is not a generalized response to wounding or tissue death.

If salicylic acid is involved in the activation of systemic as well as local plant defenses, the amount of endogenous salicylic acid in uninfected leaves of TMV-inoculated Xanthi nc (NN) tobacco should increase in parallel or before induction of PR gene expression and SAR. Indeed, salicylic acid concentrations in these leaves rose above basal levels by 48 hours, plateaued at 72 hours at five- to tenfold over basal levels, and remained relatively constant through at least 7 days after inoculation (Fig. 2A). The systemic increase in salicylic acid was followed by the appearance of PR1 mRNAs, which were first detected at 72 hours and increased in quantity thereafter (Fig. 2B). The 24-hour delay in PR1 gene expression relative to the rise in salicylic acid levels, observed in only the uninfected leaves of inoculated plants, might be due to the fact that the salicylic acid content in these leaves is lower than that in the infected leaves. The small amount of salicylic acid might also explain the relatively low steady-state quan-

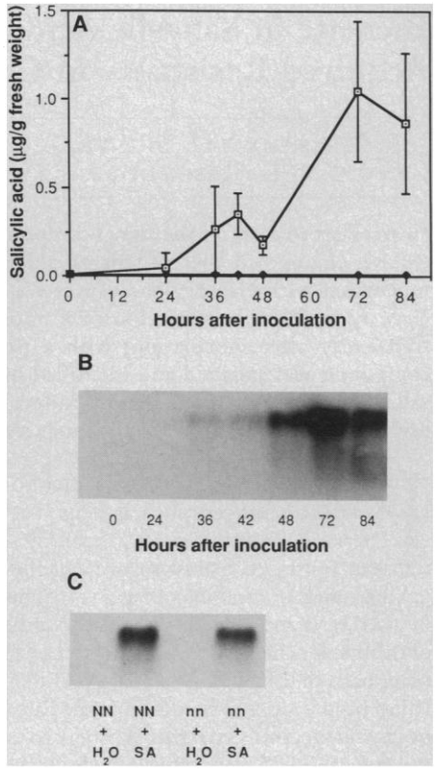

Fig. 1. Effect of TMV inoculation on the endogenous salicylic acid and PR1 mRNA in the infected leaves of Xanthi nc (NN) tobacco. (**A**) Salicylic acid levels in TMV-infected (n = 3) (⊡) and in mock-infected (n = 1) (◆) leaves. The detection limit of the assay was 0.01 μg per gram of fresh weight, and extracts in which no salicylic acid was detected were assigned this value (*20*). Two leaves of 8-week-old plants grown under a 16-hour photoperiod were inoculated with solution of TMV-U1 strain (2.8 μg/ml) in 5 mM phosphate buffer, pH 7.2. Necrotic lesions were first apparent at 42 hours. Mock-inoculated plants were inoculated with buffer alone. Leaves were harvested at the times shown, cut longitudinally, and frozen in liquid nitrogen. Half of each leaf was used for RNA analysis, and the opposite half leaf was assayed for salicylic acid content. For each time point, tissue samples from one mock-inoculated plant and three TMV-inoculated plants were individually harvested and analyzed, and the averages ± SD were plotted. All of the data shown in (A) and (B) are taken from one experiment. The experiment was repeated four times with similar results (*21*). (**B**) Northern blot analysis of steady-state levels of PR1 mRNA from TMV-infected leaves of Xanthi nc (NN) plants. Equal amounts of total RNA from all plants harvested at the given time point were pooled for the Northern blot shown here; however, RNAs from each plant were also analyzed separately to determine plant-to-plant variations. In the experiment shown, one plant exhibited increased salicylic acid levels at 24 hours and the same plant also had a detectable level of PR1 mRNA. (**C**) Induction of PR1 genes in Xanthi nc (NN) and Xanthi (nn) by exogenous salicylic acid (SA). One-centimeter leaf disks from 8- to 10-week-old plants were floated on either H_2O or 500 μM salicylic acid, pH 6.5, for 24 hours, and RNA was extracted as described (*22*).

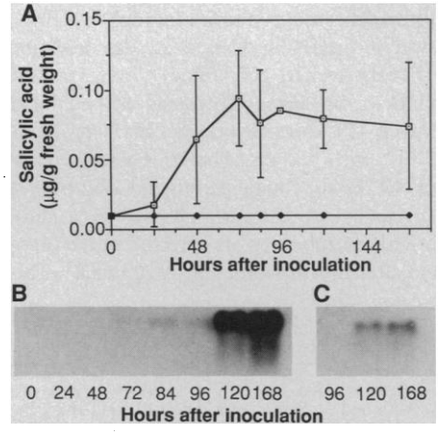

Fig. 2. Effect of TMV inoculation on the endogenous salicylic acid and PR1 mRNA in the uninfected leaves of Xanthi nc (NN) tobacco. For details, see legend to Fig. 1. Note that salicylic acid levels and time scales are different from those in Fig. 1. Upper, uninfected leaves of mock- or TMV-inoculated plants were harvested and analyzed for salicylic acid and PR1 mRNA content. The experiment was repeated three times with similar results, but all data shown are from the same experiment. (**A**) Salicylic acid levels in uninfected leaves of TMV-inoculated (n = 3) (⊡) and mock-inoculated (n = 1) (◆) plants. In TMV-inoculated Xanthi nc (NN) plants, salicylic acid was generally not detected in uninfected leaves between 0 and 24 hours, although occasionally low levels, between 0.01 and 0.03 μg per gram of fresh weight, were observed. Values are averages ± SD. (**B**) Northern blot analysis of steady-state levels of PR1 mRNA from uninfected leaves of TMV-inoculated Xanthi nc (NN) plants. (**C**) Autoradiogram of the same blot as in (B), exposed for the same time as that in Fig. 1B. Figures 1B, 2B, and 2C display lanes from the same gel, hybridized together with the same probe, and therefore can be compared.

tities of PR1 mRNAs in uninfected leaves as compared to infected leaves (Figs. 1B and 2C). No PR1 gene expression or salicylic acid increase was seen in uninfected leaves of TMV-inoculated Xanthi (nn) plants or in mock-inoculated plants of either genotype (Fig. 2A).

In 1983 Van Loon (*15*) postulated that salicylic acid acts by mimicking an endogenous phenolic signal that triggers PR gene expression and disease resistance. Our results suggest that the signal is salicylic acid itself. Susceptible Xanthi (nn) plants carry the PR genes, and these genes can be activated by treatment with exogenous salicylic acid (Fig. 1C) but not with TMV. Therefore, it is likely that infection of susceptible plants fails to trigger the signal transduction pathway that leads to salicylic acid production, resistance, and PR gene expression.

Métraux *et al.* (*16*) present independent evidence suggesting that salicylic acid plays a role in the induction of SAR in cucumber after pathogen attack. In addition, Raskin and co-workers recently demonstrated that salicylic acid is an endogenous regulator of heat and odor production in the inflorescences of some thermogenic lilies (*17–19*). These three studies suggest that salicylic acid plays a broad and important role in signal transduction in plants.

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20. Using a more sensitive assay, we have since found that basal levels range from <0.005 to 0.02 μg per gram of fresh weight, as indicated in the text.
21. Salicylic acid was extracted from 1 g, fresh weight, of leaf tissue and analyzed by high-performance liquid chromatography and spectrofluorescence as described previously (*18*). The presence of salicylic acid was confirmed by gas chromatography–mass spectroscopy. Salicylic acid recovery was 55%. The data shown were not corrected for this factor. Total leaf RNA was prepared as previously described [J. O. Berry, B. J. Nikolau, J. P. Carr, D. F. Klessig, *Mol. Cell. Biol.* **5**, 2238 (1985)] and 20 μg of each preparation was analyzed by Northern (RNA) blot and hybridization to ^{32}P-labeled PR1 cloned cDNA [J. R. Cutt, D. C. Dixon, J. P. Carr, D. F. Klessig, *Nucleic Acids Res.* **16**, 9861 (1988)].
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Increase in Salicylic Acid at the Onset of Systemic Acquired Resistance in Cucumber

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In an effort to identify the signal compound that mediates systemic acquired resistance (SAR), changes in the content of phloem sap were monitored in cucumber plants inoculated with either tobacco necrosis virus or the fungal pathogen *Colletotrichum lagenarium*. The concentration of a fluorescent metabolite was observed to increase transiently after inoculation, with a peak reached before SAR was detected. The compound was purified and identified by gas chromatography–mass spectrometry as salicylic acid, a known exogenous inducer of resistance. The data suggest that salicylic acid could function as the endogenous signal in the transmission of SAR in cucumber.

PLANTS INOCULATED WITH NECROtrophic pathogens such as fungi, bacteria, or viruses react by inducing a transient resistance against subsequent fungal, bacterial, or viral infections (*1–4*). This induced resistance can be restricted to areas of the first inoculation (*3*) but may spread to other parts of the plant to establish SAR (*1*). It has been proposed that SAR is mediated by an endogenous signal that is produced in the infected leaf and translocated in the phloem to other plant parts where it activates resistance mechanisms (*1, 2, 5*).

Cucurbitaceae have the unique property of releasing phloem sap from cut stem or petiole surfaces (*6*). We used a high-performance liquid chromatograph (HPLC) system, originally devised for the detection of hydroxylated polyamines (*7*), to analyze phloem sap after inoculation of cucumber leaves (*Cucumis sativus* L. cv. Marketer SMR 580) with either tobacco necrosis virus (TNV) or the fungal pathogen *Colletotrichum lagenarium*. A distinct increase of a fluorescing metabolite was detected in the phloem after inoculation (Fig. 1, A and B). This increase appeared before necrotization had taken place on the infected leaf and preceded the induction of resistance observed in the upper uninfected leaves, independent of the pathogen used to induce resistance (Fig. 1, C and D).

The timing of the increase was different depending on the pathogen used for the primary infection. Both resistance and the fluorescing peak appeared sooner after infection when TNV was used as an inducer. TNV produces necrosis 3 to 4 days after inoculation, whereas *C. lagenarium* produces necrosis 5 to 6 days after inoculation. The appearance of the fluorescent peak is therefore likely to be dependent on the timing of disease development inherent in the nature of the inoculated pathogen. The metabolite was also observed to increase when both necrosis on the first leaves and SAR were well established (7 to 9 days after inoculation).

Further analysis of the metabolite indicated that it consisted of several ultraviolet-absorbing components (Fig. 2). Only one of these was fluorescent and separated at the position of the initial metabolite. A preparation of this fluorescent fraction was derivatized to convert the polar compounds to their volatile trimethylsilyl derivatives (*8*), and this was used for analysis by capillary gas chromatography–mass spectrometry (GC-MS). The chemical ionization mass spectra and chromatographic retention data showed that the trimethylsilyl ester of

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