This paper was presented at a colloquium entitled “Self-Defense by Plants: Induction and Signalling Pathways,” organized by Clarence A. Ryan, Christopher J. Lamb, André T. Jagendorf, and Pappachan E. Kolattukudy, held September 15–17, 1994, by the National Academy of Sciences, in Irvine, CA.

Involvement of small GTP-binding proteins in defense signal-transduction pathways of higher plants

(cytokinins/DNA methylation/hypersensitive reaction/*rgp1*/wounding)

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ABSTRACT Small GTP-binding proteins play a critical role in the regulation of a range of cellular processes—including growth, differentiation, and intracellular transportation. Previously, we isolated a gene, *rgp1*, encoding a small GTP-binding protein, by differential screening of a rice cDNA library with probe DNAs from rice tissues treated with or without 5-azacytidine, a powerful inhibitor of DNA methylation. To determine the physiological role of *rgp1*, the coding region was introduced into tobacco plants. Transformants, with *rgp1* in either sense or antisense orientations, showed distinct phenotypic changes with reduced apical dominance, dwarfism, and abnormal flower development. These abnormal phenotypes appeared to be associated with the higher levels of endogenous cytokinins that were 6-fold those of wild-type plants. In addition, the transgenic plants produced salicylic acid and salicylic acid- β -glucoside in an unusual response to wounding, thus conferring increased resistance to tobacco mosaic virus infection. In normal plants, the wound- and pathogen-induced signal-transduction pathways are considered to function independently. However, the wound induction of salicylic acid in the transgenic plants suggests that expression of *rgp1* somehow interfered with the normal signaling pathways and resulted in cross-signaling between these distinct transduction systems. The results imply that the defense signal-transduction system consists of a complicated and finely tuned network of several regulatory factors, including cytokinins, salicylic acid, and small GTP-binding proteins.

As individual plants are unable to change their location, they have evolved numerous mechanisms that allow them to quickly respond to various environmental signals, including light, nutrients, and stresses—such as drought, pathogens, and wounding—and to adapt their behavior to the changing situation. To achieve the appropriate response, the external stimulus must be rapidly transformed to an internal signal that then triggers a whole series of biochemical and molecular pathways. This general concept postulates that external signals are transduced into chemical compounds and transmitted through a cascade of various proteins up to, for example, the DNA level. Among the numerous proteins that participate in this pathway, GTP-binding proteins play critical roles as signal transducers (1–5).

GTP-binding proteins, or G proteins, are equipped to bind GTP, which, because of the intrinsic GTPase activity of the proteins, can be hydrolyzed to GDP (3). This GTP/GDP inter-conversion is considered to enable the molecule to function as a “molecular switch,” in which the GTP-bound

form is active, and the GDP-bound form is inactive (4) and which can thus regulate the fundamental signaling pathways of organisms (5). Four major groups of G proteins have so far been distinguished: elongation factors, tubulins, membrane-bound trimeric G proteins, and small GTP-binding proteins. Among these, the latter two are known to be involved in signal transduction.

So far, >80 genes encoding small GTP-binding proteins have been documented from various organisms—including mammals, insects, yeasts, slime molds, and higher plants (6). The physiological functions of these proteins have not been fully clarified, but they have been classified into three major groups. The Ras proteins are involved in the transduction of external signals across the plasma membrane by interacting with Raf protein kinases, thus playing an intermediate role in the phosphorylation cascade (4, 5, 7). The Rho proteins are critical for cytoskeletal organization by controlling cell division (7, 8). The Rab/Ypt proteins function in intracellular transportation, or endocytosis and exocytosis, by activating individual vesicles around the endoplasmic reticulum and Golgi apparatus (7, 9). More than 30 individual genes encoding small GTP-binding proteins have been identified from higher plants—including *Arabidopsis*, pea, wheat, rice, maize, and tobacco (10). Most of them appear to belong to the Rab/Ypt subgroup, suggestive of some function in intracellular transportation, but little evidence has been provided (10). The *rha1* gene of *Arabidopsis* is predominantly expressed in developing guard cells, and Terryn *et al.* (11) conclude that it may function in vesicle transport. Expression of the *Pra2* and *Pra3* from pea, however, is down-regulated by light and mediated by phytochrome (12), suggestive of a role in developmental regulation.

We do not intend, however, to review the complex mechanisms of signal transduction here. Rather, we hope to assemble the results of our ongoing study of *rgp1*, a gene encoding a small GTP-binding protein, and to demonstrate how its gene product may be involved in the plant signal-transduction pathway.

Identification of *rgp1* Gene

Our study on *rgp1* began initially because of our interest in DNA methylation, and here we attempt to just briefly describe these findings in the hope of providing a basis for selecting *rgp1* for our further studies. In green plants, up to 30% of the total

Abbreviations: BA, *N*⁶-benzylaminopurine; JA, jasmonic acid; MeJA, jasmonic acid methyl ester; PI, proteinase inhibitor; PR, pathogenesis-related; SA, salicylic acid; TMV, tobacco mosaic virus; GTP[γ S], guanosine 5'-[γ -thio]triphosphate.

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cytosine residues of genomic DNA consists of m^5C (13), which is thought to alter the higher conformation of DNA and consequently to change the interaction between DNA and DNA-binding proteins (14, 15). As such interactions may directly affect gene expression, our preliminary study determined whether plant growth was affected by its DNA-methylation status. Analysis of the relationship between DNA methylation and dwarfism in maize (*Zea mays*) showed that the amount of m^5C in the DNA of a single-gene dwarf mutant, d5, was $\approx 8\%$ lower than that of its tall, near-isogenic counterpart (16). This relationship was further demonstrated in rice (*Oryza sativa*) seedlings treated with 5-azacytidine, a strong inhibitor of DNA methylation *in vivo* (17), in which both undermethylation and dwarfism induced by 5-azacytidine treatment were heritable. These results suggested that 5-azacytidine first induced demethylation of genomic DNA, thus causing an altered pattern of gene expression and consequently a reduction in plant height (18).

The next step in our studies was to identify gene(s) that had been affected by 5-azacytidine treatment and which, therefore, may have influenced the growth and development of the rice plants. A rice cDNA library was differentially screened with DNA probes of wild-type and 5-azacytidine-treated plants. Of the differentially expressed clones identified, one clone was the subject of further analysis (19). During growth of untreated control seedlings, expression of this clone was first observed 2 weeks after germination, reaching a maximum level between 4 and 6 weeks and gradually decreasing thereafter. In both 5-azacytidine-induced dwarf plants and their progenies, however, expression of the clone was markedly reduced throughout this entire period. As these results suggested that expression of the clone was influenced, either directly or indirectly, by DNA methylation and that the gene may be involved in some aspect of plant growth and development, the molecular structure of the gene was determined. The predicted protein encoded by the clone consisted of 226 amino acids with a relative molecular mass of 25 kDa. The putative amino acid sequence showed 62% identity to Ras-related Sp-ypt3, a small GTP-binding protein of fission yeast, whereas with other Ras-related proteins the identity ranged from 58% with ara, to 49% with Hrab1 and 45% with yeast YPT1. Such sequence similarities suggested that the gene belonged to the *ras*-related supergene family, and it was thus designated *rgp1*. Indeed, the *rgp1* gene product, expressed in *Escherichia coli*, was found to bind GTP (19).

Physiological Characteristics of Transgenic Tobacco Plants

Morphological Changes. To determine the physiological role of *rgp1*, the coding region of *rgp1* was introduced into tobacco plants in both sense and antisense orientations (20). Transformants, which contained the *rgp1* gene at one to three loci, showed distinct phenotypic changes, the most notable being a reduction in apical dominance with increased tillering, together with dwarfism, abnormal flower development, or both (Fig. 1). These morphological effects of the gene were observed in both sense and antisense transformants. Northern hybridization analysis showed that *rgp1* was expressed only in phenotypically abnormal transformants but not in apparently normal phenotypes. Furthermore, the R1 progenies from most transformants cosegregated in a 3:1 ratio for both kanamycin resistance and tillering, indicating that the observed abnormal morphology was indeed the result of the integrated *rgp1* gene. In addition, the expression of *tgf1*, a presumed tobacco homolog of *rgp1*, was markedly reduced in transformants expressing antisense *rgp1*, whereas it was apparently unaffected in transformants with the sense *rgp1*. These observations suggested that the phenotypic changes induced in antisense transformants may have been mediated by an effect on native *tgf1* mRNA, whereas, in the sense transformants, the changes may

FIG. 1. Phenotypes of transformed and untransformed tobacco plants. Control, normal untransformed plant (left); transformed plant with sense-oriented *rgp1* gene (middle); and transformed plant with antisense-oriented *rgp1* gene (right).

have been induced by overproduction of *rgp1*. This was, indeed, later confirmed by immunoelectrophoresis.

Elevated Levels of Cytokinins. The involvement of cytokinins in the maintenance of apical dominance has been well documented (21, 22) and has also been directly demonstrated by the introduction of genes related to cytokinin biosynthesis into tobacco plants (23, 24). Other analyses have also shown that apical dominance is lost when the relative levels of auxins are decreased, suggesting that apical dominance is maintained by either the appropriate balance of auxin and cytokinin levels, the sensitivity of the plant tissues to them, or a combination of the two (25). The phenotypic changes in our transgenic plants suggested to us that the *rgp1* gene product may be involved at some point of either the hormone metabolic or response pathways.

To establish whether abnormal auxin or cytokinin levels were involved in the observed phenotypic effects, levels of zeatin and zeatin riboside, which are the major constituents of native cytokinins in higher plants, were measured in transgenic plants. Whereas the zeatin and zeatin riboside content in control plants was estimated at ≈ 5 pmol/g (fresh wt), that in transformants, with either sense or antisense oriented *rgp1*, increased to 23 pmol and 20 pmol/g (fresh wt), respectively (ref. 26 and unpublished data).

Abnormal Wound Response of Transgenic Plants

Induction of Acidic Pathogenesis-Related (PR) Proteins by Wounding. To identify native tobacco genes known to be affected by cytokinins in other plants and that show altered mRNA levels in the transgenic plants, Northern analysis was done with various cDNA probes by using total RNA from wild-type and several transgenic plants expressing the sense-oriented *rgp1* gene (26). Of the 14 various genes examined, only those encoding acidic PR proteins (PR-1, PR-Q, and PR-S) showed elevated transcript levels in the transgenic plants. However, the accumulation of the PR-1 proteins was not constitutive but rather occurred only in response to wounding. Thus, when a leaf was wounded by punching out discs every 2 days, PR-1 proteins accumulated up to 600 $\mu\text{g/g}$ (fresh wt) by the 10th day. In addition, when a transgenic plant

FIG. 2. Enhanced hypersensitive reaction of transgenic tobacco with sense-oriented *rgp1*. Detached leaves were inoculated with TMV and incubated at 20°C under continuous light for the appropriate time interval. Discs containing local lesions were punched out, stained with aniline blue, and observed under UV epifluorescence. The center of the necrotic lesions is indicated by arrowheads, callose by double arrowheads, browning by small arrows, and healthy regions by large arrows. Whole local lesions, 6 days after inoculation, are shown for wild-type (W_1) and transgenic (T_1) plants, and the corresponding cross-sections are also shown (W_2 , T_2 , respectively). Cross-sections, prepared 36 hr after inoculation, were cleared with ethanol to observe necrotic lesions (W_3 and T_3). Cross-sections, prepared 6 days after inoculation, and observed by autofluorescence, show browning and healthy chlorophyll (W_4 , T_4 , and T_5). Note that while chlorophyll decay begins at the edge of the lesion in wild-type plants (small arrow), indicative of rapid spread of virus attack, the decay is limited to a small area in transgenic plants. (Bars = 200 μ m.)

was wounded by detaching lower leaves, the upper unwounded leaves also initiated PR-1 protein production up to 150 $\mu\text{g/g}$ (fresh wt) by the eighth day, indicating that PR-1 protein induction was systemic. In contrast, transcript levels of the PI-II gene, which is normally induced by wounding, were generally suppressed in the same wounded plants.

PR proteins were originally identified in hypersensitive tobacco plants carrying the *N* gene upon viral infection (27). Although the molecular functions of PR proteins are not fully clarified, they apparently are important in the plant self-defense system (28). Indeed, some PR proteins exhibit antifungal activity against certain phytopathological fungi (29–31). The PR proteins consist of both acidic and basic isoforms that differ in their tissue and organ localizations and in the signals by which they are induced (28, 30, 32, 33). The acidic PR proteins are induced by the hypersensitive reaction after tobacco mosaic virus (TMV) infection or salicylic acid (SA) treatment and, to a lesser extent, by cytokinins (32) and auxins (34), whereas the basic PR proteins are efficiently induced by wounding or ethephon (ethylene) treatment as well as TMV infection (32, 33) and are actually inhibited by auxins and cytokinins (35). Production of a large amount of acidic PR proteins in response to wounding is thus an abnormal response of the transgenic plants.

Induction of SA and Salicylic Acid- β -Glucoside by Wounding. As SA is a powerful inducer of acidic PR proteins, the question arose whether or not SA was involved in the abnormal wound induction of PR proteins in the transgenic plants. We therefore examined SA levels in young leaves after injuring the upper epidermis of leaves with carborundum (26). Samples, taken after appropriate intervals, were used to quantify endogenous SA levels. The leaves began producing SA 1 day after injury to a maximum of 180 ng/g (fresh wt) from the second day for at least an additional 6 days. In wounded transgenic plants, salicylic acid- β -glucoside was more rapidly synthesized than SA so that its level exceeded 700 ng/g (fresh wt) from before 6 hr after injury up to at least 2 days. In addition to the wounded leaves, however, unwounded adjacent leaves also produced SA and salicylic acid- β -glucoside, although their levels were $\approx 30\%$ those of the injured leaves, indicating that the wound response of transgenic plants was systemically transmitted to the neighboring leaves. We therefore concluded that the induction of SA and salicylic acid- β -glucoside by wounding constituted the abnormal response of the transgenic plants.

Enhanced Hypersensitive Reaction by TMV Infection. As exogenously applied SA can suppress TMV multiplication without affecting sensitivity to the virus (36), we tested the susceptibility of the transgenic plants to TMV infection at 20°C. Necrotic lesions in the transgenic plants were visible within 24 hr of inoculation (26), suggesting that these plants have an enhanced hypersensitive response to pathogens in comparison with control plants, which require at least 36 hr to develop visible local lesions. Six days after inoculation of the detached leaves with TMV, the average size of necrotic lesions in the wild-type and transgenic plants was 2.10 mm and 0.34 mm, respectively. Although the number of local lesions was almost the same, the amount of TMV recovered from the same number of lesions was >12 -fold higher in wild-type plants than in transgenic plants (data not shown).

Resistance of the transgenic plants was further analyzed by histochemical assays (Fig. 2). TMV-infected tobacco plants are found to have callose (β -1,3-glucan) deposited in and around necrotic lesions, and this deposition is generally regarded as proportional to the increase in systemic acquired resistance (37–39). Therefore, we first examined callose deposition as a marker for the resistance of our transgenic plants. Six days after TMV inoculation, the transgenic plants developed heavy callose deposition, as indicated by the intense bright-yellow fluorescence (Fig. 2, T_1 and T_2), whereas wild-type plants

showed almost no such deposition (W_1 , W_2). The size of brown rings, outer regions of the necrotic lesions where TMV actively multiplies, was large in the wild type plant and small or missing in the transgenic plants. This result was confirmed by cytochemical observations, in which the necrotic cells spread within 36 hr of inoculation from the upper to lower epidermis of wild-type plants (Fig. 2, W_3), whereas necrotic regions were limited to a small portion of the upper epidermis and palisade cells of the transgenic plants (Fig. 2, T_3). This situation was maintained in both wild-type and transgenic plants for at least 6 days after inoculation, as shown by an autofluorescence assay (Fig. 2, W_4 , T_4 , and T_5). These observations suggest that hypersensitive cell death occurred quickly in the transgenic plants and resulted in an enhanced hypersensitive response to prevent further viral multiplication.

Signal Crossing of Wound- and Pathogen-Signaling Pathways in Transgenic Plants

Due to its diverse biological functions, such as systemic induction of PR proteins, ion uptake, stomatal closure, and flowering of thermogenic plants, SA was suggested to function as a native regulator and even possibly as a phytohormone (40). In particular, SA is considered to be involved in various signal-transduction systems, especially those leading to systemic acquired resistance against pathogen attack (41, 42), because induced or increased SA levels followed by subsequent increases in acidic PR proteins are seen in tobacco and cucumber plants upon infection with TMV (43) or the fungal pathogen *Colletotrichum* (44), respectively. In marked contrast, SA is not induced in response to wounding (43). Thus, transduction of the wound signal is thought to follow a separate pathway from that of SA (41) and to involve jasmonic acid (JA), which is synthesized through a lipid-based signal-transduction system (45). In turn, JA may act as a direct intracellular signal intermediate and effect a subsequent change in the expression of specific sets of genes, such as those encoding proteinase inhibitors (PIs) (46, 47). In normal plants, therefore, the signal cascade involving SA is regulated quite distinctly from that involving JA and its methyl ester (MeJA).

In the transgenic plants expressing *rgp1*, however, the two normally distinct pathogen and wound signal-transduction systems appear to be modified, possibly by an unusual crossing of the signals, with the result of abnormal SA production in response to wounding. Although this disorder is clearly due to the integrated *rgp1* gene, questions arise about the mechanism by which *rgp1* modifies these signal pathways. In an attempt to address these questions, we examined, in transgenic plants, the differential expression of PR-1 and PI-II transcripts that are normally induced by SA and JA (MeJA), respectively, in wild-type plants (Fig. 3). Healthy intact leaves of both wild-type and transgenic plants produced neither of these transcripts, whereas wounding induced accumulation of PI-II transcripts in both sets of plants. In addition, however, wounding induced a large accumulation of PR-1 transcripts in transgenic plants and a trace amount in control plants. MeJA enhanced the accumulation of PI-II transcripts in both plants but severely reduced the PR-1 transcript levels induced by wounding in the wild-type plant and especially in the transgenic plant. In contrast, SA further increased transcript levels of PR-1 induced by wounding but reduced transcript levels for PI-II in both plants. N^6 -Benzylaminopurine (BA) also enhanced PR-1 transcript levels induced in both plants by wounding but either suppressed or had no effect on the PI-II transcripts in wild-type and transgenic plants, respectively. Guanosine 5'-[γ -thio]triphosphate (GTP[γ S]), a powerful inhibitor of G proteins *in vivo* (48), suppressed wound-induced PR-1 and PI-II transcript accumulation in the wild-type plant, whereas it was apparently ineffective in the transgenic plant.

FIG. 3. RNA hybridization analysis in wild-type and transgenic tobacco plants. A whole leaf from either a wild-type or transgenic plant with the sense-oriented *rgp1* was detached, cut into pieces, and floated on water or on a solution containing either 50 μ M SA, 50 μ M MeJA, 20 μ M BA, or 25 μ M GTP[γ S] at 20°C under continuous light for 40 hr and then used for total RNA isolation. As a control, total RNA was immediately isolated from untreated, freshly harvested leaves (untreated). A 30- μ g RNA aliquot was assayed for mRNA levels of actin, as an internal standard, and PR-1 and PI-II by Northern blot hybridization.

These observations clearly suggested that G proteins are indeed involved in the PR-1 and PI-II induction pathways. Because the integrated *rgp1* gene appears to overcome the inhibitory effects of GTP[γ S], we consider that small GTP-binding proteins, homologous to *rgp1*, are probably involved in these pathways. The results also show that JA and SA function antagonistically in the induction of PR-1 and PI-II transcripts. Indeed, reduction in PR-1 transcript levels is directly associated with reduced levels of SA and salicylic acid- β -glucoside after JA treatment of the transgenic plants (data not shown). Therefore, JA could inhibit SA biosynthesis. Overall, our data imply that small GTP-binding proteins, cytokinins, JA, and SA are equally necessary for correct functioning of the defense response.

Protein Transportation. Because *rgp1* belongs to the Rab/Ypt subgroup of small GTP-binding proteins, thought to be involved in intracellular transportation, the question arose whether or not the abnormal signal crossing was due to problems in protein transport. An initial immunoblot assay showed that most *rgp1* proteins in transgenic plants were located in the microsomal fraction, suggestive of some role in transportation (data not shown). Subsequently, we examined the transport of acidic PR-1 proteins, which are normally secreted into intercellular spaces, as a representative protein. Results from both intact leaves and suspension-culture cells showed that the proteins were normally transported (26), suggesting that the observed disturbance in the wound signals was not the result of a disturbance in PR protein transportation, although the transport of other proteins involved in the signal pathway could have been modified.

Are Cytokinins Involved in the Defense Response? As (i) it is generally established that some phytohormones, especially cytokinins, auxins, ethylene, and abscisic acid, are involved in the pathogen- and wound-signaling pathways (28, 49) and as (ii) our transgenic plants constitutively produce high levels of cytokinins even without wounding (data not shown), the hormonal imbalance induced by *rgp1* could be the primary cause of the observed effects. This hypothesis is corroborated by our findings that, in transgenic plants, PR-1 mRNA accumulation in response to wounding is completely suppressed by exogenous application of a cytokinin antagonist, 2-chloro-4-

cyclohexylamino-6-ethylamino-*s*-triazine (data not shown), indicating that cytokinins are a prerequisite for PR-1 expression. Furthermore, the transgenic plants are insensitive to exogenously applied BA to suppress PI-II (Fig. 3), suggestive of some sort of disturbance in the cytokinin and JA signaling pathways. However, when we compared the wound response between transgenic plants containing *rgp1* with those containing *ipt*, which constitutively produce endogenous cytokinins, we found that although the *ipt* transformants accumulated a 5-fold higher level of SA after a single wound in comparison with wild-type plants, these plants were not particularly resistant to virus infection. This result is consistent with the observation that, in TMV-inoculated leaf pieces of wild-type tobacco, exogenously applied cytokinins could not suppress viral multiplication (unpublished observation). These results suggest that cytokinins are essential, but not sufficient, for induction of the defense response. It is, therefore, attractive to speculate that cytokinins, through the action of related GTP-binding proteins, regulate the JA and SA levels so that in normal plants the external signals are correctly transmitted to the relevant transduction pathways.

Concluding Remarks—The Defense Signal Pathway Network

On the basis of the above observations, we currently propose a two-step mechanism to explain the abnormal behavior of our transgenic plants: (i) small GTP-binding proteins play an essential role in cytokinin biosynthesis and/or metabolism; and (ii) small GTP-binding proteins are also directly involved in the defense signaling pathways. Although we are unclear about the molecular mechanisms involved, we are certain that introduction of the *rgp1* gene into the host plant seriously disturbs cytokinin biosynthesis and/or metabolism, resulting in its accumulation in leaves. Such abnormally high cytokinin levels may pleiotropically affect growth and development and also possibly the defense signal pathways by, for example, sensitizing the wound-perception system. Also, the presence of excess *rgp1* in sense plants or the absence of its native tobacco homologs in antisense plants may disturb the normal signaling pathways that are mediated by numerous different small

FIG. 4. Tentative signal flows of wound and hypersensitive reaction (HR) after pathogen attack. (Upper) Wild-type tobacco plant. (Lower) Transgenic (*rgp1*) tobacco plant. Activated signals are indicated by solid letters, and suppressed signals are indicated by open letters. The signal channel involving ethylene is omitted to simplify the illustration. Is, intercellular spaces; PI, PI-II; PR, acidic PR proteins; Scop, scopoletin; Vac, vacuole; MAPK, mitogen-activated protein kinase.

GTP-binding proteins. In fact, we have observed several abnormal biochemical responses in transgenic plants, probably due to such disturbed or altered signals. For example, upon wounding, transgenic plants produced high levels of scopoletin, a derivative of coumarins, which has strong antimicrobial activity and which in wild-type plants is not induced by wounding but by the hypersensitive reaction (50). Furthermore, the activity of phenylalanine ammonia-lyase, an enzyme involved in the first step of the biosynthesis of SA and coumarin derivatives (51), is continuously increased after wounding of transgenic plants, whereas this enzyme is only transiently increased in wounded wild-type plants.

It is therefore highly probable that the defense signal pathways consist of a complicated, yet well-regulated, network of several regulatory factors including cytokinins, SA, JA, and GTP-binding proteins. In addition, we have recently identified a gene encoding a mitogen-activated protein kinase homolog, which shows increased transcript levels after exogenous application of MeJA (data not shown). Thus protein kinases also appear to be involved in these signaling pathways. A tentative model for the wound and HR signal flows, incorporating these present and previous findings, is illustrated in Fig. 4. In this model, we introduce the concept of a "signal switch," which directs the input signals to the appropriate transduction channels under the control of cytokinins and small GTP-binding proteins. In wild-type plants, the wound signal is directed to the

JA channel, and the HR signal is directed to both the JA and SA channels, whereas in transgenic plants both signals are preferentially directed to the SA channel.

We thank Dr. T. Murakami [National Institute of Agrobiological Resources (NIAR)] for histochemical analyses, Mr. S. Seo (Tsukuba University and NIAR) for SA, salicylic acid- β -glucoside, and scopoletin measurement, Dr. E. Orudjev (Biotechnology Institute) for cytokinin measurement, Professor M. Katsumi (International Christian University, Tokyo) for critical reading of the manuscript, and Ms. K. Futada (Biotechnology Institute) for manuscript preparation.

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