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Chemical Signals in Plant Resistance: Salicylic Acid

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7.1 Introduction: Systemic Acquired Resistance and Salicylic Acid

Plants are defended against pathogens by constitutive and inducible barriers. Induced resistance is expressed locally at the site of infection as well as in uninfected parts of infected plants. Induced defense responses to pathogens were already described in the first half of the 20th century (Carbone and Arnaudi, 1930; Chester, 1933; Gäumann, 1946). Some decades later, the phenomenon of induced resistance extending beyond the infected sites of a plant was studied in detail in tobacco and cucumber (Madamanchi and Kuć, 1991; Ross, 1966). The classical experimental system consists of a plant infected on the lower leaf with a necrotizing pathogen that induces a resistance response in the upper leaf toward the same or other pathogens. This resistance is referred to as systemic acquired resistance (SAR) and occurs in many di- and monocotyledonous species (Sticher et al., 1997).

The broad systemic response to pathogens and the transmission of a systemic signal are both spectacular and intriguing features of SAR. The induction of SAR by pathogens is a complex process. Elicitors released at the site of infection are recognized by corresponding plant receptors; this leads to modifications in ion homeostasis, production of reactive oxygen species (ROS), and numerous phosphorylation events (Dangl and Jones, 2001). These changes activate a signaling network leading to transcriptional events involved in various aspects of local and SAR responses. A putative signal released from the infected leaf moves to other parts of the plant where it induces defense reactions. Interestingly, besides localized infection by pathogens, colonization of roots with nonpathogenic bacteria can also induce resistance in leaves (Pieterse and van Loon, 1999; Van Loon et al., 1998). Furthermore, localized viral infections can lead to the systemic induction of post-transcriptional gene silencing, a defense mechanism to subsequent viral infections (Waterhouse et al., 2001). Environmental factors such as light or UV irradiation can also have an important impact on SAR (Genoud et al., 2002; Islam et al., 1998; Mercier et al., 2001).

SAR and its broad spectrum of protection inspired researchers to use this phenomenon for novel approaches in plant protection. For instance, nonantibiotic molecules were identified that can induce SAR on various plants under field conditions (Friedrich et al., 1996; Görlach et al., 1996; Métraux et al., 1991). The molecular responses induced during SAR also became an important target for many groups. For example, a set of proteins termed pathogenesis-related or PR-proteins and their associated genes were discovered that are locally and systemically induced in response to elicitors (Van Loon and Van Strien, 1999). Some of these PRs have antibacterial or antifungal activities, indicating a role in pathogen defense. The number of defense-related genes is much wider than originally thought, as shown by genome-wide analyses (Maleck et al., 2000; Schenk et al., 2000). Exogenously applied salicylic acid (SA) was first shown in tobacco to induce PRs and to protect against tobacco mosaic virus. Later, SA was found in plants after pathogen infection, locally and systemically, making SA an endogenous signal for SAR (reviewed in Sticher et al., 1997).

SA is found in many species and can regulate such diverse physiological processes such as thermogenesis, flowering or defense against pathogens (reviewed in Raskin, 1992). Strong correlations were found between induced resistance and endogenous SA accumulation in plant tissue after a localized pathogen infection (reviewed in Sticher et al., 1997). Further support for the importance of SA for SAR came from studies with mutants and transgenic plants that exhibit altered levels of SA. In general, plants with low endogenous SA are impaired in SAR. Conversely, mutants with constitutive high levels of SA exhibit increased tolerance to pathogens (reviewed in Métraux and Durner, 2002). Besides SA, other endogenous molecules have been identified as signals involved in the activation of resistance responses that are SA-independent. These compounds include octadecanoic acid derivatives such as jasmonic acid (JA), methyl jasmonate (MeJA), 12-oxo-phytodienoic acid (OPDA), and ethylene (ET). Interestingly, it was shown in *Arabidopsis thaliana* that SA-dependent responses can provide resistance to a defined spectrum of pathogens only (such as *Peronospora parasitica* or *Pseudomonas syringae*) while JA- and ET-dependent resistance responses seem to operate against another group (*Alternaria brassicicola, Botrytis cinerea*) (Thomma et al., 1998). Thus, a pathogen attack does not trigger a central SA-dependent cascade of reactions leading to the activation of a single set of resistance mechanisms but rather activates a complex network dependent on multiple signals, of which SA is one (Thomma et al., 1998, 2001). Some branches of this network crosstalk with each other, or interfere with pathways triggered by environmental stimuli such as light (Genoud et al., 2002). This increases the flexibility of the network to optimize the defensive reactions of the plant to a given environment. A digital approach based on Boolean logic was proposed to represent such a complex network (Genoud et al., 2001, 2002).

This chapter will focus on our state of knowledge on the biosynthesis and metabolism of SA, the various roles of SA in defense responses, SA-dependent signaling, and the SA-induced defense signaling network.

7.2 Biosynthesis and Metabolism of Salicylic Acid

Several studies have shown that SA derives from the shikimate-phenylpropanoid pathway (reviewed in Sticher et al., 1997). Depending on the species or tissues, two routes from phenylalanine to SA have been described that differ at the hydroxylation of the aromatic ring. Phenylalanine ammonia lyase (PAL) converts phenylalanine (Phe) to cinnamic acid (CA) that can be hydroxylated to form *ortho*-coumaric acid followed by oxidation of the side chain to yield SA. Alternatively, SA results from an oxidation of the side chain of CA to form benzoic acid (BA) that is hydroxylated in the *ortho* position (reviewed in Sticher et al., 1997). In tobacco, SA was postulated to be synthesized from free BA (Yalpani et al., 1993), and recent results indicate that benzoyl glucose, a conjugated form of BA, is the direct precursor of SA (Chong et al., 2001). In cucumber, potato, and rice SA is likely to derive from phenylalanine via CA and BA but the exclusive role of this route in pathogen-induced SA was never fully assessed (reviewed in Sticher et al., 1997).

Arabidopsis thaliana also produces SA locally and systemically after pathogen infection or treatment with UV-C light (Nawrath and Métraux, 1999; Summermatter et al., 1995). In *Arabidopsis*, inhibitor studies with 2-aminoindan-2-phosphonic acid (AIP), an inhibitor of PAL, indicate that the biosynthetic pathway of SA is derived from Phe and CA. AIP-treated plants have lower amounts of SA and are susceptible to *P. parasitica* (Mauch-Mani and Slusarenko, 1996). The SA-induction deficient (*sid1* and *sid2*) mutants are unable to accumulate SA and to express SAR after an infection (Nawrath and Métraux, 1999). The *sid2* mutation was localized to a gene, *ICS*, encoding isochorismate synthase (ICS) (Wildermuth et al., 2001). ICS1 includes a chorismate-binding domain. It shares 57% amino acid identity with a *Catharanthus roseus* ICS (Van Tegelen et al., 1999) and 20% identity with the bacterial ICS, and both proteins have confirmed biochemical activities (Serino et al., 1995; Wildermuth et al., 2001). The ICS1 gene is induced locally and systemically upon localized pathogen infection (Wildermuth et al., 2001). This demonstrates that SA produced by ICS is required for SAR in *Arabidopsis*. An explanation is now needed to explain the discrepancy between these results from studies with AIP-treated plants (Mauch-Mani and Slusarenko, 1996). ESTs for ICS have been annotated in soybean and tomato, making it likely that many higher plants produce pathogen-induced SA from isochorismate (Wildermuth et al., 2001). The presence of a plastid transit peptide and cleavage site in the *ICS1* gene indicates a plastid-localized synthesis of SA. Possibly, the SA pathway in *Arabidopsis* might share common ancestry with prokaryotic endosymbionts (Wildermuth et al., 2001). The presence of W-box elements in the promoter of *ICS1* suggests that WRKY transcription factors may regulate the response to pathogens or stress (Eulgem et al., 2000). The *ICS1* promoter also includes a binding site for Myb transcription factors that regulate genes for plant defense and associated secondary metabolism (Bender and Fink, 1998; Yang and Klessig, 1996). Interestingly, neither bZIP nor NF-κB motifs, typically required for the induction of *PR1* by SA, were found in the promoter of *ICS1,* suggesting a SA-independent regulation after pathogen

infection (Cao et al., 1997; Ryals et al., 1997). Indeed, wild-type expression levels of *ICS1* are observed in SA-depleted NahG plants (Wildermuth et al., 2001). Therefore, the expression of *ICS1* is likely to be under the control of a signal other than SA.

Although the site of action of SA is not known, evidence from transgenic plants expressing the *NahG* gene in the cytoplasm (Delaney et al., 1994) supports either a cytoplasmic location or at least a traffic of SA through this compartment. Interestingly, another SA-induction deficient mutant, *eds5/sid1*, was used to identify a membrane protein homologous to the bacterial multidrug and toxin extrusion (MATE) proteins (Brown et al., 1999; Nawrath et al., 2002). MATEs have recently been reported in *Arabidopsis* (Brown et al., 1999; Debeaujon et al., 2001; Diener et al., 2001; Nawrath et al., 2002). It will now be very interesting to learn more on the nature of the substrate(s) transported by EDS5/SID1.

The relative importance of CA- and ICS-derived SA for the induction of SAR needs to be investigated, since the isochorismate pathway might not be unique for *Arabidopsis* (Wildermuth et al., 2001). If both pathways really coexist in a same species, specific stimuli might selectively induce SA by one or the other pathway. In *Arabidopsis*, virulent or avirulent pathogens, ozone stress, or callus formation lead to high levels of SA while wild-type levels of SA are observed in *sid2* mutants that have an inactivated ICS (Nawrath and Métraux, 1999). This supports a unique ICS-derived pathway for pathogen, ozone and callus-induced SA formation. Possibly, wild-type basal levels of SA might derive from the CA pathway. Another source of the basal levels of SA was proposed to result from the action of a second *ICS* gene (*ICS2*), the transcripts of which remain undetected in infected or uninfected leaves of *Arabidopsis* (Wildermuth et al., 2001). Clearly, the function and regulation of CA- and ICS-derived SA needs to be clarified in *Arabidopsis* and other species where CA was proposed as a main precursor for pathogen-induced SA.

SA is also present as a conjugate, either in methylated, hydroxylated, or glycosylated form. In tobacco, volatile methyl salicylate (MeSA) is produced from SA after infection. Interestingly, MeSA can induce defense reactions upon conversion to SA (Seskar et al., 1997; Shulaev et al., 1997). It was proposed to be additive to SA for signaling within a plant and to act as a signal for communication between plants (Shulaev et al., 1997). In tobacco, a predominant and stable SA metabolite is SA-2-*O*-β-D-glucoside (SAG). The ester glucoside (GSA) was also found in tobacco (Enyedi et al., 1992). GSA was observed to accumulate rapidly and transiently after SA application (Lee and Raskin, 1998). GSA was proposed to protect the plant against phytotoxicity of high SA levels, while SAG might represent a slow release form of SA (Lee and Raskin, 1999). A UDP:glucose:SA glucosyltransferase (SAGTase) was isolated from tobacco and oats that can form both SAG and GSA (Edwards, 1994; Lee and Raskin, 1999). The tobacco SAGTase has a broad specificity for simple phenolics and its mRNA is rapidly induced upon SA treatment or inoculation with incompatible pathogens (Lee and Raskin, 1999).

7.3 Salicylic Acid and Its Various Roles in Resistance

Endogenous SA, or application of SA, or functional analogs such as BTH (benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester; BION®, ACTIGARD®) and INA (2,6-dichloroisonicotinic acid) induce the expression of a set of PR-proteins such as PR1, PR2, and PR5, the expression of which correlates with resistance (Métraux et al., 1991, Uknes et al., 1992, Ward et al., 1991). Interestingly, while some PRs have an antimicrobial activity *in vitro* and were proposed to act similarly *in planta* (reviewed in Punja, 2001) the biological function of PR1, one of the best markers for SAR, is still unknown. Some situations were also described where the induction of some PRs could be dissociated from the action of SA (Nawrath and Métraux 1999; Schaller et al., 2000). In *Arabidopsis* undergoing SAR, 31 genes linked to SAR cluster together with *PR1* (Maleck et al., 2000). This typical defense gene expression pattern is lost in SA-degrading NahG plants (Delaney et al., 1994; Gaffney et al., 1993; Maleck et al., 2000), as well as in mutants blocked in SA biosynthesis (Nawrath and Métraux, 1999). So far, it was tacitly assumed that NahG plants are only affected in SA accumulation. Several studies indicate more complex modifications that could in some cases influence the interpretation of the phenotype observed in NahG plants (Cameron, 2000; Lieberherr et al., 2003; Nawrath and Métraux 1999; Heck et al., 2003; Van Wees and Glazebrook, 2003).

SA also promotes or inhibits cell death depending on the plant pathogen interaction, environmental conditions, and genetic background of the plant cell (Greenberg et al., 2000). In *Arabidopsis*, many mutants with constitutive high PR1 expression and enhanced resistance form spontaneously HR-like lesions (Dietrich et al., 1994; Greenberg, et al., 1994; Weymann et al., 1995). In some mutants, SA-accumulation and SAR gene expression are only necessary for disease resistance, but not for lesion formation, i.e., in *lsd2* and *lsd4* (Hunt et al., 1997). In other mutants, expression of the *NahG* gene suppresses lesion formation as well as disease resistance, e.g., in *lsd6*, *lsd7*, and *ssi1* (Weymann et al., 1995; Shah et al., 1999; Greenberg et al., 2000). SA-dependent cell death has also been observed in tobacco expressing the *Cf-9* gene of tomato together with the avirulence gene *Avr9* of *P. syringae* (Hammond-Kosack et al., 1998) as well as in soybean cell cultures infected with avirulent *P. syringae* pv. *glycinea* (Shirasu et al., 1997). In TMV-infected tobacco, the expression of NahG delays the development of the HR (Mur *et al.*, 1997) and attenuates the oxidative burst after inoculation with avirulent bacteria (Mur et al., 2000).

SA-dependent cell death may also be caused by cellular dysfunction associated with superoxide production (Broderson et al., 2002; Jabs et al., 1996; Kliebenstein et al., 1999). For example, superoxide production leads to runaway cell death in the *lsd1* mutant. This might be caused by a defect in the GATA-type transcription factor LSD1 that activates the expression of a Cu/Zn superoxide dismutase (Dietrich et al., 1997). In the *snc1* mutant, an unknown additional factor besides SA was found to be needed for cell death (Li et al., 2001). In some *Arabidopsis* mutants the lesion

formation are uncoupled from SA production and SAR. This is the case in *dnd1*, *dnd2*, *and hrl1* that do not develop HR-like lesions while SA accumulation and SAR remain intact (Yu et al., 1998). In other *Arabidopsis* mutants, e.g., the *acd5* and *ddl1* mutants, SA accumulation, cell death, and disease resistance are uncoupled from each other (Greenberg et al., 2000; Pilloff et al., 2002). For example, SA or BTH induces cell death leading to an increased susceptibility to *P. syringae* and endogenous SA accumulation does not lead to SAR in *acd5* (Greenberg et al., 2000).

The prominent effect of SA on gene expression led many investigators to study its molecular mode of action. SA is unlikely to interact directly with a target site at the promoter of induced genes. Therefore, a search for protein binding sites with high affinity for SA led to the enzyme catalase (Chen et al., 1993). Binding and associated inactivation of catalase was proposed to increase intracellular H_2O_2 that could activate defense gene expression or act as an antimicrobial barrier at the site of invasion (Chen et al., 1993). This catalase inhibition hypothesis was seriously questioned (reviewed in Mauch-Mani and Métraux, 1998). SA was proposed to affect the redox status of the cells. The ability of SA to form free radicals upon inhibition of heme-containing enzymes such as peroxidase or catalase led to the "free radical" hypothesis of SA action (Durner and Klessig, 1995, 1996). Phenolic free radicals can be potent initiators of lipid peroxidation, the products of which might activate defense reactions (Farmer et al., 1998). It remains to be demonstrated that sufficient free radicals are produced in the correct time and space frames to induce defense responses. A novel protein was also found to exhibit high affinity for SA, but its relevance for the induction of SA-dependent resistance has never been completely assessed (Du and Klessig, 1997).

Another aspect of the molecular action of SA is based on its possible involvement in phosphorylation cascades. MAP kinases (MAPKs) typically compose modules of signaling equivalent to the bacterial signal-integrating phosphorelays, which are characterized by a sequence of reversible phosphorylations of the MAPK by MAPK kinases (MAPKK), subsequent to the phosphorylation of MAPKK by MAPKK kinases (MAPKKK) (Nürnberger and Scheel, 2001; Romeis et al., 2000; Wrzaczek and Hirt, 2001; Zhang and Klessig, 2001). The three successive phosphorylation events are locally assisted by a scaffold protein (see for instance Xing et al., 2002), that may also contribute to precisely target the signaling (amplifier) module to a specific location in the cell. In eukaryotes such as yeast, this type of signal transduction apparatus acts in combination with specific receptors (such as trimeric-G-coupled receptors) in the transmission of external stimuli and can be the site of crosstalk modulation by a different perceptive pathway. In plants, SA induces the activity of a protein kinase (referred to as SA-induced protein kinase, SIPK) belonging to the MAP kinase family (Zhang and Klessig, 1997). SIPK was proposed to initiate or be part of a more complex signaling cascade for the induction of defense reactions. In tobacco, the MAPKK NtMEK2 activates SIPK. This is followed by a hypersensitive reaction (HR)-like cell death and activation of the expression of 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) and L-phenylalanine ammonia-lyase (PAL), two genes encoding key enzymes of

the biosynthesis of defense-related phenolics (Yang et al., 2001). Unexpectedly, SA is not involved in the NtMEK2-mediated activation of HR (Yang et al., 2001), indicating the existence of alternative signaling cascades for SA. The existence of different MAPK cascades was also inferred from the study of the flagellin cascade in *Arabidopsis* (Asai et al., 2002). In *Arabidopsis*, H_2O_2 activates the MAPKKK ANP1 that activates the SIPK analogs AtMPK3 and AtMPK6, apparently without the implication of SA (Kovtun et al., 1998). In summary, while the activation of MAPKs by SA has been reported in some instances, many studies suggest that kinase cascades can operate without SA. Presumably, such signaling cascades would precede downstream defense responses, some of which are SA-dependent.

A possible molecular action of SA was also considered in relation to priming. This hypothesis proposes that SAR-derived signals prime or condition the plant tissue to react with a faster and more intense induction of defense reactions after an infection. Support for a role of SA in priming was first obtained in elicitor-treated cultured parsley cells (Conrath et al., 2002). The defense responses that can be primed by SA or functional analogs include the oxidative burst, the HR, the production of phenolic compounds, lignin-like polymers or phytoalexins, or the expression of defense-related genes (Conrath et al., 2002). Priming has also been observed in whole plants. *Arabidopsis* pretreated with pathogens or BTH shows an increase in the sensitivity to *P. syringae*-induced activation of the PAL gene and callose deposition, two reactions that are not induced by BTH alone (Kohler et al., 2002). Priming by BTH and pathogen infection for resistance to *P. syringae* requires the activity of the *NIM1/NPR1* gene (Kohler et al., 2002). Interestingly, in *Arabidopsis* the BTH-primed PAL expression and callose deposition could also be induced after wounding or infiltration of leaves with water, indicating that priming may be a point of crosstalk between the response to pathogens and wounding or osmotic stress (Kohler et al., 2002). The nonprotein amino acid β-aminobutyric acid (BABA) protects *Arabidopsis* from infection with *Peronospora parasitica*. BABA acts by potentiating the tissue to a stronger deposition of callose-containing papillae at the fungal infection sites. In response to infection with virulent *P. syringae*, the effect of BABA manifests itself by a potentiation of the induction of PR1 (Zimmerli et al., 2000). Interestingly, the effect of BABA against P. *parasitica* is independent of the SA, JA, and ethylene signaling pathways, whereas BABA potentiation to *P. syringae* is dependent on SA signaling (Zimmerli et al., 2000). Future experiments should elucidate the molecular mode of action of SA in priming of defense responses.

The involvement of SA as a systemic mobile signal was also repeatedly explored. Since SA was detected in the phloem sap, it was initially proposed as the primary signal for SAR that moves from the infected to the uninfected parts of the plant (Malamy et al., 1990; Métraux et al., 1990). However, grafting and leaf excision experiments indicate that while SA is a necessary component for the induction of local and systemic resistance, it is not the primary mobile signal exported from the infected leaf to other parts of the plant (reviewed in Mauch-Mani and Métraux, 1998). Radiolabeling experiments showed that SA synthesized after

inoculation can be transported from the infected to the upper leaves by the phloem before resistance was detectable (Mölders et al., 1996; Shulaev et al., 1995). These results might not be incompatible: SA produced in high amounts at infection sites could be translocated together with another primary mobile signal and induce resistance in the distal leaves. Progress in the search for a phloem-mobile signal was recently made using the *Arabidopsis dir1-1* mutant defective in systemic but not in local induced resistance. *DIR1* encodes a putative apoplastic lipid-transfer protein (Maldonado et al., 2002). Analyses of phloem exudates indicate that *dir1-1* plants are missing an essential mobile signal. The authors propose that DIR1 interacts with a lipid-derived molecule to promote long distance signaling.

SA was also found to be involved in the signal transduction pathway for virus resistance. In tobacco or in *Arabidopsis*, SA inhibits the replication or the movement of several RNA viruses, independently of SA-induced PR proteins (Chivasa et al., 1997; Murphy et al., 1999; Murphy and Carr, 2002; Naylor et al., 1998; Wong et al., 2002). In tobacco and *Arabidopsis*, SA-mediated resistance can be induced by cyanide and the mitochondrial electron transport inhibitor antimycin A (AA) or inhibited by salicylhydroxamide acid, suggesting a role of the mitochondrial alternative oxidase (AOX) in virus resistance by an action on the level of ROS in the cell (Maxwell et al., 1999; Murphy and Carr, 2002). AA, H_2O_2, and SA disrupt the normal cytochrome-dependent functions of the mitochondria, lowering the ATP levels and increasing the formation of ROS and AOX (Maxwell et al., 1999; Maxwell et al., 2002). AOX is also induced by pathogen attack, indicating that the same mechanism may act after virus infection (Simons et al., 1999). In addition, plant cells treated with the AA, SA, and H_2O_2 specifically express genes that are involved in programmed cell death. This supports the hypothesis that mitochondria transduce intracellular stress signals to the nucleus, leading to altered defense gene expression (Maxwell et al., 2002).

7.4 Regulation of the SA-Dependent Pathway Leading to PR-Gene Expression

An important element of the signal transduction pathway linking SA to defense responses is the ankyrin-repeat containing protein NPR1 (NON-expressor of PR)/NIM1 (NON-immunity) (Ryals et al., 1997; Cao et al., 1997). NPR1 function is essential for the induction of SAR by pathogens or SAR-inducers, for disease limitation after infection with virulent pathogens as well as for priming (Conrath et al., 2002). Race-specific resistance is modified by *NPR1* in some cases only (Cao et al., 1997; Delaney et al., 1995; Rate and Greenberg, 2001; Rairdan and Delaney, 2002). *NPR1* was found to control certain SA-dependent processes related to cell death and cell growth (Vanacker et al., 2001; Greenberg, 2000). In addition, NPR1 can act in a SA-independent pathway leading to ISR (Pieterse et al., 1998).

NPR1 is localized in the cytoplasm in the absence of SA and locates to the nucleus in the presence of SA, where it may act as transcriptional coactivator in a

protein complex (Kinkema et al., 2000; Weigel et al., 2001). NPR1 interacts with members of the TGA family of β-ZIP transcription factors (Deprès et al., 2000; Fan and Dong, 2002; Zhang et al., 1999; Zhou et al., 2000) that may regulate SAR positively or negatively (Lebel et al., 1998; Pontier et al., 2001). However, not all NPR1-dependent genes that consistently cluster with *PR1* in microarray experiments have TGA factor binding sites. In fact, the WRKY factor binding site is the overrepresented promoter element in the *PR1* gene cluster (Maleck et al., 2000).

The *NPR1* gene is induced after pathogen infection or SA treatment via SA-inducible members of the family of WRKY DNA-binding proteins (Robatzek and Somssich, 2002; Yu et al., 2001). Overexpression of the WRKY18 transcription factor leads to a constitutive increase of PR-protein expression that causes detrimental effects to plant growth (Chen and Chen, 2002; Robatzek and Somssich, 2002). In contrast, overexpression of *NPR1* itself leads to enhanced resistance to *P. syringae* and *P. parasitica* without leading to constitutive PR protein expression and detrimental effects (Cao et al., 1998; Friedrich et al., 2001). *NPR1* overexpression also results in an enhanced effectiveness of fungicides making concepts for combination of transgenic and chemical approaches for durable resistance attractive (Friedrich et al., 2001). Interestingly, overexpression of the *Arabidopsis NPR1* gene in rice leads to rice blast resistance, indicating that the signal transduction pathway of disease resistance is conserved between monocots and dicots (Chern et al., 2001). The search for suppressors of *NPR1/NIM1* identified the novel nucleus-localized SNI1 protein that may act as a negative regulator of SAR in wild-type plants (Li et al., 1999).

Several positive regulators of the SA-dependent pathway have been identified, such as EDS1, PAD4, NDR1, and EDS4. EDS1 and PAD4 are two proteins of unknown function containing a lipase-domain that are essential for the resistance to *P. syringae* and *P. parasitica* mediated by proteins of the TIR-NB-LRR resistance proteins (Falk et al., 1999; Feys et al., 2001; Jirage et al., 1999). The regulation of SA accumulation might require an interaction of EDS1 with PAD4 (Feys and Parker, 2000). EDS1 is necessary for the transcriptional regulation of PAD4 and both proteins are necessary for the expression of *EDS5* leading to accumulation of SA after pathogen attack and exposure to UV-C light (Nawrath and Métraux, 1999, Zhou et al., 1998). The expression of *EDS1* and *PAD4* can also be upregulated by SA; and a positive feedback loop was postulated to amplify the SA pathway (Falk et al., 1999; Jirage et al., 1999).

NDR1, a small protein containing a membrane-spanning domain, is required for resistance mediated by most R-genes of the CC-NB-LRR class (Century et al., 1997; Aarts et al., 1998). Thus, NDR1 defines a different pathway than EDS1. NDR1 contributes quantitatively to resistance depending on the respective R-gene. For example, the ability to induce cell death depends strongly on NDR1 when the RPS2 pathway is triggered; this dependence is weaker when the RPM1 pathway is activated (Century et al., 1997; Tornero et al., 2002). A link between ROS and SA production was observed in the *ndr1* mutant: SA accumulation and SAR are impaired in *ndr1* after inoculation with *P. syringae*

carrying the *avrRpt2* gene, or after treatment with ROS (Shapiro and Zhang, 2001).

Negative regulators of the SA-pathway may be identified among the large number of mutants that have constitutive PR1 or PR2 expression, high levels of SA, and an increased resistance to virulent strains of *P. syringae* and *P. parasitica*. In general, these mutants are smaller than wild-type plants and many of them also develop spontaneously HR-like lesions, as reviewed in Métraux and Durner, (2004). For example, CPR proteins act at the beginning of the SA-signaling cascade upstream of EDS1 and PAD4 and regulate defense pathway in different ways, i.e., the dwarfism may be dependent on SA, as in *cpr1*, or independent of SA, as in *cpr6* (Clarke et al., 2000; Jirage et al., 2001; Clarke et al., 2001). CPR5 also acts in the senescence pathway as well as in trichome development and has thus a very pleiotropic effect, possibly leading to plant defense only indirectly (Bowling et al., 1997; Boch et al., 1998; Kirik et al., 2001; Yoshida et al., 2002).

EDR1, a MAPKKK of the CTR1 family, is likely to function at the top of a MAP kinase cascade that negatively regulates SA-inducible defense response upstream of EDS1, PAD4 and NPR1 (Frye et al., 1998; 2001). Since the *edr1* mutant does not exhibit constitutive *PR1* expression, EDR1 might be a regulator of the priming response (Conrath et al., 2002).

7.5 The Integration of Salicylic Acid in a Network of Signal Processing

Besides SA, the phytohormones jasmonic acid (JA) and ethylene (ET) are two of the most important signaling molecules involved in defense-related responses. They are also involved in the expression of wound-responsive (WR) genes, some of which are likely to have protective properties against microbial infection. JA and ET mediate a variety of pathways that exhibit multiple forms of crosstalk interactions (reviewed in Pieterse and van Loon, 1999; Genoud and Métraux, 1999; Feys and Parker, 2000; Pieterse et al., this volume). For example, a concomitant activation of the JA and ET pathways is required in *Arabidopsis* for the induction of the antifungal plant defensin gene *PDF1.2* (Penninckx et al., 1998). The SA pathway also exhibits different types of crosstalks with the JA/ET pathways (reviewed in Reymond and Farmer, 1998; Genoud and Métraux, 1999; Genoud et al., 2001). The *Arabidopsis cpr5* and *cpr6* mutants, which have elevated levels of SA and express SAR constitutively, also express marker genes from the JA pathway (Bowling et al., 1997; Clarke et al., 1998). CPR5 and CPR6 regulate resistance through distinct pathways, and SA-mediated, NPR1-independent resistance involves components of the JA/ET-mediated pathways (Clarke et al., 2000). Similarly, the *ssi1* mutation, which bypasses the requirement of NPR1 for SAR function, makes the expression of PDF1.2 SA-dependent (Shah et al., 1999). Also, in *Arabidopsis*, the *eds4* and *pad4* mutations cause reduced SA levels in plants that exhibit a heightened response to inducers of JA-dependent gene expression (Gupta et al., 2000). Another form of crosstalk was observed in the *hrl1* mutant, where the expression of *PDF1.2* is

rendered partially *NPR1*- and SA/BTH-dependent. In *hrl1*, ET plays an essential role for the systemic expression of *PR1* and resistance to *P. syringae*, and an impairment in JA-signaling leads to exaggerated cell death and strong dwarfism (Devadas and Raina, 2002). In addition, a MAP kinase activity of *Arabidopsis* (MPK4) has recently been shown to control the repression of SAR. In the mutant *mpk4* plants, SAR is dependent on elevated SA levels, but is independent of NPR1. Interestingly, the activation of the JA-responsive genes *PDF1.2* and *THI2.1* was blocked in *mpk4* expressing NahG, suggesting the requirement of MPK4 in JA-responsive gene expression (Petersen et al., 2000).

Plants integrate information simultaneously received from various environmental stimuli, and from the fluctuating context of their organ-specific activities, developmental stage, and metabolic status. The plasticity in the response of the plant to its environment and to internal cues is also achieved through the use of alternative signaling pathways (Genoud and Métraux, 1999). For instance, SA-induced resistance to *P. syringae* is compromised in *eds4 Arabidopsis* plants when grown at 22°C and 85% relative humidity, but not when grown at 23°C and 50% relative humidity (Gupta et al., 2000). Interestingly, several targets of nitric oxide (NO) in animals, including guanylate cyclase and MAPKs (e.g., SIPK), are also modulated by NO in plants. This observation suggests that a crosstalk exists between a potential NO-signaling pathway and the SA pathway (Klessig et al., 2000).

Data from microarray analysis have recently proven to be invaluable to characterize *Arabidopsis* plants in the context of different environmental and developmental scenarios. Using a microarray prepared with 2,375 expressed sequence tags (ESTs) with a biased representation of putative defense-associated and regulatory genes, Schenk et al. (2000) characterized their expression levels in the plant after inoculation with an incompatible fungal pathogen, or treatment with SA, methyl-jasmonate (Me-JA) (a biologically active JA derivative), or ET. A substantial change in the steady-state abundance of 705 mRNAs was observed, out of which 169 genes were regulated by multiple treatments, with the largest number of coinduced or corepressed genes being responsive both to SA and Me-JA. In a recent study, Chen et al. (2002) confirm that SA- and JA/ET-pathways interact diversely (positively and negatively) to induce the expression or repression of transcription factors in *Arabidopsis* upon infection with bacterial pathogens (of the *Pseudomonas* species). In a related experiment, Maleck et al. (2000) examined transcriptional changes associated with the induction or maintenance of SAR by using a DNA microarray representing approximately 7,000 genes. Gene activity patterns were compared under 14 different SAR-inducing or SAR-repressing conditions; 413 ESTs exhibited differential expression equal to or greater than 2.5-fold in at least two SAR-relevant samples. Two different algorithms were used to generate a hierarchical "clustergram" and "self-organizing maps" (SOMs) to define groups of coregulated genes (Maleck et al., 2000). For instance, a molecular marker for the PR1 gene clustered in SOM c1, which contained 45 ESTs (from a maximum of 31 genes), suggesting that the genes in this regulon function in SAR. Significantly, these genes showed a unique expression profile, being strongly activated in secondary SAR tissue and dependent on NIM1/NPR1/SAI1. Furthermore,

the only cis-acting regulatory element present in all known promoters from SOM c1 is the binding site for WRKY transcription factors (W boxes: TTGAC). The authors proposed that NIM1/NPR1/SAI1 may mediate a WRKY-dependent derepression of PR1 regulon genes, or alternatively, that it may drive early expression of a subset of WRKY proteins that subsequently regulate other WRKY-dependent SAR target genes.

Such microarray-based studies illustrate the power of this technique for the analysis of complex signal transduction networks. Clearly, as this and other type of large-scale approaches are further exploited to elucidate the mechanisms controlling gene expression, it is necessary to simultaneously develop appropriate computational-based systems that will enable accurate integration and representation of the increasing amount of data being generated (Genoud et al., 2001).

It is also known that a crosstalk between the light signal transduction and the PR gene signaling pathways occurs in several plants. For instance, recent studies with *Arabidopsis* and maize mutants developing spontaneously HR lesions, and transgenic tomato expressing the R gene *Pto*, have suggested that light critically influences the formation of defensive cell death in plants (Dietrich et al., 1994; Martienssen, 1997; Tang, et al., 1999). Moreover, the light hypersensitive mutant of *Arabidopsis* (*psi2*) produces HR-like lesions and increased PR1 expression on leaves at high intensity of red light (Genoud et al., 1998). This indicates that a crosstalk exists between red light and/or far-red light perception and PR expression signaling pathways. The *psi2* mutant also exhibits a light-fluence-dependent amplification of SA-induced *PR1* gene expression.

We have confirmed the observations that light regulates sensitivity to SA by scoring the expression of PR genes in mutants containing no detectable phyA and B proteins (*phyA-phyB* double mutants; Genoud et al., 2002). In these plants, the expression of the PR genes elicited by either SA or BTH is strongly reduced, and the mutant's resistance to an ecotype-competent pathogen of the *Pseudomonas* group was significantly attenuated. In addition, the measured SA levels in the different mutants indicate that the endogenous level of SA is not modified by light, further suggesting that phytochrome activity modulates the perception of SA.

Other environmental stimuli have been linked to the control of SA production (i.e., they may modulate the SA-pathway upstream of SA production). In tobacco, ultraviolet (UV)-C light or ozone mimic the effect of necrotizing pathogens, inducing a transient increase in SA, in both exposed and unexposed leaves of the plants (Yalpani et al., 1994). This accumulation of SA is paralleled by a higher production of SA conjugate, also by the activation of a benzoic acid 2- hydroxylase, and by an accumulation of PR1. In correlation, an elevated SAR to a subsequent challenge with tobacco mosaic virus (TMV) has been observed. Hence, UV light, ozone fumigation, and TMV activate common, or redundant, signaling pathways leading to SA and PR-protein accumulation and SAR. As partial confirmation of these results, both UV-C and ozone treatment strongly induce the accumulation of SA and SA-conjugate in *Arabidopsis* (Nawrath and Métraux, 1999). Ozone- and superoxide-induced ROS and cell death are differently controlled by JA and ET, as shown in a description of an ozone-sensitive mutant of *Arabidopsis* (*rcd1*;

Overmyer et al., 2000). ET perception and signaling promote ozone-activated cell death while JA signaling might be responsible for the lesion containment. Thus, JA, ET, and SA might contribute to the response of plants submitted to high ozone exposure.

In barley, SA and aspirin were found to induce the accumulation of glycine betaine, an osmoprotectant produced in response to cold, drought, and osmotic stress (Jagendorf and Takabe, 2001), and SA added to the hydroponic growth solution of young maize plants under normal growth conditions provides protection against subsequent low-temperature stress. This last effect might result from the induction of antioxidative enzymes that lead to chilling resistance (Janda et al., 1999). In tobacco cells, two MAPKs, identified as SIPKs (SA-induced protein kinase) are activated in response to salt-induced hyperosmotic stress. One of these SIPKs is a 40 kD protein, that is specific for the hyperosmotic stress and is Ca^{2+}-and abscisic acid (ABA)-independent (Hoyos and Zhang, 2000), therefore the MAP kinase system could play the role of connecting the salt- and the SA-pathway. The interaction between ABA and SA is likely to differ depending on the branches of the pathways that interact, and also in function of the plant species. For instance, ABA suppresses the SA-dependent defense in tomato (Audenaert et al., 2002) and determines the basal susceptibility to *B. cinerea*. In the reaction controlling the protection against heat-stress in *Arabidopsis*, both ABA and SA (together with ET) have been shown to induce protective antioxidants (Larkindale and Knight, 2002). This has been observed in physiological experiments where ABA-insensitive mutant *abi1*, ethylene-insensitive mutant *etr1*, and SA-deficient plant NahG presented a reduction in heat-shock-induced antioxidant production with a correlated decrease in survival. The application of SA, of an ET generating substance, or ABA, have been shown to stimulate the survival of plants exposed to heat-shock; since calcium mimics this effect, Larkindale and Knight (2002) suggest that these crosstalks might be regulated by calcium signals.

7.6 Conclusions and Perspectives

Research on the role of SA in plants has witnessed a steady increase in interest since the first publications on the possible role of SA in the regulation of SAR in the early 1990s. Since then, the number of yearly publications on SA research has followed an increase that does not appear to slow down. This results from a wide recognition of the fundamental role of SA in plant defense and many aspects of its complex mode of action are keenly investigated.

Turning toward the future, breakthroughs will include the identification and characterization of additional signaling components in the SA pathway. For example, one target of research will be the regulatory process that controls the local and distal levels of SA. Another target will undoubtedly be the mode of action of SA itself, its putative binding site and the responses thereof. The response of plants to pathogens is far from a linear cascade of events but constitutes a complex network that integrates information from the internal and external plant environment. The

exploration of the properties of this network will be another major area of investigation. This approach will combine results of genome-wide expression analysis, proteomics, metabolomics, mutant studies, as well as bioinformatics. We foresee that computer simulations will be increasingly used to obtain a comprehensive overview of the results.

The advances in this fundamental knowledge will also have an important impact on agronomy. Discoveries of novel genes involved in various aspects of resistance will direct the conventional selection procedures toward new varieties with improved properties. Expression of such genes under inducible promoters will eventually allow the regulation of pathways for various defense reactions alone or in combination. The results obtained from studies of the network of resistance will establish the parameters to be taken into account and to be optimized in order to induce resistance using chemical inducers. Selection of biocontrol bacterial strains that enhance induced resistance of the plant will also profit from the knowledge on the network of information operating in the plant during interactions with pathogens. In summary, research on SA and plant defense will undoubtedly undergo very exciting developments both in our understanding of the related molecular and physiological processes, as well as in the direct or indirect application of this knowledge in agronomy.

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