



MINI-REVIEW

Role of hypersensitive cell death in conditioning elicitation competency and defense potentiation

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A plant's capacity for the recognition of an incompatible pathogen (mediated by non-host or race-specific resistance) leads initially to a nearly universal defense reaction, the programmed hypersensitive death of cells in immediate contact with the pathogen. This hypersensitive response (HR), in turn, is complemented by the expression of several other forms of induced resistance, including both local and systemic acquired resistance (LAR, SAR). In some species, LAR responses have been further delineated into discrete reactions that are immediately proximal or distal to the HR lesion [see, e.g., 12, 13, 37, 41]. Furthermore, an additional form of induced systemic resistance (ISR) has been described in some plants. Although not as fully characterized as SAR [19, 30, 38, 42, 43], ISR is often associated with host resistance induced by rhizosphere micro-organisms [31].

The spatial and temporal coordination of these various plant defense responses has been the subject of careful study in a number of systems. From these studies, it is clear that the regulation of the various cellular responses involves a very complex array and interplay of signals from the pathogen, the environment and from the host itself [see, e.g. 14, 41]. In the latter area, it is apparent that the host takes a very active role in the orchestration of its defense reactions through the generation of signals that both initiate and *condition* the responses of specific cell populations. Recent work in several plant species has suggested that signaling processes *associated with* or *initiated during* the HR may actively program or condition downstream LAR and SAR reactions. This coordinating

role of HR cell death is the subject of this mini-review. It is not the intent of this review to cover all instances of cellular conditioning. Rather, we will highlight emerging concepts by examining a few systems in which the phenomenon has been most thoroughly investigated.

It is important to differentiate cellular conditioning from simple synergy or enhancement of response. Cellular conditioning is an *induced event* that leads to an *altered state of responsiveness* of a cell or cell population to a defense signal or elicitor. As such, conditioning is the outcome of a signal perception and signal transduction pathway itself that precedes that involved in the elicitation of a defense response *per se*. Moreover, the conditioned state is often transient [10, 27], a phenomenon which has been suggested to play a role in the highly localized nature of some conditioned responses. Positive conditioning has been described in a number of systems and has been referred to as priming, activation, potentiation or competency. Among the most thoroughly studied examples are cellular competency phenomena in soybean and cucumber and defense potentiation in parsley.

Signals programming proximal cell competency in soybean

When soybean tissues are infected with an incompatible race of *Phytophthora sojae*, a series of phenylpropanoid defense responses are induced in a race-specific manner in healthy cells immediately adjacent (proximal) to the HR lesion [12]. While the cell wall glucan elicitor from *P. sojae* elicits an identical array of defense responses, it does so only in cells immediately proximal to wounded or HR dying cells. Thus, wounding or HR cell death program neighboring cells to enable their responsiveness to the glucan elicitor. This proximal cell conditioning is an induced and transient state that has been termed elicitation competency [10]. Importantly, since the glucan elicitor is itself a general (non race-specific) elicitor, we propose that it is the race-specific nature of HR cell death and the resulting proximal cell activation that is the

Abbreviations used in text: AOS, activated oxygen species; BTH, benzothiadiazole; CF-1, competency factor 1; CF-2 competency factor 2; DZ, daidzein; GT, genistein; HR, hypersensitive response; INA, 2,6-dichloroisonicotinic acid; ISR, induced systemic resistance; JAME, jasmonic acid methyl ester; MGD, malonyl-glucosyl daidzein; MGG, malonyl-glucosyl genistein; Nox II, Type II NADH oxidase; PR, pathogenesis-related; SA, salicylic acid; SAR, systemic acquired resistance; STPK, serine/threonine protein kinase; SKTI, soybean Kunitz trypsin inhibitor.

critical step that leads to race-specific expression of the proximal defense responses. The proximal defense responses include a rapid phenolic polymer deposition followed by phytoalexin (glyceollin) accumulation. Early work demonstrated that the phenolic polymer and glyceollin responses are differentially conditioned by two endogenous host competency factors, CF-1 and CF-2, respectively [10].

Activation of the CF-1 competent state, which favors phenolic polymer deposition, may be initiated by the release or accumulation of glutathione or homoglutathione, since application of these sulfhydryl reductants can mimic most aspects of endogenous CF-1 [14]. It was hypothesized that glutathione may act by releasing or activating an apoplastic protein with CF-1 activity. Indeed, we recently purified a GSH-released protein with CF-1 activity [34; D. S. Park, M. Y. Graham and T. L. Graham, unpublished]. Digestion with endoproteinase lys-C and mass spectral and sequence analysis of the polypeptide fragments led to its identification as variant A of the soybean Kunitz trypsin inhibitor (SKTI). Interestingly, SKTI is a member of a large super family of proteins that carry the “Kunitz domain”. The Kunitz-type trypsin inhibitors play central roles in a large number of regulatory processes in mammals and are a very active area of research with respect to cell activation, development and cellular response. Of particular relevance to this review are the various redox and ion-flux activities associated with proteins carrying the Kunitz inhibitor domain, which are consistent with SKTI’s identity as CF-1 and its association with the HR. Foremost of these reports is the fact that a protein with homology to tobacco tumor-related protein, containing a Kunitz inhibitor domain, was identified as one of several genes induced during the hypersensitive response in tobacco [20]. This is highly consistent with our hypothesis that CF-1 is associated with the HR in soybean. Several other activities have also been established for proteins carrying the Kunitz domain that could potentially relate to the HR. For example, the KTI protein aprotinin, which stimulates skeletal muscle differentiation [48], has been identified as the first competitive inhibitor of nitric oxide synthase [47]. Nitric oxide has just recently emerged as a potential signal involved in HR and/or disease resistance in plants [6, 7, 17]. Furthermore, spinach KTI has been found to function as a dehydroascorbate reductase (DHAR), a critical enzyme activity in redox regulation, when it is in the reduced (thiol) form [45]. Whether or not this activity plays a physiological role *in planta* [32], the activity is a reflection of the highly redox active sulfhydryl groups in the protein. In this reduced form, it is inactive as a trypsin inhibitor. Interestingly, the Kunitz domain has also been shown to be essential to the effects of the snake venom proteins, dendrotoxins, on voltage regulated potassium channels [16]. Although these various activities

are consistent with the association of SKTI with HR, how they relate to its role as CF-1 currently remains unclear. Intriguingly, the three dimensional structure of SKTI resembles that of interleukin-1- β and 1- α and fibroblast growth factor [33]. All three proteins have a very unusual β -trefoil structure formed by six two-stranded hairpins. Three of these hairpins form a barrel and the other three form a triangular array that caps off the barrel. An interesting parallel is that while interleukin 1 activates T lymphocyte recognition of antigens, a form of non-self recognition, CF-1 activates the recognition by soybean cells of the glucan elicitor from *P. sojae*, another example of non-self recognition.

CF-2, which activates glyceollin elicitation competency, is heat stable but chemically unstable to fractionation. Nonetheless, a few classes of reagents can induce the CF-2 state in cells. These include several redox dyes, including the singlet oxygen generator, rose bengal [T. L. Graham and R. Poling, unpublished] and several specific tetrazolium dyes, which we have used as redox buffers to “clamp” cells into various redox states [T. L. Graham and M. Y. Graham, unpublished]. The activation of the CF-2 competent state is illustrated in Fig. 1, in which minimally wounded, ‘snapped’ cotyledons have been treated with glucan elicitor alone [Fig. 1(A)] or glucan elicitor after pre-treatment with 10 nM rose bengal [Fig. 1(B)]. The distinctive red color is due to the accumulation of derivatives of trihydroxypterocarpan which are precursors of glyceollin [14].

The use of the tetrazolium dyes has allowed us to identify the target of their action, which appears to be an apoplastic peroxidase functioning as an NADH oxidase [T. L. Graham and M. Y. Graham, unpublished]. This activity was defined by Morre *et al.* as a type II NADH oxidase [2] and has thus been designated by us as Nox II. Nox II reduces molecular oxygen to produce superoxide, which is further dismutated to hydrogen peroxide in the presence of an obligatory, activating, phenolic co-factor [15]. Without the cofactor, Nox II forms an inactive complex with superoxide. Recent work has shown that the isoflavone genistein (GT) is the most potent phenolic activator yet identified for this enzyme [T. L. Graham, A. R. Rose and M. Y. Graham, unpublished]. Using pH microelectrodes we have demonstrated that activation of Nox II, in turn, causes a net alkalization of the apoplast. This alkalization, which is reminiscent of that which occurs during the HR, can as well be triggered by nigericin and orthovanadate, which are also very potent CF-2 mimics [T. L. Graham and M. Y. Graham, unpublished]. Taken together these results suggest that establishment of the CF-2 competent state involves shifts in redox and/or ionic potential similar to those occurring in the HR.

GT is present in the apoplast of soybean seedling tissues as a malonyl glucosyl conjugate [MGG, 11]. We have

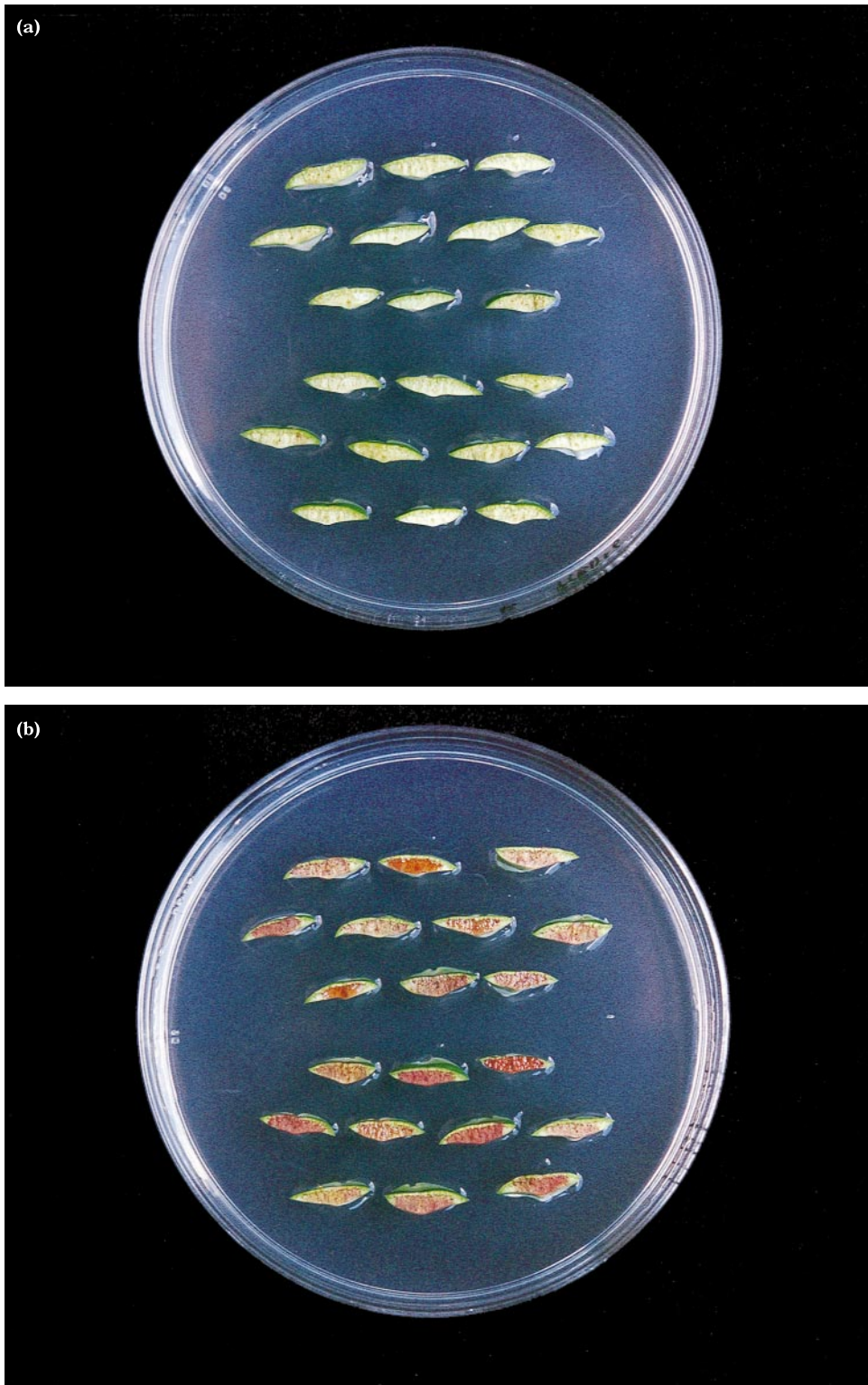


FIG. 1. Establishment of glyceollin elicitation competency in soybean. Minimally wounded cotyledon tissues were treated with either (a) glucan elicitor alone ($30 \mu\text{g}/\text{ml}^{-1}$) or with (b) glucan elicitor following pretreatment with 10 nM rose bengal. The picture was taken 20 h after treatment.

purified and characterized a highly isoflavone specific apoplastic β -glucosidase which may be responsible for the release of GT from its conjugates [18; M-C Hsieh and

T. L. Graham, unpublished]. Unconjugated GT is very susceptible to oxidative inactivation, consistent with the instability of endogenous CF-2 to fractionation. We thus

methyljasmonate or 1-aminocyclopropanecarboxylic acid to soybean cotyledon tissues indeed gives rise to the protection of cells distal from the point of application [34]. Intriguingly, the reaction of these protected tissues to compatible races of *P. sojae* is characterized by a rapid HR and is indistinguishable from an incompatible response. In contrast, salicylic acid does not lead to changes in the various phenylpropanoid defense pathways in soybean nor to local or systemic protection.

Roles of SA and jasmonic acid in potentiation of local defense in SAR responses

While the involvement of salicylic acid in the establishment of SAR is well documented [3, 5, 9, 37, 49], it also appears to play a role in the cell death program accompanying HR and additionally may be generated as an outcome of HR. More directly relevant to cellular conditioning, SA has recently been shown to potentiate elicitation of the normal array of local defense responses in SAR protected tissues. Jasmonic acid, considered a wound generated signal molecule and recently linked to ISR [35], has also been shown to have an analogous potentiating effect in some responses.

Potential role of SA in HR cell death. An analysis of various Arabidopsis cell death, lesion mimicry or necrosis mutants with and without co-expression of Nah G (which degrades SA) has been reviewed by Delaney [4]. Overall, these studies suggest that while SA is not necessary or sufficient for cell death, it may be involved in the expression of certain aspects of the cell death program. This is supported by work showing that SA strongly enhances AOS production and HR cell death in suspension cultures of soybean carrying the *Rpg2* gene for race-specific resistance to *Pseudomonas syringae* pv. *glycinea* (Psg) [39]. A separate study utilizing soybean suspension cultures [44] demonstrated that SA was essential for progression of the cell death program beyond a point between the late AOS phase (1–2 h) and the onset of cell death (5–6 h). Interestingly, the levels of SA needed to augment HR cell death are much lower than the levels needed to establish SAR and compare well with those accumulating at the site of infection with incompatible pathogens.

While part of SAR may relate to a direct systemic increase in the defense capacity of protected tissues, it is possible that the induction of normal localized defense responses to attempted infection may also be sensitized or potentiated in SAR. The most definitive work on defense potentiation in SAR has been done by examining elicitor-induced local defense responses after pre-conditioning with SA or other signal molecules.

Conditioning of elicitor responses in parsley. Responses of parsley suspension cultures to a *P. sojae* cell wall glycoprotein elicitor have proven to be a useful model system to examine these local defense responses. Potentiation in this system was first observed following pretreatment of parsley cultures with the methyl ester of jasmonic acid (JAME), which enhanced all of the characterized locally induced defense responses (formation of coumarin phytoalexins, wall esterified hydroxycinnamic acids and wall lignin-like polymers) at low elicitor concentrations [24].

Pretreatment of parsley cell cultures with derivatives of either salicylic acid (SA) or 2,6-dichloroisonicotinic acid (INA) also conditioned cells to enhance the coumarin phytoalexin and phenolic polymer responses to fungal elicitors [21, 25]. Elicitation following preconditioning also stimulated the transcriptional activation and activities of several enzymes in the biosynthetic pathway for the coumarin phytoalexins. INA, 4- or 5-chlorosalicylic acid and 3,5-dichlorosalicylic acid were more effective than other derivatives in potentiating the coumarin response.

Additional studies [22, 26] suggested that enhanced AOS production might be involved in the establishment of SA- and JAME-induced potentiation, though possibly through somewhat different mechanisms. The use of several additional pharmacological agents led to the hypothesis [25] that a complex in the plasma membrane exists that regulates hydrogen peroxide production and which requires continuous phosphorylation for function. It was further hypothesized that SA increases the activity of this system.

Finally, in a recent report from the parsley system [46], it was demonstrated that two distinct classes of genes are responsive to SA in parsley cultures. The first class, including genes encoding mannitol dehydrogenase and an anionic peroxidase, were directly responsive to relatively low levels of SA. On the other hand the induction of a second class of genes required pretreatment of cultures with SA followed by elicitor. These genes included not only genes of phenylpropanoid metabolism (PAL and 4CL), but also those for the non-SAR PR protein, PR-10, and a hydroxyproline-rich glycoprotein. Importantly, these results clearly suggest a dual role for SA in plant defense gene activation.

Conditioning of elicitor and defense responses in cucumber. Another system in which defense conditioning has been carefully studied utilizes intact cucumber seedlings. Early work demonstrated that while JAME had no effect on pathogen resistance, growing cucumber seedlings in the presence of INA, or preincubation of cut hypocotyl segments with INA, SA or 5CSA, induced resistance to *Colletotrichum lagenarium* [40]. These same hypocotyl segments also showed enhanced cell wall phenolic responses to chitosan and AOS responses to *P.*

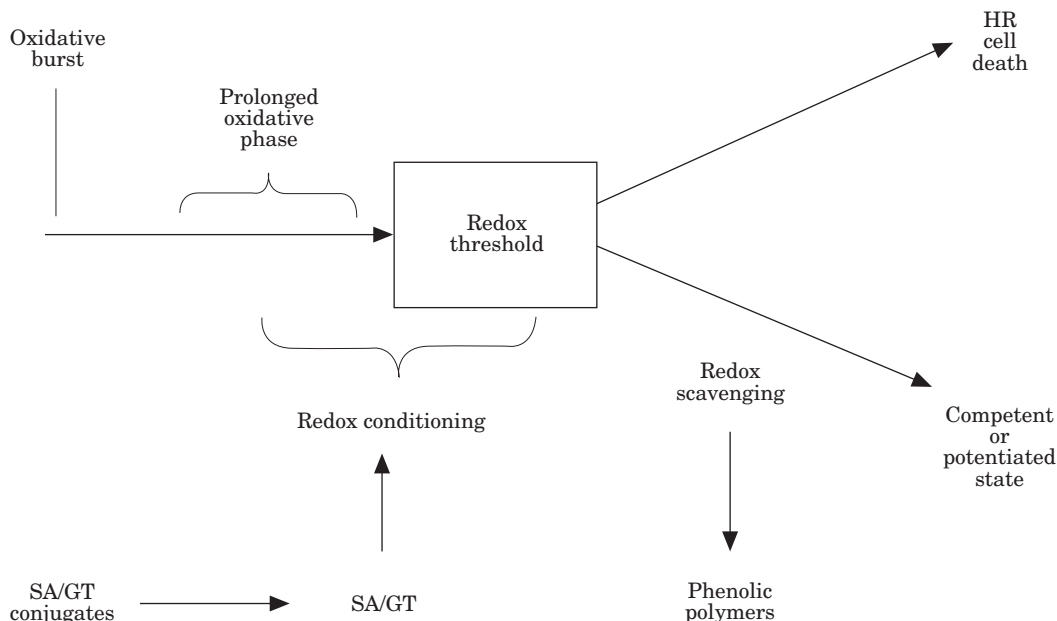


FIG. 3. General model for establishment of defense competency and potentiation. The hypersensitive cell death program progresses through the oxidative burst (occurring in minutes) and a prolonged oxidative phase (occurring over several hours) that leads to a redox threshold. The magnitude, nature and duration of the prolonged oxidative phase and the redox threshold is conditioned by SA or GT, as noted in the text and Fig. 2. With sufficient magnitude and duration, the prolonged oxidative phase leads to HR cell death. Redox scavenging, through the dismutation of superoxide and/or the utilization of hydrogen peroxide for phenolic polymer deposition “rescues” the cell from cell death and leads to the elicitation competent or defense potentiated state.

*soj*ae elicitor preparations. Further work followed the levels of class III chitinase, a PR protein, and its mRNA prior to and following infection with *C. lagenarium* after root pre-treatment of intact seedlings with INA [28]. It was demonstrated that while treatment with INA alone led to little chitinase accumulation, chitinase and its mRNA increased strongly after infection of the SAR protected plants.

In other studies, a *P. soj*ae elicitor preparation has been used on gently abraded hypocotyls. Induction of chitinase was only seen if elicitor was co-applied with INA or BTH or following long-term pretreatment of roots with INA. Thus, in cucumber, both wall phenolic and PR protein responses are potentiated in INA pre-treated plants. Further studies in cucumber [8, 27] showed that the gentle abrasion of hypocotyls induced a competent state for elicitation similar to the wound-induced competent state described earlier in the soybean system [10]. In cucumber, wounding induced competence for the production of hydrogen peroxide in response to treatment with ergosterol, chitosan, mastoparan and glucan elicitors from *P. soj*ae. In this case, as with soybeans, the establishment of this wound-associated competency was an all or nothing and transient response. Competency was further enhanced by application of INA and SA [27]. The peroxide response was also greatly enhanced in hypocotyl segments expressing a resistance response to *C. lagenarium*

induced by pretreatment of roots with INA [8]. The induced competence to elicitor was inhibited by cycloheximide or puromycin, suggesting a need for new protein synthesis.

Yet further work [H. Kauss, B. Kastner, R. Tenhaken and J. Becker, unpublished] has demonstrated that the abrasion-induced elicitation competency of hypocotyl tissues could be induced on whole plants and that optimal conditioning occurred after 4 h, rapidly falling off by 6–8 h. Again, this process is completely suppressed in the presence of protein synthesis inhibitors and also by lanthium ions and the protein kinase inhibitor, K-252a. Competence could also be partially induced by germinating spores of an albino mutant of *C. lagenarium* that cannot penetrate host tissues. Differential display results suggest that similar sets of mRNAs are induced by abrasion or spore germination and attempted infection (H. Kauss, personal communication). This suggests that wounding may mimic some early signaling response initiated by spore germination and attempted penetration.

Thus, in cucumber, potentiation of three different components of defense (wall phenolics, class III chitinase and AOS) elicited by three divergent elicitors (chitosan, *P. soj*ae glucan and cutin monomers) has been demonstrated. Wounding was also seen to induce elicitation competency. Pretreatment of plants or co-application of INA with elicitors also potentiates these various responses.

Common themes in defense conditioning

Although there are some obvious differences that emerge from the above studies, some very intriguing common themes are apparent. First is the fact that two phenylpropanoid metabolites, genistein and salicylic acid, play key signaling roles in the establishment of competency or potentiation. While SA is a metabolite common to many plants, GT is very narrowly distributed in plants, which opens the interesting possibility of evolutionary hierarchies of such signal molecules that control more or less universal or host-specific responses. Secondly, both SA and GT are “generated” during HR and both have been proposed to play a role in a discrete event in the cell death program associated with HR. While GT seems to be “generated” through release from preformed conjugates, definitive studies of a parallel turnover process in SA metabolism are lacking, although SA can be found in conjugated form. The responses conditioned by SA and GT are also similar in that they both cause a strong, but transient, potentiation of local defense responses, including the accumulations of hydrogen peroxide, phenolic polymers and phytoalexins. Finally, with both SA and GT, the mechanism of induction of potentiation or competency is hypothesized to involve regulation of AOS species, in particular the generation of hydrogen peroxide. In our lab, we hypothesize that competent cells are those which have entered into the AOS HR cell death program, but which are rescued by specific scavenging events including superoxide dismutation and phenolic polymer deposition. We hypothesize that cellular rescue is necessary for these cells to carry out the complex metabolic functions associated with local non-HR defense responses. Since many of these same events occur in SA induced potentiation and competency, it is intriguing to consider the general overall model presented in Fig. 3.

It is also very intriguing that both salicylic acid and genistein are potent signal molecules in mammalian systems. While the analgesic, anti-inflammatory and antipyretic activities of aspirin are very well known, genistein is a very potent phytoestrogen and shows selective binding affinity for mammalian estrogen receptors [29].

Finally, it is becoming clear that at least two distinct signaling pathways may lead to systemic resistance, a salicylic acid pathway leading to SAR [3, 5, 9, 37, 49] and a jasmonate/ethylene pathway leading to ISR [31, 35]. It is intriguing that both SA and JAME can also lead to defense potentiation, suggesting that local defense potentiation may play a major role in SAR and ISR. Also intriguing is the fact that while SAR and ISR are fairly highly conserved in plants, soybeans appear to have only the ISR response and may have “substituted” a local and distal GT mediated potentiation response for the SA mediated response seen in most plants. It is intriguing to

ask whether SA and GT are just representative of a hierarchy of secondary product signals that program discrete developmental or stress related responses in plants.

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