Induced resistance in plants against insects and diseases
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Priming as a mechanism behind induced resistance against pathogens, insects and abiotic stress

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Abstract: Upon treatment with a resistance-inducing agent, plants acquire an enhanced defensive capacity that results in a faster and/or stronger defence reaction at the moment the plant is exposed to biotic or abiotic stress. This phenomenon is commonly known as priming and has been associated with different forms of induced resistance. Priming accelerates and increases the plant's ability to activate the defence that is best adapted to resist the stress situation encountered. Under conditions of disease pressure, primed plants exhibit a higher fitness than non-primed plants or defence-expressing plants. Hence, the benefits of priming outweigh its costs in environments where disease occurs. Although priming has been known to occur in plants for decades, most progress in the understanding of this phenomenon has been made over the past few years. Recent insights in the mechanisms behind systemic acquired resistance (SAR), β-amino-butyric acid-induced resistance (BABA-IR), rhizobacteria-mediated induced systemic resistance (ISR), and volatile organic compound-induced resistance (VOC-IR) against insects have revealed various priming mechanisms that protect against different stresses. Whereas SAR and BABA-IR are associated with priming for salicylate (SA)-dependent defence that acts against biotrophic pathogens, ISR and VOC-IR seem to function through priming for jasmonate (JA)-dependent defence against pathogens and insects. Expression of BABA-IR and ISR against pathogenic fungi and oomycetes is also associated with an augmented formation of callose-rich papillae. This priming response depends on a largely unknown defence pathway, which involves abscisic acid (ABA) and phosphoinositide (PI) signalling, and is thought to target the cellular secretory pathway. Induction of the primed state may be mediated by an enhanced accumulation of signalling compounds, such as transcription factors (TFs) that remain inactive until the plant is exposed to stress. A Q-PCR-based transcription profiling of ~2.200 TF genes in *Arabidopsis* has revealed consistent changes in the expression of certain TF genes directly upon activation of ISR and BABA-IR. We are currently investigating the contribution of these transcription factors to the various priming responses.

Key words: priming, induced resistance, pathogens, insects, abiotic stress, maize, *Arabidopsis*.

Induced resistance in plants: distinct responses controlled by partially different signalling pathways

Plants have the ability to increase their level of basal resistance against future pathogen attack upon appropriate stimulation. This phenomenon is known as induced resistance. Based on differences in signalling pathways and spectra of effectiveness, different types of induced resistance have been defined. The classic form of induced resistance is referred to as systemic acquired resistance (SAR), and occurs in systemic plant parts upon localized infection by a necrosis-inducing pathogen (Ryals *et al.*, 1996). SAR is controlled by a signalling pathway that depends on endogenous accumulation of SA and the defence regulatory protein NPR1 (Dong, 2004) and is predominantly effective against biotrophic pathogens (Ton *et al.*, 2002). Selected strains of non-pathogenic rhizobacteria can also induce systemic resistance. This form of induced resistance is often referred to as induced systemic resistance (ISR; Pieterse *et al.*, 1996; Van Loon *et al.*, 1998). In *Arabidopsis*, ISR triggered by *Pseudomonas fluorescens* WCS417r functions independently of SA, but requires an intact NPR1 protein and sensitivity to JA and ethylene (Pieterse *et al.*, 1998). This form of ISR has a different spectrum of effectiveness than SAR and is predominantly effective against pathogens and insects that are sensitive to JA- and ET-dependent basal resistance (Ton *et al.*, 2002). A third type of induced resistance is activated upon application of the chemical *ß*-amino-butyric acid (BABA). The signalling pathway controlling BABA-induced resistance (BABA-IR) partially differs from that of SAR and ISR. Although BABA-IR against *Pseudomonas syringae* pv. *tomato* depends solely on SA and NPR1 (Zimmerli *et al.*, 2000), the BABA-IR against pathogenic fungi and oomycetes is controlled by a different defence pathway involves ABA- and phosphoinositide (PI)-dependent signalling (Ton and Mauch-Mani, 2004; Ton *et al.*, 2005). BABA-IR is effective against both biotrophic and necrotrophic pathogens, as well as some types of abiotic stress (Zimmerli *et al.*, 2000; Jakab *et al.*, 2001; Zimmerli *et al.*, 2001; Cohen, 2002; Ton and Mauch-Mani, 2004; Jakab *et al.*, 2005; Ton *et al.*, 2005). Finally, there is increasing evidence that volatile organic compounds (VOCs) that are emitted by plants upon insect infestation have the ability to induce resistance in neighbouring plants against future attack by insects and pathogens (Kishimoto *et al.*, 2005; Baldwin *et al.*, 2006). In *Arabidopsis* this VOC-induced resistance (VOC-IR) requires intact JA signalling (Kishimoto *et al.*, 2005).

The paradox of induced resistance: broad-spectrum resistance without induction of defence

For a long time, it was assumed that protection by induced resistance is based on a direct activation of defences upon application of the resistance-inducing agent. Accumulation of pathogenesis-related proteins (PRs) is an example that occurs directly upon treatment with a SAR-inducing agent. However, the suggested contribution of PRs to resistance is uncertain, and appears insufficient to explain the broad-spectrum protection by SAR (Van Loon, 1997). Moreover, both ISR and BABA-IR are not associated with direct activation of defence-related genes (Van Loon *et al.*, 1998; Jakab *et al.*, 2001). Furthermore, the phenomenon of VOC-IR has been controversial for many years, because the volatiles were only capable of triggering significant defence in neighbouring plants if applied in unnaturally high concentrations (Dicke *et al.*, 2003). Together, these results strongly suggest that most forms of induced resistance are not based on a direct activation of defence mechanisms by the resistance-inducing agent.

Priming during induced resistance

Upon pathogen infection, insect infestation, or abiotic stress exposure, plants expressing induced resistance can develop a faster and stronger activation of inducible defence responses. This capacity for augmented expression of induced defence is called priming (Conrath *et al.*, 2002). Since the first systematic investigation of priming in plant cell suspension cultures by Kauss et al. (1992), many different examples of priming have been reported in plants (Conrath *et al.*, 2006). In tobacco, (Mur *et al.*, 1996) showed that SAR-inducing amounts of SA primed for augmented expression of pathogen-inducible genes that were not responsive to SA itself. In addition, Kohler *et al.* (2002) have demonstrated that SAR-induced *Arabidopsis* activates the defence-related *PAL* gene to much higher levels after infection by *P. syringae* than non-induced control plants. The first evidence that priming plays a role in rhizobacteria-mediated ISR came from experiments with carnation (*Dianthus caryophyllus*), where induction treatment with *Pseudomonas fluorescens* WCS417r mediated an accelerated rise in phytoalexin levels upon inoculation with *Fusarium oxysporum* f.sp. *dianthi* (Van Peer *et al.*, 1991). In *Arabidopsis*, it was subsequently demonstrated that treatment with the same rhizobacteria does not directly activate defence-related genes in *Arabidopsis*, but confers a priming for enhanced expression of JA- and ET-inducible genes upon infection by *P. syringae* (Van Wees *et al.*, 1999; Verhagen *et al.*, 2004). In addition to SAR and ISR, the BABA compound has been demonstrated to act as a very potent primer for different plant defences. In *Arabidopsis*, this compound not only primes for SA-inducible *PR-1* expression (Zimmerli *et al.*, 2000; Zimmerli *et al.*, 2001), but also for enhanced formation of callose-rich papillae (Ton and Mauch-Mani, 2004; Ton *et al.*, 2005). Recently, VOC-induced resistance has been associated with priming as well. Engelberth *et al.* (2004) showed that maize plants pre-exposed to green-leafy volatiles (GLVs) from neighbouring plants exhibit an augmented production of JA, and an enhanced emission of defence-related VOCs after subsequent defence elicitation by applying caterpillar regurgitant on wounded leave areas.

The above-mentioned examples illustrate that priming is a common phenomenon that takes pace during most types of induced resistance. Because the defence arsenal in primed plants remains inactive until the plant is exposed to the defence-inducing stress, priming provides a plausible explanation for the various findings that induced resistance takes place without direct induction of defence mechanisms.

Priming during BABA-IR

The research on BABA as an inducer of resistance in *Arabidopsis* has served as a model to investigate the physiological and molecular mechanisms behind priming in plants. Although BABA occurs rarely in nature, the compound's resistance-inducing efficacy has been demonstrated in many different plant species against microbial pathogens (Jakab *et al.*, 2001; Cohen, 2002), nematodes (Oka *et al.*, 1999), aphids (Hodge *et al.*, 2005), and abiotic stress (Jakab *et al.*, 2005). In *Arabidopsis* the BABA-induced priming response partially resembles that of SAR. For example, the BABA-induced priming for enhanced *PR-1* expression upon infection with *P. syringae* is comparable to the priming for SA-inducible defences upon SAR induction (Zimmerli *et al.*, 2000). However, the observation that BABA still induced resistance against the oomycete *Hyaloperospora parasitica* in SA-nonaccumuating NahG and SA-insensitive *npr1* plants suggested a novel resistance mechanism, which may be based on an accelerated formation of callose-rich papillae at the sites where the pathogen attempted to penetrate the epidermal cell layer. This correlation between BABA-induced resistance and

augmented callose deposition was further studied in the interaction with two necrotrophic fungi, *Alternaria brassicicola* and *Plectospherella cucumerina*, both of which are unaffected by expression of SA-inducible defences (Ton and Mauch-Mani, 2004). Again, the expression of BABA-IR coincided with an augmented formation of callose-rich papillae. A causal correlation between the observed callose deposition and disease resistance was revealed in experiments with the callose synthesis inhibitor 2-deoxy-D-glucose (2-DDG). The authors showed that 2-DDG significantly reduces BABA-induced protection against *A. brassicicola*. Moreover, the callose-deficient mutant *pmr4-1* (Nishimura *et al.*, 2003) was found unable to express BABA-IR against *P. cucumerina* Hence, intact callose biosynthesis is crucial for the BABA-induced protection against both *A. brassicicola* and *P. cucumerina*. Further experiments revealed that both augmented callose deposition and BABA-IR against *A. brassicicola* and *P. cucumerina* were unaffected in *Arabidopsis* mutants impaired in camalexin synthesis, JA sensitivity, ethylene-insensitity and SA signalling (Ton and Mauch-Mani, 2004). These findings suggested that the pathway controlling priming for enhanced formation of callose-rich papillae does not involve SA-, JA-, or ET-dependent signalling. The possibility of an involvement of the phytohormone abscisic acid (ABA), as observed in connection with BABA-induced tolerance to abiotic stress (Jakab *et al.*, 2005), was investigated by testing two *Arabidopsis* mutants, *aba1-5* and *abi4-1*, for their ability to express BABA-IR against *P. cucumerina*. Interestingly, both mutants failed to (*i*) establish BABA-IR against the pathogen and (*ii*) to show augmented deposition of callose after infection. These data linked ABA signalling to the regulation of BABA-induced priming for effective deposition of callose-rich papillae (Ton and Mauch-Mani, 2004). The regulatory role of ABA in BABA-IR was supported by results from experiments with the BABA response mutant *ibs3* (impaired in BABA-induced sterility). This mutant carries a T-DNA insertion in a gene encoding the ABA biosynthetic enzyme zeaxanthin epoxidase (Ton *et al.*, 2005). In the same mutagenesis screen, the *ibs2* mutant was characterized, which carries a T-DNA insertion in the 5'-untranslated region of the *AtSAC1b* gene encoding a poly-phosphoinositide phosphatase. Both mutants were found to be reduced in the ability to express BABA-IR against *H. parasitica* and salt stress, with correlated with a reduced level of BABA-induced resistance against this pathogen (Ton *et al.*, 2005). These findings further strengthen the conclusion that PI- and ABA-dependent signalling regulate BABA-mediated priming for augmented callose deposition.

The molecular mechanisms governing ABA-dependent priming for augmented callose deposition remain to be elucidated. However, a possible role for ABA in callose deposition might be deduced from research on abiotic stress responses. It has been shown that different plant SNARE proteins were involved in mediating ABA-dependent responses (Leymann et al. 1999; Zhu et al. 2002). SNAREs have also been linked to defence responses in the plant cell wall. Mutations in two SNARE-encoding genes, *PEN1* and *ROR2*, lead to partial loss of resistance which has been reflected by enhanced penetration by non-host pathogens (Collins *et al.*, 2003; Lipka *et al.*, 2005). PEN1 and ROR2 are both thought to be involved in the transport of vesicles carrying antimicrobial components and glucan (callose) synthase proteins to sites of attempted fungal penetration. Interestingly, both these SNARE genes share high homology to the tobacco *NtSyp121* gene, which is induced by ABA (Leyman *et al.*, 1999; Collins *et al.*, 2003). Together, these results point to a prominent role of ABA signalling in the control of callose deposition by transcriptional regulation of specific SNAREs during fungal pathogen attack.

Priming during rhizobacteria-mediated ISR.

In *Arabidopsis*, ISR triggered by *Pseudomonas fluorescens* WCS417r is effective against different types of pathogens (Ton *et al.*, 2002), but it is not associated with the activation of *PR* genes (Pieterse *et al.*, 1996). Although WCS417r-mediated ISR requires intact responsiveness to JA and ET (Pieterse *et al.*, 1998), there are no consistent alterations in gene expression of JA- and ET-inducible genes in the above-ground plant parts. (Verhagen *et al.*, 2004). In addition, no alterations in the production of either JA or ET could be detected either, suggesting that ISR is based on an enhanced sensitivity to these plant hormones rather than on an increase in their production (Pieterse *et al.*, 2000). Analysis of the transcriptome of ISR-expressing *Arabidopsis* leaves after challenge inoculation with *P. syringae* DC3000 revealed 81 genes with augmented expression, indicating that the plants were primed to respond to pathogen attack (Verhagen *et al.*, 2004). The majority of these priming-responsive genes was JA- and/or ET-inducible, thus confirming earlier findings that colonization of the roots by WCS417r primes *Arabidopsis* for augmented expression of the JA- and/or ET-inducible genes *AtVSP2, PDF1.2*, and *HEL* (Van Wees *et al.*, 1999; Hase *et al.*, 2003). In order to gain more insight into the mechanisms behind priming during WCS417r-mediated ISR, a whole-genome transcriptional analysis was performed of control- and WCS417r-treated plants at different time-points after treatment with methyl jasmonate (MeJA). Interestingly, all MeJA-inducible genes displaying an augmented expression upon treatment with MeJA were found to be significantly enriched in MYC2 binding sites in their promoter regions, suggesting an important regulatory role of the MYC2 transcription factor in onset of priming (M.J. Pozo and C.M.J. Pieterse; unpublished results). Preliminary results indeed support this hypothesis, as two *Arabidopsis* mutants in the MYC2 transcription factor were found to be impaired in WCS417r-mediated ISR against *P. syringae* pv. *tomato* DC3000 (S. van der Ent; unpublished results).

Table 1: Priming for callose-rich papillae in wild-type *Arabidopsis* (Col-0) and mutants *ibs2* and *ibs3*.

Treatment	**Col-0**	***ibs2***	***ibs3***
Control	30[1]	28	33
ISR[2]	53 *	29	37
Control	31	44	36
BABA[3]	74 *	36	44

[1] Data shown are mean percentages of papillae-inducing spores in the epidermal cell layer of challenged leaves at two days after inoculation with conidiospores of *Hyaloperonospora parasitica*. Callose was visualized by aniline-blue staining of the leaves and epifluorescence microscopy, as described by Ton and Mauch-Mani (2004). Asterisks indicate statistically significant differences compared to the water-treated control plants ($\alpha = 0.05$; X^2-square test)

[2] ISR was induced by transplanting *Arabidopsis* seedlings to soil containing *Pseudomonas fluorescens* WCS417r bacteria, as described by Pieterse *et al.* (1996).

[3] BABA was applied as a soil-drench to a final concentration of 70 μM.

Hence, WCS417r-mediated ISR in *Arabidopsis* is based on MYC2-dependent priming for JA-inducible defences, which strongly supports the finding that ISR is effective against pathogens that are resisted through JA-inducible defence mechanisms (Ton *et al.*, 2002). However, ISR is also effective the oomycete *H. parasitica*, which is not inhibited by JA-inducible defences in *Arabidopsis* (Thomma *et al.*, 1998; Ton *et al.*, 2002). This suggests that ISR involves additional mechanisms of resistance than priming for JA-dependent defence alone. The observation that ISR-expressing plants, like BABA-treated plants, show an augmentation in the formation of callose-rich papillae upon infection by *H. parasitica* (Table 1), suggests that ISR and BABA-IR against *H. parasticia* are based on the same priming mechanism. Indeed, mutants *ibs2* and *ibs3*, which are both affected in the BABA-induced priming for callose (Ton *et al.*, 2005), were also impaired in the priming for callose upon treatment with WCS417r bacteria (Table 1). Hence, ISR and BABA-IR share IBS2 and IBS3 as regulatory components in their priming for enhanced deposition of callose-rich papillae.

Priming during VOC-IR

The physiological and molecular mechanisms behind VOC-induced priming are largely unknown. It seems nevertheless evident that VOC-induced priming targets JA-inducible defences (Engelberth *et al.*, 2004; Kessler *et al.*, 2006). Interestingly, VOCs from *S. littoralis*-infected maize prime for enhanced expression of only a subset of JA-inducible defence genes (J. Ton and T. Turlings, unpublished results). This suggests that JA is not the only endogenous signal that controls the VOC-induced priming response. In this context, the gaseous hormone ethylene may be an important priming factor in addition to GLVs. Ethylene emission is induced upon insect attack (Schmelz *et al.*, 2003; De Vos *et al.*, 2005), and has been shown to function as an important modulator of JA-inducible defences against insects (Harfouche *et al.*, 2006; Van Loon *et al.*, 2006). Ethylene has the ability to enhance the activation of some JA-inducible genes (Penninckx *et al.*, 1998), whereas other JA-inducible genes are unaffected or even repressed by ethylene (Shoji *et al.*, 2000; Anderson *et al.*, 2004). Furthermore, Ruther and Klein (2005) recently showed that ethylene synergizes the emission of maize sesquiterpene compounds upon treatment with high doses of the GLV Z-3-hexanol. Future experiments with mutant plants that are impaired in the production or perception of ethylene could further specify the regulatory role of ethylene in VOC-induced priming. It may also be important to compare mechanistic similarities between VOC-induced resistance and WCS417r-mediated ISR resistance, as both forms of induced resistance lead to priming for JA-inducible defence mechanisms.

Transcription factors: key regulators of priming?

In many cases, priming results in enhanced transcription of defence-related genes (Thulke and Conrath, 1998; Van Wees et al., 1999; Zimmerli et al., 2000; Kohler et al., 2002). It is therefore not unthinkable that transcription factors (TFs) play an important regulatory function in the onset of priming.

Induction of the primed state may trigger an enhanced accumulation of these regulatory proteins. Because the defence mechanisms are not directly activated upon induction of the primed state, it can be assumed that these TFs remain inactive until the plant is exposed to a specific stress-derived signal. This results in that activation of a stress-specific subset of TFs, which activate the appropriate defence-related genes. Because there was an increased presence of TFs in the primed cells, the stress-induced signal transduction develops faster in the primed plant cell, resulting in a faster and stronger activation of defence-related genes

(Figure 1). Upon exposure to another type of stress, a different set of signalling proteins becomes activated, leading to an augmentation of another defence response.

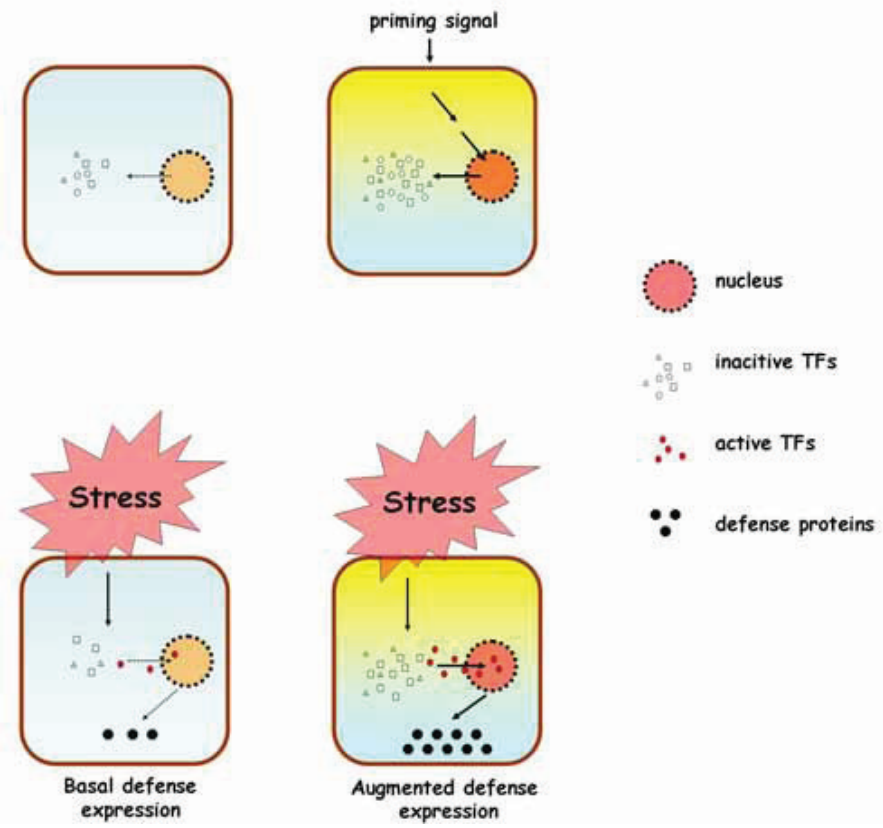

Figure 1. Model of priming for defence. In the non-induced state (left), the plant cell expresses a basal level of defence-related signalling proteins, such as transcription factors (TFs). Induction of the primed state (right) triggers an enhanced expression of these signalling proteins. In both non-induced and primed cells, these TFs remain inactive until the cell is exposed to stress. After perception of a stress-derived signal, a specific subset of TFs becomes activated, triggering the appropriate basal defence response that is marked by the transcription of defence-related genes. This defence response is expressed faster and more strongly in primed cells, due to their enhanced defence signalling potential by the TFs.

To investigate the possible role of TFs in priming during BABA-IR and ISR, the expression of ~2.200 TF genes were profiled in *Arabidopsis* upon treatment with WCS417r bacteria and BABA using real-time reverse-transcriptase PCR. To distinguish between SA-dependent and SA-independent priming mechanisms upon treatment with BABA, the *npr1-1* mutant was included, which had previously been shown to be impaired in SA-dependent priming but still capable of developing SA-independent priming for callose (Zimmerli *et al.*, 2000). Preliminary data indicate that induction of ISR and BABA-IR indeed stimulates the expression of more than 250 different defence-related TF genes. Currently, effort is being put in comparing the transcriptional patterns of these genes to those in *Arabidopsis* mutants that are impaired in specific priming responses. The biological function of the most interesting

candidate TF genes will be further investigated by studying priming phenotypes in knock-out mutants or over-expression lines of the corresponding genes.

Conclusions

Priming for enhanced defence against biotic and abiotic stress is operating via various pathways and requires specific cellular signalling components. Since the discovery of priming in plants in the 1980s, the phenomenon has been demonstrated in different plant species against pathogens, insects, and abiotic stress. Hence, priming appears to be a common feature of the plant's immune system that offers protection against wide spectrum of environmental stresses. Additionally, it has been demonstrated that various priming responses require specific cellular components (Conrath *et al.*, 2002; Ton *et al.*, 2005), which points to a specific regulation mechanism that is exclusively dedicated to priming. The emerging picture today is that priming accelerates and increases the plant's ability to activate the defence mechanism best adapted to protect against a defined stress situation. In this perspective, priming represents an important adaptation of plants to cope with environmental stress.

Priming also fits in the ecological context of induced resistance. Various studies have demonstrated that activation of inducible defences involves major costs that affect plant growth and reproduction (Heil, 2002). In case of JA-inducible defences, the costs are only affordable when the plant is actually exposed to attack by herbivores (Agrawal, 1998; Baldwin, 1998). Van Hulten et al. (2006) recently demonstrated that the fitness costs of priming in *Arabidopsis* are substantially lower than those of directly induced defence against pathogens. In addition, it was shown that the benefits of priming outweigh its costs when disease occurs. Consequently, plants in the primed state are efficiently protected against a broad spectrum of stresses without major trade-off effects on commercially and ecologically important traits, such as growth and seed set. Hence, priming is the plant's solution to the trade-off dilemma between disease protection and costs involved in direct defence activation.

Priming in plants shows phenotypic similarity to potentiation phenomena during inducible defence reactions in animals and humans, suggesting that the mode of action of priming may be of great advantage for living organisms. While the lymphocyte-based immune system in humans and animals constitutes an important adaptive defence strategy in humans and animals, priming during induced resistance may well represent an important adaptive defence strategy in plants. Apparently, adaptive immunity and induced resistance use priming as a common mechanism by which complex organisms acquire sustainable protection against environmental stress.

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