Induced Plant Defense Responses against Chewing Insects. Ethylene Signaling Reduces Resistance of Arabidopsis against Egyptian Cotton Worm But Not Diamondback Moth[1]

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The induction of plant defenses by insect feeding is regulated via multiple signaling cascades. One of them, ethylene signaling, increases susceptibility of Arabidopsis to the generalist herbivore Egyptian cotton worm (*Spodoptera littoralis*; Lepidoptera: Noctuidae). The *hookless1* mutation, which affects a downstream component of ethylene signaling, conferred resistance to Egyptian cotton worm as compared with wild-type plants. Likewise, *ein2*, a mutant in a central component of the ethylene signaling pathway, caused enhanced resistance to Egyptian cotton worm that was similar in magnitude to *hookless1*. Moreover, pretreatment of plants with ethephon (2-chloroethanephosphonic acid), a chemical that releases ethylene, elevated plant susceptibility to Egyptian cotton worm. By contrast, these mutations in the ethylene-signaling pathway had no detectable effects on diamondback moth (*Plutella xylostella*) feeding. It is surprising that this is not due to nonactivation of defense signaling, because diamondback moth does induce genes that relate to wound-response pathways. Of these wound-related genes, jasmonic acid regulates a novel β-glucosidase 1 (*BGL1*), whereas ethylene controls a putative calcium-binding elongation factor hand protein. These results suggest that a specialist insect herbivore triggers general wound-response pathways in Arabidopsis but, unlike a generalist herbivore, does not react to ethylene-mediated physiological changes.

Resistance or tolerance of plants to insect herbivores and pathogens is mediated via constitutive or induced defense mechanisms (Mauricio et al., 1997; Buell, 1998). Inducible defenses play a major role in conferring disease resistance against plant pathogens (Maleck and Dietrich, 1999), and their effects on phytophagous insects can include increased toxicity, delay of larval development, or increased attack by insect parasitoids (Baldwin and Preston, 1999). Inducible defenses are thought to compromise plant fitness less, and maybe more durable, than constitutive defense mechanisms (Agrawal, 1998).

During their evolution, specialist herbivores have explored new ecological niches and adapted to novel plant chemical defenses (Ehrlich and Raven, 1964). It is therefore not surprising that specialist herbivores are frequently attracted to secondary metabolites from their hosts. For instance, glucosinolates and their hydrolysis products are feeding and oviposition attractants for crucifer specialists (Gupta and Thorsteinson, 1960; Hicks, 1974), but deterrents for nonadapted insects (McCloskey and Isman, 1993). Specialist herbivores frequently detoxify or sequester plant defense compounds. The latter form of adaptation can even result in protection against parasitoids and predators. Differences in metabolism of plant toxins may be one reason why some induced defenses protect against generalist, but not specialist insect herbivores (Agrawal, 1999).

Several signaling pathways, including jasmonic acid (JA), salicylic acid (SA), ethylene, and perhaps hydrogen peroxide (H_2O_2; Reymond and Farmer, 1998) orchestrate the induction of defenses. The signaling molecule SA is crucial for local hypersensitive responses and systemic acquired resistance against many plant pathogens (Maleck and Dietrich, 1999). Resistance against herbivorous insects and some fungal pathogens depends on wound-response signaling via JA and ethylene (Maleck and Dietrich, 1999). In essence, tissue damage caused by insect feeding activates an octadecanoid signaling cascade that culminates in JA biosynthesis and production of antifeedant proteinase inhibitors (PIs; Broadway et al., 1986) and other putative defense molecules. Mutations that reduce JA production result in increased susceptibility to herbivores. For example, a tomato mutant unable to convert 13-hydroperoxylinolinic acid into 12-oxo-phytodienoic acid, *def1*, does not accumulate PIs

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in response to wounding and is significantly more susceptible to tobacco hornworm than wild-type plants (Howe et al., 1996). Similarly, an Arabidopsis triple mutant (*fad3-2 fad7-2 fad8*) also lacks wound-induced JA biosynthesis, and as a consequence is more susceptible to fungal gnats (McConn et al., 1997).

Unlike mechanical wounding, insect-derived elicitors are capable of inducing the emission of plant volatiles that attract predators and parasitoids to attack insect herbivores (Mattiacci et al., 1995; Alborn et al., 1997). In lima bean plants JA-induced volatile blends are similar to those induced by spider mites. However, predatory mites prefer plants that are attacked by spider mites to chemically induced plants when given the choice (Dicke et al., 1999). Thus in addition to JA, there are insect-specific signals leading to predator attraction. By contrast, JA-related defense pathways appear to be sufficient to reduce insect herbivory by increasing caterpillar parasitism in the field (Thaler, 1999), suggesting that JA is a major, but not the only component of induced defenses. In addition, insect feeding or application of gut regurgitants from hawkmoth larvae can alter gene expression, for instance, accelerating PI mRNA induction relative to mechanically wounded leaves (Korth and Dixon, 1997). Thus mechanical wounding alone cannot explain all of the physiological and biochemical changes that occur in response to insect attack.

The phytohormone ethylene is another wound-response regulator. Inhibitor studies suggest that JA- or wound-induced PI mRNA accumulation depends on ethylene (O'Donnell et al., 1996). Similarly, the *ein2* mutation of Arabidopsis blocks JA-induction of defensin (*PDF1.2*) mRNA accumulation (Alonso et al., 1999). However, antagonistic interactions between JA and ethylene regulate the antifeedant plant lectin *GS-II* in locally wounded leaves (Zhu-Salzman et al., 1998). It is significant that hawkmoth feeding results in a rise in ethylene biosynthesis that reduces JA-induced nicotine biosynthesis in *Nicotiana attenuata*, thus diminishing plant defenses (Kahl et al., 2000). In addition, SA interferes with wound-related gene expression by inhibiting the octadecanoid pathway (O'Donnell et al., 1996; Peña-Cortés et al., 1993). SA-mediated defense against pathogens apparently can lead to an increase in insect susceptibility, and vice versa (Felton et al., 1989; Stout et al., 1999). Nevertheless, spider mites cause lima bean plants to emit significant amounts of methyl-SA, in addition to JA-related volatiles (Dicke et al., 1999), suggesting that both signaling pathways operate together in that species. Perhaps the balance between different signaling pathways adjusts defense characteristics against particular insects or pathogens.

We are interested in mechanisms and regulation of plant resistance to generalist and specialist insect herbivores. Arabidopsis provides a genetically tractable model system to analyze the functional basis of plant resistance against insect herbivores. Information on many resistance mechanisms may be extrapolated from Arabidopsis to other plant species (Mitchell-Olds, 1999). It is necessary to discover the genes that are regulated by insect feeding because defense gene expression contributes to induced resistance against herbivores (Bergey et al., 1996). This paper reports the expression of plant genes that are induced by diamondback moth (*Plutella xylostella*) feeding and regulated by distinct signaling pathways. Moreover, we assessed whether mutations in the ethylene-signaling pathway alter resistance against specialist (diamondback moth) and generalist (Egyptian cotton worm [*Spodoptera littoralis*]) herbivores.

RESULTS

Characterization of Plant Gene Expression after Insect Herbivory or Mechanical Wounding

To better characterize plant responses to insect herbivory, we performed a differential gene expression screen (differential display) in Arabidopsis. Partial characterization of six genes from the differential display analysis revealed distinct patterns of regulation. We compared the effects of herbivory versus mechanical wounding on the expression of these genes, because tissue damage caused by insect chewing is known to serve as a cue for plant defense. The induction of *LOX2* and *VSP* by wounding (Bell and Mullet, 1993; McConn et al., 1997) and diamondback moth herbivory was expected from previous publications (Fig. 1A). A novel β-glucosidase 1 (*BGL1*; Fig. 1A), as well as *GST2*, *GST6*, and a putative calcium-binding elongation factor (EF) hand protein (*CaEF*) have not previously been associated with insect attack. All these genes were induced in rosette leaf tissues as a consequence of diamondback moth feeding (Fig. 1, A and B). Patterns of gene expression differed among these genes and between herbivory versus wounding treatments, suggesting that these genes were subject to separate regulation. Whereas the mRNA abundance of *VSP*, *LOX2*, *BGL1*, and *CaEF* increased more than 5-fold after 10 h of diamondback moth feeding, *GST* expression changed much less. For instance, *GST6* mRNA increased approximately 4-fold after herbivory and about 3-fold after wounding. The induction of *VSP*, *LOX2*, *BGL1*, and *GST6* after insect feeding persisted longer than after wounding, which might merely reflect the continuing tissue damage caused by diamondback moth herbivory. By contrast, the expression of *CaEF* was transient despite continuous insect feeding. *GST2* showed moderate levels of induction and greater sensitivity to transient environmental variation (Fig. 1A). We did not contrast the effects of diamondback moth versus Egyptian cotton worm herbivory because mechanical wounding induced all of these genes. However, diamondback moth and Egyptian

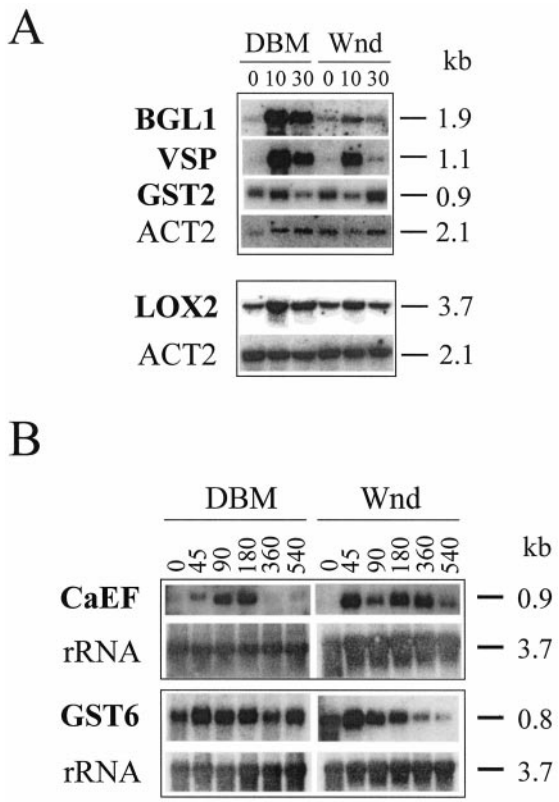

Figure 1. Regulation of Arabidopsis genes by insect feeding or wounding. Total RNA (10 μg) was extracted from rosette tissue and RNA gel blots were hybridized with probes indicated on the left. In contrast to loading controls, abbreviations of genes related to insect feeding are in bold. A, Plants were untreated, exposed to one diamondback moth (DBM) larvae per plant for 10 or 30 h, or mechanically wounded (Wnd) 10 or 30 h prior to harvest. Blots were stripped and re-probed with *ACT2*, a loading control that is constitutively expressed. Size estimates for the different mRNAs are indicated on the right. B, Plants were untreated, mechanically wounded, or diamondback moths (four larvae per plant) were applied prior to harvest at the indicated time points in minutes. Size estimates are listed on the right. A probe for 25S rRNA served as a loading control. Additional controls (not shown) found no trace of circadian or light-dependent changes in expression of these genes.

cotton worm may have contrasting effects on gene expression. The latter herbivore is known to produce volicitin, an elicitor of plant volatile emission and of indirect plant defenses (Alborn et al., 1997). Thus chemical signals from insects potentially alter the expression of the genes we analyzed as well.

JA, Ethylene, and SA Differentially Regulate Genes Induced by Wounding and Herbivory

To examine the effects of phytohormones on gene expression that relate to wounding and insect feeding in Arabidopsis, we sprayed plants with methyl-JA (MeJA), ethephon, or SA. JA is a key regulator of wound-related defense genes, such as *VSP* and *LOX2* (Fig. 2). *BGL1* mRNA was also strongly induced by MeJA. However, MeJA had little effect on either expression of *CaEF* (less than 2-fold induction) or expression of *GST2* or *GST6*. In contrast to the other genes the basal expression of *GST2* was quite variable, suggesting that *GST2* is sensitive to transient environmental variation. *GST6* and *CaEF* are wound induced (Fig. 1), suggesting that the wound-response of these genes is mediated by signals other than JA.

Ethylene is another plant hormone that participates in wound response signaling. Treatment of Arabidopsis with ethephon, a compound that slowly releases ethylene (Yang, 1969), caused reduction of *GST6* mRNA abundance (Fig. 3). *BGL1* mRNA levels showed little change in response to ethephon. Compared with the 10-fold induction by JA, there was at

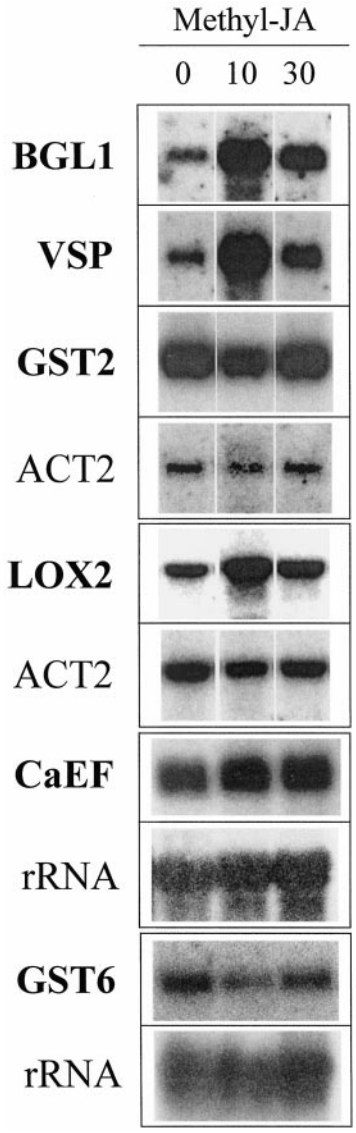

Figure 2. Regulation of stress-response genes by MeJA. Plants were untreated or sprayed with 150 μM MeJA 10 h or 30 h prior to harvest. Total RNA (10 μg) was extracted from rosette tissue and RNA gel blots were hybridized with probes indicated on the left. *ACT2* or rRNA was used as loading controls.

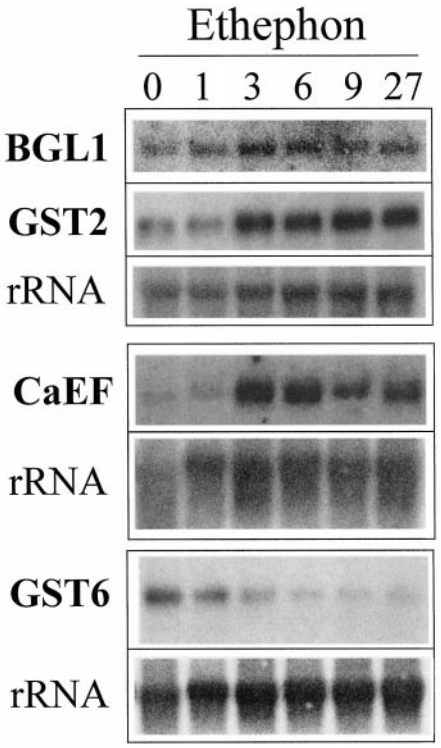

Figure 3. Regulation of stress-response genes by ethephon. Plants were untreated or sprayed with 50 μM ethephon 1, 3, 6, 9, or 27 h prior to harvest. Total RNA (10 μg) was extracted from rosette tissue and RNA gel blots were hybridized with probes indicated on the left.

most a 3-fold change in *BGL1* mRNA abundance after ethephon treatment. *CaEF* and *GST2*, two genes that were not significantly regulated by JA, were strongly induced by ethephon. It is worth mentioning that ethephon had a stronger inducing effect on *GST2* than insect feeding. Regulation by exogenous JA and ethylene appears to be negatively correlated, such that genes that respond to ethylene are not influenced by JA (e.g. *CaEF* and *GST2*) and vice versa.

SA is a signal transducer important in plant defense responses against pathogens. It caused a substantial induction of *GST2* mRNA (Fig. 4A), whereas the JA-induced genes *BGL1*, *VSP*, or *LOX2* were largely unaffected (data not shown). SA negatively regulated mRNA abundance of *CaEF* and *GST6*. Semiquantitative PCR experiments supported these RNA-blot hybridization data, suggesting that the results were specific to *GST2* and *GST6* and did not reflect confounded expression of additional gene family members (Fig. 4B). To ensure that our results were consistent with previous studies, we also confirmed SA-induction of *PR-1* (Fig. 4B), which is strongly induced by SA signaling (Uknes et al., 1992), thus demonstrating that the lack of *GST6* induction was not due to a lack of SA perception. Taken together, these results suggest that these wound-responsive genes fall into different categories based on their regulation: (a) genes that primarily respond to JA, (b) genes that essentially respond to ethylene, such as *GST2*, and (c) genes, such as *GST6*, that are regulated by other factors.

Effects of Ethylene Signaling on Insect Resistance

To estimate the contribution of a wound-signaling pathway to insect resistance, we challenged Arabidopsis mutants impaired in ethylene signaling with specialist (diamondback moth) and generalist (Egyptian cotton worm) herbivores. The amount of leaf damage in ethylene mutants or their wild-type backgrounds caused by these insects was a measure of plant resistance (Fig. 5). We specifically analyzed

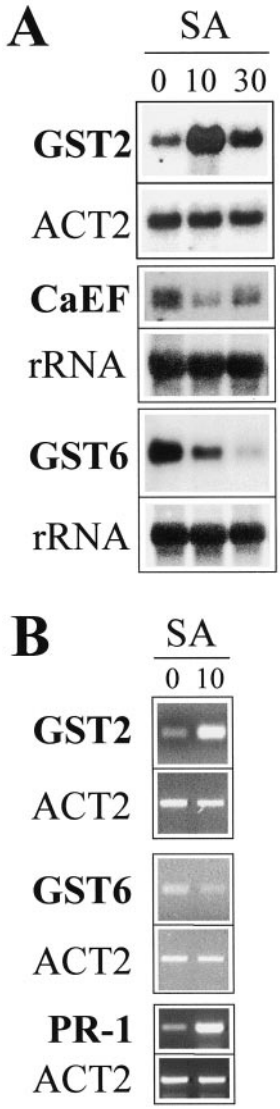

Figure 4. Regulation of stress-response genes by SA. Plants were untreated or sprayed with 5 mM SA 10 or 30 h prior to harvest. A, Total RNA (10 μg) was extracted from rosette tissue and RNA gel blots were hybridized with probes indicated on the left. B, SA-regulation of specific genes was confirmed by semiquantitative PCR. We observed more *GST2* and *PR-1* product upon SA treatment than in controls after 25, 27, or 29 PCR cycles, suggesting a real difference.

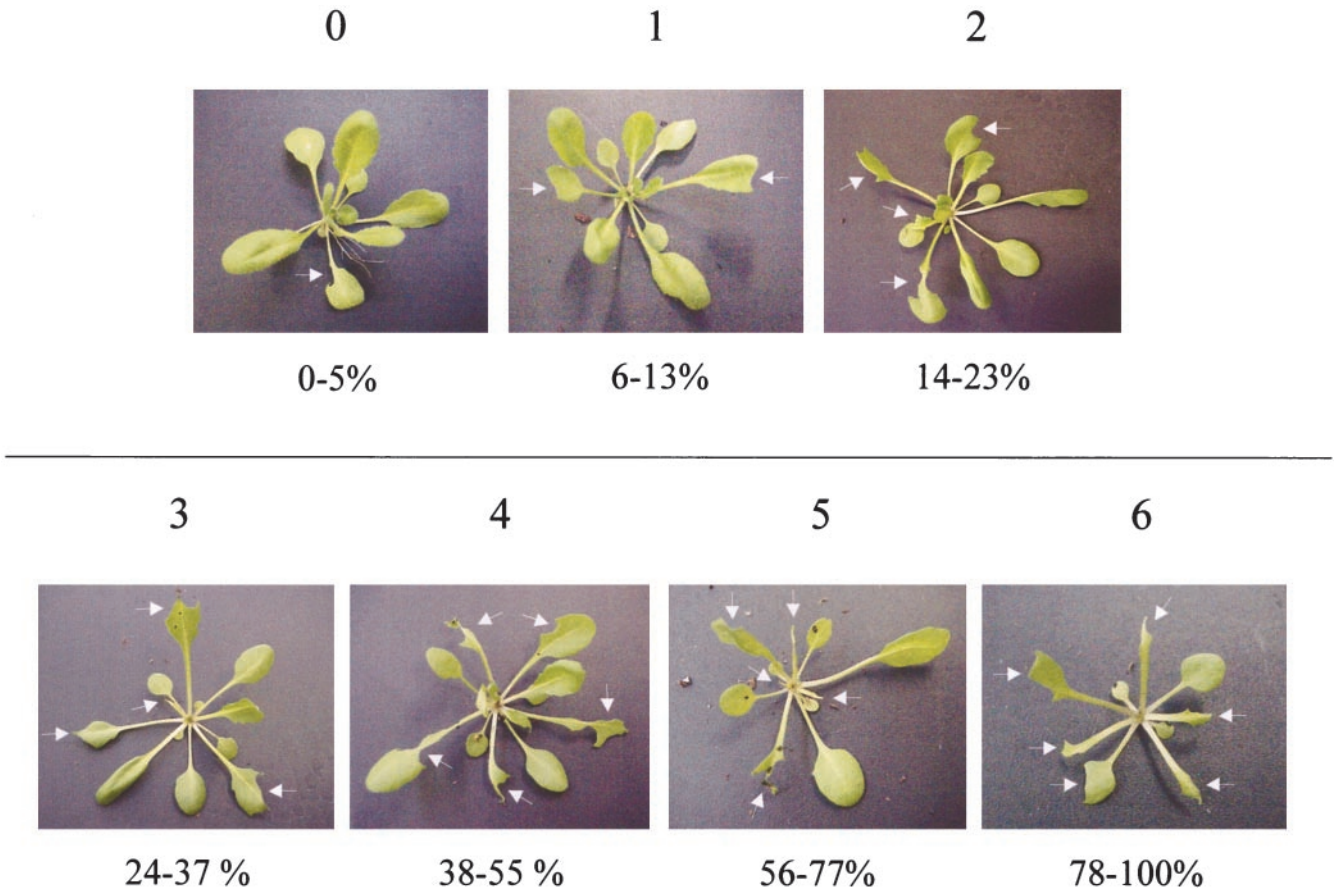

Figure 5. Measure of leaf damage caused by insect feeding on Arabidopsis. Representative examples of plants are shown that were grouped into categories (0–6) based on the amount of leaf area removed by herbivores (0%–100%). Arrows indicate leaves that were attacked.

ein2, a central component of the signaling pathway, which makes plants completely insensitive to ethylene. Another mutant, *hls1*, has an insensitive apical hook. Even though other parts of the plant remain responsive to ethylene, *hls1* does affect the growth and development of most plant tissues (Roman et al., 1995).

The *hls1-1* mutation reproducibly reduced damage by Egyptian cotton worm, suggesting that the wild-type allele confers susceptibility (Fig. 6). Consistent with this result, pretreatment of wild-type Columbia (Col)-0 and *hls1-1* mutants with ethephon increased susceptibility to Egyptian cotton worm. However, insect herbivory was also influenced by environmental variation, indicated by a significant flat effect (Table I). Moreover, the marginally significant interaction between ethephon treatment and flat suggests that the treatment effect was influenced by environmental conditions. There was no interaction between ethephon treatment and genotype (Table I; this experiment was replicated twice in separate analyses and both experiments gave identical results. Only the second experiment is reported here.). Thus the ethylene pathway apparently compromises resistance against this generalist herbivore. By contrast, damage by diamondback moth was unaffected by *hls1-1* genotype or ethylene treatment (Fig. 6).

As shown in Figure 7, the *ein2-1* mutation also enhanced resistance against Egyptian cotton worm (mixed model ANOVA; $F_{1,5} = 17.31$; $P = 0.009$), but not diamondback moth (mixed model ANOVA; $F_{1,4} = 0.015$; $P = 0.910$). The effect of *ein2-1* on resistance against the generalist herbivore was similar to *hls1-1* in magnitude (Fig. 7). We conclude that the ethylene signal transduction pathway has contrasting effects on the herbivory of different insect species.

DISCUSSION

Responses of plants against various pathogens and insects involve several signaling pathways, including SA, JA, and ethylene. This report examined the potential contribution of these pathways to defense gene expression. In addition, we determined the influence of ethylene signaling on resistance against two lepidopteran insects. We confirmed the insect-induced expression of six genes isolated via differential display and partially characterized their regulation by wounding and phytohormones (Table II). In

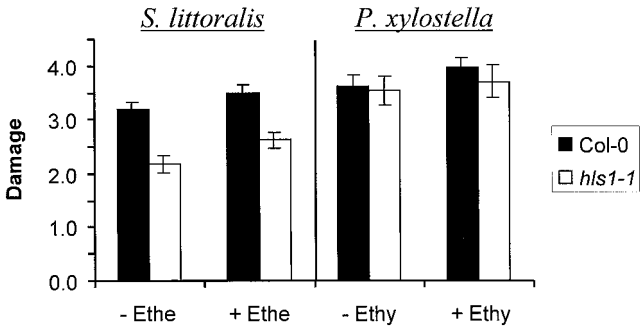

Figure 6. Ethylene perception compromises resistance of Arabidopsis to Egyptian cotton worm, but not to diamondback moth. Resistance against Egyptian cotton worm is enhanced in *hls1-1* compared with wild-type (Col-0) Arabidopsis and reduced by ethephon application. Resistance against diamondback moth is neither significantly affected by genotype nor by ethylene treatment. Damage is a measure of the amount of leaf area consumed by larvae, scored on a scale from 0 (resistant) to 6 (susceptible). Ethe, Ethephon; Ethy, ethylene. Error bars indicate SE. Statistical analysis of the Egyptian cotton worm data set is provided in Table I.

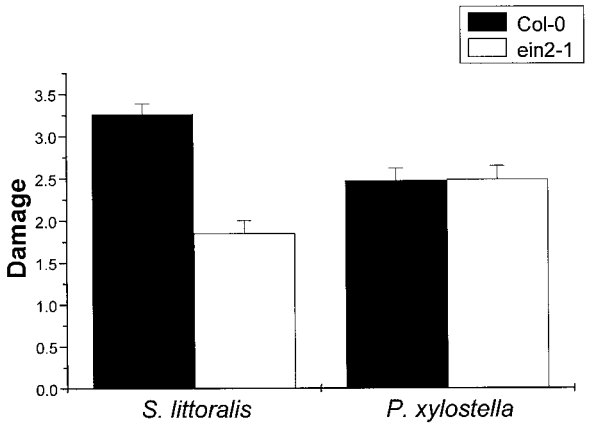

Figure 7. The *ein2-1* mutation enhances resistance against Egyptian cotton worm, but not diamondback moth relative to wild type (Col-0). Damage is a measure of the amount of leaf area consumed by larvae, scored on a scale from 0 (resistant) to 6 (susceptible). Error bars indicate SE.

Arabidopsis, JA-dependent and -independent signaling pathways mediate reactions to mechanical wounding (Titarenko et al., 1997). It is notable that similar genes are induced by biotic and abiotic stresses. This suggests either crosstalk between biotic and abiotic stress response pathways, or utilization of similar signaling cascades for different purposes (Chao et al., 1999).

JA was shown to regulate the expression of *VSP*, *LOX2*, and *BGL1*, whereas ethylene elevated the mRNA abundance of *CaEF*. We did not measure the influence of ethylene on *VSP* and *LOX2* expression because recent reports indicate that this hormone does not alter the mRNA abundance of these genes (van Wees et al., 1999). The regulation of *GST2* is reminiscent of pathogenesis-related proteins, such as hevein-like protein (Potter et al., 1993). Ethylene and SA induce both of these genes. *GST6*, however, was negatively controlled by all phytohormones we tested. In contrast to previous results (Chen et al., 1996), we did not detect an increase in *GST6* mRNA abundance in response to SA. Nonetheless, we detected an increase in *PR-1* expression upon SA treatment, demonstrating a clear difference between our experiments and Arabidopsis grown in liquid culture (Chen et al., 1996). Perhaps the rapid induction of *GST6* by insect feeding and wounding may relate to H_2O_2 signaling, because the effects of an oxidative burst caused by mechanical damage (Orozco-Cardenas and Ryan, 1999) are more immediate than regulation by phytohormones (Chen et al., 1996). For example, a soybean *GST* that is regulated by an oxidative burst in response to pathogen attack is induced within 30 min of H_2O_2 application (Levine et al., 1994). *GST* induction by wounding, independent of JA, was previously reported (McConn et al., 1997) using a probe corresponding to *GST11* (Kim et al., 1994), but its relationship to herbivory has not been tested. We found no evidence that SA plays an important role in the interaction between Arabidopsis and diamondback moth. Nor could we detect a consistent increase of free and total SA in rosette tissues as a result of larval feeding (H. Stotz, K. Weniger, T. Koch, and T. Mitchell-Olds, unpublished data). However, SA does influence other plant-insect interactions (Felton et al., 1989; Stout et al., 1999).

At least three different wound-response pathways operate in Arabidopsis when challenged by diamondback moth: (a) A JA-dependent pathway (Tita-

Table I. *ANOVA, effect of genotype (hls1-1 versus wild type), and ethephon treatment on plant resistance against S. littoralis*

DF, Degrees of freedom; MS, mean square.

Source	DF	MS	*F* Value	*P*
Genotype	1	723,052	44.44	0.0001
Ethephon	1	121,906	7.49	0.0064
Flat	3	56,550	3.48	0.0160
Ethephon × genotype	1	213	0.01	0.9089
Ethephon × flat	3	43,159	2.65	0.0482
Error	462	31,463		

Table II. *Summary of the regulation of stress-response genes by different stimuli*[a]

DBM, Diamondback moth.

Gene	DBM	Wounding	JA	Ethylene	SA
LOX2	+	+	+	0[b]	0
VSP	++	++	++	0[b]	−
BGL1	++	+	++	+	0
CaEF	++	++	0	++	−
GST2	+	0	0	+	+
GST6	+	+	−	−	−[c]

[a] +, Induction 2- to 10-fold; ++, induction more than 10-fold; 0, regulation less than 2-fold; −, repression. [b] Data referenced in Reymond and Farmer, 1998. [c] In contrast to our results, Chen et al. (1996) reported induction of GST6 by SA.

renko et al., 1997) that regulates the expression of *BGL1* in addition to *VSP* (McConn et al., 1997) and *LOX2* (Bell and Mullet, 1993); (b) an ethylene-dependent, but JA-independent pathway suggested by the induction of *CaEF* and *GST2*; and (c) a JA-independent pathway unrelated to ethylene supported by the lack of induction of *GST6*.

The functional significance of these genes for insect resistance is uncertain. However, antisense depletion of potato *LOX-H3* mRNA leads to reduced accumulation of antifeedant PIs and greater susceptibility to polyphagous insects without influencing JA-biosynthesis (Royo et al., 1999). Cosuppression experiments suggest that LOX2 contributes to wound-induced JA biosynthesis that affects downstream genes, such as *VSP* (Bell et al., 1995). Thus Arabidopsis LOX2 may also influence insect herbivory. GSTs could have consequences for insect resistance because they are multifunctional enzymes that contribute to the detoxification of xenobiotics and protection against oxidative damage (Marrs, 1996). Certain β-glucosidases are involved in defensive functions, such as cyanogenesis (Poulton, 1988). However, *BGL1* is distantly related to cyanogenic β-glucosidases (Fig. 8) and its closest relative with a known biochemical function is a zeatin-*O*-glucoside-degrading β-glucosidase from oilseed rape (Falk and Rask, 1995). Like the oilseed rape gene, *BGL1* contains a signal sequence, putative glycosylation sites, and a carboxy-terminal endoplasmic reticulum retention signal (Fig. 9). Calcium-binding EF-hand (CaEF) protein is likely to have a regulatory rather than a defensive function because members of this superfamily are involved in calcium-related cellular processes (Ikura, 1996).

Before addressing the function of individual defense genes, it is useful to determine the contribution of defense signaling pathways, such as JA, SA, and ethylene, to plant-insect interactions. Arabidopsis offers the advantage that a number of mutants are available in each pathway that can be tested for effects on insect feeding. We showed that both *hls1-1*, a mutation in a downstream component of ethylene signaling (McGrath and Ecker, 1998), and *ein2-1* reproducibly enhanced resistance against Egyptian cotton worm. These mutations had no detectable effect on diamondback moth herbivory. Ethephon treatment enhanced Egyptian cotton worm feeding, providing additional evidence for the role of ethylene signaling in susceptibility to this insect herbivore. However, we cannot exclude the possibility that other pathways influence the observed insect resistance phenotypes because *hls1-1* and *ein2-1* plants differ in their ethylene sensitivity. Nevertheless, the simplest explanation is an involvement of ethylene in insect resistance. This situation is similar to the role of *hls1* and *ein2* in pathogen resistance. According to Buell (1998), *hls1-1* exhibits enhanced susceptibility to *Xanthomonas campestris* pv *campestris*, suggesting antagonistic effects of this gene on pathogen versus

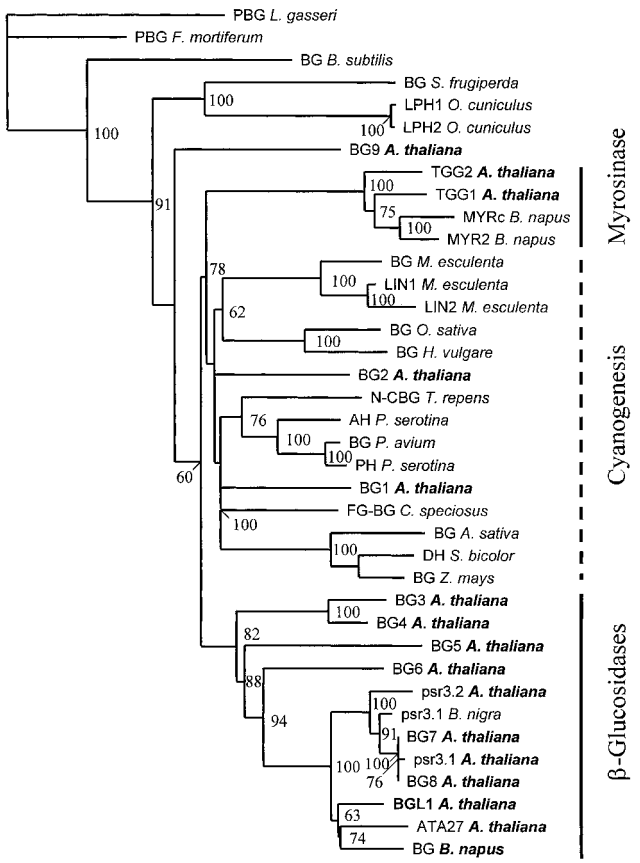

Figure 8. Consensus phylogenetic tree from genes belonging to the glucosyl hydrolase family 1 (Henrissat and Bairoch, 1993) based on coding sequence data. The tree is a majority rule consensus of 1,000 trees, each inferred from parametric distances (Lake, 1994) by the neighbor joining method (Felsenstein, 1993). Branch lengths were fitted using the Fitch-Margoliash algorithm, as implemented in PHYLIP. The numbers are percentages based on how many trees out of 1,000 supported the clades. Bar = genetic distance. *BGL1* falls into a clade of β-glucosidases from Arabidopsis and *Brassica* that is separate from myrosinases, cyanogenic β-glucosidases, and other more distantly related genes. Cyanogenesis has not been demonstrated experimentally for all of the enzymes in the middle group, and some may have alternative functions. BG, β-Glucosidases; DH, dhurrinase; FG-BG, furostanol glycoside BG; PH, prunasin hydrolase; AH, amygdlin hydrolase; N-CBG, non-cyanogenic BG; LIN, linamarase; MYR, myrosinase; TGG, thioglucosidase; LPH, lactase-phlorizin hydrolase; PBG, phospho-BG. Note that BG7 and BG8 of Arabidopsis have been mistakenly annotated as myrosinases in the databases. In contrast to myrosinases, these two genes contain the active site catalyst Glu found in β-glucosidases instead of Gln found in myrosinases. Accession numbers are available at http://vanilla.ice.mpg.de/departments/Gen/publications/stotz_tree.html.

insect resistance. By contrast, *ein2* as well as *ein2-1 hls1-1* double mutants confer tolerance to *X. c. campestris* (Buell, 1998). The reason for this difference in pathogen resistance between *ein2* and *hls1* remains to be explained. The ethylene-insensitive tomato mutant *Never ripe* exhibits enhanced tolerance to bacterial and fungal pathogens (Lund et al., 1998). Taken together, it is tempting to speculate that ethylene

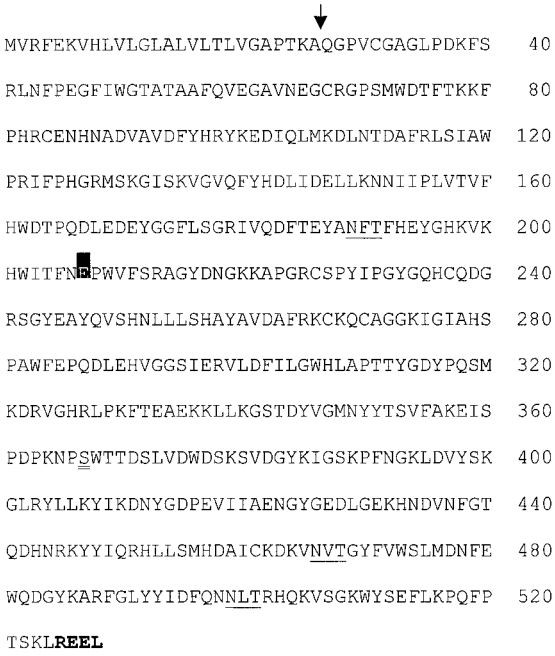

Figure 9. BGL1 encodes a predicted protein of 60.5 kD. The arrow indicates a potential cleavage site of the signal peptide. Putative *N*-glycosylation sites are underlined, a putative *O*-glycosylation site is double underlined. Residue Glu-207 is the acid catalyst that is conserved in all β-glucosidases, but not found in myrosinases. The predicted endoplasmic reticulum retention signal REEL is shown in bold.

plays a role in mediating susceptibility to both insects and pathogens.

Differences in plant resistance to specialist and generalist herbivores revealed by mutant analyses are probably due to variation in insect susceptibility to plant toxins or to manipulation of plant defense by herbivores. With respect to the tested mutants, we favor the former possibility because diamondback moth activates the ethylene pathway, as evidenced by the expression of *CaEF* and *GST2*. However, we cannot rule out quantitative differences in ethylene biosynthesis and signaling in response to diamondback moth versus Egyptian cotton worm damage. In the case of *Nicotiana attenuata*, enhancement of ethylene production by hawkmoth herbivory compared with mechanical wounding has obvious consequences for defense (Kahl et al., 2000). In conclusion, we propose the existence of insect-specific effects relating to the ethylene pathway, which are likely not caused by wounding. The differences in feeding of diamondback moth and Egyptian cotton worm on ethylene mutants and wild-type plants can be used to discover target genes and pathways that relate to a particular insect species. In addition, it may be possible to isolate insect signaling molecules that are responsible for the observed differential effects.

JA-mediated defense pathways increase resistance of Arabidopsis to generalist fungal gnat larvae (McConn et al., 1997). Our results demonstrate that ethylene compromises resistance of Arabidopsis to another generalist, Egyptian cotton worm. In other plant systems ethylene apparently interferes with JA-mediated defense responses (Kahl et al., 2000; Zhu-Salzman et al., 1998). Even though JA is thought to be the predominant defense signal against chewing insects, ethylene seems to be an important modulator of defenses in different plant species. In analogy to our results a reduction of JA-related defenses preferentially increases susceptibility to polyphagous, but not monophagous insects of potato (Royo et al., 1999). Finally, suppression of the ethylene pathway rather than enhancement of the JA-pathway could be an approach of improving plant resistance against insects. However, in addition to possible negative consequences for crop yield, altering induced resistance may modify insect associations of genetically engineered plants with manipulated JA or ethylene pathways.

MATERIALS AND METHODS

Plant and Insect Growth Conditions

The Arabidopsis ecotypes Landsberg *erecta* and wild-type Columbia (Col-0) were obtained from Lehle Seeds (Round Rock, TX). The *hookless1* (*hls1-1*) and *ein2* mutants were obtained from the Arabidopsis Stock Center (Nottingham, UK). Growth conditions for Landsberg *erecta* plants used for differential display were as described (Mitchell-Olds and Pedersen, 1998). All other Arabidopsis plants were grown in 96-celled flats at a density of 337 plant m^{-2} on a Mini-Tray:vermiculite (3:1) soil mix (Einheitserdenwerk, Fröndenberg, Germany) under 11.5-h light/12.5-h darkness at 23°C. Diamondback moth (*Plutella xylostella*) eggs were obtained from Anthony Shelton (Department of Entomology, New York State Agricultural Experiment Station, Geneva, NY) and raised on an artificial diet according to published procedures (Shelton et al., 1991). Egyptian cotton worm (*Spodoptera littoralis*) cultivation was previously published (Degenhardt and Gershenzon, 2000).

Plant Treatments

Arabidopsis plants were approximately 4 weeks old at the time of treatment and the growth stage was vegetative, prebolting. Unless otherwise indicated, a single second-instar larva of diamondback moth was allowed to feed on a plant for a given period of time. Depending upon time treatment, 5% to 10% of leaf area was removed by insect feeding. Control and treated rosette tissues were all harvested simultaneously at the same age (except in Fig. 1B). Mechanical damage was caused by crushing across a single rosette leaf per plant with a hemostat. Exogenous phytohormone applications followed published procedures to ensure comparability with previous research. Spray treatment with SA (5 mM; Sigma, St. Louis) was described by Uknes et al. (Uknes et al., 1992). Aqueous spray of MeJA (150 μM; Aldrich, Milwaukee, WI) or ethephon (2-chloroethanephosphonic acid, 50 μM; Union Carbide, Research Triangle, NC) was similar to Laudert and Weiler

(1998). Each plant received less than 300 μL of sprayed solution.

Gas fumigation of plants employed 60 mL of ethylene (Messer-Griesheim, Krefeld, Germany) to provide a brief exposure to the hormone, according to Kahl et al. (2000).

Gene Isolation

Lipoxygenase 2 (*LOX2*), β-glucosidase 1 (*BGL1*), glutathione *S*-transferase 2 (*GST2*), *GST6*, a putative calcium-binding EF-hand protein (*CaEF*), vegetative storage protein 1 (*VSP1*), and *VSP2* were isolated by differential display, based on their elevated expression in insect-challenged compared with unchallenged control plants. RNA preparations (50 μg) were treated with 2 units of fast-protein liquid chromatography-pure DNase I at 37°C for 30 min as recommended by the supplier (Pharmacia, Piscataway, NJ). RNA was extracted with phenol-chloroform, precipitated with ethanol, resuspended in RNase-free H_2O, and stored at −80°C. Lark Technologies (Houston) processed plant RNAs for the three different treatments (0, 10, and 30 h of diamondback moth herbivory) for differential display analysis. PCR products with putative differential regulation in response to insect herbivory were gel-extracted, re-amplified, and sequenced.

Gene Expression Analysis

It was typical that rosettes from nine to 12 plants were used for RNA extractions. Total RNA was isolated using TRIZOL reagent (Gibco-BRL, Gaithersburg, MD) according to manufacturer's recommendations and analyzed as described (Stotz et al., 1993). Blots were hybridized with the following probes: *BGL1* (nucleotides 959–1,636 of the cDNA), *VSP2* (696 bp from the polyA tail), *LOX2* (L23968, nucleotides 2,125–2,809), *GST2* (X75303, nucleotides 391–881), *GST6* (X95295, nucleotides 1,100–1,405), and *CaEF* (AAB80656, nucleotides 48,810–49,361). *ACT2* (ATU41998, nucleotides 1,911–2,622) or 25S rRNA (a 1.7-kb *Bam*HI fragment of the *Glycine max* gene) were used as probes to normalize for loading (Friedrich et al., 1979). Rehybridization of blots followed membrane stripping with boiling SDS (0.5%, w/v). Blots were washed with 0.2× SSC and 0.1% (w/v) SDS at 55°C. Quantification of RNA abundance was based on phosphorimaging. Superscript II (Gibco-BRL) was used for reverse transcription of total RNA according to the manufacturer's recommendations. Semi-quantitative PCR was performed according to published procedures (Kohler, 1995) with primers *ACT2F* (5′-CAGAGCGGG-AAATTGTAAGAGAC-3′) and *ACT2R* (5′-ACAAAAAGGGAAATGAAACAAACA-3′); and *PR1F* (5′-CTCAAGATAGCCCACAAGA-3′), *PR1R* (5′-TAGTATGGCTTCTCGTTCAC-3′), and *GST2F* (5′-AATATGGTTTTGCTTCAGTCA-3′). Based upon available genomic sequence, we designed gene-specific primers *GST2R* (5′-TGCCAAAGATACTCTCAAGAG-3′), *GST6F* (5′-GCAAGAAAGTCAAGGCAACCAC-3′), and *GST6R* (5′GGGCAAAAGGAAAAGAAAAGAAGT-3′). Aliquots were taken after 25 to 29 cycles and run on agarose gels.

Insect Feeding Trials

Wild-type and mutant plants were randomly assigned positions in 96-well flats. Insect feeding is a quantitative trait. To control for possible environmental or behavioral variation, we used ANOVA under replicated and randomized conditions. To induce defenses, plants were treated with phytohormones the day before they were challenged with lepidopteran larvae. One larva was applied per plant and allowed to feed for 1 to 2 d in the case of Egyptian cotton worm (third instar) or approximately 3 d in the case of diamondback moth (second instar). Leaf damage was quantified on a scale based on the percentage of leaf area removed: 0 (0%–5%), 1 (6%–13%), 2 (14%–23%), 3 (24%–37%), 4 (38%–55%), 5 (56%–77%), and 6 (78%–100%). SAS (SAS Institute, Cary, NC) and Systat (SPSS, Inc., Chicago) were used for statistical analysis. Genotype was treated as a fixed factor and flats as a random factor in mixed-model ANOVAs (testing $MS_{genotype}$ over $MS_{genotype \times flat}$).

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