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Phytochemistry 65 (2004) 1047–1055
PHYTOCHEMISTRY
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Transcriptional activation of *Igl*, the gene for indole formation in *Zea mays*: a structure–activity study with elicitor-active *N*-acyl glutamines from insects

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Received 14 November 2003; received in revised form 26 November 2003

Dedicated to Prof. Dr. Wolfgang Steglich on the occasion of his 70th birthday.

Abstract

The indole-3-glycerol phosphate lyase *Igl* is the structural gene of volatile indole biosynthesis in the tritrophic interaction in maize. The gene is activated on transcriptional level with the same kinetics and to the same level by the fatty acid–amino acid conjugates (FAC's) volicitin (17*S*)-(*N*-(17-hydroxylinolenoyl)-L-glutamine) and *N*-linolenoyl-L-glutamine. Both conjugates are present in the regurgitates of herbivorous caterpillars. Modifications of the fatty acid moiety of the FACs greatly reduces the elicitation of *Igl* and only the L-stereo-isomer of the FACs shows biological activity in the system. Volicitin treatment leads to a fast increase of *AOS* and *AOC* transcription levels and methyl jasmonate application induces *Igl* transcription. Hence, the induction of jasmonate biosynthesis appears to be an integral part of the elicitor mediated increase of *Igl* gene transcription.

Keywords: *N*-Acyl glutamines; Volicitin; Indole-3-glycerol phosphate lyase; Allene oxide synthase; Allene oxide cyclase; Jasmonate, Induced defense

1. Introduction

Plants have developed various systems for direct and indirect defenses against attack by herbivores and phytopathogens. Direct defenses include production of toxins and repellents, constitutively or in response to the attack by the herbivore or a pathogen. In contrast, indirect defenses do not rely only on immediate effects of plant derived compounds, but address and exploit the efficiency of natural enemies of the herbivores. Plants may emit so-called herbivore-induced plant volatiles that serve as a cue to direct predators into the vicinity of their prey. This plant mediated parasitoid–host communication has been termed the tritrophic interaction. Several such tritrophic interactions have been documented for agrarian systems including Lima bean (Dicke et al., 1990) cucumber, apple trees (Takabayashi and Dicke, 1996), cotton and maize (De Moraes et al., 1998). When maize is attacked by beet armyworm (*Spodoptera* sp.) larvae, the plant releases a specific blend of terpenoids and indole that is attractive to the parasitic wasp *Cotesia marginiventris*. The female wasps deposit their eggs in the caterpillars of several species of Lepidoptera, which are eventually devoured by the emerging wasp larvae. Beet armyworm oral secretions, applied to mechanically damaged sites, are sufficient to mimic the plant response to caterpillar feeding (Turlings et al., 1993). Volicitin (17*S*)-(*N*-(17-hydroxylinolenoyl)-L-glutamine) a fatty acid–amino acid conjugate (FAC), was identified as the major active elicitor in the oral secretion of the beet armyworm larvae (Alborn et al., 1997; Spiteller et al., 2001). Additional related fatty acid–amino acid conjugates with biological activity have been identified recently in the oral secretion of several Lepidoptera species (Pohnert et al., 1999a; Alborn et al.,

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; AcSA, acetylsalicylic acid; AOC, allene oxide cyclase; AOS, allene oxide synthase; EST, expressed sequence tag; FAC, fatty acid–amino acid conjugate; GAP C, glycerol phosphate dehydrogenase, cytosolic form; Gln, glutamine; Glu, glutamate; JA, jasmonate; MeJa, methyl jasmonate; PCR, polymerase chain reaction.

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doi:10.1016/j.phytochem.2003.11.021

2000; Halitschke et al., 2001; Spiteller and Boland, 2003a,b). In a great variety of plants, jasmonate (JA) induces the biosynthesis of volatile compounds that are part of the blend emitted in tritrophic interaction (Hopke et al., 1994; Dicke et al., 1999). Different mechanisms for the inducing activity are discussed. Jasmonates most probably originate from α-linolenic acid that might be released from membrane-located phospholipids by phospholipase activity. A lipoxygenases (13-LOX) catalyzes the oxygenation of the fatty acid to (13*S*)-hydroperoxy-(9*Z*,11*E*,15*Z*)-octadecatrienoic acid (13-HPOT) which is the substrate for allene oxide synthase (AOS). The AOS catalyzed dehydration of 13-HPOT generates an unstable epoxide which is converted by stereospecific cyclization catalyzed by allene oxide cyclase (AOC) to (9*S*,13*S*)-12-oxo-(10,15*Z*)-phytodienoic acid (OPDA). The reduction of OPDA and three β-oxidation steps lead to (+)-7-*iso*-jasmonate. Because linolenic acid is a precursor of JA in the octadecanoid pathway, the inducing activity of the FACs may result from supply of the substrate for JA biosynthesis. This mode of action is, however, highly questionable since the quantity of delivered fatty acids is too low to account for effective concentrations of synthesized JA (Engelberth et al., 2003). Alternatively, the intact FACs may interact with receptors that subsequently trigger the octadecanoid pathway. Moreover, FACs are amphiphilic, surface active compounds that can lead to depolarization of membranes and subsequently to an influx of Ca^{2+} (Maffei et al., 2004). The importance of a depolarization effect is supported by the observation that alamethicin, an ion channel-forming peptide belonging to the class of peptaibols, is one of the most powerful elicitors in volatile emission (Engelberth et al., 2001) although no structural similarity to FACs or JA is given.

Recently three genes, *Igl*, *stc1, tps1*, that are specifically elicited by volicitin have been isolated from maize. (Frey et al., 2000; Shen et al., 2000; Schnee et al., 2002). *Igl* encodes an indole-3-glycerol phosphate lyase (IGL) and *stc1* and *tps1* are involved in terpene biosynthesis. IGL cleaves indole-3-glycerol phosphate to form indole and glyceroaldehyde-3-phosphate. Application of volicitin leads to a rapid transient increase of specific transcripts preceding volatile emission. The availability of these target genes offers the possibility to test the structural requirements of elicitors of the volatile biosynthesis on the level of gene activation. The results may, therefore, allow conclusions on the mechanism of signal perception.

The induction of direct and indirect plant defense not only depends on elicitors, but is additionally regulated in a complex fashion by the interaction of different signaling pathways (e.g. Arimura et al., 2002). Jasmonate, ethylene and salicylate may interfere in synergistic and antagonistic manner to activate or repress specific defense responses (e.g. Engelberth et al., 2001). The complex interactions between JA, ethylene and nutrition on the volatile release in maize have been reported recently (Schmelz et al., 2003). Accordingly, besides the structure–activity study of FACs, another focus of our experiments is on the impact of these signaling pathways on transcription of *Igl*, a structural gene of volatile indole biosynthesis.

2. Results

2.1. Structural requirements for elicitation by volicitin

Fatty acid–amino acid conjugates (FACs) are commonly found in lepidopteran caterpillar regurgitates. Glutamine conjugates of linolenic acid (18:3) are present in all species investigated so far (Alborn et al., 1997; Pohnert et al., 1999a), but in *Manduca sexta* conjugates with glutamate are prevailing (Halitschke et al., 2001) Volicitin is distinguished from these by the unique hydroxyl-group in position 17; its configuration was determined to be 17*S* (Spiteller et al., 2001). To test the impact of the hydroxy function on elicitation, we applied FAC's with modified fatty acid moieties (Fig. 1) in the cut seedling assay (Fig. 2A). The plantlets were harvested two and four hours after induction and total RNA was isolated. After reverse transcription, the respective gene sequences were quantified by real time PCR. 17*R,S*-Volicitin and 18:3-L-Gln increased most effectively the *Igl* transcript level. Also 15*R,S*,16*R,S*-epoxylinoleoyl-L-Gln elicited *Igl* transcript levels significantly compared to the buffer control. No significant increase is displayed in assays with the free epoxy-fatty acid (e), the conjugate of the 15*R,S*,16*R,S*-dihydroxy-fatty acid with L-Gln (f), and the indanone (g). Application of test substances and buffer increased the *Igl* transcript transiently, the value at time point 2 h being generally higher as the 4 h value. The result clearly demonstrates that the hydroxyl group in position 17 of the fatty acid is not required for *Igl* gene induction by FAC's, no specific interaction with volicitin is necessary and the sometimes even more ubiquitous FAC 18:3-Gln can function as elicitor as well. Modification of the fatty acid moiety by introduction of epoxy- and hydroxy-groups interfered with the *Igl* gene activation. A connection between amphiphilic nature of the conjugate, decreasing in the sequence 18:3-L-Gln ⩾ volicitin > *N*-(15*R,S*,16*R,S*-epoxylinoleoyl)-L-Gln > *N*-(15*R,S*,16*R,S*-dihydroxylinoleoyl)-L-Gln, and the potency to enhance the *Igl* transcript level, can be drawn. Elicitation might require an interaction of the elicitor with the plasma membrane and subsequent membrane modification. Alamethicin is known to modify membranes massively by building voltage-dependent three-dimensional structures resulting in ion channels specific for monovalent

cations (Cafiso, 1994). When alamethicin is applied to the cut maize seedlings, the *Igl* transcript level is increased to the same level and with the same transient kinetics, as with volicitin treatment (Fig. 2B). Generation of ion-channels can mimic the application of the biologically active FAC's volicitin and 18:3-Gln with respect to the induction of the target gene *Igl*. To test whether elicitation by FAC's is based exclusively on the amphiphilic character and the ability to form micelles at very low concentrations which may lead to interference with the membrane structure, the conjugate of linolenic acid with D-glutamine was applied in the seedling assay (Fig. 2C). The natural occurring conjugate 18:3-L-Gln and the 18:3-D-Gln possess the same physicochemical properties and should therefore interfere with plasma membranes in identical manner. However, *Igl* transcript levels displayed with 18:3-D-Gln did not exceed the buffer control values, while parallel incubation with 18:3-L-Gln and 17*R,S*-volicitin demonstrated effective elicitation. This result clearly demonstrates that, besides the action of the elicitor on the plasma membrane causing an increase of $[Ca^{2+}]_{cyt}$, additional and hitherto unknown interactions are required for the transient

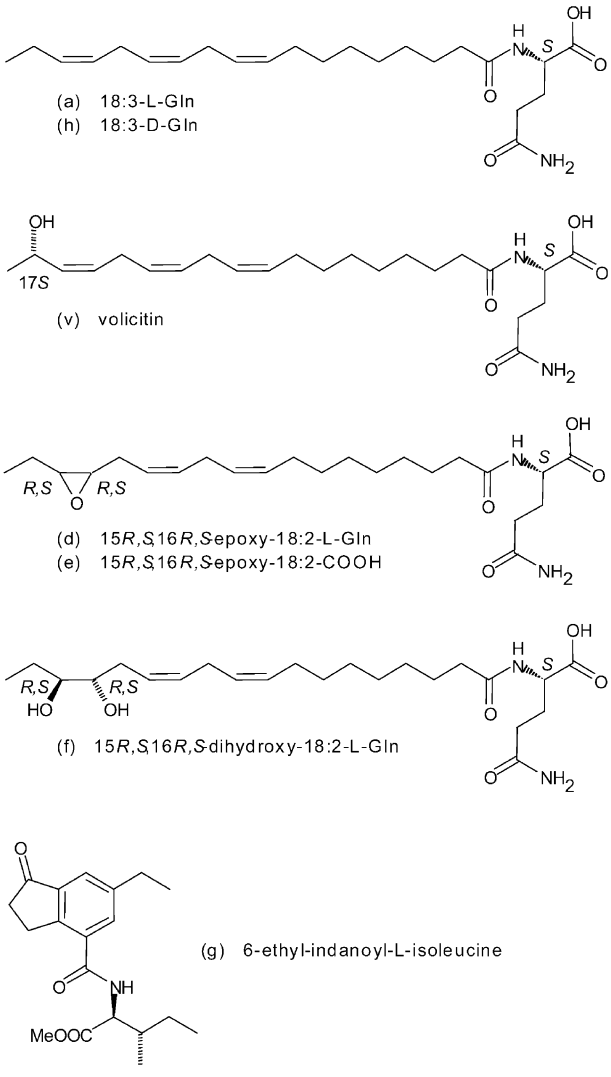

Fig. 1. Structures of *N*-acyl glutamines used for structure–activity studies. Indanoyl-L-isoleucine is a highly active synthetic analogue of the microbial phytotoxin coronatine (Greulich et al., 1995; Schüler et al., 2001).

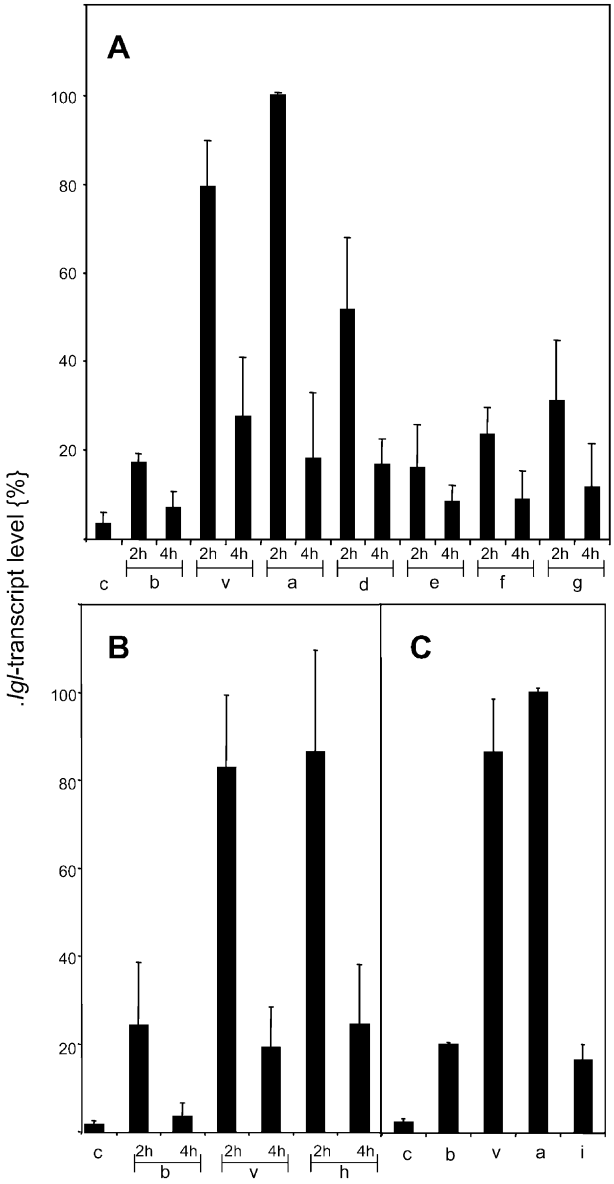

Fig. 2. *Igl* transcript elicitation by application of test substances. The level of *Igl* transcript was determined by quantitative PCR after incubation of cut seedlings in test solutions (see experimental). Incubation was for 2 and 4 h (A, B) and for 2 h (C). Mean values (±standard deviation) of two biological replicates with three plants per treatment are given. Untreated control plants (c), plants incubated in buffer (b) or volicitin (v) were included in each experiment. (A) Effect of different fatty acid–amino acid conjugates (FAC's) on *Igl* transcript level. *N*-Linolenoyl-L-Gln (a) 15,16-epoxylinolenyl-L-Gln (d), free 15,16-epoxylinolic acid (e), 15,16-dihydroxylinolenoyl-L-Gln (f), indanoyl-L-isoleucine (g). (B) Application of alamethicin (h). (C) Elicitation is dependend on the absolute configuration amino acid moiety of the FAC. *N*-Linolenoyl-L-Gln (a), *N*-linolenoyl-D-Gln (i).

increase in transcript level of *Igl*, a structural gene in the tritrophic interaction in maize.

2.2. Interaction with signaling pathways

2.2.1. Salicylic acid application has no impact on volicitin elicitation of Igl transcription

To study a putative interaction of the salicylic acid pathway and volicitin mediated *Igl* elicitation, maize plantlets were pre-incubated in acetylsalicylic acid (AcSA, 0.5 mM) as described (Engelberth et al., 2001) prior to treatment with the elicitor. Two hours after application of volicitin to buffer treated control plants and plants incubated with AcSA, RNA was isolated from these volicitin treated plants and the respective controls. *Igl* transcript levels were determined as described (Fig. 3). AcSA treatment had no significantly impact on the increase of *Igl* transcript levels. The modest decrease of the transcript level in the AcSA/buffer control and the mild increase displayed for AcSA treatment prior to volicitin application do not indicate a significant interference of AcSA with volicitin elicitation of *Igl*.

2.2.2. Volicitin elicitation and jasmonate signalling

To determine whether induction of *Igl* is mediated via activation of the octadecanoid pathway, maize plantlets were pre-treated with well-established inhibitors as described (Engelberth et al., 2001) and analyzed for *Igl* transcript levels. Phenidone interferes with the production of fatty acid hydroperoxides (Curcurou et al., 1991) whereas aristolochic acid is a potent inhibitor of phospholipase A2 and prevents the release of linolenic acid from phospholipids (Rosenthal et al., 1989; Scherer and Arnold, 1997). Pretreatment of cut plantlets in phenidone (at 1 mM) or aristolochic acid (at 0.3 mM) impeded the elicitation by volicitin while the control plantlets displayed a significant increase in *Igl* transcript level after transfer from buffer to solution containing volicitin (Fig. 4). To correlate these different treatments of the maize plantlets with their impact on the jasmonate biosynthetic pathway, we included the sequences of the maize allene oxide synthase (*AOS*) and allene oxide cyclase (*AOC*) in the analysis. *AOS* and *AOC*

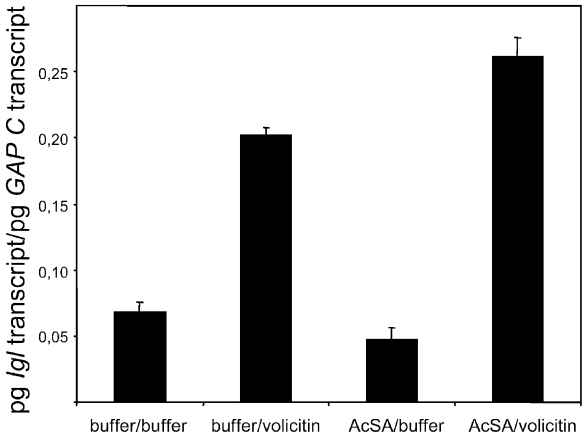

Fig. 3. Effect of acetylsalicylic acid (AcSA) treatment on *Igl* elicitation. Pretreatment with AcSA (0.5 mM) has no impact on the elicitation by volicitin. Mean values (±standard deviation) of two biological replicates with three plants per treatment are given. The average value of *GAP C* transcript was estimated as 0.090±0.12 pg and 0.093±0.011 pg.

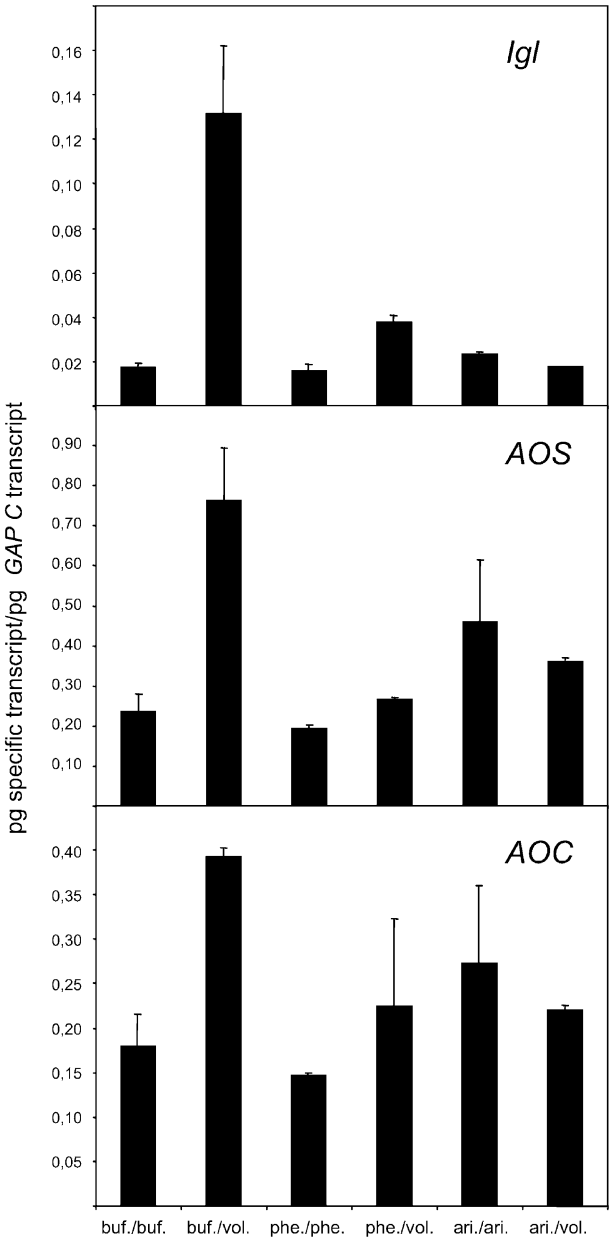

Fig. 4. Effect of inhibitors of the octadecanoid pathway on transcript levels. Plantlets were pretreated with phenidone (phe., 1 mM) and aristolochic acid (ari., 0.3 mM) or buffer (buf.). The transcript levels of *Igl*, *allene oxide synthase* (*AOS*) and *allene oxide cyclase* (*AOC*) were determined two hours after application of volicitin in treated and control plants. Mean values (±standard deviation) of two biological replicates with three plants per treatment are given, the *GAP C* transcript level was 0.086±0.14 pg and 0,093 (±0.011) pg.

transcription increase after wounding (Turner et al., 2002). In tomato the dependence of the wound response and vascular bundle-specific generation of jasmonate on AOC has been shown (Stenzel et al., 2003a). One expressed sequence tag (EST) for maize *AOS* is available in the data base, the encoded protein shares 86% amino acid identity with AOS1 from *Hordeum vulgare*. The barley enzyme has a preference for the substrate 13-HPOT and the transcription pattern of the gene is consistent with a function in jasmonate biosynthesis (Maucher et al., 2000). The highly homologous maize gene might therefore encode an identical function. Two genes are published for maize *AOC* (Stenzel et al., 2003b). Conditions for quantitative real-time PCR were established for *AOS* and *AOC*. The transcript levels of these genes of the jasmonate biosynthetic pathway were determined for the treatments with volicitin and inhibitors (Fig. 4). Both genes respond to volicitin treatment with increased transcript levels. In combined incubations with phenidone or aristolochic acid and volicitin *AOS* or *AOC* are not significantly induced. The results implicate a connection between elicitation and jasmonate signaling in the regulation of the target gene *Igl*.

The involvement of the jasmonate signaling pathway in *Igl* gene induction was further demonstrated by the application of methyl jasmonate or volicitin to wounded plantlets. Wounded plants served as controls. All three treatments, wounding, volicitin- and methyl jasmonate application, led to an increase of *Igl* transcript levels, maximum levels were, however, only reached with volicitin and methyl jasmonate (Fig. 5). These maximum transcript levels are not significantly different for the two substances. As in the cut seedling assay, wounding and volicitin application generated a transient increase and displayed the highest levels at two hours after the treatment. In contrast, the *Igl* remained at the maximal level during the eight hours of incubation with methyl jasmonate. The amount of AOS and AOC transcript was determined in parallel (Fig. 5). Both transcript levels were increased by the three treatments compared to the intact control plant. However, compared to the *Igl* gene, only a moderate elicitation of *AOS* and *AOC* by volicitin and methyl jasmonate is displayed. The transient fashion of the increase found for the *Igl* gene in the wounding reaction and volicitin elicitation is not exactly reflected by the *AOS* and *AOC* expression pattern.

2.2.3. Effect of ACC application

To test possible interactions of *Igl* elicitation and ethylene signal transduction, maize plantlets were treated with ACC to increase the internal ethylene concentration. The treatment was followed by wounding, volicitin application, and methyl jasmonate application (Fig. 6). The assay was performed with intact plants. The *Igl* transcript levels estimated following wounding and volicitin or methyl jasmonate application were not significantly influenced by ACC.

3. Discussion

FACs are frequently found in the regurgitate of lepidopteran caterpillars (Pohnert et al., 1999a; Alborn et al. 2000; Halitschke et al., 2001; Spiteller and Boland 2003a,b). Volicitin (17*S*)-(*N*-(17-hydroxylinolenoyl)-L-

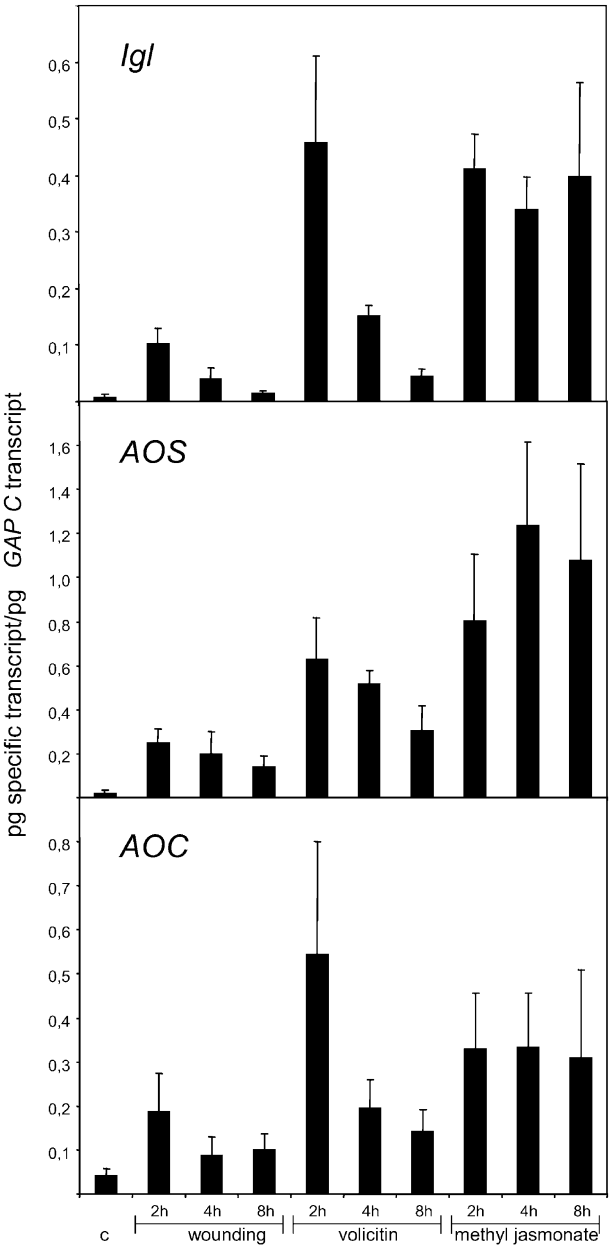

Fig. 5. Elicitation by volicitin and methyl jasmonate. Transcript levels of *Igl*, *AOS* and *AOC* were determined 2 h, 4 h and 8 h after application of buffer, volicitin and methyl jasmonate. Mean values (±standard deviation) of three to five biological replicates with three plants per treatment are given, *GAP C* transcript levels were between 0.13±0.03 pg and 0.54±0,08 pg.

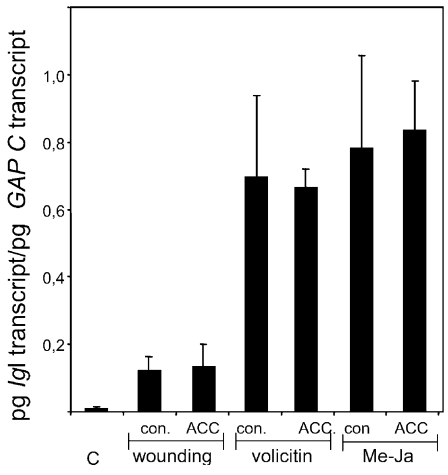

Fig. 6. Effect of exogenous 1-aminocyclopropane-1-carboxylic acid (ACC) on *Igl* transcript levels. Transcript levels were determined 2 hours after wounding and volicitin or methyl jasmonate application. ACC was applied in a concentration of 1 mM. Mean values (±standard deviation) of two biological replicates with three plants per treatment are given, *GAP C* transcript levels were 0.080±0.02 pg and 0.062±0.01 pg.

glutamine) is an FAC with limited distribution among the Lepidoptera. It differs from the more commonly found 18:3-L-Gln by its hydroxyl group in position C-17 of the fatty acid. It could be hypothesized that this feature might be essential for elicitor function in corn plants. Our experiments on the transcriptional activation of the *Igl*-gene, that catalyzes the biosynthesis of volatile indole in the tritrophic interaction, show however that this hydroxyl function is not required for transcript elicitation, since application of 18:3-Gln and volicitin results in almost identical *Igl* transcript levels. It has been shown recently, that varying amounts of 18:3-Gln are present in the regurgitate of beet armyworm larvae and can elicit volatile emission in maize (Alborn et al., 2000). It will be interesting to see whether *stc1* and *tps1*, the structural genes involved in induced terpene biosynthesis (Shen et al., 2000; Schnee et al., 2002), are also transcriptionally activated by 18:3-Gln. Volatile biosynthesis is also up-regulated in *Nicotiana attenuata* (Halitschke et al., 2001) after application of FAC-mixtures containing 18:3-L-Glu and minor amounts 18:3-L-Gln. On the contrary, leaves of Lima bean (*Phaseolus lunatus*) failed to give any volatiles response upon treatment with FAC's containing L-Gln (Koch et al., 1999). Modifications of the fatty acid moiety of the FAC, e.g. the introduction of a 15,16-epoxy-group into the linolenic acid moiety of 18:3-L-Gln reduces the efficiency of the elicitor while opening of the epoxide leading to a vicinal diol completely abolishes the biological activity of the conjugate. The same observation was made with 18:3-D-Gln which did not trigger expression of *Igl*. This finding is important and suggests that besides simple membrane depolarization and an influx of Ca^{2+} (Maffei et al., 2004) additional factors might be required that are necessary for triggering octadecanoid signaling and subsequent gene expression. Whether this involves a specific interaction of the FACs with a certain macromolecule (receptor) in the membrane, or is due to an enzymatic cleavage of the FACs resulting in a free fatty acid and L-glutamine which in turn could function as a signal, is currently unknown.

Plant defense reactions are often regulated by a cross-talk between different signaling pathways. We assayed the effect of ethylene, acetylsalicylic acid and methyl jasmonate on volicitin induced *Igl* gene transcription. Interaction or cross-talk between the jasmonate pathway, ethylene biosynthesis, or salicylate signaling was not observed, since application of acetylsalicylic acid remained without effect on the transcription level of *Igl*. Application of ACC, to increase the endogenous ethylene level, prior to volicitin treatment also had no effect on the increase of *Igl* transcripts. This is in contrast to work by Schmelz et al. (2003a) revealing synergistic effects between volicitin and ethylene on the emission of terpenoids and indole in maize. Ethylene alone had no effect on volatile production. An explanation for the discrepancy may be, that different outputs of the system are investigated, transcription of the structural gene on the one hand, and formation of volatiles on the other. It has been shown that *Igl* induction on transcriptional level precedes the emission of indole (Frey et al., 2000). The efficiency of biosynthesis is influenced by processes located downstream of transcription. Major roles may be played by the physiological status of the plant and by the availability of the substrate. Indeed, nitrogen supply has been demonstrated to be of main importance for volicitin-mediated volatile emission and the ethylene sensitivity of the system (Schmelz et al., 2003b). However, *Igl* transcription is a prerequisite for indole production.

Direct evidence for a link between jasmonate and *Igl* elicitation is given by the finding that application of MeJa increases *Igl* transcripts to the same level as volicitin incubation (Fig. 6). The involvement of octadecanoid signaling after volicitin application is further supported by the interference of the inhibitors phenidone and aristolochic acid, with induction of *Igl* transcription. It has been shown that endogenous jasmonate levels in corn plants are increased after beet armyworm feeding and after application of volicitin (Schmelz et al., 2003a,b). This observation matches our finding that volicitin transiently activates *AOS* and *AOC* transcription. AOC catalyzes the crucial step in octadecanoid and jasmonate biosynthesis (Ziegler et al., 2000). The induction of *AOS* and *AOC* expression would then account for the increase of jasmonate concentration. Therefore, the induction of jasmonate biosynthesis appears to be an integral part of the signaling cascade to

volatile emission. The observations are in line with the model outlined in Fig. 7. Interaction of FACs with the membrane (Maffei et al., 2004) resulting in Ca^{2+}-influx plus currently unknown interactions based on structural features of the FACs (L-Gln is essential) are required to express *Igl* transcripts. Specific recognition of FACs or their breakdown components transiently stimulate *AOS* and *AOC* expression leading to an increase of jasmonates. The jasmonate pulse then activates directly or indirectly *Igl* transcription. Transient *Igl* expression and indole emission is the consequence.

One major difference in the effect of volicitin and methyl jasmonate application has to be discussed. The induction of *Igl*, *AOS* and *AOC* transcription by volicitin is fast and transient, while constantly elevated transcript levels are displayed after methyl jasmonate treatment. The adjustment of constant jasmonate levels by exogenous supply is responsible for permanent *Igl* expression. This would imply that volicitin-specific endogenous jasmonate is generated transiently.

The model can also explain the alamethicin-mediated *Igl* transcript increase, since, in contrast to Lima beans (Engelberth et al., 2001), elevated jasmonate levels are found in corn plants after alamethicin treatment (Engelberth et al., 2003).

In plants, jasmonate and salicylic acid signaling pathways are often connected. A negative interference between jasmonate and salicylic acid has been shown e. g. for NPR1-dependend responses in *Arabidopsis thaliana* (Spoel et al., 2003) and for protein inhibitor synthesis in tomato leaves (Doares et al., 1995). However, the picture is complex, generally, jasmonate induces *AOS* transcription in plants, but in *Arabidopsis* thaliana salicylic acid application increases *AOS* transcription also (Laudert and Weiler, 1998). In barley salicylic acid has no effect on *AOS* transcript levels (Weichert et al., 1999) and it even inhibits transcription in flax (Harms et al., 1998). Elicitation of *Igl* was not influenced by treatment with acetylsalicylic acid indicating that salicylic acid does not interfere with jasmonate biosynthesis after volicitin elicitation in maize. Simultaneously elevated levels of jasmonate and salicylic acid may indeed persist in maize at least 4 h as demonstrated for alamethicin incubation (Engelberth et al., 2003). Alternatively, an early octadecanoid such as linolenic acid and OPDA may be also biologically active (Koch et al., 1999) in the signal cascade to *Igl* transcription. In the Lima bean the interference of salicylic acid with the biosynthetic pathway downstream to 12-oxophytodienoic acid reduces the pattern of emitted volatiles to compounds, that were only inducible by early octadecanoids (Engelberth et al., 2001). The determination of endogenous octadecanoids after volicitin elicitation is required for clarification.

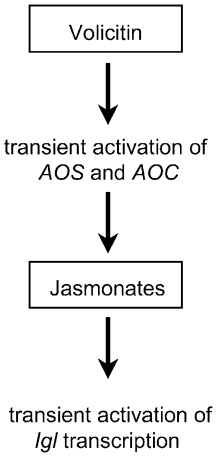

Fig. 7. Proposed model of *Igl* induction in tritrophic interaction in maize. Perception of volicitin results in transient increase of *AOC* and *AOS* transcripts. Jasmonate levels are increased and give rise to transient *Igl* transcription. Volicitin can be replaced by the fatty acid–amino acid conjugate *N*-linolenoyl-L-Gln. Whether jasmonate or an octadecanoid are effective in *Igl*-induction on transcriptional level has to be evaluated.

The investigations demonstrate that the recognition of an FAC is at the beginning of the transcriptional activation of the *Igl* gene in maize, and signal transduction is mediated by a jasmonate. These two components are common to plants that emit volatiles upon elicitation. It will be interesting to see whether and which additional common elements of the signaling cascade can be identified by future research.

4. Experimental

4.1. Chemicals

Racemic volicitin, 17*R,S*-(17-hydoxylinolenoyl)-L-glutamine was synthesized as described (Pohnert et al., 1999a). *N*-Linolenoyl-L-glutamine and *N*-linolenoyl-D-glutamine were available according to the protocol of Pohnert et al. (1999b). (15*R,S*,16*R,S*)-*N*-(15,16-Epoxylinoleoyl)-L-glutamine and (15*R,S*,16*R,S*)-*N*-(15,16-dihydroxylinoleoyl)-L-glutamine were synthesized as described by Spiteller and Boland (2003a). The synthesis of the indanone followed the protocol of Schüler et al. (2001). Methyl jasmonate was from Duchefa Biochemie BV, Harleem, The Netherlands. Acetylsalicylic acid and alamethicin was purchased from Sigma/Aldrich (Deisenhofen, Germany).

4.2. Plant sources

Maize seeds, *Zea mays*, *bx1bx1* mutant line (Hamilton, RH, 1964), planted in potting soil were maintained in growth chambers on a 16-h light, (6.00–22.00) 8-h dark cycle (22.00–6.00). Incubator temperature fluctuated between 20 °C during the dark cycle and 28 °C when lights were on. Incubation was with 11 days old seedlings.

4.3. Plant treatment

All treatments were performed between 9.00 and 10.00. For the cut seedling assay incubations were as described by Frey et al., (2000). 1 nmol of volicitin and of the other test substances were applied per plant in a volume of 500 µl. Alamethicin was applied at 10 µg ml^{-1}.

In the whole plant assay each of three leaves received two superficial damage sites using a razor to scratch the abaxial surface of the leaves perpendicular to but not including the midrib vasculature. A total of 18 µl of buffer or volicitin solution, 5 nmol per plant, was evenly distributed between the six damage sites of each plant as described by Schmelz et al. (2001). Methyl jasmonate, (30 nmol/plant) was applied in lanoline paste evenly to each wound as described by Baldwin et al. (1996), pure lanolin paste was used as a control. Three plantlets were combined for each individual treatment and the incubation series were repeated independently two to six times.

The inhibitors phenidone and aristolochic acid were used at 1 mM and 0.3 mM, respectively, as described by Engelberth et al. (2001). Pre-incubation was for 24 h. Pre-incubation experiments with acetylsalicylic acid (0.5 mM) were conducted for 13 h.

Incubation with 1 mM ACC (Sigma-Aldrich) was done in the whole plant assay. A total of 5 ml was applied to each plant by spraying 30 min before wounding and application of the test substances.

4.4. RNA isolation and quantification of transcript levels

RNA was isolated with the NucleoSpin RNA Plant kit (Macherey and Nagel, Düren). First strand cDNA synthesis and transcript quantification with the Light-Cylcler Instrument (Roche Molecular Biochemicals) was as described by Frey et al. (2000). Amplification of the *GAP C* sequence was used for normalization of the transcript levels as described. Partial sequences of the maize *AOS* and *AOC* genes were amplified from cDNA with the following primer pairs:

AOS: 5′GACCGCCTCGACTTCTACTAC3′ and 5′GAAGAGCAGCTGCTTCACCTT3′; AOC: 5′AAGGTGCAGGAGCTGTACG3′ and 5′CAGGTACGACTCCTCGTAGGT3′. The *AOC* sequences were derived from the accessions TC92158, TC92160 and *AOS* from BE511221. The PCR products were cloned in pBlueskript KS+ and the amplification of the respective gene sequence was verified by sequence analysis on an ABI 310 instrument (Perkin Elmer). The cloned sequences were used as standards in the quantification experiment. Due to the high degree of sequence identity and the extremely high GC-content it was not possible to create *AOC* gene 1 *versus* *AOC* gene 2 primer pairs that yielded efficient amplification in quantitative PCR. Therefore, we used the primer pair above that proved to amplify both genes with the same efficiency. *AOC* annealing conditions were 59° C/8 s and elongation was at 72 °C/15 s, *AOS* was amplified by annealing at 57 °C/10 s, and elongation conditions were 72 °C/19 s.

Acknowledgements

We thank Eric Schmelz and Jim Tumlinson for kindly providing volicitin, Katharina Lange for establishment of the AOC quantification system and Regina Hüttl for excellent technical assistance. Financial support by Deutsche Forschungsgemeinschaft, SSP 1152, and by the Fonds der Chemischen Industrie, Frankfurt a.M., is gratefully acknowledged.

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