Chemical Interference of Pathogen-associated Molecular Pattern-triggered Immune Responses in *Arabidopsis* Reveals a Potential Role for Fatty-acid Synthase Type II Complex-derived Lipid Signals*[S]

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We describe an experimental setup using submerged cultures of *Arabidopsis* seedlings in 96-well microtiter plates that permits chemical intervention of rapid elicitor-mediated immune responses. Screening of a chemical library comprising 120 small molecules with known biological activities revealed four compounds reducing cellulysin- or flg22-activated gene expression of the early pathogen-associated molecular patterns (PAMP)-responsive *ATL2* gene. One chemical, oxytriazine, was found to induce *ATL2* gene expression in the absence of PAMP. By monitoring additional flg22-triggered immediate early plant responses, we present evidence that two compounds, triclosan and fluazinam, interfere with the accumulation of reactive oxygen species and internalization of the activated plasma membrane resident FLS2 immune receptor. Using triclosan structure types and enzyme activity inhibition assays, *Arabidopsis* MOD1 enoyl-acyl carrier protein reductase, a subunit of the fatty-acid synthase type II (FAS II) complex, was identified as a likely cellular target of triclosan. Inhibition of all tested elicitor-triggered early immune responses by triclosan indicates a potential role for signaling lipids in flg22-triggered immunity. Chemical profiling of *eca* mutants, each showing deregulated *ATL2* gene expression, with the identified compounds revealed mutant-specific response patterns and allowed us to deduce tentative action sites of *ECA* genes relative to the compound targets.

In plants and animals, the first active defense response against microbial invasion is initiated upon the recognition of pathogen-associated molecular patterns (PAMPs).[2] These patterns, also referred to as elicitors, include components of fungal cell walls such as chitin (*N*-acetyl-chito-oligosaccharide oligomers), lipopolysaccharides (LPS) from the outer membrane of Gram-negative bacteria, flagellin, and EF-Tu (1, 2). The signaling pathways and molecular mechanisms underlying PAMP-triggered innate immunity are not completely elucidated. Genetic and biochemical evidence points to an intimate link between PAMP-triggered immunity and the production of reactive oxygen species, ligand-induced endocytosis of pattern recognition receptors (PRRs), protein phosphorylation, mitogen-activated protein kinase signaling, and transcriptional activation of the early response gene (1, 3–7).

ATL2 is a member of the *Arabidopsis ATL* gene family and has been characterized as an early and transiently activated PAMP-responsive gene (8, 9). Several members of the *ATL* gene family, each encoding RING-H2 finger domain-containing proteins, are induced by components of fungal cell walls (10) as well as by bacterium-derived flagellin (4, 11). Additionally, a tobacco cell culture expressing the tomato resistance gene *Cf9*, a member of the *ATL* tobacco family, designated *ACRE-132,* was rapidly induced within 30 min upon addition of the *Cladosporium fulvum*-derived fungal effector Avr9 in the presence of cycloheximide (12). This suggests that *ACRE-132* gene activation occurs independent of *de novo* protein biosynthesis, a typical feature of early PAMP-responsive genes. A mutational approach aimed at the identification of genetic components directing *Arabidopsis ATL2* elicitor-responsive gene induction revealed several mutants showing constitutive expression of *ATL2* (*eca,* expression constitutiva de ATL2) but failed to recover nonresponsive mutant plants (13).

Traditional forward genetic approaches have been widely used to identify and assign genes from various model organisms to specific biological processes. Major limitations of such mutational approaches are lethal mutations because of essential gene functions, pleiotropic effects caused by multifunctionality of a gene product, and redundant gene functions. Small molecules with inducing or inhibitory activities have the potential to complement mutational approaches for the dissection of biological processes because chemical intervention can be performed in a conditional, dose-dependent, and reversible manner (14). A model organism for plant biology, *Arabidopsis thaliana*, provides the potential to probe small molecule libraries for com-

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pounds acting on cellular targets that are inaccessible to conventional mutational analysis (15). Few studies have systematically explored the potential of small molecules to interfere with plant-specific processes (16–18).

Here we describe an experimental system that permits chemical manipulation of early PAMP-triggered immune responses in *Arabidopsis* seedlings. By taking advantage of several transgenic lines each carrying a fusion between an early PAMP-responsive promoter and the β-glucuronidase reporter gene, we screened a chemical library comprising 120 small molecules with known biological activities. Submerged cultures of *Arabidopsis* seedlings enabled uniform contact with and controlled exposure of the plants to the elicitors cellulysin or flg22 as well as to the tested compounds. By monitoring β-glucuronidase reporter activity histochemically and/or in quantitative fluorimetric assays, we identified compounds that either inhibit or induce early PAMP-responsive genes. The candidate compounds were subsequently tested for their ability to interfere with elicitor-induced reactive oxygen species production and PRR endocytosis. The likely cellular target of one of the inhibitory compounds, enoyl-ACP reductase, was validated by the use of structure variants and enoyl-ACP reductase activity assays. Our results indicate a possible role for signaling lipids in PAMP-triggered immune responses and provide the basis for future screening of natural compound libraries.

EXPERIMENTAL PROCEDURES

Plant Material—In this study we used *Arabidopsis* transgenic lines carrying fusions of the following 5′-regulatory gene sequences to the β-glucuronidase (*GUS*) reporter gene: *ATL2* (8), *WRKY22*, *WRKY29*, and *4CL1* (described below). *ATL2p::GUS* is expressed in a C-24 background, *WRKY22p::GUS* and *WRKY29p::GUS* in Col-0, and *4CL1p::GUS* in a *Landsberg erecta* background. The transgenic Ws-0/FLS2-GFP line used for monitoring FLS2 endocytosis is described by Robatzek *et al.* (6).

GUS Reporter Constructs—1.8 and 1.9 kb of the 5′ upstream region (relative to the ATG start codon) of the *AtWRKY22* (At4g01250) and *AtWRKY29* (At4g23550) locus, respectively, were PCR-amplified from *Arabidopsis* Col-0 genomic DNA using Gateway suitable primers, 5′-TTACCTACATGTGC-TTTGCTGTTGC-3′ and 5′-ATCGTCGGCCATTGAATT-TGGTTAC-3′ (*AtWRKY22*) and 5′-TACTCACAGAAGTC-AATAAGCGCG-3′ and 5′-CGACTATTGCTTCTAGGTC-TCCTTC-3′ (*AtWRKY29*), and cloned into the binary pJAWOHL11-GW-GUS vector resulting in a translational fusion with the *uidA* gene. 1 kb of the 5′ upstream region of the *At4CL1* (At1g51680) locus (19) was PCR-amplified from genomic *Arabidopsis* Col-0 genomic DNA using the following primers: B11F, 5′-ATTAACTGCAGACACTTTTAGCCCAT-AACTTTC-3′, and B11R, 5′-CTTGTGGCGCCATGGTAAA-TAGTAAATATTGTG-3′. Primer B11F contained a 5′ PstI restriction sequence, and primer B11R, spanning the ATG codon of the *4CL1* gene, contained one mismatch to the sequence of the *At4CL1* gene to generate an NcoI restriction site. For fusion to the *uidA* gene, the PCR product was digested with PstI and NcoI and ligated with PstI/NcoI-digested vector pBT10-TATA-GUS (20), from which the promoter::GUS cassette was excised with the restriction enzymes ClaI and SacI and

inserted into the binary vector pGPTV-Kan (21). Transformations were performed by the dip floral method (22), and transformants were selected by kanamycin resistance.

Growth Conditions and Compound Screening Procedure—The small molecule library was provided by SYNGENTA Corp. Surface-sterilized seeds were stratified for 4 days at 4 °C. Seedlings were grown for 7 days in 96-well microtiter plates (NUNC) containing ∼2–4 seeds per well in Murashige and Skoog (MS) liquid medium and 0.25% sucrose. The microtiter plates were incubated at 24 °C with continuous shaking at 100 rpm in a growth chamber (Percival AR-75L) under 16/8 light/dark conditions. After 7 days, the medium was removed by a multichannel pipette (Matrix) and replaced by MS medium containing 10 ppm of each compound dissolved in Me_2SO and incubated for 120 min. At the end of the preincubation time, seedlings were treated for 120 min using MS medium or MS medium containing 100 mg/ml cellulysin (Calbiochem) as described previously (8). FLS2-dependent immune responses were induced using MS medium containing 1 μM flg22 peptide.

GUS Expression Assay—Histochemical and fluorometric assays were performed as described (23). Samples were mounted on microscope slides using 50% glycerol and inspected with a Leica MZFLIII stereomicroscope. Protein concentrations were determined by Bradford assays using a commercial kit (Bio-Rad).

Oxidative Burst Assay—Production of reactive oxygen species was assayed by H_2O_2-dependent luminescence of luminol (24). Briefly, *Arabidopsis* leaf pieces (∼1 mm) were floated overnight in water, incubated in a solution containing 1% Me_2SO and 10 ppm compound for 30 min in the presence of luminol and peroxidase, and elicited with 1 μM flg22. Luminescence emission was measured in a luminometer (top count NXT; PerkinElmer Life Sciences).

flg22-induced Endocytosis Assay—We analyzed flg22-induced endocytosis as described previously (6). Briefly, detached leaves of Ws-0/FLS2-GFP transgenic seedlings grown under sterile conditions were preincubated in a solution containing 1% Me_2SO for 30 min. After adding the tested compounds at 10 ppm for 30 min, endocytosis was triggered by supplying the solution with 10 μM flg22 for 30 min. Microscopy was performed using the confocal laser microscope Leica TCS SP2 with a ×63 oil objective and ×4 zoom.

Enoyl-ACP Reductase Activity Assay—Enoyl-ACP reductase activity was measured as described previously (25). Total protein was extracted from ground seedlings using 10 mM sodium phosphate (pH 9.0) and 1 mM dithioerythritol. Protein concentration was determined by the Bradford assay using a commercial kit (Bio-Rad). 50 μg of protein was dissolved in the reaction buffer (10 mM sodium phosphate (pH 6.2) and 140 μM NADH). Enoyl-ACP reductase activity was monitored by the decrease of absorbance at 340 nm because of oxidation of NADH at room temperature (22 °C). 120 μM crotonoyl-CoA (Sigma) was used as substrate, whereas the endogenous NADH oxidation was measured in the absence of crotonoyl-CoA. The reaction was initiated after addition of substrate and was continuously monitored for 15 min.

RESULTS

Structurally Diverse Small Molecules Interfere with Early PAMP-responsive Gene Expression—We used a chemical library comprising 120 small molecules with known biological activities (supplemental Table 1) to identify candidate compounds that modified elicitor-responsive gene expression. We established a compound screening procedure using 7-day-old *Arabidopsis* seedlings grown in submerged culture in 96-well microtiter plates to monitor transcriptional activation of early PAMP-responsive genes (see "Experimental Procedures"). Such genes typically encode transcripts, which transiently increase in abundance within minutes upon PAMP treatment (11). We used a transgenic line carrying a fusion of the early PAMP-response gene *ATL2* promoter to the β-glucuronidase reporter gene (*ATL2p::GUS*; see Ref. 13). Although constitutive *ATL2* gene expression is mainly confined to leaf primordia and the leaf vasculature, treatment with the fungus-derived proteinaceous cellulysin elicitor (8) resulted in visible GUS expression within 2 h disseminating throughout the seedlings, which corresponds to an ~4-fold GUS activity increase in total seedling extracts (Fig. 1*A*). Only 1 of the 120 compounds tested, oxytriazine, a dihydrofolate reductase inhibitor, was capable of simulating cellulysin-mediated reporter gene expression (Fig. 1, *A* and *B*), which might be because of an agonist-like oxytriazine activity, spurious *ATL2* reporter gene activation via a PAMP-independent signaling pathway, or inhibition of a repressor blocking constitutive *ATL2* induction in the authentic cellulysin-triggered signaling pathway. Simultaneous treatment of the seedlings with oxytriazine and cellulysin did not increase *GUS* reporter gene expression above the level seen with either compound alone. In time course experiments, a more rapid reporter gene activation was detected upon treatment with the cellulysin elicitor than with oxytriazine (induction detectable within 30 and 120 min, respectively; see supplemental Fig. 1). Together this is consistent with the idea that oxytriazine either inhibits a repressor or induces a component of the authentic PAMP-triggered signaling pathway (Fig. 1, *A* and *B*).

We identified four compounds that inhibited cellulysin-triggered GUS reporter activity either strongly (~70%; triclosan) or more moderately (~50%; fluazinam, cantharidin, and fenpiclonil) (Fig. 1, *A* and *B*). Exposure of the seedlings to varying concentrations of these small molecules revealed a dose-dependent mode of action between 0.1 and 20 ppm (supplemental Fig. 2). Because histochemically detectable GUS reporter activities in leaf primordia were essentially indistinguishable in the absence or presence of each of these four compounds (Fig. 1*A*), their effect is likely to reflect inhibition of GUS reporter gene activation rather than constitutive *GUS* reporter gene transcription or GUS enzyme activity modification. Consistent with this, reverse transcription-PCR experiments using seedling-derived RNA revealed a significant reduction of cellulysin-stimulated *ATL2* endogene expression upon treatment with each of the compounds (supplemental Fig. 3). Time course experiments showed different inhibition tendency suggesting a more complex mode of action (supplemental Fig. 1). Although a 2-h preincubation period of the seedlings with triclosan and the subsequent addition of cellulysin resulted in maximal and
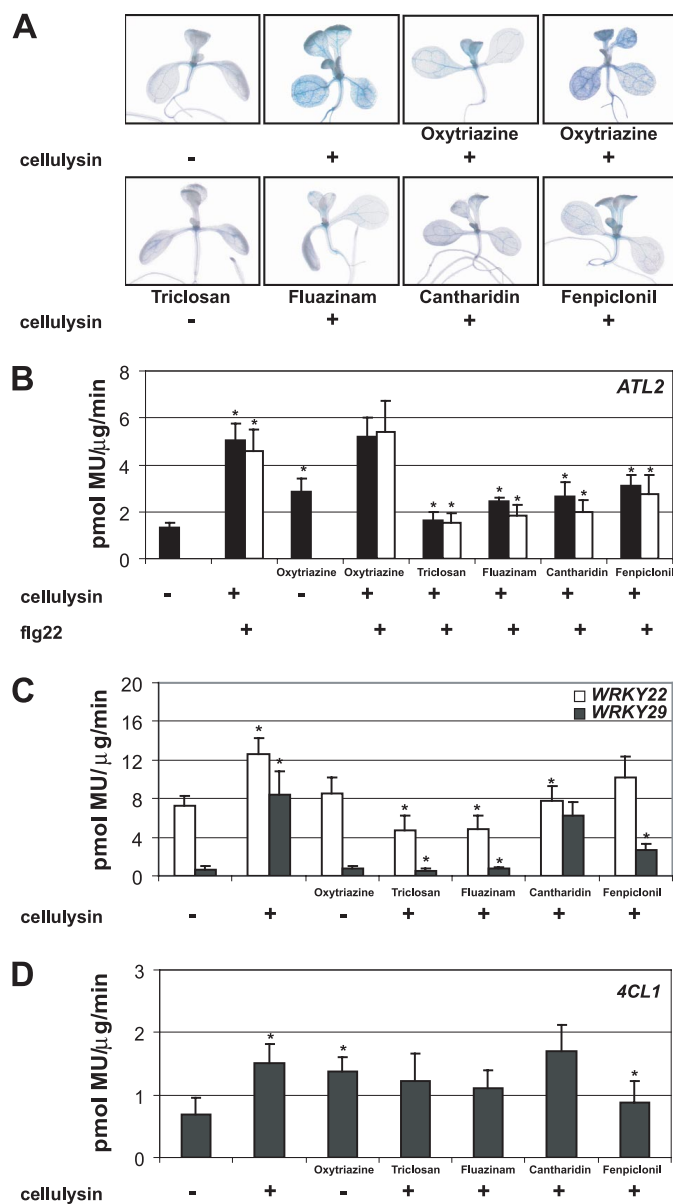

FIGURE 1. **Identification of small molecules interfering with early PAMP-responsive gene expression.** *A,* histochemical localization of *GUS* reporter gene activity. Seven-day-old *ATL2p::GUS* seedlings were preincubated for 120 min with 10 ppm of each indicated compound and induced for 120 min with MS media or MS media containing 100 mg/ml cellulysin; representative samples of three independent experiments are shown. *B,* fluorometric GUS activity assays of *ATL2p::GUS* seedlings treated with cellulysin (*black bars*) or 1 μM flg22 (*white bars*). *C,* WRKY22p::GUS (*white bars*) and WRKY29p::GUS (*black bars*). *D, 4CL1p::GUS.* Treatments in *B–D* were identical to those described in *A.* Data points shown are the mean of eight independent experiments ± S.D. Asterisks indicate a significant difference (*p* < 0.01) of GUS activity changes mediated by the indicated compounds relative to the nontreated control (in case of oxytriazine) or cellulysin/flg22-induced samples.

sustained inhibition of reporter gene activity within 30 min, both cantharidin and fenpiclonil permitted additional slight increases of reporter gene activity even 120 min after elicitor addition. Moreover, in the presence of triclosan, fluazinam, or fenpiclonil, basal GUS reporter activity remained unaffected within the 2-h preincubation period, and cantharidin significantly stimulated reporter activity in the absence of cellulysin (120-min time point; supplemental Fig. 1). This might indicate the existence of multiple cellular cantharadin targets exerting
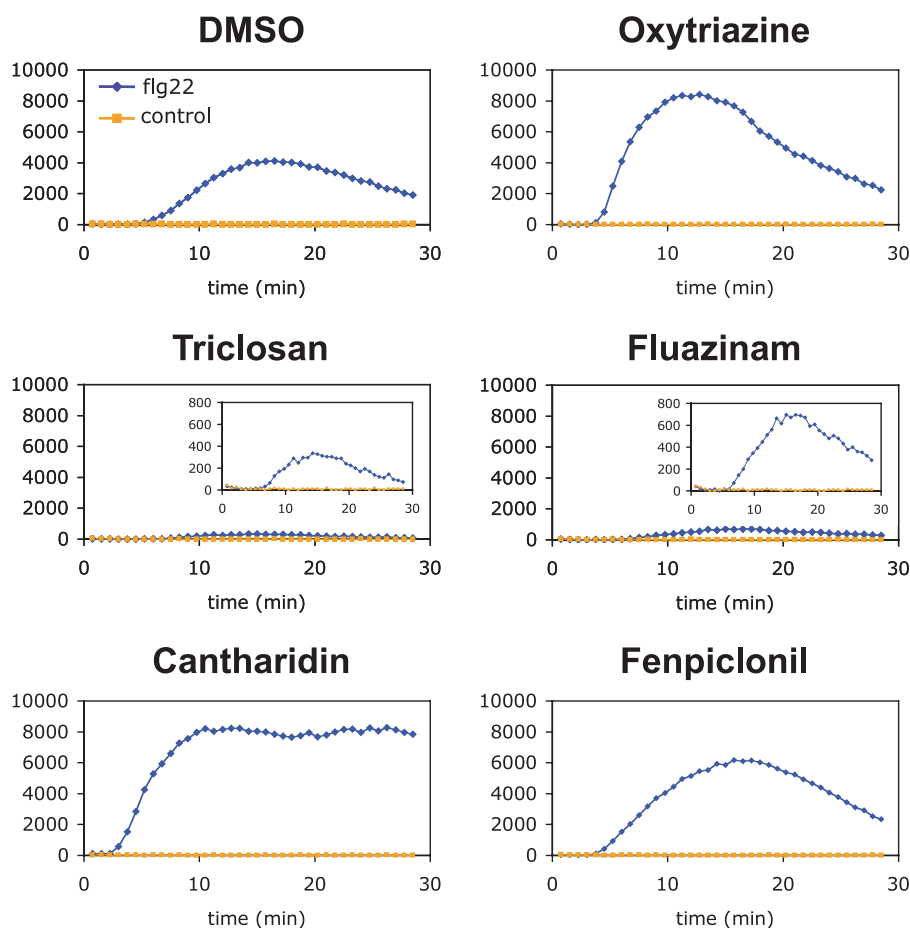
FIGURE 2. **Chemical intervention of flg22-dependent production of reactive oxygen species.** *A. thaliana* ecotype Landsberg erecta leaf pieces were incubated in a solution containing 1% Me$_2$SO (DMSO) and 10 ppm of the indicated small molecules for 30 min, supplied with luminol and peroxidase, and elicited with 1 μM flg22. Luminescence emission is given as relative light units and continuously monitored over the indicated time course. Each *graph* shows a representative measurement of three independent experiments each with six replicates.

expression in *WRKY22p*::*GUS* and *WRKY29p*::*GUS* lines, respectively (Fig. 1*C*). The *WRKY22* and *WRKY29* promoters are derived from genes encoding pathogen-responsive WRKY transcription factors (27, 28). Oxytriazine failed to induce the reporter gene in both *WRKY22p*::*GUS* and *WRKY29p*::*GUS* transgenic lines (Fig. 1*C*). In *4CL1p*::*GUS* containing transgenic lines only, fenpiclonil significantly suppressed cellulysin-induced reporter gene activity (Fig. 1*D*). Thus, elicitor-triggered *4CL1p*::*GUS* expression might occur independently of triclosan, fluazinam, and cantharidin targets. Interestingly, oxytriazine treatment also induces *4CL1p*::*GUS* induction (Fig. 1*D*), suggesting that its target(s) is shared between *4CL1* and *ATL2*. Taken together, these findings provide evidence for the existence of both common and distinct components that are recruited for early PAMP-responsive gene activation at different promoters.

Chemical Intervention of flg22-dependent Oxidative Burst—The production of reactive oxygen species ("oxidative burst") is a typical early response of plant cells to elicitor treatment (29). We assayed the well characterized flg22-induced FLS2-de-

opposite effects on constitutive and PAMP-induced *ATL2* gene expression, respectively. Taken together, of the four candidate inhibitors identified, only triclosan, fluazinam, and fenpiclonil appeared to specifically interfere with cellulysin-triggered *ATL2* gene expression.

When the *ATL2p*::*GUS*-expressing seedlings were treated with flg22, a 22-mer peptide derived from the bacterial motor protein (26), we observed a similar 4-fold reporter gene induction in comparison with cellulysin treatment (Fig. 1*B*). Each of the five compounds affecting cellulysin-stimulated *ATL2* reporter gene activity also interfered with flg22-induced reporter gene activation in an essentially identical manner (Fig. 1*B*), suggesting that these small molecules alter the activity of common PAMP-triggered signaling events shared between the two sequence-unrelated elicitors.

To determine whether the identified five compounds affect the induction of early PAMP-responsive genes other than *ATL2*, we tested additional *GUS* reporter lines. The triclosan and fluazinam inhibitors of cellulysin-stimulated *ATL2p*::*GUS* reporter activity affected in a comparable manner reporter gene activation in *WRKY22p*::*GUS* and *WRKY29p*::*GUS* transgenic lines (~60 and 80%, respectively; Fig. 1*C*). However, fenpiclonil and cantharidin did not significantly reduce reporter gene

pendent oxidative burst (26) in leaf tissue in the presence and absence of the identified small molecules to gain further insights in their potential mode of action on immediate early elicitor responses (luminescence assay; see "Experimental Procedures"). None of the five compounds, including the inducer oxytriazine, provoked an oxidative burst in the absence of flg22 (Fig. 2). Interestingly, the flg22-triggered transient oxidative burst was enhanced in the presence of oxytriazine. Thus, oxytriazine can itself induce early PAMP-responsive gene expression but cannot trigger the production of reactive oxygen species. In addition, although its effect leads to a further increase of the elicitor-induced oxidative burst, this is not the case for early PAMP-responsive gene expression (compare Figs. 1*B* and 2).

Triclosan, the most potent inhibitor of early PAMP-responsive gene expression, also inhibited the flg22-dependent oxidative burst most strongly (about 90% inhibition; Fig. 2). Tested triclosan structure types exerted similar effects (see below and supplemental Fig. 4). Likewise, fluazinam strongly inhibited the production of flg22-stimulated reactive oxygen species, but in the presence of fenpiclonil, the oxidative burst was hardly affected. Interestingly, cantharidin, which appears to exert opposite effects on constitutive and PAMP-induced *ATL2* gene expression, mediated a sustained rather than transient produc-
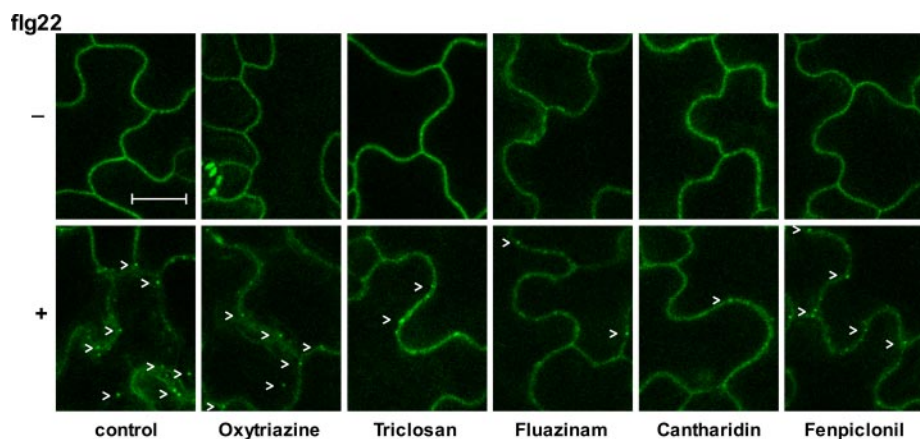
FIGURE 3. **Chemical inhibition of flg22-induced FLS2-GFP endocytosis.** Leaves of sterile-grown Ws-0/FLS2-GFP transgenic seedlings were preincubated in a solution containing 1% Me$_2$SO (*DMSO*) for 30 min. The indicated compounds were added at 10 ppm for 30 min. FLS2-GFP endocytosis was triggered by supplying 10 μM flg22 for 30 min. Micrographs show representative confocal images; *bar*, 20 μm. *Arrowheads* indicate vesicles.

tion of reactive oxygen species (Fig. 2). These complex cantharidin-mediated changes on both the flg22-dependent oxidative burst and PAMP-responsive gene expression are consistent with the presumed multiple cellular targets for this known phosphatase inhibitor and indicate that phosphatase function may be necessary for the transient behavior of the flg22-triggered oxidative burst.

Inhibition of flg22-mediated FLS2 Endocytosis—Recently, flg22-induced endocytosis of its cognate receptor kinase FLS2 has been reported (6). We addressed potential effects of the identified small molecules on PAMP receptors by monitoring FLS2 expression, subcellular localization, and flg22-triggered FLS2 activation. The latter can be assayed indirectly by flg22-dependent receptor internalization via endocytosis. *Arabidopsis* Ws-0 leaves, stably expressing the FLS2-GFP fusion protein (6), were elicited with flg22 after preincubation with the compounds, and FLS2-GFP endocytosis was observed by confocal microscopy. In the absence of flg22, none of the chemicals, including the inducer oxytriazine, led to changes in FLS2-GFP expression levels or subcellular localization within a pretreatment period of 30 min (Fig. 3). Thus, neither induction by oxytriazine nor chemical suppression of flg22-responsive gene expression by triclosan, fluazinam, cantharidin, and fenpiclonil is because of mis-localization, mis-expression, or degradation of the FLS2 receptor.

Interestingly, the inducer oxytriazine was not capable of triggering FLS2 endocytosis. This suggests that oxytriazine bypasses PAMP receptor activation to elicit PAMP-responsive gene expression (see "Discussion"). Consistent with this, flg22-mediated endocytosis of FLS2-GFP was retained in the presence of oxytriazine (Fig. 3). In contrast, flg22-mediated FLS2 endocytosis was clearly affected in the presence of the inhibitors triclosan, fluazinam, and cantharidin (Fig. 3, *lower panel*). FLS2 endocytosis was retained in the presence of fenpiclonil (Fig. 3, *lower panel*) but was significantly affected upon longer preincubation times (1.5 h; data not shown). Although the appearance of FLS2-GFP-containing "buds" at the cell membrane was still detectable in the presence of all the compounds, subsequent steps of endocytosis, such as vesicle "pinching off" and

mobile FLS2-GFP vesicle trafficking, were severely reduced (Fig. 3).

Chemical Interference of FAS II Leads to Inhibition of Early PAMP-responsive Gene Expression—Fatty acid biosynthesis is an essential metabolic pathway and involves mainly two enzyme complexes, acetyl-CoA carboxylase (ACCase), which catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, and the fatty-acid synthase complex (FAS), catalyzing *de novo* biosynthesis of fatty acids using malonyl-CoA as the elongator by sequential addition of 2-carbon units to the growing acyl chain (30). In plants, the fatty-acid synthase complex is termed FAS II and consists of eight dissociable polypeptides, each with separate enzymatic activities (Fig. 4*A*). In *Escherichia coli*, triclosan targets the *fabI* gene product, a rate-limiting subunit of the FAS II complex, the enoyl-acyl carrier protein reductase catalyzing the final reduction of the chain elongation cycle (31). Plant enoyl-ACP reductase can functionally replace its counterpart within the bacterial complex as shown by genetic complementation (32). The *Arabidopsis* enoyl-ACP reductase is encoded by *MOD1* and a point mutation in *mod1*-1 was shown to lead to decreased enzymatic activity, growth, and developmental aberrations as well as spontaneous cell death (33).

We reasoned that if MOD1 is the target of triclosan in *Arabidopsis*, then other known inhibitors of FAS II or the ACCase complex should exert similar effects on PAMP-triggered *ATL2p::GUS* reporter gene expression. However, we failed to inhibit ACCase activity *in planta* using the well characterized inhibitors dichlorofopmethyl, fluazifop acid, or tralkoxidim (Fig. 4*B*). This might be explained by the selectivity of these inhibitors against the homomeric ACCase form present in monocotyledonous plants, whereas *Arabidopsis* contains a heteromeric ACCase complex (34). Unlike this, when we targeted FAS II complex components other than enoyl-ACP reductase by treatment of the seedlings with hexachlorophene and cerulenin, we observed a significant inhibition of cellulysin-mediated *ATL2p::GUS* reporter activity (60 and 40%, respectively; Fig. 4*B*). These results point to a contribution of multiple FAS II complex components, including MOD1 to early PAMP-responsive gene expression.

MOD1 Is a Cellular Target of Triclosan and Triclosan Structure Types—Triclosan structure types inhibiting *E. coli* enoyl-ACP reductase have been described (35). Each of three tested structural triclosan analogs strongly inhibited cellulysin-mediated *ATL2p::GUS* reporter activity, although with different effectiveness, demonstrating that the effect of triclosan on reporter gene expression can be modulated by side group modifications of the structural core. The fact that triclosan and the three tested structure types each target the bacterial enoyl-ACP reductase and exert a similar effect on PAMP-triggered reporter gene expression suggests the existence of a common
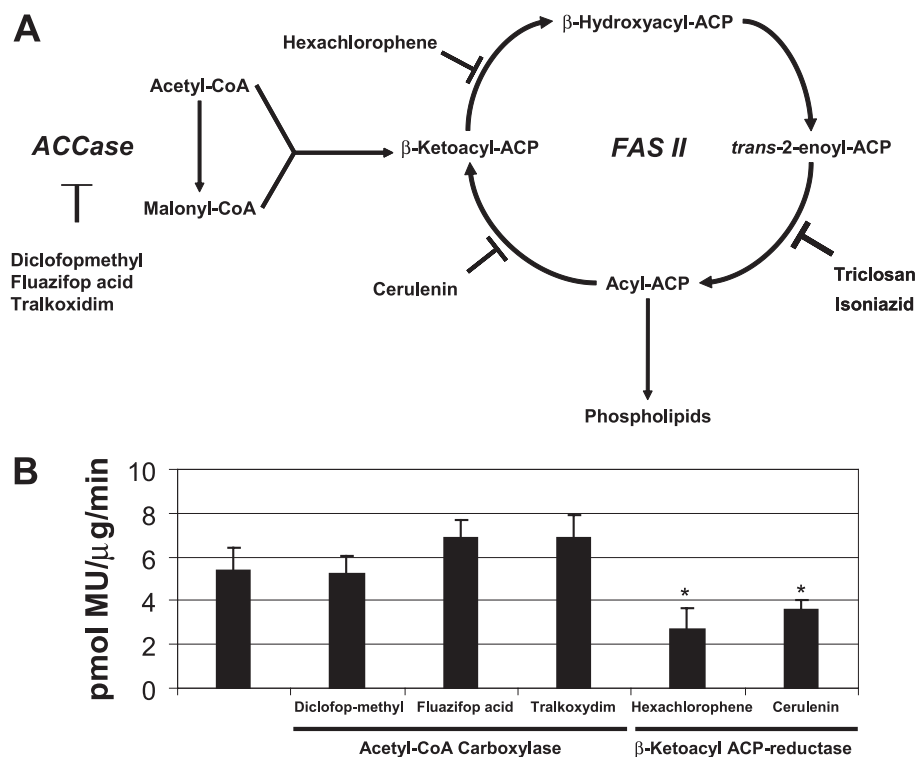
FIGURE 4. **Chemical interference of the fatty acid biosynthesis pathway.** *A,* fatty acid biosynthesis pathway scheme. Biosynthesis steps blocked by ACCase or FAS II inhibitors are shown. *B,* fluorometric GUS activity assays. Seven-day-old *ATL2p::GUS* seedlings were preincubated for 120 min with 10 ppm of each indicated ACCase or FAS II inhibitor, and immune responses were induced for 120 min with 100 mg/ml cellulysin. Data points are the mean of five independent experiments $\pm$ S.D. *Asterisks* indicate a significant difference ($p < 0.01$) of GUS activity changes mediated by the indicated compounds relative to the cellulysin-induced sample.

inducer oxytriazine revealed a significant increase of the reporter gene induction only in *eca2* plants (Fig. 6).

Next we investigated the ability of the inhibitors triclosan, fluazinam, cantharidin, and fenpiclonil to interfere with constitutive or cellulysin-induced *GUS* reporter gene expression in *eca* mutants. Upon treatment with the compounds, constitutive reporter gene expression was not affected in the mutant plants (data not shown). Remarkably, cellulysin-induced reporter gene activity in *eca4* mutants was completely unaffected by each of these inhibitors (Fig. 6), suggesting that *ECA4* acts either downstream of their cellular targets or that loss of *ECA4* leads to the activation of a spurious *ATL2* induction pathway that is insensitive to the tested compounds. In contrast, cellulysin-stimulated reporter gene activity in *eca2* plants retains its sensitivity to all but one inhibitor (cantharidin). Because the identified compounds specifically interfere with PAMP-inducible but not constitutive *ATL2*

gene expression (see Fig. 1), this could indicate that *ECA2* acts upstream of the triclosan, fluazinam, and fenpiclonil targets. Unlike this, in *eca5* plants a significant inhibition of elicitor-stimulated *GUS* reporter activity was detectable in the presence of triclosan and fenpiclonil (Fig. 6), suggesting that cellulysin-stimulated signaling is modified in *eca5* plants such that is has become insensitive to fluazinam and cantharidin targets. This hints at a potential action of *ECA5* downstream of fluazinam and cantharidin targets.

DISCUSSION

Here we described an experimental setup using 96-well microtiter plates and submerged culture conditions that permits chemical intervention of elicitor-mediated immune responses in a whole organism context. Unlike soil drench or agar media, the use of liquid media allows direct and uniform contact of all seedling organs to the chemicals, thereby minimizing the time interval needed for the compounds to reach their cellular targets (*e.g.* organ-specific uptake and intercellular as well as intracellular transport rates). This maximizes the probability to intervene with cue-dependent plant processes in a synchronous manner. By this means, we were able to shorten the pretreatment time interval with the chemicals to 120 and 30 min (GUS expression and oxidative bust/flg22-induced endocytosis assays, respectively) before adding the tested elicitors to trigger immune responses (cellulysin or flg22). Because we focused on early elicitor-triggered plant responses in this study, the total exposure time of the seedlings to the chemicals did not

cellular target *in planta*, thereby identifying *Arabidopsis* MOD1 enoyl-ACP reductase as the likely target (Fig. 5*A*; see also below). Isoniazid is also known to inhibit bacterial enoyl-ACP reductase by a mode of action that is distinct from triclosan (structurally unrelated compound; see Ref. 36). Unexpectedly, isoniazid failed to inhibit cellulysin-triggered *ATL2p::GUS* reporter activity in *Arabidopsis* (Fig. 5*A*). To test whether the differential ability of triclosan and isoniazid to inhibit reporter gene expression correlates with expected changes in MOD1 enzyme activities, we determined in dose-response experiments *in vitro* MOD1 activity in plant extracts (compare Fig. 5, *B* and *C*). Although triclosan strongly inhibited MOD1 at both 10 and 20 ppm, isoniazid failed to inhibit this enzyme activity even at 20 ppm (Fig. 5*C*). This provides direct evidence that MOD1 is a cellular target of triclosan and reveals an unexpected insensitivity of the plant enzyme to isoniazid.

Chemical Profiling of eca Mutants Suggests Tentative Action Sites of ECA Genes—Previously we described several mutants showing heightened constitutive *ATL2* expression (*eca* (13)). We utilized these mutants to obtain further insights how the identified small molecules might interfere with PAMP-responsive *ATL2* gene expression. In the hydroponic growth system used for testing chemical interference of *ATL2* promoter-driven reporter gene expression, *eca2* and *eca5* mutants showed a 2-fold and *eca4* plants more than 4-fold elevated GUS reporter activity relative to wild type (Fig. 6). In each mutant, *GUS* reporter activity could be further increased by treatment with the cellulysin elicitor. Treatment of the mutants with the
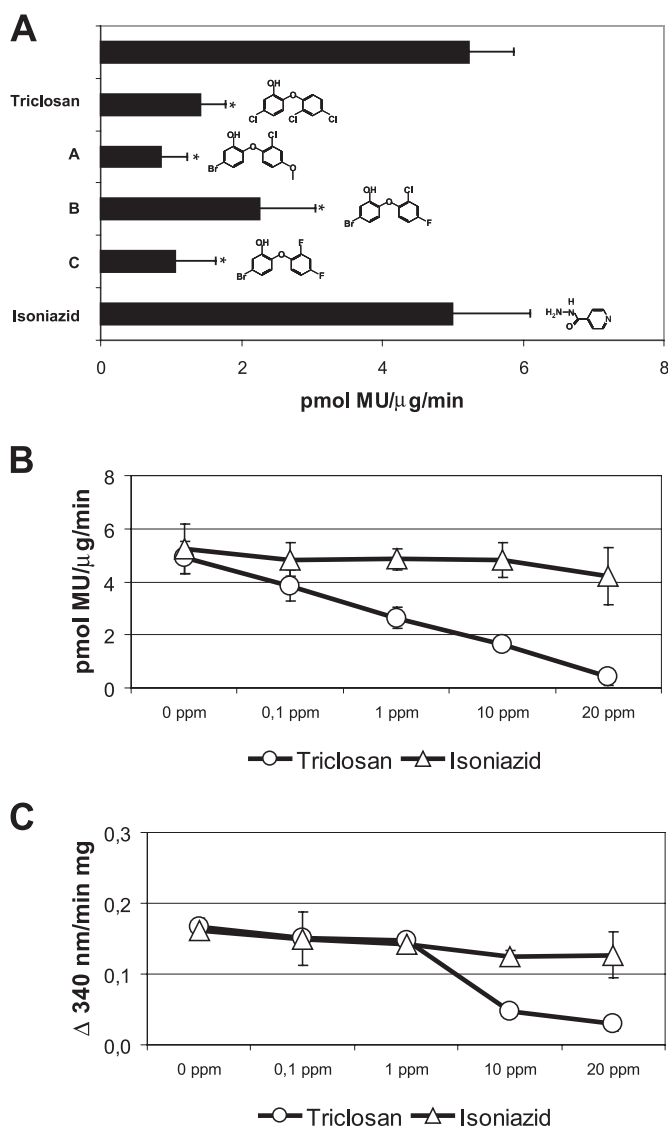
A

B

C

FIGURE 5. **Chemical inhibition of enoyl-ACP reductase.** *A,* fluorometric GUS activity assays of 7-day-old *ATL2p::GUS* seedlings preincubated for 120 min with 10 ppm of each indicated enoyl-ACP reductase inhibitor and induced for 120 with 100 mg/ml cellulysin. The chemical structures of each tested inhibitor are shown. Data points are the mean of eight independent experiments ± S.D. *Asterisks* indicate a significant difference ($p < 0.01$) of GUS activity changes mediated by the indicated compounds relative to the cellulysin-induced sample. *B,* fluorometric GUS activity assays; *C,* enoyl-ACP reductase activity assays. Seven-day-old *ATL2p::GUS* seedlings were treated for 120 min with the indicated concentrations of triclosan (○) or isoniazid (Δ). Immune responses were induced for 120 min with 100 mg/ml cellulysin. Data points are the mean of three independent experiments ± S.D.

exceed 4 h (*e.g.* for the GUS reporter gene assays). We consider this important because short exposure times can help to minimize secondary or indirect effects of a compound on a biological process. For example, triclosan has herbicidal activity after prolonged exposure (several days) of plants, which is likely because of a physiological collapse resulting from sustained fatty acid biosynthesis inhibition. We hypothesize that the observed rapid (within 30 min; supplemental Fig. 1) triclosan-dependent inhibition of PAMP-mediated immune responses (early PAMP-responsive gene expression, oxidative burst, inhibition of FLS2 internalization) indicates a signaling rather than structural role of lipids in plant immunity (see below).
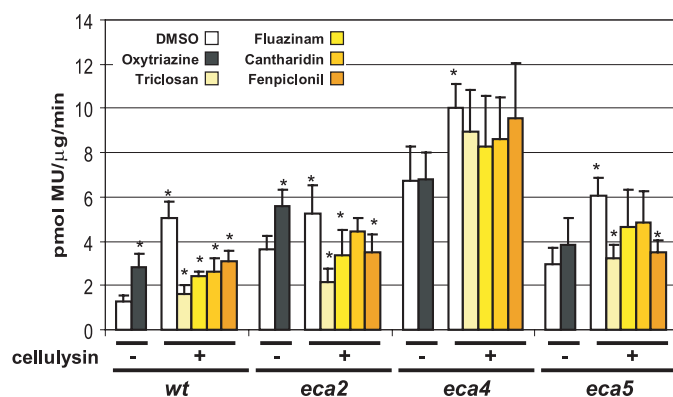

FIGURE 6. **Chemical profiling of *eca* mutants.** Fluorometric GUS activity assays of seedlings were preincubated for 120 min with 10 ppm of each indicated small molecule. Immune responses were induced for 120 min with 100 mg/ml cellulysin. Data points are the mean of seven independent experiments ± S.D. *Asterisks* indicate a significant difference ($p < 0.01$) of GUS activity changes mediated by the indicated compounds relative to the nontreated control (in case of oxytriazine) or to cellulysin-induced samples in wild type (*wt*) and *eca* mutant backgrounds.

The sole compound identified, which is able to induce normally PAMP-responsive gene induction of *ATL2* and *4CL1*, was oxytriazine, a dihydrofolate reductase (DHFR) inhibitor. DHFR is a key enzyme for the biosynthesis of purines and several amino acids (37). Interestingly, DHFR inhibition was recently shown to stimulate in *Drosophila melanogaster* gene expression of cell cycle, signal transduction, and immune response genes, indicating the participation of DHFR-derived products in different pathways/responses (38). Because simultaneous treatment of *Arabidopsis* seedlings with oxytriazine and cellulysin did not increase *ATL2p::GUS* reporter expression above the level seen with either compound alone (Fig. 1*B*) and because oxytriazine on its own neither induced an oxidative burst nor receptor internalization (Figs. 2 and 3), its inducing activity might result from inhibition of a repressor acting in a common PAMP-triggered signaling pathway downstream of receptor activation/endocytosis. We note that the inducing activity of oxytriazine is distinct from other recently identified natural compounds that hyper-induce early PAMP-responsive gene expression only in the presence of the elicitor.[3] Although oxytriazine alone was unable to provoke an oxidative burst, the chemical clearly enhanced the flg22-dependent production of reactive oxygen species (Fig. 2). One possibility to rationalize this finding is that inappropriate (*i.e.* premature) inhibition of the inferred PAMP signaling repressor generates a positive feedback signal, thereby amplifying the flg22-dependent oxidative burst. Alternatively, potentiation of the flg22-dependent oxidative burst in the presence of oxytriazine could be due to inhibition of a second cellular target that dampens the production of reactive oxygen species as soon as the oxidative machinery is activated.

In previous mutational studies of cellulysin-triggered *ATL2p::GUS* activation, we identified several *eca* mutants showing constitutive *ATL2* expression (13). However, despite the screening of large numbers of mutagenized *ATL2p::GUS* seedlings (50,000 M2 families), we were unable to recover

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[3] M. Serrano and P. Schulze-Lefert, unpublished data.

mutants that specifically failed to induce *ATL2p::GUS* expression upon cellulysin treatment. This may be due to genetic redundancy and/or lethality of presumed receptor(s) and downstream signaling factors. In this context, we consider the identification of four chemical inhibitors of early PAMP-responsive gene induction noteworthy because it indicates the potential of conditional chemical inhibition as a complementary approach to mutational analysis. Except for cantharidin that also affects cellulysin-independent constitutive *GUS* reporter gene expression, the inhibitors triclosan, fluazinam, and fenpiclonil appear to specifically inhibit flg22 or cellulysin-triggered *ATL2p::GUS* induction (Fig. 1*B* and supplemental Fig. 1). Moreover, whereas fenpiclonil reduced PAMP-triggered gene activation of reporter gene constructs *ATL2p::GUS*, *WRKY29p::GUS*, and *4CL1p::GUS*, triclosan and fluazinam were unable to inhibit cellulysin-stimulated *4CL1p::GUS* induction (compare Fig. 1, *B–D*). Likewise, oxytriazine only activated *ATL2* and *4CL1* gene expression. This reveals an unexpected diversity of factors directing gene expression at different early PAMP-responsive promoters. One possibility is that the observed differential compound sensitivity of the tested reporter gene constructs reflects differences in the composition of *cis*-acting elements in the respective 5′ regulatory sequences.

The identification of cellular targets of a small molecule is normally a challenging and long term undertaking. By monitoring multiple elicitor-triggered early plant responses in the presence or absence of the compounds (reporter gene induction, oxidative burst, and PRR endocytosis), we were able to delimit their potential site(s) of action. Of the three seemingly specific inhibitors, two, triclosan and fluazinam, impaired both FLS2 endocytosis (Fig. 3) and the flg22-induced oxidative burst (Fig. 2). Thus, the identified chemicals may decrease elicitor-responsive gene induction by interfering with endocytic processes of PAMP receptors at the cell periphery.

Perception of flg22 by the plasma membrane-localized FLS2 receptor kinase almost immediately leads to the generation of reactive oxygen species, protein phosphorylation, mitogen-activated protein kinase signaling, and transcriptional gene activation (3, 4, 39) and triggers FLS2 internalization by endocytosis (6). FLS2 endocytosis was impaired by the phosphatase inhibitor type 2A cantharidin (Fig. 3). As a potential target in this process, the kinase-associated phosphatase has been shown to interact with FLS2 (40) and is also known to play a role in receptor endocytosis (41). Because substitution of a conserved potential phosphorylation site in the FLS2 receptor kinase (Ws-0/FLS2^{T876V}) and the general kinase inhibitor (K252a) impairs both receptor endocytosis and all tested FLS2-dependent immune responses (6), the observed cantharidin sensitivity of FLS2 endocytosis points to the involvement of dynamic phosphorylation and dephosphorylation events in this process. In addition, we found in the presence of cantharidin a sustained rather than transient accumulation of reactive oxygen species upon flg22 treatment (Fig. 2). It is possible that this deregulated PAMP response is a consequence of the cantharidin-mediated inhibition of FLS2 endocytosis. For example, inhibition of endocytosis of the human TLR4 PRR recognizing bacterial LPS increased LPS signaling (7). Accordingly, plasma membrane localization of activated FLS2 would be sufficient to trigger an oxidative burst, whereas elicitor-responsive gene expression might require receptor internalization.

Triclosan was identified in this work as a potent inhibitor of all tested elicitor-triggered immune responses (elicitor-activated gene expression, FLS2 receptor endocytosis, and flg22-triggered oxidative burst). The close correlation between triclosan dose-dependent inhibition of MOD1 activity in plant extracts and reduction of elicitor-triggered *ATL2p::GUS* induction *in planta* (Fig. 5, *B* and *C*) is consistent with the idea that *Arabidopsis* MOD1 enoyl-ACP reductase is a cellular target. Because three tested triclosan structure types, each known to inhibit *E. coli* enoyl-ACP reductase, mimic *in planta* the immune response inhibition seen with triclosan (Fig. 5*A*), we conclude that MOD1 enoyl-ACP reductase is the relevant cellular target of these compounds. Why are the elicitor-stimulated immune responses sensitive to MOD1 enoyl-ACP reductase inhibition, a subunit of the FAS II complex that is localized in plastids? Since a 30-min preincubation period with triclosan was sufficient to compromise ligand-induced FLS2 receptor internalization from the plasma membrane as well as the flg22-dependent oxidative burst (Figs. 2 and 3), we consider it unlikely that MOD1 enoyl-ACP reductase inhibition results from a depletion of structural lipids. Unlike structural lipids, signaling lipids such as phosphatidic acid are present in minute amounts and are subject to rapid turnover (reviewed in Ref. 42). In tomato cell cultures, phosphatidic acid and its phosphorylated derivative diacylglycerol pyrophosphate are known to transiently accumulate within minutes upon treatment with flg22 or other elicitors (43). Likewise, phospholipase C inhibitors block the elicitor-induced oxidative burst in tobacco cells (44). Thus, we speculate that inhibition of enoyl-ACP reductase activity by triclosan affects signaling lipids, which in turn might impair elicitor-triggered immune responses. In this context it is interesting that in humans phospholipase D was recently shown to directly interact with and to stimulate the GTPase domain of dynamin, a protein mediating receptor endocytosis through GTP hydrolysis (45). Constitutive expression of a MOD1 mutant variant in *mod1*-1 plants was shown to reduce MOD1 wild type activity by 50%, leading to pleiotropic growth and developmental anomalies as well as spontaneous cell death (33). In this context it is not surprising that a 72% reduction of MOD1 activity in the presence of 10 ppm triclosan (Fig. 5*C*) has major effects on elicitor-triggered immunity. The conditional chemical interference of MOD1 activity described here illustrates the potential in revealing putative links between the activity of a multifunctional enzyme complex and signaling lipids in plant immune responses.

To obtain further information where the identified compounds might interfere with PAMP-responsive gene expression, we have taken advantage of previously identified *eca* mutants showing constitutive *ATL2* expression (13). The recessive inheritance of the mutations in *eca* plants suggests that constitutive *ATL2* gene expression results either from the elimination of repressors acting in the authentic elicitor-triggered gene induction pathway or opens spurious signaling pathways enhancing constitutive *ATL2* expression. Nonresponsiveness of *eca4* mutants to each identified inhibitor of PAMP-triggered

ATL2p::GUS reporter gene expression strongly suggests that ECA4 acts downstream of the respective compound targets (Fig. 6). However, we are unable to discriminate whether ECA4 acts in the authentic elicitor-triggered ATL2 gene activation pathway or prevents other spurious signaling pathways to act on ATL2. Nonresponsiveness of elevated ATL2p::GUS reporter activity in eca4 plants to triclosan, fluazinam, cantharidin, and fenpiclonil inhibition also rules out the possibility that under our experimental conditions the compounds generally downregulate gene expression. Unlike ECA4, we consider ECA2 as a candidate acting in the elicitor-triggered ATL2 gene induction pathway because three of the four identified inhibitors (triclosan, fluazinam, and fenpiclonil) reduced cellulysin-stimulated GUS activity in eca2 plants. Taken together these experiments illustrate the potential to assign genes of a given biological process in tentative pathways according to their sensitivity/insensitivity to chemical inhibitors in much the same way as intermutant crosses are utilized to reveal gene interactions.

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