Planta (2009) 231:143–153
DOI 10.1007/s00425-009-1032-1

ORIGINAL ARTICLE

A novel regulatory system in plants involving medium-chain fatty acids

Gretel Mara Hunzicker

Received: 13 July 2009 / Accepted: 2 October 2009 / Published online: 30 October 2009

Abstract Polyethylene glycol sorbitan monoacylates (Tween®) are detergents of widespread use in plant sciences. However, little is known about the plant response to these compounds. Interestingly, the structure of Tweens' detergents (especially from Tween 20) resembles the lipid A structure from gram-negative bacteria polysaccharides (a backbone with short saturated fatty acids). Thus, different assays (microarray, GC–MS, RT–PCR, Northern blots, alkalinization and mutant analyses) were conducted in order to elucidate physiological changes in the plant response to Tween 20 detergent. Tween 20 causes a rapid and complex change in transcript abundance which bears all characteristics of a pathogenesis-associated molecular pattern (PAMP)/elicitor-induced defense response, and they do so at concentrations which cause no detectable deleterious effects on plant cellular integrity. In the present work, it is shown that the PAMP/elicitor-induced defense responses are caused by medium-chain fatty acids which are efficiently released from the Tween backbone by the plant, notably lauric acid (12:0) and methyl lauric acid. These compounds induce the production of ethylene, medium alkalinization and gene activation in a jasmonate-independent manner. Medium-chain fatty acids are thus novel elicitors/regulators of plant pathogen defense as they have being proved in animals.

Keywords Detergent · Fatty acid · Lipid A · PAMP · sMCFAs · Tween

G. M. Hunzicker (✉)
Instituto Multidisciplinario de Biología Vegetal,
CONICET-Universidad Nacional de Córdoba,
Av. Vélez Sarsfield 299, Casilla de Correo 495,
Córdoba 5000, Argentina
e-mail: ghunzicker@imbiv.unc.edu.ar; grechenm@yahoo.com.ar

Abbreviations

PAMP	Pathogenesis-associated molecular pattern
OPR	12-Oxophytodienoate reductase
sMCFAs	Saturated medium-chain fatty acids
LRR-RK	Leucine-rich repeat receptor kinases

Introduction

Detergents are found in numerous applications. In plant biology and plant protection, they serve, e.g., as emulsifiers and wetting agents for the lipophilic plant surfaces. This action facilitates the application of chemicals, in particular the non-ionic detergents of the polyoxyethylene glycol sorbitan monoacylate group (Tween® group) such as Tween 20 (polyoxyethylene glycol sorbitan monolaurate). Surprisingly, little is known about how detergents affect organisms. The prevailing notion is that beyond a critical level detergents are toxic simply due to their destructive effect on biomembranes. However, more subtle effects have been reported for Tween detergents. The different commercial members of this group carry different fatty acid moieties. In particular, Tween 20 carries esterified lauric acid as a major component, plus several minor ones attached to the backbone (Fig. 1). Terzaghi (1986a, b) has shown that the Tween backbone acts as an acyl carrier from which the fatty acid is cleaved effectively in the plant tissue. The released fatty acids may then be used for lipid biosynthesis, resulting in membrane lipids with an altered fatty acid composition (Terzaghi 1986b). Tween carriers can be used in cell cultures (Terzaghi 1986a, b) as well as on intact plants (Terzaghi 1989). Such treatment was reported to result in feedback inhibition of fatty acid

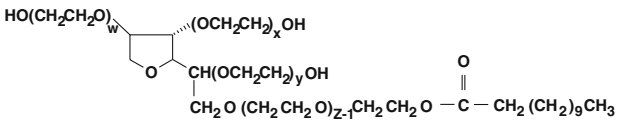

Fig. 1 Structure of Tween detergents (the sum of *w*, *x*, *z* is equal to 20). Tween 20 contains the following esterified fatty acids: 11 mol% of caprylic acid (8:0), 7.2 mol% of capric acid (10:0), 50 mol% of lauric acid (12:0), 18 mol% of myristic acid (14:0), 7.8 mol% of palmitic acid (16:0) and 6.1 mol% of stearic acid (18:0). Tween 80 contains esterified: 4.1 mol% of lauric acid (12:0), 4.1 mol% of myristic acid (14:0), 5.3 mol% of palmitic acid (16:0), 5.7 mol% of palmitoleic acid (16:1), 1.4 mol% of palmitoleic acid (16:2), 2.1 mol% of hiragonic acid (16:3), 74 mol% of oleic acid (18:1), 1 mol% of linoleic acid (18:2) and 2.6 mol% of linolenic acid (18:3) (Mittendorf et al. 1999)

biosynthesis in tobacco cells (Shintani and Ohlrogge 1995). Mittendorf et al. (1999) have shown an increased incorporation of monomers resulting from β-oxidation of the fatty acids released from Tween into polyhydroxyalkanoates in transgenic *Arabidopsis thaliana* plants overexpressing polyhydroxyalkanoate synthase. Thus, the effects of products from the Tween family on plants have so far been located at the metabolic and the enzymological level. In addition, there is some evidence showing that Tween application may also alter gene activities (Tabak and Shchelokova 1979; Huang et al. 2004). Tween 20 induced the *ALIP1* gene encoding of extracellular lipase in the yeast *Arxula adeninivorans* (Boer et al. 2005). Tween 80 induced the formation of an extracellular esterase in *Candida albicans* A-714 (Tsuboi et al. 1996). Tween 60 was found to be an inducer of the accumulation of transcripts of the proliferin gene family in murine C3H/10T1/2 cells (Parfett 1992). Tween 20 induced the accumulation of transcripts encoding the two oxophytodienoate reductases OPR1 and OPR2 (Biesgen and Weiler 1999). Moreover, 12:0, the main fatty acid in the Tween 20 detergent, has several relevant biological properties such as antifungal, antiviral, antiparasite and antibacterial activities (Hornung et al. 1994; Kristmundsdottir et al. 1999). It also induces NF_B activation when TLR2 (Toll-like receptor) is co-transfected with TLR1 or TLR6 in 293T cells (Lee et al. 2004). TLR2-1–6 are leucine-rich repeat receptor kinases (LRR-RK) essential for recognizing bacterial lipoproteins and lipopeptides and Toll-like receptor family proteins play a crucial role in human innate immunity (Takeuchi et al. 2001, 2002). In the present study, a systematic survey was carried out on the transcriptome of Arabidopsis aimed at (i) identifying those transcripts whose levels are affected by the application of Tween 20, thus estimating the extent to which Tween application might change the physiology of the plant, and (ii) identifying the potential molecular patterns of Tween action. Thus, to better understand how detergents and fatty acids affect plant metabolism, microarray analysis, hormone measurements (gas chromatography–mass spectrometry), and medium alkalinization assays in cell cultures and leaf material from different plant species were tested in this work.

Materials and methods

Plant material

Arabidopsis thaliana (L.) Heynh (Arabidopsis), ecotype C24 (seeds kindly provided by L. Willmitzer, Golm, Germany) wild type and mutant lines were grown as described by Biesgen and Weiler (1999) with the following qualifications: photon flux density 150 $\mu E\ m^{-2}\ s^{-1}$ photosynthetically active radiation. Plantlets raised under sterile conditions were kept in short days (8 h light) and were grown on agar for 22–28 days and on sterile soil thereafter. *Nicotiana benthamiana* (ZMBP, Tübingen, Germany) and *Lycopersicum esculentum* (Moneymaker, ZMBP, Tübingen, Germany) were grown on soil in long day conditions (16 h light) at 30°C.

Cell cultures

Tomato (*L. esculentum*) cell-suspension culture line Msk8 (originally provided by Koornneef, Wageningen, the Netherlands), *A. thaliana*, derived from plant tissue of accession Landsberg *erecta* plants and tobacco (*Nicotiana tabacum*) cell culture line 275N derived from pith tissue of Havanna 425 plants were provided by G. Felix (ZMBP, Tübingen, Germany) and grown on a Musrashige-Skoog-type medium as described by Felix et al. (1991). The cells were elicited 7–10 days after subculture.

Microarray analysis

Arabidopsis (Col-0) plants grown under sterile conditions for 28 days were treated with either 0.2% (v/v) Tween 20 detergent (Sigma, Munchen, Germany) or distilled water as a control. Three microarray experiments were performed, two with a collection time of 1 h after treatment (expts. Ia and Ib) and the third with a harvesting time of 2.5 h (expt. II). Total RNA from leaf material was extracted with TRIzol® (Invitrogen, Karlsruhe, Germany). Three independently isolated RNA samples were pooled for each Affymetrix GeneChip® (24 K) experiment. RNA quality and quantity control, cRNA labeling, hybridizations, expression data normalization and analysis were performed according to the Affymetrix manual and as described by Mussig et al. (2003). Clustering was performed using the MAPMAN program (Thimm et al. 2004). The complete microarray data sets are available from the GEO database,

accession no. GSE 5513. RT–PCR was employed for the validation of the microarray results with independent plant material.

Extraction of RNA and RT–PCR analysis

RNA for blotting analysis (Northern blot) was isolated from 1 g of plant material according to Barkan (1989). RNA blot analysis was performed following standard procedures (Sambrook et al. 1989) using 15 µg of total RNA. The probe utilized was *OPR1*-cDNA (EST clone, H37248), labeled with [α^{32}P]dATP. For cDNA synthesis, 0.1–1 g plant material was harvested, and the total RNA was extracted with TRIzol® (Invitrogen) according to the manufacturer's specifications. For RT–PCR reactions, 20 ng of cDNA was employed. The following forward/reverse primers were used: *RPL2* (positive control) (5′-GTGGTGCTCCTCTTGCTCGT-3′/5′-GGAGGTGCATCATC ACGCCTAAC-3′), *OPR1* (5′-ATTAGTGTGTGAGGACAAGCATG-3′/5′-GCTCTCTTTACC CCTCCAAGAC-3′), At1g05575 (5′-CCGATAATTCTTGTCTGAATC-3′/5′-CAAATGATTT CCGATGGTGAG-3′).

Tween 20 backbone production

The preparation of the Tween backbone from commercial Tween 20 was done according to Terzaghi (1986a) with the modifications described by Mittendorf et al. (1999).

Determination of fatty acids and acidic phytohormones

The technique developed by Muller et al. (2002) was applied. Leaves (500 mg) of 5–6-week-old Arabidopsis plants were harvested in 20 mL of methanol and extracted and processed in the presence of an internal standard of each 30 pmol of $[^2H]_{4-}$ SA, $[^{13}C]_{2-}$ JA, $[^2H]_{4-}$ IAA, $[^2H]_{6-}$ ABA, $[^2H]_{5-}$ OPDA for the phytohormone measurements and 50 pmol of eicosatrienoic acid (20:3) for the fatty acid determination. The samples were heated in methanol for 15 min at 50°C, they were filtered and the extract was dried under vacuum. For the pre-cleaning of the samples, 2 mL of diethyl ether was added to the dried sample. The solution was filled into a custom-made cartridge consisting of a diethyl ether-washed, silica-based aminopropyl matrix (Varian, Darmstadt, Germany), gel bed 12 mm × 1.5 mm, held in place between two 2.5 mm × 1.5 mm coated polyolefin filters (Filtrona, Reinbeck, Germany) at the tip of a 150 mm × 1.5 mm i.d. glass capillary column. The columns containing the samples were washed two times with two volumes of chloroform and one volume of isopropanol. The bound material was eluted with diethyl ether containing 2% acetic acid, dried under vacuum at 45°C and 10 mbar and redissolved in 20 µL of methanol. For methylation, 100 µL of ethereal diazomethane was added. The solution was transferred to an autosampler vial (Chromacol 05-CTV (A) 116; Fisher Scientific, Schwerte, Germany). Afterward, the samples were dried with N_2, redissolved in 30 µL of chloroform and measured with the Varian GC 3400 equipment connected to a Finnigan MAT Magnum mass spectrometer and with the Varian CP-3800 gas chromatograph connected to Varian Saturn 2000 mass spectrometer.

Alkalinization response assay

The alkalinization response was measured as described by Felix et al. (1993). The pH was continually registered in the growth medium of tomato, tobacco cell-suspension culture (2 mL). The flg22 peptide used in this work was described previously by Kunze et al. (2004).

Ethylene measurement

For assaying ethylene production, leave pieces (2-mm slices) of full expanded leaves of *A. thaliana* and *N. benthamiana* grown inside the greenhouse were placed in 6-mL glass tubes (3 leave pieces/tube; 20 mg of fresh weight per assay) containing 500 µL of water and the elicitor. The tubes were closed with rubber septa. The ethylene accumulating in the free air was measured by means of gas chromatography after 3 h of incubation.

Results

Transcriptome analysis of Tween 20-treated plants

In preliminary Northern blot experiments using the *A. thaliana* genes *OPR1*/*OPR2* (Biesgen and Weiler 1999), strongest responses were observed between 1 and 2.5 h after foliar treatment with 0.2% Tween 20 solution (approx. 1.6 mM) (Fig. 2). These conditions were thus chosen for the microarray experiments. Three experiments were carried out, two 1 h after the onset of the Tween treatment (expts. Ia and Ib) and one after 2.5 h (expt. II) in order to reveal differences between fast transient reactions and slower more permanent responses. The treated plants showed no visible signs of deterioration. They remained fully turgescent, indicating that the detergent treatment had not disintegrated cellular membranes, which would have resulted in a loss of turgor. A general observation from all array experiments was that Tween treatment substantially changed the levels of a large number of transcripts in the plants. With a few exceptions, those levels were predominantly elevated. Moreover, transcriptional expression patterns among the

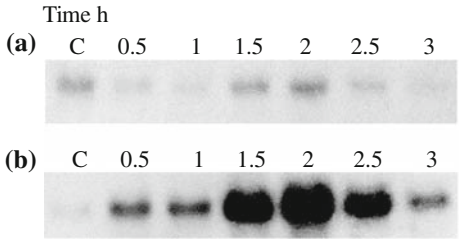

Fig. 2 RNA blot analysis of total RNA (15 μg per lane) isolated at the times indicated from leaves of Arabidopsis (C-24) plants grown under non-sterile conditions. Control samples (*lane C*) were taken before the treatment. The samples were treated with **a** distilled water and **b** 0.2% (v/v) Tween 20 detergent. The labeled *OPR1*-cDNA probe used for this experiment does not discriminate between the *OPR1* and *OPR2* isoforms due to high sequence similarity between them (91% cDNA and 71% DNA sequence identities)

microarray analyses differ between time point and experimental repetition. Results from the 1 h (Ia) and 1 h (Ib) experiments were higher than after 2.5 h suggesting a fast response of the plant to the detergent application. However, the discrepancies between 1 h (Ia) and 1 h (Ib) microarrays can be due to variations in plant material, experimental errors and conditions. Therefore, only those genes showing changes up to 2-fold and down to 0.5-fold were considered for the data analyses. It was observed that after 1 h, i.e., in both the Ia and Ib arrays, the levels of 118 transcripts were elevated (≥2-fold). In this same condition, only 12 levels were reduced (by ≥2-fold). In the 2.5 h array (expt. II), the corresponding numbers were 137 and 11 for elevated and decreased levels, respectively. Comparing the three experiments, the transcript levels of 38 genes were found elevated in all of them. This core group is represented in Table 1. Table 2 lists those genes whose transcript levels were most strongly up-regulated after 1 h but returned to normal after 2.5 h and thus representing the most dynamical responders found. The validity of the array data was checked for 25 randomly picked genes out of the 38 listed in Table 1 by RT–PCR. In all cases, the up-regulation of transcript levels was verified (data not shown, genes underlined in Table 1). As a general conclusion, it can be stated that Tween profoundly affects mRNA levels in Arabidopsis and, by inference, probably affects transcription of the corresponding genes. In order to perform a more detailed analysis, two genes were selected: *OPR1* (At1g76680) identified earlier in our laboratory (Biesgen and Weiler 1999) which is a slow responder (elevated transcript level maximum after 2.5 h) and At1g05575 which is one of the strongest affected genes with maximum transcript levels after 1 h (Table 2; Fig. 3a). This gene will in the following, for simplicity, be addressed using the lab-code assignment *11TT*.

Effects of wounding and of other detergents

Mechanical wounding leads to a rapid and transient rise of the transcript levels of the *OPR1* gene (Biesgen and Weiler 1999). The effect was confirmed in the present study (Fig. 3a), and despite the fact that application of Tween 20 did not result in visible damage of the plants, it was of interest to analyze the responsiveness of other Tween 20-inducible genes toward wounding. All of the 12 Tween-inducible genes selected for testing turned out also to be wounding-responsive (data not shown), including *OPR1* and *11TT* (Fig. 3a). However, *11TT* is an example of a gene whose transcript level is much strongly affected by Tween 20 than by wounding (26 cycles against 32 cycles, respectively), in contrast to the *OPR1* gene (26 cycles against 29 cycles, respectively), thus demonstrating that the detergent response may include a wounding-like component, but is certainly not limited to it. To bring more clarity to this point, the effects of other surfactants (detergents) were analyzed. Surfactants in general are surface active amphiphilic agents that have distinct hydrophilic (polar) and hydrophobic (non-polar) regions in their molecule. If surfactants have no electric charge present in the hydrophilic portion of the molecule (after dissociation in aqueous solution), they are referred to as non-ionic. All surfactants can lower the surface tension of water when they are added in small amounts. The particular discontinuity in the plots of surface tension against surfactant concentration can be determined for every detergent. The surfactant concentration in which this discontinuity occurs is referred to as the critical micellar concentration (CMC). When the surfactants' concentrations are below the CMC, the molecules are freely included into the water structure as monomers. However, in the CMC region, the surfactant molecules start to form their own structures, e.g., micelles (De Oude 1992). Since surfactants can have different chemical and physical characteristics, diverse detergents were tested, non-ionic: Tween 20 (CMC 0.06 mM), Tween 80 (CMC 0.012 mM), Digitonin (CMC 0.5 mM), Brij 58 (CMC 0.077 mM); zwitterionic: CHAPS (CMC 8–10 mM). The plants were sprayed with 1.6 mM detergent solution, diluted in distilled water. The results (Fig. 3b) confirm that the detergent effect cannot be attributed solely to a wounding response: (i) The promoters were induced by the detergents independently of their charge or CMC; (ii) Brij 58 showed no detectable (*OPR1*) or only a marginal (*11TT*) activity; (iii) there are pronounced and gene-specific differences in activities between the two members of the Tween group tested, Tween 20 and Tween 80.

Tween 20 and Tween 80 share a common backbone to which different fatty acids are attached (Fig. 1). The observed difference in activities between Tween 20 and Tween 80 could thus be attributable to the acyl moiety

Table 1 Genes with up-regulated (increase factor ≥ 2-fold) transcript levels common between the three independent microarrays (expts. Ia and Ib: 1 h, expt. II: 2.5 h)

Increase factor			AGI number	Gene function
Ia	Ib	II		
2.5	2	3	At1g09080	Luminal binding protein 3 (BiP-3)
8	2.5	2.8	At1g19020	Expressed protein
2.8	2	2.3	At1g19380	Expressed protein
9.2	2.8	3.5	At1g22810	DREB subfamily A-5 of ERF/AP2
3	3.7	2	At1g28010	Multidrug resistance P-glycoprotein
4	2	2.6	At1g30700	FAD-binding domain-containing protein
5.7	7.5	3.7	At1g49000	Expressed protein
4	2.1	2.3	At1g51800	Leucine-rich repeat protein kinase
3.5	2.1	2	At1g52200	Expressed protein, similar to PGPS/D12
9.2	2.1	2.5	At1g53540	17.6 kDa class I small heat shock protein
2.5	2	2.1	At1g61380	S-locus protein kinase
2	2.5	2	At1g70140	AtFH8, a group I formin
3.2	2	2	At2g23170	IAA-adenylase
4	1.2	2.1	At2g25735	Expressed protein
2.3	2.3	2.8	At2g26560	Similar to patatin-like latex allergen
45.3	6.5	3.3	At2g37430	Zinc finger (C2H2) protein (ZAT11)
2.1	2.8	2	At2g39200	MLO12 seven transmembrane
2.7	1.6	2.1	At2g39340/50	SAC3/GANP family protein
3	4.3	2.1	At2g46150	Expressed protein
2.5	2.5	2.1	At3g10720	Pectin esterase
4.9	4.3	2.8	At3g13790	β-Fructosidase (BFRUCT1)
2.5	3.7	2	At3g45650	H^+-dependent oligopeptide transporter
4.6	2.1	2.1	At3g46110	Expressed protein
3.7	3.2	2.8	At3g50900	Expressed protein
3	2.6	2.8	At3g52450	U-box domain-containing protein
2.5	2.8	2.1	At3g54420	AtEP3 class IV chitinase (CHIV)
2.3	2.1	2	At4g01700	Chitinase
3.7	2.3	2.6	At4g20830	FAD-binding domain-containing protein
4.3	2.6	2.1	At4g23190	CRK11-At-RLK3-protein kinase
4.6	4	2.3	At4g23220	Protein kinase family protein
7	7.6	2.1	At4g25350	EXS family protein
5	4.6	2.3	At5g12420	Expressed protein
7	3	3	At5g24110	WRKY30 transcription factor
3	2.5	2.3	At5g44210	AtERF-9 ethylene response factor
2.1	3.3	2	At5g44910	Toll-interleukin-resistance (TIR)
3	2	2.1	At5g46330	FLS2 receptor
6.5	7.1	3.2	At5g47330	Palmitoyl protein
3	2.6	2.1	At5g60950	Phytochelatin synthase-related

Arabidopsis plants were treated with 0.2% (v/v) Tween 20 and the controls with distilled water. Underlined, up-regulation of genes verified by RT–PCR; others, not tested

esterified to the backbone structure. The chain length of 18:1 is much longer than that of 12:0, and this has strong implications on their physical characteristics and affects parameters such as the CMC. Besides, Terzaghi (1986a, b, 1989) has shown that fatty acids can be effectively cleaved from the Tween backbone in plant cells. We thus analyzed the levels of relevant fatty acids (lauric acid 12:0, myristic acid 14:0, palmitic acid 16:0 and stearic acid 18:0) in control, wounded and Tween 20-treated leaves of Arabidopsis by GC–MS (Fig. 4). 12:0 is the main fatty acid esterified to the backbone structure in Tween 20 (approx. 50 mol%), followed by myristic acid (18 mol%) (Fig. 1). Clearly, the levels of these major fatty acids and to a lesser extent the levels of the minor fatty acid components of Tween 20 were elevated in Tween-treated leaf tissue. The effects were most striking for 12:0 which occurs only in trace amounts in non-treated leaf tissue and the increase in 12:0 content lasts for several hours. It is important to note

Table 2 Genes with up-regulated transcript levels in the Ia and Ib arrays (increase factor ≥ 2-fold after 1 h), but showing normal transcript levels in the 2.5 h array II

Increase factor		AGI number	Gene function
Ia	Ib		
24.3	2	At1g05575 (*11TT*)	Expressed protein
9.8	2.6	At2g44130/40	F-box family protein
4.9	2	At1g56060	Expressed protein
4.9	4	At3g57460	Expressed protein
4	2.6	At3g49780	AtPSK3, phytosulfokine 3
4	2.6	At4g20780	Calcium-binding protein, putative
3.7	6	At1g21120	*O*-Methyltransferase, putative
3.7	2.2	At5g02760	Phosphatase 2C family protein/PP2C
3.5	2	At1g50740	Expressed protein
3.5	2.2	At1g64380	DREB subfamily A-6 of ERF/AP2
3.5	2.5	At1g70530	Protein kinase family protein
3.5	2.5	At2g33580	Protein kinase family protein
3.5	2	At3g59220	AtPIRIN1, putative
3.5	3.2	At5g12050	Expressed protein
3.3	2.3	At1g65690	Harpin-induced protein
3.3	2.1	At2g38870	Protease inhibitor, putative
3.3	2	At4g22530	Embryo-abundant protein-related
3.3	3.3	At5g03700	PAN domain-containing protein
3	2	At1g05340	Expressed protein
3	2	At1g33590	Disease resistance protein-related
3	2.3	At1g67930/20	Golgi transport complex protein-related
3	4.6	At5g41900	Hydrolase, alpha/beta fold family protein
2.8	5.7	At1g02360	Chitinase
2.8	3.3	At1g30730/20	FAD-binding domain-containing protein

Arabidopsis plants were treated with 0.2% (v/v) Tween 20 and the controls with distilled water

that wounding changed none of the levels of the analyzed saturated fatty acids compared to controls.

Role of saturated medium-chain fatty acids (sMCFAs) in inducing transcript accumulation

The results presented so far do not allow discerning whether the activity of the Tween 20 was due to the intact detergent structure or caused by fatty acids released from the backbone. Thus, the effect of applying the Tween backbone alone to Arabidopsis plants was compared side by side with the intact Tween molecule and the free fatty acids (Fig. 5). The results for both markers (*OPR1* and *11TT*) show that the backbone alone was inactive while the free fatty acids were active. Particularly, 12:0 exhibited the same or even higher activity compared to the intact Tween 20 molecule. Changes in the fatty acid level in the plant after Tween 20 treatment were measured next (Fig. 4). Since the experimental protocol does not distinguish between endogenously synthesized fatty acids and those taken up from the sprayed solution, the data are present as ratios over plants sprayed with water. Moreover, because the plant material was not washed before measurement, it is possible that a fraction of the measured fatty acid came from the Tween 20 and not from the tissue.

Moreover, in order to asses whether the induction of the genes analyzed in the present work is reflected by changes in the hormone situation of the plant, GC–MS experiments were performed. Quantitative analysis by GC–MS/MS (Muller et al. 2002) measuring the levels of jasmonic acid (JA), its biosynthetic precursor 12-oxophytodienoic acid (OPDA) and, as a reference, salicylic acid (SA) revealed no detectable changes in the levels of none of these compounds in Tween 20-treated plants. Meanwhile, as expected, the levels of JA and OPDA, but not that of SA, increased strongly and transiently after wounding (Fig. 6). It was thus considered unlikely that the pronounced activity of 12:0 (and, by inference, of the other sMCFAs) occurred through JA. In order to check this finding, mutants deficient in JA biosynthesis were studied.

Effect of fatty acids and detergents in a JA-deficient mutant

RT–PCR performed with homozygous plant material kindly provided by C. Böttcher (Ruhr-Universität

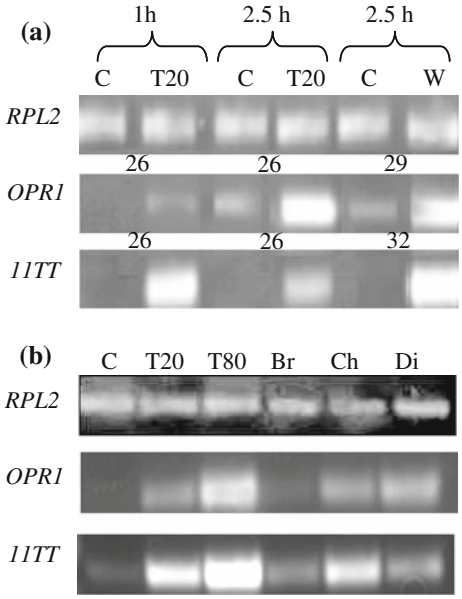

Fig. 3 RT–PCR performed with Arabidopsis (Col-0) plants grown under sterile conditions. *RPL2*, a housekeeping gene, provided the loading control. The plants were treated with distilled water as a control (*lane C*), with 1.6 mM detergent solution, or they were wounded with a hemostat (*lane W*). **a** The harvesting times were 1 and 2.5 h for the Tween 20 samples, and 2.5 h for the wounded samples. Number of cycles per gene and treatment: *RPL2*, 27; *OPR1*, 26 cycles for Tween 20 (*T20*) and 29 for wounding (*W*); *11TT*, 26 cycles for Tween 20 and 32 for wounding. **b** Effects of different detergents, Tween 20 (*T20*), Tween 80 (*T80*), Brij 58 (*Br*), CHAPS (*Ch*), Digitonin (*Di*), on the transcript levels of *OPR1* and *11TT* after 1 h of treatment

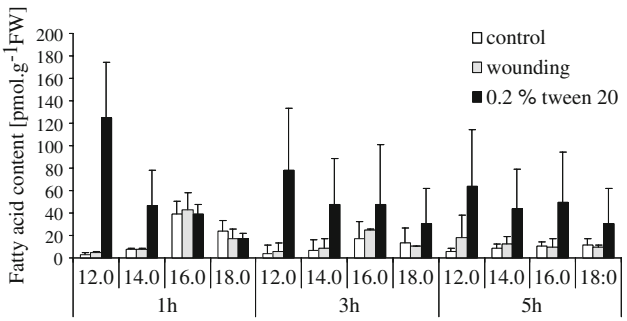

Fig. 4 Fatty acid analysis. GC–MS analysis was performed for the quantification of fatty acids (lauric acid, 12:0; myristic acid, 14:0; palmitic acid, 16:0; stearic acid, 18:0) from leaves of 5–6-week-old Arabidopsis (Col-0) plants grown under non-sterile conditions. Plants were sprayed with distilled water as a control, with 0.2% Tween 20, or they were wounded with a hemostat. The samples were measured after 1, 3 and 5 h. The *bars* indicate the standard error of the mean of two independent experiments, with two repetitions each

Germany) from a double *aos*/*opr3* mutant (JA-biosynthetic enzymes) resulted from the crosses of *aos* (Park et al. 2002) and *opr3* (Sanders et al. 2000) loss activity mutants, clearly showed that transcript levels of both markers (*OPR1* and *11TT*) increased in the double mutant after treatment with

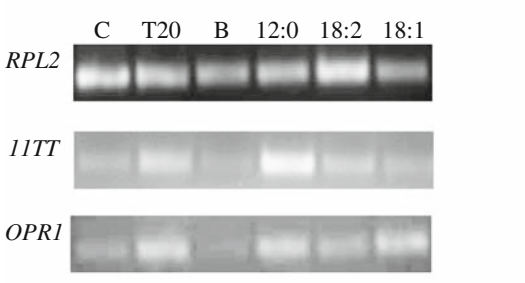

Fig. 5 Response of *OPR1*- and *11TT*-transcript levels either to the complete Tween 20 molecule or to partial structures. Arabidopsis plants (Col-0, 5–6-week old, grown in the greenhouse) were sprayed with 1.6 mM solutions of Tween 20 (*T20*), Tween backbone (*B*), lauric acid (*12:0*), linoleic acid (*18:2*) or oleic acid (*18:1*) in 40% of acetone. Controls were sprayed with 40% acetone (*lane C*). The samples were harvested after 1 h of incubation and were analyzed by RT-PCR, conditions as in Fig. 3

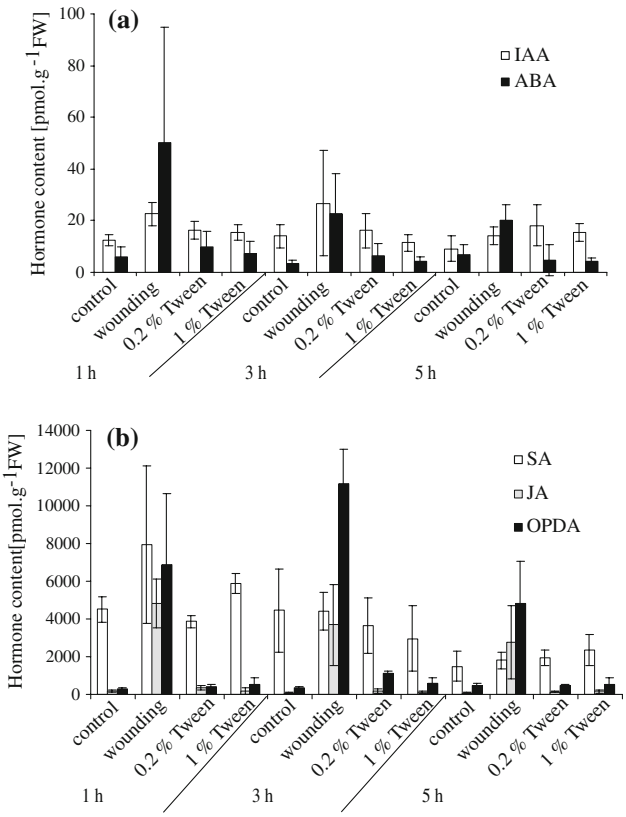

Fig. 6 GC–MS/MS quantification of endogenous plant hormones in leaves of 5–6-week-old Arabidopsis (Col-0) grown in the greenhouse. The plants were sprayed with distilled water as a control, with 0.2% Tween 20, or they were wounded with a hemostat. The samples were measured after 1, 3 and 5 h. **a** *IAA* indole acetic acid, *ABA* abscisic acid; **b** *SA* salicylic acid, *JA* jasmonic acid, *OPDA* 12-oxophytodienoic acid. The *bars* indicate the standard error of the mean of two independent experiments, with two repetitions each

Tween 20, Tween 80 and lauric acid (Fig. 7). Thus, their induction must be independent of the JA-signaling system. Interestingly, *11TT* was slightly induced by wounding in

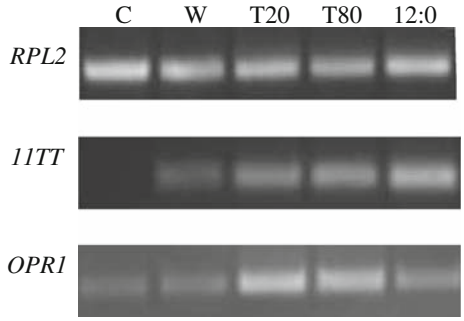

Fig. 7 RT-PCR analysis of *OPR1*- and *11TT*-transcript levels in the *aos/opr3* double mutant. The plants (grown under sterile conditions for 28 days) were exposed to different treatments, distilled water (*lane C*) was sprayed as a control or they were wounded with a hemostat (*W*). The mutants were sprayed with 1.6 mM of Tween 20 (*T20*), Tween 80 (*T80*) or lauric acid (*12:0*). The samples were collected after 1 h

the double mutant indicating that at least part of the wound response was also independent of JA.

Ethylene and alkanization induction

Lauric acid and methyl laurate induced defense responses in Arabidopsis and other plant species. Leaf tissues (Fig. 8a) tested showed increased biosynthesis of ethylene after treatment with flg22 used as a positive control (400 nM), 12:0, methyl laurate and Tween 20 solution (10 μM). Afterward, minor concentrations of these solutions were tested in tobacco, Arabidopsis and tomato. The highest ethylene induction was obtained in tobacco samples with methyl laurate (Fig. 8b) being 100 nM the minimum concentration needed for the induction. However, no significant ethylene production was detected after 12:0 treatment at small concentrations. Other plant defense-related response to elicitors can be measured as a pH shift (alkalinization) in extracellular medium of plant cell cultures. Thus, the pH in different cell culture varieties was monitored upon flg22 (c+), methyl laurate addition. Results show that methyl laurate triggers a rapid alkalinization of the medium of tobacco and tomato (Fig. 9) but not in Arabidopsis cells (data not shown). These results were consistent with those found for ethylene, suggesting that 12:0 and methyl laurate are plant elicitors.

Discussion

The results presented in the previous section, in particular those genes affected by Tween 20 (Tables 1, 2), show the characteristics of a typical innate, pathogenesis-associated molecular patterns (PAMPs)/elicitor-like immune response. Such a response occurs as an immediate reaction of a plant

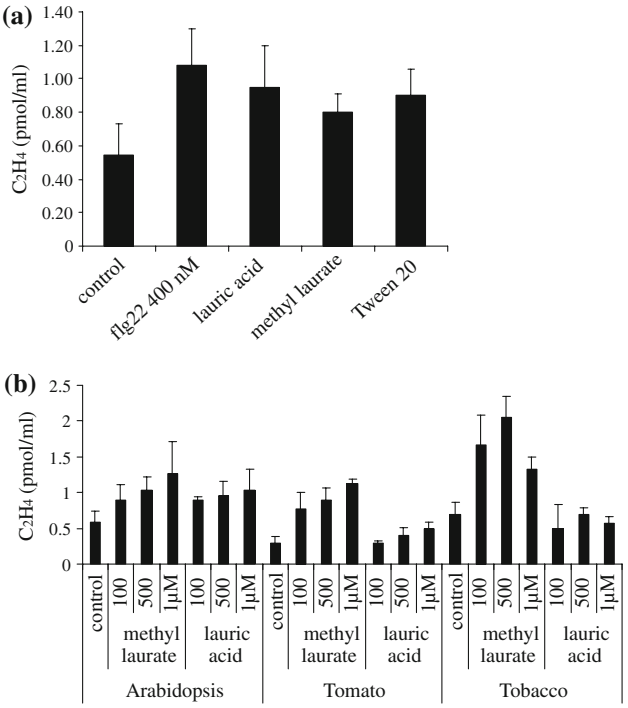

Fig. 8 Induction of ethylene biosynthesis in leaf tissue. **a** Leaf pieces from *Arabidopsis thaliana* were mock treated (controls water with DMSO 25%) or treated with 10 μM methyl laurate or lauric acid and 400 nM flg22. **b** Leaf pieces from *A. thaliana*, *Lycopersicon esculentum* (tomato), *Nicotiana benthamiana* (tobacco) were treated with 100 nM, 500 nM and 1 μM methyl laurate or lauric acid. Ethylene was measured after 3 h. Standard deviation of $n = 4$ replicates

to pathogen challenge (Nurnberger et al. 2004) and can be associated to the fatty acids by which Tween 20 carries into the plant. PAMPs, also called elicitors, are mixtures of general microbial components by which the plant recognizes an invading pathogen. Actions of this type are triggered, e.g., by flagellin fragments (Felix et al. 1999), lipopolysaccharides (Dow et al. 2000), peptidoglycans (Gust et al. 2007) or chitin (Baureithel et al. 1994). Some elicitors are recognized by cellular receptors, notably by LRR-RK and nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins (Nurnberger et al. 2004; DeYoung and Innes 2006). The repertoire of the genes involved in the PAMP or the Tween/FA response is very similar. Identities include receptor-like kinases as the mammalian Toll-like receptor homolog (At5g44910), the flagellin rapidly (*FLARE*) elicited genes encoding Lys-RLK (receptor-like kinase) At2g33580, DUF26-RLKs (At4g 23220, At4g23190) (Navarro et al. 2004), and FLS2 (At5g46330) (Zipfel et al. 2004). FLS2 is a LRR-RK receptor essential for flagellin perception and response transmission (Gómez-Gómez et al. 1999). A mutant containing a point mutation in this receptor (*fls2-17*, Gómez-Gómez and Boller 2000) showed a strong down-regulation

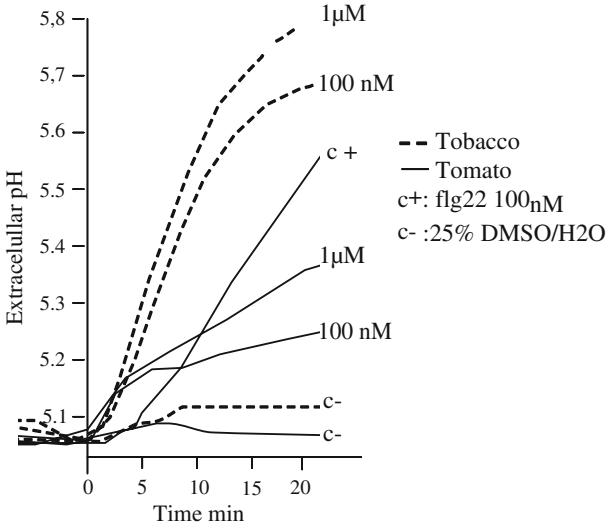

Fig. 9 Extracellular pH in *Lycopersicon esculentum* (tomato, *continuous line*) and *Nicotiana benthamiana* (tobacco, *dashed lines*) cells after treatment with 1 µM and 100 nM of methyl laurate. As a positive control (c+), 100 nM of flg22 peptide was used and as a negative control (c−), 25% DMSO in water

of the At1g05575 (*11TT*) transcript level. We have shown the transcript level of At1g05575 to be strongly and rapidly up-regulated by Tween/12:0. Thus, At1g05575 could be a link between flagellin and lipid elicitation but, in order to clarify this point, further investigation is needed. PAMPs trigger a complete MAP (mitogen activate protein) kinase signaling cascade and lead to the activation of some of the WRKY transcription factors (Nurnberger et al. 2004). The MAP kinase MKK1 (At4g26070) transcript was found up-regulated in the arrays, as was WRKY30 (At5g24110). Other defense-related genes whose transcript levels were found elevated include patatin (At2g26560), the LRR proteins (At1g51880, At1g33590) and a protein related to harpin-induced-1 (At1g6590), the chitinases encoded by At4g01700, At3g54420, and At1g02360, a pectin esterase (At3g10720), a putative protease inhibitor (At2g38870), and several genes identified by Gechev and Hille (2005) as H_2O_2 responsive (At3g13790, At2g26560, AT1g53540, AT1g52200, AT1g19020 and At2g37430). Reactive oxygen species play a crucial role in early stages of plant pathogen defense including hypersensitive responses (Foyer and Noctor 2005). Among the other up-regulated transcripts, many encode putative or known regulatory functions.

In order to understand these effects, we should consider that Tweens are mixtures of similar molecules all having the same polyethylene glycol sorbitan backbone. The main difference among these molecules is that they carry different fatty acids, being a different fatty acid which is dominant in each mixture (cf. Fig. 1). Tween 20, e.g., contains mainly 12:0 (50 mol%), the remainder being other saturated fatty acids from 8:0 to 18:0. Tween 80 contains mainly unsaturated fatty acids (16:1, 16:2, 16:3, 18:1, 18:2, 18:3) with a preponderance of oleic acid (18:1, 74 mol%). One of the latter, linolenic acid (18:3) is known to be convertible to JA in plants (Vick and Zimmermann 1984; Weber et al. 1997). JA is mainly a regulator of herbivore defenses which is produced when plant tissue is wounded. It is also an inducer of secondary metabolites, toxic for certain microorganisms (Gundlach et al. 1992). Other fatty acids (e.g., palmitic acid) may be converted to linolenic acid by chain elongation and desaturase reactions. These facts combined with the finding that some of the tested Tween 20-inducible transcripts were also wound-inducible suggested, at first, that the observed Tween 20 induction occurred via JA. However, this possibility can be clearly excluded using an Arabidopsis mutant bearing a double block in JA biosynthesis (Fig. 7). sMCFAs such as 12:0 do not act via conversion to JA. Consequently, it has been observed that some PAMPs-induced reactions occur independent of the JA system (Zipfel et al. 2004). This evidence and the lack of activity shown by Tween backbone (Fig. 5) suggest that sMCFAs are a new class of PAMPs that are either active by themselves or through metabolites other than jasmonates. Results obtained with the cell cultures and leaf material from different plant species showing a positive answer to ethylene production (Fig. 8; Table 1, At1g22810, At5g44219, At1g64380) and medium alkalanization (Fig. 9) after sMCFAs treatment also support this hypothesis. Interestingly, sMCFAs are also endogenous metabolites of plants. However, their level as free acids in plant cells is exceedingly low. Traces amount of 12:0 can be detected in phloem exudates of Arabidopsis plants (S. Hoffmann-Benning, Michigan State University, East Lansing, USA; personal communication). sMCFAs are components in the cell membranes of microorganisms, notably bacteria, but also fungi. They occur in the lipid A fraction (the most potent stimulator of innate immunity responses in mammals) of lipopolysaccharides of gram-negative bacteria (Fensom and Gray 1969). It is thus conceivable that plants use sMCFAs as one of the components of PAMPs to mount a rapid defense. Besides the genes tested in this work (Fig. 5), 12:0 induced phospholipases among another tested genes. 12:0 is also able to induce a NAD+ oxidoreductase from *Pisum sativum* (Saffert et al. 2000) and P450-12:0-dependent monooxygenases which are responsible for ω-hydroxylations in different plant species and may have a role in stress-response reactions (Saulan et al. 1978; Benveniste et al. 1982; Tijet et al. 1998; Imaishi et al. 2000). Finally, transgenic plants overproducing 12:0 did not show accumulation of this product in the leaves or in their lipids. A possible explanation for this absence of accumulation is its degradation through peroxisomal β-oxidation cycles (Eccleston et al.

1996; Hooks et al. 1999). Pathogenesis responses in these plants will be an interesting point to investigate.

The results presented here showed that Tween 20 acts as a carrier for saturated fatty acids which, in turn, are elicitors of a typical innate immune response. The use of Tweens causes changes in the plant physiology even when the detergent is used at low levels which do not visibly damage the plant. An important implication of the present findings is that when using Tweens in experiments checking defense reactions, test compounds may turn out inactive against the response elicited by the detergent itself.

Acknowledgments From the Ruhr-Universität Germany, I would like to thank P. Duchting for her assistance with the GC–MS, J. Schab for the Tween 20 backbone preparation, and especially to Prof. Dr. E. Weiler for his guidance and laboratory space. I would also like to thank Dr. A. Baruj (Atomic Center, Bariloche, Argentina) for his help with the manuscript, Prof. Dr. G. Felix (ZMBP, Tübingen, Germany) for the cell cultures and flg22 peptides and Dr. C. Mussig (MPI, Golm, Germany) for his help with the array analysis. This work was supported by the Deutsche Forschungsgemeinschaft (European Graduate College 795 grant to G.H.).

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