The synthetic cationic lipid diC14 activates a sector of the Arabidopsis defense network requiring endogenous signaling components

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Running title

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SUMMARY

Natural and synthetic elicitors have significantly contributed to the study of plant immunity. Pathogen-derived proteins and carbohydrates, that bind to immune receptors allowed fine dissection of some defense pathways. Lipids of different nature that act as defense elicitors have also been studied, but their specific effects have been less characterized, and their receptors have not been identified. In animal cells, nanoliposomes of the synthetic cationic lipid 3-tetradecylamino-tert-butyl-N-tetradecylpropionamidine (diC14) activate the TLR4-dependent immune cascade. We here investigated if this lipid induces Arabidopsis defense responses. At the local level, diC14 activated early and late defense gene markers (*FRK1*, *WRKY29*, *ICS1* and *PR1*) acting in a dose dependent manner. This lipid induced the salicylic acid (SA)-, but not jasmonic acid (JA)-, dependent pathway and protected plants against *Pseudomonas syringae* pv. *tomato* (*Pst*), but not *Botrytis cinerea*. diC14 was not toxic for plant or pathogen, and potentiated pathogen-induced callose deposition. At the systemic level, diC14 induced *PR1* expression and conferred resistance

against *Pst*. diC14-induced defense responses required the signaling protein EDS1 but not NDR1. Curiously, the lipid-induced defense genes expression were lower in *fls2/efr/cerk1* triple mutant while still unchanged in the single mutants. The amidine headgroup and chain length were important for its activity. Given the robustness of the responses triggered by diC14, its specific action on a defense pathway and the requirement of well-known defense components, this synthetic lipid is emerging as a useful tool to investigate initial events involved in plant innate immunity.

INTRODUCTION

Plants detect potential pathogenic microbes at different cellular levels. At the cell surface, pattern recognition receptors (PRRs) perceive pathogen/microbe associated molecular patterns (PAMPs) or compounds released during infection (damage-associated molecular patterns, DAMP) (Albert et al., 2010), to activate PRR-triggered immunity (PTI) (Macho and Zipfel, 2014; Zhang and Thomma, 2013). This warning system confers broad-spectrum defenses against non-adapted invaders, but fails to counteract successful pathogens that deliver effectors to suppress PTI (Block and Alfano, 2011). Inside plant cells, effectors can be recognized by host resistance proteins (R), to therefore induce effector-triggered immunity (ETI), a second layer of defense that provides race-specific resistance (McDowell and Simon, 2008).

PRRs are single pass transmembrane proteins carrying a ligand-binding ectodomain. At the intracellular level, these proteins contain either a kinase domain (receptor-like kinases; RLK), or a short tail lacking kinase function (receptor-like proteins; RLPs). Most PRRs act in concert with other receptors forming active multi-component complexes that signal defenses

upon ligand recognition (Macho and Zipfel, 2014; Zhang and Thomma, 2013). PRRs that bind known ligands were identified in various plant species. In Arabidopsis, flagellin and the flagellin-derived peptide flg22 bind to the FLS2 (flagellin sensing 2) receptor (Chinchilla et al., 2006), elongation factor Tu (EF-Tu) binds to EFR (EF-Tu receptor) (Zipfel et al., 2006), chitin binds to CERK1(chitin elicitor receptor kinase 1) (Miya et al., 2007), and peptidoglycans (PGNs) bind to LYM1 and LYM3 (lysin-motif proteins 1, 3) (Willmann et al., 2011). In turn, R proteins contain a central nucleotide-binding site (NBS) and a C-terminal leucine-rich repeat (LRR), and based on their N-terminus they are classified into two subfamilies. One of them includes homologues of Drosophila Toll / mammalian Interleukin receptors (TIR), while the second group contains receptors with a coiled-coil (CC) domain. TIR-NBS-LRR and CC-NBS-LRR proteins function through distinct signaling cascades, with the first group requiring the lipase-like protein EDS1 (enhanced disease susceptibility 1) and the second one, the NDR1 (non-race-specific disease resistance 1) protein (Aarts et al., 1998).

ETI and PTI trigger some common responses, such as the increase of reactive oxygen species, nitric oxide and salicylic acid (SA), activation of mitogen-activated protein kinase cascades, alteration of cell wall, and induction of pathogen-responsive genes (Asai et al., 2002; Block and Alfano, 2011; Dempsey et al., 2011; Tsuda et al., 2013). In most cases, ETI is faster and stronger than PTI, and generates cell death associated to the hypersensitive response (HR) (Mur et al., 2008). Both defense programs induce systemic acquired resistance (SAR), which protects the entire plant against further microbial infections (Dempsey and Klessig, 2012; Shah and Zeier, 2013). Interestingly, some endogenous signaling components, such as the EDS1 protein, participate in ETI and PTI (Rietz et al., 2011) reinforcing the notion of some convergence between them (Tsuda et al., 2013).

The use of elicitors allowed uncovering many key features of the plant immune programs. Bacteria-derived elicitors, including peptides from flagellin (flg22) and EF-Tu (elf18), and

the sugar moiety of cell wall derived PGNs, assisted the identification and characterization of their corresponding receptors. The Xanthomonas outer membrane protein Ax21 is other well-studied natural PAMP, previously suspected to bind the XA21 receptor (see Macho and Zipfel, 2014). Elicitors derived from fungi and oomycetes include the EIX protein (ethylene-inducing xylanase), the 13-aminoacid peptide from cell-wall transglutaminase (Pep13), as well as the cellulose binding domains of cell wall proteins, beta-glycans and chitin. PRRs that bind these elicitors have also been identified, including the tomato EIX receptor (EIX2), and the Arabidopsis and rice chitin receptors (CERK1 and CEBip, respectively) (Bar and Avni, 2009; Kaku et al., 2006; Miya et al., 2007). In contrast, the effects of lipid elicitors present in bacteria, fungi or oomycetes have been less characterized, and their receptors have not been described so far. Rhamnolipids and surfactin stimulate the SA and JA pathways protecting plants against *Botrytis cinerea*, *Hyaloperonospora arabidopsidis* and *Pseudomonas syringae* (Bais et al., 2004; Ongena et al., 2007; Sanchez et al., 2012). Massetolide A triggers induced systemic resistance, but not SA signaling, and enhances defenses to *Phytophthora infestans* (Tran et al., 2007). Arachidonic acid activates JA-mediated responses and represses the SA-pathway, affecting resistance to aphids and necrotrophic as well as biotrophic pathogens (Savchenko et al., 2010). Ergosterol induces alkalinization of cell growth medium and might activate a host receptor since its application desensitizes host cells to a second treatment (Granado et al., 1995). At present, the best characterized lipid elicitor is lipopolysaccharide (LPS), which induces defenses in mono- and dicots (Newman et al., 2002; Sun et al., 2012; Zeidler et al., 2004). LPS potentiates the oxidative burst, nitric oxide generation, callose deposition, defense gene expression, and local and/or systemic pathogen resistance, although not all these responses are observed in all systems (Newman et al., 2002; Newman et al., 2007; Sun et al., 2012). The lipid A moiety is conserved in many plant-associated bacteria and acts itself as a PAMP, while the oligosaccharide core and O-specific chains also trigger defenses. Moreover, in Arabidopsis lipid A and oligosaccharide domains display agonistic

effects on defense gene expression (Madala et al., 2012). The mechanisms involved in LPS perception by plants are unknown, and receptors for this elicitor have not been described yet. In animals, LPS is detected by intracellular NOD-like receptors and the surface Toll-like receptor 4 (TLR4) which requires accessory proteins (Chen et al., 2009).

In mammals, lipid-based nanoparticles stimulate the immune system (Landesman-Milo et al., 2012). In mouse and human bone marrow-derived dendritic cells, the cationic lipid diC14 (3-tetradecylamino-tert-butyl-N-tetradecylpropionamidine) activate the TLR4-dependent pathways leading to secretion of the cytokines TNF-α, IL-6, IFN-β, IP-10 and IL-12p40, and induction of CD80/CD86 expression of co-stimulatory factors (Jacquet et al., 2005; Tanaka et al., 2008; Wilmar et al., 2012). This cytokine secretion pattern is reminiscent of the TLR4-dependent cytokine pattern induced by bacterial LPS, the natural ligand of TLR4 (Tanaka et al., 2008). So far, the effect of synthetic cationic lipid-based nanoparticles on plant immunity had not been analyzed. We here describe the ability of diC14 nanoparticles to stimulate the Arabidopsis defense signaling cascades. A single application of the lipid induces long-term defenses and pathogen resistance at local and systemic levels. Activation of these responses requires endogenous defense components such as EDS1.

RESULTS

diC14 induces SA-dependent gene expression

We tested if a single application of diC14 nanoliposomes was sufficient to elicit Arabidopsis defense responses. Leaves infiltrated with different lipid concentrations (5, 40, 100 µg diC14 ml^{-1} in HEPES 0.2 mM) were collected at 8 and 24 h post treatment and used to monitor defense gene expression by sqRT-PCR. *WRKY29* (transcription factor) and *FRK1* (flg22-induced receptor-like kinase *1*) were used as PTI markers, *ICS1* (isochorismate

synthase 1) and *PR1* (pathogen related 1) as SA-sensitive genes, and *PDF1.2* (plant defensin 1.2), *VSP2* (vegetative storage protein 2) and *LOX2* (lipoxygenase 2) as JA-responsive genes (Asai et al., 2002; Dempsey et al., 2011). In parallel, mock (HEPES 0.2 mM)-treated leaves were used to control the effects of mechanical stress associated to the inoculation process.

diC14 nanoliposomes induced the expression of all PTI/SA gene markers (Fig. 1a). In different experiments, *WRKY29* was consistently activated at 8 hours post infiltration (hpi) even with the lowest lipid dose. *FRK1*, *ICS1* and *PR1* were induced at 8 and 24 hpi in a dose-dependent manner, and *PR1* showed the strongest overall response. In turn, *VSP2* was induced at 8 hpi by low lipid concentrations (5 and 40 µg ml^{-1}), and *PDF1.2* and *LOX2* responded to both diC14 and mock treatment in similar manner (Fig. 1b). These results indicated that diC14 produced a strong and sustained activation of the SA pathway (at least 24 h post-treatment), while it induced the JA pathway in a weak and transient manner.

diC14 enhances resistance to Pst DC3000

To examine the physiological relevance of the previous responses, we tested if diC14 (20 µg ml^{-1}) conferred resistance to hemi-biotrophic pathogens. In this assay, leaves were first treated with the lipid, 24 h later they were challenged with *Pseudomonas syringae* pv *tomato* DC3000 (*Pst*; 10^5 cfu ml^{-1}), and 3 days post inoculation (dpi) were excised to quantify bacterial content. As a control, leaves pre-treated with mock solution were used to determine pathogen growth. As shown in Figure 2a, diC14 restricted *Pst* proliferation *in planta*. At 3 dpi mock- and diC14-treated leaves contained 1.4 x 10^5 and 9.2 x 10^3 cfu cm^{-2}, respectively. Interestingly, the lipid did not affect bacterial growth *in vitro* (Fig. S1, see Supporting information), suggesting that in the plant, it reduced pathogen proliferation by activating defense responses.

Tissues treated with diC14 (20 µg ml^{-1}), that were able to activate SA-responsive genes from 8 hpi (Fig. 1a), evidenced no cell death or damage at 8 or 24 hpi (Fig. 2b). In contrast,

tissues challenged with a classical inducer of SA-defenses and cell death responses, the avirulent bacteria *Pst AvrRpm1*, triggered cell death since 8 hpi (Fig. 2a).

diC14 primes callose deposition

Reinforcement of cell wall through callose deposition is a classical PTI marker, usually accompanied by activation of *Callose Synthase 12* gene (*CalS12*) (Dong et al., 2008). Callose deposits reach high levels in tissues treated with the *Pst hrpC*$^-$ mutant lacking the capacity to inject effectors though type III secretion system (TTSS) (Hauck et al., 2003), and lower levels in tissues treated with *Pst* whose effectors may inhibit their generation (DebRoy et al., 2004).

We tested if diC14 nanoliposomes (20 µg ml^{-1}) activated callose deposition at the infiltration site. Lipid-treated leaves were sampled at 0, 12 and 24 hpi and stained with aniline blue to detect callose deposits (Cecchini et al., 2011). diC14 itself was unable to induce this response (Fig. 3a), but slightly activated the *CalS12* gene expression (Fig. 3b). Interestingly, when diC14 was combined with *Pst*, the lipid potentiated the effect of pathogen in both responses. At 12 and 24 hpi callose deposits increased 100% and 25%, respectively, in tissues simultaneously inoculated with lipid and bacteria compared to those only treated with pathogen (Fig. 3a).

We wondered if the potentiation effect of the lipid was caused by inhibition of effector secretion. To test this possibility, we performed two experiments. First, we quantified callose deposits in tissues simultaneously treated with diC14 and *Pst hrpC*$^-$. The lipid maintained a potentiation effect under this condition (130% increase in callose deposits at 24 hpi, in diC14 + *Pst hrpC*$^-$ with respect to *Pst hrpC*$^-$; Fig. S2, see Supporting information). Next, we evaluated if diC14 reduced the capacity of *Pst AvrRpm1* to trigger ETI in Col-0 plants, where intracellular recognition of the TTSS effector *AvrRpm1* by the host R protein RPM1 leads to cell death. As shown in Figure 3c, cell death was not reduced by co-inoculation of diC14

with the avirulent bacteria. These results indicate that diC14 does not impair the TTSS of *Pst*, suggesting that the lipid sensitizes the tissues to rapidly trigger callose deposition upon PAMPs sensing.

diC14 requires EDS1 to activate defenses

To learn more about the action of diC14, we tested whether lipid-induced *FRK1* and *PR1* activation required endogenous defense signaling components. First, we used the triple mutant *fls2/efr/cerk1* lacking the FLS2, EFR and CERK1 receptors (Gimenez-Ibanez et al., 2009). At 24 hpi, the *fls2/efr/cerk1* mutant showed lower capacity than wild type plants to induce *FRK1* and *PR1* by diC14 (Fig. 4a; Fig. S3a upper panels; see Supporting information), accumulating lower levels of both transcripts in lipid-treated tissues (Fig. S3a lower panels; see Supporting information). However, the mutant retained the ability to activate both genes during stimulation of ETI by *Pst AvrRpm1* (Fig. S3b; see Supporting information). Next, we evaluated the effect of diC14 on the *fls2*, *efr*, and *cerk1* single mutants and observed that all of these plants activated *FRK1* and *PR1* as control plants (Fig. 4b).

Finally, we assessed how the absence of EDS1 and NDR1 affected the induction of *FRK1* and *PR1* by diC14. Whereas EDS1 participates on both PTI and ETI, NDR1 is only involved on ETI (Aarts et al., 1998; Wiermer et al., 2005). Interestingly, the *eds1-2* mutant abolished *PR1* induction by diC14. In addition, *eds1-2* plants reduced diC14-mediated *FRK1* activation (Fig. 4c). In contrast, *ndr1-1* plants maintained the capacity to activate both genes in response to diC14 (Fig. 4d).

These results indicate that the function of EDS1, but not NDR1, is required for full activation of *FRK1* and *PR1* by diC14 nanoliposomes.

diC14 induces systemic resistance

To test the effect of diC14 on systemic defenses, we treated two leaves per plant with diC14 or mock solution, and 8 and 24 h later we sampled systemic untreated leaves to analyze *PR1* and *FRK1* expression. Both gene markers were clearly induced at 24 hpi demonstrating the effect of diC14 on systemic tissues (Fig. 5a), which could derive from generation of a systemic signal or mobility of the lipid in the plant (see Discussion). To assess the susceptibility of these systemic tissues, we treated plants with diC14 as before. One day later we inoculated distal leaves with *Pst* (10^5 cfu ml^{-1}) to determine bacterial content at 3 days post infection. A single application of diC14 was sufficient to induce SAR, as indicated by 10 times reduction in pathogen content in diC14- respect to mock- pre-inoculated plants (Fig. 5b). DIR1 is an Arabidopsis lipid-transport protein necessary to induce systemic *PR1* expression after local inoculation of avirulent bacteria (Maldonado et al., 2002). To test if diC14 requires DIR1 to signal *PR1* and *FRK1* activation, we quantified these transcripts in distal tissues of the null mutant plant *dir1-1*. The mutant behaved as the control plant (Fig. 5c) indicating that DIR1 is dispensable for these effects.

Features of the diC14 molecule affecting defense induction

Finally, we evaluated which features of the diC14 molecule (charge, hydrophilic moiety or chain length) affected its capability to activate plant defenses. Nanoliposomes of diC14 and other four synthetic related lipids (Table S1, see Supporting information) were infiltrated in different sets of plants (20 µg ml^{-1} of each lipid) to test their effect on *FRK1*, *ICS1* and *PR1*, expression. diC16, a longer tailed derivative that conserves the cationic amidine group but contains 16 instead of 14 carbon residues in each lipid chain, had also an effect albeit much smaller than diC14, since it activated *FRK1* at 8 but not 24 hpi, and weakly induced *ICS1* and *PR1* at these time points (Fig. 6a). However, diC16 did not enhance resistance against *Pst*, since pre-treatment with this lipid did not reduce pathogen growth (Fig. 2a). Neither was diC16 able to enhance systemic resistance to *Pst* (Fig. 5b). These results

indicated that structural differences in the amidine molecule can alter the lipid capacity of defense activation.

To assess the role of the hydrophilic lipid moiety, we compared *FRK1*, *ICS1* and *PR1* expression in samples treated with diC14, two cationic lipids (DMTAP, DMDAP) and one neutral lipid (DMPC), all sharing 14-C acyl chains (Table S1, see Supporting information). None of these lipids, except diC14, activated the gene markers in a strong or sustained manner (Fig. 6b) suggesting that the amidine chemical function is required for gene induction. Moreover, pre-inoculation of DMTAP, DMDAP or DMPC on leaf tissues did not confer resistance against *Pst* (Fig. S4; see Supporting information). In addition, diC16 which does possess an amidine chemical function, did not produce robust *PR1* expression, suggesting that lipid chain length was also important for this effect.

DISCUSSION

This study examines how diC14 impacts on the Arabidopsis immune pathways. The lipid proved to be a good inducer of local and systemic defenses. A single application of diC14 produced rapid (8 hpi) and durable (24 hpi) activation of *FRK1*, *WRKY29*, *ICS1* and *PR1* genes in treated tissues. Gene induction was dose-sensitive, increasing with lipid concentration. The extent of *PR1* activation was similar to that produced by *Pst AvrRpm1* infection (Fig. S5, see Supporting information). Thus, diC14 appears to activate signaling cascades that normally function in disease resistance, using amplification events to mount a range of lasting defenses. Supporting this possibility, several defense marker genes were induced in parallel and remained up-regulated for at least 24 h. In addition, diC14 boosted pathogen-induced defenses by potentiating *Pst*-mediated callose deposition. Similarly, LPS and rhamnolipids activate SA-sensitive genes for at least 24 h (Sanchez et al., 2012; Zeidler et al., 2004), and LPS primes both the synthesis of antimicrobial compounds, and the expression of defense genes induced by bacterial infection (Newman et al., 2002).

Lipid elicitors characterized to date have different effects on defense. Rhamnolipids and surfactin, mostly used as mixtures of compounds, activate SA- and JA-dependent pathways enhancing resistance to biotrophic and necrotrophic pathogens (Bais et al., 2004; Ongena et al., 2007; Sanchez et al., 2012; Varnier et al., 2009). Riboflavin (Dong and Beer, 2000) and ultrashort cationic lipopeptides (Brotman et al., 2009), used as pure compounds, also activate both pathways. Extracts of LPS induce the SA cascade protecting plants against hemi/biotrophic pathogens (Sun et al., 2012; Zeidler et al., 2004). Under particular conditions, massetolide A (Tran et al., 2007), surfactin (Bais et al., 2004; Ongena et al., 2007), ultrashort cationic lipopeptides (Makovitzki et al., 2006), and rhamnolipids (Varnier et al., 2009) display antimicrobial activity, whereas none of these elicitors cause death or toxicity on plant cells at the lowest concentration that induces defense (Dong and Beer, 2000; Makovitzki et al., 2007; Newman et al., 2007; Ongena et al., 2007; Tran et al., 2007; Varnier et al., 2009). Meanwhile, diC14 induced SA-sensitive defenses signaled by EDS1, but not NDR1, and protected plants against *Pst*, having a mild and transient effect on the JA pathway and no consequences on resistance to *Botrytis cinerea* (Fig. S6, see Supporting information). diC14 was not toxic for bacteria or plant cells. Comparing the effects of diC14 with those of other lipid elicitors, LPS is the one with the greatest similarity. However, both compounds have differential actions (only LPS activates callose deposition). Hence, diC14 appears to be a nontoxic lipid elicitor that can be used as a pure compound to study early signaling events that activate plant immunity.

The mechanisms underlying diC14-mediated plant defense activation are unknown. At the local level, the lipid targets particular signaling cascades. Based on our data and other published studies, we can envision different effects. Surfactin binds to the plasma membrane (PM) of tobacco cells, showing high affinity for phospholipids. Its insertion into the

membrane may either disturb lipid compartmentalization, or generate curvature constrains, thus affecting mechanosensitive channels or proteins involved in defense signaling (Henry et al., 2011). Cryptogein alters the PM of tobacco cells by modifying lateral compartmentalization and biophysical properties (fluidity), suggesting the generation of signaling platforms in the cell surface (Gerbeau-Pissot et al., 2014). In addition, cryptogein (Stanislas et al., 2009), chitin (Fujiwara et al., 2009) and flg22 (Keinath et al., 2010) modify the protein composition of detergent resistant PM (DRM) fractions. In the latter case, 64 proteins (including RLKs-like, FLS2, H^+-ATPAses, and others) are enriched in this fraction 15 min after elicitation. Therefore, interaction of diC14 with plant PM may alter the organization, compartmentalization or composition of this membrane to somehow boost the activity of the defense components targeted by this lipid.

Eventually, as suggested for animal cells (Tanaka et al., 2008), diC14 may bind a plant receptor. Cationic lipids sharing structural similarities with diC14 (charge, acyl chain length or headgroup), such as DMTAP, DMDAP, DMPC and diC16 were unable to stimulate significantly defense genes or confer resistance against *Pst* as diC14, arguing against broad-range effects of cationic lipids on plant immunity. Low affinity receptors that perceive lipid PAMPs/DAMPs might be present in plants. Lipid elicitors require higher concentrations than peptide elicitors to activate JA/SA/PTI markers (rhamnolipids: 200 µg ml^{-1}, 300 µM approx; diC14: 5-40 µg ml^{-1},10-75 µM approx.; peptide elicitors: subnanomolar concentration) (Boller and Felix, 2009; Sanchez et al., 2012). The dose of LPS that triggers plant PTI (5-100 µg ml^{-1}) is higher than the one stimulating the TLR4 pathway in animal cells (pg-ng ml^{-1}) (Zeidler et al., 2004). Competition experiments analyzing internalization of labeled molecules suggested the existence of a low affinity LPS receptor in tobacco cells (Gross et al., 2005). However, this possibility was questioned by other studies (Zeidler et al., 2004). On the other hand, lipid receptors operate in plant-insect interactions. The linolenic acid derivative volicitin from beet armyworm caterpillar elicits defenses in maize, displaying high affinity

interaction (Kd=1.3 nM) with a PM protein. Such interaction is reversible and saturable and it involves near 3000 binding sites per cell (Truitt et al., 2004). Therefore, it is feasible that plants use receptors to detect pathogen-derived lipids, as they are essential components of fungal (ergosterol) or bacterial (LPS) plasma membranes that can function as PAMPs/DAMPs. Moreover, diC14 may mimic the effect of such PAMPs/DAMPs.

diC14-mediated *PR1* and *FRK1* activation was lower in the *fls2/efr/cerk1* mutant, but not in the *fls2*, *efr* or *cerk1* single mutants, indicating that these proteins do not function by themselves as the lipid receptor. This is consistent with the notion that PRRs with leucine-rich repeats at the ectodomain (FLS2, EFR) bind proteins or peptides, while those with lysine motifs (CERK1) bind chitin or PGN (Macho and Zipfel, 2014). Interestingly, the *fls2/efr/cerk1* mutant was able to induce *PR1* and *FRK1* during *Pst AvrRpm1*-mediated ETI activation, suggesting that the low response of these genes to diC14 could not be due to a general effect of the plant on activation of these genes. As mentioned before, diC14 may stimulate defenses at different levels. We do not know why diC14-induced defense gene expression is weaker in the *fls2/efr/cerk1* mutant. There is much evidence suggesting that PRR association is necessary for elicitor-triggered defenses. In rice, hetero-oligomeric complexes formed by dimers of the binding receptor OsCEBiP (chitin-elicitor binding protein) and the non-ligand-binding receptor OsCERK1 signal chitin perception (Macho and Zipfel, 2014). In Arabidopsis, perception of flg22 requires heterodimerization of FLS2 with the co-receptor BAK1 (Sun et al., 2013), while recognition of PGN requires the ligand-binding receptors LYM1/LYM3 and CERK1, which does not bind itself to the elicitor (Willmann et al., 2011). However to date, there is no direct link between the PRRs FLS2, CERK1 and EFR related to activation of defense responses.

At the systemic level diC14 induces defenses against pathogens, as also do LPS, flagellin, surfactin, fengycin and massetolide A (Mishina and Zeier, 2007; Ongena et al., 2007; Tran et al., 2007). This may result from the generation of plant signals acting at the systemic level in

response to diC-14, as well as from lipid movement to systemic tissues. Even if diC14 could be transported to other leaves the second possibility seems unlikely since its capacity to activate defense genes is reduced at low dose (5 *vs* 40 µg mL^{-1}) and may be negligible once the inoculum (40 µg mL^{-1}) was diluted into the plant. Concerning the involvement of plant systemic signals, diC14 may use any reported mobile SAR signal, like methyl salicylate (MeSA), glycerol-3-phosphate (G3P), dehydroabietinal (DA), azelaic acid (AzA) and pipecolic acid (Pip) (Dempsey et al., 2012; Shah and Zeier, 2013). However, diC14 does not require DIR1, at least exclusively, for systemic *PR1* activation. This lipid transport protein participates in MeSA, G3P, DA and AzA mobilization (Shah and Zeier, 2013), suggesting *a priori* that these signals would not be involved. Assuming that DIR is capable of transporting Pip, it would be interesting to determine whether diC14 uses Pip to induce SAR, as suspected for LPS and flagellin which cause Pip accumulation (Navarova et al., 2012).

EXPERIMENTAL PROCEDURES

Plant material

Arabidopsis thaliana (Col-0 and Ws) wild type, and *dir1-1*, *fls2* (Zipfel et al., 2004), *efr* (*efr1*; SALK_044334) (Zipfel et al., 2006), *cerk1* (*cerk1-2*; GABI_096F09) and *fls2/efr/cerk1* (Gimenez-Ibanez et al., 2009), *ndr1-1* (Shapiro and Zhang, 2001) and *eds1-2* (Bartsch et al., 2006) mutants were grown in soil under cycles of 8 h light and 16 h dark at 23ºC for 8 weeks in incubators with strict hygiene.

Pathogen growth and inoculation

Pseudomonas syringae pv *tomato* DC3000 strains (virulent, *hrpC*⁻ mutant, or avirulent *AvrRpm1*) were grown on King's B medium supplemented with kanamycin (50 μg mL^{-1}) and rifampicin (100 μg mL^{-1}). Bacterial pathogens were inoculated into leaf tissues (Pavet et al., 2005) at 10^5 colony-forming units (cfu) mL^{-1} for bacterial growth curves, and 10^7 cfu mL^{-1} for all other studies. Bacterial growth curves were performed as previously reported (Pavet et al., 2005). *Botrytis cinerea* B05.10 was obtained from Dr. F. Pieckenstain (INTECH-CONICET, Buenos Aires), and used for plant inoculation as described previously (Rossi et al., 2011). Fungal proliferation was analyzed by trypan blue staining (Pavet et al., 2005).

Liposome preparation

diC14 and diC16 (3-hexadecyl-amino-tert-butyl-N-hexadecyl-propionamidine) nanoliposomes were synthesized as described before (Ruysschaert et al., 1994).The same protocol was used to prepare liposomes of diC14, DMTAP, DMDAP, and DMPC (Avanti Polar lipids): lipid films (formed after resuspension of lipids into chloroform and evaporation of solvent under nitrogen flux), and HEPES 10 mM pH 7.2, were independently incubated at

55-60°C for 10 minutes. Then the buffer was added to the lipids without mixing at a final concentration of 1 mg ml^{-1} and incubated for additional 20 min at 55-60 °C. A similar protocol was used to prepare diC16 liposomes, but both incubation steps were made at 60-65°C. After incubation, all resuspended lipids were vortexed for 1 min to obtain liposomes. Next, all lipids were diluted in water at 20 µg ml^{-1} and infiltrated in leaves by needleless syringe. In experiments from Figure 1 diC14 was also used at 5, 40 and 100 µg ml^{-1}. Mock treatment included infiltration of HEPES 0.2 mM treated under identical conditions.

Cell death and callose analysis

Trypan blue and aniline-blue staining were used to quantify plant cell death and callose deposition, respectively (Cecchini et al., 2009).

Gene expression

Reverse transcription was performed by using 2 µg of total RNA treated with RQ1 DNAsa (Promega), random hexamer primers, and MMLV reverse transcriptase (Promega) to synthesize cDNA. sqPCR was performed with Taq polymerase (Promega) as follows: 3 min at 95 °C, *n* cycles of 35 sec at 95 °C, 35 sec at 60 °C and 45 sec at 72 °C. *GapC* (GADPH C subunit; *At3g04120*) was used as reference gene. Primers, number of cycles and annealing conditions are listed in Table S2 (see Supporting information), where cycle numbers correspond to the exponential amplification phase for each gene. qPCR was performed with Promega Master Mix, as follows: 10 min at 95 °C; 40 cycles of 35 sec at 95 °C, 30 sec at 60 °C and 30 sec at 72 °C. Reaction efficiency was in the range of 97-105% for all analyzed genes including *UBQ5* (Ubiquitin 5; *At3g62250*) used as internal control. The relative expression of *FRK1* and *PR1* in each sample was calculated by the $2^{-\Delta Ct}$ method using *UBQ5* as reference gene (ΔCt = Ct target-Ct reference). The $2^{-\Delta\Delta Ct}$ method was used to evaluate

FRK1 and *PR1* expression respect to *UBQ5* in diC14-treated samples, by normalizing to mock-samples [ΔΔCt = (Ct target-Ct reference) elicited - (Ct target-Ct reference) mock].

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SUPPORTING INFORMATION LEGENDS

Fig. S1 diC14 does not reduce *Pst* proliferation *in vitro*. *Pst* was inoculated at $5x10^6$ cfu ml^{-1} in liquid KB medium supplemented with diC14 (20 µg ml^{-1}) or HEPES 0.2 mM solution (mock). Bacterial content was determined by plating aliquots of culture at 0, 2, 4, 8, 10, 12 and 24 hpi.

Fig. S2 diC14 potentiates callose deposition induced by *Pst hrpC*$^-$. Leaves were infiltrated with diC14 (20 µg ml^{-1}), *Pst hrpC*$^-$ (10^7 cfu ml^{-1}) or diC14+*Pst hrpC*$^-$. (a) Callose deposits were detected by aniline blue staining at 12 and 24 hpi. Scale bar, 0.5 mm. (b) Amount of deposits per field (4.4 mm^2) determined with ImageJ software as indicated in Figure 3. Values indicate mean ± standard error (12 pictures from 6 leaves). *: significant differences between *Pst* and diC14+*Pst* treatments ($p< 0.05$ by *t* test).

Fig. S3 *FRK1* and *PR1* expression in wild type and *fls2/efr/cerk1* plants treated with diC14 (a) or *Pst AvrRpm1* (b). (a) Upper panels: RT-qPCR comparing diC14-mediated gene activation in each plant, relative to mock-treatment (ΔΔCt method), at 7 and 24 hpi. Different letters indicate significant differences among samples ($p< 0.05$; two-way ANOVA followed by Tukey test). Insets show the indicated samples in different scale. Lower panels: RT-qPCR comparing the *FRK1* and *PR1* mRNA levels in both plants, relative to *UBQ5* mRNA content (ΔCt method), at 24 hpi. *: significant differences respect to Col-0 samples ($p< 0.05$ by *t*

test). (b) RT-sqPCR (left) or RT-qPCR (right) assays used to compare the response of both plants to mock or *Pst AvrRpm1* (10^{-7} cfu ml^{-1}) (*Avr*) treatments at the indicated time points. One representative experiment from two independent assays is shown for each study.

Fig. S4 Leaves pre-treated with HEPES 0.2 mM (Mock), diC14, DMTAP, DMDAP or DMPC (20 µg ml^{-1} each), were inoculated 24 h later with *Pst* (10^5 cfu ml^{-1}) to quantify bacterial content at 3 days post-infection. Values represent mean ± SD of two technical replicates. Similar results were obtained in two independent infection experiments. *: significant differences between mock and diC14 treatments ($p< 0.05$ by *t* test).

Fig. S5 *PR1* induction by diC14 and *Pst AvrRpm1*. *PR1* expression was analyzed as described in Figure 1, in leaves infiltrated with diC14 (20 µg ml^{-1}) or *Pst AvrRpm1* ($2x10^7$ cfu ml^{-1}). Two independent experiments produced similar results.

Fig. S6 diC14 does not protect against *Botrytis cinerea* infection. Leaves were infiltrated with diC14 (20 µg ml^{-1}) or mock solution (HEPES 0.2 mM) and inoculated 24 h later with *Botrytis cinerea* (10^4 and 10^3 conidia ml^{-1}; three sites for each concentration). Trypan blue staining was used to monitor fungal proliferation at 3 dpi.

Table S1 Chemical structure of the lipids used in this work.

Table S2 Primers and conditions used on RT-PCR experiments.

FIGURE LEGENDS

Fig. 1 Expression of SA- and JA- sensitive genes in diC14-treated leaves analyzed by sqRT-PCR. The PTI and SA-gene markers *WRKY29*, *FRK1*, *ICS1* and *PR1* (a), and the JA-sensitive genes *VSP2*, *LOX2* and *PDF1.2* (b), were studied. *GapC* was used as internal control. Untreated (0 hpi) and diC14-treated (5, 40 or 100 µg ml^{-1} HEPES 0.2 mM) samples were analyzed at 8 and 24 hours post-infiltration (hpi), using HEPES 0.2 mM as control (0 µg ml^{-1} diC14; mock inoculation). One representative from three independent biological experiments is shown.

Fig. 2 diC14 induces local resistance to *Pst*. (a) Leaves pre-treated with HEPES 0.2 mM (mock), diC14 or diC16 (20 µg ml^{-1} each), were inoculated 24 h later with *Pst* (10^5 cfu ml^{-1}) to quantify bacterial content at 3 days post-infection. Values represent mean ± SD of two technical replicates. Similar results were obtained in two independent infection experiments. *: significant differences between mock and diC14 treatments ($p< 0.05$ by *t* test). (b) Cell death was evaluated by trypan blue staining on leaves sampled at 8 or 24 hours post infiltration (hpi). The effects of mock solution (HEPES 0.2 mM), diC14 (20 µg ml^{-1}) and *Pst AvrRpm1* (10^7 cfu ml^{-1}) are compared. Similar results were obtained in three independent experiments.

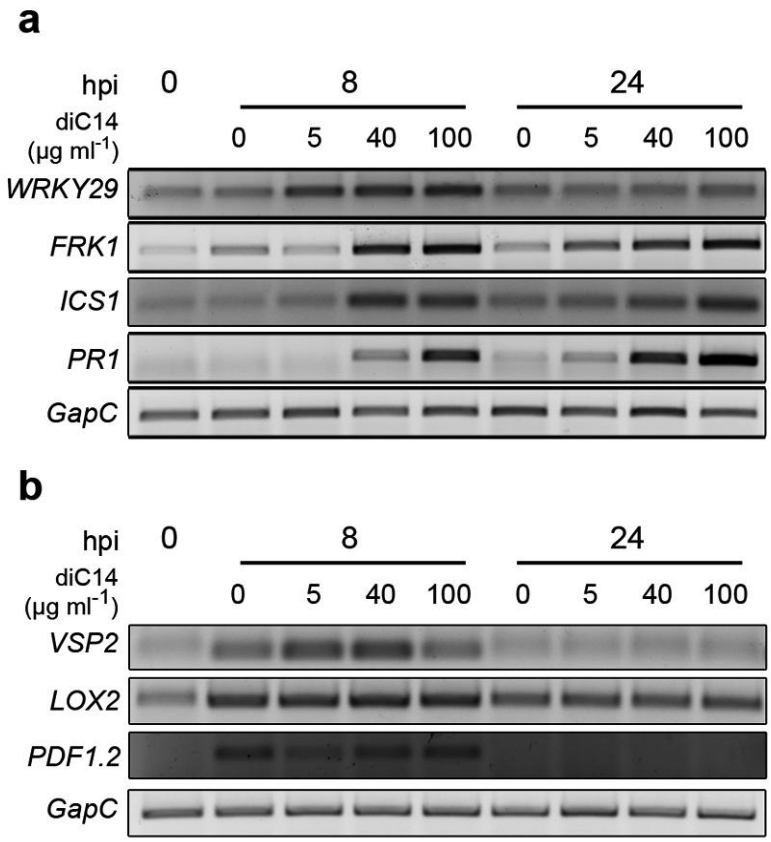
Fig. 3 diC14 potentiates callose deposition. (a) Left: callose deposits detected by aniline blue staining at 12 and 24 hpi with diC14 (20 µg ml^{-1}), *Pst* (10^7 cfu ml^{-1}) or diC14+*Pst*. Scale bar, 0.5 mm. Right: number of callose deposits per field (4.4 mm^2) determined with ImageJ software, values indicate mean ± SE (12 pictures from 6 leaves). *: significant differences between *Pst* and diC14+*Pst* treatments ($p< 0.05$ by *t* test). (b) sqRT-PCR used to determine

the *CalS12* transcript levels at 24 hpi in samples described in (a). Mock: HEPES 0.2 mM. (c) Trypan blue staining of tissues treated with diC14 (20 µg ml^{-1}), *Pst AvrRpm1* (10^7 cfu ml^{-1}) or diC14+*Pst AvrRpm1* analyzed at 24 hpi.

Fig. 4 Plant signaling components required to activate defenses by diC14. *FRK1* and *PR1* expression was evaluated as described in Figure 1. Leaves treated with mock solution or diC14 (20 µg ml^{-1}) were sampled at 0, 8 or 24 hpi. Responses of wild type plants were compared with those of *fls2/efr/cerk1* (a), *fls2, efr, cerk1* (b), *eds1-2* (c), or *ndr1-1* (d) mutants. One representative experiment from three independent assays is shown. RT-qPCR assays confirming the reduced capacity of the *fls2/efr/cerk1* mutant to induce *FRK1* and *PR1* by diC14 are shown in Fig S3a (see Supporting information).

Fig. 5 diC14 induces systemic resistance. Two leaves per plant were infiltrated with mock solution, diC14 or diC16 (20 µg ml^{-1} each). One day later three untreated secondary leaves were used to evaluate either gene expression (a,c) or pathogen resistance (b). Expression of *PR1* and *FRK1* genes was studied as described in Figure 1. (b) Leaves infiltrated with *Pst* (10^5 cfu ml^{-1}) were analyzed at 3 days post-infection, as described in Figure 2. One representative experiment from two biological replicates is shown. *: significant differences between mock and diC14 treatments ($p< 0.05$ by *t* test). (c) Responses of wild type (Ws) and *dir1-1* mutant plants are compared.

Fig. 6 Effect of other synthetic lipids on *FRK1*, *ICS1* and *PR1* activation. Gene expression was monitored as in Figure 1. All lipids were used at 20 µg ml^{-1}. The structure of diC16 (a), DMTAP, DMDAP and DMPC (b) are described in Table S1 (see Supporting information).



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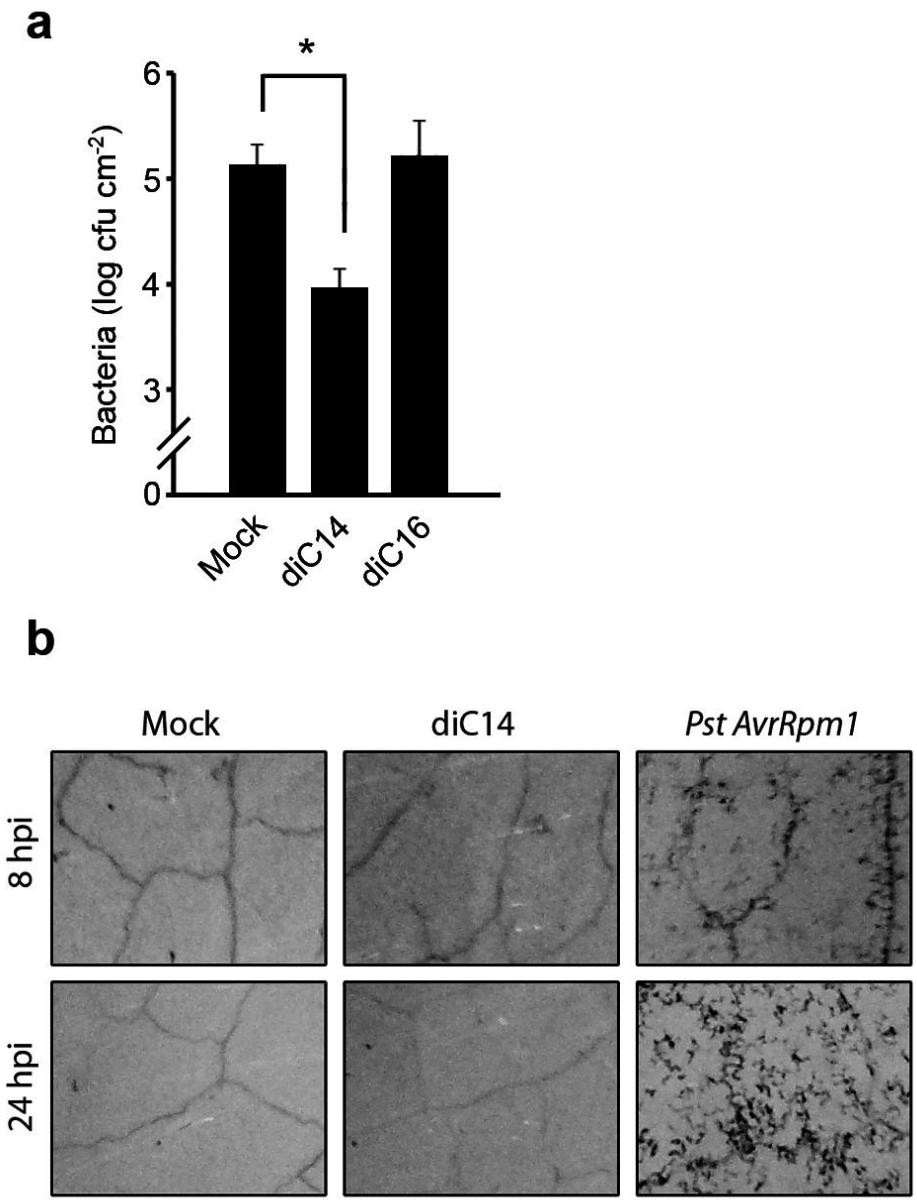

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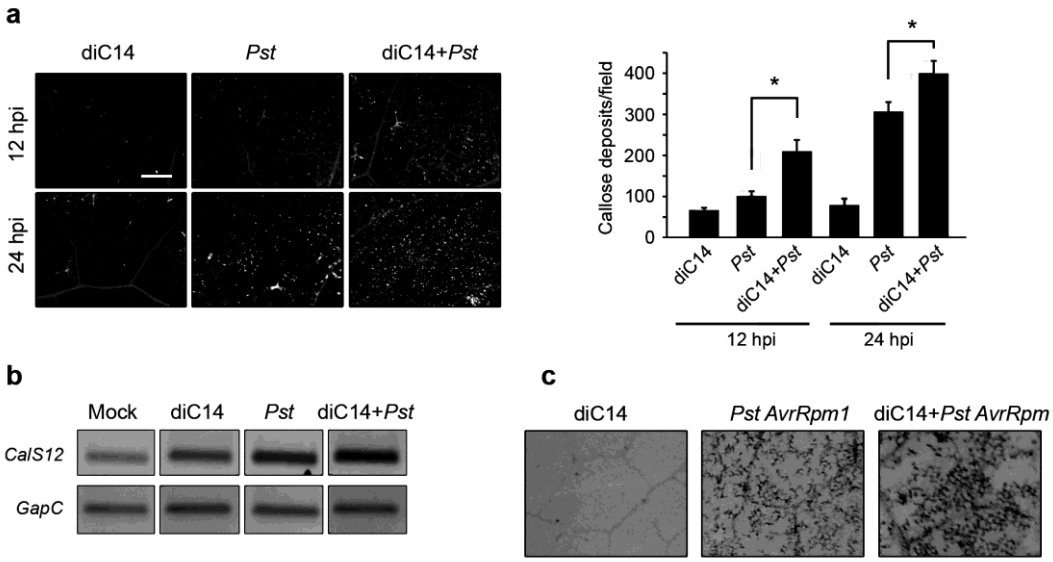

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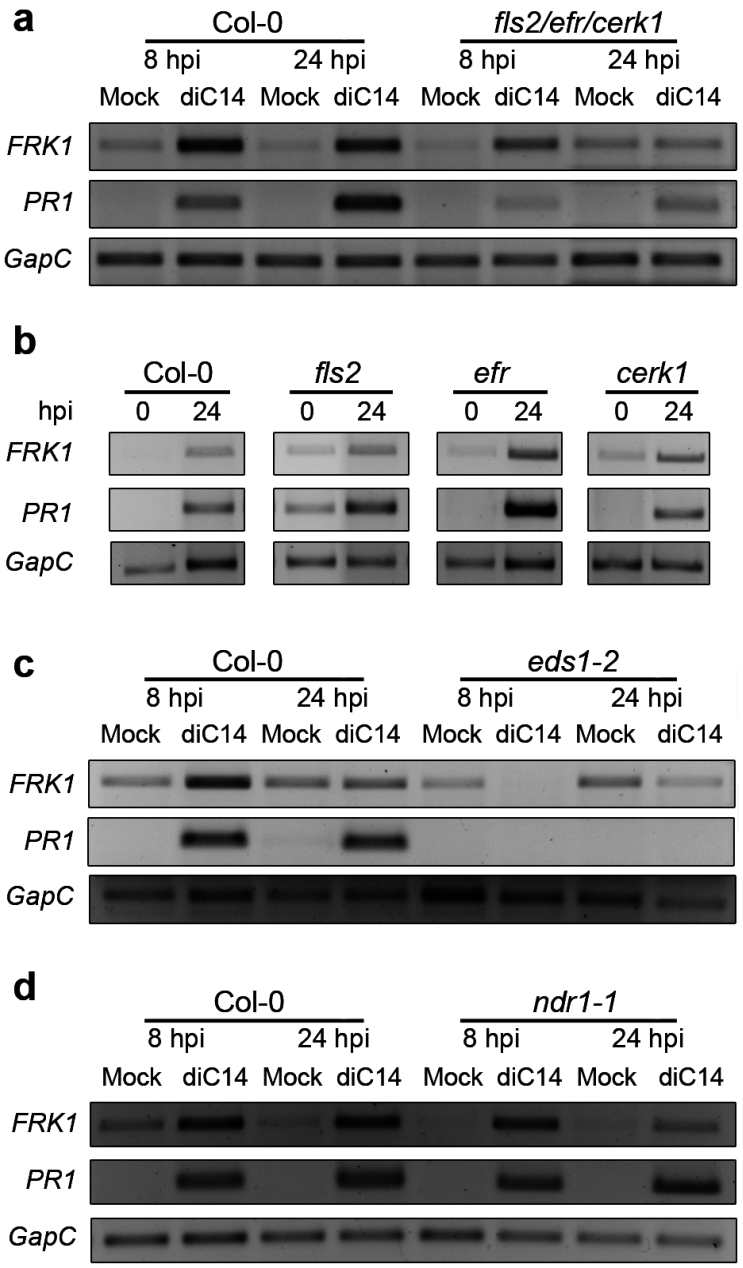

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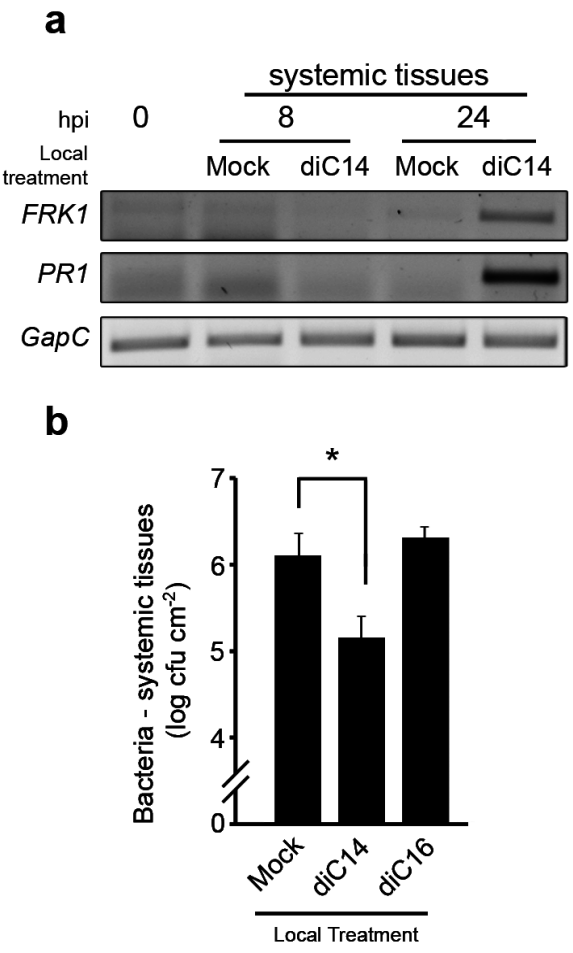

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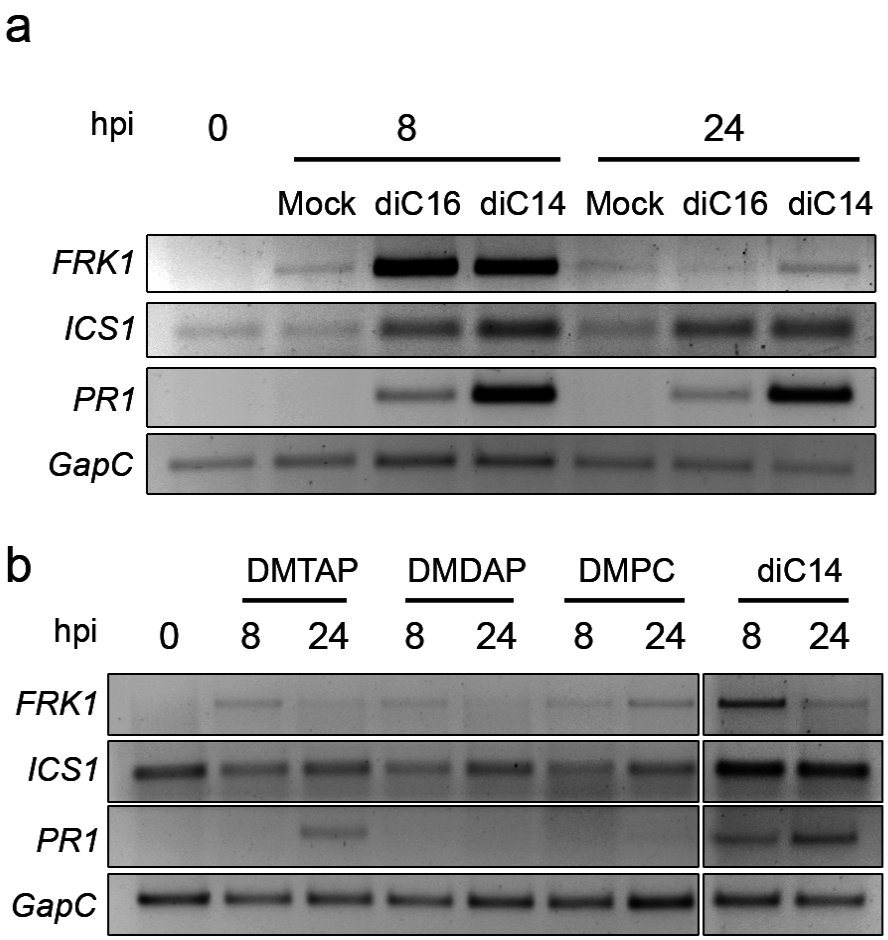

MPP_12252_F6