MPK6, sphinganine and the LCB2a gene from serine palmitoyltransferase are required in the signaling pathway that mediates cell death induced by long chain bases in Arabidopsis

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Summary
• Long chain bases (LCBs) are sphingolipid intermediates acting as second messengers in programmed cell death (PCD) in plants. Most of the molecular and cellular features of this signaling function remain unknown.
• We induced PCD conditions in Arabidopsis thaliana seedlings and analyzed LCB accumulation kinetics, cell ultrastructure and phenotypes in serine palmitoyltransferase (spt), mitogen-activated protein kinase (mpk), mitogen-activated protein phosphatase (mkp1) and lcb-hydroxylase (sbh) mutants.
• The lcb2a-1 mutant was unable to mount an effective PCD in response to fumonisin B1 (FB1), revealing that the LCB2a gene is essential for the induction of PCD. The accumulation kinetics of LCBs in wild-type (WT) and lcb2a-1 plants and reconstitution experiments with sphinganine indicated that this LCB was primarily responsible for PCD elicitation. The resistance of the null mpk6 mutant to manifest PCD on FB1 and sphinganine addition and the failure to show resistance on pathogen infection and MPK6 activation by FB1 and LCBs indicated that MPK6 mediates PCD downstream of LCBs.
• This work describes MPK6 as a novel transducer in the pathway leading to LCB-induced PCD in Arabidopsis, and reveals that sphinganine and the LCB2a gene are required in a PCD process that operates as one of the more effective strategies used as defense against pathogens in plants.

Introduction
Programmed cell death (PCD) is a necessary and recurrent process during the plant life cycle, participating in differentiation, immunity and senescence (Greenberg, 1996; Turner et al., 2007; Mur et al., 2010; Peet et al., 2010). Recently, sphingolipids have been revealed as PCD mediators in plants (Shi et al., 2007; Wang et al., 2008; Pata et al., 2009), as known previously in yeast and mammalian cells (Liu K et al., 2005; Hannun & Obeid, 2008). Given the vast and complex repertoire of sphingoid compounds revealed by new analytical tools (Markham et al., 2006; Markham & Jaworski, 2007) and by the existence of a plethora of enzymes that catalyze sphingolipid metabolism (Supporting Information Fig. S1) (Chen et al., 2009), the assignment of precise signaling roles to specific sphingoid species and a knowledge of their mechanisms of action in PCD and in other cell functions are particularly challenging in plants.
As signaling molecules, phosphorylated sphingoid bases, or long chain bases (LCBs-P), regulate stomatal closure (Ng et al., 2001; Coursol et al., 2003, 2005; Townley et al., 2005; Worral et al., 2008), whereas long chain bases (LCBs) and ceramides regulate PCD (Shi et al., 2007; Wang et al., 2008; Lachaud et al., 2010). In this regard, evidence of the involvement of sphingoid compounds has been known for some time. For example, fumonisin B1 (FB1) is a mycotoxin that disrupts sphingolipid biosynthesis (Fig. S1) and elicits PCD (Stone et al., 2000; Merrill et al., 2001). In Arabidopsis, FB1-induced PCD is triggered by increased LCB levels, as demonstrated by a mutation in the \( LCB1 \) gene (Shi et al., 2007) that encodes one of the two subunits of serine palmitoyltransferase (SPT), a key enzyme in the de novo synthesis of sphingolipids (Gable et al., 2002) (Fig. S1). Sphingolipids in plants also participate in the PCD of defense against pathogens, called the hypersensitive response (HR-PCD), as shown by mutants exhibiting a premature cell death phenotype and other defense responses (Brodersen et al., 2002; Liang et al., 2003).

However, despite all the work carried out on this issue, PCD elicited by LCBs is largely unknown in terms of the molecular components of the pathway and of the changes to the cell during this process.

We hypothesized that LCBs might induce mitogen-activated protein kinase (MAPK) cascades leading to PCD induced by FB1, based on the following evidence: (1) FB1 increases MPK activity in animal cells (Wattenberg et al., 1996; Pinelli et al., 1999); (2) infection or treatment with elicitors leading to HR-PCD induces the activation of salicylic acid (SA)-induced (SIPK) and wound-induced (WIPK) protein kinases in Nicotiana tabacum (Zhang et al., 1998, 2000) and of MPK6 and MPK3 (SIPK and WIPK Arabidopsis orthologs, respectively) (Desikan et al., 2001; Ren et al., 2008); (3) Arabidopsis plants overexpressing MEK4 and MEK5 (that are MAPKK) show an HR-PCD-like phenotype and a prolonged activation of the MPK3 and MPK6 kinases (MEK4 and MEK5 downstream targets) (Ren et al., 2002); and (4) the rice MPK6 (OsMPK6) ortholog of AtMPK6 is activated by a sphingolipid elicitor from a blast fungus (Lieberherr et al., 2005).

In this study, we induced PCD conditions in Arabidopsis seedlings using FB1 in several genetic backgrounds in order to investigate how LCB accumulation kinetics are associated with molecular and cell death features. Our results reveal that the \( LCB2a \) gene, the dihydroxylated LCB sphinganine \( (d18:0) \) and MPK6 are important contributors to the LCB-induced PCD, which can be physiologically manifested as an HR-PCD. We also found that LCB- and MPK6-induced PCD involves extensive disorganization of endomembranes and features of autophagic cell death. MPK6 is the first kinase described as a transducer in the LCB-mediated PCD in plants.

### Materials and Methods

#### Plant growth conditions and treatments

For the germination and growth of Arabidopsis thaliana (L.) plants, 3-wk-old Arabidopsis seedlings were transferred to Gamborg plates supplemented with FB1 (Sigma-Aldrich Corp., St Louis, MO, USA) and incubated at 22°C under constant light (11.5 \( \mu \)mol m\(^{-2} \) s\(^{-1} \)) for the indicated concentrations and times. For myriocin (Sigma-Aldrich Corp.) treatment, seedlings were transferred to solid medium supplemented with 100 nM myriocin and exposed during 5 d before FB1 treatment. The details are described in Methods S1.

#### Isolation of T-DNA insertion lines and construction of mutant lines

The previously characterized T-DNA insertional mutants \( lcb2a-1 \) (SALK_061472), \( lcb2a-2 \) (GABI 216-D07), \( lcb2a-3 \) (SAIL_706-A05) (Dietrich et al., 2008) and \( sbh1-1 \) (SALK_090881) (Chen et al., 2008) were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH, USA). RNAi-silenced \( mpk3 \) plants were generated by transgenesis as described in Methods S1. An \( mpk6-2 \) homozygous line was recovered by self-crossing heterozygous seedlings from the SALK_073907 insertional mutant collection stock (see details in Methods S1). The \( mkk1 \) single mutant and \( mpk3 \) \( mkp1 \) and \( mpk6 \) \( mkp1 \) double mutants were kindly donated by Marina A. González Besteiro from the Roman Ulm Laboratory (University of Geneva, Switzerland) (Ulm et al., 2001). The presence of T-DNA insertions was verified as described in Methods S1.

#### Detection of nuclear DNA fragmentation

DNA from controls and seedlings treated with FB1 for 5 d was stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich Corp.) and the free 3'-OH groups in the DNA were subjected to terminal deoxynucleotidyl transferase-mediated UTP nick end labeling (TUNEL) reaction, as described in Methods S1.

#### Ultrastructural analysis

FB1-treated and untreated seedlings were fixed, dehydrated, embedded, sectioned and observed as described in Methods S1.

#### LCB analysis

Seedlings were collected after treatment with 10 \( \mu \)M FB1 for 0, 1, 4, 8, 12 and 72 h, frozen in liquid nitrogen and...
later lyophilized. Quintuplicates of these treatments were independently performed and processed. LCBs-P were extracted from 30 mg of the lyophilized tissue (dry weight), followed by separation, identification and quantification through reverse-phase high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (HPLC ESI-MS/MS), as described in Markham & Jaworski (2007).

Protein extraction, kinase assays and immunoblot analyses

Soluble protein extracts were obtained from seedlings and subjected to in-gel kinase activity assays, and immunoblots were performed as described in Methods S1.

Treatment with exogenous LCBs

Three-wk-old *Arabidopsis* wild-type (WT) or mutant seedlings grown in Petri dishes (3.5 cm in diameter) were sprayed with 5 μM d18:0 (Sigma-Aldrich Corp.), 5 μM t18:0 (Sigma-Aldrich Corp.) or 5 μM d18:0-P (Avanti Polar Lipids, Inc., Alabaster, AL, USA) solution (0.7 ml per dish). All compounds were dissolved in 0.04% (v/v) Silwet L-77 (Chemtura Corporation México, S. de RL de C.V. México, D.F., México). Controls sprayed with Silwet solution were included. Seedlings were kept under continuous light (11.5 μmol m⁻² s⁻¹) at 22°C and phenotypic changes were recorded photographically.

Bacterial inoculation and *in planta* growth determination

Fresh cultures of *Pseudomonas syringae* pv. *tomato avrRpm1* were obtained and resuspended in 10 mM MgCl₂. The suspension [10⁷ colony-forming units (cfu) ml⁻¹] was sprayed over the Petri dishes (2.0 ml per 8-cm-diameter dish) containing the *Arabidopsis* WT, *lcb2a-1* or *mpk6* mutant seedlings, which had been exposed, or not, to 10 μM FB1 for 12 h (under continuous light, 11.5 μmol m⁻² s⁻¹). Seedling samples were collected at the indicated times and ground in 10 mM MgCl₂; serial dilutions were plated in Kings B medium supplemented with 40 μg ml⁻¹ rifampicin and 50 μg ml⁻¹ kanamycin, incubated at 29°C and the number of colonies was counted after 48 h (Yang et al., 2002).

Results

Two approaches used to generate low or high accumulation of endogenous LCBs

To gain an insight into the pathway between LCB accumulation and PCD, we used two systems that allowed an increase or decrease in endogenous LCBs in *Arabidopsis* seedlings. In one system, the pharmacological approach, seedlings were exposed to FB1 to generate an accumulation of LCBs that eventually produced tissue cell death (Fig. S1). Moreover, myriocin, an SPT inhibitor, was used to decrease LCB synthesis and to prevent LCB accumulation (Fig. S1). FB1 treatment caused generalized cell death in the WT seedling tissues, which was prevented by pre-treatment with myriocin (Fig. 1a), suggesting that FB1 decreased the viability of WT seedlings through the production of high LCB levels.

Another strategy to decrease LCBs was of genetic nature. *Arabidopsis* contains three genes encoding SPT subunits: one LCB1 subunit (At4g36480) and two LCB2 subunits sharing 86% identity: LCB2a (At5g23680) and LCB2b (At3g48780). LCB2a and LCB2b are functionally redundant when interacting with the LCB1 monomer to form SPT (Dietrich et al., 2008). Because *LCB2a* gene expression is higher than *LCB2b* in all plant organs analyzed thus far (Dietrich et al., 2008), we made the *LCB2a* contribution to LCB generation and accumulation in response to FB1 a focus of this study. We examined three independent T-DNA *Arabidopsis* mutants for *LCB2a* (Dietrich et al., 2008) in relation to FB1 response: *lcb2a-1* (SALK_061472), *lcb2a-2* (GABI 216-D07) and *lcb2a-3* (SAIL_706-A05). On exposure to FB1, all mutants showed a slight decrease in growth, some chlorosis and flowering as a stress response (phenotype designated as FB1-resistant), but did not show the extensive tissue death seen in WT (Fig. 1b). These results indicate that these mutants may be useful as a tool to examine the molecular bases of PCD in comparison with WT seedlings which, on FB1 exposure, show an increase in LCB levels.

To substantiate the phenotypic cell death, we examined DNA fragmentation, a hallmark of PCD. Fragmented DNA was not detected in WT and *lcb2a-1* control seedlings (Fig. 2a, sections 1–3, 7–9), but FB1-treated WT seedlings showed a higher proportion of free 3′-OH groups, indicating fragmentation of DNA (Fig. 2a, sections 4–6). By contrast, *lcb2a-1* mutants showed few cells with DNA nicks (Fig. 2a, sections 10–12), implying that this mutant displays very low PCD features at the molecular level. These results were in agreement with the PCD appearance shown by the FB1-treated WT and mutant seedlings (Fig. 1b).

PCD induced by LCB accumulation involves changes in the organization and integrity of organellar membranes

In order to characterize the PCD features as a product of LCB buildup at a subcellular level, we used transmission electron microscopy (TEM) (Fig. 2b). WT seedlings showed typical cell morphology with large vacuoles restricting the cytoplasm to the periphery, and crescent-shaped
chloroplasts with well-formed thylakoid membranes (Fig. 2b, sections 1–3). By contrast, FB1-treated WT seedlings showed features of cells undergoing PCD: interiorized and rounded chloroplasts, indicating loss of tonoplast integrity (Fig. 2b, sections 4, 5); few starch grains (Yao & Greenberg, 2006); and a remarkable disintegration of the thylakoids (Fig. 2b, section 5). In addition, we detected vesicles with inner structures resembling organellar debris, as reported in plant systems undergoing PCD (Liu Y et al., 2005; Mino et al., 2007) (Fig. 2b, section 6). The first visible ultrastructural signs of cell injury in WT seedlings occurred as early as 36 h post-FB1 treatment (Fig. S2).

In contrast with WT cells, disruption of the LCB2a gene affected chloroplast morphology, in particular an increase in the size and number of rounded plastids (Fig. 2b, section 7). However, no dramatic changes were observed in thylakoid organization, starch granules or tonoplast integrity in the lcb2a-1 mutant exposed to control or FB1 conditions (Fig. 2b, sections 7–9 and 10–12, respectively). These results indicate that cell death promoted by an assumed LCB accumulation is coincident with DNA degradation and extensive organellar membrane damage. However, the FB1 resistance of the lcb2a-1 mutant was particularly intriguing, as no differences in growth and development, when compared with WT plants, were observed (Dietrich et al., 2008). Therefore, an approach to analyze the dynamics of endogenous LCBs was undertaken.

Differential accumulation of LCBs during PCD elicitation in Arabidopsis seedlings

To compare the FB1-induced accumulation of specific LCBs in WT and lcb2a-1 seedlings, we used a protocol that included the selective extraction of free LCBs and their subsequent separation and quantification by HPLC ESI-MS/MS (Markham & Jaworski, 2007). LCB profiling included the free nonphosphorylated and phosphorylated (LCB-P) forms of sphinganine (d18:0), 4-hydroxysphinganine (t18:0), 8-sphingenine (d18:1), 4,8-sphingadienine (d18:2) and 4-hydroxy-8-sphingenine (t18:1). Table S1 shows the determinations derived from five replicates for each treatment.

The levels of the 10 LCB species in seedlings grown under control conditions were very low during the 72-h time span studied, both in WT and the lcb2a-1 mutant (Fig. S3, Table S1), in agreement with the report by Markham et al. (2006). It was clearly observed that, on FB1 treatment, LCB levels increased as a function of time in absolute amounts, with higher levels in WT than in the lcb2a-1 mutant. d18:0, d18:0-P, t18:0 and t18:0-P showed major increases on FB1 treatment (Fig. 3, Table S1). The accumulation kinetics of d18:0 and t18:0 in WT showed gradual and significant increases starting at 4 h and reaching similar absolute amounts at 72 h of FB1 exposure (Fig. 3a,b). At this time, although d18:0 had increased 619-fold (compared with the WT, 0-h value), the t18:0 increase was 113-fold. With regard to d18:0-P and t18:0-P, a slight increase was observed from 4 to 12 h, but a marked increase was attained at 72 h of FB1 exposure, corresponding to 7970-fold and 952-fold, respectively, when compared with the 0-h value (Fig. 3c,d). The results of FB1 exposure of lcb2a-1 seedlings showed several differences from the WT treatment. There was no increase in d18:0 levels at 4 h, and the levels accumulated between 8 and 12 h were very low when compared with the corresponding WT seedlings treated with the toxin; a high value was reached at 72 h, when...
the increment was 141-fold (compared with the lcb2a-1, 0-h value) (Fig. 3a). In contrast, t18:0 increased significantly from 4 to 72 h (41-fold at the latter time point), but at levels lower than those of the corresponding WT FB1-treated samples (Fig. 3b). d18:0-P and t18:0-P attained similar levels to their nonphosphorylated forms, but with different increments (2186- and 452-fold, respectively) at 72 h (Fig. 3c,d).

From the four LCBs most affected by FB1 treatment, the clearest differences between WT and the lcb2a-1 mutant were in the d18:0 and d18:0-P contents, and in the earlier increase in d18:0 in WT, suggesting that the timing and levels of d18:0 or d18:0-P were the cause of the differences in the PCD phenotypes observed in WT and the lcb2a-1 mutant. The fact that d18:0 equalled t18:0 levels at 72 h in both WT and the lcb2a-1 mutant suggests that FB1 preferentially favors the endogenous accumulation of d18:0, although important increases in d18:0-P, t18:0 and t18:0-P were also found. In order to elucidate the function of t18:0 in the PCD elicited by FB1, we evaluated its contribution using a mutation in one of the two LCB hydroxylase genes (SBH1). It was found that the sbb1-1 mutant contained smaller amounts of trihydroxy-LCBs and elevated levels of total LCBs when compared with WT plants, although the SBH2 gene was functional (Chen et al., 2008). The rationale was that, if the trihydroxy-LCBs were main contributors to PCD, the sbb1-1 mutant would show FB1 resistance. The results in Fig. 1(c) show that LCB-induced cell death in sbb1-1 mutants yields a similar appearance to WT plants, which were sensitive to FB1, implying that a decrease in trihydroxy-LCB levels does not inhibit PCD. This finding indicates that increased levels of trihydroxy-LCBs are not essential triggers in PCD, and is consistent with the concept that dihydroxy-LCBs, such as d18:0, may be the primary mediators of LCB-induced PCD.

**MPK6 participates in the PCD signaled by LCBs**

Recently, LCBs have been revealed as important players in HR-PCD, one of the most studied cases of PCD in plants (Takahashi et al., 2009; Lachaud et al., 2010; Peer et al., 2010) and in which MAPK cascades are involved. In order to evaluate the participation of MPKs in the signalling transduction pathway mediated by LCB accumulation, an in-gel MPK activity assay was performed in WT seedlings exposed to FB1. As shown in Fig. 4(a), FB1 induced an increase in the activity of an MPK of c. 46 kDa throughout the interval of 15–90 min when compared with the control. From the MPKs in Arabidopsis, MPK6 and MPK3 have been repeatedly described as components of pathogen signaling pathways (Asai et al., 2002; Wang et al., 2007; Ren et al., 2008; Beckers et al., 2009). Thus, these two MPKs were selected in this study as possible candidates to be part of the LCB pathway that leads to PCD. Two Arabidopsis mutants were used: a T-DNA insertion line in...
the MPK6 gene and a MPK3 loss-of-function RNAi mutant, hereby referred to as mpk6 and mpk3, respectively. The mpk6 mutant has been described as a null allele of MPK6 with a T-DNA insertion within the fourth exon. This mutant shows impairments in embryo, leaf and floral development (Bush & Krysan, 2007; Wang et al., 2007) and in ethylene synthesis (Liu & Zhang, 2004). mpk6 and mpk3 mutants showed undetectable transcript or protein levels (Fig. 4b). Mutant viability on FB1 treatment was then evaluated. The mpk6 mutant exhibited resistance to FB1, similar to that observed in lcb2a-1 seedlings (Fig. 4c). An intermediate viability was seen in FB1-treated mpk3 plants, with cell death symptoms less severe than in WT plants, but more pronounced than in mpk6 plants. These results strongly indicate the participation of MPK6 in the LCB-induced PCD.

To confirm that toxin resistance in the MPK mutants was related to the suppression of molecular PCD events, DNA fragmentation was also analyzed (Fig. 5a). FB1-treated mpk6 seedlings presented low levels of DNA fragmentation, similar to those exhibited by the lcb2a-1 mutant (Fig. 2a). By contrast, the mpk3 mutant presented more DNA fragmentation than mpk6, but less than WT. These results confirm that MPK6 participates in the pathway that triggers LCB-induced PCD and, moreover, suggest that both MPKs are not completely redundant in this PCD scheme.

As judged by TEM, the mpk6 and mpk3 mutants exhibited changes in chloroplast morphology (Fig. 5b, sections 1, 2, 7, 8) and in a larger number of mitochondria compared with the WT and lcb2a-1 lines (Fig. 5b, sections 3, 9). In addition, the FB1-treated mpk3 mutant showed several subcellular alterations: chloroplasts with smaller starch granules and disorganized grana with intact membranes (Fig. 5b, section 5); vacuoles with loss of turgor (Fig. 5b, section 5) that included membrane vesicles containing cytoplasmic materials and organelles resembling autophagosomes (Fig. 5b, section 6); and cytosol containing a large number of vesicles (Fig. 5b, section 6). By contrast, the FB1-treated mpk6 seedlings only showed slight alterations in membrane and cell structure (Fig. 5b, sections 10–12), and chloroplasts contained fewer but larger starch granules compared with untreated seedlings (Fig. 5b, section 10 vs section 7).

In order to confirm that LCBs and MPK6 were in the same signaling pathway leading to PCD, several approaches...
were examined. First, d18:0, t18:0 or d18:0-P was sprayed on WT, mpk3 and mpk6 seedlings (Fig. 6). Both WT and mpk3 seedlings were highly susceptible to d18:0 addition, whereas the mpk6 mutant was resistant, as observed previously (Figs 1a, 4c). Treatment of WT and mpk3 with t18:0 and d18:0-P produced weak chlorosis, whereas mpk6 seedlings showed a healthy phenotype similar to the control, indicating that d18:0 was unable to launch the PCD pathway and that, even when d18:0 was added to this mutant, the absence of MPK6 prevented cell death emergence. As a very important point, Fig. 6 shows that exogenously added d18:0 induces a clear PCD phenotype in WT, which is not driven by t18:0 or d18:0-P. It should be noted that constant exposure of whole seedlings to FB1 leads to a generalized PCD that is accompanied by defense responses (Asai et al., 2000). To provide additional support for the hypothesis that LCBs and MPK6 are involved in the same route that orchestrates PCD, we used the mpk6 mutant. MKP1 is an Arabidopsis cytosolic dual specificity phosphatase (DSP) that physically interacts with MPK3, MPK4 and MPK6 (Ulm et al., 2001, 2002), and is able to inactivate the MPK6 phosphorylated form (Ulm et al., 2001). MPK6 is the main MPK needed for the establishment of the defense response. Treatments of mpk1, mpk3 mpk1 and mpk6 mpk1 mutants with t18:0 or d18:0-P did not produce significant changes, results that are in agreement with the phenotypes observed in WT and the single mutants.

In addition, to assess the association between LCB elevation and MPK6 activation, an in-gel MPK activity assay was performed (Fig. 7a). WT seedlings exposed to exogenously added FB1, d18:0 or t18:0 showed heavy myelin basic protein (MBP) labeling, corresponding to MPK6 activity and not to MPK3, as demonstrated by the molecular masses and activities shown in the mpk3 and mpk6 null mutants (Fig. 7a). Indeed, t18:0 showed the highest MPK6 activation, followed by d18:0 and FB1. This result provides direct proof which clearly demonstrates that LCBs selectively use MPK6 and not MPK3 as their transducer towards PCD.

From a consideration of the results that independently link MPK6 or LCBs to an HR-PCD (Menke et al., 2004; Ren et al., 2006; Takahashi et al., 2009), a supplementary strategy to support the involvement of LCBs and MPK6 in the same pathway leading to PCD was undertaken. We induced an HR-PCD through an exposure of WT, lcb2a-1 and mpk6 seedlings to Pseudomonas syringae pv. tomato avrRpm1, a bacterial strain that is avirulent towards Arabidopsis and therefore produces a resistance response involving HR-PCD (Tao et al., 2003). Seedlings were pretreated with FB1 for 12 h, and then exposed to the bacterial pathogen. Fig. 7(b) shows that FB1 treatment caused a decrease in bacterial proliferation in the WT and lcb2a-1 mutant seedlings when compared with the controls (non-FB1 treated). By contrast, the same treatment did not affect
the bacterial proliferation in mpk6 seedlings. These results indicate that an increase in LCBs and the intervention of MPK6 are required in the route that unchains PCD, but, more importantly, that the signaling route so configured decisively contributes to this strategy of defense against pathogens.

**Discussion**

**Role of specific SPT genes and specific LCBs in PCD elicitation**

**Role of the LCB2a gene** In this study, we have reported that LCB2a is required for the manifestation of PCD induced by FB1 based on the observation that, when the LCB2a gene is absent, a significant attenuation in the levels of all analyzed LCB species is reached on FB1 exposure and very scarce signs of PCD are shown. Shi et al. (2007) found that a mutation in the LCB1 gene contributed to lower LCB accumulation and therefore to FB1 resistance. This finding makes sense, as this gene is present in only one copy in Arabidopsis and the mutant used was only partially devoid of LCB1 transcripts (indeed, total loss of this gene would have led to lethality; see Chen et al., 2006). Thus, as a result of this partial decrease in the LCB1 gene, a diminished SPT activity produced minor increases in LCB on FB1 addition, and therefore a toxin-resistant phenotype. However, this is not the case in the present study, in which we evaluated one of the two LCB2 isogenes. We can assign the functional significance of the LCB2a gene in PCD elicitation to its higher transcription when compared with the LCB2b gene (Dietrich et al., 2008). Consequently, the lcb2a-1 mutant, which displays an FB1-resistant phenotype, maintains an SPT activity only through the formation of an LCB1/LCB2b dimer which, as a result of lower LCB2b expression, may lead to an inability to achieve high levels of LCBs. The majority of SPT complexes that generate LCBs in WT plants are composed of LCB1⁄LCB2a subunits, and therefore are committed to the increased formation of LCBs that mediate PCD, supporting the role of SPT as a PCD regulator in Arabidopsis. The nonfunctional redundancy of the Arabidopsis LCB2a⁄b subunits has been described recently in vertebrates, which contain an alternative SPT subunit (SPTCL3) analog to LCB2 that uses shorter acyl chain-CoA to synthesize C-16 sphinganine (Hornemann et al., 2009). The importance of the differential expression of SPT genes under stress conditions has recently been illustrated in the case of the LCB2 gene in Nicotiana benthamiana, whose overexpression is induced by the nonhost pathogen Pseudomonas cichorii, enhancing PCD (Takahashi et al., 2009).

In addition to the contribution of the LCB1/LCB2a–SPT complex to the high accumulation of d18:0 sphinganine, FB1 could be exerting a differential inhibition on the two proposed ceramide synthase (CS) activities: CSI (which uses d18:0 as substrate) would be sensitive to FB1, whereas...
CSII (which uses t18:0) (Chen et al., 2009) would show less FB1 inhibition. Therefore, the increase in d18:0 concentration, acting as a second messenger in the PCD route, is generated by a complex physiological regulation of different enzymatic reactions involving sphinganine metabolism.

**Role of d18:0** We postulate that d18:0 is the main LCB responsible for signaling in the LCB-induced PCD on the basis of three lines of evidence. First, the correlation between the high levels reached by this LCB in WT seedlings on FB1 treatment and the PCD phenotype, a correlation that is not achieved by the *lcb2a-1* mutant. Second, the addition of exogenous d18:0 to WT seedlings, which results in a clear PCD phenotype, is barely shown by t18:0. Third, the *Arabidopsis* double mutant *sbb1 sbb2*, which is defective in the two LCB hydroxylases and shows a remarkable increase in dihydroxylated LCBs, manifests necrotic spots and premature death (Chen et al., 2008), supporting the concept that the accumulation of sphinganine (d18:0) produces PCD. This interpretation is strengthened by a very recent study reporting that the mutant *sbb1-1*, which shows decreased levels of t18:0, but increased total LCB levels, fails to show susceptibility to infection by *Pseudomonas syringae* in an HR-PCD model (Peer et al., 2010). Moreover, these authors provide evidence suggesting that the sustained increase in t18:0 on avirulent bacterial infection originates from hydroxylase activity on the *de novo*-synthesized d18:0, as ceramide species seem unchanged. In this sense, our data confirm this view and indicate that the LCB species involved in FB1

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**Fig. 6** MPK6 operates downstream of long chain bases (LCBs) in the fumonisin B1 (FB1)-induced programmed cell death through an activation mainly mediated by d18:0. Wild-type (WT), *mpk3*, *mpk6*, *mkp1*, *mpk3 mkp1* and *mpk6 mkp1* mutant seedlings were exposed to exogenous d18:0 (5 μM), t18:0 (5 μM), d18:0-P (5 μM) or 0.04% Silwet (control) solutions under continuous light. Photographs were taken after 4 d of exposure. (See the Materials and Methods section for experimental details.)
PCD elicitation, and which evoke a defense reaction, are de novo synthesized, indicating that these signaling species are not derived from the degradation of complex lipids, as is the case for the classic signaling mediated by lyso-glycero-phospholipids (Wang, 2004). Endorsing our interpretation of d18:0 but not t18:0 as an operator in signaling towards PCD, we cite the work of Lachaud et al. (2010), in which the external administration of d18:0 to tobacco BY2 cells induced an increase in Ca^{2+} concentration in the cytosol, and later in the nucleus, which was essential for the expression of an apoptotic-like PCD. Despite the aforementioned arguments in favor of d18:0 as a main contributor to PCD elicitation, the observed high accumulation of t18:0 and the phosphorylated forms of d18:0 and t18:0 under FB1 exposure prompted us to explore their role in PCD unchaining. With regard to the participation of t18:0 in PCD, the LCB increases in WT FB1-treated seedlings were lower than those of d18:0-associated with PCD expression, weakening the possible cardinal role of t18:0 in PCD elicitation. In addition, we found that the sshb1-1 hydroxylase mutant (Chen et al., 2008) was able to express an FB1-induced PCD phenotype, indicating that t18:0 is not the leading intermediate of PCD. Supporting this interpretation, we must mention the results from Chen et al. (2008) with the Arabidopsis sshb1 sshb2 mutant, which is enriched in dihydroxylated LCB species, but contains no trihydroxylated LCBs, and nonetheless shows spontaneous PCD lesions, implying that high levels of trihydroxylated LCBs do not elicit PCD by themselves.

We cannot discount a role of d18:0 and t18:0 phosphorylated forms in PCD, as we determined that d18:0-P and t18:0-P increased in WT seedlings to very important levels after 72 h of FB1 exposure (c. 8000- and 1000-fold, with respect to the 0-h value, respectively). However, the external addition of d18:0-P showed meager PCD symptoms. It must be acknowledged that, in plants, most of the available evidence on LCBs as second messengers has been reported for the phosphorylated LCB forms. Stomatal closure under drought conditions is driven by the activity of ion channels in the plasma membranes of guard cells (Schroeder et al., 2001), and is induced by Ca^{2+} mobilization and elevation in d18:1-P levels (a desaturated form of d18:0) in Commelia communis (Ng et al., 2001). However, Michaelson et al. (2009) reported that the ablation of the Δ4-desaturase gene did not affect guard cell closure in Arabidopsis. Thus, t18:0-P could fulfill this role, as t18:0 is a substrate of sphingosine kinase (SphK) (Coursol et al., 2005; Worrall et al., 2008). This is an LCB kinase involved in abscisic acid (ABA) signaling of stomatal closure (Coursol et al., 2005; Worrall et al., 2008), which could generate d18:0-P in the case of BY2 cells (Lachaud et al., 2010) or t18:0-P in the case of guard cells (Coursol et al., 2005; Worrall et al., 2008), and could potentially trigger Ca^{2+} elevation. In animal cells, SphK2, a nuclear SphK, has a proapoptotic role, producing LCBs-P (Maceyka et al., 2005). The importance of the maintenance of low LCBs-P levels has been manifested by the FB1 hypersensitivity of the LCB lyase mutant in Arabidopsis (Tsegaye et al., 2007).

MPK6 as a transducer in PCD orchestrated by LCBs

In this work, we have demonstrated that MPK6 has a regulatory role in PCD induced by LCBs. We have provided four lines of experimental evidence to support this finding. First, our results with the mpk6 mutants showed that LCB-induced PCD requires the mediation of MPK6 downstream of LCB accumulation, as the elevation of endogenous LCBs induced by FB1 did not produce severe changes in the
It is noteworthy to point out that such rapid MPK6 activation in LCBs that may not be well detected by HPLC ESI-MS is very sensitive to small changes in the endogenous levels of LCB or FB1 addition suggests that this MAPK cascade is appealed to in cases of immunity-related PCD as previously reported (Ren et al., 2006), this functional redundancy of MPK6 and MPK3 does not apply to the route of PCD instrumented by LCB elevation, same that selectively requires MPK6, as derived from our experiments.

Another important result was that MPK6, but not MPK3, was essential to LCB-induced PCD. This finding is in contrast with multiple other cases in which both kinases, which are very close homologs, show high functional redundancy (Colcombet & Hirt, 2008). These kinases may be differentially expressed under circumstances that involve specific requirements, such as time of response, cellular type or abundance. For example, MPK3, but not MPK6, regulates either pathogen-induced or H$_2$O$_2$-induced stomatal closure (Gudesblat et al., 2007) and shows transcriptional regulation (Walley et al., 2007; Pitzschke et al., 2009). By contrast, MPK6, but not MPK3, is essential in gametogenesis and the development of reproductive organs, but no control of its gene expression has been found (Bush & Krysan, 2007). However, although activation of both MPK3 and MPK6 is involved in cases of immunity-related PCD as previously reported (Ren et al., 2006), this functional redundancy of MPK6 and MPK3 does not apply to the route of PCD instrumented by LCB elevation, same that selectively requires MPK6, as derived from our experiments.

With the exclusion of LCBs, MPK6 upstream elements in this PCD model are unknown. As many possible activators have been proposed, these may include transducers, such as proteins/kinases, carrying pleckstrin homology domains that can recognize FB1 or LCBs (Wang et al., 1991; Popescu et al., 2009). G proteins are among the possible initial signaling factors as, in rice, the Arabidopsis MPK6 ortholog that is induced by a sphingolipid elicitor (Bush et al., 2009). These rapid responses are common in signaling molecules, such as phosphatidic acid species, which increase within 10 min of ABA addition (Zhang et al., 2009).

The observed subcellular features were consistent with autophagic cell death, as reported for FB1-induced PCD in Arabidopsis, and which has been related to the activity of vacuolar processing enzymes (VPEs) (Kuroyanagi et al., 2005). It is possible that some of the effects of MPK6 are related to the activity of VPEs, as mutations in the four VPE genes preserve the membrane integrity of FB1-treated cells (Kuroyanagi et al., 2005).

Cell features of LCB-induced PCD

The features of cells undergoing PCD as a consequence of LCB accumulation are practically unknown. We observed DNA fragmentation and the loss of integrity of organelar membranes. The incorporation of LCB overload and the decrease in complex sphingolipids may account for membrane disruption; however, oxidative stress may also be responsible for some PCD-driven cell perturbations (Levine et al., 1994), as it has been reported that LCB-induced PCD is mediated by downstream H$_2$O$_2$ generation (Shi et al., 2007).

It has been reported that the overexpression of NtMEK$^{DD}$ (a MAPKK) in tobacco leads to a prolonged activation of SIPK, WIPK and Ntr6, inhibiting CO$_2$ fixation and generating excess energy excitation (Liu et al., 2007). Mutations in MPK3 and MPK6 seem to be important in starch metabolism, as mutants of these kinases showed chloroplasts with starch granules of large dimensions. As SIPK and WIPK are the tobacco orthologs of Arabidopsis MPK6 and MPK3, respectively, it is possible that a loss of function of these MPKs in Arabidopsis favors carbon fixation, increasing carbohydrate synthesis and starch deposition.

The observed subcellular features were consistent with autophagic cell death, as reported for FB1-induced PCD in Arabidopsis, and which has been related to the activity of VPEs (Kuroyanagi et al., 2005). It is possible that some of the effects of MPK6 are related to the activity of VPEs, as mutations in the four VPE genes preserve the membrane integrity of FB1-treated cells (Kuroyanagi et al., 2005).
The absence of PCD observed in the mpk6 mutant reveals that LCB accumulation per se is not sufficient to promote the breakdown of DNA and cell membranes, as MPK6 seems to be required. A feasible explanation for this finding is that MPK6 is responsible for reactive oxygen species generation at levels leading to PCD. This is consistent with the available evidence that associates MPK6, reactive oxygen species formation and PCD (Liu et al., 2007).

General model

LCB-induced PCD in Arabidopsis seedlings results from three factors: the activity of the LCB1/LCB2a–SPT complex, the increase in sphinganine (d18:0) and the activation of MPK6 (Fig. 8). Sphinganine accumulation is favored by the efficient de novo synthesis of its precursor by the LCB1/LCB2a–SPT complex and by the predominant inhibition of FB1 over CSI. The elevation of LCBs as a second messenger targets a transducer element that leads to downstream MAPK activation through MPK6, amplifying death signals. An additional effect of FB1 on the same transducer element reached by d18:0 cannot be excluded, because of the structural similarities between the two molecules (as shown for CS that recognizes both FB1 and dihydrosphinganine; see Wang et al., 1991). The participation of enzymes that use d18:0 or its derivatives as substrates is essential for a tight and differential regulation of LCB levels. The model includes the role of Ca2+ as an inducing PCD factor in the signaling pathway unchained by LCB elevation (Lachaud et al., 2010) and SA generation downstream of LCB signaling (Fig. 8).

A considerable body of evidence has confirmed the participation of MPK6 in HR-PCD. More recent experimentation has associated sphingolipids with PCD signaling. In the present work, our results indicate that both MAP cascades and sphingolipids are transducers in the same PCD pathway, and that this operates as one of the strategies used as defense against pathogens (HR-PCD). In addition, evidence has been provided linking the participation of specific LCBs, SPT complexes and MPK forms in LCB-induced PCD, contributing to the deciphering of cell death transduction pathways in plants.

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References


**Supporting Information**

Additional supporting information may be found in the online version of this article.

**Fig. S1** Modification of long chain bases during *de novo* synthesis of sphingolipids in plants.

**Fig. S2** Programmed cell death promoted by fumonisin B1 (FB1) is already observed at 36 h.

**Fig. S3** Accumulation kinetics of the less abundant free long chain bases (LCBs) and phosphorylated LCBs (LCBs-P) in wild-type (WT) and lcb2a-1 seedlings exposed to fumonisin B1 (FB1).

**Table S1** Accumulation kinetics of free long chain bases and phosphorylated long chain bases in wild-type (WT) and lcb2a-1 mutant seedlings treated or not with fumonisin B1 (FB1).

**Methods S1** Plant material and growth conditions, isolation of T-DNA insertion lines and construction of mutant lines, detection of nuclear DNA fragmentation, ultrastructural analysis, protein extraction and kinase assays, and immunoblots of MPK6 and MPK3.

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