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The grapevine (*Vitis vinifera*) LysM receptor kinases VvLYK1-1 and VvLYK1-2 mediate chitooligosaccharide-triggered immunity

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Summary

Chitin, a major component of fungal cell walls, is a well-known pathogen-associated molecular pattern (PAMP) that triggers defense responses in several mammal and plant species. Here we show that two chitooligosaccharides, chitin and chitosan, act as PAMPs in grapevine (*Vitis vinifera*) as they elicit immune signaling events, defense gene expression, and resistance against fungal diseases. To identify their cognate receptors, the grapevine family of LysM receptor kinases (LysM-RKs) was annotated and their gene expression profiles characterized. Phylogenetic analysis clearly distinguished three *V. vinifera* LysM-RKs (VvLYKs) located in the same clade as the *Arabidopsis* CHITIN ELICITOR RECEPTOR KINASE1 (AtCERK1), which mediates chitin-induced immune responses. The *Arabidopsis* mutant *Atcerk1*, impaired in chitin perception, was transformed with these three putative orthologous genes encoding VvLYK1-1, -2 or -3 to determine if they would complement the loss of AtCERK1 function. Our results provide evidence that VvLYK1-1 and VvLYK1-2, but not VvLYK1-3, functionally complement the *Atcerk1* mutant by restoring chitooligosaccharide-induced MAPK activation and immune gene expression. Moreover, expression of *VvLYK1-1* in *Atcerk1* restored penetration resistance to the non-adapted grapevine powdery mildew (*Erysiphe necator*). On the whole, our results indicate that the grapevine VvLYK1-1 and VvLYK1-2 participate in chitin- and chitosan-triggered immunity and that VvLYK1-1 plays an important role in basal resistance against *E. necator*.

Introduction

Plants are constantly exposed to potentially pathogenic microbes such as bacteria, fungi, oomycetes or viruses. However, plants have developed effective immune systems triggering various defense reactions against invading pathogens upon the perception of pathogen-associated molecular patterns (PAMPs; Dodds and Rathjen, 2010). The recognition of these conserved microbial signatures is mediated by pattern recognition receptors (PRRs), which also detect plant endogenous molecules released by hydrolytic enzymes during interaction with the pathogen, and called damage-associated molecular patterns (DAMPs; Boller and Felix, 2009; Boutrot and Zipfel, 2017). PRRs have a characteristic structure defined by the presence of a ligand-binding ectodomain, a single transmembrane domain and, for some of them, an intracellular kinase domain. The structure of the ectodomain determines binding

specificity: PRRs containing a leucine-rich repeat ectodomain mostly bind peptides, such as flagellin or elongation factor Tu (EF-Tu) from bacteria, whilst lysine motif (LysM)-containing PRRs preferentially bind carbohydrates such as chitin or peptidoglycans from fungi and bacteria, respectively (Boutrot and Zipfel, 2017; Trdá et al., 2015). PAMP perception by PRRs leads to PAMP-triggered immunity (PTI), which is characterized by a wide range of defense responses including the production of reactive oxygen species (ROS), calcium influx, mitogen- activated protein kinase (MAPK) phosphorylation and expression of defense-related genes (Yu et al., 2017).

Several distinct microbial patterns are composed from *N*-acetylglucosamine (GlcNAc) residues, including fungal chitin or bacterial peptidoglycan (PGN) present in microbial cell walls (Gust et al., 2012). Chitin, and its derivatives, are representative PAMPs from fungal cell walls known to induce immune responses in both monocots and dicots, indicating the presence of a conserved mechanism to perceive these chitooligosaccharides in a wide range of plant species (Shinya et al., 2015). In plants, chitin elicits a variety of defense responses including the activation of the phenylpropanoid pathway and production of pathogenesis-related (PR) proteins such as peroxidases, chitinases or thaumatin-like proteins (Boller and Felix, 2009; Kaku et al., 2006; Miya et al., 2007). Chitosan, a deacetylated derivative of chitin, is also a potent elicitor of plant immunity (Aziz et al., 2006; Povero et al., 2011). In grapevine, chitosan elicits phytoalexin production, chitinase and glucanase activities leading to resistance against *Botrytis cinerea* and *Plasmopara viticola*, the causal agents of grey mould and downy mildew, respectively (Aziz et al., 2006).

The mechanism of chitin perception and signaling in plant cells was first characterized in rice with the identification of the chitin-elicitor binding protein, CEBiP (Kaku et al., 2006), which contains three extracellular LysM motifs and is anchored to the plasma membrane via a glycosylphosphatidylinositol (GPI)-anchor (Gong et al., 2017). Chitin perception in rice triggers the formation of a heterodimer complex between OsCEBiP and OsCERK1, a protein which contains an intracellular kinase domain required for signal transduction. Thus, two LysM proteins are required for chitin perception and signaling in rice (Hayafune et al., 2014; Shimizu et al., 2010). In *Arabidopsis thaliana*, AtCERK1/LYK1, a homolog of OsCERK1, has been shown to play a crucial role in both chitin signaling (Miya et al., 2007; Wan et al., 2008) and bacterial PGN perception (Gimenez-Ibanez et al., 2009; Willmann et al., 2011). Homodimers of AtCERK1/LYK1 were shown to directly bind long chain chitin oligomers (Liu et al., 2012). However, more recent data suggest that other members of the LysM-RK

gene family in *Arabidopsis* may also be involved in chitin perception. For example, Cao et al (2014) proposed that AtLYK5 (and/or AtLYK4), which have inactive kinase domains, may be the primary receptors for chitin, and that chitin perception may result in the formation of an AtLYK5-AtLYK1 heterotetramer, triggering intracellular signal transduction.

The majority of commercially grown grapevine cultivars are derived from the species *Vitis vinifera*, which is highly susceptible to cryptogamic diseases, such as downy mildew (*Plasmopara viticola*), grey mould (*Botrytis cinerea*) and powdery mildew (*Erysiphe necator*). These two last pathogens are ascomycete fungi containing chitooligosaccharides in their cell walls. These diseases cause significant losses to viticultural production and control of these pathogens is heavily dependent on frequent fungicide application. The level of fungicide application has serious economic, environmental and potential health implications and has driven research efforts into alternative strategies (Trouvelot et al., 2014; Walters et al., 2013). Among them is the generation of new resistant varieties by introgression of downy and powdery mildew resistance (*R*) genes from wild North American grapevine species (Qiu et al., 2015). However, whilst *R*-gene triggered resistance is very effective at controlling pathogens, widespread use of *R*-genes may impose a selection pressure on parasites to evolve and evade R protein recognition, thereby compromising the durability of this control strategy (Jones and Dangl, 2006). Thus, characterization of new PRRs in a given plant species by identifying their cognate PAMPs and understanding their involvement in disease resistance may provide more durable and broad-spectrum immunity (Piquerez et al., 2014), notably by promoting a PTI-based crop protection (Boutrot and Zipfel, 2017; Wiesel et al., 2014).

In this study, we have investigated whether two chitooligosaccharides, chitin and chitosan, are active PAMPs in grapevine. We also report on the functional characterization of members of the *VvLYK* gene family, with particular focus on three orthologs of *AtCERK1/LYK1* and *OsCERK1*, designated *VvLYK1-1*, *VvLYK1-2* and *VvLYK1-3*. By functional complementation of the *Arabidopsis Atcerk1* mutant, we demonstrate that *VvLYK1-1* and *VvLYK1-2* are involved in the chitooligosaccharide-induced immune responses in *V. vinifera*. Moreover, *VvLYK1-1* was demonstrated to confer basal resistance against the grapevine powdery mildew *E. necator* when expressed in *A. thaliana*.

Results

Chitooligosaccharides trigger immune responses and induced resistance in grapevine

Chitooligosaccharides with a degree of polymerization (DP) ranging from 6 to 8 (hexamer to octamer) are the most effective at triggering ROS production and defense gene expression in rice and *Arabidopsis*, respectively (Miya et al., 2007; Petutschnig et al., 2010). In grapevine, chito-oligosaccharides with a MW of 1500 (*i.e.* DP6) were shown to be the most effective at triggering phytoalexin production and expression of chitinase and glucanase, compared to chitooligosaccharides with a MW of 3000 and 10,000 (*i.e.* DP13-45) (Aziz et al., 2006). In this study, chitooligomers with a DP of 6 were used to test if their perception by grapevine triggers immune responses similar to that commonly observed in *Arabidopsis* or rice. To also investigate the importance of the degree of acetylation (DA), the early signaling events and defense gene expression induced by chitin hexamer (DA 99.9% and DP 6) or deacetylated chitosan hexamer (DA 0.1% and DP 6) were characterized in *V. vinifera* cell suspensions.

Contrary to what has been previously observed in *Arabidopsis* (Albert et al., 2006; Miya et al., 2007), chitin DP6 did not induce any oxidative burst in grapevine cells (Fig. S1) whereas flg22 triggered the expected positive response (Trdá et al., 2014). Similarly, the fully deacetyled chitosan DP6 did not elicit any H_2O_2 production in grapevine cell suspension (Fig. S1).

However, chitin DP6 induced a rapid and transient phosphorylation of two MAPKs with relative molecular masses of 45 and 49 kDa, which was not observed in water-treated control cells (Fig. 1a). Interestingly, chitosan DP6 also activated the phosphorylation of these two MAPKs but for a longer period (Fig. 1a). In parallel, treatment of grapevine cells with unpurified crab shell chitin NA-COS-Y, previously used to elicit ROS production and defense gene expression in *Brassica* species (Lloyd et al., 2014), was also shown to activate these two MAPKs (Fig. S2).

In response to chitooligosaccharide treatment, the expression of defense genes known to be induced by different PAMPs in grapevine (Aziz et al., 2003; Dubreuil-Maurizi et al., 2010; Poinssot et al., 2003; Trdá et al., 2014) was examined by qPCR. One hour post-treatment (hpt), both chitin DP6 and chitosan DP6 markedly induced the expression of four selected grapevine defense genes (Fig. 1b) encoding an acidic chitinase (*CHIT4C*), a stilbene

synthase (*STS*), a phenylalanine ammonia lyase (*PAL*) and a respiratory burst oxidase homolog D (*RBOHD*).

To further characterize the immune responses triggered by chitooligosaccharides, we also investigated the efficacy of chitin- and chitosan-induced resistance in grapevine. Leaf discs were treated with chitin DP6 and chitosan DP6 for 48 h prior to inoculation with either the necrotrophic fungus *B. cinerea* or with the biotrophic oomycete *P. viticola*. Chitin treatment induced a low but significant resistance against these pathogens (Fig. 1c, d), whilst chitosan treatment significantly reduced *B. cinerea* lesion diameter and *P. viticola* sporulation (Fig. 1c, d). Indeed, the reduced susceptibility to *P. viticola* infection, triggered by chitosan, was comparable to that obtained by pretreatment with the β-1,3-glucan sulfated laminarin (PS3), a potent resistance inducer in grapevine (Gauthier et al., 2014).

Phylogenetic analysis and characterization of grapevine LysM-RKs (VvLYKs)

The results of Fig. 1 demonstrate that grapevine cells are capable of detecting chitooligosaccharides, suggesting the presence of a perception system. To identify the CERK1/LYK1 ortholog(s) in grapevine, genes encoding LysM-RKs were identified from the reference genome of *Vitis vinifera* cv Pinot Noir PN40024 (Jaillon et al., 2007). A previous annotation of VvLYK family based on the 8x grapevine genome predicted 12 gene family members (Zhang et al., 2009). However, our re-annotation of the *VvLYK* gene family, based on the most recent version of the 12x genome, predicts the presence of 15 putative genes encoding VvLYK proteins in the *V. vinifera* genome (Table S1). A maximum-likelihood phylogenetic tree indicated that out of these 15 LysM-RKs, 3 grapevine proteins are located in the same clade as the *Arabidopsis* AtCERK1/LYK1 and the rice ortholog OsCERK1 (Fig. 2a), proteins that have been shown to be involved in chitin perception/signaling. These proteins, designated as VvLYK1-1, VvLYK1-2 and VvLYK1-3, share 60%, 57% and 56% amino acid identity with AtCERK1/LYK1, respectively (Table S2). VvLYK1-1 and VvLYK1-2 also show the highest percentage of amino acid identity with the rice chitin co-receptor OsCERK1 (Table S2).

The expression profile of each putative *VvLYK* gene was analyzed using RNA-Seq and microarray data obtained from time course infection experiments of leaves and berries with *E. necator* and *B. cinerea* (Kelloniemi et al., 2015), respectively. In response to inoculation with the fungal pathogen *E. necator*, only *VvLYK1-1* and *VvLYK1-3* were clearly up-regulated across the entire 24h period, while *VvLYK1-2* and *VvLYK6* were transiently

induced at 6 hpi (Fig. 2b). During *B. cinerea* infection, in the clade of *VvLYK1s* only *VvLYK1-3* was slightly induced in ripe susceptible berries (Fig. 2b). Interestingly, *VvLYK4-1/2* (detected using the same Nimblegen probe), *VvLYK5-1* and *VvLYK6* were strongly up-regulated in berries during infection by *B. cinerea* (Fig. 2b). *VvLYK2* expression is also much higher in ripe berries than green berries, suggesting that it could have an as yet unknown function during grape berry ripening. *VvLYK3-1* seemed to be repressed during the infection by both pathogens. Of note, we found *VvLYK3-2*, *VvLYK3-3*, *VvLYK7*, *VvLYK8* and *VvLYK9* to only be expressed at very low levels or were undetectable in the tissues examined (Fig. 2b). However, we cannot rule out the possibility that these genes are expressed at detectable levels in other tissues, such as roots or flowers, or in response to other biotic stresses.

As AtCERK1 and OsCERK1 are key components that mediate chitin-triggered signaling in *Arabidopsis* (Miya et al., 2007; Wan et al., 2008) and rice (Hayafune et al., 2014; Shimizu et al., 2010), we undertook further analysis of the three putative grapevine orthologs *VvLYK1-1*, *VvLYK1-2* and *VvLYK1-3*. Sequencing of the cloned full-length coding sequences (CDS) from *V. vinifera* cv Cabernet Sauvignon revealed that genes *VvLYK1-1*, *-2* or *-3* consist of open-reading frames of 1845, 1878 and 1866 bp, respectively (Table S1). All three VvLYK1 proteins contain a similar domain structure with a signal peptide, three extracellular LysM motifs, a single transmembrane domain and a RD-type intracellular kinase domain (Fig. 2c and Fig. S3). Interestingly, the amino acids E110 and E114, shown to be involved in the binding of the N-acetyl moieties of $(GlcNAc)_5$ in AtCERK1/LYK1 (Liu et al., 2012) are mutated in the 3 VvLYK1 proteins (Fig. S3). All three VvLYK1 protein sequences share a high degree of identity (Fig. S3) and the kinase domains of VvLYK1-1 and VvLYK1-2 possess the highest identity with the kinase domains of AtCERK1/LYK1 and OsCERK1 (Fig. S3, Table S2).

All three VvLYK1 proteins have a predicted N-terminal signal peptide (Fig. S3). Confocal analysis of the *Atcerk1* mutant expressing a *VvLYK1-1-GFP* fusion expression construct showed a GFP signal co-localized with the red fluorescence of the plasma membrane-specific probe FM4-64 (Brandizzi et al., 2004) (Fig. 2d). Furthermore, when plasmolysis was triggered by the addition of 1M NaCl, VvLYK1-1-GFP fluorescence followed the movement of the plasma membrane away from the plant cell wall (Fig. 2d). Both observations are consistent with VvLYK1-1 being localized to the plasma membrane.

VvLYK1-1 restores chitin-induced MAPK activation and *FRK1* expression in the *Atcerk1* mutant

To investigate whether VvLYK1-1, VvLYK1-2 or VvLYK1-3 are capable of activating chitooligosaccharide-triggered defenses, expression constructs comprising each native *VvLYK1* coding sequence (i.e. no C-terminal tag) under the control of a constitutive *35S* promoter were introduced into the *Atcerk1* mutant. Semi-quantitative PCR was performed on the leaves of T2 transgenic lines to test for the presence of the *VvLYK1-1*, *VvLYK1-2* or *VvLYK1-3* transcripts. Six transgenic lines were positively identified as expressing the *VvLYK1-1* transgene and five transgenic lines were identified for *VvLYK1-3* (Fig. 3a). However, analysis of five independent transgenic lines, confirmed to contain the *VvLYK1-2* construct by genomic PCR, indicated that transgene expression was either undetectable (lines #2, #12, #14) or at very low levels (lines #5 and #10) compared to *VvLYK1-1* and *VvLYK1-3* transgene expression (Fig. 3a). The failure to positively identify lines highly expressing *VvLYK1-2* suggested that this gene is potentially lethal when expressed under a strong constitutive *35S* promoter. This was confirmed by agro-infiltration of the *p35S::VvLYK1-2* construct into *N. benthamiana* leaves resulting in patchy necrosis after 48 h compared to leaf segments infiltrated with *Agrobacterium* alone (Fig. S4). As *VvLYK1-2* induces necrosis when over-expressed, this suggests that it may have a crucial function in defense and its expression needs to be tightly regulated *in planta*. Based on these results, *Arabidopsis* lines transformed with the *p35S::VvLYK1-2* construct were excluded from complementation analysis using a constitutive expression system.

Transgenic *Atcerk1/p35S::VvLYK1-1* and *Atcerk1/p35S::VvLYK1-3* lines were first examined for restoration of early chitin-induced events by analysing the phosphorylation of MAPKs in two independent lines following treatment with chitin (NA-COS-Y; Lloyd et al., 2014) for 10 min prior to protein extraction. Figure 3b shows that chitin treatment triggered the phosphorylation of two MAPKs, with relative molecular weights of 43 and 47 kDa, in WT Col-0 seedlings but no MAPK phosphorylation was observed in *Atcerk1*, in agreement with the previous report of Miya et al. (2007). Chitin-induced MAPK activation was restored in the two independent *p35S::VvLYK1-1* lines *#2* and *#5* (Fig. 3b) but no MAPK phosphorylation was detected in protein samples extracted from the two independent *p35S::VvLYK1-3* lines #1 and #2 (Fig. 3b).

The expression of the defense gene encoding flagellin-induced receptor kinase 1 (*FRK1)* was also investigated 2 h after chitin treatment. Chitin induced a high level of expression of *FRK1* in WT Col-0 that was totally suppressed in the *Atcerk1* mutant (Fig. 3c). *FRK1* expression was partly restored in the two *p35S::VvLYK1-1* lines *#2* and *#5*, but remained close to the basal level in the two *p35S::VvLYK1-3* lines *#1 and #2* (Fig. 3c). Taken together, these results indicate that over-expression of *VvLYK1-1* can restore chitin-triggered immune responses in *Atcerk1* but *VvLYK1-3* cannot.

VvLYK1-1 expression restores penetration resistance in *Atcerk1* against the non-adapted powdery mildew *Erysiphe necator*

In addition to testing for complementation of MAPK activation and defense gene expression, the ability of *VvLYK1-1* and *VvLYK1-3* to restore resistance against a non-adapted grapevine powdery mildew pathogen in the *Atcerk1* mutant was also determined. *Arabidopsis thaliana* is a non-host for the fungus *E. necator*. Although a proportion of *E. necator* spores placed onto a Col-0 leaf will successfully penetrate the epidermal cell wall and form a haustorium under the first appressorium, the pathogen is unable to complete its life cycle on this host (Feechan et al., 2013).

Fig. 4a shows that the *Atcerk1* mutant is significantly more susceptible to penetration by *E. necator* than the WT Col-0, showing the important role of AtCERK1 in non-host resistance against non-adapted powdery mildew species. More precisely, the penetration rate of WT Col-0 leaves by *E. necator* spores ranged from 35-43 % with a mean at 39 % whereas in the *Atcerk1* mutant, the penetration rates ranged from 76-88% with a mean of 82% which approaches the rate of penetration by the adapted powdery mildew species *E. cichoracearum* on Col-0 (Feechan et al., 2013). Constitutive expression of *VvLYK1-1* in the *Atcerk1* mutant significantly reduced the mean penetration rates of *E. necator* in the leaves of all *Atcerk1/p35S::VvLYK1-1* lines to levels comparable to the penetration rates on WT Col-0 plants (31 to 40%; Fig. 4a). As additional negative controls, T2 lines #3 and #4 that had been generated through the same transformation procedure but had lost the introduced *VvLYK1-1* transgene through segregation, showed mean penetration rates of *E. necator* similar to *Atcerk1* (77 to 80%; Fig. 4a). This demonstrates that the complementation of penetration resistance in the *Atcerk1/p35S::VvLYK1-1* lines is a result of *VvLYK1-1* expression and is not related to the transformation process. In contrast, expression of *VvLYK1-3* in the five

independent *Atcerk1/VvLYK1-3* lines did not significantly reduce *E. necator* mean penetration rates (58 to 72%; Fig. 4b) in comparison to the *Atcerk1* mutant or the negative T2 control lines (#9 and #10; Fig. 4b).

Together, the ability of VvLYK1-1 to restore MAPK activation, the expression of *FRK1* and penetration resistance against *E. necator* in the *Atcerk1* mutant background suggests that VvLYK1-1 mediates chitin sensing and might be important for grapevine defense against *E. necator*.

The inducible expression of *VvLYK1-2* also restores chitin-triggered responses in the *Atcerk1* mutant

Due to the toxicity of constitutively expressed *VvLYK1-2*, new constructs were generated in which *VvLYK1-2* expression is driven by an inducible promoter. The pABindGFP vector (Bleckmann et al., 2010) permitted the inducible expression of a C-terminally tagged VvLYK1-2-GFP fusion protein regulated by the β-estradiol *LexA* promoter in the *Atcerk1* mutant background. Two independent hygromycin-resistant T3 lines *Atcerk1/LexA::VvLYK1-2-GFP* #27 and #28 were selected to be homozygous and containing only one copy of the transgene.

Following β-estradiol treatment, confocal microscopy confirmed the presence of the VvLYK1-2-GFP protein at the cell periphery (Fig. 5a) suggesting a localization at the plasma membrane similar to VvLYK1-1 (Fig. 2d).

To investigate whether VvLYK1-2 can also restore chitin-induced signaling and immune responses in the *Atcerk1* mutant, MAPK activation and defense gene expression were analyzed. Fig. 5b shows that β-estradiol pre-treatment alone did not induce MAPK phosphorylation in the WT Col-0 or in the *Atcerk1* mutant. However, β-estradiol pre-treatment followed by a chitin treatment lead to the restoration of MAPK phosphorylation in the two independent *Atcerk1*/*LexA::VvLYK1-2-GFP* lines #27 and #28 (Fig. 5b). Similarly, the chitin-induced expression of the defense gene *FRK1* was also restored in both lines *Atcerk1/pLexA::VvLYK1-2-GFP* #27 and #28 (Fig. 5c). These data indicate that VvLYK1-2, like VvLYK1-1, also restores MAPK activation and immune gene expression in the *Atcerk1* mutant. Unfortunately, the use of this transient β-estradiol-inducible expression system did not permit us to obtain reproducible results concerning the putative role of VvLYK1-2 in the resistance against *E. necator*.

VvLYK1-1 and VvLYK1-2 expression restore chitosan-triggered responses in the *Atcerk1* mutant

To further characterize these new grapevine PRRs, we also tested the responses triggered by chitosan in *Atcerk1/VvLYK1* transgenic lines (Fig. 6). Like chitin (Fig. 3), chitosan was able to strongly induce the phosphorylation of MAPKs in WT Col-0 and this signaling pathway was highly compromised in the *Atcerk1* mutant (Fig. 6a). Expression of *VvLYK1-1* in the *Atcerk1* mutant also restored chitosan-induced MAPK activation but *VvLYK1-3* did not (Fig. 6a). Similarly, the chitosan-induced expression of the defense gene *FRK1* was also restored at the WT or higher level in both lines *Atcerk1/p35S::VvLYK1-1* #2 and #5 whereas the *FRK1* transcript level in lines *Atcerk1/p35S::VvLYK1-3* #1 and #2 was comparable to the one in *Atcerk1* (Fig. 6b). Of note, the *FRK1* expression level in *Atcerk1* treated by chitosan is significantly higher than in water condition (Fig. 6b). Chitosan-induced phosphorylation of MAPKs (Fig. 6c) and *FRK1* defense gene expression (Fig. 6d) were also complemented in the two independent lines *Atcerk1/pLexA::VvLYK1-2-GFP* #27 and #28. Thus VvLYK1-1 and VvLYK1-2 also restore chitosan-triggered responses in *Atcerk1*.

Discussion

Chitin is a well-known PAMP which elicits typical immune responses in *Arabidopsis* (Cao et al., 2014; Miya et al., 2007; Petutschnig et al., 2010; Wan et al., 2008) and a number of other plant species (Akamatsu et al., 2013; Ao et al., 2014; Felix et al., 1998; Hayafune et al., 2014; Kaku et al., 2006; Liu et al., 2016; Shimizu et al., 2010; Zeng et al., 2012). However, little is known about chitin perception in grapevine. Here we clearly demonstrate that chitin and chitosan, its deacetylated derivative, trigger grapevine immune responses such as phosphorylation of MAPKs and the expression of defense genes including *CHIT4C*, *STS*, *PAL* and *RBOHD*. Up-regulation of genes encoding chitinases and PAL was also observed in *Arabidopsis* and rice upon chitin treatment (Kaku et al., 2006; Miya et al., 2007). Surprisingly, these chitooligosaccharides did not induce any detectable H_2O_2 production in grapevine, in contrast to *Arabidopsis* (Miya et al., 2007) and rice (Hayafune et al., 2014). However, this lack of H_2O_2 production clearly does not prevent the phosphorylation of MAPKs showing independence between these two pathways, in accordance with results previously obtained in *N. benthamiana* and Arabidopsis (Segonzac et al., 2011; Xu et al., 2014).

We also show that chitin enhances the resistance of grapevine leaves to the necrotrophic fungus *B. cinerea* and the obligate biotrophic oomycete *P. viticola* (Fig. 1), as previously demonstrated following treatment with chitosan, flg22 or sulfated β-1,3-glucan (Aziz et al., 2006; Gauthier et al., 2014; Trdá et al., 2014). Similarly, treatment of rice plants with chitin reduced the susceptibility to the fungal pathogen *Magnaporthe oryzae* (Tanabe et al., 2006). More recently, chitin treatment was also shown to reduce the susceptibility of *Arabidopsis* to the bacterial pathogen *Pseudomonas syringae* pv tomato (*Pto*) DC3000 and the fungus *Alternaria brassicicola* (Cao et al., 2014). All of these results confirm that stimulation of plant immune responses with PAMPs can trigger enhanced resistance against different plant pathogens.

The degree of acetylation (DA) of chitooligosaccharides appeared to have no effect on the amplitude of the immune responses in grapevine although the duration of the chitosan-triggered MAPK activation was longer (Fig. 1). Similarly, in *Arabidopsis*, the DA of chitooligosaccharides had no effect on the activation of PAL (Cabrera et al., 2006). In contrast, in wheat, chitosan oligomers with a DA of 50% were better able to induce PAL activity than those possessing a DA of 0% (Vander et al., 1998). Thus, the structure/activity of chitooligomers might differ depending on the plant species used (Yin et al., 2016).

The activation of MAPKs and defense gene expression in grapevine cells treated with chitin demonstrates that grapevine possesses the cognate PRRs. Zhang et al. (2009) previously proposed the grapevine LYK family to be comprised of 12 members. We undertook a re-examination of the predicted *LYK* gene family in grapevine in combination with published EST data and our own RNA-Seq data. This revealed a number of errors in the original Zhang et al. (2009) predictions both in terms of the predicted ORFs and gene number. For example, the previously annotated single *VvLYK10* gene (Zhang et al., 2009) was found to contain a tandemly arrayed *LYK* gene pair. The two ORFs encode proteins with a 74% amino acid similarity to each other and homology to AtLYK5 (49% and 51% amino acid similarity). Tandem *LYK* gene pairs have also been identified in legume and poplar plants (Zhang et al., 2009). We are therefore proposing a new annotation scheme for the grapevine *VvLYK* gene family which uses a naming convention based on sequence similarity to *Arabidopsis LYK* gene family (Fig. 2a, Table S1).

In *Arabidopsis*, AtCERK1/LYK1 has been demonstrated to play a key role in chitin-induced signaling. Grapevine encodes three putative orthologs of AtCERK1/LYK1, designated VvLYK1-1, VvLYK1-2 and VvLYK1-3. Our data demonstrates that the constitutive expression of *VvLYK1-1* or the inducible expression of *VvLYK1-2* in the *Atcerk1*

mutant restores chitooligosaccharide-induced immune responses such as MAPK activation and expression of the defense gene *FRK1*. Thus our results demonstrate that these two independent grapevine proteins are functional orthologs of AtCERK1/LYK1, suggesting duplication events during the evolution of the ancestral genome of *V. vinifera* (Jaillon et al., 2007). VvLYK1-1 and/or VvLYK1-2 also restore chitosan perception by the *Atcerk1* mutant, as indicated by MAPK activation, suggesting that in grapevine the same PRRs can mediate both chitin and chitosan signaling. Similarly, an AtCERK1 protein band shift was detected in Arabidopsis after treatment with chitin or chitosan and the ectodomain of AtCERK1 has been shown to bind chitosan DP6 (Petutschnig et al., 2010). However, the fact that ROS production in Arabidopsis is induced by chitin DP6 but not by the fully deacetylated chitosan DP6 (Fig. S1) confirmed previous results of Gubaeva (2017) indicating that some downstream signaling events may be divergent. The fact that a weak signal for MAPKs activation and a significant *FRK1* transcript accumulation are observed in the *Atcerk1* mutant following chitosan treatment (Fig. 6) is considered together with previous results demonstrating AtCERK1/LYK1-independent defense gene expression (Povero et al., 2011), suggests that different AtLYK proteins may be involved in detecting different chitooligosaccharides. A preliminary investigation of the response of five different *Atlyk* mutants to chitosan DP6 treatment shows that MAPK activation is weaker in the *Atlyk5* and *Atlyk3* mutants compared to WT Col-0 (Fig. S5) suggesting that the AtLYK3 and AtLYK5 proteins might also participate in the perception of chitosan oligomers in combination with AtCERK1/LYK1.

Interestingly, we were unable to obtain *Arabidopsis* lines with high levels of constitutively expressed *VvLYK1-2*. Furthermore, we observed an induction of cell death following transient expression of *VvLYK1-2* in tobacco (Fig. S4), confirming gene toxicity. Cell death in response to heterologous LysM-RK expression in *N. benthamiana* has previously been observed when *AtCERK1* was fused with the yellow fluorescent protein variant, sYFP2 and transiently expressed under the control of a *35S* promoter (Pietraszewska-Bogiel et al., 2013), demonstrating the importance of regulating *LYK* expression levels.

In order to confirm the results obtained from complementation studies in Arabidopsis, we also attempted to confirm the function of *VvLYK1-1* and *VvLYK1-2* in chitin and chitosan perception in grapevine by generating grapevine transgenics in which these genes had been silenced. However, no transformed calli were recovered in three independent agrobacterium-mediated transformations of somatic grapevine embryos with *p35S::antisense-VvLYK1-1* and

p35S::antisense-VvLYK1-2 constructs whereas parallel control transformations with a *p35S::GFP construct* were successful (data not shown).

VvLYK1-1 expression in the *Atcerk1* mutant background was demonstrated to restore non-host resistance against grapevine powdery mildew suggesting that VvLYK1-1 may participate in anti-fungal basal resistance also in grapevine. As the *Atcerk1* mutant is more susceptible to the non-adapted pathogen *E. necator*, it also indicates that AtCERK1/LYK1 plays a role in the non-host resistance against this grapevine pathogen. Paparella *et al.* (2014) previously showed that an *Atlyk3-1* mutant was more resistant to *B. cinerea* suggesting that AtLYK3 negatively regulates certain immune responses such as the production of phytoalexins, suggesting that different members of the AtLYK gene family may play a role in the basal resistance of *Arabidopsis* against different fungal pathogens. It is interesting to note that *VvLYK4-1/2*, *VvLYK5-1* and *VvLYK6* genes are highly up-regulated during *B. cinerea* infection of grapevine berries (Fig. 2). Thus, it is plausible that other members of the large VvLYKs family may exhibit specificity to the different ligands released during the interactions of grapevine with this kind of pathogen.

Cao et al. (2014) recently showed that AtLYK5 is able to bind chitin at a greater affinity than AtCERK1 and that chitin perception leads to the formation of an AtCERK1-AtLYK5 dimer which is required for AtCERK1 phosphorylation. These observations led them to propose that AtLYK5, and not AtCERK1, is the primary receptor for chitin perception and which has been proposed to be responsible for the activation of defense responses (Cao et al., 2014). Thus, one explanation of our complementation data is that VvLYK1-1 or VvLYK1-2 could dimerize with AtLYK5 in the presence of chitin, but that the interaction is not sufficiently effective to obtain a full restoration of MAPK activation and defense gene expression back to wild-type levels. This suggests the existence of molecular complexes for chitooligosaccharides perception in grapevine, as previously shown for rice (Hayafune et al., 2014) and *Arabidopsis* (Cao et al., 2014).

In summary, we present a re-annotation of the *VvLYK* gene family and demonstrate that two *AtCERK1/LYK1* orthologs, *VvLYK1-1* and *VvLYK1-2*, are involved in chitooligosaccharide signaling. Elucidating components of PAMP-triggered immunity in grapevine opens the possibility of developing grapevine varieties with durable resistance against fungal pathogens. *E. necator* has adapted to successfully infect grapevine by evolving host-specific effector proteins that target and re-programme the signalling pathways that lead to PAMP-triggered immunity. The introduction of PRRs from a closely related species that can function in *V. vinifera* but are not modulated by *E. necator*'s specific effector suite has

the potential to restore PAMP-triggered immunity against this adapted pathogen (Heath, 2000; Lee et al., 2016). The proof of concept for this approach was demonstrated by the expression of the *Arabidopsis* PRR EFR in *N. benthamiana*, tomato, rice and wheat plants which conferred greater resistance against a range of phytopathogenic bacteria (Lacombe et al., 2010; Lu et al., 2015; Schoonbeek et al., 2015; Zipfel et al., 2006). Several components of the chitin-signalling network are known targets of numerous pathogen effector proteins (Gimenez-Ibanez et al., 2009; Mentlak et al., 2012; van den Burg et al., 2006; van Esse et al., 2007; van Esse et al., 2008; Yamaguchi et al., 2013; Zeng et al., 2012), supporting the hypothesis that the chitin-signalling network is an excellent candidate for enhancing the grapevine immune response.

Thus, further experiments will be necessary to gain a better understanding of how grapevine cells specifically perceive different chitooligosaccharides *via* these complex receptors and to determine the role of each member of the *VvLYK* multigene family, particularly during its interactions with both beneficial and pathogenic microbes.

Experimental procedures

Plant, cell culture and fungal materials

Arabidopsis thaliana wild-type (WT) Columbia (Col-0), mutant *Atcerk1* (GABI-Kat_096F09, allele *Atcerk1-2*; (Gimenez-Ibanez et al., 2009) or transgenic lines *Atcerk1/35S::VvLYK1-1/3* and *Atcerk1/LexA::VvLYK1-2-GFP* were grown under a 10/14-h day/night cycle at 20/18 °C (Trdá et al., 2014). For *in vitro* culture, *Arabidopsis* plants were grown on solid or in liquid half Murashige and Skoog (MS) medium including Nitsch vitamins (M0256; Duchefa, Haarlem, the Netherlands) supplied with 10 g L^{-1} sucrose. Seedlings were grown at 20 °C (day) or 18 °C (night) with a 14-h photoperiod.

Grapevine (*V. vinifera* cvs Cabernet Sauvignon and Marselan) cuttings were grown in a greenhouse until they had developed 6-8 leaves. The second and third youngest adult leaves from each plant were used for experiments, as previously indicated (Steimetz et al., 2012). Grapevine cells (*V. vinifera* cv. Gamay) were cultivated as described in Vandelle et al. (2006). For all experiments, 7-day-old cultures were diluted twice with new medium 24 h prior to use.

Grapevine powdery mildew *(E. necator* - isolate APC) was maintained detached leaves of *V. vinifera* cv. Cabernet Sauvignon as previously described (Donald et al., 2002). Grapevine downy mildew (*P. viticola* – isolate collected from a Burgundy vineyard) was routinely maintained on *V. vinifera* cv. Marselan plants as previously described (Steimetz et al., 2012).

Elicitors

Chitin and chitosan hexamer, with a degree of acetylation (DA) of 99.9 % and 0.1 %, respectively, were provided by Elicityl (Crolles, France). They were extracted from exoskeletons of crustaceans, hydrolyzed, purified by chromatography and finally their degree of polymerization (DP) and DA were verified by ^{1}H NMR analysis. The crab shell chitin NA-COS-Y (Lloyd et al., 2014), was obtained from Yaizu Suisankagaku Industry Co. (Yaizu, Japan). All the above mentioned chitooligosaccharides were dissolved in sterile ultrapure water (pH 8.5) at a concentration of 1 or 10 mg mL^{-1}. Sulfated laminarin (PS3), used as a potent inducer of grapevine resistance (Gauthier et al., 2014), was provided by Goëmar Laboratories and dissolved in sterile ultrapure water.

The flagellin-derived flg22 peptide from *Xanthomonas campestris* pv *campestris* strain 305 (QRLSSGLRINSAKDDAAGLAIS) was purchased from Proteogenix and dissolved in sterile ultra-pure water at 1 mM, as previously described (Trdá et al., 2014).

MAPK activation

Grapevine cells were equilibrated as described in Dubreuil-Maurizi et al. (2010), then treated with chitooligosaccharides (100 µg mL^{-1}) or water (as control) and harvested at 0, 5, 10, 20, 40 and 60 min post-treatment. MAPK activation was detected after immunoblotting of the extracted proteins using anti-p42/44-phospho-ERK antibody (Cell Signaling, Danvers, MA, USA). Transfer quality and homogeneous loading were checked by Ponceau red staining.

For *Arabidopsis* plantlets, 10- to 15-day-old liquid-grown seedlings were equilibrated for 24 h in fresh half MS medium. β-estradiol (10 µM) was added 1 h before elicitor treatment (1 mg mL^{-1}) for inducible transgenic lines *Atcerk1/LexA::VvLYK1-2-GFP*. Seedling samples were harvested 10 min after chitin or chitosan treatment.

Analysis of defense gene expression by quantitative polymerase chain reaction (qPCR)

For defense gene expression kinetics using grapevine cell suspensions, the cell culture density was adjusted to 0.1 g FWC mL^{-1} with NN medium, 16 h prior to experiment. Cells were then treated with 100 µg mL^{-1} chitooligosaccharides or water (as control) and harvested at 1 h post-treatment by filtration on GF/A filters.

For *Arabidopsis*, 10- to 15-day-old seedlings grown on solid half MS medium were transferred in liquid medium 2 d before treatment in a 24-well microtiter plate. β-estradiol (10 µM) was added 1h before treatment with 1 mg mL^{-1} of chitooligosaccharides for 2 h.

For both cells and seedlings, tissues were briefly ground before the addition of TRIzol® (Life Technologies). RNA extraction was then carried out following the manufacturer's instructions (Invitrogen). Reverse transcription was performed using Superscript III (Invitrogen) for cells or M-MLV reverse transcriptase (Invitrogen) for seedlings, following the manufacturer's protocol. Real-time qPCR was carried out as described previously (Trdá et al., 2014), except that a 1:100 dilution of cDNA was used. The relative transcript level was calculated using the comparative ΔΔCt method (Livak and Schmittgen, 2001) with the previously validated grapevine *VvEF1α* (Dubreuil-Maurizi et al., 2010; Reid et al., 2006) or the Arabidopsis *At4g26410* (Czechowski et al., 2005) housekeeping gene as internal control for normalization (*AtOLI* in Table S3).

Confocal microscopy

Confocal microscopy was performed using a Leica TCS SP2-AOBS confocal laser scanning microscope with a 40X oil-immersion objective (numerical aperture 1.25; Leica, Nanterre, France). Inducible transgenic lines were sprayed with 200 µM β-estradiol, 4 h before visualization. Leaf segments were mounted in ultra-pure water or in 1 M NaCl solution for plasmolysis experiments. For FM4-64 staining, samples were incubated in 8 µM FM4-64 solution in water for 10 min prior to observation. Fluorescent markers were visualized at 488 nm. GFP and FM4-64 emissions were bandpass filtered at 500–525 nm and 616–694 nm, respectively.

Botrytis and downy mildew assays

Leaves from the second and third adult top leaves of at least 3 grapevine plants were first sprayed on both sides with elicitor solution in 0.1% surfactant (Deshcofix) or surfactant alone (control) for 48h.

For *B. cinerea* infection assays, 36 leaf discs (1.9 cm diameter) were incubated on moist Whatmann paper and inoculated on the upper surface with 1000 conidia in a 20 µL-droplet of potato dextrose broth (PDB), ¼ diluted. Inoculated discs were placed in a plastic box maintained in 100% humidity under a 10/14 h day/night cycle at 20/18°C. Infection intensity was assessed 3 d post-inoculation (dpi) by measuring the macerated lesion diameter.

For *P. viticola* infection, the lower leaf surface was sprayed with a freshly prepared suspension (2.10^4 sporangia mL^{-1}) and plants were maintained in 100% humidity for 2 h. Leaf discs (1 cm diameter) were cut, transferred onto moist Whatmann paper in a plastic box and maintained in 100% humidity under a 10/14 h day/night cycle at 20/18°C. Infection intensity was assessed at 8 dpi by measuring the sporulating area using image analysis Visilog 6.9 software (Kim Khiook et al., 2013).

Powdery mildew penetration assay on transgenic *Arabidopsis*

Four-week-old *Arabidopsis* plants were used to assess powdery mildew penetration efficiency. Two leaves per plant were infected with *E. necator* using a fine paintbrush. Detached leaf material was sampled 48 hpi and stained with trypan blue according to Koch and Slusarenko (1990). Fungal structures were visualized using a Zeiss (Göttingen, Germany) Axioscop 2 light microscope. A minimum of 100 germinated spores were scored on each leaf. Successful penetration of epidermal cells (% penetrated cells) was indicated by the presence of a haustorium or a secondary hyphae.

Phylogenetic analysis of the VvLYK family

Proteins were aligned with the CLUSTAL W program (Tables S1, S2). The Maximum Likelihood phylogenetic tree was generated with the MEGA7 software (Kumar et al., 2016), using a bootstrapping of 1000 replications.

Expression analysis of *VvLYK* genes in pathogen-infected grape tissues

Young glossy *V. vinifera* cv. Cabernet Sauvignon leaves of similar developmental stage (~6 cm in diameter) were inoculated with *E. necator* conidia as described previously (Donald et al., 2002). The leaves were incubated at 23°C under a 16 h light/8 h dark cycle and sampled at 0, 6, 12 and 24 hpi into liquid nitrogen. Total RNA was extracted from two independent leaves each time point using the Spectrum Plant Total RNA Kit (Sigma-Aldrich) and DNase-treated according to the manufacturer's instructions. RNA quantity and quality

were assessed using a 2100 Bioanalyzer (Agilent Technologies). Library construction and Illumina RNA sequencing (single end, 100 bp reads) were carried out at the Australian Genome Research Facility (Melbourne, Australia). Reads were mapped to the coding sequences of each predicted *VvLYK* cDNA sequence using CLC Genomics Workbench v6.0.1. Reads were normalized according to (a) length of the VvLYK reference sequence and (b) mean relative expression of *V. vinifera* cv Cabernet sauvignon housekeeping genes: elongation factor 1-alpha (XM_002284888); glyceraldehyde-3-phosphate dehydrogenase (XM_002263109), phosphoenolpyruvate carboxylase (XM_010658735), to produce a Relative Expression Value (REV) at each time point of infection.

For microarray data, RNA was extracted from grape berries infected with *B. cinerea* then microarray hybridization and data analysis were performed as described in Kelloniemi *et al*. (2015). All microarray expression data are available at GEO under the entry GSE65969.

Generation of the *Atcerk1/VvLYK1* transgenic lines

The coding sequences of *VvLYK1-1*, *VvLYK1-2* and *VvLYK1-3* from *V. vinifera* cv. Cabernet Sauvignon were amplified from grapevine leaf cDNA prepared as previously described (Feechan et al., 2013). Gene-specific primers were designed with 5'-*Xho* I or *Xba* I restriction sites to facilitate sub-cloning (Table S3). Amplified products of the expected size were cloned into pCR-BLUNT vector and verified by sequencing. The coding sequences were subcloned into pART7 vector (Gleave, 1992) between the *35S* promoter and *OCS* terminator sequences. The *35S-VvLYK1-OCS* expression cassettes were subcloned as *Not* I fragments into the binary vector pART27 and then transferred into *Agrobacterium tumefaciens* strain EHA-105 for *Arabidopsis* transformation or *A. tumefaciens* strain GV3101 for agroinfiltration experiments (Williams et al., 2016).

The GFP-tagged constructs were amplified using primers designed to replace the stop codon with an Ala codon (GCC nucleotides, Table S3). PCR products of the expected size were first directionally subcloned into pENTR™/D-TOPO® vector (Invitrogen), then inserted into Gateway expression vectors by using Gateway LR Clonase™ II enzyme mix (Invitrogen). The three full-length coding sequences of *VvLYK1-1*, *VvLYK1-2* and *VvLYK1-3* were cloned into pK7FWG2 (kanamycin resistance) to obtain a constitutive overexpression construct (*p35S::VvLYK1-1/-2/-3-GFP*) or in pABindGFP (Bleckmann et al., 2010; hygromycin resistance) for a β-estradiol inducible gene expression (*pLexA::VvLYK1-2-GFP*).

The *Arabidopsis Atcerk1* mutant (Gimenez-Ibanez et al., 2009) was transformed using the floral dip method (Clough and Bent, 1998). Antibiotic resistant transgenic plants were screened in the T1 generation as described previously (Zipfel et al., 2006).

For analysis of *VvLYK1-1/-2/-3* transgene expression in the T2 generation, seed collected from selfed T1 lines was sown into soil and plants grown in a controlled growth chamber under a 10/14h day/night cycle at 24°C. Leaf material (~50 mg) was sampled from individual T2 segregating lines and the presence of the *VvLYK1-1/-2/-3* transgene confirmed by genomic PCR. Positive lines were resampled for total RNA extraction, cDNA synthesis and semi-quantitative PCR analysis of *VvLYK1-1*, *VvLYK1-2* or *VvLYK1-3* transcript expression using primers listed in Table S3.

Accession numbers

Vitis vinifera cv. Cabernet Sauvignon sequences: *VvLYK1-1* (MF177032), *VvLYK1-2* (MF177033), *VvLYK1-3* (MF177034). *VvLYK* sequences fused with Cter-GFP tag: *VvLYK1-1-GFP* (MF537036), *VvLYK1-2-GFP* (MF537037) and *VvLYK1-3-GFP* (MF537038).

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Author Contribution

DB, CV, LJD performed most of the experiments; LT, FB, CZ and BP conceived the original screening and research plans; JC, AC, BD and LS provided technical assistance; MCH, FB, CZ and BP supervised the experiments, LT, DB, LJD and PT designed the experiments and analysed the data; DB, LJD, IBD and BP conceived the project and wrote the article with contributions of all the authors; FB, MA and CZ supervised and complemented the writing. The authors declare no conflict of interest.

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Figure Legends

Fig. 1. Chitin and chitosan induced defense responses and resistance to pathogens in grapevine.

(**a**) Activation kinetics of two mitogen-activated protein kinases (MAPKs) detected by immunoblotting with an antibody raised against the human phosphorylated extracellular regulated protein kinase 1/2 (α-pERK1/2) in grapevine cells treated with chitin DP6 (100 µg mL^{-1}), chitosan DP6 (100 µg mL^{-1}) or water (negative control). Homogeneous loading was checked by Ponceau red staining. (**b**) Expression of defense genes encoding an acidic chitinase (*Chit4C*), a stilbene synthase (*STS*), a phenylalanine ammonia lyase (*PAL*) and a respiratory burst oxidase homolog D (*RbohD)* measured by quantitative polymerase chain reaction (qPCR) 1h post-treatment with chitin DP6 (100 µg mL^{-1}), chitosan DP6 (100 µg mL^{-1}) or water. Values represent the mean of triplicate data ± SE (n=3) from one experiment out of three and data were normalized by the housekeeping gene *EF1α* and compared with water (negative control), set as 1. Asterisks (*) indicate statistically significant differences between water and chitooligosaccharide treatment, using an unpaired heteroscedastic Student's t test ($P<0.05$). (**c**) Development of *B. cinerea* at 3 d post-inoculation (dpi) on grapevine leaf discs treated 48h before with chitin DP6 (1 mg mL^{-1}), chitosan DP6 (1 mg mL^{-1}) or flg22 (10 µM) previously solubilized in Dehscofix 0.1% and compared with control (adjuvant : Dehscofix 0.1%). Values represent the mean of lesion diameters ± SE ($n \geq 36$ discs from 3 different plants) from one representative experiment out of three. (**d**) Sporulation caused by *P. viticola* at 8 dpi on grapevine leaf discs treated 48h before inoculation with chitin DP6 (100 µg mL^{-1}), chitosan DP6 (100 µg mL^{-1}) or 2.5 mg mL^{-1} sulfated laminarin (PS3) previously solubilized in Dehscofix 0.1% and compared with control (adjuvant : Dehscofix 0.1%). Sporulating leaf area was evaluated by image analysis Visilog 6.9 software (Kim Khiook et al., 2013). Values represent the mean of percentage of sporulating area ± SE (n=30 discs from 3 different plants) from one representative experiment out of three. Asterisks indicate a statistically significant difference between control and the elicitor treatment (Student's t-test; *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$). A representative leaf disc for each treatment is shown. Similar results were obtained in at least three independent experiments.

Fig. 2. Phylogenetic analysis and characterization of grapevine LysM-RKs (VvLYKs).

(a) Maximum-likelihood phylogenetic tree drawn with MEGA 7 (Kumar et al., 2016) showing the relationship between the Arabidopsis proteins AtCERK1/LYK1 and AtLYK2-5 (red), the rice OsCERK1 (blue) and the most similar protein sequences of *Vitis vinifera* (black). Sequences used for the phylogenetic analysis were: AtCERK1/LYK1 (NP_566689), AtLYK2 (OAP05017), AtLYK3 (NP_175606), AtLYK4 (NP_179957), AtLYK5 (NP_180916), OsCERK1 (A0A0P0XII1), VvLYK1-1 (XP_010657225), VvLYK1-2 (XP_010655366), VvLYK1-3 (XP_010655365), VvLYK2 (XP_019080819), VvLYK3-1 (XP_002283628), VvLYK3-2 (XP_019074828), VvLYK3-3 (XP_002272814), VvLYK4-1 (XP_002269408), VvLYK4-2 (XP_010649202), VvLYK5-1 (XP_002277331), VvLYK5-2 (MF177034), VvLYK6 (XP_002280070), VvLYK7 (XP_002269472), VvLYK8 (XP_002281880) and VvLYK9 (XP_002276830).

(b) *VvLYK* expression profiles during *E. necator* or *B. cinerea* infection. Results are expressed as Relative Expression Values. Colour range has been made independently from RNA-Seq or microarray data. (n.d. = no full-length transcript detected in RNA Seq; _=no specific probe available in microarray). (c) Schematic structure of AtCERK1/LYK1, OsCERK1, VvLYK1-1, VvLYK1-2 and VvLYK1-3 based on the multiple alignment realized with T-coffee (Fig. S2). (d) Subcellular localization of VvLYK1-1-GFP in the line *Atcerk1/p35S::VvLYK1-1-GFP*. Leaves of *Arabidopsis thaliana* expressing VvLYK1-1-GFP were incubated with the plasma membrane dye FM4-64. Confocal microscopy imaging revealed the green GFP-tagged VvLYK1-1 (1), the red FM4-64 labelled plasma membrane (2) and the co-localization of both probes in *Arabidopsis* leaves (3). (4) NaCl (1M) induced plasmolysis and confocal microscopy imaging revealed that VvLYK1-1-GFP fluorescence followed the plasma membrane shrinking (5). Bars, 20 µm.

Fig. 3. VvLYK1-1 restores chitin-induced immune responses in *Atcerk1*.

(**a**) Semi-quantitative RT-PCR analysis of *VvLYK1-1*, *VvLYK1-2* and *VvLYK1-3* expression in leaf tissue of independently transformed *Atcerk1* lines. AtEF1α (At5g60390) was used as an internal control. (**b**) Activation of two mitogen-activated protein kinases (MAPKs) 10 min after chitin treatment (1 mg mL^{-1}) detected by immunoblotting with an antibody raised against the human phosphorylated extracellular regulated protein kinase 1/2 (α-pERK1/2). Homogeneous loading was checked by Ponceau red staining. Similar results were obtained in three independent experiments. (**c**) Relative expression of a defense gene encoding flagellin-induced receptor kinase1 *(FRK1)* measured by qPCR, 2h after chitin treatment (1 mg mL^{-1}). Data show a representative experiment from three independent biological ones. Means of the triplicate data were normalized by the housekeeping gene *At4g26410* and expressed as a percentage of the chitin-treated WT Col-0, set as 100%. Asterisks (*) indicate statistically significant differences between water and chitin treatment whereas hash marks (#) indicate statistically significant differences between WT or transgenic line and *Atcerk1*, using an unpaired heteroscedastic Student's t test ($P<0.05$).

Fig. 4. VvLYK1-1 expression restores penetration resistance against the non-adapted powdery mildew *Erysiphe necator* in *Atcerk1*.

Penetration efficiency (*i.e.* haustorium formation) of the non-adapted powdery mildew pathogen *E. necator* on *Arabidopsis* WT (Col-0), *Atcerk1* mutant and eight independent transgenic *Atcerk1* lines transformed with the *VvLYK1-1* construct (**a**) or seven lines transformed with the *VvLYK1-3* construct (**b**). One hundred germinated conidia were scored per leaf, with three leaves inoculated per line. Each data point represents the mean of three independent experiments ± SE. WT Col-0 and transgenic lines were compared to the mutant *Atcerk1* with a Student *t*-test (***, $P<0.001$). (+) lines expressing the transgene. (-) lines with no detectable *VvLYK1* transcripts.

Fig. 5. The inducible expression of VvLYK1-2 also restores chitin-triggered responses in *Atcerk1*.

(**a**) Subcellular localization of *VvLYK1-2-GFP* visualized by confocal microscopy 4h post-treatment with β-estradiol (βE). DIC, differential interference contrast. Bars, 20 µm. (**b**) Activation of two mitogen-activated protein kinases (MAPKs) detected 10 min after chitin treatment (1 mg mL^{-1}) by immunoblotting with an antibody raised against the human phosphorylated extracellular regulated protein kinase 1/2 (α-pERK1/2).

Homogeneous loading was checked by Ponceau red staining. Similar results were obtained in three independent experiments. (**c**) Relative expression of the defense gene encoding flagellin-induced receptor kinase1 *(FRK1)* measured by qPCR, 2h post-chitin treatment (1 mg mL^{-1}). Data show a representative experiment from three independent biological ones. Means of the triplicate data were normalized by the housekeeping gene *At4g26410* and expressed as a percentage of the transcript level in WT Col-0 plants treated by chitin + β-estradiol, set as 100%. Asterisks (*) indicate statistically significant differences between water and chitin treatment whereas hash marks (#) indicate statistically significant differences between WT or transgenic line and *Atcerk1*, using an unpaired heteroscedastic Student's t test ($P<0.05$). For (**b**) and (**c**), inducible transgenic lines *Atcerk1/LexA::VvLYK1-2-GFP* were treated 1h before chitin treatment with 10 µM β-estradiol (βE).

Fig. 6. VvLYK1-1 and VvLYK1-2 expression restores chitosan-triggered responses in the *Atcerk1* mutant. (**a, c**) Activation of two mitogen-activated protein kinases (MAPKs) detected 10 min after treatment with chitosan DP6 (1 mg mL^{-1}) by immunoblotting with an antibody raised against the human phosphorylated extracellular regulated protein kinase 1/2 (α-pERK1/2). Homogeneous loading was checked by Ponceau red staining. (**b, d**) Expression of a defense gene encoding the flagellin-induced receptor-like protein kinase 1 (*FRK1*) measured by qPCR 2 h after chitosan treatment. Data show an average of three biological experiments that were normalized by housekeeping gene *At4g26410* and compared with Col-0 treated with chitosan, set as 100%. Asterisks (*) indicate statistically significant differences between water and chitosan treatment whereas hash marks (#) indicate statistically significant differences between WT or transgenic line and *Atcerk1*, using an unpaired heteroscedastic Student's t test ($P<0.05$). (**c, d**) All lines were pretreated 1h before elicitor treatment with β-estradiol (βE; 10 µM), when indicated.

Supporting Information Legends

Fig. S1. Chitin and chitosan DP6 did not induce similarly ROS production in Arabidopsis and grapevine cells. Relative H_2O_2 production in **(a)** *A. thaliana* or **(b)** *V. vinifera* cells 20 min post-treatment with chitin DP6 (100 µg mL^{-1}), chitosan DP6 (100 µg mL^{-1}) and flg22 (1 µM) treatments or water (negative control). ROS production was measured by chemiluminescence of luminol according to Trdá et al. (2014) and results are expressed relatively to flg22 (positive control=100%). Values are means ± SE from three independent experiments (n=3). Asterisks indicate a statistically significant difference between control and the elicitor treatment (Tukey's pairwise test; *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

Fig. S2. The crab shell chitin also induced defense responses in grapevine cells. Activation kinetics of two mitogen-activated protein kinases (MAPKs) in grapevine cells treated with 100 µg.mL^{-1} of the unpurified crab shell chitin NA-COS-Y (Lloyd et al., 2014). MAPKs were detected by immunoblot with an antibody raised against the human phosphorylated extracellular regulated protein kinase 1/2 (α-pERK1/2). Fifteen µg of proteins were loaded in each lane. Homogeneous loading was checked by Ponceau red staining. This result shows one representative experiment out of three.

Fig. S3. Alignment of AtCERK1/LYK1, the rice OsCERK1 and its putative orthologs in grapevine (VvLYK1-1/-2/-3). Protein sequences were aligned with T-Coffee. Black and gray shading representing identical and positive amino acids, respectively, was visualized with Boxshade. The predicted signal peptide, the lysin motifs (LysM), the transmembrane region and the serine/threonine (S/T) kinase are shown. The residues of the chitin-binding site in AtCERK1/LYK1 LysM2 are indicated by asterisks and their conservation is highlighted in the grades of green. The residues E110, E114 and I141 bind to N-acetyl moieties of $(GlcNAc)_5$, while Q109, T112, Y113, A138, T139, N140, P142 and L143 interact with hydroxyl and hydroxymethyl groups of glucose part (Liu *et al.*, 2012). Conserved Cys residues (in red) form disulfide bridges (indicated by arrows). The RD type of kinase is highlighted in blue. SNPs between the NCBI reference sequence from *V. vinifera* cv Pinot Noir (PN40024) and cv Cabernet-Sauvignon are highlighted in purple (VvLYK1-1: Q99H, L220I, A221S, H264Q, S265T, S313T, D514E, K543R, P547A; VvLYK1-2: K67T, E73K, G95R, I186V, E216G, R279G, H574Q; VvLYK1-3: F116Y, I142L, V250A, A493E, L564F) or in orange when compared to cv Gamay (VvLYK1-1: no SNP; VvLYK1-2: no SNP; VvLYK1-3: -224A, V250A, A493E, I517V, Q577L). Residues S266, S268, S270, S274 and T519 (indicated by ●) were found to be phosphorylated in AtCERK1/LYK1 after chitin treatment (Petutschnig *et al.*, 2010). A: Ala, C:Cys, D:Asp, E:Glu, F:Phe, G:Gly, H:His, I:Ile, K:Lys, L:Leu, M:Met, N:Asn, P:Pro, Q:Gln, R:Arg, S:Ser, T:Thr, V:Val, W:Trp, Y:Tyr.

Fig. S4. Necrosis observed in response to the over-expression of *VvLYK1-2* in *Nicotiana benthamiana*. Symptoms observed 2d after leaf infiltration with (1) *A. tumefaciens* GV3101 alone (negative control), (2) *A. tumefaciens* GV3101 containing the construct $RPV1_{TIR1-193}$ (positive control; Williams et al. 2016), (3) *A. tumefaciens* GV3101 containing the construct *pART27-VvLYK1-2.*

Fig. S5. Immunodetection of MAPKs in Arabidopsis mutants *Atlyk1-5* in response to chitosan. Plants of the different Arabidopsis *Atlyk* mutants have been treated with 1 $mg.mL^{-1}$ chitosan DP6 for 10 min before protein extraction. Activation of the two MAPKs was detected by immunoblot with an antibody raised against the human phosphorylated extracellular regulated protein kinase 1/2 (α-pERK1/2). Fifteen µg of proteins were loaded in each lane. Homogeneous loading was checked by Ponceau red staining. Similar results were obtained in three independent experiments.

Table S1. The *Vitis vinifera* VvLYK family contains 15 putative genes in the grapevine genome.

Table S2. Percentage of amino acid identity or similarity between VvLYK1-1/-2/-3 and AtCERK1/LYK1 or OsCERK1.

Table S3. Primers used in this study. For each primer, gene family, name used (ID), sequence (5'-> 3') and usage are reported. Underlined cacc is used for Gateway directional TOPO cloning. Letters in bold indicate restriction sites added to the 5' end of gene-specific primer sequences to facilitate cloning.

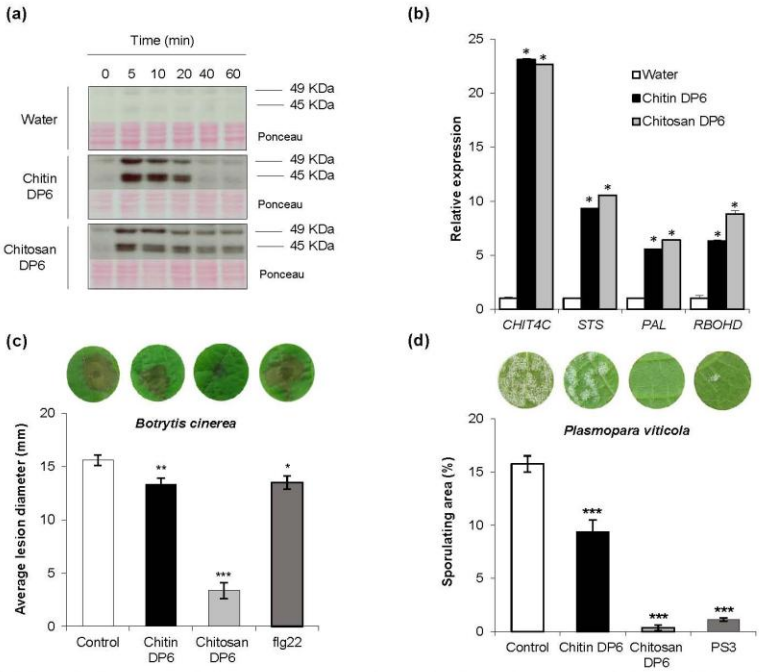

Fig. 1 Chitin and chitosan induced defense responses and resistance to pathogens in grapevine. (**a**) Activation kinetics of two mitogen-activated protein kinases (MAPKs) detected by immunoblotting with an antibody raised against the human phosphorylated extracellular regulated protein kinase 1/2 (α-pERK1/2) in grapevine cells treated with chitin DP6 (100 µg mL^{-1}), chitosan DP6 (100 µg mL^{-1}) or water (negative control). Homogeneous loading was checked by Ponceau red staining. (**b**) Expression of defense genes encoding an acidic chitinase (*Chit4C*), a stilbene synthase (*STS*), a phenylalanine ammonia lyase (*PAL*) and a respiratory burst oxidase homolog D (*RbohD*) measured by quantitative polymerase chain reaction (qPCR) 1h post-treatment with chitin DP6 (100 µg mL^{-1}), chitosan DP6 (100 µg mL^{-1}) or water. Values represent the mean of triplicate data ± SE (n=3) from one experiment out of three and data were normalized by the housekeeping gene *EF1α* and compared with water (negative control), set as 1. Asterisks (*) indicate statistically significant differences between water and chitooligosaccharide treatment, using an unpaired heteroscedastic Student's t test (P<0.05). (**c**) Development of *B. cinerea* at 3 days post-inoculation (dpi) on grapevine leaf discs treated 48h before with chitin DP6 (1 mg mL^{-1}), chitosan DP6 (1 mg mL^{-1}) or flg22 (10 µM) previously solubilized in Dehscofix 0.1% and compared with control (adjuvant : Dehscofix 0.1%). Values represent the mean of lesion diameters ± SE (n ≥ 36 discs from 3 different plants) from one representative experiment out of three. (**d**) Sporulation caused by *P. viticola* at 8 dpi on grapevine leaf discs treated 48h before inoculation with chitin DP6 (100 µg mL^{-1}), chitosan DP6 (100 µg mL^{-1}) or 2.5 mg mL^{-1} sulfated laminarin (PS3) previously solubilized in Dehscofix 0.1% and compared with control (adjuvant : Dehscofix 0.1%). Sporulating leaf area was evaluated by image analysis Visilog 6.9 software (Kim Khiook et al., 2013). Values represent the mean of percentage of sporulating area ± SE (n=30 discs from 3 different plants) from one representative experiment out of three. Asterisks indicate a statistically significant difference between control and the elicitor treatment (Student's t-test; *, P < 0.05, **, P < 0.01, ***, P < 0.001). A representative leaf disc for each treatment is shown. Similar results were obtained in at least three independent experiments.

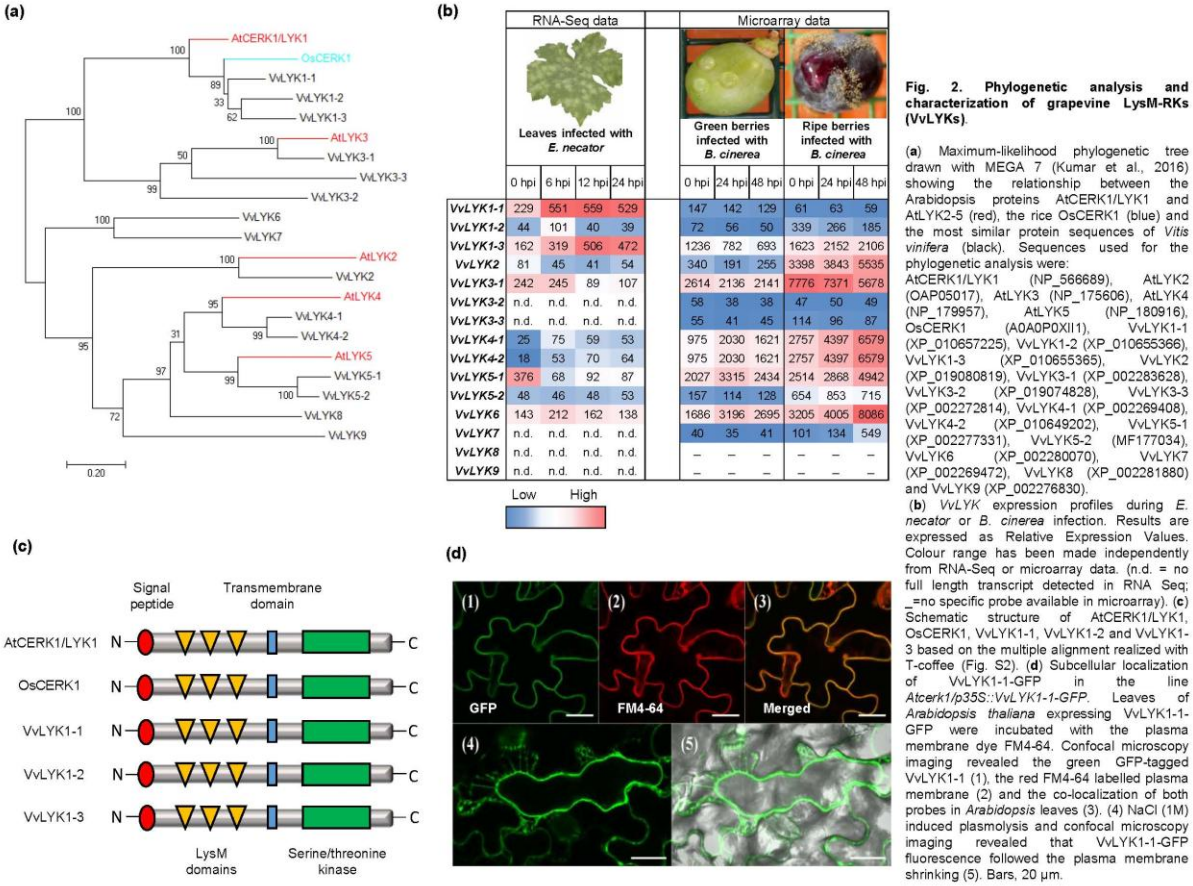

Fig. 2. Phylogenetic analysis and characterization of grapevine LysM-RKs (VvLYKs).

(**a**) Maximum-likelihood phylogenetic tree drawn with MEGA 7 (Kumar et al., 2016) showing the relationship between the Arabidopsis proteins AtCERK1/LYK1 and AtLYK2-5 (red), the rice OsCERK1 (blue) and the most similar protein sequences of *Vitis vinifera* (black). Sequences used for the phylogenetic analysis were: AtCERK1/LYK1 (NP_566689), AtLYK2 (OAP05017), AtLYK3 (NP_175606), AtLYK4 (NP_179957), AtLYK5 (NP_180916), OsCERK1 (A0A0P0XII1), VvLYK1-1 (XP_010657225), VvLYK1-2 (XP_010655366), VvLYK1-3 (XP_010655365), VvLYK2 (XP_019080819), VvLYK3-1 (XP_002283628), VvLYK3-2 (XP_019074828), VvLYK3-3 (XP_002272814), VvLYK4-1 (XP_002269408), VvLYK4-2 (XP_010649202), VvLYK5-1 (XP_002277331), VvLYK5-2 (MF177034), VvLYK6 (XP_002280070), VvLYK7 (XP_002269472), VvLYK8 (XP_002281880) and VvLYK9 (XP_002276830).

(**b**) *VvLYK* expression profiles during *E. necator* or *B. cinerea* infection. Results are expressed as Relative Expression Values. Colour range has been made independently from RNA-Seq or microarray data. (n.d. = no full length transcript detected in RNA Seq; _=no specific probe available in microarray). (**c**) Schematic structure of AtCERK1/LYK1, OsCERK1, VvLYK1-1, VvLYK1-2 and VvLYK1-3 based on the multiple alignment realized with T-coffee (Fig. S2). (**d**) Subcellular localization of VvLYK1-1-GFP in the line *Atcerk1/p35S::VvLYK1-1-GFP*. Leaves of *Arabidopsis thaliana* expressing VvLYK1-1-GFP were incubated with the plasma membrane dye FM4-64. Confocal microscopy imaging revealed the green GFP-tagged VvLYK1-1 (1), the red FM4-64 labelled plasma membrane (2) and the co-localization of both probes in *Arabidopsis* leaves (3). (4) NaCl (1M) induced plasmolysis and confocal microscopy imaging revealed that VvLYK1-1-GFP fluorescence followed the plasma membrane shrinking (5). Bars, 20 µm.

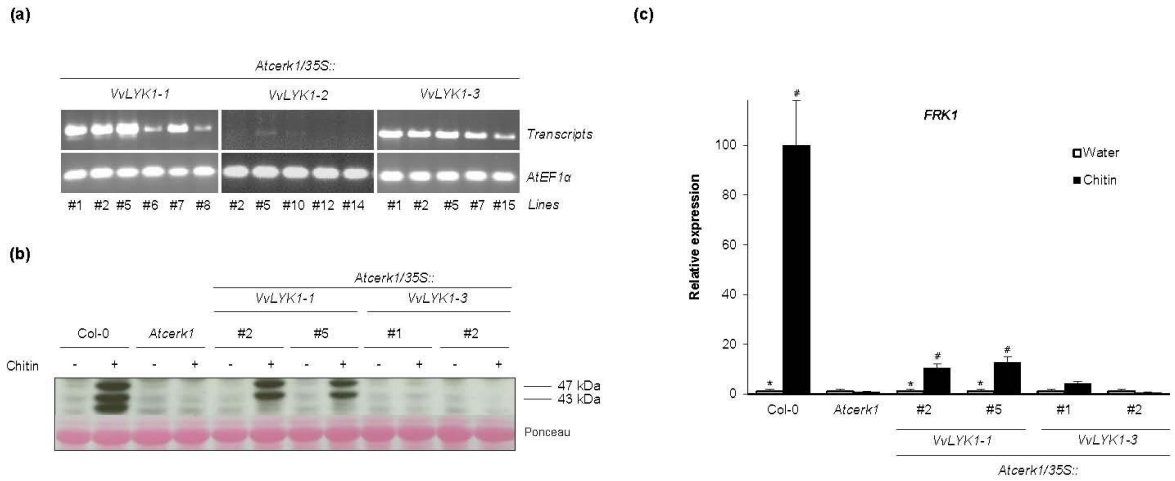

Fig. 3. VvLYK1-1 restores chitin-induced immune responses in *Atcerk1*. (**a**) Semi-quantitative RT-PCR analysis of *VvLYK1-1*, *VvLYK1-2* and *VvLYK1-3* expression in leaf tissue of independently transformed *Atcerk1* lines. *AtEF1α* was used as an internal control. (**b**) Activation of two mitogen-activated protein kinases (MAPKs) 10 min after chitin treatment (1 mg mL^{-1}) detected by immunoblotting with an antibody raised against the human phosphorylated extracellular regulated protein kinase 1/2 (α-pERK1/2). Homogeneous loading was checked by Ponceau red staining. Similar results were obtained in three independent experiments. (**c**) Relative expression of a defense gene encoding flagellin-induced receptor kinase1 *(FRK1)* measured by qPCR, 2h after chitin treatment (1 mg mL^{-1}). Data show a representative experiment from three independent biological ones. Means of the triplicate data were normalized by the housekeeping gene *At4g26410* and expressed as a percentage of the chitin-treated WT Col-0, set as 100%. Asterisks (*) indicate statistically significant differences between water and chitin treatment whereas hash marks (#) indicate statistically significant differences between WT or transgenic line and *Atcerk1*, using an unpaired heteroscedastic Student's t test ($P<0.05$).

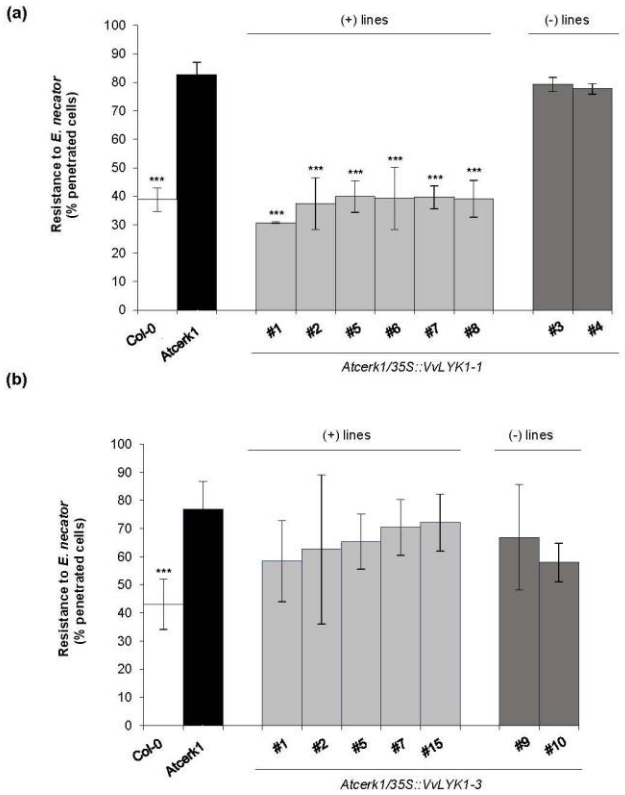

Fig. 4. VvLYK1-1 expression restores penetration resistance against the non-adapted powdery mildew *Erysiphe necator* in *Atcerk1*. Penetration efficiency (*i.e.* haustorium formation) of the non-adapted powdery mildew pathogen *E. necator* on Arabidopsis WT (Col-0), *Atcerk1* mutant and eight independent transgenic *Atcerk1* lines transformed with the *VvLYK1-1* construct (**a**) or seven lines transformed with the *VvLYK1-3* construct (**b**). One hundred germinated conidia were scored per leaf, with three leaves inoculated per line. Each data point represents the mean of three independent experiments ± SE. WT Col-0 and transgenic lines were compared to the mutant *Atcerk1* with a Student's *t*-test (***, P<0.001). (+) lines expressing the transgene. (-) lines with no detectable *VvLYK1* transcripts.

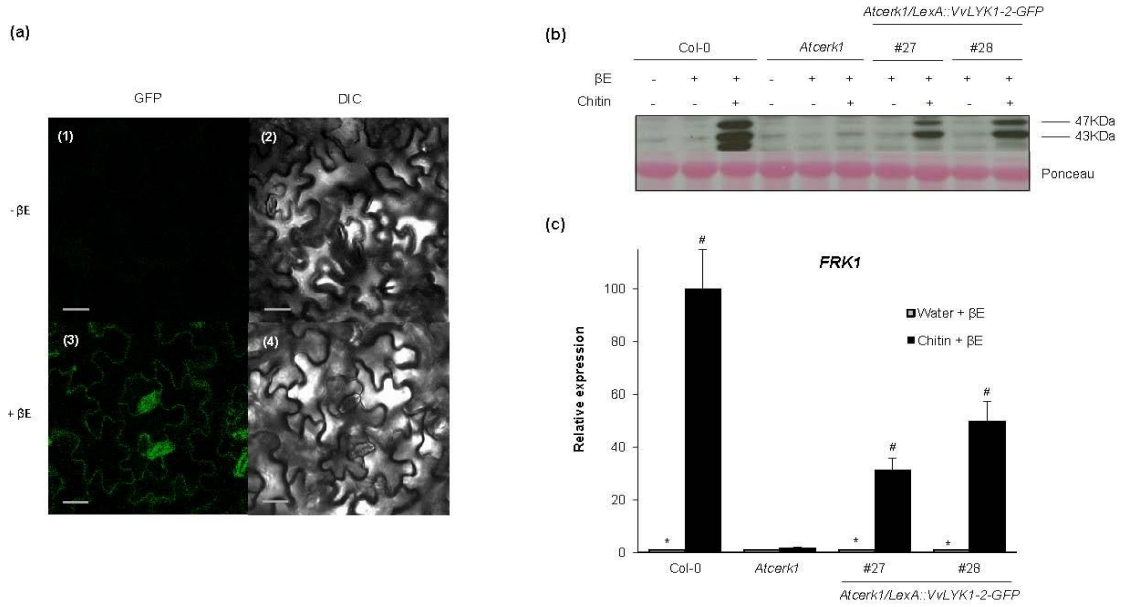

Fig. 5 The inducible expression of VvLYK1-2 also restores chitin-triggered responses in *Atcerk1*. (**a**) Subcellular localization of *VvLYK1-2-GFP* visualized by confocal microscopy 4h post-treatment with β-estradiol (βE). DIC, differential interference contrast. Bars, 20 µm. (**b**) Activation of two mitogen-activated protein kinases (MAPKs) detected 10 min after chitin treatment (1 mg mL^{-1}) by immunoblotting with an antibody raised against the human phosphorylated extracellular regulated protein kinase 1/2 (α-pERK1/2). Homogeneous loading was checked by Ponceau red staining. Similar results were obtained in three independent experiments. (**c**) Relative expression of the defense gene encoding flagellin-induced receptor kinase1 *(FRK1)* measured by qPCR, 2h post-chitin treatment (1 mg mL^{-1}). Data show a representative experiment from three independent biological ones. Means of the triplicate data were normalized by the housekeeping gene *At4g26410* and expressed as a percentage of the transcript level in WT Col-0 plants treated by chitin + β-estradiol (βE), set as 100%. Asterisks (*) indicate statistically significant differences between water and chitin treatment whereas hash marks (#) indicate statistically significant differences between WT or transgenic line and *Atcerk1*, using an unpaired heteroscedastic Student's t test ($P<0.05$). For (**b**) and (**c**), inducible transgenic lines *Atcerk1/LexA::VvLYK1-2-GFP* were pre-treated, 1h before chitin treatment, with 10 µM β-estradiol (βE).

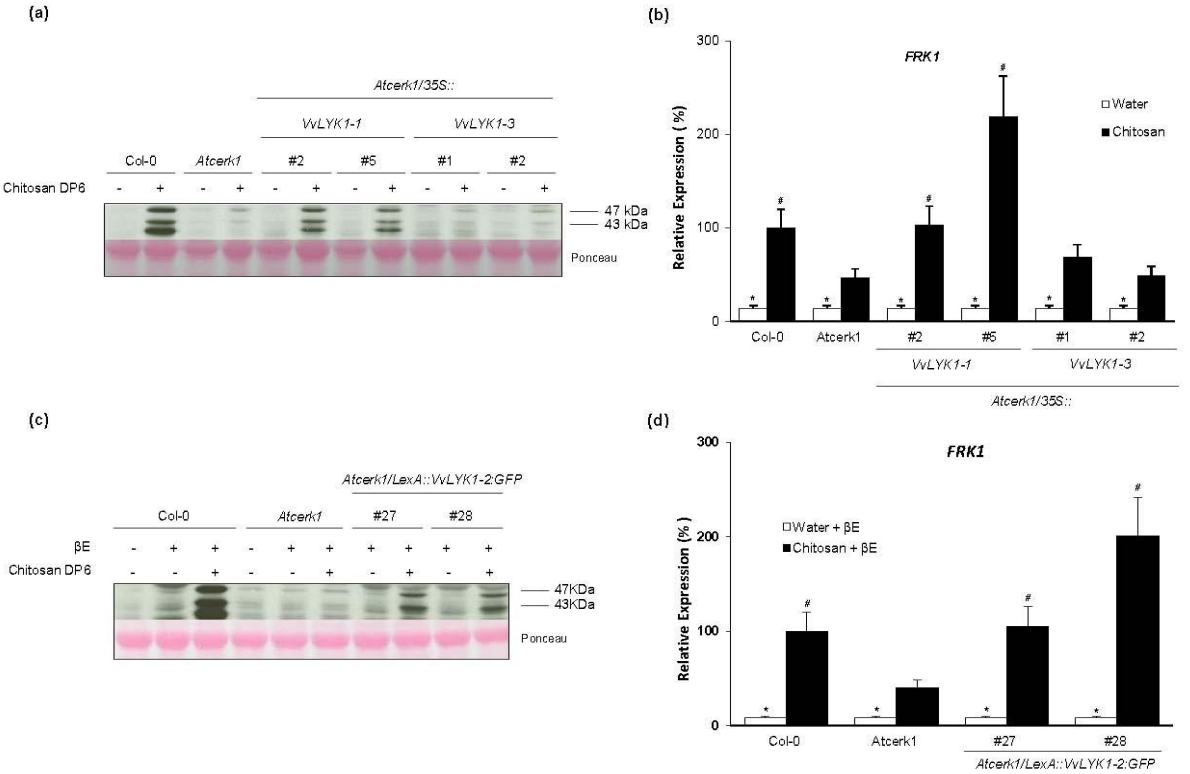

Fig. 6. VvLYK1-1 and VvLYK1-2 expression restores chitosan-triggered responses in the *Atcerk1* mutant. (**a, c**) Activation of two mitogen-activated protein kinases (MAPKs) detected 10 min after treatment with chitosan DP6 (1 mg mL^{-1}) by immunoblotting with an antibody raised against the human phosphorylated extracellular regulated protein kinase 1/2 (α-pERK1/2). Homogeneous loading was checked by Ponceau red staining. (**b, d**) Expression of a defense gene encoding the flagellin-induced receptor-like protein kinase 1 *(FRK1)* measured by qPCR 2 h after chitosan treatment. Data show an average of three biological experiments that were normalized by housekeeping gene *At4g26410* and compared with Col-0 treated with chitosan, set as 100%. Asterisks (*) indicate statistically significant differences between water and chitosan treatment whereas hash marks (#) indicate statistically significant differences between WT or transgenic line and *Atcerk1*, using an unpaired heteroscedastic Student's t test (P<0.05). (**c**, **d**) All lines were pretreated 1h before elicitor treatment with β-estradiol (βE; 10 µM), when indicated.