Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Chitosan triggers actin remodelling and activation of defence genes that is repressed by calcium influx in grapevine cells

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ARTICLE INFO

Keywords:
Actin
Calcium
Chitosan
Defence
Grapevine
Phytoalexins

ABSTRACT

Defence to pathogens must be specific. In the past, we have dissected early signalling deployed by bacterial elicitors in a grapevine cell system. In the current work, we asked, how defence of fungi differs. Fungal diseases of grapevine pose great challenges for global viticulture and require massive plant protection measures. Plant cells are able to sense chitin, a central component of fungal cell walls and respond by activation of basal defence. We, therefore mapped early defence responses evoked by chitosan, a chitin fragment able to bind to chitin receptors. We found an activation of calcium influx, monitored by extracellular alkalinisation due to a co-transport of protons, remodelling of actin (but not of microtubules), and the activation of transcripts for phytoalexin synthesis, jasmonate-signalling, salicylate signalling, and chitinase. Interestingly, Gadolinium, an inhibitor of calcium influx, can inhibit extracellular alkalinisation in response to chitosan, while the induction of the phytoalexin synthesis transcripts was specifically promoted. In contrast, both DMSO and benzyl alcohol, compounds known to modulate membrane fluidity, partially inhibited the transcript responses to chitosan. We discuss these data with a model, where chitosan deploys signalling culminating in activation of defence related transcripts, but at the same time activates calcium influx that negatively feeds back on the same signal chain, which might be a mechanism to achieve a temporal signature that is rapid, but transient.

1. Introduction

Since its domestication, around 8000 years ago, Grapevine (*Vitis vinifera*) has been bred for fast growth and sweet fruits. This was achieved on expense of stilbene accumulation, the major phytoalexins in grapevine (Duan et al., 2015). In consequence, viticulture in Europe almost collapsed, when from the mid 19th century new pathogens were introduced from North America. In addition to the oomycete *Plasmopara viticola*, the causative agent of Downy Mildew of Grapevine, it was mainly the ascomycete *Erysiphe necator* (Pearson, 1988) accounting for the tremendous intensity of chemical plant protection characteristic for viticulture. For instance, US viticulture spends around 100 Mio US$ per year for fungicides (Gianessi and Reigner, 2005), and in European viticulture, the average of fungicide applications exceeds 10 cycles per year (Sattler et al., 2007).

As for other plants, Grapevine commands two levels of innate immunity (Chang and Nick, 2012). A broadband basal immunity evoked by molecular patterns shared by entire groups of organisms, so called Pathogen Associated Molecular Patterns (PAMP). For instance, flagellin, a component of bacterial flagella can elicit a PAMP-triggered immunity (PTI), culminating in cellular adaptation to contain pathogen development. In grapevine, this is achieved by the accumulation of antimicrobial, phenolic compounds, the stilbenes. Pathogens that are adapted to a host, can often silence PTI by means of effectors. In a host that has co-evolved with the pathogen, effectors can be sensed by specific receptors, such that a second tier of defence is deployed, so called effector-triggered immunity (ETI) that culminates in a Hypersensitive Response, linked with the activation of specific metacaspases that execute the cellular suicide (Gong et al., 2019a). For grapevine, American wild species that have co-evolved with *P. viticola* or with *E. necator* are endowed with ETI, while the domesticated grapevine, *V. vinifera*, which evolved without contact to these pathogens, is susceptible,

Abbreviations: **BA**, Benzyl Alcohol; **DMSO**, Dimethyl Sulfoxide; **ETI**, Effector-Triggered Immunity; **JAZ1**, Jasmonate-ZIM-domain 1; **ROS**, Reactive Oxygen Species; **PAL**, Phenylalanine Ammonia Lyase; **PAMP**, Pathogen-Associated Molecular Pattern; **PTI**, PAMP Triggered Immunity; **StSy**, Stilbene Synthase; **RS**, Resveratrol Synthase; **DPI**, Di-Phenylene Iodonium.

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https://doi.org/10.1016/j.plantsci.2022.111527
Received 31 May 2022; Received in revised form 28 October 2022; Accepted 30 October 2022

because its PTI is quelled by the pathogen effectors. Comparative studies conducted in cell lines from the *V. vinifera* variety Pinot Noir and the wild American species *V. rupestris* revealed that the early signalling of the two forms of immunity share several events, such as an influx of calcium ions, activation of apoplastic burst by the NADPH oxidase Respiratory oxidative burst Homologue, reorganisation of the cytoskeleton, activation of a MAPK cascade, and activation of transcripts of phytoalexin synthesis (Chang and Nick, 2012). However, the temporal signature of these events differs. While activation of basal immunity by the flagellin fragment flg22 triggers first a calcium influx that is then followed by oxidative burst, the order of these inputs is reversed in response to the elicitor harpin that triggers a cell-death related form of defence. There are qualitative differences as well. For instance, only flg22, but not harpin, can activate the accumulation of jasmonates (Chang et al., 2017). In summary, grapevine cells can deploy different defence response patterns, depending on the type of trigger, but also on genetic differences.

For the response to *E. necator*, the causative agent of Powdery Mildew, several of these genetic factors have been genetically mapped and are already used to breed for resistance against this disease. Originally, these resistance factors were found in wild *Vitis* species from America, and the strain specificity of these factors supports the notion that they indeed represent a form of effector triggered immunity (Jones and Dangl, 2006). However, recently, further resistance factors have been located in wild species from China, such as *V. pseudoreticulata*. These resistance factors seem to act independently of a Hypersensitive Response, and thus obviously cannot be the product of co-evolution between host and pathogen, and rather seem to emerge from a more efficient activation of basal immunity (a short discussion is given in (Jiao et al., 2016).

This leads to the question, how the two forms of immunity (basal immunity or PTI versus ETI) interact, when they are both activated simultaneously. This would be the case, for instance, if *E. necator* infects *V. rupestris*, a wild Grapevine from North America, capable of a Hypersensitive Response to this pathogen. Since *E. necator*, as Ascomycete, is also endowed with a cell wall containing chitin, one of the most important fungal PAMPs (Felix et al., 1993), the launch of ETI should be accompanied or preceded by chitin-induced basal immunity.

Chitin is actually the most abundant polymer in nature, since it is the major building block of cell walls in fungi, and the exoskeleton of arthropodes, but also the annelids. It is absent from plants, which means that plant chitinases are exclusively acting in the defence against fungal or invertebrate attack (Jalil et al., 2015). Chitin itself is not soluble, but its breakdown product chitosan, generated by de-acetylation, acts as a vigorous elicitor of plant defence upon binding to the chitin-elicitor binding protein (CEBiP) located in the plasma-membrane (Kaku et al., 2006). This receptor harbours three extracellular lysine motif domains and a glycosyl phosphatidyl inositol anchor at the cytoplasmic side, but requires a binding partner, the chitin elicitor receptor kinase 1 (CERK1) for signalling (Shimizu et al., 2010). The binding of the ligand involves dimerisation of CEBiP and then activates CERK1 signalling (Hayafune et al., 2014). The signalling involves a rapid influx of calcium (Felix et al., 1993), and interactions with other kinases, culminating in activation of a MAPK cascade that conveys the signal to the nucleus (Huang et al., 2020). As a result, pathogenesis-related proteins such as chitinases accumulate (reviewed in (Boller et al., 2009)). In grapevine, the induction of chitinase and glucanase activities along with phytoalexin accumulation correlates with resistance against *Botrytis cinerea* causing Grey Mold, and *Plasmopara viticola*, the causal agent of Downy Mildew (Trotel-Aziz et al., 2006).

In the past, we have mapped defence signalling for grapevine cell cultures (Chang and Nick, 2012; Chang et al., 2017) and were able to define overlapping, but distinct signal chains for the bacterial elicitors flg22 (triggering basal immunity, not accompanied by cell death) and harpin (triggering cell-death related immunity). In the current study, we used the same system to get insight into the responses to chitosan and how they relate to the signalling deployed by bacterial elicitors. While we find a large extent of overlap, there is a clear sign reversal with respect to the effect of calcium influx, which seems inhibitory in the context of chitosan signalling, while it is activating in the context of flg22 signalling. Furthermore, we observe a response of actin filaments as it is otherwise seen during cell-death related defence. We arrive at a model, where the different elicitors share the elements that convey the signalling, but differ with respect to their temporal signatures.

2. Materials and methods

2.1. Cell lines

We used for this study two grapevine suspension cells deriving from the North American wild species *V. rupestris* originally derived from pith parenchyma from non-lignified young shoots with internodes (Seibicke, 2002). One cell line was not transformed, the other line expressed a GFP fusion with the *Arabidopsis thaliana* β-tubulin 6 (AtTuB6) under control of the constitutive CaMV-35S promoter (Guan et al., 2015). To visualise the response of actin filaments, we used a cell line in the background of *V. vinifera* cv. Chardonnay expressing the actin-binding domain of *Arabidopsis thaliana* fimbrin 1 in fusion with GFP (Akaberi et al., 2018). All cell lines were cultivated in liquid MS medium in the dark at 25 °C at weekly subcultivation intervals as described in (Wang and Nick, 2017).

2.2. Elicitors and chemicals

If not stated otherwise, chitin signalling was elicited by 25 mg· L^{-1} chitosan (low molecular weight, 75% deacetylated, Sigma-Aldrich, Deisenhofen, Germany) dissolved in 1% of acetic acid, using 1% acetic acid as solvent control. If not stated otherwise, the responses were assessed at 60 min after elicitation. To address the role of potential signalling components, these components were pharmacologically modulated by pre-incubation for 30 min prior to elicitation. The role of calcium influx was probed by 100 μM of $GdCl_3$ (Sigma-Aldrich, Deisenhofen); that of apoplastic oxidative burst by 10 μM of Diphenylene Iodonium (DPI, Sigma-Aldrich, Deisenhofen), a blocker of the NADPH oxidase Respiratory burst oxidase Homologue (Chang et al., 2011), that of membrane fluidity by 10 mM of Benzyl Alcohol (BA, Carl Roth, Karlsruhe), a compound that renders biomembranes more fluid (Wang and Nick, 2017), and that of MAPK signalling by the specific mitogen-activated protein kinase inhibitor PD98059 (Sigma-Aldrich, Deisenhofen) in a concentration of 50 μM (Chang and Nick, 2012). To test potential effects of the solvent DMSO (DPI, BA, PD98059), controls testing the effect of pre-incubation with 1% DMSO were included as well as a negative control with medium. The cells were used at the onset of the cell expansion phase, at day 4 after subcultivation. All experiments were conducted in three independent biological series.

2.3. Measuring extracellular alkalinisation

Calcium influx as one of the earliest measurable defence responses can be monitored through a co-import of protons as extracellular alkalinisation (Felix et al., 1993). Extracellular alkalinisation was measured by a pH meter (pH 12, Schott Handylab) with a pH electrode (LoT 403-M8-S7/120, Mettler Toledo) to explore changes of extracellular pH. Prior to the actual measurement, the cell suspension was kept on a shaker for about 60 min till the pH was stable indicating completed pre-equilibration. Then chitosan was added to a final concentration of 25 mg·L^{-1} and the pH recorded over one hour in the dark. The response is transient, such that this time interval is sufficient to capture the entire response. Since the amplitude of the response depends on the number of cells (Suppl. Fig. S1), a volume of 4 ml corresponding to 0.8 g fresh weight was used for the measurements. Each condition was measured in at least four independent experimental series. Fig. 1.

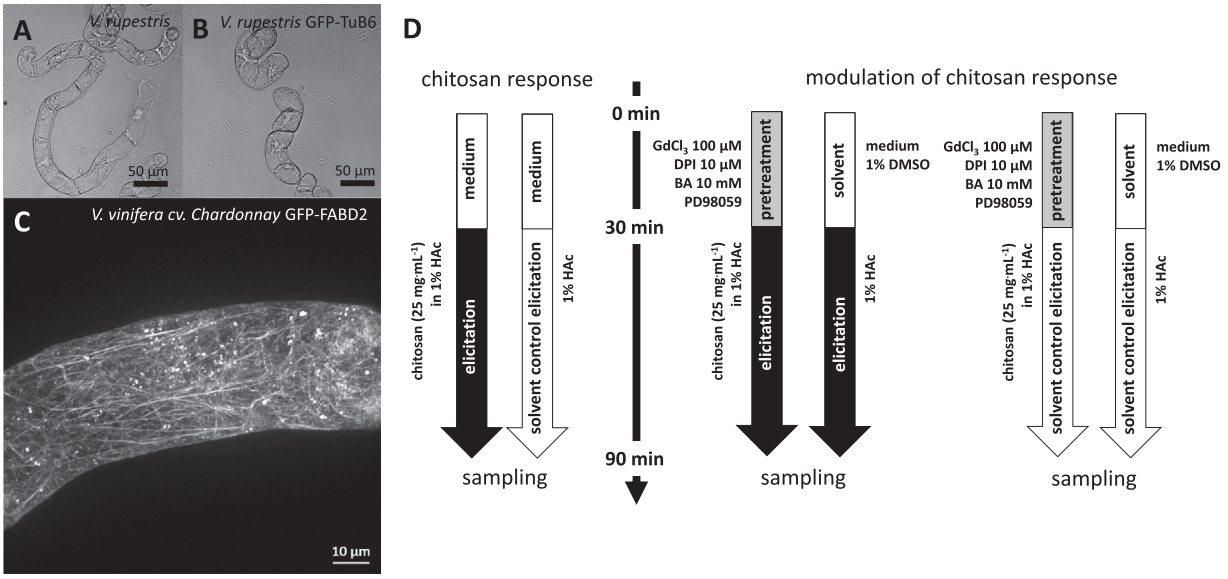

Fig. 1. Experimental material and experimental design. Expanding cells of the suspension lines *V. rupestris* **(A)**, *V. rupestris* GFP-TuB6 **(B)**, and GFP-FABD2 **(C)**, at day 4 after subcultivation in the differential inferference contrast **(A, B)**, or collecting the GFP signal **(C)**. **D** Experimental design to probe for the chitosan response itself (left) and for the pharmacological modulation of the chitosan response (right). Note that, for each inhibitor pretreatment, the respective solvent control is given in the corresponding line.

2.4. Visualisation and quantification of actin responses in grapevine cells

The responses of actin filaments were followed in the *V. vinifera* cv. Chardonnay FABD2-GFP line (Akaberi et al., 2018) in response to 25 $mg \cdot L^{-1}$, that were in some experiments pre-treated for 30 min with either 200 nM DPI or 1% of DMSO as solvent control prior to elicitation with chitosan. The fluorescently labelled actin filaments were visualised by spinning disc confocal microscopy on a AxioObserver Z1 (Zeiss, Jena, Germany) inverted microscope equipped with a spinning disc scan head (Yokogawa CSU-X1 Spinning Disk Unit, Yokogawa Electric Corporation, Tokyo, Japan), and a cooled digital CCD camera (AxioCamMRm; Zeiss). Actin bundling has often been quantified using the skew of the intensity distribution, but for elicitor-induced remodelling in cells, this approach is not sensitive enough. A discussion is given in (Wang et al., 2021a; Guan et al., 2014). In brief, depletion of cortical filaments in the periphery occurs concomitantly with bundling of transvacuolar cables. We used, therefore, two probing lines parallel to the long cell axis, and collected the intensity profiles parallel to the long axis of the cell, once in the centre, crossing the nucleus, once in the periphery, avoiding the nucleus using a line width of 10 pixels and the spline averaging option (ImageJ, https://imagej.nih.gov/ij/). The standard error over the profile reflects the difference between peaks (actin filaments) and troughs (the region between filaments). As long as the intensity histogram remains in the linear range, this method is independent of the absolute level of intensity and, thus, robust against variations of laser power, exposure time, or exposure gain. Nevertheless, all images were recorded at the same parameters by inactivating the automatic image acquisition routine of the software (ZEN, Zeiss, Jena). Around 5–10 cells per data point were used for the quantification.

2.5. Measuring expression of defence-related genes by real-time qPCR

The RNA was isolated using the universal RNA Purification Kit (Roboklon, Berlin, Germany) according to the protocol of the manufacturer. The extracted RNA was treated with an RNase-free DNase (Qiagen, Hilden, Germany) to remove any potential contamination by genomic DNA. The mRNA was reversely transcribed into cDNA using the M-MuLV cDNA Synthesis Kit (New England Biolabs, Frankfurt am Main, Germany) according to the instructions of the manufacturer. To prevent RNA degradation, RNase inhibitor (New England Biolabs, Frankfurt am Main, Germany) was added. The amount of RNA template was adjusted to 1 µg. To assess the defence response elicited by chitosan, we measured the steady-state transcript levels for the phytoalexin synthesis genes *PHENYLALANINE AMMONIA LYASE* (*PAL*), *RESVERATROL SYNTHASE* (*RS/STS47*), *STILBENE SYNTHASE* (*STS27*), the salicylate response gene *PATHOGENESIS RELATED 1* (*PR1*) and the jasmonate signalling gene *JASMONATE ZIM DOMAIN 1* (*JAZ1*), as well as the PR gene *CHITINASE 4* (*CHIT4*) by quantitative real-time RT-PCR using the primers listed in Table S1 of the supplementary material. The Real-Time RT-PCR was performed in a Bio-Rad CFX detection system (CFX96 Touch™ Real-Time PCR Detection System, Bio-Rad, München) according to the instructions of the manufacturer with the following programs: initial strand separation at 95 °C for 3 min followed by 39 cycles of strand separation at 95 °C for 15 s; annealing and elongation at 60 °C for 40 s. Values for relative transcript abundance were calculated using elongation factor 1α and actin as internal standards (Reid et al., 2006). The recorded C_t values were then used to estimate the steady-state level of the respective transcript either using the $2^{-\Delta\Delta Ct}$ method to quantify induction over the control of the respective gene, or the $2^{-\Delta Ct}$ to compare steady-state transcript levels across different genes (Livak and Schmittgen, 2001). Statistical significance was tested by a Student's t-test.

3. Results

3.1. Chitosan-induced apoplastic alkalinisation depends on calcium influx

Activation of calcium influx is one of the earliest defence responses in plant cells. To record calcium influx is far from trivial. While there exist fluorescent calcium dyes such as Fura, these have to be microinjected and the quantification has to rely on ratiometric measurements and image analysis of individual cells that have then to be compared statistically, a highly error-prone approach. Therefore, since the 1990ies, it has become a common practice in plant pathology to use extracellular alkalinisation caused by the co-transport of protons as proxy for calcium influx (Felix et al., 1993). Recently, the feasibility of this method in the same system (suspension cells of *V. rupestris*) has been verified using the membrane permeable fluorophore chloro-tetracycline (Nick et al.,

2021). Thus, the use of extracellular alkalinisation as readout for calcium influx is valid also for the cell line used in the current study. Here, we had observed earlier that this response is induced by the bacterial elicitors flg22 and harpin (Chang and Nick, 2012). We tested, therefore, whether chitosan would be able to deploy a similar response. In fact, extracellular pH increased rapidly from about 20 s after addition of 25 $mg \cdot L^{-1}$ chitosan, reaching a peak of around 0.8 pH units at 15 min, and dissipating slowly after this time point (Fig. 2**A**). The solvent control (1% acetic acid) did not show any significant pH response (Fig. 2**B**). To verify, whether the extracellular alkalinisation was caused by a co-transport of protons (Ding and Pickard, 1993), we pre-treated with 100 μM of $GdCl_3$, an inhibitor of mechanosensitive calcium channels. In fact, this reduced the amplitude of the response by a factor of four, to 0.2 pH units (Fig. 2**C**). Again, $GdCl_3$ alone did not elicit any significant pH response (Fig. 2**D**). To further probe for the specificity of this chitin response, we addressed the role of NADPH oxidases that are important stress inputs and can be specifically blocked by diphenylene iodonium (DPI). In *V. rupestris* cells, the activation of defence genes in response to bacterial elicitors can be effectively blocked by 10 μM of DPI [5 Chang]. However, the extracellular alkalinisation in response to chitosan was not affected (Fig. 2**E**). The amplitude (>0.8 pH units) was not significantly different from that seen for chitosan alone (Fig. 2**A**). Again, the solvent for DPI (1% DMSO) was only inducing a tiny (around 0.05 pH units) response. Thus, chitosan can induce a rapid and vigorous calcium influx that depends on Gd-sensitive calcium channels, but does not require NADPH oxidases.

3.2. *Chitosan rapidly induces defence-related transcripts requiring calcium influx*

To probe, whether the rapid calcium influx triggered by chitosan is followed by activation of defence-related transcripts, we selected two phytoalexin synthesis marker genes, *Phenylalanine Ammonia Lyase* (*PAL*) as the first committed step of the phenylpropanoid pathway, and *Resveratrol Synthase* (*RS*, *VvSTS47*) as member of the stilbene synthase family with a high responsiveness to fungal infection (Khattab et al., 2021), as well as the jasmonate signalling gene *JASMONATE ZIM DOMAIN* (*JAZ1*), which is a proxy for active jasmonate, a marker for PTI (Chang et al., 2017). We observed a rapid (1 h after elicitation) and strong accumulation for the two phytoalexin synthesis transcripts with inductions of around 30-fold over the solvent control (Fig. 3**A, B**). In contrast, the induction of *JAZ1* was much less pronounced with only around 4-fold (Fig. 3**C**). Likewise, the inductions of PR1 (around 2-fold) and STS27 (around +25%) were quite modest (Supplementary Fig. S2).

In the next step we tested, whether calcium influx, as immediate response to chitosan, is required for this induction of transcripts. We tested this, again, by pre-treatment with 100 μM of $GdCl_3$, which had effectively eliminated extracellular alkalinisation. To our surprise, we observed that this pre-treatment stimulated the accumulation of transcripts strongly, to around 100-fold of the control level in case of PAL (Fig. 4**A**), and even to around 150-fold in case of RS (Fig. 4**B**). Compared to the induction by chitosan alone, this represented a clear enhancement of response amplitude, by around 3.4-fold in case of *PAL*, and even 4.5-fold in case of *RS*. Interestingly, this enhancement was also seen for the stilbene synthase STS27 (boosted by a factor of 2.5-fold over the level seen with chitosan alone (Supplementary Fig. S2). Even $GdCl_3$ alone, without chitosan elicitation, stimulated these transcripts beyond the levels seen for chitosan.

Again, *JAZ1* showed a qualitatively different behaviour (Fig. 4**C**). Neither was the induction by chitosan enhanced by pretreatment with $GdCl_3$, nor did $GdCl_3$ in the absence of chitosan induce any response of these transcripts. Likewise, the salicylate response genes PR1 did not show any enhancement by $GdCl_3$ (Supplementary Fig. S2). Instead, the induction by chitosan was inhibited by $GdCl_3$.

3.3. *Chitosan-triggered transcript responses are blocked by DMSO*

To probe for the role of NAPD oxidases for chitosan induced gene expression, we pretreated with DPI before elicitation. At first sight, DPI seemed to be effective, since we observed that the activation of the

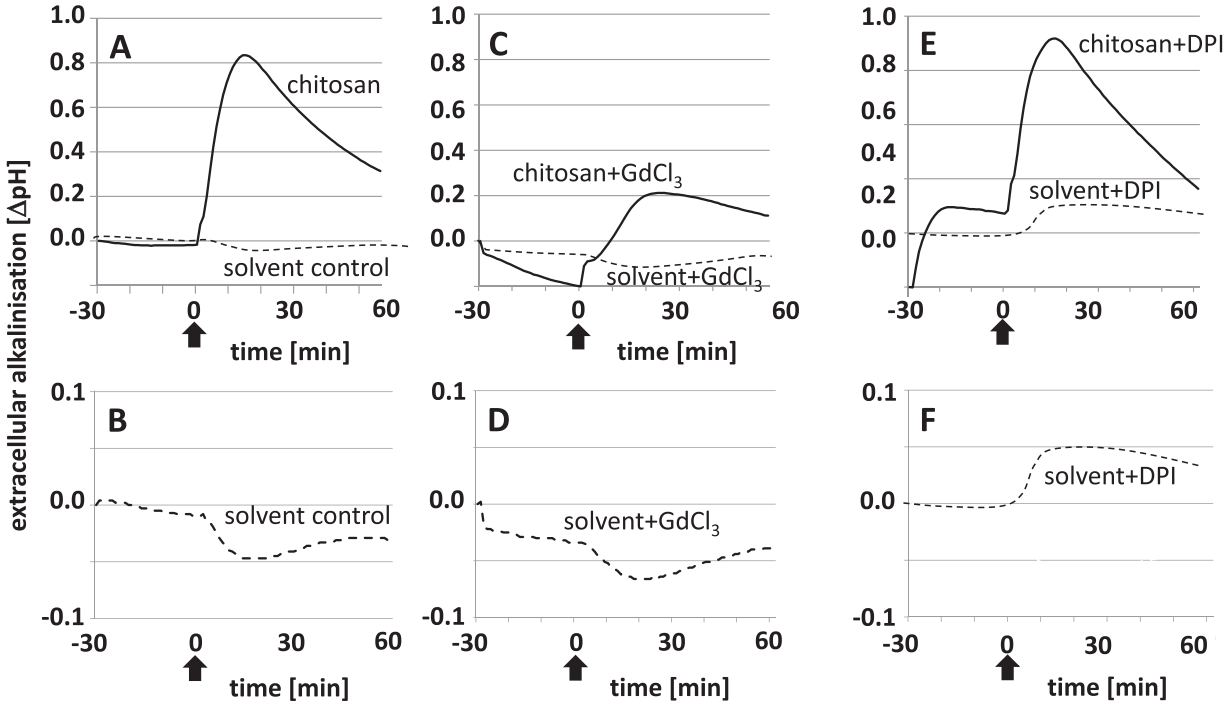

Fig. 2. Representative time courses of extracellular alkalinisation. Cells were preincubated for 30 min, at time 0 min, chitosan (25 $mg \cdot L^{-1}$ in 1% Hac) or the respective volume of 1% Hac as solvent control were added (arrows). **A, B** preincubation with water, **C, D** preincubation with 100 μM $GdCl_3$ **E, F** preincubation with 10 μM DPI.

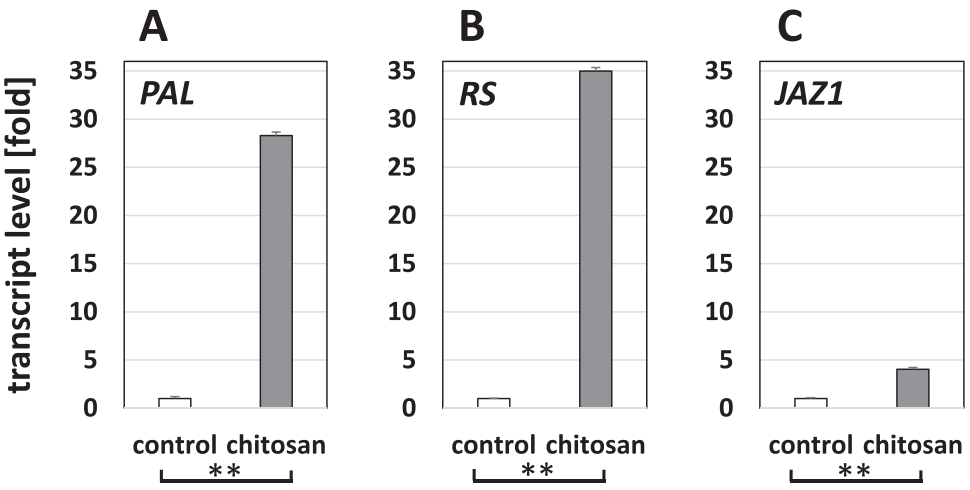

Fig. 3. Steady-state transcript levels for Phenylalanine Ammonia Lyase (PAL), Resveratrol Synthase (RS), a specific member of the stilbene synthase family, and Jasmonate-ZIM-domain 1 (JAZ1) scored after 1 h of elicitation with chitosan (25 $mg \cdot L^{-1}$) as compared to the control. C_t values from real-time qPCR were normalised to using elongation factor 1α and actin as internal standards. Data represent mean±SE from three independent experiments in technical triplicates. ** significant at $P < 0.01$ using a Student's t-test.

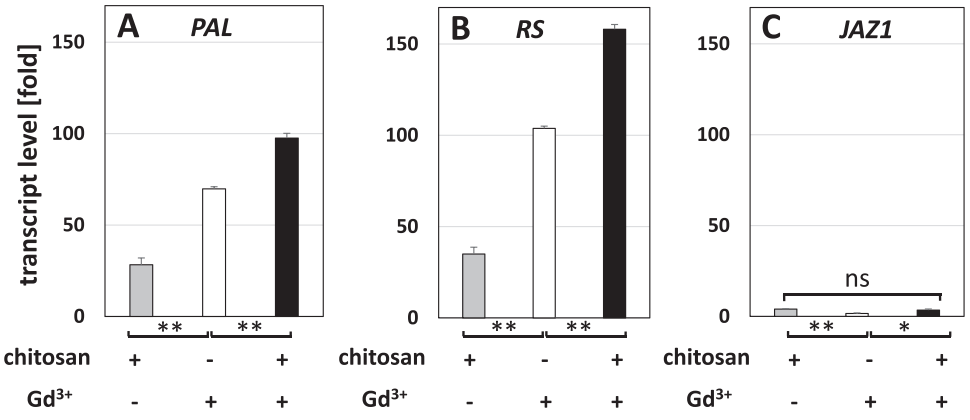

Fig. 4. Effect of calcium-channel blocker $GdCl_3$ on the induction of transcripts for PAL, RS, and JAZ1 by chitosan. $GdCl_3$ (100 μM) was added 30 min prior to elicitation by 25 $mg \cdot L^{-1}$ chitosan. Transcripts were scored after 1 h of elicitation with chitosan (25 $mg \cdot L^{-1}$) as compared to the control. C_t values from real-time qPCR were normalised to elongation factor 1α and actin as internal standards. Data represent mean±SE from three independent experiments in technical triplicate. * significant at $P < 0.05$, ** significant at $P < 0.01$, n.s. not significant using a Student's t-test.

phytoalexin synthesis transcripts by chitosan was strongly inhibited. The induction of *PAL* was reduced to 27% of the value seen without DPI pre-treatment (Fig. 5**A**), that of *RS* even to 16% (Fig. 5**B**). Again, the behaviour of *JAZ1* was deviant. Here, the induction by chitosan (which was much lower than that of *PAL* and *RS*) was only mildly inhibited by DPI to 59% (Fig. 5**C**). When the cells were just treated with DPI alone without subsequent elicitation by chitosan, we observed a slight (PAL 3-fold, RS 1.9-fold, JAZ1 1.2-fold) elevation of transcript levels over the untreated, non-elicited control. A closer look on these apparent effects of DPI revealed that the inhibition of chitosan induction was not due to DPI, but due to the solvent, DMSO because the inhibition seen in the solvent control (1% DMSO alone without DPI) was as strong as that seen for DPI. So, absence or presence of DPI did not make any difference as long as the cells encountered the solvent (Fig. 5). Thus, similar to the pattern seen for calcium influx, NADPH oxidases seemed dispensible for the induction of defence transcripts by chitosan. However, the strong inhibition of any induction by the 1% DMSO required as solvent for DPI would mask any possible effect of DPI itself.

When we probed for a potential role of MAPK cascades by using the inhibitor PD98059, we obtained a similar pattern (Fig. 6). The induction of *PAL* by chitosan was reduced to 28% of the value seen in the absence of the inhibitor (Fig. 6**A**). Again, for RS, the reduction was even more

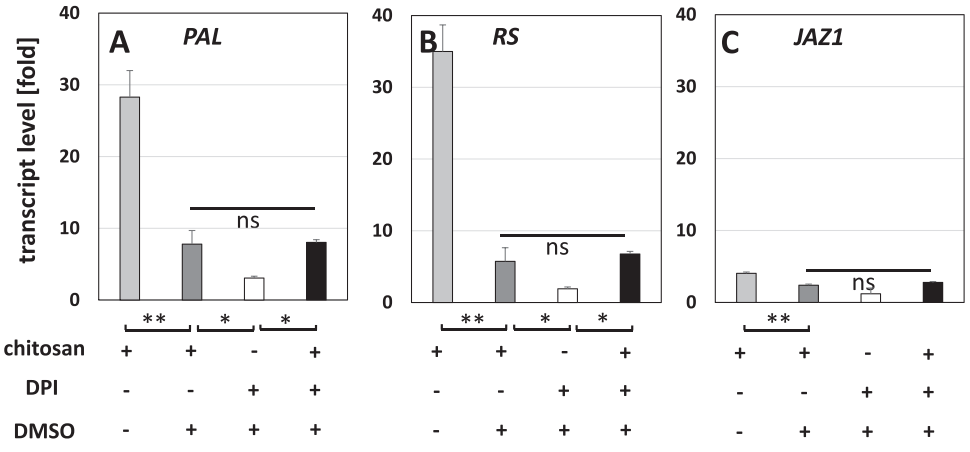

Fig. 5. Effect of the Respiratory burst oxidase Homolgoue inhibitor Diphenylene Iodonium (DPI) and of 1% DMSO on the induction of transcripts for PAL, RS, and JAZ1 by chitosan. DPI (10 μM) was added 30 min prior to elicitation by 25 $mg \cdot L^{-1}$ chitosan. Transcripts were scored after 1 h of elicitation with chitosan (25 $mg \cdot L^{-1}$) as compared to the control. C_t values from real-time qPCR were normalised to elongation factor 1α and actin as internal standards. Data represent mean±SE from three independent experiments in technical triplicate. * significant at $P < 0.05$, ** significant at $P < 0.01$, n.s. not significant using a Student's t-test.

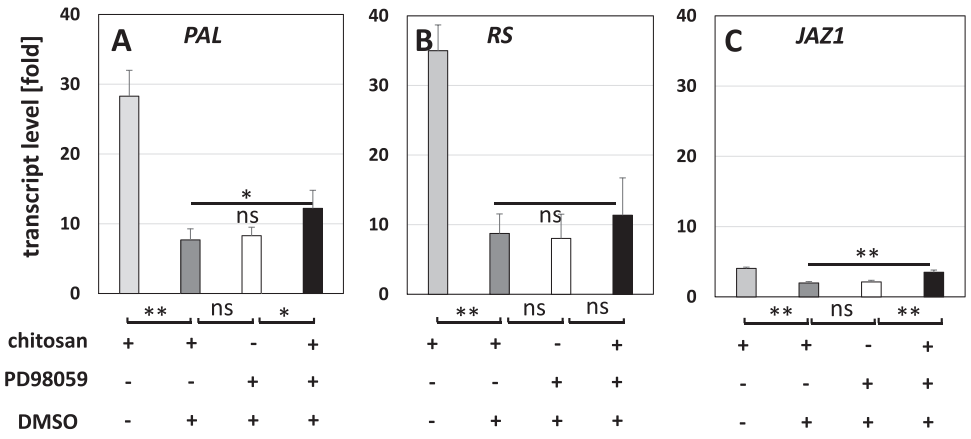

Fig. 6. Effect of the MAPK-signalling inhbitior PD98059 on the induction of transcripts for PAL, RS, and JAZ1 by chitosan. PD98059 (50 μM) was added 30 min prior to elicitation by 25 mg·L^{-1} chitosan, DMSO as solvent control was administered at 1%. Transcripts were scored after 1 h of elicitation with chitosan (25 mg·L^{-1}) as compared to the control. C_t values from real-time qPCR were normalised to using elongation factor 1α and actin as internal standards. Data represent mean±SE from three independent experiments in technical triplicates. * significant at $P < 0.05$, ** significant at $P < 0.01$, n.s. not significant using a Student's t-test.

pronounced decreasing to 22% (Fig. 6**B**), and for *JAZ1* it was weaker, down to 52% (Fig. 6**C**). Again, this apparent inhibition by PD98059 was the same as that seen for the solvent (1% DMSO) alone. Similar to the DPI treatment, the inhibition by the solvent masks any potential effect of PD98059 itself.

In summary, while an effect of DPI or of PD98059 can neither be confirmed, nor denied, these experiments show that 1% DMSO is silencing chitosan-induced gene expression very efficiently, leading to the question, whether membrane fluidity (DMSO is not only a widely used solvent, but also known as membrane rigidifier) is relevant for chitosan-triggered gene expression.

3.4. *Chitosan-triggered transcript responses depend on membrane fluidity*

To further test the possibility that DMSO might modulate chitosan-triggered gene expression due to its rigidifying effect o membrane fluidity, we tested the effect of Benzyl Alcohol (BA), a membrane fluidiser (Wang and Nick, 2017). Again, the induction of phytoalexin synthesis genes *Phenylalanine Ammonia Lyase* (*PAL*), and *Resveratrol Synthase* (*RS*), and *JASMONATE ZIM DOMAIN* (*JAZ1*) was monitored by qPCR (Fig. 7). BA inhibited the induction of all three transcripts significantly (*PAL*: by 49%; *RS*: by 28%, *JAZ1*: by 49%). Thus, the effect of BA was very similar to that of DMSO (compare Fig. 7 with Figs. 5, 6). Both compounds inhibited the induction of defence transcripts by chitosan (whereby this inhibition was more pronounced for DMSO compared to BA). This parallel effect of BA and DMSO appears paradox, since their effect on membrane fluidity is just opposed (BA acting as fluidiser, DMSO acting as rigidifier).

3.5. *Induction of chitinase 4 by chitosan is not blocked by Gd^{3+}*

During the preceding experiments we saw different regulatory patterns for *PAL* and *RS* on the one hand, and *JAZ1*, especially when calcium influx was inhibited by $GdCl_3$. While the induction of *PAL* and *RS* by chitosan was amplified under these conditions (Fig. 4**A, B**), this was not seen in case of *JAZ1* (Fig. 4**C**). This led to the question, which pattern would be observed for the induction of chitinase as central downstream response. We focussed on chitinase 4 as most abundant chitinase (Colas et al., 2012) and observed that this transcript was rapidly induced (Fig. 8) by chitosan by a factor of 7.2 fold over the resting level. While this induction appears weaker than that seen for PAL and RS, it has to be considered that this induction starts from a higher

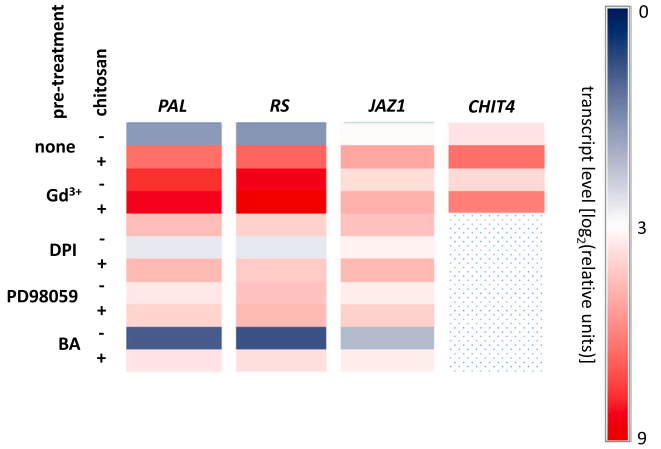

Fig. 8. Relative expression of Chitinase 4 (CHIT4) relative to PAL, RS, and JAZ1 1 h after elicitation by chitosan (25 mg·L^{-1}). C_t values from real-time qPCR were normalised to elongation factor 1α and actin as internal standards. Steady-state transcript levels were calculated from these ΔCt values.

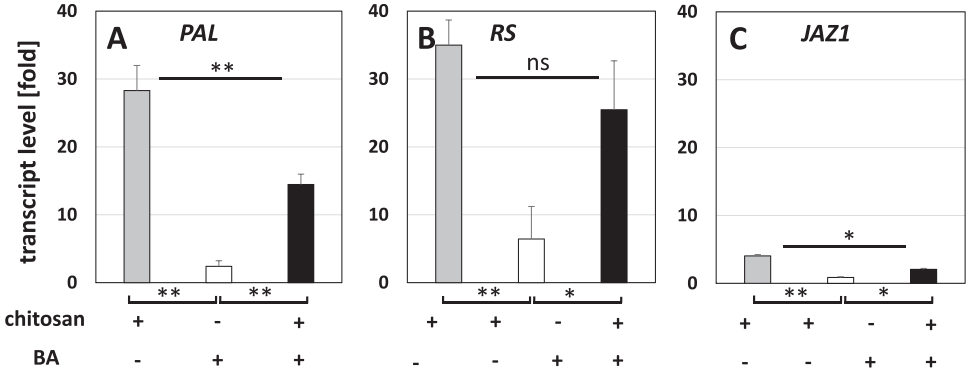

Fig. 7. Effect of the membrane fluidiser benzyl alcohol (BA) on the induction of transcripts for PAL, RS, and JAZ1 by chitosan. BA (10 mM) was added 30 min prior to elicitation by 25 mg·L^{-1} chitosan, DMSO as solvent control was administered at 1%. Transcripts were scored after 1 h of elicitation with chitosan (25 mg·L^{-1}) as compared to the control. C_t values from real-time qPCR were normalised to elongation factor 1α and actin as internal standards. Data represent mean±SE from three independent experiments in technical triplicates. * significant at $P < 0.05$, ** significant at $P < 0.01$, n.s. not significant using a Student's t-test.

(about 4-fold) resting level. Unlike for PAL and RS, pre-incubation with Gd^{3+} did not amplify the induction of chitinase 4, but rather caused a mild reduction (by 23% as compared to the levels seen for chitosan alone). Thus, chitinase 4 transcripts follow rather the pattern seen for *JAZ1*, not that seen for *PAL* and *RS*.

3.6. Chitosan causes actin remodelling

The cytoskeleton responds to pathogen attack by rapid remodelling (for a review on actin filaments see (Day et al., 2011), for a review on microtubules see (Hardham, 2013)). In previous work, we could show in grapevine cells that the actin remodelling was blocked by DPI and, thus, dependent on the NADPH oxidase Respiratory burst oxidase Homologue (RboH, (Chang et al., 2015; Wang et al., 2021b, 2021c). Thus, making use of grapevine cell lines, where either microtubules or actin filaments were tagged by GFP, we probed for a potential remodelling of the cytoskeleton in response to chitosan at 60 min, i.e., the same time point, when we had scored the transcripts. While cortical microtubules did not show any response whatsoever (Supplementary Fig. S3), we observed a substantial remodelling of actin filaments. In the controls, actin was organised as fine cortical meshwork (Fig. 9**A, B**). In cells that had been elicited with chitosan, this meshwork was depleted, instead cables of bundled actin emanated from the nuclear envelope (Fig. 9**C, D**). This central contraction could be quantified by quantitative image analysis from intensity profiles collected against a probing line in the direction of the main cell axis (Fig. 9**E, F**) and was found to be significant, independently of, whether the probing line was positioned in the cell periphery (Fig. 9**E**) or laid straight across the cell centre (Fig. 9**F**). This contraction was also observed in the cell periphery, if the cells were pre-treated with 1% DMSO for 30 min prior to administering chitosan. Likewise, a pretreatment with 10 μM DPI did not impinge on the induction of actin remodelling, if the cell periphery was considered (Fig. 9**E**). However, the remodelling of actin in the cell centre in response to chitosan was moderately, but significantly ($P < 0.05$) reduced after a pre-treatment with DPI, albeit the solvent, 1% DMSO, produced a similar effect (Fig. 9**F**). It should be noted that DPI alone caused already a partial remodelling in the cell centre, and chitosan did not change this effect of DPI. Thus, the remodelling of actin in response to chitosan is partially inhibited by DPI, but also by the solvent of DPI. The only effect of DPI beyond its solvent is a partial remodelling of actin in the cell centre, which afterwards remains resilient to chitosan. Despite this partial effect of the inhibitor alone, the actin remodelling induced by chitosan does not require the activity of RboH, but it seems to depend on membrane fluidity, which is reduced by DMSO.

4. Discussion

To get insight into the specificity of defence signalling, we mapped early defence responses triggered by chitosan, a fungal elicitor to see commonalities and differences with the responses deployed by bacterial elicitors, studied earlier in the same system, a grapevine cell culture system. We found that the fungal elicitor chitosan deployed the same set of responses as we have seen earlier in the same cells, when we used the bacterial elicitor flg22. However, the two elicitors differ fundamentally, when the role of this early calcium influx in gene activation is probed by Gd^{3+}. While we had seen in our previous work that calcium influx triggered by the bacterial elicitor flg22 is necessary to evoke activation of defence genes, we see for chitosan-induced gene activation that calcium influx acts as a negative regulator. This qualitative difference must derive from events different from calcium influx. In this context, we report that modulation of membrane fluidity (no matter, whether rigidification by treatment with DMSO or fluidisation by treatment with Benzyl Alcohol) quells chitosan induced gene expression. Furthermore, actin filaments show a remodelling. While defence related remodelling of actin is normally depending on the NADPH oxidase RboH, we see that the chitosan induced remodelling is independent of RboH. These observations stimulate the following questions that will be used to structure the discussion: 1. What is the role of calcium influx in chitosan signalling? 2. How can we integrate chitosan signalling into the signalling models developed for the bacterial elicitors flg22 (PTI) and harpin (ETI). 3. How is specificity generated in basal immunity?

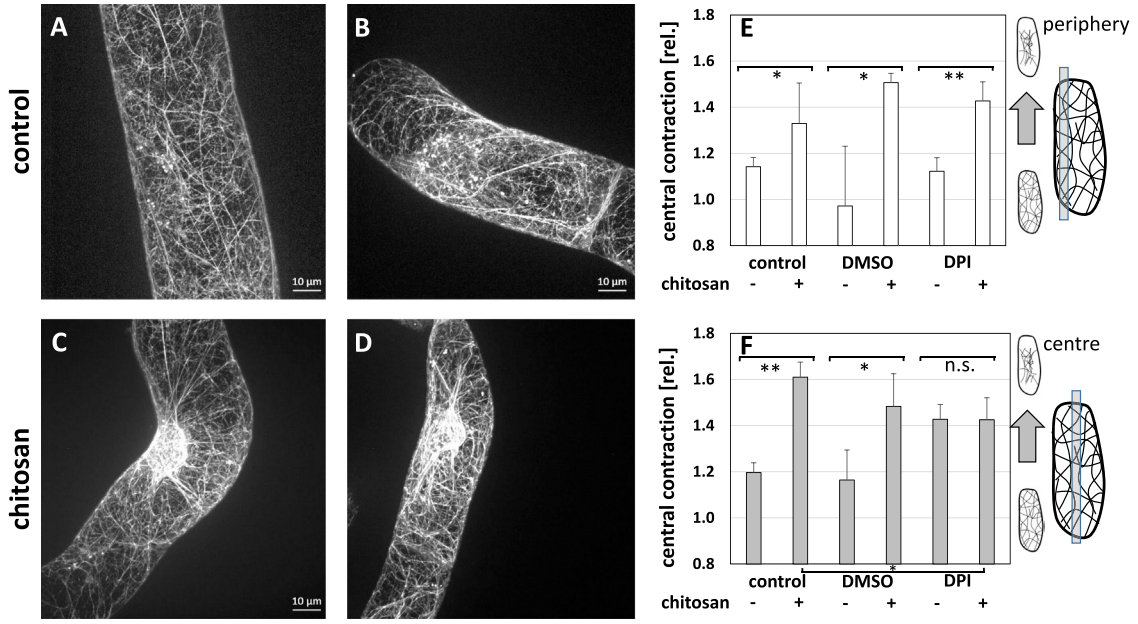

Fig. 9. Response of actin filaments to elicitation by chitosan monitored in the grapevine actin-marker line *V. vinifera* cv. Chardonnay FABD2-GFP line. Representative images collected by spinning-disc confocal microscopy for untreated control cells (**A, B**) and cells imaged 1 h after elicitation with 25 $mg \cdot L^{-1}$ chitosan (**C, D**). Quantification of the actin remodelling in the cell periphery (**E**) and in the centre (**F**) of cells in response to chitosan (scored at 1 h) with or without pretreatment for 30 min with either DMSO (1%) as solvent control or with DPI (10 μM). Data represent mean±SE from 5 to 10 individual cells per data point. * significant at $P < 0.05$, ** significant at $P < 0.01$, n.s. not significant using a Student's t-test.

4.1. Calcium influx – One signal, two meanings?

Calcium influx belongs to the earliest events that can be observed, when defence is deployed (for review see (Lecourieux et al., 2006)), and can be conveniently followed by virtue of extracellular alkalinisation due to co-transport of protons (Felix et al., 1993). In fact, we observe that the pH response triggered by chitosan can be partially suppressed by pretreatment with Gd^{3+} ions (Fig. 2**C**) that specifically impair calcium influx channels (Ding and Pickard, 1993). In the same cellular system, a comparable calcium influx was also observed in response to bacterial elicitors, such as flg22, or harpin, and in both cases inhibition by Gd^{3+} could be demonstrated as well (Chang and Nick, 2012). This leads to the question, how different elicitors (representing different microbes and hence demanding a different type of defence reaction) can be discriminated by the plant cell even though that they deploy the same early signalling response, calcium influx. For flg22, a bacterial PAMP that activates basal immunity and harpin, a bacterial elicitor evoking a cell-death related defence response, the specificity was shown to correlate with differences in the time course. While the alkalinisation response for flg22 was immediate, the response was delayed by about 5–10 min in case of harpin (Chang and Nick, 2012). Instead, the formation of reactive oxygen species by the NADPH oxidase RboH was swifter and more pronounced for harpin as compared to flg22. In case of chitosan, the alkalinisation was as swift and immediate as reported for flg22. Furthermore, chitosan elicitation led to the rapid and strong induction of defence related transcripts (Fig. 3), which is also observed in response to flg22. If calcium influx is the cause for this transcript induction, it should be possible to disrupt the response by Gd^{3+}. In fact, this implication had been confirmed for flg22 induced gene responses (Chang and Nick, 2012). However, for chitosan, the effect of Gd^{3+} was just inversed. Here, the induction of PAL and RS was not inhibited, but even amplified (Fig. 4**A, B**). Thus, the effect of Gd^{3+} on chitosan signalling is a mirror image of that seen for flg22 signalling. This inversion is difficult to reconcile with a linear model (as it had been proposed for flg22 (Chang and Nick, 2012), where chitosan, by eliciting calcium influx, and transduced further by MAPK signalling, causes the induction of defence genes. Instead, one needs to assume a bifurcation of signalling upstream of calcium influx, where chitosan on the one hand induces gene expression by a path that is independent of calcium influx, while, in parallel, activating calcium influx that turns now from a causative into a repressing signal. This model of a bifurcation is further corrobated by the observation that a further member of the stilbene synthase family (*STS27*) showed the enhancement by Gd^{3+} indicative of a repression by calcium influx, while, on the other hand, the induction of the salicylate response gene PR1 by chitosan was inhibited by Gd^{3+}, telling that calcium influx is here a necessary condition for signalling (Supplementary Fig. S2).

While the elicitors flg22 and chitosan are ligands to different receptors, they both compete for the chitin elicitor receptor kinase 1 CERK1 (Gong et al., 2019b), which implies that the two signalling chains should cross-talk. In fact, CERK1 can be recruited by a complex with BAK1 for the flg22 receptor FLS2 (Chinchilla et al., 2007), which will activate calcium influx. However, in combination with a different partner, LYK5, CERK1 can bind chitosan as ligand and deploy a phosphorylation cascade (Gong et al., 2019b). A physical interaction of CERK1 with a calcium channel has already been shown in the context of salt stress (Espinoza et al., 2017). Thus, when the calcium channel is blocked by Gd^{3+}, this might release CERK1 from its complex with BAK and FLS2, such that it is now available for chitosan signalling. A similar role of CERK1 as switch between different signal transduction chains has been recently reported for rice, where it can, in a complex with CeBiP, induce defence reactions, while, upon interaction with Nod Factor Receptor 5, symbiotic signalling is initiated (Zhang et al., 2021), and might, therefore, be a general principle.

Further, more indirect, evidence for the inhibitory activity of calcium influx upon chitosan-induced gene activation comes from the experiments with DMSO (Figs. 5, 6) and Benzyl Alcohol (Fig. 7). In the same system, cells of *V. rupestris*, we had observed earlier that DMSO can activate a rapid and strong alkalinisation which was blocked by Gd^{3+} ions supporting that it was driven by calcium influx. In contrast, alkalinisation was seen only after a lag of 5–10 min in response to Benzyl Alcohol (Nick et al., 2021). Benzyl alcohol can insert into lipid bilayers and weaken the lateral interactions between lipids, such that fluidity of the membrane increases (Ebihara et al., 1979), while DMSO has the opposite effect, as shown by fluorescence polarisation of 1,6-diphenyl-1, 3,5-hexatriene in protoplasts of alfalfa (Örvar et al., 2000). A straightforward explanation for the effect of DMSO upon chitosan-induced gene expression might be the enhanced activity of calcium influx upon rigidification of the membrane by DMSO. In fact, the effect of DMSO on alkalinisation was blocked by by Gd^{3+} ions supporting that it was driven by calcium influx (Nick et al., 2021). On the other hand, in the same study, DMSO was seen to suppress actin remodelling in response to the bacterial elicitor harpin. Since harpin acts through RboH, membrane fluidity seems to be required for the activity of RboH (Chang et al., 2011).

When CERK1 can switch between different partners, calcium influx might convey different signals: In response to flg22, it acts as primary signal reporting a bacterial infection. In response to chitosan, it acts as secondary signal (preceded by a phosphorylation cascade initiated from the CERK1-CBiP complex) reporting a fungal infection. A testable implication of this hypothesis would be that defence genes that are specific for defence against bacteria and fungi are differentially activated by these inputs and respond differently to Gd^{3+} pre-treatment. The fact that Gd^{3+} does not inhibit the induction of chitinase 4 transcripts by chitosan (Fig. 8) indicates that this implication might hold true.

Further support for parallel signal chains comes from the observation that the responses of the defence-related transcripts followed two patterns: while the chitosan response of the phytoalexin synthesis genes PAL and RS was amplified by Gd^{3+}, this was not only absent for CHITINASE 4, but also for JAZ1, a central regulator of basal immunity (Fig. 4**C**). This leads to the question, at what level the two regulatory cascades differ.

The stilbene synthases, responsible for the synthesis of resveratrol, the central phytoalexin of grapevine, are repressed by the transcription factor WRKY8 through interaction with the transcription factor MYB14 (Jiang et al., 2019). This repression is released upon activation of defence signalling, when MYB14 binds to MYB15 and, thus, can deploy stilbene synthesis (Höll et al., 2013). Also PAL, as first committed step of phenylpropanoid synthesis, is transcriptionally activated by recruiting a MYB factor by virtue of a specific ACII motif in its promoter (Craven-Bartle et al., 2013). A comparison of different accessions of *Vitis sylvestris* (the ancestor of domesticated grapevine) showed that activation of the MYB14 promoter correlated with the amplitude of stilbene synthases (Duan et al., 2016). This promoter was activated by jasmonic acid. Calcium influx was necessary and sufficient for this activation. Instead, the activation of JAZ transcripts is brought about directly by JA-Ile (reviewed in (Kazan and Manners, 2008), without the need for second messengers such as calcium.

4.2. Actin remodelling – A signal specifier?

In addition to calcium influx, we also observed a remodelling of actin as early response to chitosan (Fig. 9). This response was specific, since we did not see any significant response of microtubules (Supplementary Fig. S3). Actin remodelling is often observed in a defence context, and has often been interpreted in the context of forming callosic plugs around fungal penetration sites (reviewed in (Day et al., 2011)), but actin remodelling is also often heralding ensuing programmed cell death in many life forms (reviewed in (Franklin-Tong and Gourlay, 2008)), In grapevine cells, actin remodelling was identified as early event in cell-death related defence in response to the bacterial elicitor harpin, and elimination of actin by Latrunculin B was found to mitigate the

cell-death response (Chang et al., 2015). However, actin remodelling does not necessarily culminate in cell death, but can be integrated into basal immunity as well – recently, we demonstrated that aluminium ions, mediated through the RboH NADPH oxidase can induce actin remodelling that is not linked with programmed cell death (Wang et al., 2022). This actin response is followed by the activation of phytoalexin synthesis genes along with isochorismate synthase, a gene involved in the biosynthesis of salicylic acid. Actin remodelling was found to be necessary and sufficient for these responses that clearly fell into the realm of basal immunity. Interestingly, actin remodelling had also been found to be necessary and sufficient for the cell-death related defence in response to the bacterial elicitor harpin (Chang et al., 2015). This means that actin remodelling as such represents a crucial event in both types of innate immunity, basal immunity as well as cell-death related immunity. Hence, actin remodelling as such can not be a part of the machinery initiating or executing cell death, but must be an event upstream of the decision to deploy programmed cell death.

The difference might be linked with RboH and calcium influx. Actin remodelling depends on activation of RboH and, thus, can be inhibited by DPI in grapevine cells (Wang et al., 2022), but also in other systems, such as tobacco BY-2 (Eggenberger et al., 2017). However, while for induction by harpin, the actin response is followed by programmed cell death (Chang et al., 2015), this is not the case, if actin remodelling is triggered by aluminium (Eggenberger et al., 2017), nor, if actin is triggered by chitosan (this work). What delineates harpin triggered actin remodelling from the other two cases is the presence of a late extracellular alkalinisation that follows the oxidative burst mediated by RboH. While this proxy for calcium influx is rapid in case of PTI as it is triggered by flagellin (Chang and Nick, 2012) or by chitosan (this work), it is late in case of harpin (Chang and Nick, 2012; Chang et al., 2015). For aluminium as trigger, it is absent (Wang et al., 2022). Although it might appear astonishing that a signal (calcium influx) can assign a different "meaning" to actin remodelling, depending on the timing, it should be mentioned that cases, where the response to a signal depends on its "signature" (its pattern in time and space) are described progressively. For instance, whether accumulation of jasmonates during salt stress lead to cellular adaptation or to necrosis, depends on the temporal pattern of jasmonate accumulation (reviewed in (Ismail et al., 2014)). How the temporal signatures of oxidative burst and calcium are processed into differential defence responses, remains a rewarding research question.

4.3. *Specificity – A matter of modular combination?*

Although basal immunity is acting over a broad range of pathogens, it must have a certain specificity. For instance, activation of a chitinase is a very efficient approach to block a fungal attack, but will not help against phytopathogenic bacteria. On the other hand, phytoalexins that ward off fungi, often have also antibacterial activity, as is the case for resveratrol (Vestergaard and Ingmer, 2019). Thus, the signalling deployed by different PAMPs, while partially overlapping, must diverge at some point. To explain our data, we propose a working model that is based on modular combination (Fig. 10). Hereby, calcium-dependent signalling, if deployed alone, signals a bacterial attack. If calcium signalling is accompanied by CERK1 dependent signalling, this stands for a fungal attack. In both cases, basal immunity (*JAZ1*), as well as the phytoalexin synthesis machinery (*PAL*, *RS*) become active. However, chitinase is generated only in case of fungal attack. The actin remodelling in response to chitosan is quite certainly a parallel phenomenon.

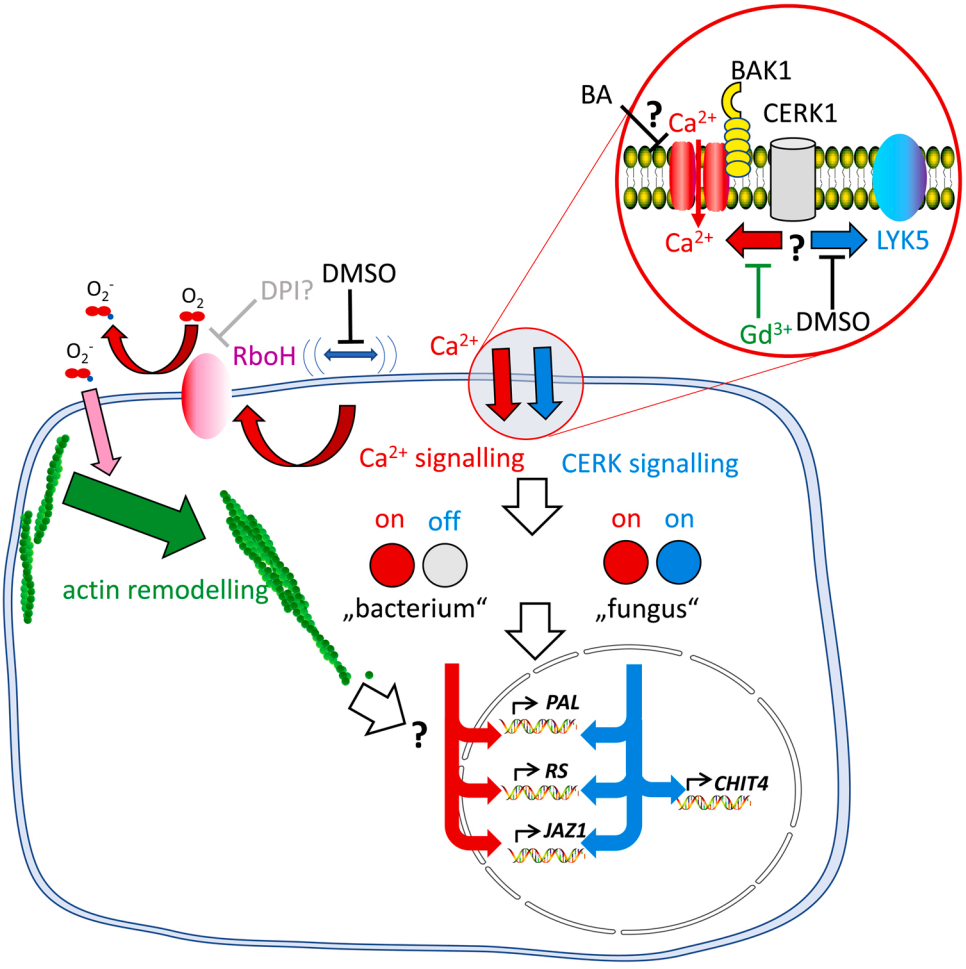

Fig. 10. Working model for the defence signalling triggered by chitosan in grapevine cells. The deregulation of gene expression by Gd3 + is explained by competition of calcium influx and chitin receptor LYK5 for the kinase CERK1. The combination of calcium influx and CERK-dependent signalling can be used to distinguish a bacterial from a fungal attack. The effect on actin seems indirect, probably through calcium activation of oxidative burst. Actin remodelling might feed back on defence signalling. The effect of DMSO as rigidifier and BA as fluidiser of the membrane might be due to interaction with different perceptive events at the membrane. The competence of different genes to the two signal chains differs. Question marks indicate open points, where future research should attach.

The calcium influx has been shown to activate the apoplastic oxidative burst through RboH (Chang and Nick, 2012), and we could show recently that this oxidative burst is necessary and sufficient for actin remodelling (Wang et al., 2022). In the same study, we could show that actin remodelling activates defence genes as well as salicylic acid synthesis and response genes. To what extent this will modulate defence gene expression in response to chitosan, remains to be elucidated. We tried to address the role of RboH with the specific inhibitor DPI, but observed that the solvent, DMSO, already exerted some effect on actin remodelling (similar to the effect of DMSO on chitosan dependent gene activation). As pointed out above, this effect is caused by the drop of membrane fluidity evoked by DMSO which will impair the activity of RboH (Chang et al., 2011). During the current work, we have found three points that indicate such signal divergence: First, the role of calcium influx – an event observed for the fungal elicitor chitosan as well as for the bacterial elicitor flg22 – acts inversely with respect to the tested phytoalexin synthesis transcripts (PAL, RS). In case of flg22, calcium influx is supportive, it is suppressive in case of chitosan. Second, the transcript for CHITINASE4, does not display this suppressive effect of calcium influx, while the transcripts for PAL and RS do. Third, actin remodelling triggered by the bacterial elicitor harpin is followed by PCD, while actin remodelling triggered by chitosan is not. Thus, the consequences of a given signalling event seem to depend on the context (of other signalling events?).

For the two bacterial elicitors flg22 (triggering PTI) and harpin (triggering an ETI-like response), our previous work could show that the relative timing correlates with the resulting output. While flg22 deploys calcium influx first, and RboH dependent oxidative burst, the sequence is reversed in case of harpin (Chang and Nick, 2012), and although both elicitors trigger phytoalexin synthesis genes in a similar manner, the resulting output differs, since in case of flg22 the glycosylated form of resveratrol, α-piceid, is produced, while harpin leads to the accumulation of the aglycon and its oxidative oligomers. In case of chitosan, the activation of calcium influx is as swift as it is for flg22, which would suggest that also the subsequent steps (calcium binding proteins, activation of MAPK cascades, transcriptional activation) should initiate swiftly.

Thus, a swift calcium influx cannot account for the specific differences in gene activation between flg22 and chitosan. A second input, which modulates defence, is the RboH dependent oxidative burst. This can be addressed by blocking this burst by the specific inhibitor DPI. Based on promoter-reporter assays using the promoter for MYB14, the activator of STS, induction by flg22 was shown to be completely eliminated by DPI (Duan et al., 2016). For chitosan, we were not able to detect any specific effect of DPI beyond that of the solvent. Thus, it might be the subsequent oxidative burst that defines the gene responses to a swift calcium influx. However, the activation of CERK1 signalling upon binding of chitosan might contribute as well (Hayafune et al., 2014). While the molecular details remain to be elucidated, the concept proposed to explain specificity would be that of a combinatorial signature of different input signals.

4.4. Conclusions

The current work was motivated by the question, how specificity is generated during innate immunity. Using a grapevine cell system, which during the past years had been mapped in detail for the responses to bacterial elicitors, we tested the responses to chitosan as fungal PAMP. We observed that many events overlapped, for instance calcium influx or the activation of phytalexin-synthesis related transcripts. However, the way, how these events were recruited for chitosan signalling was qualitatively different from flagellin-triggered signalling. The role of calcium influx was a complete mirror image – promotive for flagellin-triggered signalling, suppressive for chitosan-triggered signalling. We also observe actin remodelling, as it is often heralding programmed cell death, but this response seems to be independent of oxidative burst generated by the NADPH oxidase RboH. Our data are compatible with a model, where individual signalling events, such as calcium influx, or actin remodelling, are differently recruited in response to different triggers. Specificity would then be generated in a modular fashion by differential recombination. The molecular reflection of such a modular model would be the differential recruitment of co-receptors, such as CERK1 to either flagellin or chitosan signalling. Specificity by redistribution would also explain, why one pathway is silent, when the other pathway is on – it is basically zero-sum game, maintained by a limited abundance of signalling factors required for both pathways.

4.5. Outlook

Chitosan signalling overlaps with the flagellin-triggered pathway of defence, but results in a different and specific output. The modular interaction of early signalling must at one point culminate in differential downstream signalling. To pinpoint the branching point, it would be interesting to trigger both upstream pathways by combining chitosan with flg22, either simultaneously or in sequence to see, whether the two elicitors act antagonistically or synergistically on individual events of the pathway that might be probed using inhibitors. The de-repression of chitosan signalling by Gd^{3+} discovered in the current study would provide a very specific and salient diagnostic tool to map this interaction.Furthermore, the transcriptional cascade upstream of phytoalexin synthesis genes might be addressed, with MYB14 and WRKY33 being prime candidates. Last, but not least, the role of actin filaments for chitosan dependent gene expression might be probed using Latrunculin B, a specific and potent drug eliminating actin filaments.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgements

This work was supported by a DAAD-STIBET fellowship to KGS and the European Fund for Regional Development (Interreg Upper Rhine, project DialogProTec).

Author Contributions

KGS conceived and conducted the experiments, MR mentored the experimental work, PN supervised the project, generated the graphs and wrote the paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2022.111527.

References

S. Akaberi, H. Wang, P. Claudel, M. Riemann, B. Hause, P. Hugueney, P. Nick, Grapevine fatty acid hydroperoxide lyase generates actin-disrupting volatiles and promotes defence-related cell death, J. Exp. Bot. 69 (2018) 2883–2896.

T. Boller, G. Felix, A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors, Annu. Rev. Plant Biol. 60 (2009) 379–406.

X. Chang, E. Heene, F. Qiao, P. Nick, The phytoalexin resveratrol regulates the initiation of hypersensitive cell death in Vitis, PLoS ONE 6 (2011), e26405.

X. Chang, M. Riemann, Q. Liu, P. Nick, Actin as deathly switch? How auxin can suppress cell-death related defence, PloS One 10 (2015), e0125498.

X. Chang, M. Seo, Y. Takebayashi, Y. Kamiya, M. Riemann, P. Nick, Jasmonates are induced by the PAMP flg22 but not the cell death-inducing elicitor Harpin in *Vitis rupestris*, Protoplasma 254 (2017) 271–283.
X.L. Chang, P. Nick, Defence signalling triggered by Flg22 and harpin is integrated into a different stilbene output in vitis cells, Plos One 7 (2012), e40446.
D. Chinchilla, C. Zipfel, S. Robatzek, B. Kemmerling, T. Nürnberger, J.D. Jones, G. Felix, T. Boller, A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence, Nature 448 (2007) 497–500.
S. Colas, D. Afoufa-Bastien, L. Jacquens, C. Clément, F. Baillieul, F. Mazeyrat-Gourbeyre, L. Monti-Dedieu, Expression and in situ localization of two major PR proteins of grapevine berries during development and after UV-C exposition, PLoS One 7 (2012), e43681.
B. Craven-Bartle, M.B. Pascual, F.M. Cánovas, C. Avila, A. Myb, transcription factor regulates genes of the phenylalanine pathway in maritime pine, Plant J. 74 (2013) 755–766.
B. Day, J.L. Henty, K.J. Porter, C.J. Staiger, The pathogen-actin connection: a platform for defense signaling in plants, Annu. Rev. Phytopathol. 49 (2011) 483–506.
J.P. Ding, B.G. Pickard, Mechanosensory calcium-selective cation channels in epidermal cells, Plant J. 3 (1993) 83–110.
D. Duan, D. Halter, R. Baltenweck, C. Tisch, V. Tröster, A. Kortekamp, P. Hugueney, P. Nick, Genetic diversity of stilbene metabolism in *Vitis sylvestris*, J. Exp. Bot. 66 (2015) 3243–3257.
D. Duan, S. Fischer, P.R. Merz, J. Bogs, M. Riemann, P. Nick, An ancestral allele of grapevine transcription factor MYB14 promotes plant defence, J. Exp. Bot. 67 (2016) 1795–1804.
L. Ebihara, J.E. Hall, R.C. Macdonald, T.J. Mcintosh, S.A. Simon, Effect of benzyl alcohol on lipid bilayers-comparison of bilayer systems, Biophys. J. 28 (1979) 185–196.
K. Eggenberger, P. Sanyal, S. Hundt, P. Wadhwani, A.S. Ulrich, P. Nick, Challenge integrity: the cell-permeating peptide BP100 interferes with the actin-auxin oscillator, Plant Cell Physiol. 58 (2017) 71–85.
C. Espinoza, Y. Liang, G. Stacey, Chitin receptor CERK1 links salt stress and chitin-triggered innate immunity in arabidopsis, Plant J. 89 (2017) 984–995.
G. Felix, M. Regenass, T. Boller, Specific perception of subnanomolar concentrations of chitin fragments by tomato cells: induction of extracellular alkalinization, changes in protein phosphorylation, and establishment of a refractory state, Plant J. 4 (1993) 307–316.
V.E. Franklin-Tong, C.W. Gourlay, A role for actin in regulating apoptosis/programmed cell death: evidence spanning yeast, plants and animals, Biochem J. 413 (2008) 389–404.
L.P. Gianessi, N. Reigner, The Value of Fungicides in U.S. Crop Production, Croplife Foundation, 2005 (Washington, DC).
B.Q. Gong, J. Guo, N. Zhang, X. Yao, H.B. Wang, J.F. Li, Cross-microbial protection via priming a conserved immune co-receptor through juxtamembrane phosphorylation in plants, Cell Host Microbe 26 (2019b) 810–822.
P. Gong, M. Riemann, D. Dong, N. Stoeffler, G. Gross, A. Markel, P. Nick, Two grapevine metacaspase genes mediate ETI-like cell death in grapevine defence against infection of *Plasmopara viticola*, Protoplasma 256 (2019a) 951–969.
X. Guan, G. Buchholz, P. Nick, Actin marker lines in grapevine reveal a gatekeeper function of guard cells, J. Plant Physiol. 171 (2014) 1164–1173.
X. Guan, G. Buchholz, P. Nick, Tubulin marker line of grapevine suspension cells as a tool to follow early stress responses, J. Plant Physiol. 176 (2015) 118–128.
A.R. Hardham, Microtubules and biotic interactions, Plant J. 75 (2013) 278–289.
M. Hayafune, R. Berisio, R. Marchetti, A. Silipo, M. Kayama, Y. Desaki, S. Arima, F. Squeglia, A. Ruggiero, K. Tokuyasu, A. Molinaro, H. Kaku, N. Shibuya, Molecular basis of CEBiP-OsCERK1 activation, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) E404–E413.
J. Höll, A. Vannozzi, S. Czemmel, C. D'Onofrio, A.R. Walker, T. Rausch, M. Lucchin, P. K. Boss, I.B. Dry, J. Bogs, The R2R3-MYB transcription factors MYB14 and MYB15 regulate stilbene biosynthesis in *Vitis vinifera*, Plant Cell 25 (2013) 4135–4149.
C. Huang, Y. Yan, H. Zhao, Y. Ye, Y. Cao, Arabidopsis CPK5 phosphorylates the chitin receptor LYK5 to regulate plant innate immunity, Front. Plant Sci. 11 (2020) 702.
A. Ismail, S. Takeda, P. Nick, Life and death under salt stress: same players, different timing? J. Exp. Bot. 65 (2014) 2963–2979.
S.U. Jalil, M. Mishra, M.I. Ansari, Current view on chitinase for plant defence, Trends Biosci. 8 (2015) 6733–6743.
J. Jiang, H. Xi, Z. Dai, F. Lecourieux, L. Yuan, X. Liu, B. Patra, Y. Wei, S. Li, L. Wang, VvWRKY8 represses stilbene synthase genes through direct interaction with VvMYB14 to control resveratrol biosynthesis in grapevine, J. Exp. Bot. 70 (2019) 715–729.
Y. Jiao, W. Xu, D. Duan, Y. Wang, P. Nick, A stilbene synthase allele from a Chinese wild grapevine recruits salicylic acid signalling for efficient defence, J. Exp. Bot. 67 (2016) 5841–5856.
J.D.G. Jones, J.L. Dangl, The plant immune system, Nature 444 (2006) 323–329.
H. Kaku, Y. Nishizawa, N. Ishii-Minami, C. Akimoto-Tomiyama, N. Dohmae, K. Takio, E. Minami, N. Shibuya, Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 11086–11091.
K. Kazan, J.M. Manners, Jasmonate signaling: toward an integrated view, Plant Physiol. 146 (2008) 1459–1468.
I.M. Khattab, V.P. Sahi, R. Baltenweck, A.M. Grondard, P. Hugueney, E. Bieler, M. Dürrenberger, M. Riemann, P. Nick, Ancestral chemotypes of cultivated grapevine with resistance to Botryosphaeriaceae related Dieback allocate metabolism towards bioactive stilbenes, N. Phytol. 229 (2021) 1133–1146.
D. Lecourieux, R. Ranjeva, A. Pugin, Calcium in plant defence-signalling pathways, N. Phytol. 171 (2006) 249–269.
K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method, Methods 25 (2001) 402–408.
P. Nick, P. Guan, W.J. Shi, M. Riemann, Dissecting the membrane-microtubule sensor in grapevine defence, Hortic. Res. 8 (2021) 260.
B.L. Örvar, V. Sangwan, F. Omann, R.S. Dhindsa, Early steps in cold sensing by plant cells: role of actin cytoskeleton and membrane fluidity, Plant J. 23 (2000) 785–794.
R.C. Pearson, Powdery mildew, in: R.C. Pearson, A.C. Goheen (Eds.), Compendium of Grape Diseases, APS, St. Paul, 1988.
K.E. Reid, N. Olsson, J. Schlosser, F. Peng, S.T. Lund, An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development, BMC Plant Biol. 6 (2006) 27.
C. Sattler, H. Kächele, G. Verch, Assessing the intensity of pesticide use in agriculture, Agric., Ecosyst. Environ. 119 (2007) 299–304.
Seibicke, T., Untersuchungen zur induzierten Resistenz an Vitis spec. PhD thesis. University of Freiburg (2002).
T. Shimizu, T. Nakano, D. Takamizawa, Y. Desaki, N. Ishii-Minami, Y. Nishizawa, E. Minami, K. Okada, H. Yamane, H. Kaku, N. Shibuya, Two LysM receptor molecules CEBiP and OsCERK1, cooperatively regulate chitin elicitor signaling in rice, Plant J. 64 (2010) (2010) 204–214.
Pl Trotel-Aziz, M. Couderchet, G. Vernet, A. Aziz, Chitosan stimulates defense reactions in grapevine leaves and inhibits development of botrytis cinerea, Eur. J. Plant Pathol. 114 (2006) 405–413.
M. Vestergaard, H. Ingmer, Antibacterial and antifungal properties of resveratrol, Intern. J. Antimicrob. Agents 53 (2019) 716–723.
H. Wang, M. Riemann, Q. Liu, J. Siegrist, P. Nick, Glycyrrhizin, the active compound of the TCM drug Gan Cao stimulates actin remodelling and defence in grapevine, Plant Sci. 302 (2021a), 110712.
H. Wang, M. Riemann, Q. Liu, J. Siegrist, P. Nick, Glycyrrhizin, the active compound of the TCM drug Gan Cao stimulates actin remodelling and defence in grapevine, Plant Sci. 302 (2021b), 110712.
L. Wang, P. Nick, Cold sensing in grapevine - which signals are upstream of the microtubular "thermometer", Plant Cell Environ. 40 (2017) 2844–2857.
R. Wang, D. Dong, C. Metzger, X. Zhu, M. Riemann, M. Pla, P. Nick, Aluminium can activate grapevine defence through actin remodelling, Horticult. Res. (2021c).
R. Wang, D. Dong, C. Metzger, X. Zhu, M. Riemann, M. Pla, P. Nick, Aluminium can activate grapevine defence through actin remodelling, Horticult. Res. (2022).
C. Zhang, J.M. He, H.L. Dai, G. Wang, X.W. Zhang, C. Wang, J.C. Shi, X. Chen, D.P. Wang, E.T. Wang, Discriminating symbiosis and immunity signals by receptor competition in rice, Proc. Natl. Acad. USA 118 (2021), e2023738118.