Protoplasma
https://doi.org/10.1007/s00709-026-02206-w

RESEARCH

Priming of defence signalling in grapevine cells depends on actin filaments

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Received: 30 September 2025 / Accepted: 28 April 2026

Abstract
Defence priming has attracted significant attention as a potential sustainable strategy to enhance plant immunity. In this study, we show that pre-treatment with aluminium ions can amplify the response of transcripts for phytoalexin synthesis and their transcriptional regulators to the bacterial elicitor harpin, although these transcripts are not induced by aluminium alone. The priming effect of aluminium depends on actin, and is accompanied by activation of genes linked with aluminium tolerance. As a result, the cell-death response to harpin is mitigated, again depending on actin filaments. We discuss these findings in the context of a working model, where actin remodelling (triggered by aluminium) can promote defence signalling independently of a Hypersensitive Response and discuss potential implications for the use of priming in sustainable viticulture.

Keywords Actin filaments · Aluminium · Grapevine · Priming · Phytoalexins · *Vitis vinifera* L.

Introduction

Throughout its entire history, life has been accompanied by parasitism. Taxonomic estimates indicate that about 30–50% of described species are host-associated and live inside other organisms (de Meeûs and Renaud 2002). As to be expected, systems to detect and ward off a pathogen attack, have developed early in evolution. The outcome of a host-pathogen interaction is usually depending on time. Resistance usually means that the host can deploy protective responses before the pathogen has the chance to advance its development. Our concepts of immunity have been biased by the adaptive immunity of vertebrates. While this type of immunity is powerful and specific, it is confined to the vertebrates and neither found in invertebrates, nor in plants (for review see Swann 2014). The predominant type of immunity is innate (for reviews see Melillo et al. 2018), which means that the perceptive structures are genetically hardwired, and, therefore, do not require the learning process that is characteristic for inducible immunity. This learning process needs considerable time, and, therefore, even vertebrates do not rely exclusively on inducible immunity. In naive animals, innate immunity is responsible for the early, immediate response to a pathogen. Only in future encounters, the trained immune system can command the specific response rapidly enough to fight down the intruder. Thus, inducible immunity displays a pronounced memory of previous experiences with the respective pathogen. Since inducible immunity benefits from experience, the question arises, whether innate immunity is able to learn from previous encounters as well. This seems to be the case indeed, a phenomenon that is often referred to as priming (Reimer-Michalski and Conrath 2016).

The immune-stimulating effects of non-protein amino acids, such as β-aminobutyric acid (BABA), or benzo(1,2,3)thiadiazole-7-carbothioic acid (BTH) have often been discussed in the context of priming, because these compounds mimic salicylic acid, a hormone that is often formed in a defence context (for a classical review

Handling Editor: Martina Sombetzki

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Published online: 25 May 2026

see Conrath et al. 2002). It is interesting to consider, what molecular mechanisms are involved here. A study, where *Arabidopsis thaliana* was "primed" with BTH against the bacterial pathogen *Pseudomonas syringae*, demonstrated that the priming treatment induced the expression of FLS2, the receptor for the bacterial elicitor flagellin, and its co-receptor BAK1 (Tateda et al. 2014). Here, the priming effect was achieved by boosting the perception to pathogen-associated molecular patterns (PAMPs) which should result in a higher sensitivity to pathogen attack and a swifter defence response. Along a similar line, BTH amplified the defence responses of parsley cells against an oomycete elicitor, such that otherwise non-inducive elicitor concentrations were sufficient to activate the transcripts needed for the synthesis of coumarin phytoalexins (Katz et al. 1998). Again, this priming effect was linked to an increase of sensitivity. Likewise, a third example, where potatoes were primed for resistance against the causative agent of Potato Blight, *Phytophthora infestans* by aluminium, was linked with changes in signalling (Arasimowicz-Jelonek et al. 2014), in this case, it was the systemic signalling through nitric oxid that was amplified. While it should be kept in mind that pre-treatment with mimics of defence-related hormones represents just an operational approximation to real-world priming, one can conclude that priming seems to target the perceptive part of defence, rather than its downstream execution. From the point of view of resource allocation, this seems rational, because signalling components are not bulk molecules, but typically occur in only a small number, while boosting a down-stream executor of defence, for instance, an enzyme, represents a much larger investment that is likely to bind resources that otherwise would be available for growth and development. The strategy to amplify pathogen perception rather than defence execution allows to draw analogies to vertebrate immunity, where immunity memory is embodied in a small, but rapidly inducible population of memory cells (for review see Ratajczak et al. 2018).

For the immobile plant cells, such a mechanism would not be feasible. Here, the memory must act on the level of the individual cell. Pathogen-associated molecular patterns (PAMPs) are recognised by receptors localised in the plasma membrane (for review see Boller and Felix 2009) that activate rapid signalling responses including calcium influx, phosphorylation cascades, and an apoplastic oxidative burst, driven by the membrane located NADPH oxidase Respiratory burst oxidase Homologue (RboH). These early events culminate in activation of phytoalexin synthesis genes and basal immunity, also known as PAMP triggered immunity (PTI). During co-evolution with their hosts, some pathogens have developed so-called effectors that can silence components of PAMP signalling or response. Some hosts, in turn, can sense these effectors by cytoplasmic proteins harbouring a leucine-rich repeat and a nucleotide-binding site. When these proteins bind the effectors, they can re-install defence, often linked with a Hypersensitive Response (HR). In other words, in contrast to basal immunity, where the cell survives, here, the attacked cell undergoes Programmed Cell Death, thus, arresting the pathogen attack and saving its neighbours from infection (for review see Balint-Kurti 2019). It should be noted that the perceptive events differ with respect to subcellular localisation – PAMP signalling is initiated by binding of ligands at the apoplastic face of the plasma membrane, HR signalling is initiated inside of the cell requiring that pathogen proteins have successfully breached the membrane of the host. This difference in the localisation of the perceptive event bears on the potential mechanism of defence signalling priming. Priming targeted to PAMP triggered basal immunity is expected to alter the abundance or activity of the membrane located receptors, or to amplify the primary signalling at the membrane, such as calcium influx or oxidative burst. Priming targeted to the HR, would more likely alter the expression of the intracellular sensing of the effectors, or the signalling activating Programmed Cell Death.

A comparative study in grapevine cells, where basal immunity or HR can be triggered by different bacterial elicitors (Chang and Nick 2012) revealed that RboH-dependent oxidative burst is a common signalling event. However, the resulting type of defence response depends on the timing of this oxidative burst. When it follows calcium influx, basal immunity ensues, when it precedes calcium influx, defence is accompanied by Programmed Cell Death.

These considerations lead to the hypothesis that priming might be linked with a modulation of RboH activity. In grapevine cells, this can be experimentally achieved by aluminium ions, followed by actin remodelling and activation of defence transcripts including salicylic acid synthesis and response (Wang et al. 2022). In the current study, we explore, whether aluminium ions can prime the response of grapevine cells to the bacterial elicitor, and whether this response depends on actin. Furthermore, we test the possibility that signalling from organelles to the nucleus, so-called retrograde signalling, might be a target of this priming effect.

Materials and methods

Cell cultivation and priming by aluminium

Suspension cultures of *Vitis rupestris* were grown in a modified Murashige-Skoog liquid at weekly subcultivation as described previously (Maisch and Nick 2007). For

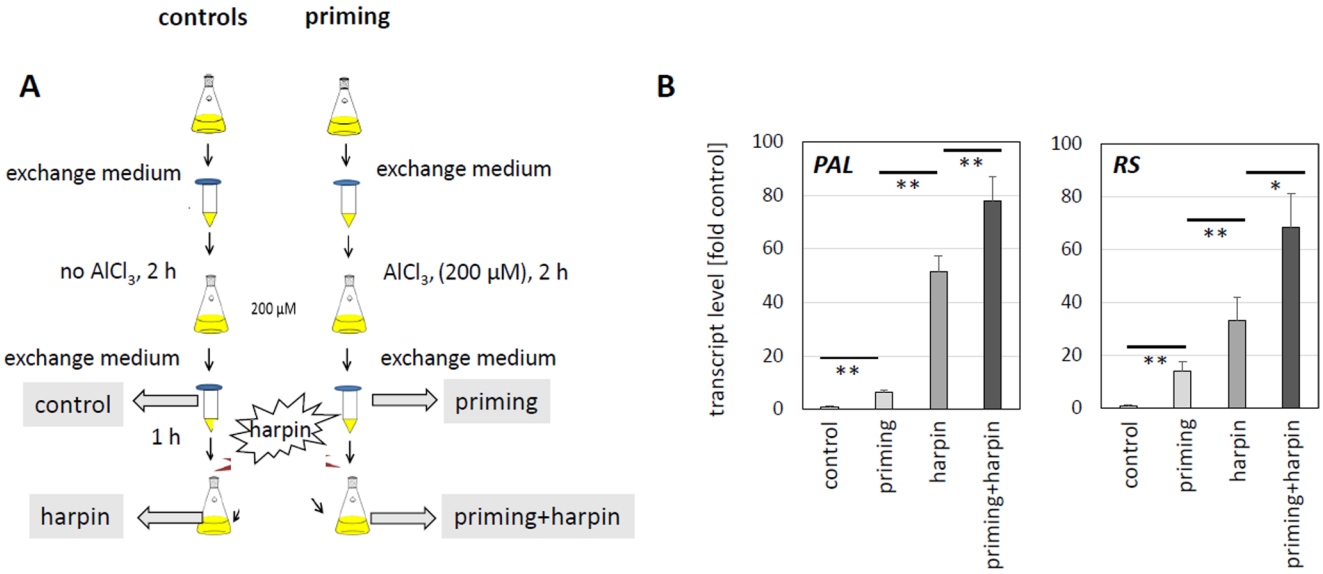

Fig. 1 Priming of the phytoalexin transcript response by aluminium in suspension cells of *V. rupestris*. **A** Operationalisation of priming. To ensure the solubility of $AlCl_3$, the treatment had to occur in minimal medium at low pH requiring medium exchange prior and subsequent to Al treatment. Therefore, the control was treated in the same manner, but omitting Al. After approximately 1 h, harpin (9 µg·mL^{-1}) was added to both the control group and the aluminum-pretreated group to determine whether defence signaling had been activated. **B** Steady-state transcript levels for the phytoalexin synthesis genes *Phenylammonium Lyase* (*PAL*) and *Resveratrol Synthase* (*RS*, *STS47*) for the three set-ups shown in **A**. Data represent mean values and standard errors from three independent experimental series with three technical replications for each biological replicate. Transcript levels are calibrated to *EF 1α* as the internal standard. Asterisks indicate significant differences with * $P<0.05$, and ** $P<0.01$

priming by aluminium (Fig. 1A), cells were collected by sterile filtration through a bottle-top filter (Nalgene) and resuspended in 3% sucrose, acidified to pH 4.5 using 1 M HCl and supplemented with 3 mM $CaCl_2$ and 200 µM $AlCl_3$ (Sigma-Aldrich, Deisenhofen, Germany). The cells were then returned to the shaker for another 2 h before elicitation with harpin 9 µg·mL^{-1} (ProAct, 1% w/v harpin protein, Plant Health Cure, Ojsterwijk, Netherlands). Controls included a treatment in the same acidified medium, but without aluminium (Fig. 1A). In both cases, sampling prior and after the harpin treatment were used to separate the immediate effect of priming from its combination with elicitation. In some experiments, the actin-eliminating drug Latrunculin B (sequestering G-actin from integration into the growing filament, Sigma-Aldrich, Deisenhofen, Germany), or the actin-stabilising drug phalloidin (Sigma-Aldrich, Deisenhofen, Germany) were added to the pre-treatment with aluminium. Harpin itself directly triggers cell death in the tissues, so using aluminum as a pre-elicitor before harpin treatment.

Analysis of gene expression

The steady-state transcript levels of the selected genes (*PAL*, *RS*, *MYB14*, *MYBA*, *MYBF1*, *3AT*, *WRKY22*, *AOX1a and NAC017*) were quantified using quantitative real-time PCR (qRT-PCR). Details on oligonucleotide primer sequences and gene accession numbers are specified in Supplemental Table 1 as described in Wang et al. (2022). The results were expressed according to Livak and Schmittgen (2001) either as $2^{-\Delta\Delta CT}$ to give the fold-induction as compared to the control or as $2^{-\Delta CT}$ to show relative expression levels of different transcripts. Data represent three independent experimental series, each in technical triplicates.

Determination of cell mortality

Cell mortality was determined by the Evans Blue Dye Exclusion assay (Gaff and Okong'O-Ogola 1971) with minor modifications as described in Wang et al. (2022). Mortality values represent mean and standard errors from three biological replicates, each comprising 500 individual cells.

Statistical analysis

Statistical analyses were performed using SPSS software (version 26.0). For normally distributed data, two-group comparisons were conducted with Student's t-test (independent samples t-test). For multiple-group comparisons, one-way analysis of variance (ANOVA) was first applied, followed by Tukey's honestly significant difference (HSD) post-hoc test if the ANOVA was significant ($P<0.05$).

Results

Priming with aluminium significantly amplifies induction of defence genes.

Aluminium activates Respiratory Burst Oxidase Homologue (RboH), a central regulator of grapevine immunity, and can stabilize microfilaments and partially preserve them even in the presence of latrunculin B (Wang et al. 2022). We therefore assessed whether aluminium exposure alters defence gene induction. For this purpose, harpin from the phytopathogen Erwinia amylovora was used as the elicitor, which triggers a hypersensitive response in *Vitis rupestris* (Chang and Nick 2012). To monitor the defence response, we scored the steady-state transcript levels of *Phenyl Ammonium Lyase* (*PAL*), the key enzyme for the entire phenylpropanoid pathway, and *Resveratrol Synthase* (*RS*), synonymous with *Stilbene Synthase 47* (*STS47*), a member of the enzyme family controlling the synthesis of stilbenes, the central phytoalexins of grapevine. The priming treatment itself induced the basal transcript levels of both transcripts significantly by around one order of magnitude (Fig. 1B). However, compared to the elicitation by harpin alone, the effect of priming was much weaker. For *PAL*, harpin yielded some 50-fold of induction, for *RS*, the induction was 35-fold. When harpin was administered after priming by aluminium, the response was amplified for both transcripts. For *PAL*, the induction was 78-fold, for *RS*, it was 68-fold. The induction for this combined treatment was exceeding the values expected for a mere addition of the individual factors. For instance, for *PAL*, a mere addition of the induction by priming and the induction by harpin would yield an induction of 57-fold. For *RS*, the respective value would be 47-fold. Thus, priming and elicitation act not additively, but synergistically. Regarding the potential effect of pH on gene expression, we would like to clarify that Al^{3+} addition caused only a minimal decrease in pH (approximately 0.1 unit; Wang et al. 2022; Suppl. Fig. S3), which is negligible and does not create a meaningful difference between the two groups. Moreover, our previous published results showed that such a slight pH fluctuation has no obvious effect on the expression of the target defense genes. Therefore, the observed changes in defense gene expression can be attributed to Al^{3+} treatment rather than pH variation.

Priming targets transcriptional regulators of phenylpropanoid metabolism

To get insight into the mechanism mediating the priming effect of phytoalexin synthesis genes, we probed transcript levels for key regulators of phenylpropanoid metabolism (Wang et al. 2022) (Fig. 2A). These included *MYB14*, a key transcriptional activator for the expression of Stilbene Synthases (STS), generating the crucial phytoalexin *trans*-resveratrol, *MYBF1*, activating the expression of Flavonol Synthase (FLS), producing quercetine, and *MYBA*, activating the expression of 3-Anthocyanin Alkyl Transferase (3AT), generating cyanidine-type anthocyanins. With exception of *MYBA*, we observed a very clear synergistic interaction between priming and elicitation. For *MYB14* (Fig. 2B), this synergy was more pronounced as compared to its downstream target Resveratrol Synthase (Fig. 1B). For *MYBF1* (Fig. 2C), the priming itself was even inhibitory, while it amplified the response to harpin by threefold. For *MYBA* (Fig. 2D), no such synergy was observed. Here, harpin induced the transcript to the same degree, irrespective of the priming pre-treatment. Interestingly, the downstream target of this transcription factor, *3AT* (Fig. 2E), displayed the most pronounced synergy of elicitation and priming among all tested transcripts. Thus, the regulators of the key enzymes, controlling the bifurcation into the stilbene and the quercetine branches, are prominent targets of priming, while for the cyanidine-type anthocyanins, the priming addresses the enzyme *3AT*, rather than its regulator *MYBA*.

Actin filaments are necessary for induction of defence genes by harpin

Since aluminium, used for priming, is causing a remodelling of actin that is depending on the NADPH oxidase Respiratory burst oxidase Homologue, (Wang et al. 2022). We wondered, whether priming could be abolished by Latrunculin B, a compound eliminating actin filaments. Indeed, using again the phytoalexin synthesis genes *PAL* and *RS* we observed a drastic effect of Latrunculin B on the expression of these genes (Fig. 3). Latrunculin B alone induced a significant upregulation (around 15-fold) of *PAL* transcripts, a level that was maintained when the priming aluminium treatment was administered alone. In contrast, the strong induction by harpin was completely suppressed, no matter, whether this elicitor was given alone or following a priming treatment with aluminium. For *RS*, the pattern was similar – after pre-treatment with Latrunculin B, harpin was unable to elicit any activation of transcripts. Minor differences were seen for the induction by Latrunculin B alone (which was weaker than for *PAL*), and an inhibition of transcript accumulation in response to aluminium by this inhibitor (which was not observed for *PAL*). These data show clearly that actin filaments are necessary for gene induction by harpin. Latrunculin B also overrules the effect of priming on this induction by harpin.

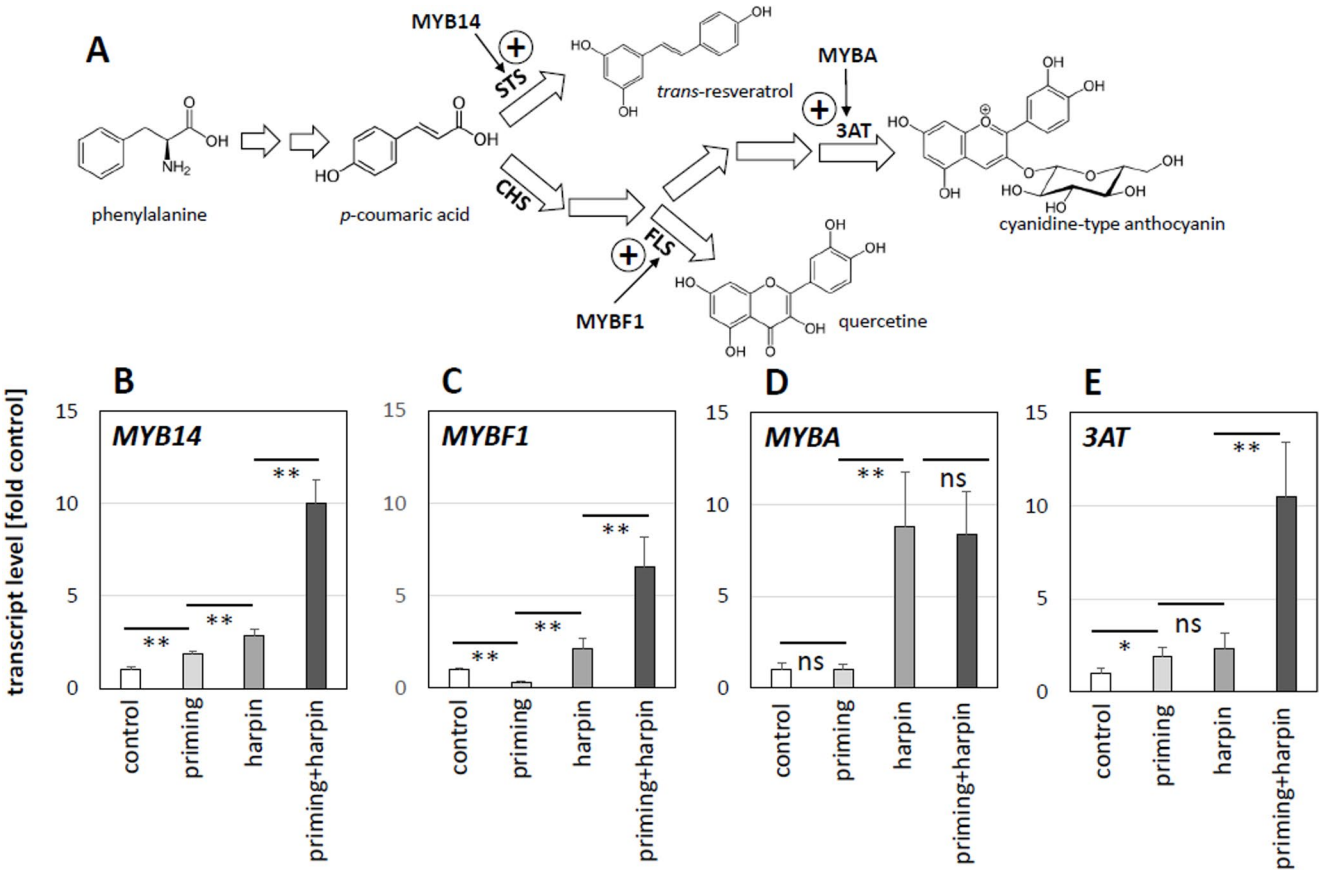

Fig. 2 Priming of regulators for phenylpropanoid synthesis by aluminium in suspension cells of *V. rupestris*. **A** Simplified pathway showing the activities of the gene products investigated in this experiment. STS Stilbene Synthase, CHS Chalcone Synthase, FLS Flavonol Synthase, 3AT 3-Anthocyanin Alkyl Transferase. **B-E** Steady-state transcript levels for *MYB14* (**A**), *MYBF1 / MYB12* (**B**), *MYBA* (**C**), and its target *3AT* (**D**). Priming with 200 µM of $AlCl_3$ for 2 h, elicitation by harpin (9 µg·mL^{-1}) for an additional hour. Data represent mean values and standard errors from three independent experimental series with three technical replications for each biological replicate. Transcript levels are calibrated to *EF 1α* as the internal standard. Asterisks indicate significant differences with * $P<0.05$, and ** $P<0.01$, ns non-significant

Priming of the stilbene synthase regulator *MYB14* depends on actin

Since the priming of resveratrol synthase was suppressed by Latrunculin B (Fig. 3), we wondered, whether this was reflected in the behaviour of *MYB14*, a crucial transcriptional activator of stilbene synthases (Höll et al. 2013). In fact, the strong synergistic effect of priming and elicitation by harpin was completely eliminated by pre-treatment with Latrunculin B. This is even more significant, considering that Latrunculin B by itself significantly promoted the expression of these transcripts. In the next step, we asked, whether actin-dependent priming is a general feature of genes, whose expression is promoted by Latrunculin B. However, when we followed the behaviour of *NAC17*, a transcription factor involved in retrograde signalling of mitochondria to the nucleus (Meng et al. 2019), we observed a completely different pattern. Here, Latrunculin B alone, as well as priming alone yielded a strong induction of these transcripts, while the induction by harpin was comparable to that seen in *MYB14* (Fig. 4). In sharp contrast to *MYB14*, the response to harpin was not promoted by preceding priming, irrespective of pre-treatment by Latrunculin.

Thus, the induction of *MYB14* transcripts by harpin is strongly amplified by preceding priming. This amplification is strictly dependent on actin filaments, because it can be eliminated by Latrunculin B. The priming effect is not a general feature of actin-dependent genes, because *NAC17*, whose expression is strongly induced, when actin filaments are removed by Latrunculin B, does not show any priming effect of the response to harpin.

Priming correlates with induction of genes involved in aluminium tolerance

The observed priming, developed within two hours, might be a direct effect of aluminium. However, since aluminium

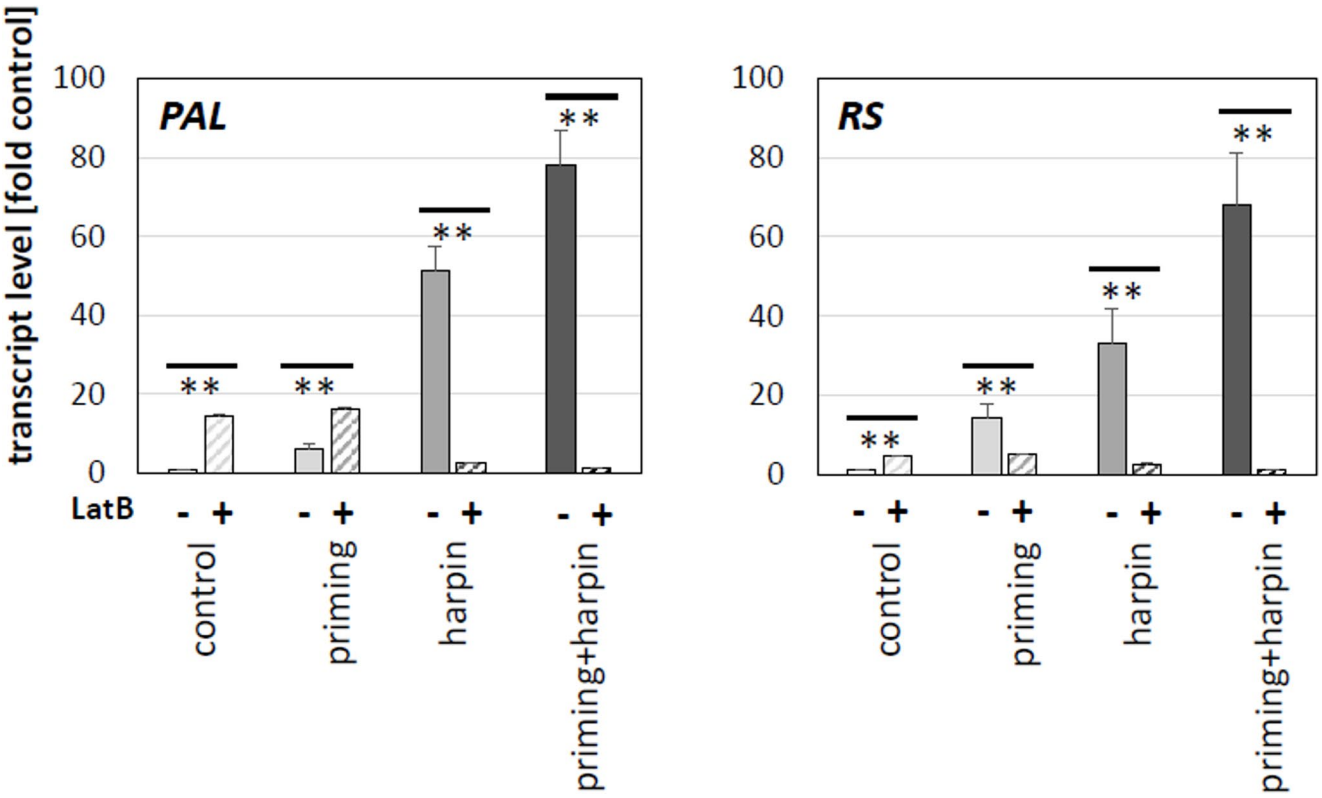

Fig. 3 Effect of pretreatment with Latrunculin B (LatB, 1 µM, 2 h) on the priming of *Phenylammonium Lyase* (*PAL*) and *Resveratrol Synthase* (*RS*) by aluminium in suspension cells of *V. rupestris*. Steady-state transcript levels are shown as manifold of the untreated control cells. Priming with 200 µM of $AlCl_3$ for 2 h, elicitation by harpin (9 µg·mL^{-1}) for an additional hour. Data represent mean values and standard errors from three independent experimental series with three technical replications for each biological replicate. Transcript levels are calibrated to *EF 1α* as the internal standard. Asterisks indicate significant differences with ** $P<0.01$

represents an abiotic stressor, the priming might also be an indirect consequence of the adaptive responses to aluminium stress. To probe this, we monitored two of these responses. The transcription factor WRKY22 activates citrate secretion into the apoplast sequestering aluminium ions (Li et al. 2018), and the alternative oxidase allows to evade oxidative stress by circumventing a shunt flow of electrons at complex III (Panda et al. 2013). For both genes, transcripts accumulated in response to the priming treatment (Fig. 5). The response was more rapid for *WRKY22*, where transcripts were significantly elevated already after 1 h around 3-fold and increased more slowly afterwards to around 5-fold over the resting level. In case of *AOX1*, transcript levels at 2 h, the time point, where the physiological effect of priming was already fully manifest, were just beginning to be induced, and increased afterwards to more than 15-fold after 6 h. Thus, the priming effect of aluminium clearly precedes the accumulation of *AOX1* transcripts (not to speak about AOX1 protein), clearly precluding any role of *AOX1* for priming. For *WRKY22*, the more rapid induction of the transcript might at first sight appear congruent with a role in priming. However, it should be kept in mind that WRKY22 is just a regulator, that first needs to activate the expression of the citrate transporter FRDL4, which not only needs to be expressed, but also has to act for a certain time to exert a significant effect, before any relevant metabolic change can be built up. Thus, the time course of these aluminium adaptive genes rather supports a scenario where aluminium induces priming directly, and not indirectly through adaptive responses to aluminium-induced stress.

Priming can mitigate harpin-induced mortality depending on dynamic actin.

The defence-response triggered by harpin is linked with a hypersensitive response culminating in programmed cell death observable from one day after elicitation. We wondered whether priming by aluminium would modulate this response (Fig. 6). Cellular mortality in untreated cells was between 10 and 15%, which was significantly induced to almost 25% one day after elicitation with harpin. Priming

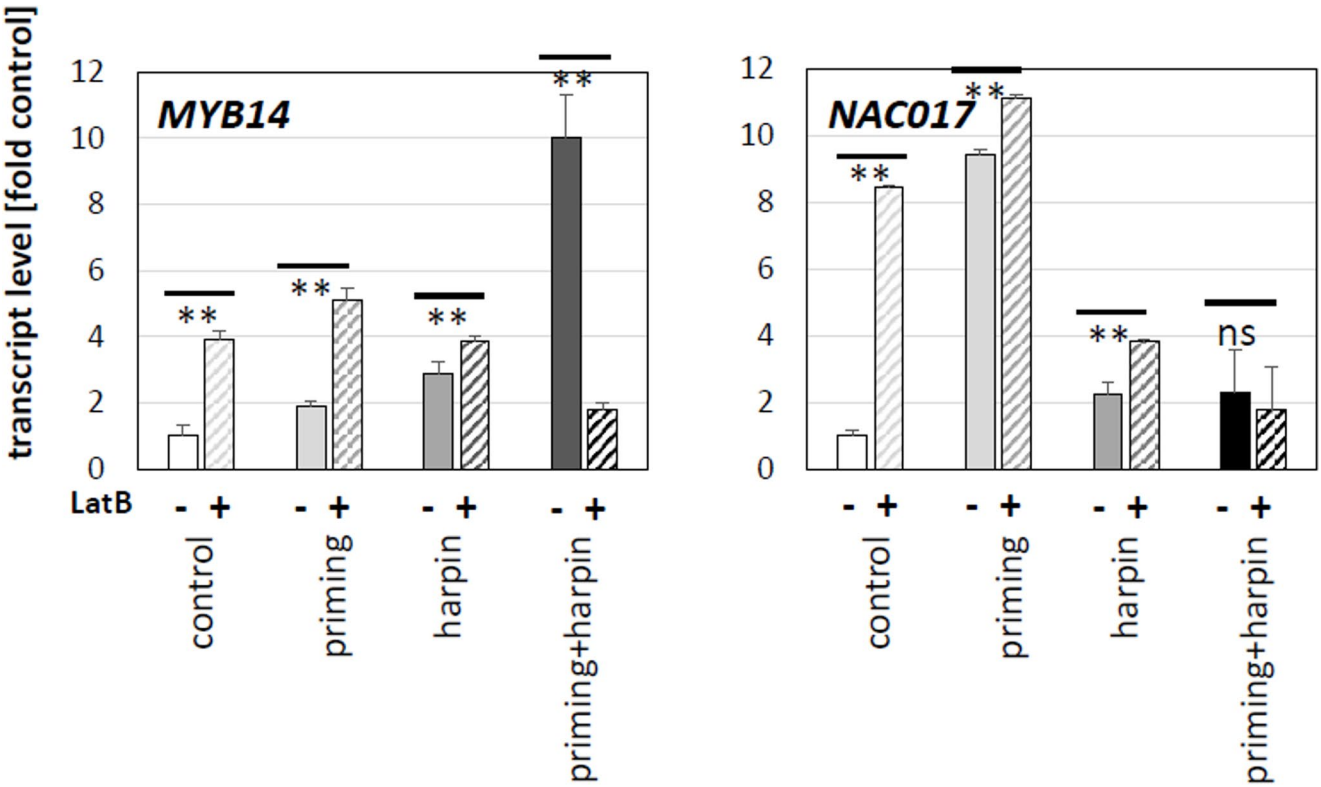

Fig. 4 Effect of pretreatment with Latrunculin B (LatB, 1 µM, 2 h) on the priming of *MYB14* (activator of StSY) and *NAC017* (involved in mitochondrial retrograde signalling) by aluminium in suspension cells of *V. rupestris*. Steady-state transcript levels are shown as manifold of the untreated control cells. Priming with 200 µM of $AlCl_3$ for 2 h, elicitation by harpin (9 $\mu g \cdot mL^{-1}$) for an additional hour. Data represent mean values and standard errors from three independent experimental series with three technical replications for each biological replicate. Transcript levels are calibrated to *EF 1α* as the internal standard. Asterisks indicate significant differences with ** $P<0.01$, ns non-significant

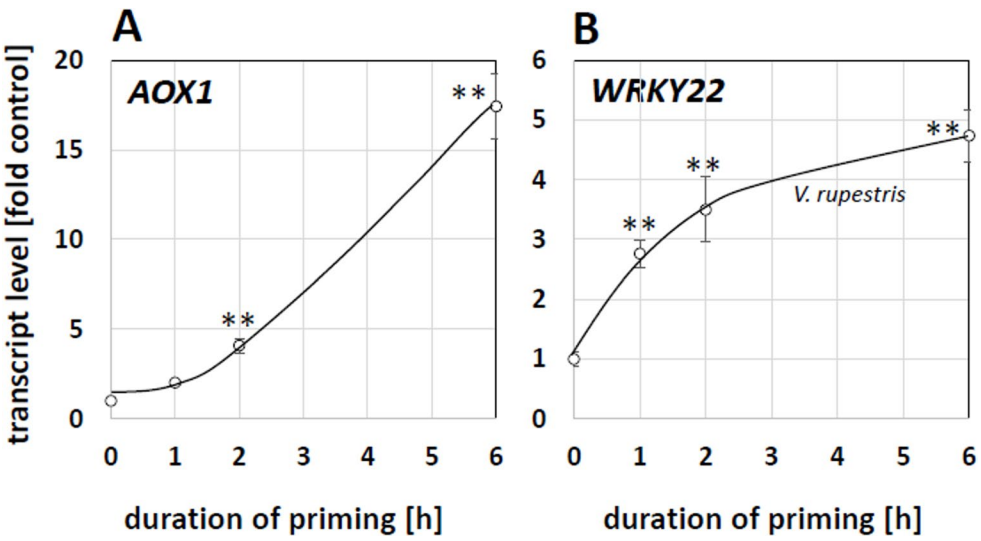

Fig. 5 Time course of transcript induction for the Alternative Oxidase (*AOX1*) and the transcription factor *WRKY22* in response to priming with 200 µM of $AlCl_3$ in cells from *V. rupestris*. Steady-state transcript levels are either given as induction over the resting level prior to priming using the $-\Delta\Delta C_t$ method. Data represent mean values and standard errors from three independent experimental series with three technical replications for each biological replicate. Transcript levels are calibrated to *EF 1α* as the internal standard. Asterisks indicate significant differences over the ground level with * $P<0.05$, and ** $P<0.01$

itself did not yield a significant increase of mortality, but significantly mitigated the mortality induced by harpin down to 15%, i.e., to a level comparable to the controls.

During our previous work (Wang et al. 2022), we had shown that aluminium induces a remodelling of actin filaments. This stimulated the question, whether the mitigating effect of aluminium priming on harpin-induced mortality would depend on actin filaments. In one experiment, actin filaments were eliminated by pretreatment with Latrunculin B (Fig. 6, left). While this treatment did

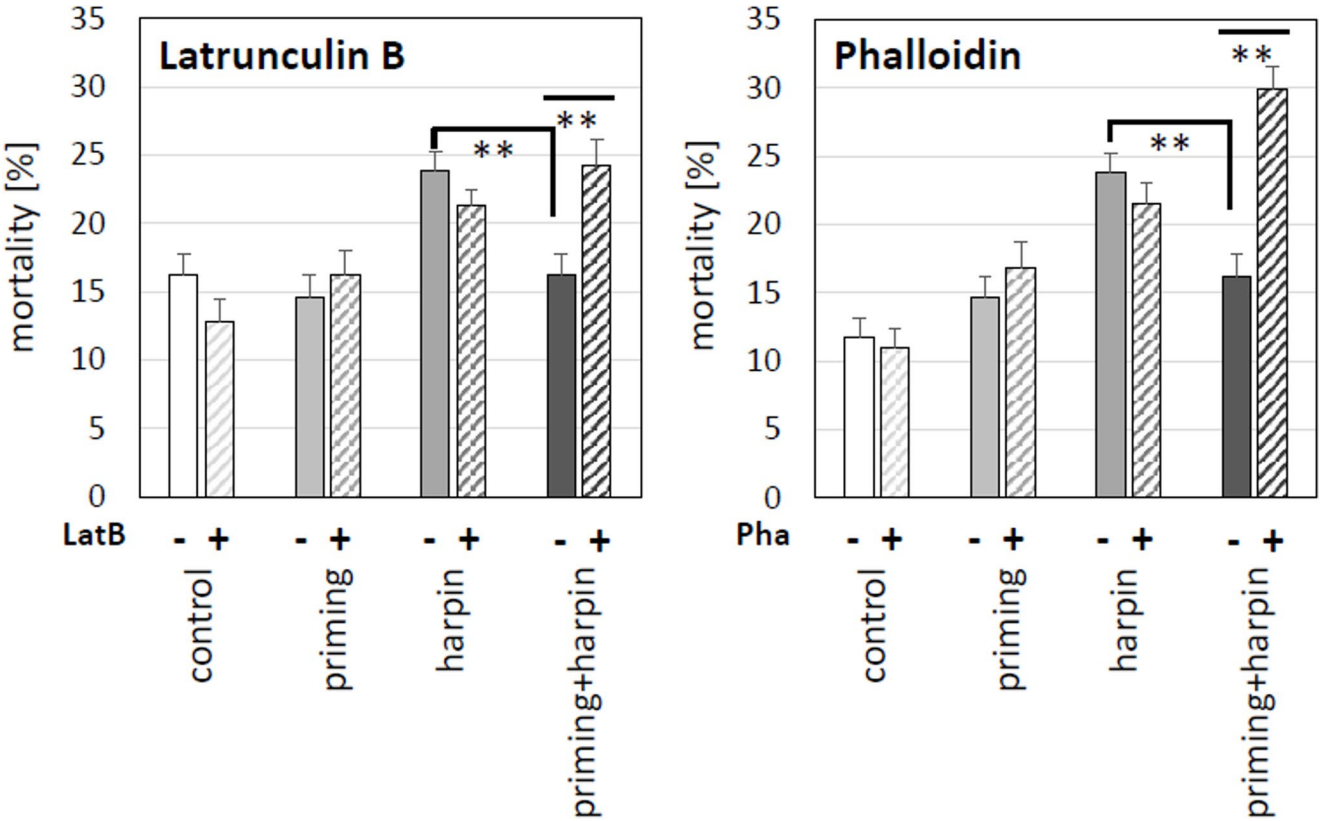

Fig. 6 Effect of pretreatment with Latrunculin B (LatB, 1 µM, 2 h) or Phalloidin (Pha, 1 µM, 2 h) on priming-associated mitigation of harpin-induced mortality in suspension cells of *V. rupestris*. Priming with 200 µM of $AlCl_3$ for 2 h, elicitation by harpin (9 $\mu g \cdot mL^{-1}$) for an additional day Data represent mean values and standard errors from three independent experimental series. Asterisks indicate significant differences with ** $P<0.01$

not prevent harpin-induced mortality, it completely abolished the mitigation caused by priming prior to harpin elicitation. This effect was not caused by a potential cytotoxicity of Latrunculin B as such, because mortality in controls treated by Latrunculin B alone or in cells that had been primed, but not subsequently elicited by harpin was not different from the respective condition without Latrunculin B.

In the next step, we asked, how Phalloidin, a compound stabilising actin filaments by suppressing their turnover, would influence the effect of priming on mortality. Interestingly, the pattern (Fig. 6, right-hand graph) was very similar to that seen for Latrunculin B. Phalloidin could not suppress harpin-induced mortality, but it eliminated the mitigation by aluminium priming, although phalloidin, in the absence of harpin, failed to cause any effect on mortality.

Thus, suppression of harpin-induced mortality by priming does not only require the presence of actin filaments, but these filaments also need to be dynamic.

Discussion

Priming of plant immunity can be operationalised as experimental system, where defence responses are modulated by a memory of preceding experience. In other words, priming is the differential in the response to an elicitor between a naive cell as compared to a cell that had been exposed to this elicitor earlier. In the current study we asked the question, whether activation of the NADPH oxidase Respiratory burst oxidase Homologue (RboH) as central input for stress signalling would modulate subsequent responses to the bacterial elicitor harpin, triggering cell-death related defence in our experimental model, suspension cells deriving from the wild American grapevine species *Vitis rupestris*. As priming agent, we use aluminium ions that can induce RboH and evoke a remodelling of actin filaments. In fact, we can show that the induction of central phytoalexin synthesis genes by harpin is enhanced by preceding priming with aluminium, linked with a corresponding priming

of their transcriptional activators. This priming effect is depending on actin, accompanied by activation of genes linked with aluminium tolerance, and mitigates harpin-induced mortality (again depending on actin filaments). These findings provide clear evidence for a priming effect of aluminium and stimulate a couple of questions on the underlying mechanism that are discussed in the following: (1) What can we deduce on the relationship between priming and cell-death related defence? (2) What can we conclude on the role of actin filaments in priming ? (3) What is the potential of these findings for application in the context of sustainable viticulture?

Priming by aluminium and basal immunity antagonise Hypersensitive Response.

The bacterial elicitor harpin is produced by the phytopathogenic bacterium *Erwinia amylovora*, the causative agent of Fire Blight in fruit trees and evokes a Hypersensitive Response (Wei et al. 1992). This type of defence is efficient against biotrophic pathogens, but inappropriate for a necrotrophic pathogen, such as *E. amylovora*. Thus, this pathogen uses harpin to hijack host defence for its own purpose, driving the host cell into suicide, such that it can be more easily scavenged. In grapevine cells, harpin can activate several aspects of a Hypersensitive Response, including apoplastic oxidative burst, calcium influx, activation of defence genes, and accumulation of stilbene phytoalexins (Chang and Nick 2012). While these responses can also be observed in the context of basal immunity, their temporal sequence differs – for harpin oxidative burst precedes calcium influx; for flg22, the order is reversed. There are, however, also qualitative difference between the two tiers of immunity: The bioactive isoleucine conjugate of jasmonic acid (JA-Ile), along with its precursor, jasmonic acid, accumulate upon stimulation with flg22, but are not found, when the cells are elicited with harpin (Chang et al. 2017). Conversely, in tobacco BY-2, the hypersensitive response evoked by harpin can be mitigated by addition of jasmonic acid (Akaberi et al. 2018).

Thus, the absence of jasmonate signalling might channel defence into the cell-death related form (as it is triggered by harpin). In contrast, active jasmonate signalling would mitigate cell-death favouring basal immunity, where the cell deploys defence responses, but stays alive. This hypothesis is consistent with the well documented antagonism between jasmonates and salicylic acid. While dicot plants deploy jasmonate signalling for their defence against herbivory and necrotrophic pathogens, salicylic acid is pivotal to the defence against biotrophic pathogens. This functional divergence is usually linked with a clear antagonism between the two phytohormones (reviewed in Thaler et al. 2012). This seems connected to the antagonism between the two master regulators of jasmonate and salicylate dependent gene expression, *MYC2* and *NPR1*, respectively. On the one hand, *MYC2* can inhibit SA-inducible genes (reviewed in Thaler et al. 2012). On the other hand, *NPR1* can suppress JA-inducible genes (Nomoto et al. 2021).

A prominent example is coronatine, a highly potent jasmonate mimic, employed by the biotrophic bacterium *Pseudomonas syringae*. This highly potent jasmonate mimic coronatine is used to suppress the Hypersensitive Response of the host *Arabidopsis thaliana* (Brooks et al. 2005).

In the current study, we observe that priming by aluminium mitigates harpin-induced cell death, a phenomenon that can also be produced by adding jasmonic acid (Akaberi et al. 2018). In a previous study (Wang et al. 2022), we have dissected the defence-related events induced by aluminium, that can significantly up-regulate the expression of *PR1*, a well-established marker gene for the salicylic acid pathway, as well as *ICS* encoding isochorismate synthase, the key synthetase for salicylic acid biosynthesis, and the outcome from this study allows to infer several aspects of priming, moreover, since the cell line and the conditions were exactly identical:

Aluminium activates RboH, which will generate apoplastic ROS. This oxidative burst is necessary and sufficient to induce remodelling of actin filaments. Actin remodelling in turn activates a MAPK cascade culminating in the activation of phytoalexin biosynthesis. In addition to key enzymes of the stilbene biosynthesis, aluminium also activates *ISOCHORISMATE SYNTHASE 1* (*ICS1*), the rate-limiting enzyme for salicylic acid biosynthesis. In fact, *PR1*, a characteristic readout of SA, is activated as well. Accumulation of SA often heralds defence-related cell death, but this does not seem to be the case for aluminum-induced priming. It might be the calcium influx that makes the difference – in case of cell-death related immunity, the activation of RboH is followed by an influx of calcium (Chang and Nick 2012), in case of aluminium, it is not (Wang et al. 2022).

The rapid calcium influx triggered by the PTI-inducing elicitor flg22 has been shown, by genetic and pharmacological ablation, to be necessary for RboH mediated oxidative burst, while RboH activity is not required for calcium influx (Marcec and Tanaka 2021). The influx of calcium will not only activate RboH through binding to its cytosolic calcium-binding motifs (reviewed in Marcec et al. 2019), but also open the Voltage Dependent Anion Channel 1 at the mitochondrial membrane, such that calcium

enters the matrix, causing accumulation and release of mitochondrial ROS from calcium-dependent activation of Voltage Dependent Anion Channel 3 (reviewed in Ravi et al. 2023). The differential signature would then be presence (defence) or absence (aluminium priming) of a mitochondrial oxidative burst in addition to RboH-dependent apoplastic ROS.

An implication of this signature hypothesis would be that aluminium should induce responses avoiding a mitochondrial oxidative burst. Our observation that transcripts for *AOX1* are strongly induced during priming (Fig. 5A) are consistent with this mitochondrial signature hypothesis. In fact, experiments with tobacco mutants, where *AOX1* was either knocked down or overexpressed, demonstrated that this enzyme, which can uncouple electron transport from proton gradient and, thus, from ATP synthesis, can prevent mitochondrial oxidative burst (Jayawardhane et al. 2020).

However, the oxidative signature might act in concern with other signatures. Especially calcium signatures are relevant here (for a recent comprehensive review see Wang et al. 2024). While rapid, but transient calcium increase through influx channels in the plasma membrane is a hallmark of basal immunity, hypersensitive responses are heralded by constitutive elevation of calcium originating from endogenous stores, such as mitochondria. Whether a cell decides to activate basal immunity or whether it goes for programmed cell death, might, thus, depend on the interaction between calcium and ROS signature in a modular fashion. The oxidative burst initiated by RboH in response to aluminium lacks the subsequent calcium influx (Wang et al. 2022), while in case of elicitation by harpin, the oxidative burst is followed by a strong influx of calcium (Chang and Nick 2012). The cellular decision between basal immunity versus Hypersensitive Death might, therefore, be modular. There is accumulating evidence that modular combination between stress input signals represents a general principle, by which plants prioritise and structure their responses to the complex and multifaceted challenges by their environment (for review see Nick 2024).

The induction of *AOX1* is preceded by a strong induction of transcripts for *WRKY22*. This transcription factor is established as regulator of basal immunity (reviewed in Eulgem et al. 2000). It quells SA-responsive and promotes JA-responsive gene expression (Kloth et al. 2016). On the other hand, *WRKY22* is induced by SA (Dong et al. 2003), which would establish a negative feedback loop. By inducing *WRKY22*, SA would with a delay of a few hours not only prevent additional SA signalling, but simultaneously promote JA signalling, thus, culminating in a transient SA signature. This mechanism might explain, why aluminium, although it activates *ICS1* as key step of SA synthesis, which is followed by induction of the SA-responsive *PR1* (Wang et al. 2022), does not trigger hypersensitive cell death, but rather, possibly through WRKY22-dependent activation of jasmonate signalling, switches the defence machinery towards basal immunity. In the next stage of the study, we will measure the levels of jasmonic acid and salicylic acid, which will make the findings more convincing.

Priming versus hypersensitive response – a signalling role for actin?

Aluminium can induce a remodelling of actin filaments (Ahad and Nick 2007), and for grapevine cells this remodelling has been shown to induce defence genes, including genes for the synthesis of stilbene phytoalexins (Wang et al. 2022). This leads to the question, whether actin is involved in priming as well. Since Latrunculin B disrupts the priming effect for *PAL* and *RS*, actin filaments can be concluded to be a necessary factor for priming by aluminium. This is reflected in the pattern of *MYB14*, the crucial activator for stilbene biosynthesis genes (Höll et al. 2013; Duan et al. 2016). The specificity of this response is highlighted by the fact that *NAC017* as further stress-inducible transcription factor that is responsive to aluminium, did not exhibit a priming effect, if probed with harpin. Since also phalloidin can suppress priming, actin filaments need not only to be there, but also have to be dynamic.

From our previous study (Wang et al. 2022), we know that actin remodelling in response to aluminium requires oxidative burst through the NADPH oxidase Respiratory burst oxidase Homologue in the plasma membrane. This burst not only induces the activation of phytoalexin-synthesis genes, depending on MAPK signalling, but also causes a strong accumulation of transcripts for *Isochorismate Synthase 1*, a key enzyme for Salicylic Acid biosynthesis. The induction of *PR1*, a typical output of Salicylic Acid signalling, is further evidence for Salicylic Acid activation in response to aluminium.

Salicylic Acid is usually considered a hallmark for a Hypersensitive Response (for review see Alvarez 2000). However, this is not necessarily the case. For instance, the induction of stilbene synthases in the Chinese wild grapevine *V. pseudoreticulata* is linked with a strong inducibility by Salicylic Acid that is independent of ETI, but rather acts through integration of Salicylic Acid into basal immunity (Jiao et al. 2016). Similarily to Salicylic Acid, actin remodelling, while often heralding Programmed Cell Death (for review see Smertenko and Franklin-Tong 2011), can also occur in contexts, linked with cell expansion rather than defence and Hypersensitive Response

(Waller et al. 2002; Nick et al. 2009). Here, a dynamic pool of actin was linked with growth, while bundled actin was a proxy for growth arrest.

This leads to the question, whether the role of actin (Hypersensitive Death in some cases, activation of basal immunity without subsequent Hypersensitive Death in others) might depend on its interaction with Salicylic Acid. In case of priming, the actin response to aluminium precedes the formation of Salicylic Acid, since the induction of *Isochorismate Synthase 1* (*ICS1*) transcripts, the formation of the respective enzyme and the accumulation of the respective metabolic product are expected to take some time (Wang et al. 2022). Moreover, this activation seems not to be direct, but requires the induction of specific WRKYs, such as AtWRKY28, that have been shown to bind to the *ICS1* promoter by Chromatin immunoprecipitation and quantitative PCR (van Verk et al. 2011). Experiments, where Latrunculin B specifically can activate genes of the Salicylic Acid biosynthesis and signalling, place actin upstream (Matoušková et al. 2014). For the interaction between *Pseudomonas syringae* and *Arabidopsis thaliana*, the disruption of actin was shown to increase resistance, confirming that, indeed, actin and Salicylic Acid act here in the context of a Hypersensitive Reaction (Leontovyčová et al. 2019).

Whether actin remodelling culminates in Hypersensitive Cell Death or in priming restraining this cell death might depend on the accompanying context, for instance, the presence of calcium. In fact, the activation of chitinase by salicylic acid can be blocked by EGTA, chelating calcium ions, or by verapamil, an inhibitor of calcium influx, in carrot cells (Schneider-Müller et al. 1994). Likewise, an evolutionarily conserved mechanism leads to the release of calcium from mitochondria in response to salicylic acid (Yoshida et al. 1992). Priming by aluminium does not induce calcium influx (Wang et al. 2022). Furthermore, the induction of AOX1 transcripts, indicates that mitochondrial calcium release is avoided in consequence of priming. Metacaspases as executors of Hypersensitive Cell Death are activated by calcium (van Midden et al. 2021), providing a mechanism to explain, why the effect of actin remodelling depends on the status of salicylic acid.

Thus, the cellular decision between a defence response that employs cell death versus priming a defence response that avoids cell death involves actin and salicylic acid in a combinatorial manner. This would be a further example for the modular nature of plant stress signalling (reviewed in Nick 2024).

Can priming be integrated into sustainable viticulture? Viticulture belongs to the parts of agriculture with the highest fungicide load. Alone for the European Union, around 68,000 tons per year are needed for grapevine corresponding to some 70% of total fungicide consumption (Pirrello et al. 2023). Alternatives to chemical plant protection would have a great impact on the ecological footprint of viticulture. In fact, introgression of resistance factors from wild North American species has initiated soon after the first epidemic spread of Downy and Powdery Mildew of Grapevine in Europe (for review see Eibach et al. 2009). However, an off-taste from these American resistance donors, called foxiness, has severely impaired consumer acceptance and development of this strategy (recently reviewed in Foria et al. 2022). Only after many decades of back-crossing and selection, the (desired) fungal resistance could be uncoupled from foxiness, giving rise to the modern so-called PiWi (for Pilz-Widerstandsfähig, fungal resistant) varieties that have grown into an important element of organic viticulture in Germany, France, and Switzerland with steady and substantial growth rates on the market, especially among the young generation. While breeding of novel varieties has been successful, there is need for alternative strategies that are feasible for the traditional varieties with their long cultural history and high consumer acceptance, but low disease resistance. Priming of basal immunity might provide such an alternative approach.

Aluminum-based formulations such as aluminium ethyl phosphite have been used in the past to contain oomycete-borne diseases especially Downy Mildew of Grapevine (Dercks and Creasy 1989) or Black Shank of Tobacco (Nemesthoty and Guest 1990). While the mode of action has remained enigmatic, in both cases, induction of phytoalexins was observed. A straightforward working hypothesis would be that these formulations act through priming of basal immunity. A caveat against the excessive such compounds is the risk of aluminium accumulation in the soil leading to root toxicity (for a classic review see Foy et al. 1978). However, if these compounds indeed act through priming, it should be possible to strongly reduce the ecological footprint, because the aluminium would act as a signal rather than a poison, and the durability of the priming effect should allow for a low number of treatment cycles. The findings of the current and our previous (Wang et al. 2022) study indicate that the crucial point about priming is the induction of RboH while leaving calcium influx silent. There might be alternative compounds able to uncouple these two stress inputs avoiding the issue of aluminium toxicity.

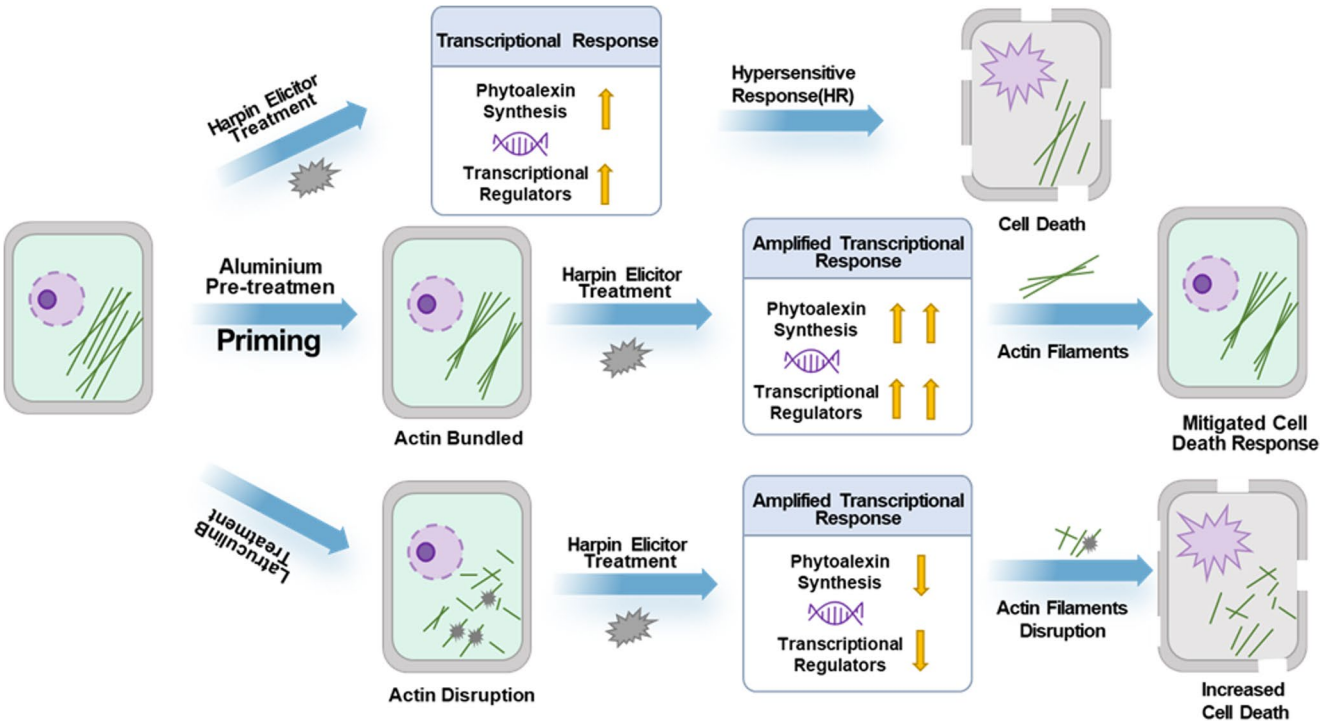

Fig. 7 The working model of Aluminium primed grapevine defence through actin filaments

Conclusion

In our previous work (Wang et al. 2022), we could show that aluminium ions, through remodelling of the actin cytoskeleton, can activate the expression of phytoalexin biosynthesis genes. Now, we demonstrate that aluminium can induce, in addition, defence signalling priming. The priming effect becomes evident upon elicitation with harpin, a bacterial virulence factor of *Erwinia amylovora*. Here, priming by aluminium can synergistically enhance the expression of defence genes and mitigate the programmed cell death induced by this elicitor (Fig. 7). The priming depends on dynamic actin and the use of priming to modulate immunity signalling also offers promising implications for sustainable viticulture in the face of environmental challenges.

Acknowledgements We would like to express our gratitude to the China Scholarship Council (CSC) for their support of Ruipu Wang and Pingyin Guan.

Author contributions In this collaborative research effort, Ruipu Wang conducted all experimental work and drafting the initial manuscript. Pingyin Guan contributed significantly by assisting Ruipu Wang with specific experiments, thereby enhancing the overall quality of the research. Michael Riemann provided essential guidance throughout the experimental process and supplied necessary materials, ensuring that the project progressed smoothly. Peter Nick offered invaluable academic guidance, facilitated the exchange of ideas, refined the manuscript, and provided financial support, all of which were crucial for the successful completion of this study. Together, this team exemplifies a strong collaborative spirit, leading to impactful scientific contributions.

Funding Open Access funding enabled and organized by Projekt DEAL. This work was mainly supported by the Interreg Upper Rhine project (Vitifutur), co-financed by the European Union/European Regional Development Fund (ERDF), the German Federal Agency for Agriculture (Programme for Sustainable Agriculture, BÖLN), the Guizhou Provincial Basic Research Program (Natural Science) No.[2024] Youth Program 156, Guizhou Provincial Basic Research Program (Natural Science) No.[2025] General Program 650, the Guizhou University Doctoral Fund (Guidarenjihezi [2022]40), and the Guizhou University Fundamental Research Project [2023] No. 32.

Data availability The data supporting this study are available from the corresponding author upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.

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