Impact of cold exposure on pathogen resistance in *Arabidopsis thaliana*

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Declaration of authorship

I hereby declare that I alone am responsible for the content of my doctoral dissertation and that I have only used the sources or references cited in the dissertation.

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Index

Summary

The present study investigated the effect of a preceding, short cold treatment (4 °C, 24 h) on the resistance of *Arabidopsis thaliana* against hemibiotrophic *Pseudomonas syringae* pv. *tomato* (*Pst*) and necrotrophic *Botrytis cinerea*. For this aim, two different experimental cold setups were compared. In the first setup (cold pre-treatment; CT), the pathogen infection occurred directly after the cold treatment. The second experimental setup (cold priming; CP) included a memory phase between the cold treatment and the infection, whereby the impact of a priming memory on the resistance against pathogens was investigated. Arabidopsis benefited from the cold treatment and exhibited significantly increased resistance against *Pst* and *B. cinerea*. For plant defense against *Pst*, cold priming and cold pre-treatment led to increased resistance, while against *B*. *cinerea* only cold pre-treatment resulted in transient resistance increase.

To identify the immune signaling pathways responsible for the increased resistance, transcript analyses and pathogen growth experiments were conducted with Arabidopsis (Accession: Col-0) and selected mutant lines. The experiments demonstrated that cold priming-mediated resistance in Arabidopsis against virulent *Pst* is independent of the plant immune regulator Enhanced Disease Susceptibility 1 (EDS1) and does not alter transcript levels of pathogen-triggered *Pathogenesis-related 1* (*PR1)*, *Isochorismate Synthase 1* (*ICS1)*, *FLG22-induced receptor-like Kinase 1* (*FRK1)*, and *NDR1/HIN1-like 10* (*NHL10)*. These results, but also the observation that cold priming does not confer resistance against avirulent *Pst avrRPS4* and *Pst avrRPM1*, indicated that cold-mediated resistance against *Pst* is mainly independent from effector-triggered immunity (ETI), hypersensitive response (HR), PAMPs-triggered immunity (PTI) and salicylic acid (SA) signaling.

Cold pretreatment-mediated resistance against *B*. *cinerea* was also without cold signatures on the transcript levels of *Pathogenesis-related 1* (*PR1)*, *Pathogenesis-related 4* (*PR4*), *Plant Defensin 1.2a* (*PDF1.2a)* and *Phytoalexin Deficient 3 (PAD3)*. This suggested that cold exposure-mediated resistance against *B*. *cinerea* is independent of SA signaling, jasmonic acid (JA) signaling, and camalexin biosynthesis. Increased accumulation of reactive oxygen species (ROS) was observed in Arabidopsis leaves after the cold pre-treatment and subsequent *B*. *cinerea* infection indicating a central function of ROS in the cross-talk between the prior cold and pathogen infection. Besides other functions, ROS contribute to lignin and callose formation. Enhanced callose formation and lignification could be detected immediately after the cold exposure. In contrast, *B. cinerea* did not alter plant

lignin amounts and pathogen-triggered callose deposition was not further affected by prior cold treatment.

We extended our analyses by investigating the impact of cold-pre-treatments on the establishment of systemic aquired resistance (SAR). The induction of SAR in cold-pretreated plants was successful, but the prior cold treatments did not provide additional benefits for SAR against *Pst*. Interestingly, a syringe infiltration with the mock solution instead of the SAR-triggering primary *Pst* inoculation enhanced the susceptibility of Arabidopsis after a cold pre-treatment against *Pst*.

The most pivotal finding of this work demonstrated that the chloroplast-localized ROS-scavenging enzymes stromal ascorbate peroxidase (sAPX) and thylakoid ascorbate peroxidase (tAPX) are essential for the wildtype-like enhanced resistance against *Pst* and *B. cinerea* after prior cold treatments. CP-mediated resistance was also confirmed in the immunocompromised null mutant *enhanced disease susceptibility 1* (*eds1-2)* crossed with *stroma ascorbate peroxidase* knockout (*eds1 sapx*). This work, furthermore, showed that sAPX is essential for cold-induced callose formation and dispensable for pathogen-induced callose. Finally, *sapx*, but not *thylakoid ascorbate peroxidase* knockout (*tapx*), showed a different ROS generation pattern, with significantly more ROS being generated than in cold-treated *sapx* or compared to wildtype plants without cold pre-treatment. These results highlight the relevance of the chloroplast antioxidant system in cold sensing and its impact on the plant immune system.

Zusammenfassung

In der vorliegenden Studie wurde die Auswirkung einer vorangehenden, kurzen Kältebehandlung (4 °C, 24 h) auf die Resistenz von *Arabidopsis thaliana* gegenüber zwei unterschiedlichen Pathogenen untersucht: dem hemibiotrophen Bakterium *Pseudomonas syringae* pv. *tomato* (*Pst*) und dem nekrotrophen Pilz *Botrytis cinerea*. Für die Kältebehandlungen wurden zwei verschiedene Versuchsanordnungen verglichen. Zum einen, eine Anordnung, bei der die Infektion direkt nach der Kältebehandlung erfolgte (Kältevorbehandlung; CT). Zum anderen ein Versuchsaufbau, der eine stressfreie Memoryphase zwischen Kältebehandlung und der Infektion beinhaltete und es ermöglichte die Auswirkung von Kältepriming auf die Resistenz gegen Krankheitserreger zu untersuchen (Kältepriming; CP). Bei der Abwehr beider Pathogene profitierte Arabidopsis von der Kältebehandlung und zeigte eine signifikant erhöhte Resistenz. Kältevorbehandlung und Kältepriming führten bei der Interaktion mit *Pst* zu einer erhöhten Resistenz, während bei der Infektion mit *B. cinerea* nur eine vorangehende Kältebehandlung zu einer transient erhöhten Resistenz führte.

Um für die durch Kälte erhöhte Resistenz verantwortliche Immunsignalwege zu identifizieren, wurden Transkriptionsanalysen und Pathogenwachstumsexperimente mit Arabidopsis (Accession: Col-0) und genetischen Vergleichslinien durchgeführt. Die Experimente zeigten, dass die durch Kältepriming vermittelte Resistenz von Arabidopsis gegen *Pst* unabhängig von dem Abwehrregulator Enhanced Disease Susceptibility 1 (EDS1) ist und ebenso nicht mit veränderten Transkriptspiegeln von *Pathogenesis-related 1* (*PR1*), *Isochorismate Synthase 1* (*ICS1*), *FLG22-induced receptor-like Kinase 1* (*FRK1*) und *NDR1/HIN1-like 10* (*NHL10*) korreliert. Diese Ergebnisse deuten an, dass die kältevermittelte Resistenz gegen *Pst* unabhängig von klassischen Abwehrsignalen (Effektor ausgelöste Immunität (ETI), Hypersensitive Antwort (HR), PAMPs ausgelöste Immunität (PTI) und Salicylsäure (SA) Signaling) ist. Dies wurde durch die Beobachtung unterstützt, dass Kältepriming bei der Abwehr gegen avirulente *Pst avrRPS4* und *Pst avrRPM1* keine zusätzlichen Resistenzvorteile bietet.

Die durch Kältevorbehandlung vermittelte Resistenz gegenüber *B. cinerea* ging nicht mit Änderungen auf Transkriptspiegelebene von bekannten Abwehrsignalgenen wie *Pathogenesis-related 1* (*PR1*), *Pathogenesis-related 4* (*PR4*), *Plant Defensin 1.2a (PDF1.2a)* und *Phytoalexin Deficient 3* (*PAD3*) einher. Dies deutet darauf hin, dass die durch Kälteeinwirkung vermittelte Resistenz gegen *B. cinerea* unabhängig von SA Signaling, Jasmonsäure

(JA) Signaling und der Camalexin-Biosynthese ist. Allerdings wurde in Arabidopsis-Blättern nach der Kältevorbehandlung und der anschließenden *B. cinerea*-Infektion eine erhöhte Akkumulation reaktiver Sauerstoffspezies (ROS) beobachtet, was auf eine zentrale Funktion von ROS bei der Wechselwirkung zwischen einer vorherigen Kältebehandlung und Pathogeninfektion hindeutet. Neben anderen Funktionen tragen ROS zur Bildung von Lignin und Callose bei. Eine verstärkte Callosebildung und Lignifizierung konnte unmittelbar nach der Kälteexposition festgestellt werden. Im Gegensatz dazu veränderte *B. cinerea* die Ligninmenge der Pflanze nicht, und die durch den Erreger ausgelöste Callosebildung wurde durch eine vorherige Kältebehandlung nicht weiter beeinträchtigt.
Desweiteren wurde untersucht, welchen Einfluss eine Kältevorbehandlung oder ein Kältepriming auf die Induktion einer systemischen erworbenen Resistenz (SAR) hat. Die Etablierung von SAR in kältevorbehandelten Pflanzen war erfolgreich, wurde aber durch die vorangegangene Kälteexposition nicht weiter verstärkt. Interessanterweise, erhöhte zudem eine Spritzeninfiltration mit der Mock-Lösung nach einer unmittelbaren Kältevorbehandlung die systemische Anfälligkeit von Arabidopsis gegen *Pst*.
Die zentralen Erkenntnisse dieser Arbeit zeigten, dass die in den Chloroplasten lokalisierten, ROS abbauenden Enzyme stromale Ascorbatperoxidase (sAPX) und thylakoidäre Ascorbatperoxidase (tAPX) für die wildtypähnliche Ausprägung der Resistenz gegen *Pst* und *B. cinerea* nach vorheriger Kältebehandlung essentiell sind. Die CP vermittelte Resistenz wurde auch in der immungeschwächten *eds1*-2 Linie, welche mit der Knockoutlinie von sAPX (*sapx*) gekreuz wurde (*eds1 sapx*), nachgewiesen. Darüber hinaus zeigte diese Arbeit, dass sAPX für die kälteinduzierte Callose essentiell und für die pathogeninduzierte Callose dagegen entbehrlich ist. Schließlich zeigt *sapx*, aber nicht die *thylakoidäre ascorbatperoxidase* Knockoutlinie *tapx*, ein anderes ROS-Bildungsmuster, wobei im Gegensatz zum Wildtyp ohne Kältebehandlung signifikant mehr ROS gebildet wurde als bei den kältebehandelten Pflanzen. Diese Ergebnisse heben hervor, wie das chloroplastidäre antioxidative Schutzsystem eine zentrale Verbindungsstelle zwischen der Kältewahrnehmung im Chloroplasten und dem pflanzlichen Immunsystem darstellt.

1. Introduction

1.1 What makes a microorganism a pathogen?

Plants are exposed to many biotic interactors. Their biological surface, for instance, provides an important habitat for microorganisms such as bacteria and fungi. Approximately 4 x 10^8 km^2 of the terrestrial earth surface are covered with foliage (phyllosphere) and about 10^{26} bacteria in total or 5.4 10^8 bacteria cm^{-2} leaf surface live in the phyllosphere (Remus-Emsermann et al., 2014; Leveau, 2006). Most microorganisms in the phyllosphere are non-pathogenic and have a neutral or even beneficial impact on plant fitness (Vogel et al., 2016). Plant pathogens are the origin of diseases that cause global losses of 20 – 30 % in major crops such as wheat, potato, rice and maize (Ristaino et al., 2021). To minimize crop losses due to diseases, a fundamental understanding of plant-pathogen interactions is crucial. How plants differentiate between pathogenic and neutral or beneficial microorganisms remains an open question (Vogel et al., 2016). Miebach et al. (2024) suggest a relationship between microbial population density and the induction of plant defense responses: higher microbial population densities induce immune response-related genes in plants, independent whether the microorganisms are described to be pathogenic or non-pathogenic. To study plant-pathogen interactions, model organisms have been established, with *Arabidopsis thaliana* is frequently used as a plant model host (Mauch-Mani & Slusarenko, 1993). The gram-negative, rod-shaped, and (hemi-)biotrophic bacterium *Pseudomonas syringae* pv. *tomato* (*Pst*) was established as a model pathogen for Arabidopsis (Katagiri et al., 2002). Most Arabidopsis accessions, including Col-0, are susceptible to infection with high doses of *Pst* and the plants develop symptoms such as chlorosis or leaf collapse. *Pst* enters plant tissues through natural openings such as stomata and propagate within the intercellular apoplast. However, most microorganisms are unable to enter and propagate within the apoplast due to constitutive and induced plant defense systems (Xin et al., 2018). In addition to bacterial toxins, *Pst* possesses a highly conserved Type III secretion system (T3SS) which enables the secretion of at least 29 different proteins into the plant cell. For instance, the *Pst*-secreted proteins AvrPtoB and HopN1 are particularly important in suppressing the plant's immune response. HopM1 and AvrE induce an aqueous apoplast, which is essential for a proper development of *Pst in planta* (Xin et al., 2018; Cunnac et al., 2011; Chang et al., 2005). *Botrytis cinerea* is another plant pathogen that is suitable as a model organism. It is one of the best-studied necrotrophic fungal plant pathogens. Unlike *Pst*, which has a biotrophic phase, *B*.

cinerea does not manipulate living plant cells but kills them to evade plant immune response and obtain the plant's resources (van Kan et al., 2006). Additionally, *B*. *cinerea* can actively penetrate plant cells, allowing fungal growth beyond the apoplast. Hence, physical barriers, such as the plant cell wall, play a significant role in defending against pathogens like *B*. *cinerea* (Bi et al., 2023).

1.2 Local pathogen perception in plants

1.2.1 PAMPs-triggered immunity - first defense layer

Plants can recognize pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs). PRRs are usually anchored in the plasma membrane and induce PAMP-triggered immunity (PTI) after extracellular PAMP recognition (Bigeard et al., 2015) (Fig. 1). One well-studied example of PAMP recognition is the pathogen derived 22 amino acid epitope flg22 of flagellin. When flg22 binds to the PRR flagellin-sensitive 2 (FLS2) receptor, it triggers the association of FLS2 with the co-receptor BRI1-associated receptor kinase (BAK1) leading to the activation of the plant's innate immune response (Chinchilla et al., 2006; Gómez-Gómez & Boller, 2000). In addition to flg22 other PAMPs, such as chitin or the elongation factor TU (EF-Tu), damage-associated molecular patterns (DAMPs) can also trigger PTI. DAMPs are plant degradation products like cutin monomers or cell wall damage products generated by pathogen-secreted enzymes (Bigeard et al., 2015). While *B*. *cinerea* and many other necrotrophic pathogens are exclusively detected by PRRs and thus only PTI is relevant for disease suppression, the interaction of *Pst* with Arabidopsis comprises further elements of pathogen detection and immune activation (Mengiste, 2011).

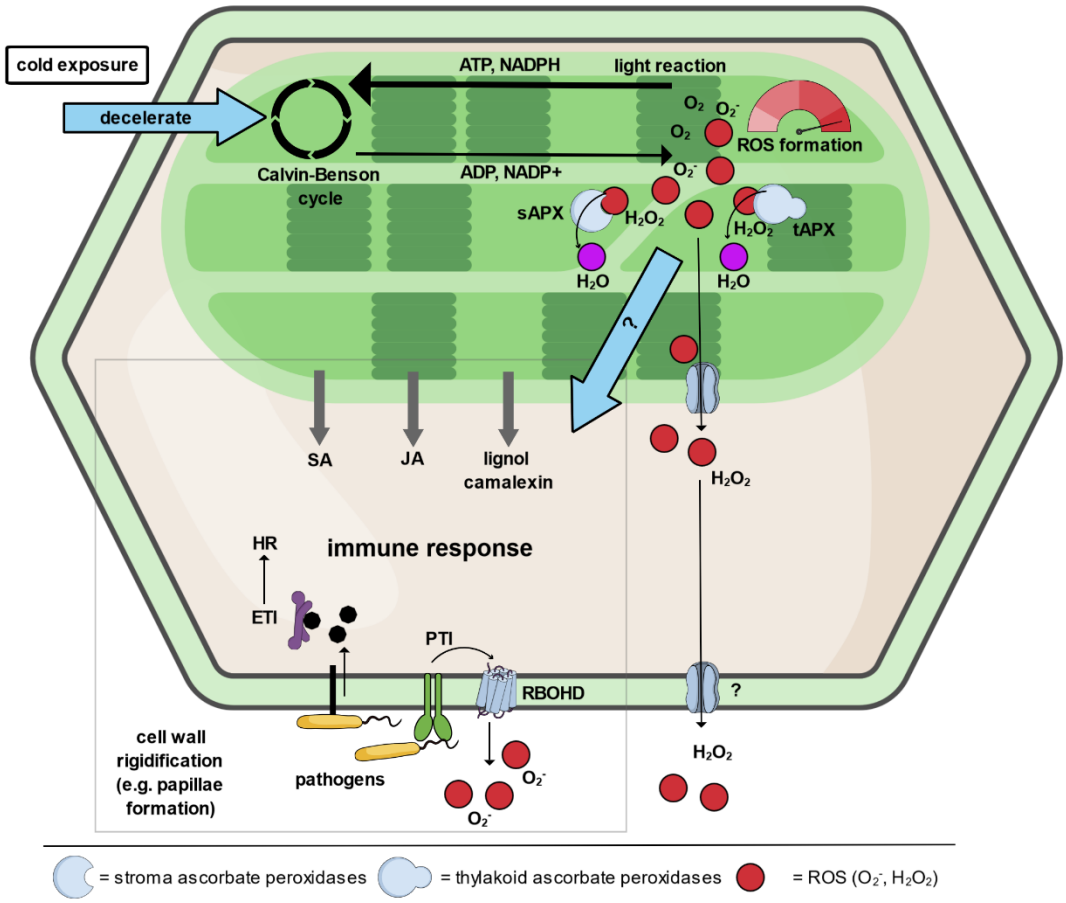

Figure 1: Cold perception in chloroplasts and pathogen responses (grey box). Cold exposures repress the enzymatic Calvin-Benson cycle (sink) stronger than the photosynthetic light reactions (source). This imbalance leads to enhanced generation of reactive oxygen species (ROS) in chloroplasts (Section 1.5). Excess excitation is transferred to O_2 forming O_2^-, which rapidly reacts to H_2O_2 (Section 1.5). H_2O_2 accumulates in chloroplasts or migrates via aquaporins into the cytosol or other cell compartments and potentially also enters the apoplast (Section 1.5). The ROS scavenging enzymes sAPX and tAPX, amongst others, reduce H_2O_2 into H_2O in the plastids (Section 1.6). The overall changes in ROS levels have the potential to impact the plant immune response, which finally affects the resistance against invading pathogens: Plasmamembrane anchored receptors recognize conserved pathogen patterns that induce PAMP-triggered immunity (PTI) along with an apoplastically directed ROS burst by the respiratory burst oxidase homolog D (RBOHD) (Section 1.2.1 & 1.3.2). Pathogens secrete effectors into the plant cell to manipulate plant physiology, and these effectors can be recognized by intracellular receptors, leading to effector-triggered immunity (ETI) and often to a hypersensitive response (HR) (Section 1.2.2 & 1.3.5). Salicylic acid (SA) and jasmonic acid (JA) are essential phytohormones for the plant immune response, and their precursors are synthesized in chloroplasts (Section 1.3.3 & 1.3.4). The precursors of lignol and camalexin are also biosynthesized in chloroplasts. Camalexin acts as an antimicrobial compound and lignol as a cell wall reinforcer (Section 1.3.6 & 1.3.7). Another cell wall reinforcement, called papillae, can be formed directly at potential pathogen entry sites (Section 1.3.6).

1.2.2 Effector-triggered immunity - second defense layer

Virulent and host adapted pathogens secrete proteins, known as effectors, into the apoplast or directly into plant cells to manipulate the host and suppress PTI (Büttner, 2016) (Fig. 1). A successful suppression of the plant innate immune system affects the susceptibility of a plant and is called effector-triggered susceptibility (ETS) (Jones & Dangl, 2006). To overcome ETS, plants have a second layer of immunity, which is particularly important for biotrophic pathogens (Glazebrook, 2005). In an avirulent pathogen-host interaction, plants can recognize effectors secreted by pathogens using intracellular nucleotide-binding leucine-rich repeat immune receptors (NLRs). This recognition triggers a strong and robust plant response known as effector-triggered immunity (ETI) (Lolle et al., 2020) (Fig. 1). Gram-negative bacteria, such as *Pst*, can use the type III secretion system (T3SS) to release effectors into the plant cell. (Coburn et al., 2007; Cunnac et al., 2009). In the case of a defective T3SS, T3SS-dependent effectors can no longer be secreted into the plant cell, which leads to a loss of virulence. One example is the modified *Pst hrcC*$^{-}$ strain, which cannot form a functional T3SS and therefore does not cause disease symptoms in Arabidopsis (Hauck et al., 2003). While *Pst* can repress immune responses in the Arabidopsis accession Col-0 and is therefore a virulent pathogen that enables ETS. Other bacterial strains induce ETI in Col-0 (Velásquez et al., 2017). For instance, the bacterial strain *Pseudomonas syringae* pv. *maculicola* expresses the bacterial effector avrRPM1, which is recognized by RPM1, an NLR receptor with a coiled-coil (CC) domain (CNL). The CNL receptor RPM1 detects the interaction of the effector avrRPM1 with the host target RPM1-interactiong protein 4 (RIN4), leading to activation of an immune response (Mackey et al., 2002). Another subgroup of NLRs, besides CNLs, are receptors with a toll-interleukin-1 receptor-like domain (TNLs). P*seudomonas syringae* pv. *pisi* expresses the bacterial effector avrRPS4. Arabidopsis induces ETI after sensing avrRPS4 with the TNL receptor pair Resistance to *Ralstonia solanacearum* 1 (RRS1) and Resistance to *Pseudomonas syringae* 4 (RPS4) through a decoy WRKY domain, which is homologous to the actual WRKY domain containing plant targets of avrRPS4 (Le Roux et al., 2015; Sarries et al., 2015; Hinsch & Staskawicz, 1996). All TNL receptors require the nucleocytoplasmic immune regulator Enhanced Disease Susceptibility 1 (EDS1) to initiate ETI following effector perception (Dongus & Parker, 2021). In contrast, only a few cases of CNL-mediated ETI are known which require EDS1 for downstream signaling (Xiao et al., 2005; Chandra-Shekara et al.,

2004). The null mutant *eds1-1* has a significant higher susceptibility against *Hyaloperonospora arabidopsidis* and *Pseudomonas syringae* emphasizes the important role of EDS1 for the plant immune response against pathogens with a biotroph phase (Cui et al., 2017).

1.3 Local immune response in plants

1.3.1 The role of chloroplasts in plant immunity

In addition to the outstanding role of chloroplasts as the site of photosynthesis and as a cold sensor (section 1.5), plastids are also a major synthesis site for precursors of immune signalling and response metabolites like salicylic acid (SA), jasmonic acid (JA), lignin, camalexin and glucosinolates (Ruan et al., 2019; Mucha et al., 2019; Liu et al., 2018; Gigolashvili et al., 2009; Wildermuth et al., 2001). The fact that chloroplasts are essential synthesis hubs of immune response-related metabolites underlines the outstanding role of chloroplasts for the interaction with plant pathogens (Fig. 1). Chloroplasts are one of the main sources of ROS in the plant cell and the reactive oxygen species (ROS) generated in chloroplasts or redox changes in chloroplasts modify potential signaling pathways important for the immune response (Yang et al., 2021; Asada, 2006). Chloroplasts employ plastid-to-nucleus retrograde signaling as an important cell communication strategy, in which ROS can act as a signal modifier. One well-described example of the modifying role of ROS is the phosphatase SAL1-phosphoadenosine-5′-phosphate (SAL1-PAP) retrograde signaling pathway (Chan et al., 2016). Chloroplast localized SAL1 show decreased activity after chloroplast derived ROS accumulation leading to PAP formation. The mobile signal compound PAP migrates in the nucleus and effects expression of stress responsive genes. Accordingly, mutant lines of *sal1* show enhanced susceptibility against avirulent, (hemi)biotroph pathogens and hypersusceptibility against a nectroroph pathogen as well as altered phytohormone levels of SA, JA, and glucosinolates (Li & Kim, 2022; Ishiga et al., 2017). Another example is the volatile compound β-cyclocitral (βCC), a ROS-induced degradation product of β-carotene. βCC enhances the transcription of *Isochorismate Synthase 1* (*ICS1)*, increases SA levels and activates Nonexpressor of Pathogenesis-related Genes 1 (NPR1) (Li & Kim et al., 2022). These examples demonstrate that altering ROS levels in chloroplasts can significantly affect plant immune responses through retrograde signaling.

Some pathogens secrete effectors into the plant cell of wich some can enter chloroplasts and target chloroplast localized proteins. In addition, other secreted compounds can also affect chloroplasts. One example of chloroplast-localized pathogen effectors that has been extensively studied is HopK1. HopK1 is secreted by *P*. *syringae* and has been shown to suppress or dampen programmed cell death (PCD) and the ROS burst and therefore has a significant impact on virulence (Li et al., 2014; Jamir et al., 2004). The effector Pst_12806, which is secreted by *Puccinia striiformis* f. sp. *Tritici*, targets a subunit of the cytochrome b_6f complex, ultimately leading to impaired photosynthesis and reduced accumulation of ROS (Xu et al., 2019). After pathogen recognition, plants can utilize stromules to rearrange their chloroplasts toward the pathogen surface (Savage et al., 2021). *N*. *benthamiana* employs this strategy to fight *Phytophthora infestans*, a fungus that forms haustoria inside the plant cell. The pathogen utilizes the AVR3a effector to inhibit the formation of stromules, thus reducing the contact surface with chloroplasts (Savage et al., 2021). PTI-induced defense mechanisms include the reduction of non-photochemical quenching (NPQ), which could lead to increased ROS accumulation in chloroplasts (Göhre et al., 2012). Infection by *S*. *sclerotiorum* and *B*. *cinerea* results in the secretion of oxalic acid, which acidifies the host plant tissue and manipulates the chloroplast-localized xanthophyll cycle. This manipulation leads to an accumulation of zeaxanthin at the expense of violaxanthin, ultimately increasing NPQ. Furthermore, ROS formation and callose formation are altered by secreted oxalic acid (Bi et al., 2023; Zhou et al., 2015). In summary, these examples show that some pathogens manipulate plant ROS homeostasis directly in the chloroplast, suggesting an important role for chloroplast-mediated ROS in the plant immune response. Finally, ROS produced in the chloroplast, which reaches among other organelles the cell nucleus via stromules, is thought to positively impact the induction of programmed cell death (PCD) (Serrano et al., 2016; Zurbriggen et al., 2009). Mitogen-activated protein kinase 3 (MPK3) and mitogen-activated protein kinase 6 (MPK6), important for pathogen resistance (Galetti et al., 2011; Menke et al., 2004), are thought to contribute to chloroplast-mediated PCD by inhibiting photosynthesis, leading to increased ROS accumulation. This indicates a regulatory role for MPK3 and MPK6 in chloroplast-mediated PCD (Su et al., 2018).

1.3.2 Apoplastic ROS

Apoplastic ROS is an essential signaling compound and is involved in one of the earliest defense responses after PAMP recognition, which occurs within minutes (Torres, 2010). Plants utilize various enzymes capable of apoplastic ROS production, including lipoxygenases, peroxidases and superoxide dismutases (SOD). Peroxidases, for example, produce ROS for lignol polymerization, contributing to the maintenance of cell wall rigidity. However, the regulatory mechanisms for class III peroxidases are relatively uncharacterized (Rivas et al., 2024; Podgórska et al., 2021). This chapter focuses on the key hub for apoplastic ROS production, the plasmamembrane-localized NADPH oxidase (respiratory burst oxidase homolog, RBOH) (Rivas et al., 2024). Arabidopsis provides 10 different isoforms of RBOH, with high similarity in the C-terminal and membrane-spanning domains, but a more divergent N-terminal domain at the cytosolic side (Sagi & Fluhr, 2006). RBOH utilizes cytosolic NADPH for electron transfer to apoplastic oxygen and produces superoxide radicals, which dismutate spontaneously or via SOD to hydrogen peroxide. Hydrogen peroxide can propagate apoplastic long-distance signaling by activating ROS production in neighboring cells via RBOHD, or it can translocate through aquaporins such as plasma membrane intrinsic protein 2;1 (PIP2;1) into the symplasm (Rivas et al., 2024). While some RBOH fulfill specific functions, such as RBOHC's role in root hair formation, RBOHD and RBOHF are pleiotropic and stress-inducible proteins, responding to pathogens, abiotic stress signaling and stromatal closure (Morales et al., 2016). In particular, RBOHD is responsible for pathogen-induced apoplastically directed ROS production (Nühse et al., 2007; Torres et al., 2002). RBOHD can be activated through PAMPs as part of the PTI response (Fig. 1). For example, the PAMP flg22 induces a complex formation of Flagellin-sensing 2 (FLS2) and Brassinosteroid Insensitive 1-associated Receptor Kinase (BAK1), leading to an immediate rise of intracellular Ca^{2+}. This, in turn, activates Calcium-dependent Protein Kinase 5 (CPK5), which finally phosphorylates the N-terminus of RBOHD, inducing the activation of RBOHD and an apoplastic ROS burst (Dubiella et al., 2013; Sun et al., 2013; Miller et al., 2009). Both PTI and ETI can trigger an RBOHD-dependent transient and rapid ROS burst upon recognition of PAMPs/pathogens (Yuan et al., 2021). Although the RBOHD-dependent ROS burst is a component of PAMP-induced signaling, the contribution of RBOHD to plant resistance remains elusive. This is because infection with *B. cinerea* or *Pst* in the *rbohD* mutant leads to susceptibility similar to that of the wild type (Marino et al., 2012). While the antimicrobial properties of H_2O_2 against fungi, oomycetes and bacteria have been sufficiently proven in *in vitro* experiments (Juven

& Pierson, 1996; Peng & Kuc et. al., 1992), there is a lack of evidence for an antimicrobial effect of H_2O_2 *in planta*.

1.3.3 The phytohormone salicylic acid

The phytohormone SA, an important mediator of plant defense responses, originates from two independent pathways in higher plants (Garcion et al., 2008). Plants initiate SA biosynthesis with the educt chorismate in chloroplasts via an isochorismate synthase (ICS) (Fig. 1). Alternatively, plants utilize the PAL pathway for basal SA levels (Huang et al., 2020). In Arabidopsis, biotic stress primarily induces the biosynthesis of *ICS1*-dependent SA, while the PAL pathway only contributes to 5 – 10 % of SA production (Wildermuth et al., 2001). A central positive regulator of SA-responsive immune signaling is NPR1. In the resting state, NPR1 is present in the cytoplasm as an oligomer and SA accumulation leads to cellular redox changes and the reduction of NPR1. This step is mediated by thioredoxin H-type 3 (TRX-h3) and thioredoxin (TRX-h5), which reduce Cys156 of NPR1 and disassemble the oligomer into the active dimer. Following NPR1 release from the oligomer and other post-translational changes, NPR1 translocates into the nucleus after binding SA (Zavaliev & Dong, 2024; Kumar et al., 2022). In the nucleus, NPR1 recruits transcription factors and induces the expression of the immune response related protein Pathogenesis-Related 1 (PR1) next to other PR proteins (Chen et al., 2019; Zhang et al., 1999). Despite its antimicrobial activity, the biological function of PR1 is still elusive (Han et al., 2023). Although the exact mode of action of an SA-induced immune response is not fully understood, it has been shown that *npr1* and *ics1* (*sid2)* mutants have a significantly higher susceptibility against *Pst* and *Pseudomonas syringae* pv. *maculicola ES4326*, emphasizing the importance of SA-signaling against pathogens with a biotroph growing phase (Yoo et al., 2022). SA also contributes to the resistance against necrotrophic *B*. *cinerea* in Arabidopsis. Arabidopsis resistance against *B*. *cinerea* is independent of NPR1 and ICS1, but plants expressing salicylate hydroxylase (NahG, SA-degrading enzyme) or treated with a PAL inhibitor showed increased susceptibility, suggesting a more important role for the PAL pathway in *B*. *cinerea*-Arabidopsis interaction (Ferrari et al., 2003).

1.3.4 The phytohormone jasmonic acid

The phytohormone JA and its derivative methyl esters (MeJA) and isoleucine conjugates (JA-Ile) are known as jasmonates. In addition to a variety of regulatory functions in plant growth and development, JA improves the stress tolerance of plants against abiotic (e.g. cold and drought) and biotic stress conditions (e.g. herbivores and necrotrophic pathogens) (Fig. 1). Its biosynthesis is initiated in the plastids by the release of α-linolenic acid from plastid membrane derived galactolipids, and finally, cis-(+)-OPDA is translocated to the peroxisome and biosynthetic steps in the plastid involve phospholipase A1 (PLA1), 13-lipoxygenase (13-LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC) (Ruan et al., 2019; Scherer et al., 2010). In the next steps, cis-(+)-OPDA is converted to (+)-7-iso-JA with the participation of OPDA Reductase 3 (OPR3), acyl-CoA oxidase (ACX), multifunctional protein (MFP) and 3-ketoacyl-CoA thiolase (KAT) (Wang et al., 2021b; Huang et al., 2017). (+)-7-iso-JA translocates to the cytoplasm where it is conjugated to isoleucine (Ile) to form JA-Ile by the action of Jasmonate Resistance 1 (JAR1). JA-Ile is transported to the nucleus where JA-Ile initiates JAZ degradation after JA-Ile recognition via Coronatine-insensitive Protein 1 (COI1) leading to the activation of transcription factors that initiate JA signaling (Wang et al., 2021b; Li et al., 2017). After its biosynthesis, JA can be metabolized into 12 different jasmonates, which differ in their biological activity. For example, MeJA is the biologically inactive form of JA, while JA-Ile is considered the most biologically active form (Wang et al., 2021b; Wasternack & Song, 2017). Disruption of JA signaling significantly affects plant resistance against necrotrophic pathogens. The *coi1-1* or *jar1-1* mutant lines are highly susceptible against the necrotrophic pathogens *B. cinerea* or *A. brassicicola* (Ferrari et al., 2007; Ferrari et al., 2003; Thomma et al., 1999; Thomma et al., 1998). The necrotrophic pathogen *Sclerotinia sclerotiorum* exhibits enhanced virulence in *coi1-1* and *coi1-2* mutants, confirming the crucial role of JA signaling in combating necrotrophic pathogens (Guo & Stotz et al., 2007). An immune response involving JA signaling often leads to cross signaling with the phytohormone ethylene (ET) (Yang et al., 2019). In general, JA signaling works synergistically with ET in defending against necrotrophic pathogens. For instance, the interaction between JA and ET activates the expression of the antifungal protein Plant Defensin 1.2 (PDF1.2) and thereby resists infection by necrotrophic pathogens (Pieterse et al., 2012; Zhu et al., 2011; Penninckx et al., 1996). In addition, JA acts as an antagonist of SA and activation of the JA pathway negatively impacts the SA response. Conversely, deletion of *COI1* results in increased SA accumulation and enhances resistance against the biotrophic pathogen *Pst* (Li et al., 2019).

Another example of the antagonistic roles of the phytohormones JA and SA is the phytotoxin coronatine (COR) secreted by *Pst* for full virulence. Coronatine mimics jasmonates and suppresses the SA-dependent immune response (Brooks et al., 2005).

1.3.5 Hypersensitive response

The hypersensitive response (HR) is a rapid localized cell death at the side of pathogen infection in plants and a type of programmed cell death (PCD) (Lam, 2004). HR is induced mainly via ETI/R-gene mediated resistance while PTI alone is usually not sufficient to trigger HR (Balint-Kurti, 2019) (Fig. 1). One well-described example of effector-mediated HR is the cytoplasm-localized HopZ-activated Resistance 1 (ZAR1) resistosome (Shi et al., 2020; Wang et al., 2019). ZAR1 is inactive in the resting state until pathogen delivered effecors like HopZ1 of *Pst* mediate ADP release of the ZAR1 complex leading to the primed state. Thereafter, the ZAR1 complex binds deoxyadenosine triphosphate (dATP) or adenosine triphosphate (ATP), initiating structural remodeling and forming a pentamer, which is the active resistosome. It is assumed that the resistosome disrupts plasma membrane integrity by pore formation, which ultimately leads to cell death and simultaneously activates defense genes (Shi et al., 2020; Wang et al., 2019). HR is associated with increased resistance against biotrophic pathogens, as these pathogens obtain their nutrients from living cells. While HR is not effective against necrotrophic pathogens or supports the disease spread as its lifestyle requires dead host tissue (Balint-Kurti, 2019). The phytotoxic HR-inducing protein 1 (Hip1) secreted by *B*. *cinerea* elicits an HR-like response after PTI, suggesting that HR is not only a feature of ETI (Jeblick et al., 2023).

1.3.6 Pathogen interaction at the plant cell wall

Pathogens can enter their host plant through wounds or stomata. In many cases, however, fungal pathogens in particular penetrate directly the host by mechanical force through the cuticle and epidermis (Toruño et al., 2016; Hématy et al., 2009). In addition, pathogens such as *B*. *cinerea* form specialized cells called apressoria and infection cushions (IC) that support host penetration for initial epidermal penetration and the subsequent infection process (Bi et al., 2023). Therefore, IC are particularly important for enhanced pathogen secretion of phytotoxins, cutinases, and various types of plant cell wall degrading enzymes (PCWDE of cellulose, hemicellulose, and pectin) that aid in cell penetration (Choquer et al., 2021). These findings suggest that the plant cell wall serves as acrucial

physical barrier, protecting the host against invading pathogens. Additionally, (1,3)-β-glucan callose acts as a transient physical barrier, safeguarding plant cells against pathogen penetration.. Upon recognizing PAMPs or DAMPs, the callose synthase *Powdery Mildew Resistant 4* (*GSL5/PMR4)* promotes pathogen-induced plant callose deposition between the plasma membrane and the cell wall in Arabidopsis (Wang et al., 2021a). While definitive evidence is still missing, it is assumed that callose as a part of the so-called papillae strengthens the cell wall penetration resistance. The formation of papillae directly at the contact sites of fungal hyphae in infected plant tissue supports the hypothesis that papillae are involved in enhancing cell wall penetration resistance (Hückelhoven, 2007; Nishimura et al., 2003; Jacobs et al., 2003) (Fig. 1). Studies have shown that increased callose accumulation correlates with enhanced resistance against *B*. *cinerea* in *A*. *thaliana* and *S*. *lycopersicum* (Sanmartínetal et al., 2020; Nie et al., 2017). In addition to papillae formation, plant PRRs for chitin and flg22 perception are also localized in the plasmodesmata. Pathogen recognition or SA-treatments can induce callose deposition at the plasmodesmata in an EDS1 and NPR1 dependent manner. Callose deposition at the plasmodesmata reduces the size exclusion limit, thereby potentially minimizing the cell-to-cell transfer of compounds that could enhance pathogen virulence, such as effectors or phytotoxins. However, reports on pathogen-plasmodesmata interaction are limited, except for plant viruses (Iswanto et al., 2022; Tilsner et al., 2016). In contrast to *B*. *cinerea*, *Pst* is an extracellular pathogen and is therefore not dependent on cell wall penetration. However, infection of Arabidopsis with *Pst* and *Pst hrcC*$^{-}$ leads to strong callose deposition in the non-virulent interaction, whereas only 5 % callose deposition could be detected in the virulent interaction, indicating a T3SS effector-induced repression of callose deposition (Hauck et al., 2003). *Pst* secreted effectors such as HopM1, AvrPto, and AvrE1 have been identified to suppress PAMP-induced callose deposition (Wei & Collmer, 2018; Hauck et al., 2003). Another, but irreversible synthesized cell wall rigidification compound is lignin which serves as physical barrier against pathogens (Ma, 2024). Lignin can enhance plant resistance by limiting the influx of toxins into the plant cell and protecting other cell wall components from cell wall-degrading enzymes (Eynck et al., 2012). For instance, infections with *Erwinia carotovora* result in elevated H_2O_2 production and increased lignification in *Brassica rapa* ssp. *pekinensis* (Zhang et al., 2007). Mycelium extracts of *Botrytis cinerea*, *Phoma exigua* and *Fusarium oxysporum* f. sp. *lini* enhance expression of key enzymes for lignol biosynthesis in cell cultures of *Linum usitatissimum* (Hano et al., 2006).

Bacterial infection with *Pst* leads to lignification, which is markedly higher in plants infected with avirulent ETI/HR-inducing strains than with virulent *Pst* strains (Fig 1). In addition, the intensity of lignification correlates with enhanced resistance (Lee et al., 2019). Lignin precursors, such as hydroxycinnamaldehydes and hydroxycinnamic acids, possess antimicrobial properties (Barber et al., 2000). Lignin formed after abiotic or biotic treatments is also called "stress lignin" or "stress-induced lignin" and is to be distinguished from developmental lignin, e.g. in vascular tissue. The lignin composition of stress-induced lignin differs depending on the stress application. However, much is unclear about the regulation of stress-induced lignin and requires further research (Nakamura et al, 2020; Cesarino, 2019).

1.3.7 Camalexin and its role in plant immune response

Arabidopsis thaliana utilizes tryptophan provided by chloroplasts as a precursor for synthesizing indole glucosinolates and camalexin, which are crucial for the plant's immune system (Yang et al., 2020). Camalexin has a major impact on plant resistance against invading pathogens and is a significant sink of tryptophan (Mucha et al., 2019; Ferrari et al., 2007; Thomma et al., 1999) (Fig. 1). Camalexin biosynthesis involves several cytochrome P450 enzymes, glutathione transferases (GSTs) and γ-glutamyl peptidase1 (GGP1). The final biosynthesic step requires the unique cytochrome P450 enzyme Phytoalexin Deficient 3 (PAD3) (Mucha et al., 2019). *PAD3* expression is wildtype-like in *NahG*, *npr1*, *coi1* and *jar1* mutant lines after *B. cinerea* infection, indicating SA-, JA-, and ET-independent *PAD3* expression (Ferrari et al., 2007). In contrast, a regulatory role of MKP3/MPK6 along with the transcription factor WRKY33 was discovered for camalexin biosynthesis when ET and JA are synergistically involved after *B. cinerea* infection. Camalexin is an important antimicrobial compound against various biotrophic and necrotrophic pathogens and improves resistance against protists (*Plasmodiophora brassicae*), fungi (*Botrytis cinerea*), and oomycetes (*Phytophthora brassicae*) (Nguyen et al, 2022; Lemarié et al., 2015; Schlaeppi et al, 2010; Ferrari et al, 2007; Ferrari et al, 2003; Thomma et al, 1998). Camalexin is also induced during infection with bacterial pathogens (*Pst* and *Pseudomonas syringae* pv*. maculicola ES4326*), but camalexion accumulation does not alter resistance against these strains (Glazebrook & Ausubel et al., 1994).

1.3.8 Systemic immune response (systemic acquired resistance)

Upon local pathogen recognition, systemic signals propagate from the affected local site throughout the plant, establishing a prepared state in the systemic, unaffected tissues to exhibit enhanced resistance in response to a subsequent secondary infection. This phenomenon is referred to as systemic acquired resistance (SAR) and it aligns with priming in the conceptual framework, as both responses involve the alteration of a stress response following a prior stress treatment (Vlot et al., 2021, Conrath et al., 2015). SAR induces systemic broad-spectrum resistance against (hemi-)biotrophic pathogens after SA-dependent PTI or ETI activation at local infection sites (Vlot et al., 2021; Conrath et al., 2015; Spoel & Dong, 2012; Vlot et al., 2009). NahG-expressing plants do not accumulate SA locally or systemically and are lacking a systemic SAR response, indicating that SA is an essential phytohormone for SAR induction. Due to the mobility of SA/methyl-SA (MeSA) in apoplastic and vascular tissues, SA and its derivatives contributes to systemic SAR signaling, but is not the sole cause of SAR (Lim et al., 2016; Vlot et al., 2009; Park et al., 2007). The *sid2-1* mutant, which is defective in pathogen-triggered SA accumulation, shows a weaker but still significant SAR response on bacterial titer level (Bernsdorff et al., 2016). Therefore, other components besides SA are relevant for SAR. A main regulator of SAR besides SA is pipecolic acid (Pip) and the putative bioactive derivative N-hydroxypipecolic acid (NHP) (Hartmann et al., 2018 ; Chen et al., 2018). Pip is synthesized in chloroplasts from L-lysine via two reaction steps involving the aminotransferase AGD2-like Defense Response Protein 1 (ALD1) and the reducing factor SAR-Deficient 4 (SARD4) (Vlot et al., 2021). After leaving chloroplasts through EDS5 (Dmitrij et al., 2019), Pip is converted to NHP in the cytoplasm by the Flavin-dependent Monooxygenase 1 (FMO1) (Hartmann et al., 2018). Mutations in ALD1 and FMO1 abolish SAR, whereas SAR is compromised but not abolished in a SARD4-deficient mutant (Ding et al., 2016; Návarová et al., 2012; Mishina & Zeier, 2006). However, the essential role of SARD4 in Pip/NHP biosynthesis is probably not the cause for the reduced but still significant SAR induction in *sard4*, since Pip was not detected in the systemic leaves of the *sard4* mutant after SAR induction. (Ding et al., 2016; Návarová et al., 2012; Mishina & Zeier, 2006). One identified regulatory pathway of Pip/NHP biosynthesis is the MAPK pathway. MAPK 3 and MAPK 6 regulate Pip biosynthesis through an interaction of the transcription factor WRKY33 with the ALD1 promoter (Wang et al., 2018). Another major compound of SAR signaling is ROS. One example for the role of ROS was revealed by a study about the pathogen-inducible Calcium-dependent Kinase 5 (CPK5): CPK5 activates apoplastic directed ROS generation through

RBOHD leading to the expression of SAR-responsive genes, accumulation of SA and NHP and enhance SAR (Guerra et al., 2020; Dubiella et al., 2013). In addition, ROS formation leads to fragmentation of C18 membrane lipids into C9 dicarboxylic acid (AzA) (Vlot et al., 2021). It is suggested that AzA acts as a further SAR signal because it is pathogen inducible, systemically mobile and exogenous AzA application stimulates defense responses in a SA-Pip/NHP-dependent manner (Jung et al., 2009). In summary, SAR signaling in the plant is based on SA, Pip/NHP and ROS, among other factors such as Aza or EDS1 (Zeier, 2021). Plants are also able to propagate SAR from plant-to-plant. Infected plants emit volatile organic compounds (VOCs) such as pinenes and camphene that are recognized via Legume Lectin-like Protein1 (LLP1) which leads to ROS- and Pip-accumulation, enhanced expression of SAR-associated genes and emission of further VOCs in signal receiving plants (Wenig et al., 2019; Riedlmeier et al., 2017).

1.4 Cold acclimation and cold priming

In temperate or boreal climates low to freezing temperatures affect plant growth and development. Low temperatures (< 10 °C) suppress growth, whereas freezing temperatures (< 0 °C) lead to drought stress and mechanical wounding. Consequently, plants of cold climates have evolved strategies including cold acclimation to acquire increased freezing tolerance while experiencing low temperatures (Leuendorf et al., 2020; Penfield, 2008). Cold exposure is immediately sensed through membrane rigidification, Ca^{2+}-signaling, and imbalance in photosynthetic electron transport. Among multiple other cell responses, cold-inducible transcripts and phytohormones respond to cold within hours. Although cold sensing and responding starts quickly, it takes a few days of adjustment for full cold acclimation (Baier et al., 2019; Zhao et al., 2017; Ensminger et al., 2006). Freezing temperatures cause a reduction in water potential within plant cells, resulting in a substantial loss of osmotically active water. To prevent subsequent drought stress, the plant synthesizes cryoprotectants such as soluble sugars, proline, and polyamine. Soluble sugars, including sucrose, glucose, and fructose, are particularly effective in preventing mechanical damage caused by the formation of ice crystals within the cells (Satyakam et al., 2022; Xin & Browse, 2000). Because maintenance of cold acclimation is cost-intensive (Jackson et al., 2004; Browse & Lange, 2004), plants inititate a fast deacclimation process after temperatures increase again (Zuther et al., 2015). Next to long-term cold acclimation processes that are useful for plants to survive freezing temperatures in the winter, short period temperature drops in the spring are also stressful for plants (Baier et al., 2019).

Therefore, plants need further adjustment strategies. One such strategy is called cold priming. Priming is a modified stress response in which an initial sequential and transient stress recognition leads to an earlier, faster, stronger, or more sensitive reaction during a subsequent stress experience (trigger). Priming can be a consequence of very different types of stressors and both stress stimuli (priming and triggering) can be of the same (cis-priming) or different origin (trans-priming). Furthermore, priming implies a stress-free memory phase between the two stress treatments (Hilker et al., 2016). Cold priming (24 h at 4 °C) leads to a different expression pattern of cold and pleiotropic stress-responsive genes like the *Zinc Finger of Arabidopsis thaliana 10* (*ZAT10*, AT1G27730), increased ROS generation and revealed *tAPX* as a priming mediator, whereas cold priming memory was lost after 7 days (van Buer et al., 2019). The crucial function of tAPX as a mediator for cold priming is likely attributed to its hypothetic localization at photosystem I, where tAPX-dependent H_2O_2 detoxification impacts H_2O_2 signaling (van Buer et al., 2019; van Buer et al., 2016;). In addition, cold priming builds a stress memory that modifies the stress response to the triggering stress without additional metabolic adjustment, which occurs in cold-acclimatized plants (Baier et al., 2019; Hilker et al., 2016).

1.5 Cold perception in chloroplasts

Chloroplasts as the sites of photosynthesis are highly sensitive to the balance of redox potential energy generated in the light reaction and the main metabolic sink, the Calvin cycle. This balance, known as photostasis, can become disrupted because the light reaction is less temperature-dependent than the downstream reactions of the Calvin cycle (Ensminger et al., 2006). Abiotic stress, such as cold exposure, reduce the sink's ability to utilize the total redox potential energy, resulting in excess energy and increased formation of ROS (Ensminger et al., 2006; Huner et al., 1993) (Fig. 1). ROS include free radicals such as superoxide anion (O_2-), hydroxyl radical (-HO), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2). In addition to the toxic properties of high ROS levels, which can destroy lipids, DNA, and proteins, ROS are essential signaling molecules in the plant and induce nuclear transcriptional changes, inhibition of phosphatases or trigger protein redox switches (e.g. cystein residues) and activation of the Mitogen-activated Protein Kinase (MAPK) pathway (Ugalde et al., 2021; Magder, 2006; Apel & Hirt, 2004). H_2O_2 produced by chloroplasts after photo-oxidative stress migrates via aquaporines into the cytosol and other cell compartments (Ugalde et al., 2021) (Fig. 1), indicating putative cold-

induced redox changes and downstream signaling changes that are not restricted to chloroplasts. In summary, chloroplasts function as a cold sensor.

1.6 Role of chloroplast localized sAPX and tAPX for ROS homeostasis

As mentioned in section 1.5, cold exposure disrupts photostasis and leads to increased ROS generation in chloroplasts (Ensminger et al., 2006; Huner et al., 1993). In order to counteract toxic ROS accumulation, the plant has several chloroplast-localized ROS scavenging systems. Enzymatic ROS scavenging systems in chloroplasts includes ascorbate peroxidases (APX) (Miyake et al., 1993), 2-Cys Peroxiredoxins (2CPA) (Baier & Dietz, 1997), glutathione peroxidases (Chang et al., 2009), and superoxide dismutases (SOD) (Asada, 1999). Ascorbate is the most abundant water-soluble redox compound in plants, highlighting the importance of APX as a ROS scavenging system (Ishikawa & Shigeoka, 2008). APX reduces H_2O_2 to H_2O with the help of ascorbate (Groden & Beck, 1979) (Fig. 1). Nine nuclear-encoded APX isoforms are located in different cellular compartments (cytosol, chloroplasts, mitochondria, and peroxisomes) in Arabidopsis. Two of the nine APX isoforms from Arabidopsis are catalytically active and localized in chloroplasts, of which one is soluble in the stroma (sAPX) and one is bound to the thylakoid membrane (tAPX) (Fig. 1). One additional APX isoform was identified in the thylakoid lumen, although its function remains unknown (Ishikawa & Shigeoka, 2008). Under high-light stress, which has a comparable impact on photostasis as cold exposure (Kameoka et al., 2021), knockout of *sAPX* or *tAPX* leads to lower expression of H_2O_2-responsive genes, higher H_2O_2 accumulation in leaves and more oxidized proteins. In contrast, knockout of *sAPX* and *tAPX* show wild-type-like expression of other APX isoenzymes and chloroplast-localized antioxidant enzymes without stress application (Kameoka et al., 2021; Maruta et al., 2010).

1.7 Cold acclimation at the plant cell wall

Temperatures below the freezing point lead to the formation of extracellular ice crystals, resulting in dehydration of the cell and thereby, destruction of the membrane. The cell wall acts as a physical barrier, protecting against ice ingress and controlling its spread, making it important for freezing tolerance and a potential site for cold acclimation (Takahashi et al., 2021). The plant cell wall is composed of cellulose, which provides mechanical strength. Hemicellulose cross-links the cellulose fibrils to enhance this function.

Pectin is another essential component that is also considered to play a role in the structural integrity of the cell wall (Tenhaken, 2015). Additionally, the cell wall of Arabidopsis contains 14 % proteins (Zablackis et al., 1995). It was shown that increased pectin content is associated with cold acclimation in oilseed rape, while other cell wall polysaccharides remain unaffected (Kubacka-Zębalska & Kacperska, 1999). This effect was also observed in a frost-tolerant pea genotype after cold acclimation in comparison to a frost-sensitive genotype (Baldwin et al., 2014). β-1,4-galactan, a polysaccharide that is a part of pectin, shows a higher abundance in spinach (*Spinacia oleracea* "Progress"), Japanese mustard spinach (*Brassica rapa* var. *Perviridis* "Hamatsuduki"), crown daisy (*Glebionis coronaria* "Satoakira"), and *Arabidopsis thaliana*, which correlates with enhanced frost tolerance. Additionally, these cold acclimation-induced changes are associated with lower cell wall extensibility and higher rigidity in Arabidopsis (Takahashi et al., 2024). Taken together, these results suggest that pectin plays an important role in altering the mechanical properties of the plant cell wall, which is crucial during cold acclimation. Cellulose and hemicellulose exhibit cold exposure-dependent regulation, but with contrasting results, making it difficult to determine their specific roles in cold acclimation (Le Gall et al., 2015). Lignin is another putative compound for cold acclimation in plants . Lignin is synthesized primarily in the secondary cell wall of specialized cells, such as tracheal elements and endodermal cells. However, it can also be synthesized in the mesophyll/epidermis after stress recognition (Barros et al., 2015; Cabané et al., 2004). The biosynthesis of lignin begins with phenylalanine from the phenylalanine ammonia-lyase (PAL) pathway in chloroplasts. The product cinnamic acid is utilized to synthesize three main monolignols that are formed in multiple steps in the cytoplasm (sinapyl alcohol, S-unit; coniferyl alcohol, G-unit; and p-coumarcyl alcohol, H-unit). These lignol monomers are transported into the apoplast and polymerized to lignin via peroxidases and laccases (Liu et al., 2018). In *Brassica napus*., PAL activity significantly increases after cold acclimatization, and lignin precursors, such as hydroxycinnamic acids, are also significantly more abundant (Solecka & Kacperska, 2003; Solecka et al., 1999). In Arabidopsis, exposure to as little as 12 hours of cold is enough to significantly upregulate several genes responsible for lignin biosynthesis (Hannah et al., 2005). It is suggested that pectin and lignin may work together to strengthen the cell wall and are involved in the response to cold exposure. However, the exact molecular mechanisms, including cell wall acclimation, that contribute to cold tolerance are still largely unknown (Takahashi et al., 2024).

1.8 Pre-exposures to abiotic changes affecting plant pathogen defence

While the plant immune system and many host pathogen interactions are conceptually understood (Albert et al., 2020; Lolle et al., 2020; Jones et al., 2016), the impact of abiotic stress on host pathogen interactions is less understood. Evidence suggests that the antagonistic phytohormones SA and JA together with ROS play an important role in the crosstalk between abiotic and biotic stresses but further research is needed to fully understand the concept of stress crosstalk (Ku et al., 2018; Fujita et al., 2006). Environmental factors such as the infection timepoint or photoperiodic stress significantly impacts the susceptibility of Arabidopsis against *Pst*, whereby, for example, the enhanced resistance of Arabidopsis correlates with increased SA accumulation in the case of an early infection event during the day (Lajeunesse et al., 2023; Griebel und Zeier 2008). Some studies have investigated the impact of different temperatures during infection on the susceptibility of Arabidopsis against *Pst*. It has been shown that higher temperatures (28 – 30 °C) dampen ETI and HR but enhance PTI, which is linked to stronger susceptibility against *Pst*. Conversely, temperatures of about 23 °C result in decreased susceptibility of Arabidopsis against *Pst* . One possible explanation for the enhanced susceptibility at higher temperatures is the plant's compromised capacity to accumulate SA after infection, which is also associated with weaker *ICS1* and *PR1* expression (Huot et al., 2017; Menna et al., 2015). In addition, exposure to cold (4 °C) for more than a week leads to significant enhanced SA accumulation in Arabidopsis even in the absence of a pathogen infection (Scott et al., 2004; Kim et al., 2013). There are limited reports about the impact of a prior abiotic stressor on resistance against *Pst*. A total of 7 stress treatments (1.5 h per day) with heat stress (38 °C), cold stress (4 °C) or salt stress leads to increased resistance against *Pst* in Arabidopsis and the effect of the increased resistance is lost after 7 days. Increased resistance in response to such repetitive stress treatments correlates with upregulation of PTI-responsive genes and is mediated by the epigenetic regulator Histone Acetyltransferase 1. Interestingly, a heat stress treatment is not sufficient to enhance resistance against the necrotrophic pathogen *B*. *cinerea* (Singh et al., 2014). Another report shows that a 10-hour cold exposure during the night phase is sufficient to transiently increase the H_2O_2 content and resistance of Arabidopsis against *Pst* in a SA-dependent manner. However, the cold-primed memory upon only 10 h cold exposure is already lost after 12 hours (Wu et al., 2019). In summary, the examples show that SA is elevated during extended exposure to cold, or at cooler temperatures during infection, and could therefore contribute to increased resistance againt pathogens with a biotrophic phase (Huot et al., 2017; Kim et al., 2013; Scott

et al., 2004). In contrast, the effect of brief cold stress on plant immune response is less well understood (Wu et al., 2019; Singh et al., 2014). Moreover, there are limited studies on the impact of short-term cold stress on the interaction of plants and necrotrophic pathogens such as *B*. *cinerea*.

1.9 Aim of the work

In the context of rising temperatures due to climate change, cold temperatures and frost in particular continue to present a significant challenge for plants in our growing regions (Leuendorf et al., 2020; Wersebe et al., 2019; Penfield, 2008).

The primary objective of this thesis was to elucidate the impact of a brief cold exposure (4°C, 24h) on the resistance against *Pst* (Chapter I, II) and *B*. *cinerea* (Chapter III). Both pathogens employ distinct infection strategies for disease progression. We compared two experimental setups with and without a memory phase to ascertain whether Arabidopsis benefits from a memorized cold exposure when colonized by pathogens. In addition, the impact of such cold pre-treatments on the establishment of SAR was determined (Chapter IV).

The second aim of this work (Chapter I-III) was to determine the role of the chloroplast as a cold sensing hub, whereby cold stress-induced ROS formation may affect plant immune signaling pathways. Therefore, we hypothesized that the chloroplast plays an important role in fine-tuning plant resistance after prior cold exposure, which involves the chloroplast-localized ROS scavenger's sAPX and tAPX for cold sensing and crosstalk with plant pathogen defence systems. Finally, we aimed to identify plant immune signaling pathways and metabolites which are affected at the interplay of cold stress signaling and pathogen defense activation.

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Chapter I: Cold Exposure Memory Reduces Pathogen Susceptibility in Arabidopsis Based on a Functional Plastid Peroxidase System

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2.1 Abstract

Chloroplasts serve as cold priming hubs modulating the transcriptional response of *Arabidopsis thaliana* to a second cold stimulus for several days by postcold accumulation of thylakoid ascorbate peroxidases (tAPX). In an attempt to investigate cross-priming effects of cold on plant pathogen protection, we show here that such a single 24-h cold treatment at 4°C decreased the susceptibility of *Arabidopsis* to virulent *Pseudomonas syringae* pv. *tomato* DC3000 but did not alter resistance against the avirulent *P. syringae* pv. *tomato avRPM1* and *P. syringae* pv. *tomato avrRPS4* strains or the effector-deficient *P. syringae* pv. *tomato* strain *hrcC*$^{-}$. The effect of cold priming against *P. syringae* pv. *tomato* was active immediately after cold exposure and memorized for at least 5 days. The priming benefit was established independent of the immune regulator Enhanced Disease Susceptibility 1 (EDS1) or activation of the immune-related genes *NHL10*, *FRK1*, *ICS1* and *PR1* but required thylakoid-bound as well as stromal ascorbate peroxidase activities because the effect was absent or weak in corresponding knock-out-lines. Suppression of *tAPX* postcold regulation in a conditional-inducible *tAPX-RNAi* line led to increased bacterial growth numbers. This highlights that the plant immune system benefits from postcold regeneration of the protective chloroplast peroxidase system.

2.2 Introduction

Plants have evolved sophisticated molecular networks that respond differently to simultaneous or sequentially experienced stress events than to single stress situations (Saijo and Loo 2020; Zhang and Sonnewald 2017). The combination of two sequential and transient stress events in which the exposure to a prior stress leads to earlier, faster, stronger, or more sensitive responses during the subsequent triggering stress defines a priming scenario (Crisp et al. 2016; Hilker et al. 2016). Although plants lack a nervous system and an antibody-based adaptive immune system, the plant capacity for a stress memory is evident and well described (Conrath 2011; Crisp et al. 2016; Gourbal et al. 2018; Hilker et al. 2016). The molecular priming memory is formed during a stress-free interphase (lag or memory phase), when primary stress responses are lost (Hilker et al. 2016). Such a priming memory, which subsequently modifies the response to a later triggering stimulus, can result from chromatin modifications but also be imprinted by preparatory formation or persistence of key signaling metabolites and proteins which are kept in an inactive form during the stress-free memory phase (Baier et al. 2019; Conrath 2011; Crisp et al. 2016). The priming and the subsequent stress events can be of the same nature (*cis*-priming) or differ from each other (*trans*-priming) (Hilker et al. 2016). The phenomenon of systemic acquired resistance (SAR) is an intensively studied example of priming in plants, in which a pathogen infection leads to improved and preactivated immune responses in distant, uninfected tissues (Conrath 2011). SAR requires long-distance signaling and provides long-lasting protection against a broad range of pathogens (Fu and Dong 2013; Shah and Zeier 2013). Pathogen-induced priming leads to a transcriptional reprogramming in uninfected plant tissues, including chromatin opening and modification and preactivation of immune-related genes (Baum et al. 2019; Gruner et al. 2013; Jaskiewicz et al. 2011).

In contrast to multiple and ternary stress concepts, the dual plant–pathogen interaction based on the plant innate immune system is broadly and conceptually understood (Albert et al. 2020; Jones et al. 2016; Lolle et al. 2020). Plants detect pathogens through recognition of pathogen-associated molecular patterns (PAMPs) by cell surface-exposed pattern recognition receptors (PRRs). PRR activation induces defense responses, summarized as PRR- or PAMP-triggered immunity (PTI), and is efficient against a broad range of pathogens (Albert et al. 2020). Host-adapted and virulent pathogens suppress PTI by secreting virulence proteins (so-called effectors) into the host cells with the aim to manipulate cellular physiology and to suppress innate immunity (Büttner 2016). This process strongly

affects the susceptibility of the plant against pathogens and is designated accordingly as effector-triggered susceptibility (ETS) (Jones and Dangl 2006). A further layer of pathogen defense comprises intracellular nucleotide-binding leucine-rich repeat immune receptors (NLRs) that intercept the presence or activity of pathogen virulence effectors and initiate plant responses summarized as effector-triggered immunity (ETI) (Lolle et al. 2020). Two structurally different N-terminal domains, Toll-interleukin1 receptor-like and coiled-coiled, form two major groups of plant NLRs: TNLs and CNLs, respectively (Jacob et al. 2013). For instance, the CNL Resistance to *Pseudomonas syringae* pv. *maculicola* 1 (RPM1) detects the presence of the bacterial effector avrRPM1 by sensing its virulence activity on the RPM1-Interacting Protein 4 (RIN4) (Mackey et al. 2002). An alternative scenario is described by immune activation through the TNL receptor pair Resistance to *Ralstonia solanacearum* 1 (RRS1) and Resistance to *P. syringae* 4 (RPS4) (Griebel et al. 2014). When the bacterial effector AvrRps4 from *P. syringae* pv. *pisi* is expressed in the otherwise virulent *P. syringae* pv. *tomato* DC3000, the TNL pair RPS4/RRS1 traces avrRPS4 interference by using the integrated WRKY domain in RRS1 as a decoy and trap and turns ETS into ETI (Hinsch and Staskawicz 1996; Le Roux et al. 2015; Sarris et al. 2015). Although the bacterial effectors and corresponding NLRs inducing ETI are numerous, all TNL receptors share the requirement of the nucleocytoplasmic immune regulator Enhanced Disease Susceptibility 1 (EDS1) for all identified signaling responses (Dongus and Parker 2021).

The outcome of a dual plant–pathogen interaction is determined at the genetic level and by the repertoire of available plant immune receptors. In addition, environmental factors such as light or temperature shape the plants' capacity to defeat pathogens (Roeber et al. 2021; Saijo and Loo 2020; Velásquez et al. 2018). Lower temperatures often lead to simultaneous activation of plant responses required for cold protection and pathogen resistance, indicating plant cross-tolerance of cold and pathogen defense (Saijo and Loo 2020). At the transcriptional level, even after a several-day-long cold period, reacclimation to precold conditions occurs fast and within hours (Byun et al. 2014; Zuther et al. 2015). Although a single 10-h cold exposure during the night phase is not sufficient to prime plant resistance against the hemibiotrophic bacterial pathogen *P. syringae* pv. *tomato* for several days (Wu et al. 2019), daily repetitive cold treatments of 1.5 h (in the dark) result in a memorized and enduring effect on *in planta P. syringae* pv. *tomato* growth numbers and a preactivation of PTI responses (Singh et al. 2014).

Ascorbate peroxidases (APX) scavenge H_2O_2 by using ascorbate as an electron donor (Groden and Beck 1979). *Arabidopsis thaliana* has nine nuclear-encoded *APX* genes that translate into isoforms with different subcellular localizations (cytosol, peroxisomes, chloroplasts, and mitochondria) (Ishikawa and Shigeoka 2008). Most plants have two active chloroplast APX, of which one is soluble in the stroma (sAPX) and one is anchored in the thylakoid membrane (tAPX) (Ishikawa and Shigeoka 2008; Miyake and Asada 1992; Pitsch et al. 2010). Chloroplastic APX differ from other APX by containing two additional sequence insertions (Pitsch et al. 2010; Wada et al. 2003). At the functional level, active chloroplast APX have a faster half-inactivation time (15 s) compared with cytosolic isoforms (>40 min) when the ascorbate levels are low (<10 µM) (Kitajima 2008). tAPX is part of a first layer to scavenge photosynthesis-generated H_2O_2 at the thylakoids, while sAPX provides downstream antioxidant protection in the stroma (Asada 1999). Chloroplastic APX activity is suggested to be central for increased abiotic stress tolerance by avoiding oxidative damage and supporting retrograde signaling (Caverzan et al. 2012; Maruta et al. 2016; Pandey et al. 2017). However, extreme phenotypes in loss-of-function mutants of single genes could not be observed, probably because of strong functional compensation by other antioxidant enzymes and low molecular weight antioxidants (Kangasjärvi et al. 2008; Maruta et al. 2016).

As shown recently, 4-week-old *A. thaliana* Columbia-0 (Col-0) plants memorize a 24-h exposure (including day and night phase) to mild cold (4°C) for up to 7 days (van Buer et al. 2019). Such cold priming leads to modified activation of cold-stress-responsive genes during a second cold treatment and requires reincreasing expression of *tAPX* after cold priming (Bittner et al. 2020; van Buer et al. 2016, 2019). Consistently, conditional overexpression of *tAPX* establishes a cold memory in the absence of the priming cold treatment (van Buer et al. 2019). Levels of neither reactive oxygen species (ROS) nor ascorbate are altered in cold-primed plants 5 days after the end of the initial cold exposure compared with control plants (van Buer et al. 2016). Here, we investigated whether a single, memorized cold exposure lasting 24 h (including day and night phase) affects plants pathogen resistance against *P. syringae* pv. *tomato*, given that such a cold priming event specifically alters gene regulation during a second cold exposure or a high light treatment in a trigger-specific manner (Bittner et al. 2020, 2021; van Buer et al. 2016, 2019). Further-

more, we analyzed whether plastid APX contribute to cold priming against pathogens because tAPX was shown to be a cold priming mediator during a subsequent cold exposure (van Buer et al. 2016, 2019).

2.3 Results

2.3.1 Cold exposure of 24 h reduces *Arabidopsis* immune susceptibility for up to 5 days in an EDS1-independent manner.

To study whether cold priming affects the plant immune response, we challenged cold-pretreated 4-week-old *Arabidopsis* Col-0 plants (4°C, 24 h) after a recovery period of 5 days by infiltration with different strains of the bacterial pathogen *P. syringae* pv. *tomato* DC3000 (Fig. 1A). In addition to the virulent *P. syringae* pv. *tomato*, avirulent *P. syringae* pv. *tomato* strains were used that additionally express either the bacterial effector avrRPM1 or avrRPS4. Both strains are recognized *in planta* by intracellular immune receptors and activate ETI immune responses (Grant et al. 1995; Hinsch and Staskawicz 1996). The nonvirulent *P. syringae* pv. *tomato* strain *hrcC*$^-$ is deficient of a functional type three secretion system (Roine et al. 1997) and provides information about the plant's resistance without the involvement of type three secretion system–dependent bacterial effectors. Cold-primed Col-0 plants (4°C, 24 h) showed significantly reduced titers of the virulent *P. syringae* pv. *tomato* strain 3 days after infiltration compared with naive control plants when the inoculation was performed 5 days after cold exposure (Fig. 1A). Bacterial titers of the avirulent ETI-inducing *P. syringae* pv. *tomato* strains *avrRPM1* and *avrRPS4* and the nonvirulent PTI-inducing strain *hrcC*$^-$ (Fig. 1A) did not differ in cold-primed and control plants. This indicates that, specifically, plant defense against virulent pathogens benefits from a prior cold exposure while plant resistance against avirulent and nonvirulent pathogens is not affected. Consistent with the results of Wu et al. (2019), *P. syringae* pv. *tomato* infections immediately (1 h) after 24 h of cold exposure (4°C) resulted in reduced bacterial growth in Col-0 (Fig. 1B). Our experiment showed that the effect lasted for 5 days, during which the plants did not experience cold (Fig. 1B). Hence, the priming effect of the 24-h cold treatment counteracted pathogen growth in the plants not only during the period when cold regulation of gene expression weakens out but also later, when the priming effect on the cold sensitivity was established (van Buer et al. 2019). Equal density of the starting inoculum and the infiltration efficiency was confirmed by

measuring bacterial titers 3 h after plant infiltration in control and cold-primed plants (Supplementary Fig. S1).

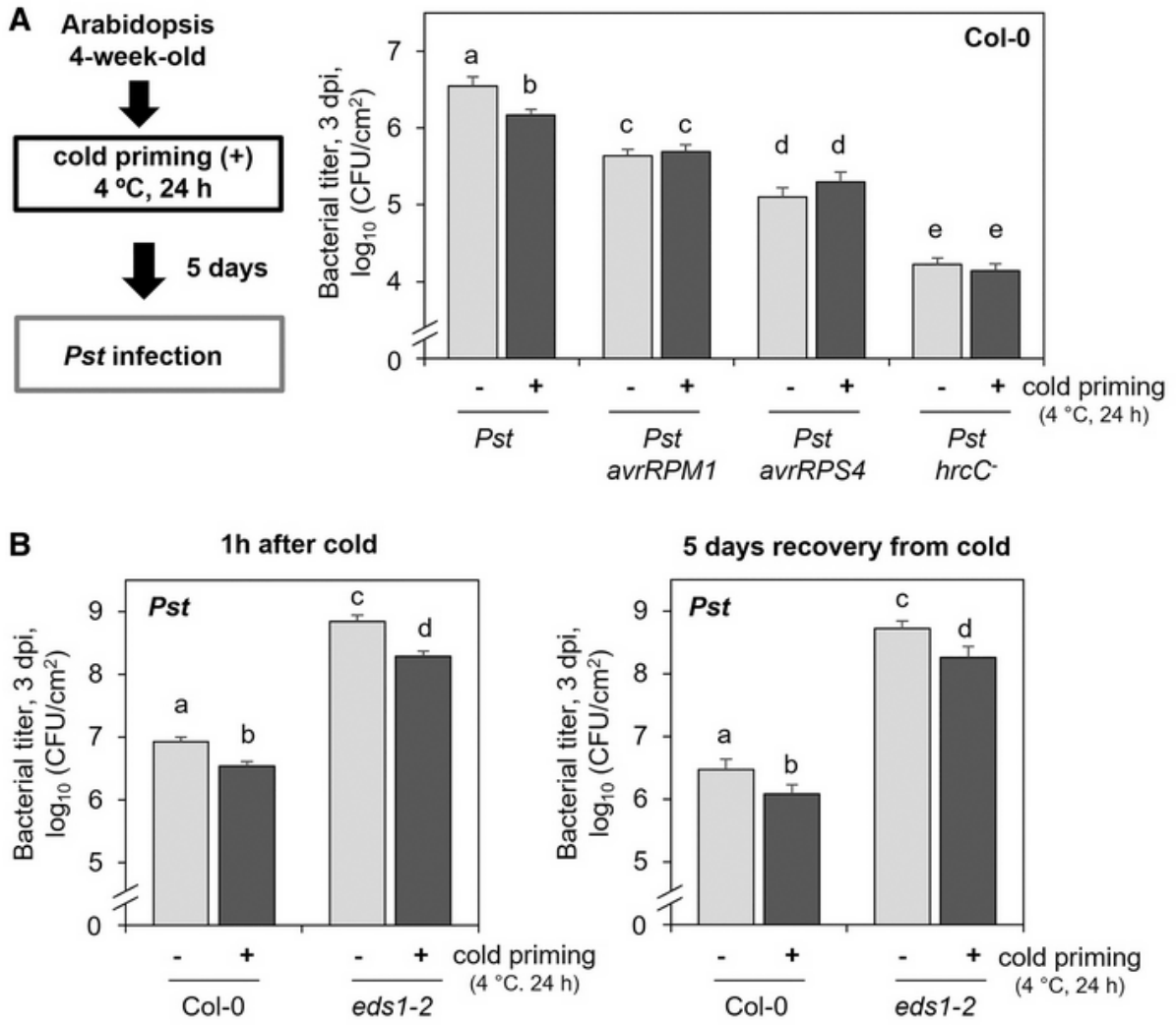

Figure 1: Bacterial growth of *Pseudomonas syringae* pv. *tomato* (*Pst*) in Col-0 and *eds1-2* after a 24-h cold exposure. **A,** Four-week-old *Arabidopsis thaliana* plants were exposed to a 4°C cold priming treatment for 24 h (+). At 5 days after cold exposure, plants were infiltrated with *P. syringae* pv. *tomato* strains to determine the plant's level of resistance and susceptibility. Bacterial titers (log_{10}-transformed) of virulent *P. syringae* pv. *tomato*, avirulent *P. syringae* pv. *tomato avrRPM1* and *avrRPS4*, and the type-three-secretion-deficient *P. syringae* pv. *tomato hrcC*$^-$ strains in cold-primed (+) and control (−) Col-0 plants were determined at 3 days post inoculation (dpi). Bars represent means and standard errors calculated from three independent experiments, each with six to eight biological replicates using a mixed linear model. **B,** Bacterial titers of *P. syringae* pv. *tomato* (log_{10}-transformed) in cold-primed (+) and control plants (−) of Col-0 and *eds1-2* null mutants were determined 3 dpi. Plants were infiltrated with *P. syringae* pv. *tomato* (optical density at 600 nm = 0.001) 1 h (left panel) or 5 days (right panel) after the cold exposure. Bars represent means and standard errors calculated from five independent experiments each, with five to six biological replicates using a mixed linear model. Different letters above the bars denote statistically significant differences (adjusted $P < 0.05$; two-tailed t tests).

To distinguish cold priming regulation from induced basal immunity and resistance responses, we included the strongly immune-compromised *eds1-2* null mutant (Bartsch et al. 2006) into our analyses. EDS1 is an essential molecular plant immune regulator that

contributes to resistance mediated by cell-surface receptors and intracellular TNLs and, hereby, is an integral part of TNL-driven immune signaling in PTI, ETI, and SAR (Breitenbach et al. 2014; Dongus and Parker 2021). The enhanced susceptibility of *eds1-2* against *P. syringae* pv. *tomato* was significantly reduced compared with the level of control plants when the plants were inoculated immediately or after 5 days of recovery from the cold treatment (72 and 65% reduction of bacterial titers, respectively) (Fig. 1B). Cold exposure and its memorization weakened plant susceptibility independent or downstream of EDS1-mediated immune signaling.

2.3.2 Cold response and recovery is functional in immune-impaired *eds1.*

Priming and memory concepts require the perception and response of a first (priming) stimulus which initiates the formation of a molecular memory for future stresses (Hilker et al. 2016). We compared initial cold response and recovery effects between Col-0 and *eds1-2* at the transcriptional level of selected genes directly after 24 h of exposure to 4°C (0 days) and after 3 days. For this analysis, we harvested plant leaves of the same size and developmental status as used for bacterial infiltrations. We selected four genes based on recent work on cold *cis*-priming: *Cold-Regulated Gene 15A* (*COR15A*; At2g42540), *Zinc Finger of Arabidopsis thaliana 10* (*ZAT10*; At2g27730), *Bon-Associated Protein 1* (I; At3g61190), and *Phenyl Ammonia Lyase 1* (*PAL1*; At2g37040) (van Buer et al. 2016). *COR15A,* which encodes a protein protecting the inner envelope of chloroplasts against freezing damage, is strongly induced in the cold and quickly (within 24 h) reset at optimal growth temperatures (Steponkus et al. 1998; Zarka et al. 2003). The same applies to the ROS-induced and pleiotropic stress-responsive genes *ZAT10*, *BAP1*, and *PAL1* (van Buer et al. 2016, 2019). Cold induction of *COR15A* and *ZAT10* reached a similar level in Col-0 and *eds1-2* at the end of the cold exposure and was reset to control rates within 3 days (Fig. 2). This is similar for *BAP1* regulation in Col-0 and *eds1-2*, although at an overall lower level in *eds1-2*. The conversion of L-phenylalanine to cinnamic acid by PAL1 is a key enzymatic step for a multitude of phenlypropanoids such as anthocyanins, flavonoids, hydroxycinnamates, and monolignols but also for the synthesis of basal amounts of the plant hormone salicylic acid (SA) (Ding and Ding 2020). However, pathogen-induced SA is mainly metabolized by iso-chorismate synthase 1 (ICS1), and its gene expression is strongly induced after pathogen attack (Hartmann and Zeier 2019). We could not detect a clear and significant upregulation of *ICS1* at the end of cold priming exposure and after

3 days of reacclimation; however, *ICS1* transcripts were rather reduced after 24 h at 4°C in *eds1-2* (Fig. 2).

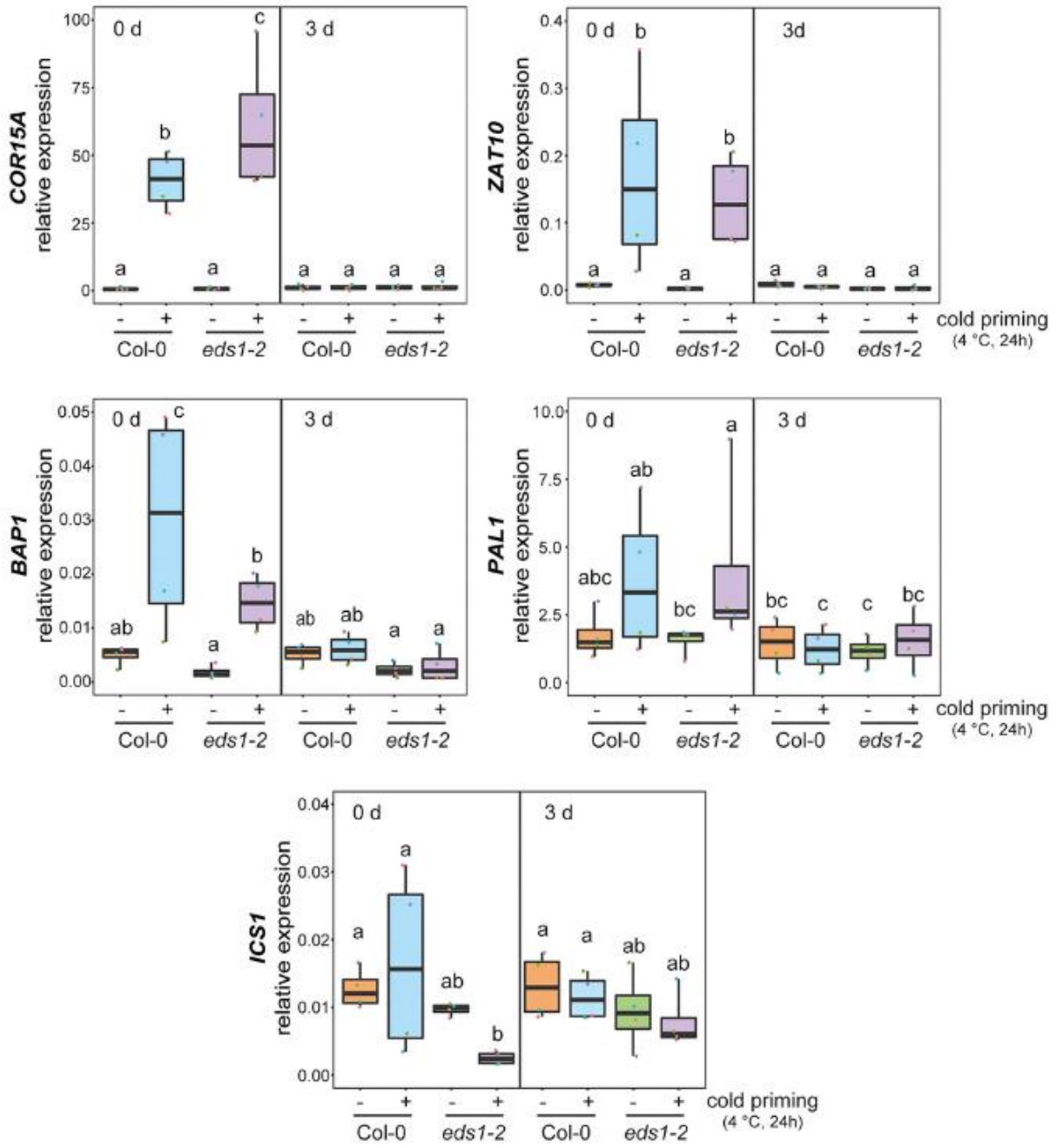

Figure 2: Postcold expression of stress-responsive genes in Col-0 and *eds1-2*. Transcript levels of *COR15A*, *ZAT10, BAP1, PAL1*, and *ICS1* in leaves of Col-0 and *eds1-2* null mutants immediately (0 days) and 3 days after end of cold exposure (+; 4°C, 24 h) were determined by quantitative real-time PCR. Transcript levels in leaves harvested from control plants (−) of the same age are also shown and were determined in four independent experiments as relative expression to the geometric mean of three reference genes (*ACT2*, *YLS8*, and *RHIP1*). Different letters denote statistically significant differences (analysis of variance and least significant difference, $P < 0.05$).

In all of the samples, transcripts of SA-responsive immune marker gene *Pathogenesis-Related 1* (*PR1*) remained at low and basal levels and were not detectable. Overall, this analysis demonstrated that cold signaling during and after exposure to 4°C is perceived and processed in Col-0 and in plants lacking the central immune regulator EDS1. Although infections directly after cold exposure might benefit from overlapping with postcold deacclimation of gene expression, infections 5 days after priming required a molecular memory, because cold-induced genes were already reset for at least 2 days (Fig. 2) (Zuther et al. 2015). The low responsiveness of *ICS1* and *PR1* during and after the postcold phase distinguished cold priming-reduced susceptibility from SAR, in which a first infection leads to an upregulation of immunity genes (including *ICS1* and *PR1)* in the noninfected systemic tissue (Bernsdorff et al. 2016; Gruner et al. 2013; Hartmann and Zeier 2019). The *eds1-2* null mutant, which is impaired in establishing SAR (Breitenbach et al. 2014), showed reduced susceptibility in response to cold priming (Fig. 1). This indicates different regulation of SAR and cold-primed pathogen resistance.

2.3.3 Pathogen-induced transcript levels of selected *PTI* genes and SA signaling are independent from crosstalk with the cold priming memory.

Two recently published transcriptomic data sets showed that, 5 days after 24 h of cold exposure, gene expression patterns differ only marginally (Bittner et al. 2020, 2021). To differentiate transcriptional cold priming effects from preactivation of genes in *P. syringae* pv. *tomato*-induced SAR, we had a closer look at the transcript ratios of all genes from gene ontology (GO) group "SAR" (GO:0009627) at the end of the 5-day recovery phase from both data sets (Bittner et al. 2020; Bittner et al. 2021) and compared with data after 24 h cold exposure (Bittner et al. 2020) and with gene regulation data in a SAR-induced state (Gruner et al. 2013). Although genes from the GO term group "SAR" showed strong differential expression in the SAR data set, differential expression after 5 days recovery from cold exposure or after 24 h cold exposure (4°C) was weak or absent (Supplementary Fig. S2). This analysis confirmed that preactivation of SAR-related genes is not a central component reduced susceptibility by cold priming.

Next, we tested transcript levels of selected immune-related genes as indicators for a stronger and primed activation upon *P. syringae* pv. *tomato* infection. Transcriptome dynamics upon infections with virulent *P. syringae* pv. *tomato* are established rather late (between 16 and 24 h after infection), whereas ETI-inducing pathogens trigger mainly

identical transcriptional changes already between 4 and 6 h (Mine et al. 2018). Hence, we tested the expression levels of PTI-triggered gene *NDR1/HIN1-like10* (*NHL10*, also known as *YLS9*; At2g35980) and mitogen-activated protein kinase-specific target gene *FLG22 Induced Receptor Kinase 1* (*FRK1*; At2g19190) (Boudsocq et al. 2010) 0, 6, and 24 h after *P. syringae* pv. *tomato* infection in leaves of cold-primed and control plants. At the time of pathogen infiltration, no cold-priming-related preactivation of *NHL10* and *FRK1* was detectable (Fig. 3). *NHL10* and *FRK1* were significantly induced 24 h after infiltration in Col-0 but remained at very low basal amounts in *eds1-2* (Fig. 3). Expression levels in cold-primed and control plants did not differ in time or intensity (Fig. 3). Next, we tested for cold-priming-responsive expression profiles of SA-biosynthetic *ICS1* and the SA-responsive *PR1*. *ICS1* and *PR1* were significantly induced at 24 h but not at 6 h after the pathogen treatment in Col-0 (Fig. 3). The induction levels did not differ between cold-primed and control samples (Fig. 3), indicating that pathogen-triggered SA production and signaling were not cold-primed and, therefore, not causative for the cold-reduced susceptibility. This conclusion is further supported by the requirement of functional EDS1 for a robust activation of SA-related immune pathways upon infection with virulent pathogens (Cui et al. 2017; Rietz et al. 2011). Cold priming did not affect *ICS1* and *PR1* levels downstream or independent of EDS1 because induced transcripts in Col-0 were absent or low in cold-pretreated and inoculated *eds1-2* (Fig. 3).

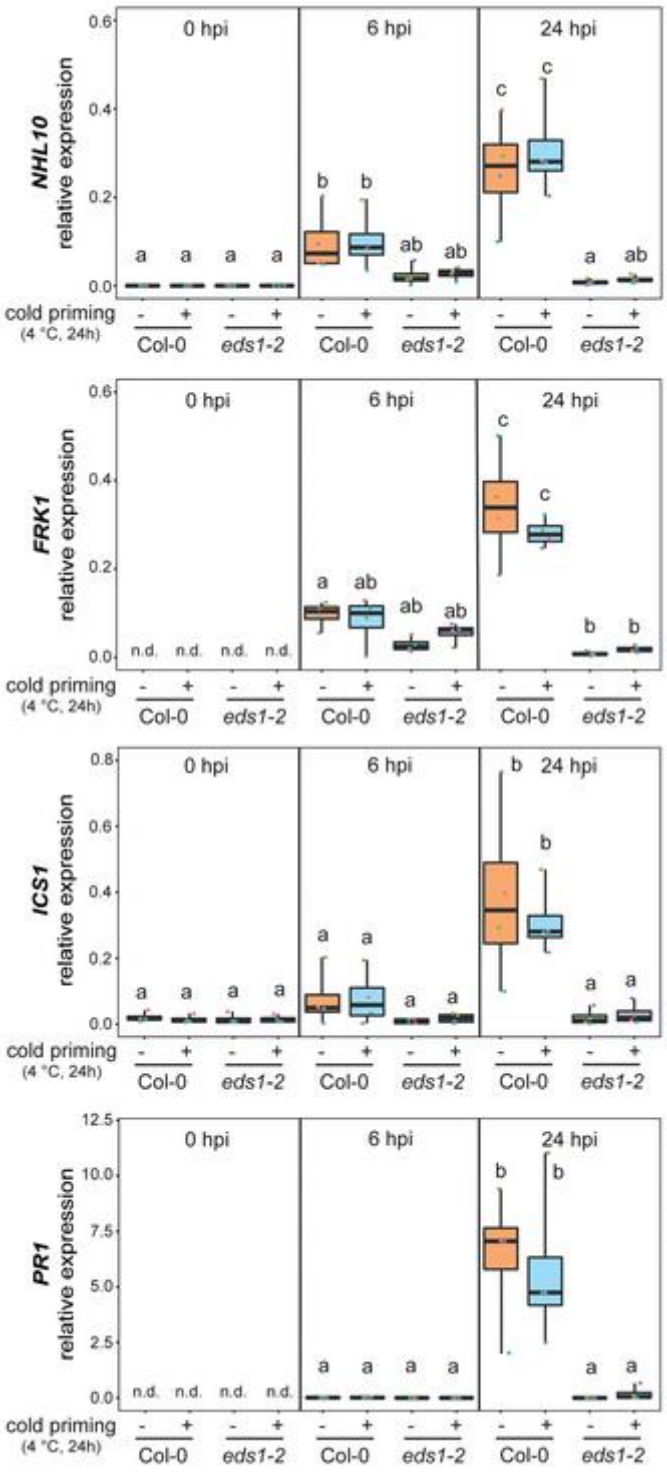

Figure 3: Transcript levels of selected pathogen-responsive genes during *Pseudomonas syringae* pv. *tomato* infection upon cold priming. Transcript levels of pathogen-associated molecular pattern-triggered immunity marker genes *NHL10* and *FRK1*, salicylic acid (SA) biosynthetic *ICS1*, and SA-responsive *PR1* in leaves of Col-0 and *eds1-2* null mutants after infiltration with *P. syringae* pv. *tomato* (optical density at 600 nm = 0.005). Infiltrations were done 5 days after end of cold treatment with cold-primed plants (+; 4°C, 24 h) or control plants (−). Transcript levels were determined at the time of *P. syringae* pv. *tomato* infiltrations (0, 6, and 24 h post inoculation [hpi]) using quantitative PCR in four independent experiments as relative expression to the geometric mean of three reference genes (*ACT2*, *YLS8*, and *RHIP1*). Different letters denote statistically significant differences (analysis of variance and least significant difference, $P < 0.05$); n.d. = not detectable.

2.3.4 Postcold and *P. syringae* pv. *tomato*-triggered regulation of *tAPX* and *sAPX* transcripts.

Recently, tAPX was described for its role in establishing the memory that controls cold regulation of gene expression after cold priming for several days (van Buer et al. 2016, 2019). Hereby, postcold accumulation of *tAPX* transcripts was essential for the memory function (van Buer et al. 2016, 2019). In addition to tAPX, *Arabidopsis* expresses sAPX (Ishikawa and Shigeoka 2008), which evolved from the same ancestral gene as *tAPX* and still has a highly similar catalytic subunit (Pitsch et al. 2010). We compared regulation of *tAPX* and *sAPX* after cold exposure and after subsequent *P. syringae* pv. *tomato* infection in leaves of cold-primed and control plants of Col-0 and *eds1-2* (Fig. 4). Our data confirmed previously described postcold induction of *tAPX* in Col-0 (van Buer et al. 2016) and showed that this memory phase lasting process was also functional and significant in *eds1-2* (Fig. 4A). *sAPX* was upregulated during a 24-h cold phase of 4°C and quickly readjusted to precold levels at normal temperature (van Buer et al. 2016) (Fig. 4A). Cold-induced *sAPX* upregulation was less pronounced in *eds1-2* (Fig. 4A). Similar to cold exposure, infiltration of leaves with *P. syringae* pv. *tomato* reduced *tAPX* amounts in Col-0 between 3 and 24 h after infection in control and cold-primed plants (Fig. 4B). This effect was marginally less in *eds1-2* (Fig. 4B). At 24 h after *P. syringae* pv. *tomato* treatment, *sAPX* transcripts were enhanced in Col-0. Pathogen induction of *sAPX* was weaker in *eds1-2* and only marginal in cold-primed *eds1-2* (Fig. 4B). Therefore, our data reveal a regulatory similarity between cold- and pathogen-responsive cellular plant stress management: EDS1-dependent upregulation of *sAPX* and repressive regulation of *tAPX*. The pathogen-responsive diametrical regulation of *tAPX* and *sAPX* is further supported by other published transcriptomic studies; for example, after infiltration with *P. syringae* pv. *tomato* and ETI-inducing *P. syringae* pv. *tomato* strain *avrRPM1* (Mine et al. 2018) and EDS1-dependent ETI-inducing *P. syringae* pv. *tomato* strain *avrRPS4* (Bhandari et al. 2019) in the *P. syringae* pv. *tomato*-primed SAR state (Gruner et al. 2013); and detectable, even though less pronounced, after treatment with the bacterial PAMP flg22 peptide (Birkenbihl et al. 2017) (Supplementary Fig. S3).

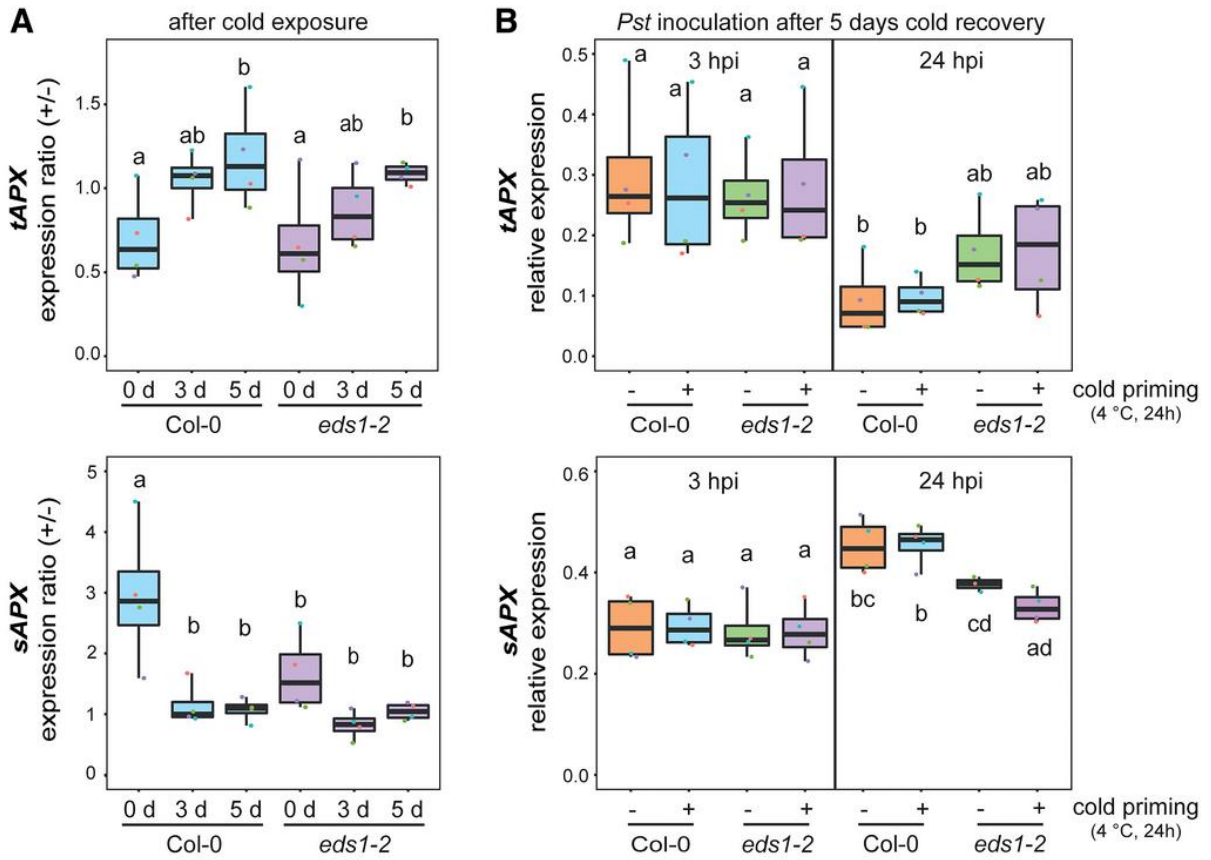

Figure 4: Transcript regulation of plastid ascorbate peroxidases (APX) encoding APX soluble in the stroma (*sAPX*) and thylakoid-bound APX (*tAPX*) genes during postcold phase and after *Pseudomonas syringae* pv. *tomato* (*Pst*) infection. **A,** Postcold (4°C, 24 h) transcript levels of *tAPX* (upper panel) and *sAPX* (lower panel) in leaves of Col-0 and *eds1-2* null mutants immediately (0 d), 3 days (3 d), and 5 days (5 d) after the end of cold treatment. Transcript levels are shown as ratios from samples of cold-primed leaves (+) compared with samples from untreated control plants (−) of the same age. **B,** Transcript levels of *tAPX* (upper panel) and *sAPX* (lower panel) in leaves of Col-0 and *eds1-2* null mutants 3 and 24 h post inoculation (hpi) with *P. syringae* pv. *tomato* (optical density at 600 nm = 0.005) of cold-primed (+) or control (−) plants. Plants were infected 5 days after the end of the cold treatment. Transcript levels were determined in four independent experiments as relative expression to the geometric mean of three reference genes (*ACT2*, *YLS8*, and *RHIP1*). Different letters denote statistically significant differences (analysis of variance and least significant difference, $P < 0.05$).

2.3.5 Cold priming-reduced pathogen susceptibility requires plastid APX.

To test whether *tAPX* is required not only for cold priming of ROS-responsive genes during cold triggering (van Buer et al. 2019) but also for beneficial responses during *P. syringae* pv. *tomato* infections, we included *tapx*- and *sapx*-knockout (KO) lines (Kangasjärvi et al. 2008) in our analysis. Although altered phenotypes in both lines can be observed under stress conditions, both lines show wild-type-like phenotypes under normal and stable laboratory conditions, probably through compensation by other antioxidant compounds (Kangasjärvi et al. 2008; Maruta et al. 2010). Here, cold-primed (4°C, 24 h) and control plants of both KO lines and Col-0 were infiltrated with *P. syringae* pv. *tomato* 5

days after the priming stimulus. The bacterial titers measured in control plants revealed that *tAPX* did not contribute, per se, to the degree of plant pathogen susceptibility and basal resistance (Fig. 5A) and that *sapx*-KO lines were not significantly more resistant against *P. syringae* pv. *tomato* (Fig. 5A). The cold priming effect on reducing bacterial titers was strong in Col-0, weak in *tapx*-KO, and absent in *sapx*-KO (Fig. 5A). Although cold triggering responses specifically required *tAPX* but not *sAPX* (van Buer et al. 2019), cold-primed pathogen susceptibility was supported by functionality of both plastid APX variants. Consequently, the cold memory that reduces pathogen susceptibility can be postulated to be more generally controlled by plastid APX activity than priming of the cold responsiveness. We have recently shown that cold-induced accumulation of *tAPX* transcripts affects cold regulation of cold-primable genes (van Buer et al. 2019). To test whether postcold *tAPX* affects plant susceptibility, we used an estradiol-responsive *tAPX-inducible RNA interference* (*iRNAi*) line (Maruta et al. 2012; van Buer et al. 2019). We suppressed postcold regulation of *tAPX* by spraying cold-primed plants after the initial cold exposure and, 4 days later, with an estradiol or a mock solution before *P. syringae* pv. *tomato* was infiltrated the following day. Both the estradiol and the mock treatment after cold exposure resulted in increased susceptibility in the Col-0 and *tAPX-iRNAi* lines compared with the cold-primed-only control group (Fig. 5B). However, the bacterial numbers were specifically enhanced by the estradiol treatment in cold-primed *tAPX-iRNAi* (Fig. 5B). To confirm the effect of the estradiol treatment on reducing *tAPX* transcript abundance in *tAPX-iRNAi,* we monitored the *tAPX* transcripts at the time point of infection (Supplementary Fig. S4). We concluded that the regeneration of the plastid antioxidant protection by the *tAPX* postcold induction supports plastid functions during a subsequent infection with *P. syringae* pv. *tomato*, which again results in a perturbation of plastid APX at the transcriptional level (Fig. 4; Supplementary Fig. S3). When *tAPX* transcript recovery was suppressed after the initial cold phase, plants infected with *P. syringae* pv. *tomato* showed increased pathogen susceptibility, as determined by bacterial numbers (Fig. 5B; Supplementary Fig. S4). We suggest that the regeneration of the *tAPX* pool and the restabilization of the plastid antioxidant protection after the cold stress phase provides benefits for plant fitness and defense during a subsequent pathogen infection.

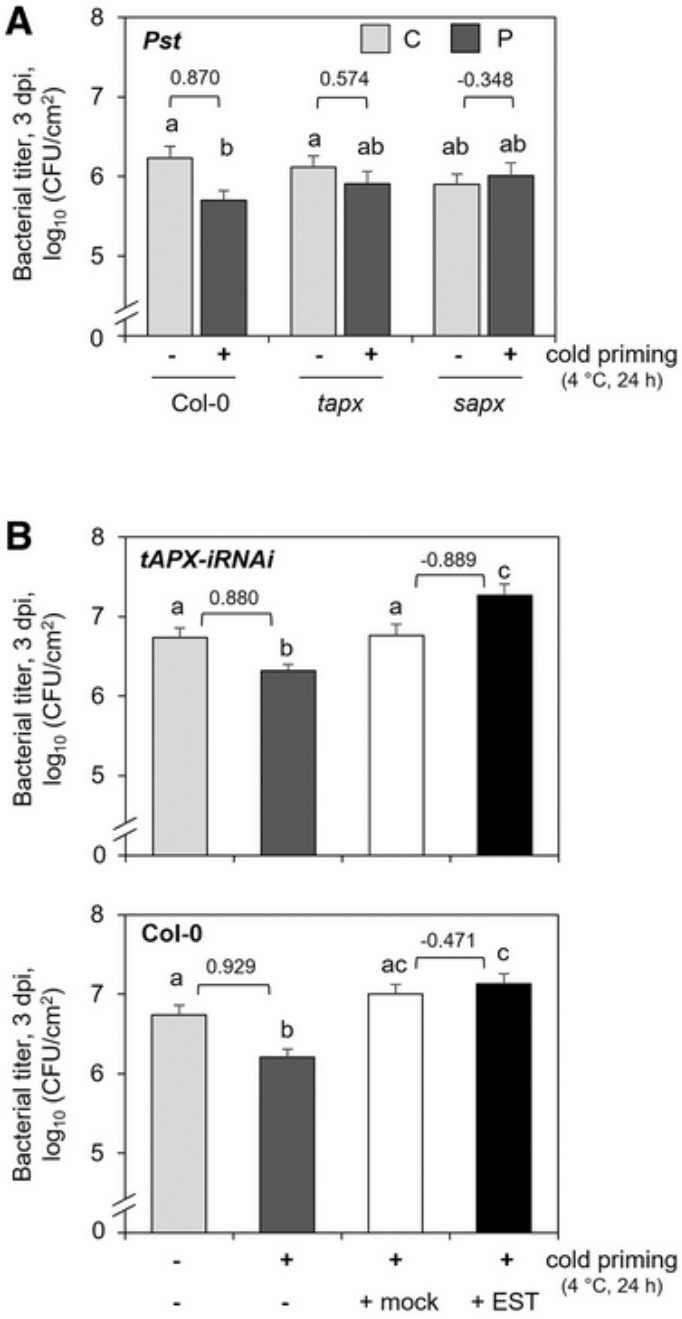

Figure 5: Bacterial growth of *Pseudomonas syringae* pv. *tomato* (*Pst*) in cold-primed Col-0, thylakoid-bound ascorbate peroxidase (*tAPX*), and APX soluble in the stroma (*sAPX*) knock-out (KO) lines and after conditional repression of *tAPX*. **A,** Cold-primed (+) and control (−) Col-0, *sapx*-KO, and *tapx*-KO plants were infiltrated with *P. syringae* pv. *tomato* (optical density at 600 nm [OD_{600}] = 0.001) 5 days after cold exposure (4°C, 24 h). Bacterial titers (log_{10}-transformed) were measured 3 days post inoculation (dpi). Bars represent means and standard errors calculated from five independent experiments, each with five to six biological replicates using a mixed linear model. **B,** Cold-primed (+) and control (−) Col-0 and *tAPX-inducible RNA interference* (*iRNAi*) plants were infiltrated with *P. syringae* pv. *tomato* (OD_{600} = 0.001) 5 days after cold exposure (4°C, 24 h). For conditional repression of *tAPX* transcript in *tAPX-iRNAi*, plants were sprayed with a mock or an estradiol (EST) solution after the cold treatment and after 4 days. Bacterial titers (log_{10}-transformed) were measured 3 dpi. Bars represent means and standard errors calculated from four independent experiments, each with six to eight biological replicates using a mixed linear model. Different letters above the bars denote statistically significant differences within each graph (adjusted $P < 0.05$; two-tailed t tests). Numbers between two bars show the effect size between two means according to Cohen's d.

2.4 Discussion

When plants are exposed to simultaneously or sequentially occurring combined abiotic and biotic stress situations, responses often differ compared with single and individual stresses (Zhang and Sonnewald 2017). The outcome of different combined stresses can result in a trade-off situation or enable cross-tolerance (Saijo and Loo 2020). Cross-tolerance upon two sequentially applied stresses disconnected by a stress-free interphase, which enables recovery and requires memorization of the first stressor, is a characteristic feature of the *trans*priming phenomenon (Hilker et al. 2016). Here, we showed that a single 24-h cold exposure primed the susceptibility of *A. thaliana* Col-0 against the virulent plant pathogen *P. syringae* pv. *tomato* for up to 5 days (Figs. 1 and 5). This cold-priming-reduced pathogen susceptibility was independent from plant immunity pathways controlled by EDS1 but it required the chloroplast-located sAPX and tAPX (Fig. 5).

The immune system of *A. thaliana* benefits from the experience of cold temperatures. A single 24-h cold (4°C) exposure resulted in immediate decrease of pathogen susceptibility (shown by infection 1 h after cold), and was also robust for 5 to 8 days (shown by infection 5 days after cold) (Fig. 1), although the initial transcriptional cold response (e.g., *COR15A*, *ZAT10*, *BAP1*, and *PAL1*) was quickly reset to precold levels (Fig. 2). As shown recently by Wu et al. (2019), a shorter 10-h cold treatment applied during the night phase also decreases pathogen susceptibility in *A. thaliana*. However, the shorter night stimulus is only transiently memorized for up to 12 h (Wu et al. 2019). Repetitive application of seven 1-h cold periods (one treatment per day) equally results in reduced growth of *P. syringae* pv. *tomato* and entraines protection for 7 days (Singh et al. 2014). Our study showed that, already, a single cold treatment lasting 24 h, including day and night phase, primed and established a molecular stress memory lasting for 5 to 8 days (Fig. 1). Whereas repetitive cold treatments resulted in enhanced activation of PTI-responsive genes *FRK1* and *NHL10* upon triggering with type three secretion–deficient *P. syringae* pv. *tomato* strain *hrcC*$^{-}$ (Singh et al. 2014), the single 24-h cold priming stimulus did not reveal a priming pattern for *FRK1* and *NHL10* transcripts. Activation of *FRK1* and *NHL10* without priming signatures excludes a cross-stress memory formation similar to that with the repetitive cold stimuli and suggests a PTI-independent memory (Fig. 3).

In summary, our data showed that cold priming memory formation was independent from the central plant immune regulator EDS1: (i) bacterial growth was reduced in cold-

primed *eds1* null mutants (Fig. 1), (ii) initial cold sensing in *eds1* was wild-type-like leading to *COR15A, ZAT10, BAP1*, and *PAL1* activation (Fig. 2), and (iii) EDS1-dependent transcript activation of selected immune-related genes (*NHL10, FRK1, ICS1*, and *PR1*) did not show a cold priming signature or an activation downstream of EDS1 (Fig. 3). Several EDS1-dependent responses have been well described for their effects in reducing plant susceptibility to virulent pathogens: EDS1, together with its homolog and heteromeric complex partner PAD4, boosts SA biosynthesis (Cui et al. 2017) and promotes tocopherol production in an SA-independent manner upon *P. syringae* pv. *tomato* infection (Stahl et al. 2019). Furthermore, EDS1 is required for the plant immune *cis*-priming SAR by contributing to signal generation in primary infected leaves and signal perception in the systemic uninfected tissues (Breitenbach et al. 2014). Based on the functionality of cold-priming-reduced susceptibility in *eds1-2*, we conclude that the molecular mechanisms of cold priming memory formation are independent from EDS1-controlled immune activation and are established regardless of SA production during *P. syringae* pv. *tomato* infection or SAR signaling. In addition, the indispensable requirement for EDS1 in TNL-mediated immunity (Dongus and Parker 2021; Griebel et al. 2014) excludes the possibility that (post)cold activation of TNL immune receptor signaling is causative for the reduced susceptibility in cold-primed plants. Several articles have described an induction of SA under low-temperature conditions (Kim et al. 2013, 2017). As during pathogen attack, cold-induced SA derives from the plastid isochorismate pathway but SA levels are not increasing before 7 days at 4°C (Kim et al. 2013, 2017). Cold exposure lasting 24 h was not sufficient to significantly increase *ICS1* transcript levels (Fig. 2) or enhance SA levels; these required a constant cold exposure of at least 1 week (Kim et al. 2013; Wu et al. 2019). In contrast to the independence of EDS1 during mild cold exposure (4°C, 24 h) and cold memory formation, induction and activation of the EDS1 complex at temperatures below 4°C negatively affected freezing tolerance in an SA-dependent manner (Chen et al. 2015).

Chloroplasts can be considered as an important cellular origin of cold sensing and priming: cold exposure supports chloroplast ROS production by an imbalance between the Calvin-Benson cycle and photosynthetic electron transport (Ensminger et al. 2006; Huner et al. 1993) and leads to reduced activation of chloroplast-to-nucleus redox-sensitive genes during a second cold phase (van Buer et al. 2016, 2019). tAPX, the main regulator of cold priming memory in chloroplasts, is transcriptionally activated during the postcold

phase (van Buer et al. 2016, 2019) in an EDS1-independent manner (Fig. 4). Gene expression regulation upon cold triggering specifically benefits from tAPX activation but not from sAPX (van Buer et al. 2019). Bacterial growth rates in *tapx-KO* were wild-type-like and not affected, except for the missing cold memory response (Fig. 5). Similar to cold exposure, infections with virulent pathogens resulted in reduced *tAPX* transcript levels (Fig. 4). Conditional silencing of *tAPX* transcripts during the memory phase increased plant susceptibility and *P. syringae* pv. *tomato* titers during a subsequent infection (Fig. 5). Whereas cold priming responses during a second cold exposure were solely regulated by *tAPX* (van Buer et al. 2016, 2019), pathogen triggering of cold-primed plants also required functional *sAPX* for memory effects (Fig. 5). This suggests that the *sAPX* upregulation during the initial cold phase and the *tAPX* increase during the subsequent recovery phase contribute to cold-priming-reduced susceptibility against *P. syringae* pv. *tomato*. tAPX and sAPX jointly scavenge plastid ROS and, therefore, contribute to the plastid antioxidant protection and redox-mediated signaling and communication between cellular compartments. We assume that the regeneration of the plastid APX pool and the restabilization of the plastid antioxidant protection after the initial cold stress phase supports the plant's fitness and primes defense activation by altering cellular redox communication in response to a pathogen infection.

2.5 Material and Methods

2.5.1 Plant material and cultivation.

A. thaliana var. Col-0 plants, *eds1-2* null mutant (Bartsch et al. 2006), T-DNA knockout lines *tapx* and *sapx* (Kangasjärvi et al. 2008), and *tAPX-iRNAi* (Maruta et al. 2012) were used in this study. All lines are in the Col-0 background. Plants were cultivated in round pots (6 cm in diameter) containing a soil mixture (14:14:5) of Topferde (Einheitserde, Sinntal-Altengronau, Germany), Pikiererde (Einheitserde, Sinntal-Altengronau), and Perligran Classic (Knauf, Germany) supplemented with dolomite lime (Deutsche Raiffeisen-Warenzentrale, Germany) at 0.5 g liter^{-1} and in a controlled environmental chamber with a day and night temperatures of 20 ± 2 and 18 ± 2°C, respectively; a cycle of 10 h of light (100 to 110 µmol photons m^{-2} s^{-1}; Lumilux Cool White fluorescence stripes, Osram, Germany) and 14 h of darkness; and a constant relative humidity of 60 ± 5% after stratification at 4°C for 2 days.

2.5.2 Cold treatments.

Cold treatments were performed as previously described (van Buer et al. 2016, 2019). Four-week-old plants were exposed to cold 2.5 h after onset of light by transferring them to a growth chamber with a constant temperature of 4 ± 2°C but otherwise identical aeration, illumination, and air humidity as in the 20°C chamber. After a continuous cold exposure for 24 h (comprising a full day and night phase), the plants were placed back to the 20°C chamber, labeled, and randomized with the noncold-treated control plants.

2.5.3 Estradiol treatments.

For estradiol-sensitive conditional silencing of *tAPX* in *tAPX-iRNAi*, plants were sprayed with 100 µM estradiol (Roth, Karlsruhe, Germany) dissolved in 0.8% (vol/vol) dimethyl sulfoxide (DMSO) and supplemented with 0.01% (vol/vol) Tween 20 as described recently (Bittner et al. 2021; van Buer et al. 2019). Control plants were sprayed with a mock solution (0.8% [vol/vol] DMSO and 0.01% [vol/vol] Tween 20) without estradiol. The treatments were performed immediately after the cold exposure (= 5 days before bacterial infiltration) and repeated after 4 days.

2.5.4 Cultivation and inoculation of bacteria.

P. syringae pv. *tomato* DC3000, *P. syringae* pv. *tomato* strains carrying either the avirulence gene *avrRpm1* or *avrRPS4*, and type three secretion system–deficient *P. syringae* pv. *tomato* strain *hrcC*⁻ were grown for 24 h at 28°4 C on nutrient-yeast extract glycerol agar (NYGA) solid medium containing the appropriate antibiotics. Bacterial cultures were suspended in 10 mM $MgCl_2$ and diluted to optical density at 600 nm (OD_{600}) of 0.001 for bacterial growth assays or 0.005 for gene expression analyses. The bacterial suspensions were infiltrated from the abaxial side into the leaves with a needleless syringe. For transcript analyses, control plants were mock treated with 10 mM $MgCl_2$. Bacterial inoculations were performed 3.5 h ± 0.5 h after onset of light and either 1 h or 5 days after the end of cold treatment, as indicated. The three youngest but fully-grown leaves of each plant were selected for infiltration.

2.5.5 Bacterial growth assays.

In planta bacterial titers were determined at the indicated time point after infiltration by combining three leaf discs for one sample and shaking in 10 mM $MgCl_2$ with 0.01% (vol/vol) Silwet L-77 at 28°C for 1 h. From each sample, a dilution series was spread in 15-µl spots on NYGA plates with appropriate antibiotics and incubated for 2 days at 28°C. CFU per leaf surface area were calculated for each sample. Statistical analysis of bacterial growth data was described previously (Tsuda et al. 2009). Log_{10}-transformed data from all independent experiments were analyzed using the lme4 package in the R environment and the following model was fitted to the data: $\log_{10} CFU_{gyr} = GY_{gy} + R_r + e_{gyr}$, where GY = genotype–treatment interaction, R = biological replicate, and e = residual. The mean estimates were used as the modeled log_{10}-transformed bacterial titers and were compared using two-tailed *t* tests. To correct for multiple hypothesis testing, the Benjamini-Hochberg method was applied.

2.5.6 Quantitative real-time PCR analysis.

For transcript analyses using quantitative real-time PCR (qRT-PCR), plant material was harvested from leaves of the same age and developmental status as the ones used for pathogen infiltrations. Each sample included leaves from at least two plants. Total RNA was extracted from frozen leaves using the Gene Matrix Universal RNA Purification Kit (EURx, Gdansk, Poland). cDNA was synthesized using the High-Capacity Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, U.S.A.) and Oligo dT16V primer according to the manufacturer's instructions using 1 µg of RNA for a 20-µl reaction. qRT-PCR assays were performed in technical triplicate on the CFX96 real-time system (Bio-Rad, Hercules, CA, U.S.A.) as described previously (van Buer et al. 2016) using SYBR Green (Sigma-Aldrich, Germany) and OptiTaq Polymerase (EURx) and a cycling program of 95°C for 5 min; followed by 40 cycles at 95°C for 15 s, 60°C for 30 s, and, finally, 72°C for 30 s. All qRT-PCR primers are listed in Supplementary Table S1. The cycle threshold (Ct) values were determined using the CFX Manager software and relative expression values (ΔCt) of genes of interest were analyzed against the geometric mean of the *actin 2* (*ACT2*), *yellow leaf specific protein 8*) (*YLS8*), and *RGS1-HXK1 interacting protein 1* (*RHIP*) transcript levels as reference. The relative expression ($2^{-\Delta Ct}$) was determined based on three technical replicates in four independently performed experiments. Box plots of the summarized data were generated using the R package gglpot2 and showed the median, the distance between the upper quartile ($q_n = 0.75$) and lower quartiles ($q_n = 0.25$), and the raw values

of each experiment as dots. For statistical analysis, basic R environment and the agricolae package were used.

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The author(s) declare no conflict of interest.

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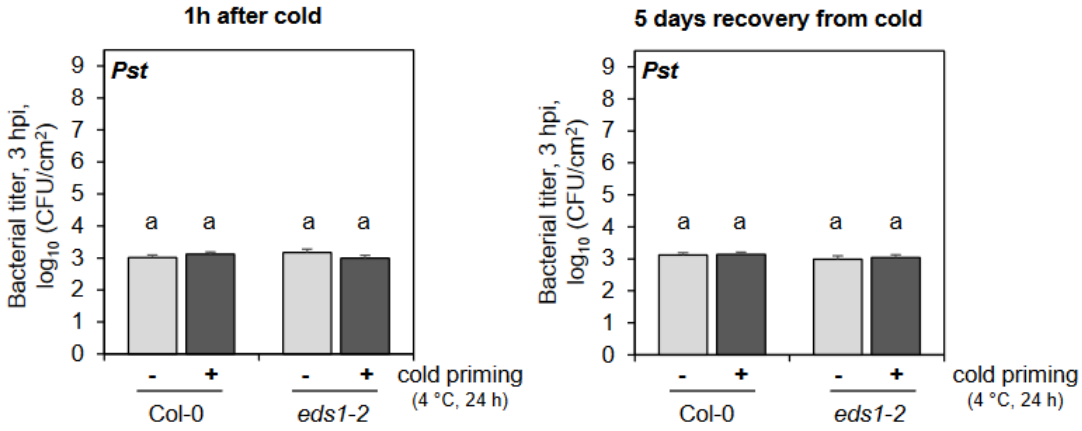
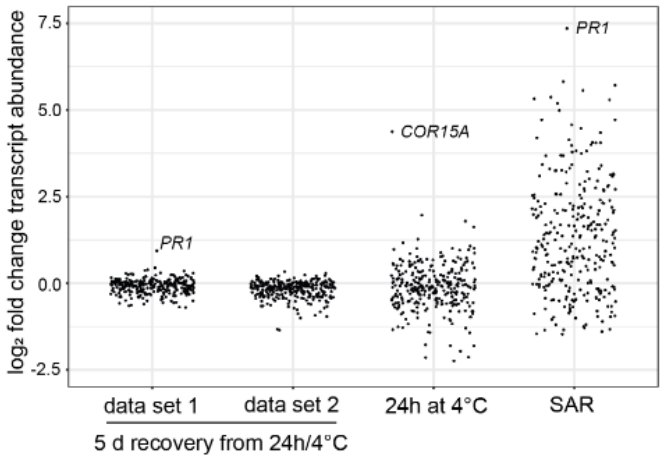
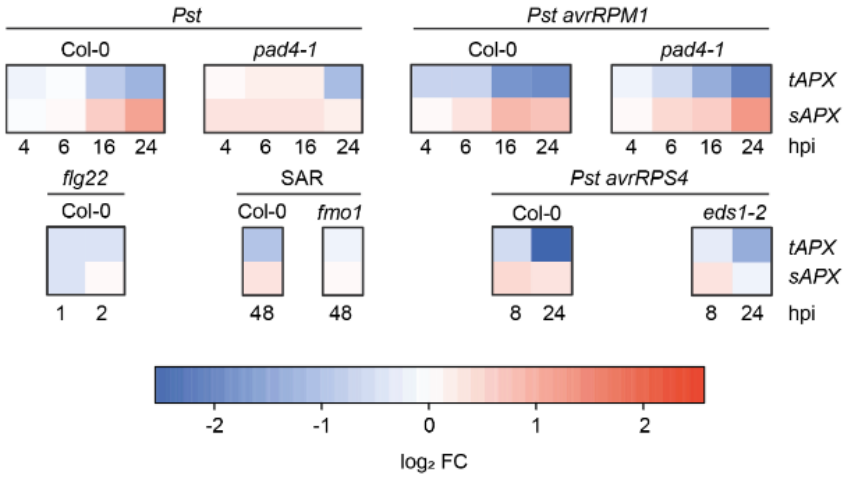
2.9 Supplements



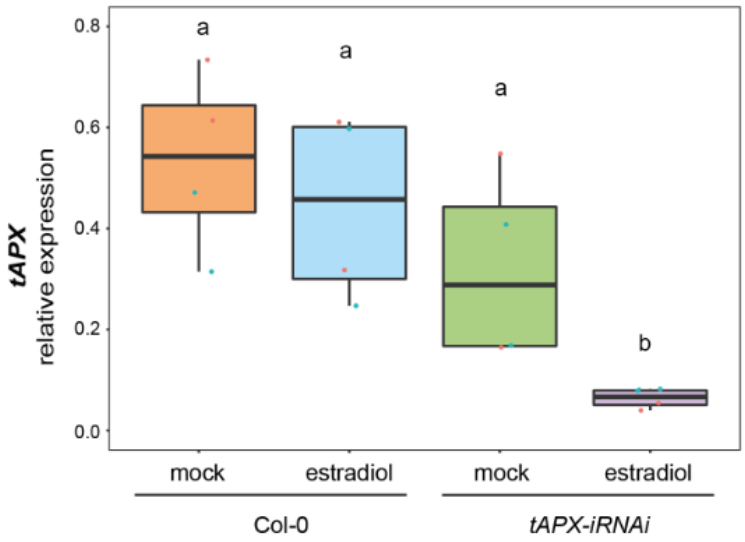
Supplementary Fig. S1. Bacterial titers of *Pst* (log_{10}-transformed) in cold-primed (+) and control plants (-) of Col-0 and ***eds1-2*** null mutants were determined 3 hpi. Plants were infiltrated with *Pst* (OD_{600} = 0.001) 1 hour (left panel) or 5 days (right panel) after cold exposure (4°C, 24h) .Bars represent means and standard errors calculated from 3 independent experiments each with 3 biological replicates using a mixed linear model. Different letters above the bars denote statistically significant differences (adjusted $p < 0.05$; two-tailed t-tests).



Supplementary Fig. S2. Analysis of transcript changes from genes of gene ontology group "Systemic Acquired Resistance/SAR" (GO:0009627) in public data sets. Transcript changes of 295 genes were compared in 4 different data sets: (i) data set 1 (Bittner al., 2020) and data set 2 (Bittner et al., 2021) represent the effect of SAR genes regulation 5 days after recovery from a 24h lasting 4°C cold exposure compared to transcript levels in non-treated control plants, (ii) SAR genes regulation directly after a 24h 4°C cold exposure compared to control conditions (Bittner et al. 2020), (iii) SAR genes in a SAR-induced state 48 hpi with *Pst* compared to mock treatment (10 mM $MgCl_2$) (Gruner et al. 2013).



Supplementary Fig. S3. Analysis *of tAPX/sAPX* transcript changes in public data sets in context of transcriptional immune reprogramming: after infiltration with *Pst* and *Pst avrRPM1* (Mine et al., 2018), *Pst avrRPS4* (Bhandari et al., 2019), in the *Pst*-primed SAR state (Gruner et al., 2013) and after treatment with the bacterial PAMP flg22 peptide (Birkenbihl et al., 2017). *sAPX/tAPX* transcript regulation is supported by the EDS1/PAD4 node when triggered by *Pst* and *Pst avrRPS4*, and FMO1 in the SAR-induced state. Log_2 fold changes (FC) are shown comparing the immune trigger with mock treatments. Visualization was performed using the heatmap tool from http://www.heatmapper.ca/expression/ .



Supplementary Fig. S4. *tAPX* transcript levels in Col-0 and *tAPX-iRNAi* 5 days after cold priming (4°C, 24 h) and after spray treatment with a mock or an estradiol solution after the cold treatment and again after 4 days. *tAPX* transcript levels were determined with qPCR in two independent experiments from two samples and calculated as relative expression to *YLS8* as a reference gene. Different letters denote statistically significant differences (ANOVA/LSD, $p < 0.05$).

Supplementary Tab. S1. qRT-Primer used in the study

Gene	Gene name	Forward primer	Reverse primer
AT1G27730	*ZAT10*	TCACAAGGCAAGCCACCGTAAG	TTGTCGCCGACGAGGTTGAATG
AT1G74710	*ICS1*	GCTTGGCTAGCACAGTTACAGC	CACTGCAGACACCTAATTGAGTCC
At1g77490	*tAPX*	GCTAGTGCCACAGCAATAGAGGAG	TGATCAGCTGGTGAAGGAGGTC
At2g14610	*PR1*	TTCTTCCCTCGAAAGCTCAA	AAGGCCCACCAGAGTGTATG
AT2G19190	*FRK1*	GCCAACGGAGACATTAGAG	CCATAACGACCTGACTCATC
AT2G35980	*NHL10*	TTCCTGTCCGTAACCCAAAC	CCCTCGTAGTAGGCATGAGC
AT2G37040	*PAL1*	GCAGTGCTACCGAAAGAAGTGG	TGTTCGGGATAGCCGATGTTCC
At2g42540	*COR15A*	AACGAGGCCACAAAGAAAGC	CAGCTTCTTTACCCAATGTATCTGC
At3g18780	*ACT2*	AATCACAGCACTTGCACCAAGC	CCTTGGAGATCCACATCTGCTG
At3g61190	*BAP1*	ATCGGATCCCACCAGAGATTACGG	AATCTCGGCCTCCACAAACCAG
At4g08390	*sAPX*	AGAATGGGATTAGATGACAAGGAC	TCCTTCTTTCGTGTACTTCGT
AT4G26410	*RHIP*	GAGCTGAAGTGGCTTCCATGAC	GGTCCGACATACCCATGATCC
AT5G08290	*YLS8*	TTACTGTTTCGGTTGTTCTCCATTT	CACTGAATCATGTTCGAAGCAAGT

Chapter II: Cold priming on pathogen susceptibility in the Arabidopsis *eds1* mutant background requires a functional *stromal Ascorbate Peroxidase*

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Contribution to the publication:

Concept: 30 %
Experiments: 100 %
Writing: 45 %

3.1 Abstract

24 h cold exposure (4°C) is sufficient to reduce pathogen susceptibility in *Arabidopsis thaliana* against the virulent *Pseudomonas syringae* pv. *tomato* (*Pst*) strain even when the infection occurs five days later. This priming effect is independent of the immune regulator Enhanced Disease Susceptibility 1 (EDS1) and can be observed in the immune-compromised *eds1-2* null mutant. In contrast, cold priming-reduced *Pst* susceptibility is strongly impaired in knock-out lines of the stromal and thylakoid ascorbate peroxidases (sAPX/tAPX) highlighting their relevance for abiotic stress-related increased immune resilience. Here, we extended our analysis by generating an *eds1 sapx* double mutant. *eds1 sapx* showed *eds1*-like resistance and susceptibility phenotypes against *Pst* strains containing the effectors avrRPM1 and avrRPS4. In comparison to *eds1-2*, susceptibility against the wildtype *Pst* strain was constitutively enhanced in *eds1 sapx*. Although a prior cold priming exposure resulted in reduced *Pst* titers in *eds1-2,* it did not alter *Pst* resistance in *eds1 sapx*. This demonstrates that the genetic *sAPX* requirement for cold priming of basal plant immunity applies also to an *eds1* null mutant background.

3.2 Short Communication

Plants have evolved strategies for improved stress responses based on prior stress experiences. One such strategy that differs from acclimation and adaptation but requires a molecular stress imprint or memory is defined as priming.[1,2] A diverse set of stimuli has been shown for being effective in priming the plant immune system against pathogens.[3,4] This includes abiotic changes and pretreatments with altered environmental conditions as a consequence of activated cross-tolerance. Several short (1.5 h) and repetitive cold (4°C) or heat (38°C) treatments increase the resistance of *Arabidopsis thaliana* (Arabidopsis) against the hemi-biotrophic, virulent pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*).[5] Improved plant resistance was also observed when the light period the day prior to the *Pst* infection is extended from 8 h to 16-32 h as a consequence of photoperiod stress.[6] A 24 h pre-exposure of Arabidopsis to an extended or continuous light phase increases the ability for a strong apoplastic production of reactive oxygen species, boosts pathogen-driven salicylic acid accumulation and signaling, and reduces the capability of *Pst* for inducing so-called water-soaking leasions.[7,8]

Recently, we showed that a 24 h cold exposure (4°C) is sufficient to prime plant immunity for an infection with *Pst* occurring 5 days later and resulting in reduced bacterial titers in cold pre-treated Arabidopsis plants (accession: Col-0) compared to naïve control plants.[9] This effect is independent of the plant immune regulator Enhanced Disease Susceptibility 1 (EDS1) and can be observed in the highly susceptible null mutant *eds1-2*.[9] In contrast, cold priming did not lead to reduced bacterial titers when *Pst* strains delivering the pathogen effector proteins avrRPM1 or avrRPS4 were used.[9] When detected by the host, these strains initiate strong and robust plant immune responses in the context of effector-triggered immunity (ETI).[10–13] While avrRPS4-tiggered ETI is strongly EDS1-dependent, defense activation triggered by the recognition of avrRPM1 is mainly EDS1-independent.[14] EDS1 is part of a small family of nucleocytoplasmic lipase-like proteins.[15–18] Together with its other family members Phytoalexin-Deficient 4 (PAD4) and Senescence-Associated Gene 101 (SAG101), EDS1 forms exclusive heterodimers and functions as a central regulator of ETI, basal immunity, and systemic acquired resistance.[17,19–21] Intracellular immune receptors containing Toll-Interleukin 1 receptor (TIR) domains catalyze ribosylated nucleotide second messengers that specifically bind either to EDS1-PAD4 or to EDS1-SAG101 heterodimers and initiate complex activation.[22–25] Mobilized EDS1 complexes

contribute to the activation of pathogen-triggered transcriptional defence reprogramming and cell death, and boost accumulation of immune enhancing metabolites, such as salicylic acid and pipecolic acid derivatives.[20,21,26]

As mentioned above, EDS1-dependent signaling is dispensable for cold priming-enhanced *Pst* resistance. However, functional plastid ascorbate peroxidases (APX) are indispensable.[9] Two APX isoforms reside in the chloroplasts of Arabidopsis and most tracheophytes: a soluble stromal APX (sAPX) and thylakoid-bound APX (tAPX).[27–29] While tAPX specifically resides in the plastids, sAPX is dual targeted to the chloroplast stroma and the mitochondrial matrix.[30,31] Based on homologies, a further plastid APX-like protein, named TL29, was identified with location to the thylakoid lumen, but does not possess peroxidase activities.[32,33] The interplay of tAPX and sAPX provides two spatial layers for scavenging photosynthesis-related H_2O_2 in the plastid.[27,28] In this context, sAPX and tAPX have mainly redundant functions for photooxidative protection under abiotic stress situations in mature plants.[34–36] However, also distinct roles are reported. Photoprotection in seedlings rather requires sAPX, while tAPX functions in leaves as central regulator of cold priming mediated-repression of core stress-responsive genes during a second cold phase.[35–38] In the priming control, tAPX-mediated suppression of chloroplast NADPH dehydrogenase subunits resulting in less cyclic electron transport provides a source for altered chloroplast-to-nucleus stress signaling.[39] While cold priming-reduced *Pst* susceptibility is significantly weakened in *tapx-knockout* (KO) lines compared to Col-0, *Pst* titers are similar in cold-pretreated and control *sapx*-KO (hereafter: *sapx*) plants indicating a stronger contribution of sAPX.[9]

To test, whether cold priming-reduced *Pst* susceptibility requires plastid ascorbate peroxidases also in the background of the null mutant *eds1-2*, we generated an *eds1 sapx* line (Fig. 1A) using the *eds1-2* null mutant and the *sapx* line (SALK_083737).[9,35,40] We tested EDS1 and sAPX protein abundance in the *eds1 sapx* line using a plastid APX serum[37] and a commercial EDS1 antibody.

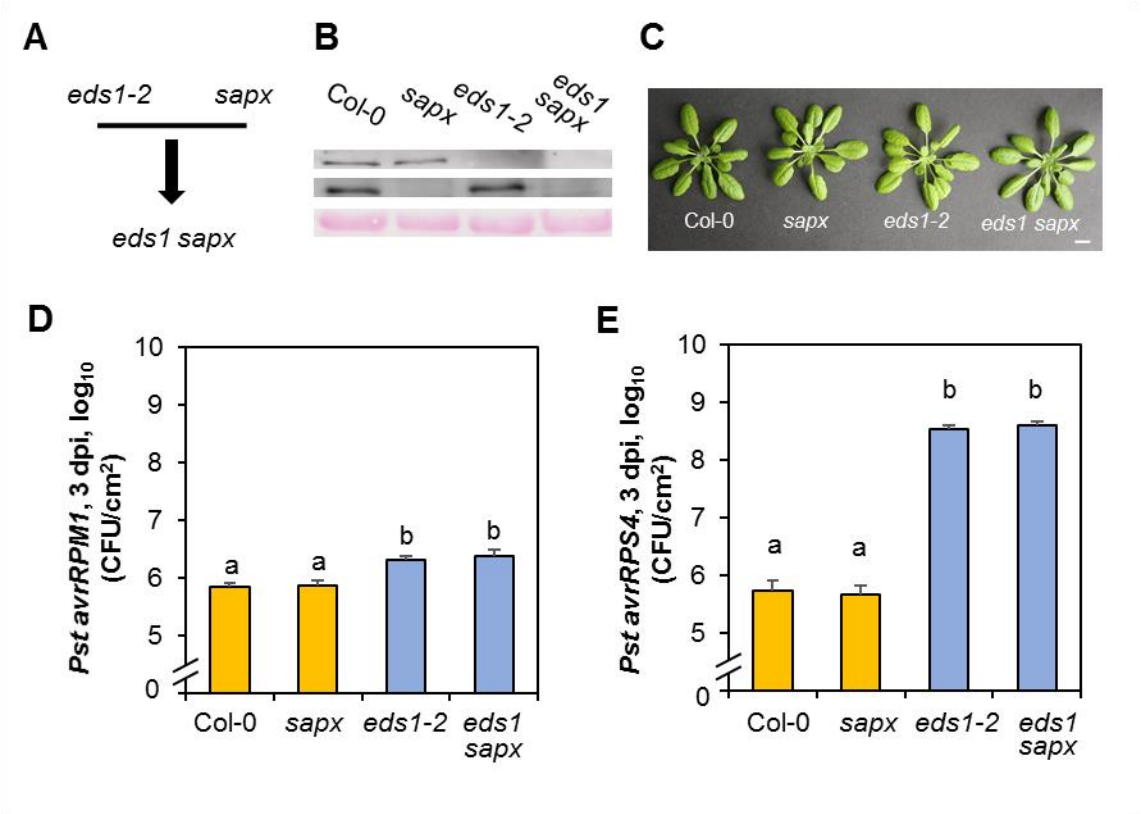

Figure 1: Generation and first analyses of an *eds1 sapx* double mutant line. **(A)** The *eds1-2* null mutant was crossed with the T-DNA-inserted *sapx*-knockout line (*sapx*) to receive an *eds1 sapx* line. **(B)** Protein detection of stromal Ascorbate Peroxidase (sAPX) and Enhanced Disease Susceptibility1 (EDS1) in leaf extracts of *eds1 sapx* and corresponding single lines. Ponceau S staining of the Rubisco large subunit (rbcL) is shown as loading control. **(C)** Representative picture of rosettes of 5-week-old plants. Scale bar = 1 cm. **(D,E)** Pathogen-related immune phenotyping of *eds1 sapx* line and parental single lines (5-week-old) was verified by leaf syringe infiltration using *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) strains (OD_{600} = 0.001 in 10 mM $MgCl_2$) delivering either the EDS1-independent effector avrRPM1 (D) or the EDS1-dependent effector avrRPS4 (E). Bacteria were re-isolated 3 days post infection (dpi) and colony-forming units per leaf disk area (CFU/cm^2) were determined. Bars show mean of log_{10}-transfromed CFU/cm^2 and standard error (n = 18 from 3 independent experiments). Different letters above the bars denote statistically significant differences (Tukey HSD, $P < 0.05$).

(AS13 2751, Agrisera, Sweden) confirming (in addition to prior genotyping) lack of EDS1 and sAPX in the *eds1 sapx* line (Fig. 1B). Growth and developmental phenotype of 5-week-old plants did not differ between *eds1 sapx* and parental lines (Fig. 1C).

Next, we analysed the impact of sAPX for EDS1-dependent and -independent immunity. For this purpose, we infiltrated the *eds1 sapx* double line either with *Pst avrRPM1* or *Pst avrRPS4*. We could neither detect differences in bacterial titers between the wildtype Col-0 and the *sapx* nor between *eds1-2* and *eds1 sapx* (Fig. 1 D,E). The bacterial titers of *Pst avrRPM1* determined three days after inoculation were equally ~0.5 log_{10} higher in *eds1-2* and *eds1 sapx* compared to Col-0 (Fig. 1D). In contrast, bacterial numbers of *Pst avrRPS4*

were ~3 log_{10} higher in *eds1-2* and *eds1 sapx* than in Col-0 and *sapx* (Fig. 1E). This demonstrates that under stable conditions sAPX does not affect plant immunity and that *eds1 sapx* largely resembles the immune phenotype of *eds1-2*.

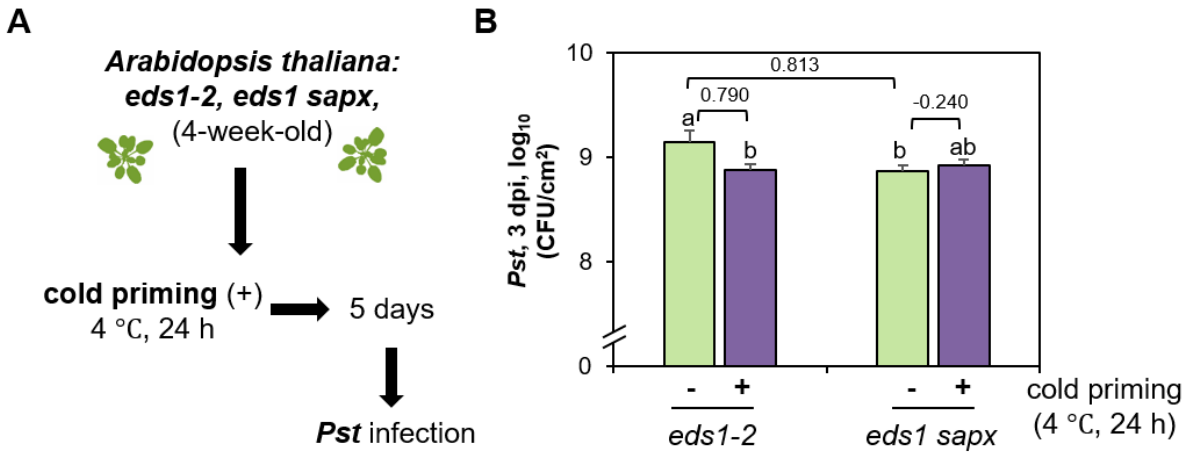

Figure 2: *Pst* titers in *eds1 sapx* after prior cold exposure **(A)** Experimental design: 4-week-old *eds1-2* and *eds1 sapx* Arabidopsis plants were cold-treated (4 °C) for a full light/dark phase (10 h /16 h) of 24 h starting 2.5 h after the onset of light. After 5 d back at normal temperature conditions (day/night: 22 °C/ 20 °C), plants were infiltrated with *Pst* (OD_{600} = 0.001). **(B)** Bacterial titers of *Pst* (log_{10}-transformed) in (+) cold-primed and (-) control *eds1-2* and *eds1 sapx* plants were determined 3 days post infection (dpi). Bars represent means of log_{10}-transformed colony-forming units (CFU/cm^2) and standard errors calculated from 3 independent experiments (n = 15-18). Different letters above the bars denote statistically significant differences (Tukey HSD, $P < 0.05$). Numbers between two bars show the effect size between two means according to Cohen's d.

Our main aim with this study was to investigate whether sAPX is not only required for cold priming-reduced *Pst* susceptibility in Col-0 but also in *eds1-2*. We repeated the cold priming experiments from our recent study[9] in the exact same way, but compared this time *eds1-2* and *eds1 sapx* (Fig 2A). As shown before[9], a 24 h lasting cold exposure reduced the enhanced susceptibility of *eds1-2* when the *Pst* inoculation was performed 5 days later (Fig. 2B). *Pst* titers were lower in cold-primed *eds1-2* than in the non-primed control group (Fig. 2B). *Pst* numbers in *eds1 sapx* were already significantly lower without a pre-cold exposure (Fig. 2B), which was similar but not significant between Col-0 and *sapx* in our earlier study[9]. The cold priming exposure did not further alter *Pst* titers in *eds1 sapx*. This confirms our recent finding, (i) that a prior cold exposure does not alter *Pst* susceptibility when sAPX is lacking. (ii) It additionally shows, that the requirement of sAPX for cold priming-reduced susceptibility also exists in *eds1-2*. (iii) It further highlights, that in the absence of sAPX, *Pst* susceptibility in *eds1 sapx* is constitutively reduced to the level of

cold-primed *eds1-2*. As outlined above, EDS1 is required for many different pathogen responses, but not the main player in the cold priming signalling cascade. The generated *eds1 sapx* line provides the opportunity to further analyse the cold priming signalling response on plant immunity in the absence of well-known and strong EDS1-dependent defence responses.

3.3 Acknowledgments

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3.5 References

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Chapter III: Cold exposure transiently increases resistance of *Arabidopsis thaliana* against the fungal pathogen *Botrytis cinerea*

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Concept: 75 %
Experiments: 90 %
Writing: 45 %

4.1 Abstract

A sudden cold exposure (4°C, 24 h) primes resistance of *Arabidopsis thaliana* against the virulent biotrophic pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) for several days. This effect is mediated by chloroplast cold sensing and the activity of stromal and thylakoid-bound ascorbate peroxidases (sAPX/tAPX). In this study, we investigated the impact of such cold exposure on plant defence against the necrotrophic fungus *Botrytis cinerea*. Plant resistance was transiently enhanced if the *B. cinerea* infection occurred immediately after the cold exposure, but this cold-enhanced *B. cinerea* resistance was absent when the cold treatment and the infection were separated by 5 days at normal growth conditions. Plastid ascorbate peroxidases partially contributed to the transient cold-enhanced resistance against the necrotrophic fungus. In response to *B. cinerea*, the levels of reactive oxygen species (ROS) were significantly higher in cold-pretreated Arabidopsis leaves. Pathogen-triggered ROS levels varied in the absence of sAPX, highlighting the strong capacity for sAPX-dependent ROS regulation in the chloroplast stroma. The cold-enhanced resistance against *B. cinerea* was associated with cold-induced plant cell wall modifications, including sAPX-dependent callose formation and significant lignification in cold-treated Arabidopsis leaves.

4.2 Introduction

Abiotic factors, such as temperature, fluctuate strongly in most environments. In temperate regions, short temperature drops in spring are common and occur in irregular patterns. The frequency and risk of such late-spring frosts has increased in Europe and Asia within the last 60 years (Zohner et al., 2020). Sudden frost (< 0°C) often causes extracellular ice crystal formation, damages the plasma membrane, and reduces osmotically active water in the plant cells (Xin and Browse, 2000; Satyakam et al., 2022). Already chilling temperatures (0°C – 10°C) affect plant performance and growth. Such softer sudden cold exposures cause energy imbalances between the photosynthetic light reaction and the Calvin-Benson cycle and lead to the formation of reactive oxygen species (ROS) in chloroplasts (Huner et al., 1993; Ensminger et al., 2006), but also modify susceptibility of plants against pathogens. Several days after transition to 4°C, *Arabidopsis thaliana* (Col-0 accession) plants activate plant immune responses, such as salicylic acid production, and enhance immune gene expression even in the absence of pathogens (Kim et al., 2013; Kim et al., 2017). Daily repetitive 1.5 h cold treatments entrain and single 8-24 h lasting pre-exposures to 4°C prime plant resistance against subsequent infections with the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) (Singh et al., 2014; Wu et al., 2019; Griebel et al., 2022). In contrast to acclimation that describes plant adjustments to a sustained environmental change, entrainment results from repetitive and regular external cues, and priming is the outcome of an initial and single stress stimulus that influences plant response to a subsequent stress exposure (Hilker et al., 2016; Baier et al., 2019).

Botrytis cinerea is a necrotrophic pathogenic fungus and causes grey mould disease on plants (Williamson et al., 2007). It kills plant host cells as part of its infection strategy and can infect numerous plant species (Glazebrook, 2005; Williamson et al., 2007; Bi et al., 2023). Upon spore germination, *B. cinerea* forms specialized cells, so-called appressoria, to penetrate host epidermal tissues and cells (Bi et al., 2023). The fungal pathogen produces various cell wall-degrading enzymes, toxins, and oxalic acid to turn the host's defence into susceptibility (Williamson et al., 2007; Nakajima and Akutsu, 2014). Plants employ an interconnected two-layered defence system against pathogen threats (Jones and Dangl, 2006; Jones et al., 2016; Ngou et al., 2021; Yuan et al., 2021): First, pattern recognition receptors (PRRs) anchored in plant membranes identify pathogen-associated molecular patterns (PAMPs) and initiate pattern-triggered immunity (PTI) (Zipfel, 2014; Albert et al., 2020). The PAMP chitin, for instance, is a major component of fungal cell walls and is detected by a receptor-like kinase known as Chitin Elicitor Receptor Kinase 1

(CERK1) (Miya et al., 2007). Second, intracellular receptors intercept pathogen virulence factors, so-called effectors, and activate effector-triggered immunity (ETI) (Jones et al., 2016; Lolle et al., 2020). This second layer, however, plays a negligible role against necrotrophic fungal pathogens and is rather important for defence against biotrophs (Liao et al., 2022).

Plant defence responses against *B. cinerea* include the generation of ROS, the biosynthesis of phytoalexins, such as camalexin, but also a fine-tuned activation of the plant hormones jasmonic acid (JA) and salicylic acid (SA), callose deposition, and cell wall modifications (Thomma et al., 1998; Ferrari et al., 2003; Veronese et al., 2006; Ferrari et al., 2007; Ramírez et al., 2011; Birkenbihl et al., 2012; Yang et al., 2018). Callose is a central component of papillae, which are locally and transiently formed plant cell wall modifications at the site of infection (Jacobs et al., 2003; Nishimura et al., 2003). The polymer lignin strengthens the plant cell walls and lignification is often enhanced in response to distinct biotic, but also abiotic stress treatments (Eynck et al., 2012; Cesarino, 2019; Lee et al., 2019; Nakamura et al., 2020; Ma, 2024).

ROS are important sub- and intra-cellular signalling molecules but also mediate cell-to-cell signalling (Miller et al., 2009; Ugalde et al., 2021; Peláez-Vico et al., 2024). In photosynthesis, ROS generation by the light-driven electron transport is unavoidable (Smirnoff and Arnaud, 2019; Foyer and Hanke, 2022). Plants neutralize these ROS by a highly efficient chloroplast antioxidant system. Tightly functionally interacting with superoxide dismutases, chloroplast ascorbate peroxidases (APX) utilize ascorbate as an electron donor to detoxify H_2O_2 (Groden and Beck, 1978). Thylakoid-bound APX (tAPX) is part of a first layer of protection and scavenges photosynthesis-related H_2O_2 directly at the thylakoid membrane (Asada, 1999; Jardim-Messeder et al., 2022). Stromal APX (sAPX) provides downstream antioxidant protection in the plastid stroma (Asada, 1999; Jardim-Messeder et al., 2022). Increasing evidence suggests that both plastid APX have additional functions in chloroplast energy metabolism and signalling (Kangasjarvi et al., 2008; Maruta et al., 2010; van Buer et al., 2016; Maruta et al., 2016; van Buer et al., 2019; Seiml-Buchinger et al., 2022). tAPX specifically regulates cold priming-mediated repression of core stress-responsive genes during a subsequent cold exposure (van Buer et al., 2019). In response to the initial cold, tAPX promotes the suppression of the chloroplast NADPH dehydrogenase subunits and alters chloroplast-to-nucleus stress signalling (Seiml-Buchinger et al., 2022). While tAPX transcripts decrease in the cold and slowly rise again during the post-cold phase, sAPX transcripts are upregulated in the cold and are quickly reset at normal

growth temperatures (van Buer et al., 2016; Griebel et al., 2022). Previous work showed that both plastid APX contribute to cold priming-enhanced resistance against the bacterial pathogen *Pst* (Griebel et al., 2022). Even in the immune-compromised null mutant *enhanced disease susceptibility 1-2* (*eds1-2*), which is competent to establish cold priming-mediated *Pst* resistance, lack of sAPX abolishes cold priming-enhanced resistance against *Pst* (Griebel et al., 2022; Schütte et al., 2024).

Here, we investigated whether a single cold exposure (4°C, 24 h) impacts not only resistance of Arabidopsis against the virulent bacterial pathogen *Pst* but also against the necrotroph fungus *B. cinerea*. In addition, we analysed the role of plastid APX for cold exposure-enhanced fungal resistance and clarified their contribution for (post-)cold and *B. cinerea*-triggered ROS and callose deposition.

4.3 Results

4.3.1 Cold pretreatment of Arabidopsis transiently enhances resistance against *Botrytis cinerea*

A 24 h cold exposure (4°C) of Arabidopsis Col-0 plants results in enhanced resistance against the bacterial hemibiotrophic pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) if the inoculation occurs immediately after the cold treatment, but also 5 days later (Griebel et al., 2022). Here, we studied the impact of such a prior cold exposure (4°C, 24 h) on plant resistance against the necrotrophic fungal pathogen *Botrytis cinerea*. Two different experimental setups were compared (Fig. 1A): (i) The first one consisted of the cold pretreatment (4°C, 24 h) and an immediately (2 h) subsequent infection (CT), (ii) the second included a stress-free period of 5 days at regular growth conditions (day/night: 20°C/18°C) between the initial cold exposure and the inoculation, and was therefore designated as cold priming (CP) setup. While the infection in the CT setup might be affected by the post-cold deacclimation, the infection 5 days after the cold exposure requires a priming memory, because cold-responsive genes and metabolites, such as soluble sugars and proline, are already reset after three days of deacclimation (Byun et al., 2014; Zuther et al., 2015; Griebel et al., 2022)

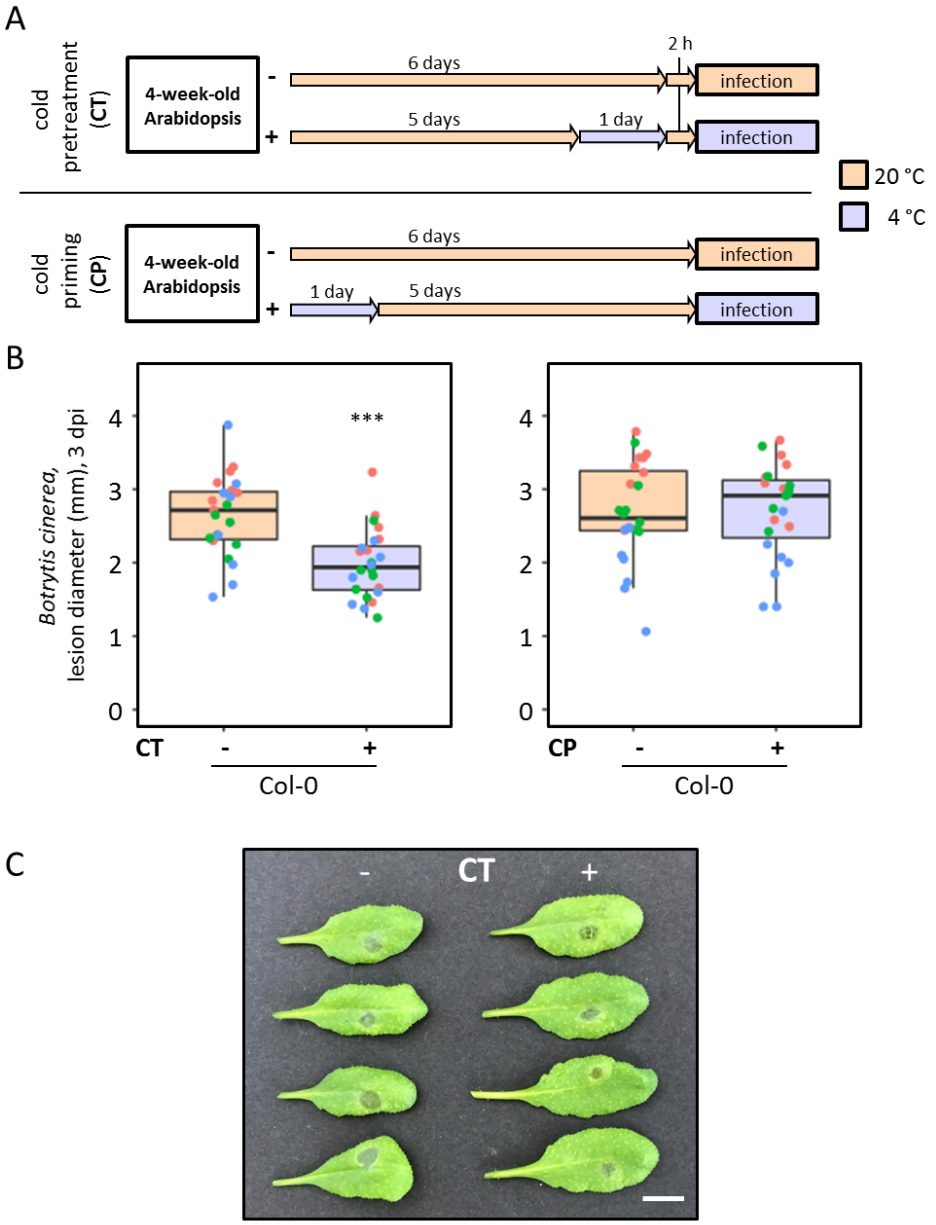

Figure 1*:* Impact of a prior cold exposure on resistance against *Botrytis cinerea* in *Arabidopsis thaliana*. **(A)** 5-week-old *Arabidopsis thaliana* (accession Col-0) plants were cold-pretreated (CT; 4°C, 24 h) and drop-inoculated (5 x 10^4 spores ml^{-1}) with *Botrytis cinerea* (*B.c.*) after 2 h at 20°C. For the cold priming setup (CP), 4-week-old Col-0 plants were cold-exposed (4°C, 24 h). Plants were drop-inoculated (5 x 10^4 *B.c.* spores ml^{-1}) after a memory phase of 5 days at normal growth conditions. **(B)** Lesion diameters of *B. c.*-infected plants were measured 3 days post inoculation (dpi) after preceding CT-treatment (left panel) or PT-treatment (right panel). Data points are shown from three independent experiments indicated by different colors (n= 23-24). Box plots show the median (central line) and asterisks denote statistically significant differences (*P* ≤ 0.05 (*), 0.01 (**), 0.001 (***), two-tailed *t-test*). **(C)** Representative picture of cold-pretreated (CT, +) and control (CT, -) Col-0 leaves with *B. c.* lesions at 3 dpi. Scale bar indicates 1 cm.

To study the impact of such prior cold treatments on resistance against B. cinerea, we analysed plant lesion sizes 3 days after drop inoculations with B. cinerea spores immediately and 5 days after the 24 h lasting cold exposure (Fig. 1B,C). Arabidopsis Col-0 exhib-

ited significant smaller lesion diameters compared to naïve control plants, if the cold exposure immediately preceded the inoculation (Fig. 1B,C; CT). By contrast, lesions diameters in the CP group, with a 5-day memory phase after the cold, were similar to those in the control group (Fig. 1C). This indicates that a directly preceding cold exposure enhances the resistance of Col-0 against B. cinerea, but the effect is not sufficiently long-lasting to restrict fungal growth several days later.

4.3.2 Plastid ascorbate peroxidases contribute to cold-enhanced resistance against *Botrytis cinerea*

Recently, we showed that cold-enhanced resistance against the bacterial pathogen *Pst* requires the plastid ascorbate peroxidases sAPX and tAPX (Griebel et al., 2022; Schütte et al., 2024). We repeated the experiment with *B. cinerea* including previously used *sapx* and *tapx* knockout lines (Kangasjarvi et al., 2008). In addition, we reduced the experimental set-up to the immediate cold pretreatment as this was effective in enhancing resistance against *B. cinerea* (Fig. 1). Again, cold-pretreated Col-0 plants showed significantly smaller *B. cinerea* lesions compared to the control group at 3 days post inoculation (dpi) (Fig. 2). In contrast, the difference in lesion sizes between cold-pretreated and control *sapx* and *tapx* plants was less pronounced and remained at a non-significant level (Fig. 2). This demonstrates that the availability of functional plastid peroxidases partially contributes to cold-enhanced resistance against the necrotrophic pathogen *B. cinerea*.

Next, we tested transcript levels of selected *B. cinerea*-triggered defence genes for cold- or APX-related differential activation patterns. *Pathogenesis-related 1* (*PR1*) is responsive to SA, while *Plant Defensin 1.2* (*PDF1.2*) and *Pathogenesis-related 4* (*PR4*) belong to the group of pathogen-triggered genes that depend on JA signalling (Thomma et al., 1998). *Phytoalexin-deficient 3* (*PAD3*) encodes the cytochrome P450 enzyme CYP71B15 which catalysis the final biosynthetic step of the phytoalexin camalexin (Zhou et al., 1999; Schuhegger et al., 2006). A prior cold treatment did not result in altered *PR1*, *PDF1.2*, *PR4* and *PAD3* transcript levels 2 hours after the cold exposure (0 dpi) in the plant lines Col-0, *sapx,* and *tapx* (Fig. 3). Consequently, the cold pre-treatment had no impact on the level of the analysed transcripts prior to *B. cinerea* infection. (Fig. 3).

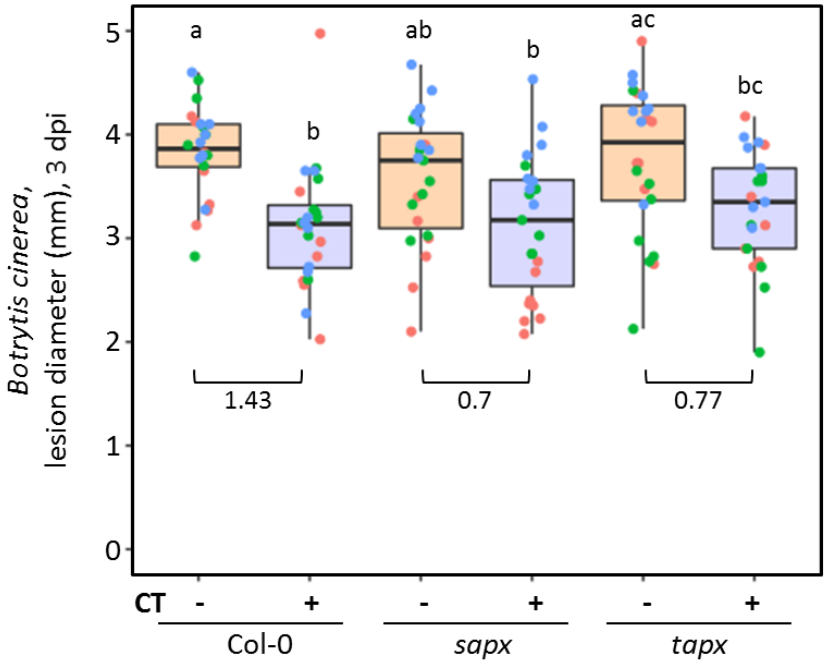

Figure 2: Impact of a cold pretreatment (CT; 24 h at 4°C) on resistance against *Botrytis cinerea* (*B.c.*) in Col-0 and in knockout lines of *stromal ascorbate peroxidase* (*sapx*) and *thylakoid ascorbate peroxidase* (*tapx*). Leaves of cold-pretreated (CT, +) and control (CT, -) plants were drop-inoculated (5 x 10^4 *B.c.* spores ml^{-1}) and the lesion diameter was measured 3 days post inoculation (dpi).). Data points are shown from three independent experiments indicated by different colours (n = 23-24). Box plots show the median (central line) and letters denote statistically significant differences (Tukey-HSD; $P \leq 0.05$). Numbers between two boxes show the effect size between two means according to Cohen's d.

At 1 dpi and 2 dpi after inoculation with *B. cinerea,* relative expression of *PR1*, *PDF1.2a*, *PR4*, and *PAD3* was increased. However, the prior cold treatment did not significantly impact *B. cinerea*-triggered transcript levels in Col-0, *sapx*, and *tapx* (Fig. 3). Since these genes are strongly connected with SA, JA, or camalexin pathways, it indicates that cold-enhanced resistance against *B. cinerea* does not correlate with altered SA and JA signalling or increased biosynthesis of camalexin.

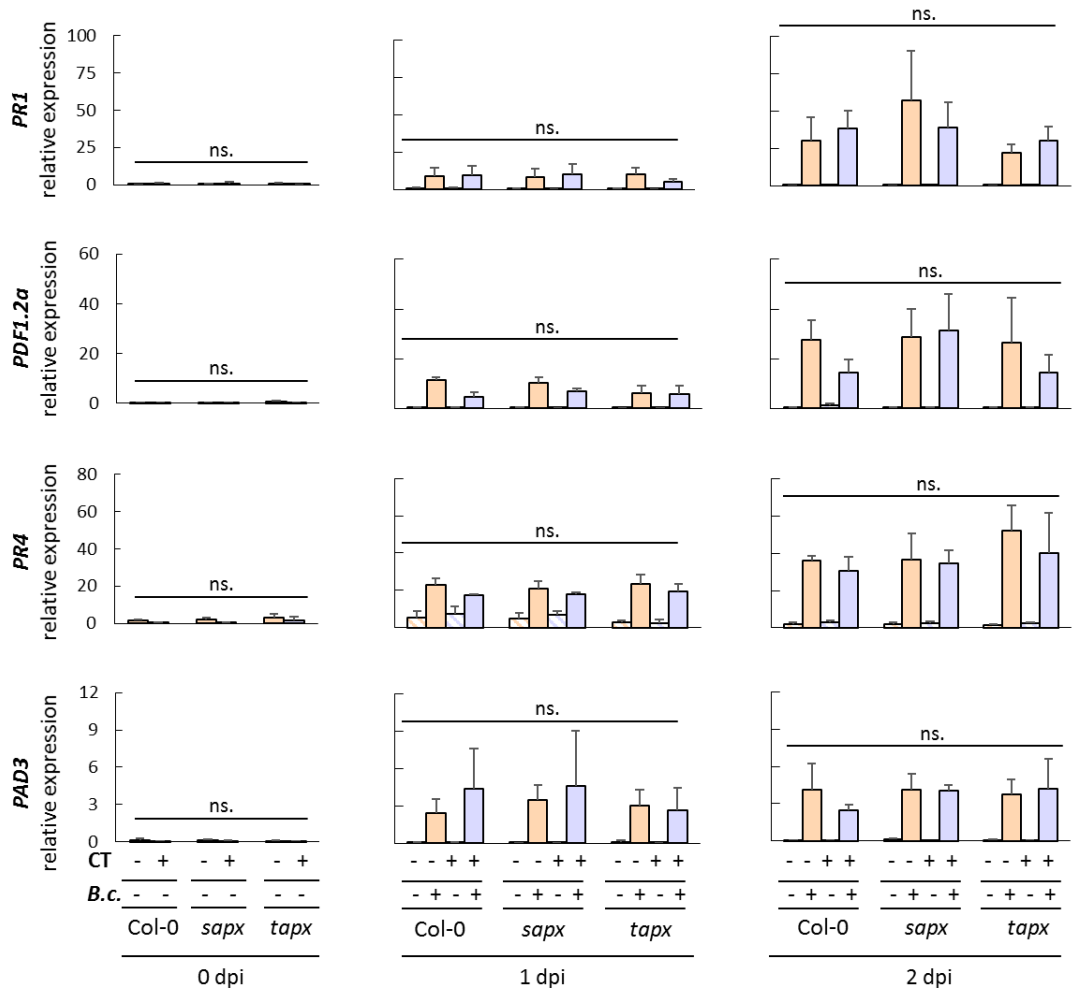

Figure 3: *Botrytis cinerea* (*B.c.*)-triggered transcripts of *PR1*, *PDF1.2a*, *PR4*, and *PAD3* in Col-0, *stromal ascorbate peroxidase* knockout (*sapx*) and *thylakoid ascorbate peroxidase* knockout (*tapx*) lines. Transcript levels in leaves were analysed after cold pretreatment (CT +; 4°C, 24 h) and subsequent *B.c.* spray infection (+; 2 x 10^5 *B.c.* spores ml^{-1}) at 0 days post inoculation (dpi), 1 dpi, and 2 dpi. Mock spraying (-; Vogel buffer) was used as infection treatment control. Transcript levels were determined by quantitative real-time PCR and were calculated as relative expression to the geometric mean of the reference genes *YLS8* and *RHIP1.* Bars show the mean of 3 independent experiments (n = 3) and the standard deviation. For mock treatments, only 2 independent experiments were performed and analysed (n = 2). No significant differences were observed for the comparison between the *B.c.*-treated samples (not significant, ns.; Fisher`s LSD test, $P \leq 0.05$).

4.3.3 *B. cinerea*-triggered ROS generation is enhanced in cold-pretreated Col-0 and in the absence of sAPX

sAPX and tAPX have central functions in scavenging photosynthesis-related H_2O_2 in the plastids (Asada, 1999). At the end of a 10 or 24 h cold exposure to 4 °C, H_2O_2 is significantly enhanced in Arabidopsis Col-0 leaves (van Buer et al., 2016; Wu et al., 2019). We analysed H_2O_2 levels by staining leaves with 3, 3′-diaminobenzidine (DAB) (Bittner et al., 2020). At the time point of inoculation (2 hours after the cold; 0 dpi), we could not detect increased H_2O_2 in cold-pretreated or naïve Col-0, *sapx*, and *tapx* (Fig. 4A). This is consistent with a rapid downregulation of cold-triggered ROS in Arabidopsis under optimal growth conditions given that enhanced ROS levels are detectable directly after the cold pretreatment (van Buer et al., 2016). 1 dpi after *B. cinerea* treatment, H_2O_2 remained at a low, not detectable level in Col-0, but it was significantly increased, if the plants where cold-treated before (Fig. 4A,B). Similarly, the *tapx* line showed enhanced H_2O_2 accumulation after *B*. *cinerea* infection (1 dpi) in prior cold-exposed leaves (Fig. 4A,B). In contrast to Col-0 and *tapx*, we detected strong *B. cinerea*-induced H_2O_2 accumulation in non-cold-treated *sapx* at 1 dpi, and the prior cold exposure even resulted in a weaker pathogen-triggered ROS accumulation in *sapx* (Fig. 4A,B).

To prove our results for Col-0 and *sapx* with an independent method, we used the luminol-based leaf disc assay for H_2O_2/ROS quantification (Bisceglia et al., 2015) and measured luminescence within a time frame of 1 h at 1 dpi. Although the differences were overall less pronounced, we observed the same trend as from the DAB staining (Fig. 4C): (i) *B. cinerea*-triggered ROS production was stronger in cold-pretreated Col-0 than in Col-0 that were constantly grown at normal growth conditions. (ii) Without a prior cold, *B. cinerea*-triggered ROS signals were significantly higher in *sapx* compared to Col-0 ($p \leq 0.001$, t-test). (iii) Cold-pretreated *sapx* showed lower *B. cinerea*-triggered ROS levels than not cold-treated *sapx* plants suggesting that the antioxidant system overcompensates for the lack of sAPX.

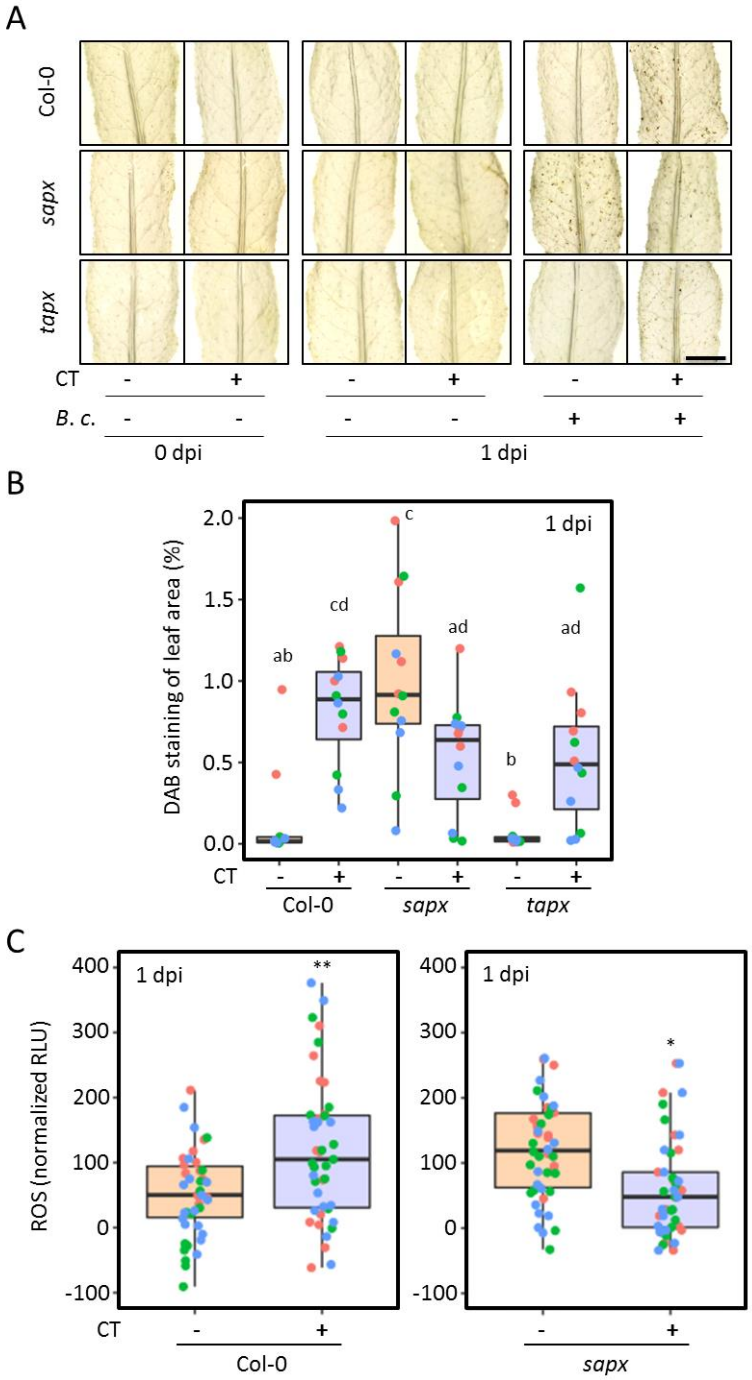

Figure 4: Reactive oxygen species (ROS) after cold pretreatment (CT +; 24 h at 4°C) and *Botrytis cinerea* spray infection (2 x 10^5 spores ml^{-1} (+), Vogel buffer (-)) in leaves of Col-0, *stromal ascorbate peroxidase* knockout (*sapx*) and *thylakoid ascorbate peroxidase* knockout (*tapx*) lines. **(A)** Hydrogen peroxide accumulation at 0 days post infection (dpi) and 1 dpi visualized with DAB staining. Representative pictures are shown. Scale bar indicates 0.5 cm. **(B)** Quantification of DAB-stained H_2O_2. Pictures from (A) were analysed for the percentage of DAB-stained area of the leaf area. For each treatment and genotype 12 leaves from 3 independent experiments were analysed. Data points from independent experiments are shown in different colours. Box plots show the median (central line) and different letters denote statistically significant differences (Tukey-HSD: $P \leq 0.05$). **(C)** Quantification of *B.c.*-triggered H_2O_2 with the luminol assay 1 dpi in Col-0 and *sapx*. Data points show the total amount of measured relative light units (RLU) counted within 1 hour relative to the non-infected control samples Data points from 3 independent experiments are shown in different colours (n = 46-48). Box plots show the median (central line) and asterisks denote statistically significant differences (two-tailed *t-test*, $P \leq 0.05$ (*), 0.01 (**), 0.001 (***)).

4.3.4 Cold-induced callose deposition requires sAPX

Callose, a β-1,3-glycan polymer, is transiently synthesized between the plasma membrane and the cell wall and contributes to the physical barrier (papillae) against pathogen attacks (Nishimura et al., 2003; Zavaliev et al., 2011; Ellinger and Voigt, 2014; Schneider et al., 2016). Callose biosynthesis and degradation in the neck region of plasmodesmata restricts their permeability and the transport of signalling compounds for intercellular communication (Zavaliev et al., 2011; German et al., 2023). In response to pathogens, callose deposition is initiated following detection of conserved pathogen-associated molecular patterns (Gómez-Gómez et al., 1999; Iriti and Faoro, 2009; Luna et al., 2011). However, callose can also be triggered by abiotic changes, such as cold (Wu et al., 2019). In some cases, increased callose accumulation correlates with enhanced resistance against *B. cinerea* in *Arabidopsis* (Nie et al., 2017; Sanmartín et al., 2020). We analysed callose deposition 2 h after the 24 h cold exposure (0 dpi) and in response to *B. cinerea* infection at 1 dpi. At 0 dpi, callose deposition in Col-0, but not in *sapx*, was significantly enhanced in cold-pretreated plants (Fig. 5 A,B). We could not anymore detect the cold-responsive callose in the non-infected Col-0 at 1 dpi. After infection with *B. cinerea* (Fig. 5 A,B), an increased callose deposition was observed in Col-0 and *sapx.* However, the number of *B. cinerea*-triggered callose spots was not affected by the cold treatment. We concluded that sAPX-promoted cold-triggered callose deposition correlates with enhanced resistance against *B. cinerea*.

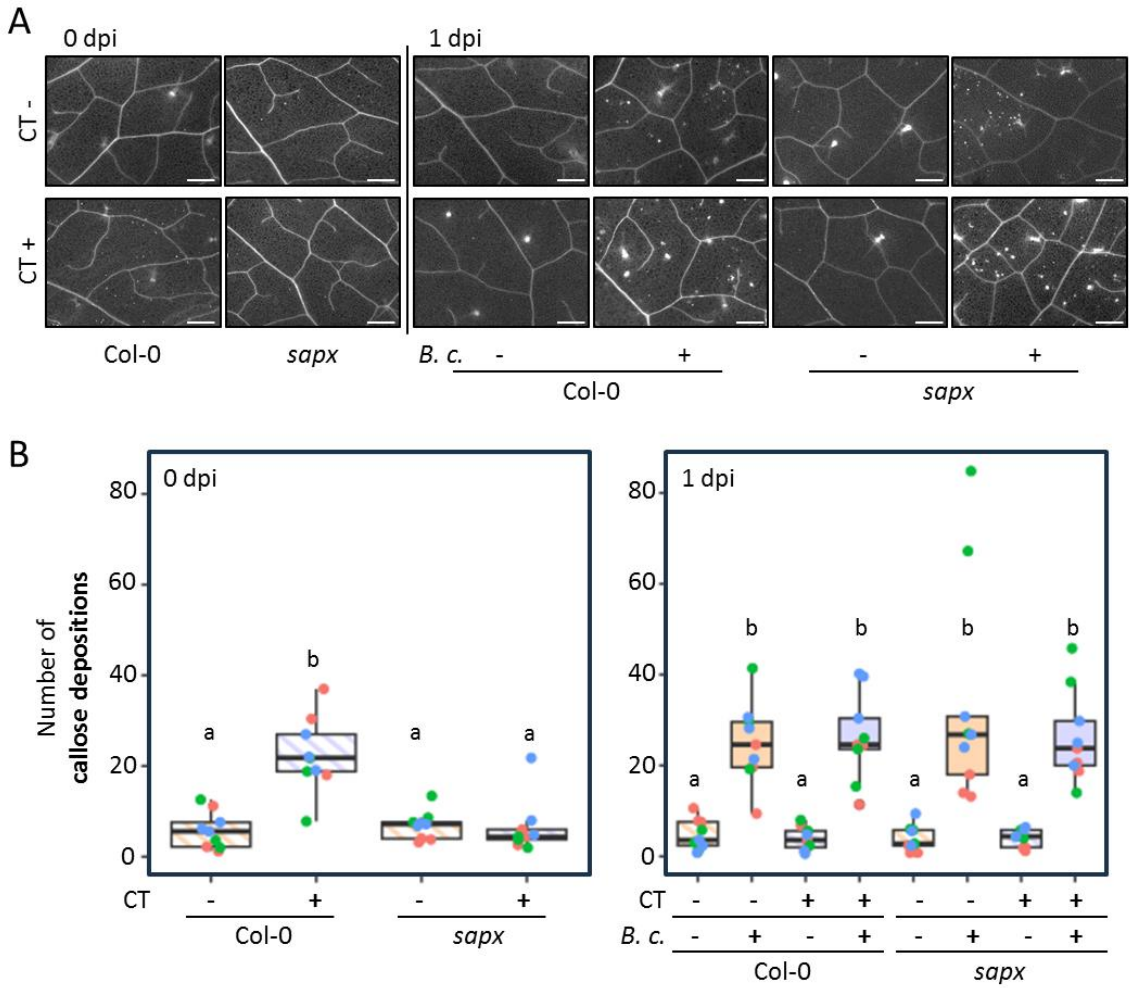

Figure 5: Callose formation after cold treatment (CT +; 4°C, 24 h) and subsequent *Botrytis cinerea* spray inoculation in Col-0 and *stromal ascorbate peroxidase* knockout (*sapx*). **(A)** Pictures of aniline blue-stained callose in Arabidopsis leaves immediately after CT (+, 0 days post inoculation (dpi)) and 1 dpi after subsequent *B. cinerea* infection (+) or mock infection (-) at 1 dpi. Scale bar = 500 µm. **(B)** Quantification of the callose depositions from (A). Each data point (biological replicate) represents the mean of 5 randomly analysed leaf areas (7 mm^2). Counts from three independent experiments (n = 9) are shown and indicated by different colors. Box plots show the median (central line) and different letters denote statistically significant differences and different letters denote statistically significant differences (Tukey-HSD, $P \leq 0.05$).

4.3.5 Cold exposure enhances lignin content in cell walls

To analyse lignification of the cell wall in response to cold and *Botrytis cinerea*, we measured the lignin contents of extracted cell wall residues (CWR) using the acetyl bromide (AcBr) method (Moreira-Vilar et al., 2014; Chezem et al., 2017) in Col-0 and *sapx* leaves after prior cold exposure and 2 days after additional *B*. *cinerea* infection. The lignin content in the leaves of Col-0 significantly increased in response to cold (Fig. 6). Even at 2 dpi, the lignin ratio was still enhanced in cold-pretreated Col-0 samples, but not further altered in the *B. cinerea*-inoculated samples. This demonstrated that solely cold exposure but not fungal invasion was responsible for the significantly higher lignin content in Col-0 leaves. In contrast, the lignin content in *sapx* did not show any cold-related signatures (Fig. 6). Besides, lignin content measurements from *sapx* resulted in high variation and did not provide a decent basis for further interpretations. Taken together, we concluded that cold exposure but not *B. cinerea* infection promoted plant lignification. Pathogen-triggered lignification was reported before, but mainly after inoculation with bacterial pathogens or in the response of resistant plants to otherwise necrotrophic fungi (Menden et al., 2007; Eynck et al., 2012; Lee et al., 2019; Kim et al., 2020; Jeon et al., 2023). We assume that the absence of *B. cinerea*-triggered lignification is not caused by fungal lignin degradation as it was reported earlier that *B. cinerea* is most likely not capable of degrading lignin (Hörmann et al., 2013).

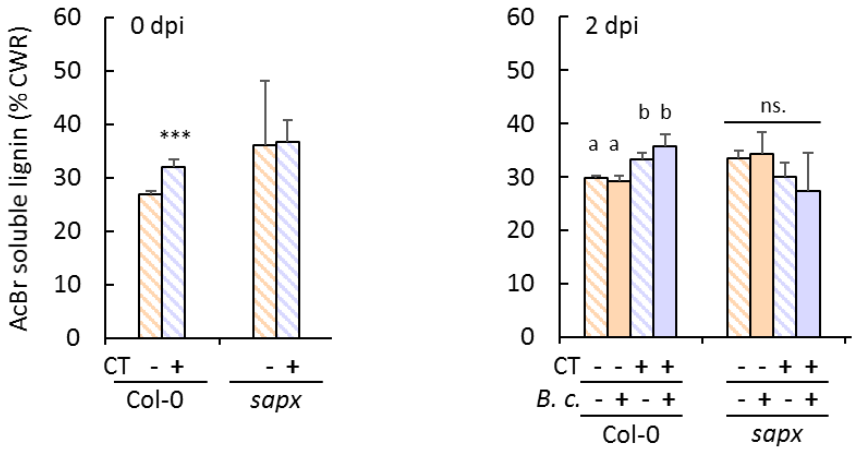

Figure 6: *Lignin content in cell walls of leaves of Col-0 and stromal ascorbate peroxidase knockout (sapx) after prior cold pretreatment (CT +; 4 °C, 24 h) and Botrytis cinerea (B.c) spray infection (2 x 10^5 spores ml^{-1} (+), Vogel buffer (-)). Bars show the mean percentage (%) of acetyl bromide-soluble lignin in the fraction of isolated cell wall residues (CWR) from three independent experiments (n = 4 – 5). Left panel: data from samples analyzed immediately after the cold exposure at the day of infection (0 dpi), two-tailed t-test, P ≤ 0.05 (*), 0.01 (**), 0.001 (***). Right panel: samples analyzed at 2 dpi. Different letters denote statistically significant differences within each genotype (Tukey-HSD, P ≤ 0.05). AcBr = acetyl bromide; CWR = cell wall residues; dpi = days post inoculation*

4.4 Discussion

A single 8-24 h lasting pre-exposure to cold conditions (4°C) boosts immunity in 4-week-old Arabidopsis Col-0 against a subsequent infection with the hemibiotroph bacterial pathogen *Pst* (Wu et al., 2019; Griebel et al., 2022). Also, daily repetitive 1.5 h cold (4°C) exposures enhance plant resistance against *Pst* (Singh et al., 2014). Much less is known about the impact of cold pretreatments on plant immunity against necrotrophic pathogens. Here, we investigated the effects of preceding cold exposures on the susceptibility against the necrothrophic fungus *B. cinerea*. When *B. cinerea* (B05.10) spore inoculation was performed on cold-pretreated plants, we observed a reduction of disease lesion diameters (Fig. 1B,C). This indicates that Arabidopsis Col-0 benefits from a temporally close and timely limited cold pretreatment with an immunity boost against a broad spectrum of virulent pathogens with biotrophic and necrotrophic lifestyles. Interestingly, a milder cold exposure to only 12°C but for a longer time period of 3 days results in enhanced susceptibility of 2-week-old Arabidopsis seedlings against *B. cinerea* and *Pst* (Garcia-Molina and Pastor, 2024).

Short but more severe cold exposures and other abiotic stress treatments can be memorized in Arabidopsis for up to 5-7 days (Ding et al., 2012; Singh et al., 2014; van Buer et al., 2019). If such a memory of an experienced stress exposure results in an improved response during a subsequent stress exposure, this is defined as priming (Hilker et al., 2016). However, when cold exposure and pathogen inoculation are separated by 5 days at normal growth conditions, only plant resistance against the hemibiotroph *Pst* is potentiated (Griebel et al., 2022; Schütte et al., 2024) but not plant defence against the necrotrophic *B. cinerea* (Fig. 1). Hence, the cold priming memory is beneficial for defence against virulent hemibiotrophic *Pst* but not supportive for the defence against the necrotrophic fungus *B cinerea*. Host resistance mechanisms vary according to the lifestyle of the pathogen (Liao et al., 2022). We assume that defence responses enhanced by a cold priming memory rather include signal pathways beneficial for immunity against virulent biotrophs.

Recently, we reported that the effects of cold priming on plant resistance against bacterial pathogens relies on the availability of plastid APX (Griebel et al., 2022; Schütte et al., 2024). Here, we showed that the transient cold-enhanced resistance against *B. cinerea* immediately upon cold exposure was also stronger when plastid APX were present (Fig. 2). How do plastid APX contribute to plant pathogen defence subsequent to an abiotic stress exposure? APX scavenge ROS by using ascorbate as an electron donor (Asada,

1999). At the end of a 10-24 h lasting cold exposure (4°C), 4-week-old Arabidopsis have transiently enhanced ROS amounts (van Buer et al., 2016; Wu et al., 2019). When we measured ROS 2 h after the cold at the time point of inoculation (Fig. 4A, 0 dpi), we could not anymore observe the cold-triggered ROS burst. This indicates a fast reacclimation to a precold ROS homeostasis at normal growth conditions. Transient ROS waves are often observed in plant stress signalling (Lamb and Dixon, 1997; Apel and Hirt, 2004; Miller et al., 2009; Peláez-Vico et al., 2024). In response to a post-cold *B. cinerea* inoculation, cellular and apoplastic ROS generation was stronger in cold-pretreated than in control plants at 1 dpi. (Fig 4). In our study, higher *B. cinerea*-triggered ROS levels in cold-pretreated Col-0 plants correlated with higher resistance (Fig. 1,4). However, the contribution of plant ROS in defence against necrotrophic pathogens is complex (Torres et al., 2006). For instance, Pogány et al. (2009) suggested that ROS generated by plasma-membrane located NADPH oxidase RBOHD support plant resistance during the early phase of defence against necrotrophic fungi but enhance susceptibility at later stages. Also, transgenic *Nicotiana tabacuum* lines with less chloroplastic ROS triggered by *B. cinerea* at later stages of infections (3 dpi) showed enhanced resistance against this necrotrophic fungus (Rossi et al., 2017). We suggest that the enhanced ROS levels observed already at 1 dpi in our study of cold-pretreated Arabidopsis are connected with smaller fungal lesions and a strengthened plant resistance. Interestingly, sAPX- but not tAPX,-deficient lines responded with stronger *B. cinerea*-triggered ROS generation even without a cold-pretreatment (Fig. 4). This indicates that sAPX scavenges a significant proportion of *B. cinerea*-triggered ROS in the wildtype, but also in *tapx*, and that *tAPX* contributes less than *sAPX* to the amount of fungal-triggered plant ROS amounts. In addition, the enhanced ROS levels in *sapx* lines matched with a slightly, but not significantly, improved resistance of control *sapx* against *B. cinerea* (Fig. 3). In contrast, the weakened fungal-triggered ROS response of cold-treated *sapx* plants compared to cold-treated Col-0 or control sAPX was connected with weaker cold-enhanced resistance (Fig. 2,4). The measured ROS levels in *tapx* did not differ from Col-0. We suggest, that the stromal ROS protection with contribution from sAPX but without tAPX is sufficient to result in a wildtype-like plastid ROS homeostasis. In contrast, tAPX, but not sAPX, was identified as a specific mediator of cold priming-dependent altered activation of stress-related transcripts during a second cold exposure and affects cold priming-dependent regulation of chloroplast NADPH dehydrogenase activity (van

Buer et al., 2016; van Buer et al., 2019; Seiml-Buchinger et al., 2022). Our ROS measurements (Fig.4) support the concept of distinct functions for sAPX and tAPX on plastid ROS control and redox signalling in response to cold pretreatments.

Among other functions, the interplay of apoplastic ROS and peroxidases plays pivotal roles in modifying and remodelling plant cell walls (Kärkönen and Kuchitsu, 2015). Cell wall modifications also occur in responses to abiotic stress exposures, such as cold (Le Gall et al., 2015). In biotic stress interactions, plant cell walls are physical barriers and provide protection against the invasion of pathogens (Underwood, 2012). Necrotrophic fungi secrete a large repertoire of cell wall degrading enzymes to facilitate their successful infection. To counteract this, plants sense pathogens by monitoring cell wall integrity and activate defence pathways including remodelling of cell walls (Bellincampi et al., 2014; Lee et al., 2019; Pontiggia et al., 2020; Wolf, 2022; Kim et al., 2023). Precursors of lignin derive from the phenylalanine ammonia-lyase (PAL) pathway and Arabidopsis *PAL1* is induced during cold exposure (Rohde et al., 2004; Olsen et al., 2008; van Buer et al., 2016; Griebel et al., 2022). We observed an enhanced lignification in Arabidopsis Col-0 after the 24 h lasting cold exposure (Fig. 6), which might hamper fungal penetration and support plant resistance. By contrast, degradation of lignin or additional lignification was not detectable at 2 dpi with *B. cinerea* (Fig. 6). It was already proposed by Hörmann et al. (2013) that *B. cinerea* is not able to degrade lignin. Avirulent PTI/ETI-triggering *Pst* strains and to a lower extent the virulent *Pst* strain, promote plant lignification as part of induced plant immune responses (Lee et al., 2019). Lignin enhances disease resistance against the hemibiotrophic *Pst* (Lee et al., 2019) and might also contribute to resistance against the necrotrophic fungus *B. cinerea* (Fig. 1, 6).

The most prominent plant cell wall modification in response to pathogen detection is the deposition of callose (Nishimura et al., 2003; Ellinger and Voigt, 2014). Our findings (Fig 5) corroborate previous observations (Wu et al., 2019) that cold exposure triggers callose formation in Arabidopsis leaf tissue (in the absence of pathogens). Cold-triggered callose formation was absent in *sapx*, but pathogen-triggered callose deposition did not depend on sAPX (Fig. 5). This indicates an additional and novel contribution for plastid sAPX in cold-triggered callose formation and distinguishes cold and pathogen-responsive pathways for callose deposition.

In summary, we conclude that also resistance to necrotrophic pathogens benefits from a short preceding cold treatment with increased resistance that correlates with stronger ROS formation, cold stress-induced lignification and callose deposition.

4.5 Material and Methods

4.5.1 Plant material and cultivation

Experiments were carried out with *Arabidopsis thaliana* accession Columbia-0 (Col-0) and described knockout lines *sapx* and *tapx* (Kangasjarvi et al., 2008). All lines are in Col-0 background. Plants were cultivated in round pots (Ø 6 cm) on a substrate composed of Topferde (Einheitserde, Germany), Pikiererde (Einheitserde, Germany), Perligran Classic (Knauf, Germany) in a 14:14:5 ratio supplemented with 0.5 g liter^{-1} dolomite lime (Deutsche Raiffeisen-Warenzentrale, Germany). After sowing, seeds were stratified at 4°C for 2 days and seedlings were pricked out approximately 8 days after stratification. Plants were grown in a controlled environmental chamber at constant humidity (60 ± 5 %) with 10 h of light (100 – 120 µmol photons m^{-2} s^{-1}; Luminol Cool White fluorescence stripes, Osram, Germany) and a temperature of 20°C ± 2°C during the day and 18°C ± 2°C during the night (14 h).

4.5.2 Cold stress treatments

Cold treatments were performed as previously described (van Buer et al., 2016; van Buer et al., 2019; Griebel et al., 2022). Briefly, four-week-old plants were exposed to cold 2.5 h after onset of light by transferring them to a growth chamber with a constant temperature of 4 ± 2°C but otherwise identical aeration, illumination, and air humidity as in the 20°C chamber. After a continuous cold exposure for 24 h (comprising a full day and night phase), the plants were placed back to the 20°C chamber, labelled, and randomized with the non-cold-treated control plants. After 2 h (CT, cold treatment set-up) or 5 days (CP, cold priming set-up) at normal growth conditions (20°C), plants were used for pathogen infection assays

4.5.3 *Botrytis cinerea* infection

Inoculations with the *Botrytis cinerea* strain B05.10 were performed 4.5 h after onset of light. For drop inoculation assays, spore suspensions were adjusted to 5 x 10^4 spores ml^{-1} potato dextrose broth (PDB, 6 g l^{-1}). Spray-inoculation was carried out with spore suspensions adjusted to 2 x 10^5 spores ml^{-1} in Vogel buffer (sucrose 15 g l^{-1}, tri-sodium citrate 2.5 g l^{-1}, K_2HPO_4 5 g l^{-1}, $MgSO_4$ x 7 H_2O 0.2 g l^{-1}, $CaCl_2$ x 2 H_2O 0.1 g l^{-1}, NH_4NO_3 2 g l^{-1}, pH 6). The adjusted spore suspension was incubated under gently agitation at room temperature for 4 h to allow germination. Drop inoculation was conducted by pipetting a 6 µl

droplet next to the midrib of a fully expanded leaf. The mean of four drop-inoculated leaves of one plant represents one biological replicate in the lesion diameter experiments. Spray inoculations were carried out by evenly spraying the leaf surface with the spore suspension. Spore-free Vogel buffer was used for mock spray treatments as control. Infected plants were kept as described above except for an additional high humidity environment created by watering and light permeable coverage of the pots.

4.5.4 Quantitative real-time PCR analysis

Transcript analyses were performed using quantitative real-time PCR (qRT-PCR). One sample (= one biological replicate) consisted of 4 pooled plant rosettes. Plant samples were harvested at the indicated time points. RNA extractions, cDNA syntheses and qRT-PCR assays from ground plant material were performed as previously described (van Buer et al., 2016; Griebel et al., 2022). A mix of oligo(dT)16 primers and random primers was used for cDNA syntheses. The qTower^3 G instrument (Analytik Jena, Germany) was used for qRT-PCR assays. Primers for genes of interest and reference genes are listed in Table 1. Values of cycle thresholds were determined using qPCRsoft (Analytik Jena, Germany) and relative expression of transcripts of interest were calculated using the ΔCT-method against the geometric mean of two reference genes *Yellow Leaf Specific Protein 8* (*YLS8*) and *RGS1-HXK1 Interacting Protein 1* (*RHIP1*).

Table 1. Primers used in this study

Gene	Gene name	Forward primer	Reverse primer
AT5G44420	***PDF1.2a***	TTTGCTTCCATCATCACCCTTA	GCGTCGAAAGCAGCAAAGA
AT2G14610	***PR1***	TTCTTCCCTCGAAAGCTCAA	AAGGCCCACCAGAGTGTATG
AT3G04720	***PR4***	GCGGCAAGTGTTTAAGGGTGAAG	CGTTGCTGCATTGGTCCACTATTC
AT3G26830	***PAD3***	CTTTAAGCTCGTGGTCAAGGAGAC	TGGGAGCAAGAGTGGAGTTGTTG
AT5G08290	***YLS8***	TCATTCGTTTCGGCCATGACTGG	ACGCAAGCACCTCATCCATCTG
AT4G26410	***RHIP1***	GAGCTGAAGTGGCTTCCATGAC	GGTCCGACATACCCATGATCC

4.5.5 DAB-staining

To analyse hydrogen peroxide abundance after cold exposure and *B. cinerea* infection, 3,3′-diaminobenzidine (DAB) was used as previously described (Bittner et al., 2020). 1 mg ml^{-1} DAB was dissolved in phosphate-buffered saline (PBS; 73 mM NaCl, 10 mM, Na_2HPO_4, 3 mM KCl, 2 mM KH_2PO_4, pH 7.2). A minimum of 4 leaves from 3 plants per treatment was harvested and collected in a tube with DAB-staining solution. The collected leaves were infiltrated with the staining solution by applying a gentle vacuum for 5 min in a desiccator. Afterward, samples were incubated overnight in the dark. The background of stained leaves was removed in a 1:1:3 mixture of acetic acid, glycerol, and ethanol before analysis and image acquisition. The intensity of DAB staining per leaf was calculated using ImageJ (Schneider et al., 2012; Bittner et al., 2020).

4.5.6 Luminol-based ROS assay

ROS was measured by horseradish peroxidase (HRP)-catalyzed luminol oxidation in the presence of H_2O_2. 21 h after *B. cinerea* spray-inoculation, leaf discs (Ø 4 mm) from at least 3 plants per treatment were collected in $_{dd}H_20$-filled petri dishes and incubated for 3 h under normal growth conditions to minimize wounding response during the measurement. Thereupon, leaf discs were transferred to 96-well plates. 150 µl reaction solution (200 µM luminol, 0.04 µg HRP ml^{-1} $_{dd}H_20$) was added to each well. Chemiluminescence was measured with a CLARIOstar^{PLUS} plate reader (BMG Labtech, Germany) and is shown as the sum of all counted relative light units (RLU) within 1 h of measurement. RLU of inoculated samples were normalized to the corresponding mean of the non-infected mock treatments ($RLU_{treatment} - RLU_{mock\ treatment}$ = normalized RLU) within each independent experiment.

4.5.7 Callose quantification

Callose was stained with aniline blue (Carl Roth GmbH, Germany) according to Schenk and Schikora (2015) with the alteration of an extended aniline blue incubation overnight. One biological replicate of the callose quantification was defined as the mean of five randomly imaged field of views (7 mm^2) from one leaf. Leaves were taken from three different plants and the experiments were repeated three times independently. Monochrome pictures were taken using a AxioImager Z2 microscope (Zeiss, Germany) equipped with a Zeiss filter 49 (G 365/ FT 395 / BP 445/ 50) and an Axiocam 712mono (Zeiss, Germany)

camera. Images were acquired with the software ZEN blue (Zeiss, version 3.3) and numbers of callose depositions were manually counted.

4.5.8 Lignin quantification

Quantifications of lignin were performed according to Moreira-Vilar et al.(2014) with modification from Chezem et al. (2017). 14 leaf discs (Ø 8 mm) from at least 3 plants were pooled to one sample and defined as one biological replicate. Samples were frozen in liquid nitrogen and ground in 2 ml tubes, each containing 2 x 4 mm glass beads, using a swing mill (Retsch, Germany) at 30 Hz for 2 min. Ground samples were vacuum-dried using a Concentrator plus (Thermofisher, Germany) and the dried leaves were ground again as described above. Next, cell wall residues (CWR) were twice extracted in an ultrasonic bath with each of the following solvents for 15 min: 1 ml methanol, 1 ml PBS with 0.1 % (v/v) Tween 20, 1 ml ethanol, 1 ml chloroform/methanol (1:1 ratio) and at least 1 ml acetone. After each extraction step, samples were centrifuged at 16.000 g for 10 min and the supernatant was discarded. CWR were dried at 45°C using a Concentrator plus and ground with a swing mill (2 ml tubes, each containing 2 x 4 mm glass beads) at 30 Hz for 10 min. Approximately 3 mg of CWR from each sample was used for the spectrometric analysis. For this purpose, 500 µl of 25 % (v/v) acetylbromid in glacial acetic acid was added to each sample and incubated at 50°C with rotation (800 rpm) for 2 h. After dissolving the lignin, samples were cooled on ice and centrifuged at 20.000 g for 15 min. 125 µl of the lignin-containing supernatant was mixed with 500 µl glacial acetic acid and 250 µl of 5 M hydroxylamine HCl / 2 M NaOH (1:9 ratio). Photometric measurements were done in a single glass cuvette (10 mm) using an Ultrospec 2100 pro at 280 nm and a sample without CWR as blank. Acetylbromid soluble lignin content was calculated with the extinction coefficient ε of 23.35 mg cm^{-1} l^{-1} (Chang et al., 2008).

4.5.9 Statistical analyses and boxplot design

The statistical analysis was conducted using Excel for Student's t-tests, basic R environment for ANOVA and the follow-up Tukey-HSD test, the R agricolae package for LSD-Fisher test after prior ANOVA. Box plots of the summarized data were generated using the R package ggplot2 and show the median, the distance between the upper quartile (qn = 0.75) and lower quartiles (qn = 0.25), and the values of each data point as dots. Data points form independent experiments are shown in different colour.

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Chapter IV: Impact of cold exposure on systemic acquired resistance *in Arabidopsis thaliana*

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Publication in preparation.

Contribution to the publication:

Concept: 85 %
Experiments: 100 %
Writing: 75 %

5.1 Abstract

Plants face numerous environmental challenges, including pathogen attacks and abiotic stresses like cold temperatures. Understanding how these factors interact is crucial for improving plant resilience. Here, we investigated the effects of cold exposure on systemic acquired resistance (SAR) in *Arabidopsis thaliana* challenged with *Pseudomonas syringae* pv. *tomato* (*Pst*). We demonstrated that a 24-hour cold exposure at 4 °C immediately before *Pst* infection (CT) led to significantly enhanced systemic *PR1* expression. Although the induction of SAR in cold-pretreated plants was successful, cold pre-treatment and the increased systemic *PR1* expression did not translate into further enhanced systemic resistance against *Pst*. Moreover, cold priming (CP), with a 3-day memory period between cold exposure and infection, did not show additive effects on SAR-induced gene expression or additional benefits of cold priming at the level of systemic *Pst* resistance . Interestingly, we observed a transient increased systemic susceptibility against *Pst* in cold pretreated plants in response to mock infiltration, suggesting a potential alteration in immune signaling pathways. This effect was independent of SAR and might be associated with a stronger wound response. However, we proved that cold-pretreated plants are fully competent in establishing a SAR resonse.

5.2 Introduction

Plants are exposed to a constantly changing environment that hosts a diversity of microbes. Some microbes have the potential to become pathogenic and cause diseases in the plant. Abiotic changes cannot only be a challenge in themselves; they also impact the plant's resistance against pathogens (Saijo et al., 2020). One abiotic factor is cold, and plants have developed strategies, including cold acclimation, to avoid cold-induced damage such as ice crystal formation in plant cells (Satyakam et al., 2022). Even a single, brief cold experience increases the plant's tolerance against cold. This phenomenon is defined as cold priming (Baier et al., 2019). Priming, as a result of a first stress experience, impacts performance on a future stress stimulus (trigger) with a stress-free phase (memory phase) between both stimuli (Hilker et al., 2016). Priming can be caused by all different types of stressors. In addition to cold or abiotic stress priming, systemic acquired resistance (SAR) is a form of biotic priming due to pathogen infection. Within SAR, a local pathogen infection induces systemic long-distance signaling that results in an improved and pre-activated immune response in systemic, non-infected tissues against a broad spectrum of hemibiotrophic pathogens. (Shah & Zeier et al., 2013; Conrath, 2011).

Pathogen recognition at the infection site involves the detection of pathogen-associated molecular patterns (PAMPs) by plasma membrane-anchored pattern recognition receptors (PRRs) and initiates so called PAMP-triggered immunity (PTI) (Bigeard et al., 2015). Another local recognition layer of the plant immune system comprises intracellular nucleotide-binding leucine-rich repeat immune receptors (NLRs), which recognize effectors released by pathogens and activate effector-triggered immunity (ETI) (Lolle et al., 2020; Büttner et al., 2016). Activation of PTI and ETI at the local infection site triggers SAR (Vlot et al., 2021; Conrath et al., 2015; Mishina & Zeier, 2007). The establishment of SAR requires signaling by salicylic acid (SA) and by pipecolic acid (Pip) or its derivative N-hydroxy-pipecolic acid (NHP) in the local and the systemic tissue. Arabidopsis mutant lines with impaired biosynthesis/accumulation of SA or Pip/NHP fail to establish SAR (Chen et al., 2018; Vlot et al., 2009). SA is mobile in apoplastic and vascular tissues and one component of the long-distance SAR signaling metabolites. NHP accumulates in systemic tissues 1 day after infection and thereby, prior to the systemic increase of Pip. This indicates that NHP is transported over long distances from the local infected tissue and is therefore a central mobile SAR signal (Chen et al., 2018; Hartmann et al., 2018; Vlot et al., 2009). In addition to SA and Pip/NHP, a number of other compounds were suggested to contribute to SAR signaling, including mitogen-activated protein kinase 3 and 6 (MAPK 3, MAPK 6),

reactive oxygen species (ROS), azelaic acid (AzA) and volatile organic compounds like pinenes (Guerra et al., 2020; Wenig et al., 2019; Wang et al., 2018; Riedlmeier et al., 2017; Dubiella et al., 2013; Jung et al., 2009). The successful activation of SAR appears with increased systemic expression of *pathogenesis-related* (*PR*) genes, including *PR1*, *PR2*, and *PR5*, as well as *Phytoalexin Deficient 3* (*PAD3*). *PAD3* is crucial for biosynthesis of the antimicrobial compound camalexin (Bektas & Eulgem, 2014; Gruner et al., 2013; Návarová et al., 2012). In total, the transcriptional changes due to SAR involve the upregulation of over 3400 genes and the downregulation of about 2900 genes indicating a large and complex reprogramming process (Bernsdorff et al., 2016).

Our understanding of how abiotic factors such as cold affect the establishment of SAR is limited. Recently, it was demonstrated that exposing *Arabidopsis thaliana* (Col-0) to a single 24-hour cold exposure (4 °C), either immediately (1 hour after cold exposure) or after a subsequent 5-day stress-free memory phase, is sufficient to increase local resistance against the virulent, hemibiotrophic pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) (Griebel et al., 2022). Additionally, no link was found between SAR signaling and cold exposure mediated enhanced resistance against a local *Pst* infection (Griebel et al., 2022). While it has not been demonstrated that there is a link between SAR and cold exposure enhanced resistance against local *Pst* infection in Col-0, it is possible that a single short cold stress could potentiate the outcome of SAR against *Pst*. Therefore, we investigated whether cold exposures affect the establishment of SAR in Arabidopsis plants.

5.3 Results

5.3.1 *Pst*-triggered, systemic *PR-1* expression is enhanced when a cold exposure immediately precedes the infection

Recently, we demonstrated that transcript patterns of genes belonging to gene ontology group of SAR only marginally overlap with genes regulated in response to a 24 h cold exposure (Griebel et al. 2022). To analyze the impact of such prior cold treatments on transcript regulations within the *Pst*-triggered SAR response, we investigated systemic transcript abundance of the SA-inducible *PR1* and *Flavin-dependent Monooxygenase 1* (*FMO1*), which is crucial for the biosynthesis of the SAR mediator NHP (Chen et al., 2018; Hartmann et al 2018; Zhang et al., 1999). We either applied a 24 h lasting cold exposure immediately before the SAR-triggering *Pst* infection (CT) or 3 days earlier (CP) to elucidate effects of a longer preceded cold exposure (Fig. 1A). As SAR is transcriptionally established in the systemic tissue after two days (Bernsdorff et al., 2016; Gruner et al., 2013),

we removed the SAR-inducing leaves 2 days after infection (Fig. 1A) to achieve a uniform cutoff of the SAR signal (local infection) in all treatments. We analyzed *PR1* and *FMO1* transcripts at 1, 2, 4, and 6 dpi. At 2 dpi, the systemic transcript abundance of *PR1* in Col-0 control plants (CT - and CP -) significantly increased compared to the corresponding mock treatments (two-tailed t test: p-value ≤ 0.05) (Fig.1 B,C). This indicates the successful establishment of the SAR response at the transcriptional level. At 4 dpi and 6 dpi, the increase in *PR1* transcripts was again back to pre-activation levels and the levels in non-induced, mock-treated samples. Transcripts of *PR1* and *FMO1* were not enhanced in the cold-pre-exposed plants of both experimental setups at 1 dpi, 4 dpi, or 6 dpi, suggesting that cold exposure does not induce an earlier or prolonged SAR response (Fig. 1 B,C). However, *Pst*-triggered SAR resulted in significantly higher *PR1* expression at 2 dpi when the exposure to cold immediately preceded the infection (CT), but not when the cold treatment was applied 4 day earlier (CP).

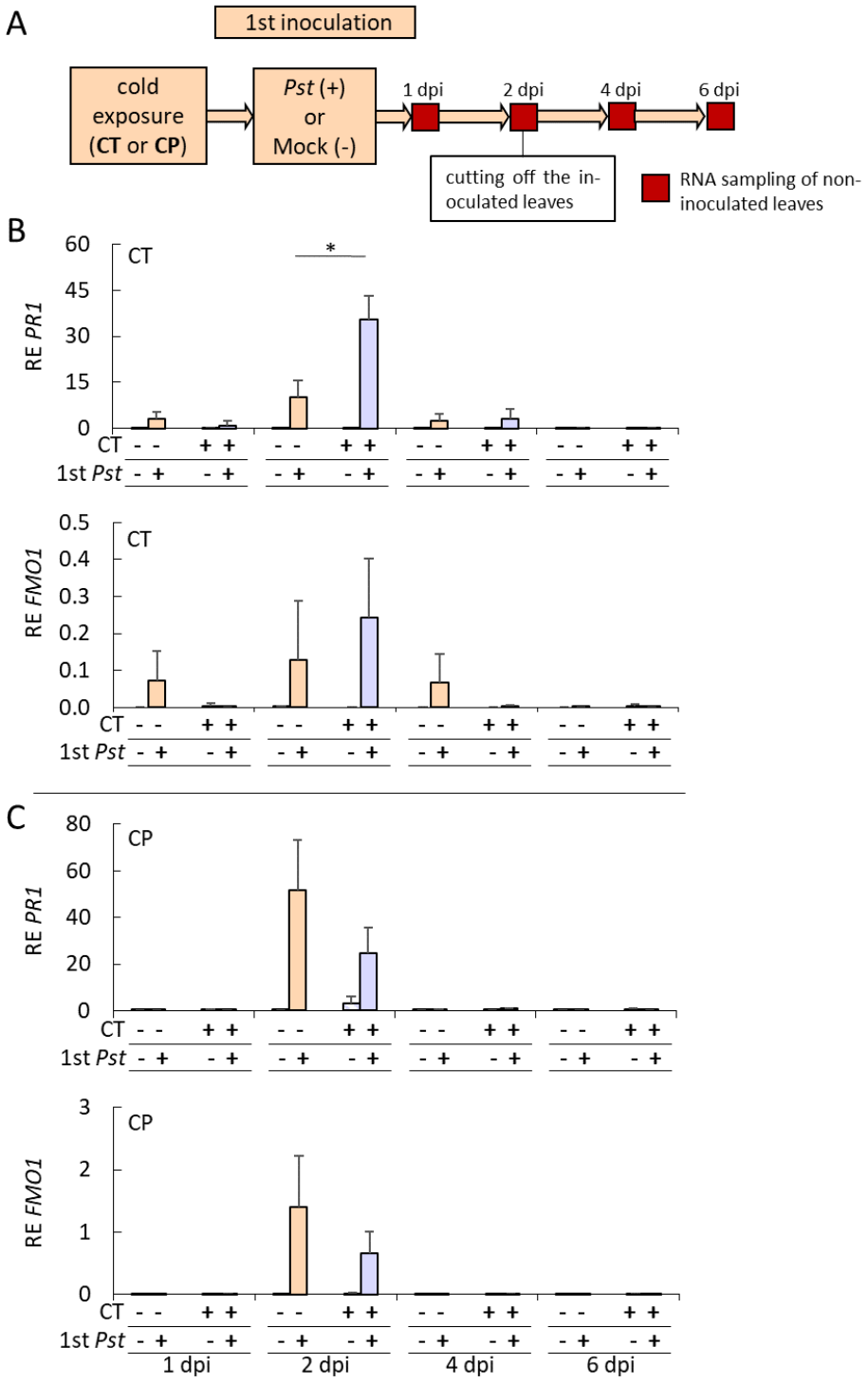

Figure 1: Relative expression (RE) of pathogen-responsive genes after cold exposure and SAR induction in non-inoculated (systemic) leaves of Col-0. **(A)** Experimental set-up for transcript analysis upon SAR induction (first *Pst* inoculation) after cold exposure (4 °C) for 24 h either as an immediate pre-treatment (CT) or with a 3-day long stress-free period between cold exposure and infection, called cold priming (CP). Systemic, uninfected leaves were harvested 1 day post inoculation (dpi), 2 dpi, 4 dpi and 6 dpi. Infected leaves were cut for a uniform SAR signal termination at 2 dpi. **(B & C)** Transcript levels of *PR1* and *FMO1* are analyzed after CT (B) or CP (C) and subsequent *Pst* inoculation (OD_{600} = 0.002) or mock (10 mM $MgCl_2$) infiltration at 1dpi, 2 dpi, 4 dpi, and 6 dpi. The quantification of transcripts was determined by qPCR. The results were calculated as relative expression (delta-Ct) against the geometric mean of the reference genes *YLS8* and *RHIP1*. Bars show mean and standard deviation. (B) CT: N = 3 (3 independent experiments). (C) CP: N = 2-3 (2-3 independent experiments). Statistical differences are calculated for each day separately (excluding samples from mock treatments): two-tailed t test, $p \leq 0.05$).

Due to the significantly increased expression of *PR1* in SAR-induced Col-0 leaves 2 dpi after an immediately preceding cold treatment (CT), we investigated further SAR-inducible transcripts in both cold exposure experimental setups (Fig. 1). We analyzed *Isochorismate Synthase 1* (*ICS1*), which is crucial for pathogen-induced SA biosynthesis, and *PR2*, another SAR marker transcript (Bernsdorff et al., 2016; Wildermuth et al., 2001). *PAD3* is tightly regulated with camalexin biosynthesis but can be independent of SA signaling (Ferrari et al., 2007; Zhou et al., 1999). The cold pre-treatments, with or without memory phase, did not significantly affect the transcript levels of *ICS1*, *PR2*, and *PAD3* in SAR-induced leaves two days after *Pst* infection (Fig. 2). If the cold treatment was applied 3 d before the SAR-inducing *Pst* infiltration (CP), transcript levels of *ICS1* were lower than in non-cold-treated, SAR-induced leaves. This weaker *ICS1* activation in CP leaves was, however, not significant.

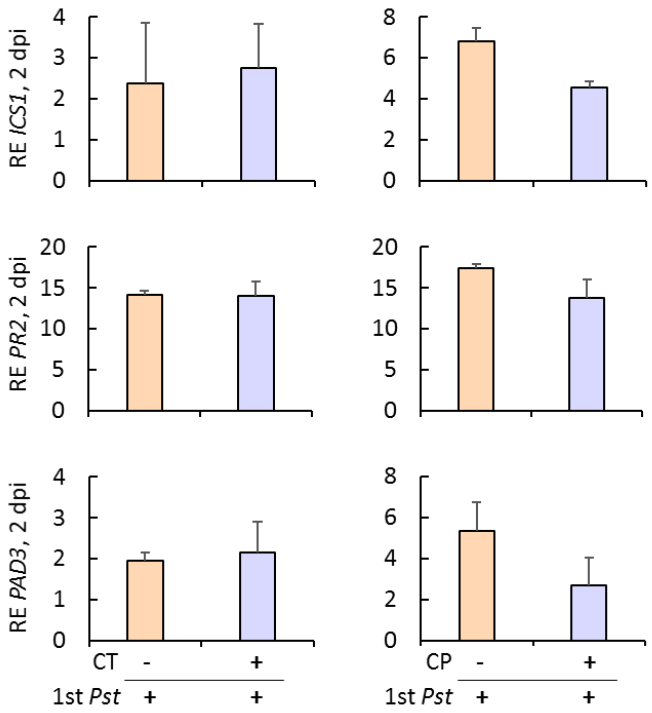

Figure 2: Relative expression (RE) of pathogen-responsive genes after SAR-induction leaves of Col-0. Transcript levels of *ICS1*, *PR2* and *PAD3* were analyzed after the cold pre-treatment (CT) or cold priming (CP) and subsequent *Pst* inoculation (OD_{600} = 0.002) or mock treatment (10 mM $MgCl_2$) at 2 days post inoculation (dpi). The quantification of transcripts was determined by qPCR. The results were calculated as relative expression (delta-Ct) using the mean of the reference genes *YLS8* and *RHIP1*. Bars show mean and standard deviation. N = 2 (2 independent experiments): two-tailed t test, $p \leq 0.05$, no significant differences determined.

5.3.2 Cold pretreatment transiently enhances systemic susceptibility against *Pst* in response to a prior mock infiltration

Next, we wanted to test the relevance of cold treatments for SAR at the decisive level of bacterial titers. Again, the two different experimental setups were compared (Fig. 3A): the cold pre-treatment (CT, 4 °C for 24 h), followed by the first SAR inducing *Pst* inoculation 1 hour after the cold pre-treatment or after a 3-day memory phase between the identical cold treatment and the first *Pst* infection (CP). Afterwards, a subsequent second *Pst* inoculation was performed in upper non-infected leaves to quantify the SAR response on bacterial titers at 3 dpi (Fig. 3A). In addition to Col-0, we included the *flavin-dependent monooxygenase 1* (*fmo1-1)* mutant, in which SAR is abolished and a first *Pst* infection does not result in enhanced resistance against a subsequent infection (Mishina & Zeier, 2006) (Fig. 3B, C). In the control plants (CT -, CP -) of Arabidopsis Col-0, a significant lower bacterial titer was detected in the plants with a first *Pst* infection compared to the mock treatment. Taken together, with the absence of enhanced resistance in the *fmo1-1* mutant, the results indicate a successful SAR response in the control Col-0 plants of both experimental setups (Fig. 3 B, C).

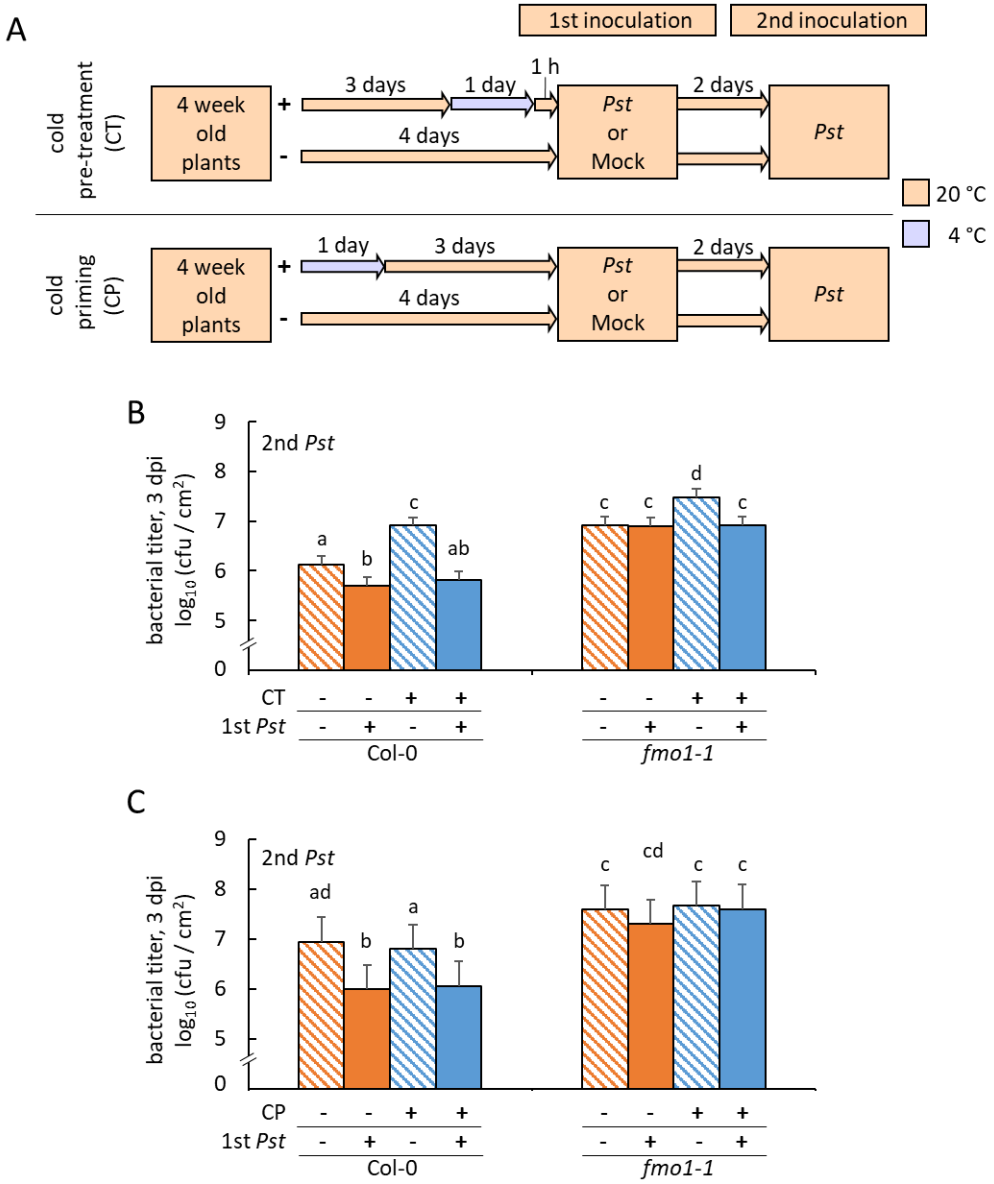

Figure 3. Bacterial titers of secondary, systemic *Pst* inoculation after cold pre-treatment (CT) or cold priming (CP) and subsequent first SAR inducing *Pst* infection in Col-0 and *flavin-dependent monooxygenase 1* (*fmo1-1*). **(A)** Experimental setup to determine the impact of cold treatments (CT or CP) on the SAR response against *Pst*. **(B)** Bacterial titers of second *Pst* inoculation (OD_{600} = 0.001) after CT and subsequent first Pst inoculation (OD_{600} = 0.005; +) or mock treatment (-) in Col-0 and *fmo1-1* 3 days post inoculation (dpi). **(C)** Bacterial titers of second *Pst* inoculation (OD_{600} = 0.001) after CP and subsequent first *Pst* inoculation (OD_{600} = 0.005; +) or mock infiltration (-) in Col-0 and *fmo1-1* 3 dpi. Bars represent means of log_{10}-transformed colony-forming units (cfu / cm^2) and standard errors calculated from 2 independent experiments (n = 12) using a mixed linear model. Different letters above the bars denote statistically significant differences (two-tailed t tests, adjusted P < 0.05).

The enhanced resistance induced by SAR was present in Col-0 plants after both cold treatments. Surprisingly, the cold-pretreated (CT) Col-0 and *fmo1-1* plants showed a significantly higher *Pst* titer after the mock (10 mM $MgCl_2$) infiltration compared to the mock-treated control plants (CT -) (Fig. 3 B). The mock infiltration-mediated enhanced systemic

susceptibility was only transiently as we did not observe this in the CP experimental setup with 3 days between the cold exposure and the first infiltration (Fig. 3 C). In contrast to the local immune response against *Pst*, where Col-0 shows enhanced resistance after a prior cold treatment or a cold treatment with memory phase (Griebel et al., 2022), an additional SAR response or mock inoculation leads to the loss of the cold-induced increased resistance against *Pst* in Col-0 (Fig. 3).

5.4 Discussion

The aim of this study was to elucidate the impact of preceding cold treatments on SAR in *Arabidopsis thaliana* (Col-0) plants challenged with *Pst*. Prior cold exposure, with and without a 5-day memory phase before the infection, has been demonstrated to enhance resistance in Arabidopsis against *Pst* (Griebel et al., 2022). Based on these recent results, we determined whether immediate cold pre-treatment or an earlier cold priming results in an earlier, prolonged, or stronger SAR response, ultimately leading to improved systemic resistance against *Pst*.

At the level of selected transcripts, no earlier, prolonged, or stronger SAR response was observed in systemic, non-infected leaves following both cold exposure treatments and a primary *Pst* infection (Fig. 1, 2). However, a significant enhanced *PR1* expression was detectable in the CT experimental setup at 2 dpi and indicates a stronger SAR response following a cold pre-treatment (Fig. 1). Enhanced *PR1* expression often correlates with increased resistance against *Pst* (Cheng et al., 2022; Lim et al., 2014; Kloek et al., 2001). However, in our experimental CT setup, increased *PR1* expression (2 dpi) did not seem to correlate with enhanced resistance against *Pst* (Fig. 1, 3). At the level of bacterial titers, resistance against *Pst* was equally pronounced in SAR-induced tissues of non-cold and cold-pretreated plants. Though, systemic resistance upon a primary mock infiltration in the absence of pathogens caused a higher plant susceptibility when the infiltration immediately followed the prior cold exposure (CT, Fig. 3B). It is unlikely that the increased susceptibility in cold-pretreated and mock-infiltrated plants is induced by cold alone, as we have previously shown that the cold exposure confers higher resistance against *Pst* (Griebel et al., 2022). The cold-enhanced resistance observed in Col-0 against local *Pst* infection in Griebel et al. (2022) was not replicated after additional mock infiltration or SAR-inducing *Pst* inoculation. In addition, mock-infiltration of cold-pretreated leaves did not results in altered transcript levels of SA or SAR-responsive genes *(PR1*, *FMO1*) (Fig. 2). We suggest that mock infiltration may elicit a stronger JA-mediated wound response in

cold pre-treated plants (Fig. 4) (Liu et al., 2015). Wounding induces an electric signal through membrane potential changes, which leads to the formation of bioactive JA in systemic, non-wounded tissue within minutes. This ultimately activates JA-dependent wound responses, such as the protection of the plant against herbivores (Lee & Seo, 2022; Savatin et al. 2014; Koo et al., 2009). Enhanced JA signaling can increase the susceptibility of Arabidopsis against hemibiotrophic pathogens like *Pst* (Glazebrook, 2005), which could be a putative explanation for the increased susceptibility in cold pre-treated and mock treated Col-0 plants (Fig. 3 B). Furthermore, our findings demonstrate that this effect is independent of FMO1, as we also observed mock treatment-enhanced susceptibility in cold-pretreated *fmo1-1* (Fig. 3 B). Consequently, it can be concluded that the mock-induced increased susceptibility against *Pst* must occur independently of known SAR signals. Nevertheless, the indications for an enhanced wound response after a cold pre-treatment requires further investigations. For instance, repeating the bacterial growth experiments with JA-insensitive lines would be appropriate to further analyze the role of the wound response, since JA is not essential for the establishment of SAR induction against *Pst* (Attaran et al., 2009).

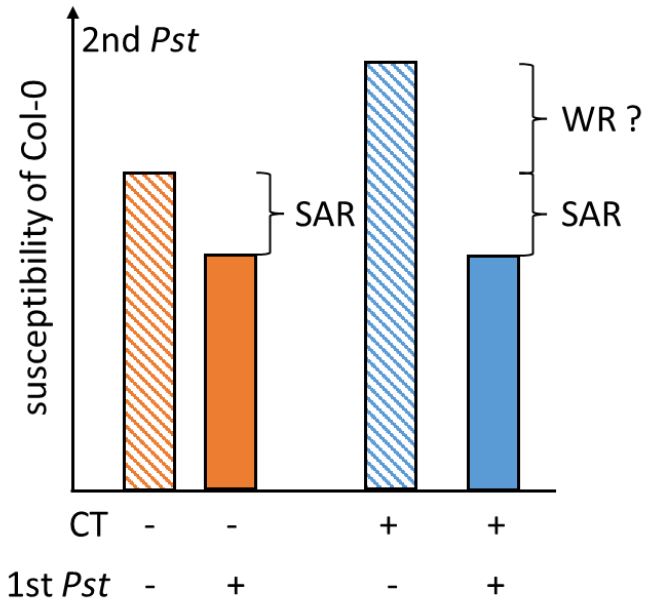

Figure 4: Model of susceptibility degree of *Arabidopsis thaliana* after a cold pre-treatment (CT, 4 °C for 24 h) and subsequent SAR-inducing first *Pst* infection: The establishment of systemic acquired resistance (SAR) is in cold-pretreated plants neither impaired nor does it additionally benefit form the prior cold. Mock infiltration after a cold-pre-treatment, instead of the primary *Pst* infection, leads to an enhanced susceptibility against *Pst*. This might result from a putative cold-mediated enhanced wound response (WR) in Col-0.

Furthermore, we hypothesize that the mock-triggered susceptibility levels observed after both cold treatments (CT and CP; Fig. 4) in Col-0 indicate altered immune signaling pathways, depending on SAR activation during the cold deacclimation phase or a later cold memory requiring phase .

We observed a precipitous decline in *PR1* and *FMO1* transcripts to mock levels already two days after the infected leaves were severed (Fig. 1, 4 dpi)., suggesting a fast deactivation of SAR. This observation seems to contradict the definition of SAR as a long-lasting effect (Li et al., 2023; Conrath, 2006; Mou et al., 2003). However, *Colletotrichum lagenarium* only induces systemic resistance in cucumber scions when the infected leaf is left on transplanted rootstocks. Once the infected leaf is removed from the rootstock, the transplanted scion is no longer able to benefit from systemic resistance, suggesting that SAR is abolished after local SAR-inducing infection is removed (Dean & Kuc, 1986). In Arabidopsis, *P. syringae* pv. *maculicola* infection triggers SAR at the level of bacterial titers for up to 4 days when the primary infected leaves remain on the plant (Baum et al., 2019). In conclusion, it can be postulated that the maintenance of SAR may be prolonged in the sustained presence of local infection. However, further time series experiments would be beneficial in order to ascertain the duration of SAR maintenance.

We defined the experimental set-up with a stress-free phase of 3 days between the cold exposure and the first SAR-inducing *Pst* infection as cold priming (CP). In such a similar set-up, we showed before that the memorized cold exposure has a beneficial impact on plant resistance against *Pst* (Griebel et al., 2022). By definition, a beneficial effect of the first stressor during a following stress application is a requirement for the priming phenomenon (Hilker et al., 2016). However, our findings did not show a positive effect of a several days earlier cold exposure on SAR against *Pst* at the level of bacterial titers. Nevertheless, we cannot rule out the possibility that SAR establishment in cold-primed leaves benefits from the prior cold exposure at levels distinct from immunity against *Pst*. SAR is established at the expense of growth and photosynthesis (Bernsdorff et al., 2016). Based on this, it would be interesting to study whether the preceding cold treatment leads to a beneficial balance between growth and systemic immunity for the plant.

While we know that abiotic factors like light are essential for SAR induction (Griebel & Zeier, 2008; Zeier et al., 2004), little attention has been paid to the impact of temperature on SAR. Recent work revealed that elevated temperatures of 28 °C during the infection period abolishes SAR in Col-0 due to suppressed Pip accumulation, but the crosstalk re-

mains elusive (Shields et al., 2023). In contrast, this study provides results that SAR induction can be achieved not only in naïve plants, but also in plants that experienced a cold stress treatment before the SAR-triggering pathogen infection.

5.5 Materials and Methods

5.5.1 Plant material and cultivation

Experiments were conducted using *Arabidopsis thaliana* accession Columbia-0 (Col-0) and the T-DNA knockout line *fmo1-1* (Bartsch et al., 2006; Mishina & Zeier, 2006).
For cultivation and growth conditions see section 4.4.1 (Chapter III, 4.5.1).

5.5.2 Cold treatments

Experiments were conducted using the cold priming experimental setup with a shortened memory phase of 3 days between the cold treatment and the first *Pst* inoculation.
Further informations can be found in section 4.4.2 (Chapter III, 4.5.2).

5.5.3 Cultivation and inoculation of bacteria

Pst were cultivated overnight at 28 °C on NYGA-plates (5 g peptone, 3 g yeast extract, 2 % glycerol, 1,5 % agar l^{-1}, rifampicin 100 ng ml^{-1}) prior to infiltration, the bacteria were suspended in 10 mM $MgCl_2$ and a dilution of OD_{600} = 0.002 was used for SAR induction when analyzing systemic transcript levels. 10 mM $MgCl_2$ was used for the mock infiltration. For the bacterial growth assay, OD_{600} = 0.005 was used for first *Pst* inoculation and 0.001 for second *Pst* inoculation. A needleless syringe was used to infiltrate the bacterial suspension abaxial into the leaves. For the bacterial growth assays, younger leaves above the leaves of the first *Pst* inoculation were selected for the second infiltration. The time of infection was approximately 3 h after the onset of light.

5.5.4 Bacterial growth assays

To analyse the outcome of SAR, *Pst* titers of the secondary *Pst* infection were determined at 3 dpi. Three infected-leaf discs (Ø 8 mm) of one plant were combined for one sample (= one biological replicate). Harvested leaf discs (samples) were incubated while shaking at 28 °C for 1 h in 10 mM $MgCl_2$ with 0.01 % (v/v) Silwet-77. After the bacteria have been extracted out of the leaves, each sample was diluted several times and spread in 15 µl spots on rifampicin –containing (100 µg ml^{-1}) NYGA-plates . The plates were incubated at

28°C for two days and the log_{10}-transformed colony forming units (CFU) per cm^2 leaf were calculated (Griebel et al., 2022).

5.5.5 Transcript analysis

Transcript analyses were performed using quantitative real-time PCR (qRT-PCR). One sample (= one biological replicate) consisted of 9 non-infected leaves of 3 different plants. RNA extraction, cDNA synthesis, and qRT-PCR were carried out as described in section 4.4.4. (Chapter III), using primers (Tab. 1) for genes well-known for showing enhanced systemic transcription upon SAR induction (Bernsdorff et al 2016; Gruner et al., 2013).

Table 1: Primers used in this study.

Gene	Gene name	Forward primer	Reverse primer
AT1G19250	***FMO1***	CTCCATGATAGGCCTAACCAAAGC	AAACTACGGCACGCAGAAGAGAG
AT1G74710	***ICS1***	GCTTGGCTAGCACAGTTACAGC	CACTGCAGACACCTAATTGAGTCC
AT2G14610	***PR1***	TTCTTCCCTCGAAAGCTCAA	AAGGCCCACCAGAGTGTATG
AT3G26830	***PAD3***	CTTTAAGCTCGTGGTCAAGGAGAC	TGGGAGCAAGAGTGGAGTTGTTG
AT3G57260	***PR2***	AGCTTCCTTCTTCAACCACACAGC	TGGCAAGGTATCGCCTAGCATC
AT4G26410	***RHIP1***	GAGCTGAAGTGGCTTCCATGAC	GGTCCGACATACCCATGATCC
AT5G08290	***YLS8***	TCATTCGTTTCGGCCATGACTGG	ACGCAAGCACCTCATCCATCTG

5.5.6 Statistical analysis

The statistical analysis was performed using Excel for two-tailed t test and the basic R environment for ANOVA and the follow-up Tukey-HSD test. The combined analysis of log_{10}-transformed bacterial titers from independent experiments was conducted using a linear mixed model, as previously described (Griebel et al., 2022).

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6. General discussion

6.1 Arabidopsis benefits from brief cold exposure with enhanced sustained resistance against *Pseudomonas syringae* and transiently increased resistance against *B. cinerea*

The objective of this thesis was to analyze the impact of a prior 1-day cold exposure on the pathogen resistance in the model plant *Arabidopsis thaliana* with the aim to gain a deeper understanding of the interconnection between cold stress signaling and plant immunity. For this aim, two distinct cold exposure treatments were compared: (i) an experimental setup with a cold pre-treatment (CT) to investigate the effect of a 1-day long cold exposure for an immediately subsequent pathogen infection, and (ii) a cold priming (CP) experimental setup to investigate the effects of a 1-day cold exposure with an additional memory phase between initial cold treatment and pathogen infection (Chapter III, Fig. 1 A). The objective of this memory phase was to ascertain whether the potential impact of a prior cold treatment on plant pathogen defence could be retained by the plant over an extended period. To determine the resistance of *Arabidopsis thaliana* against different pathogens, the hemibiotrophic bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC 3000 (*Pst*) and the necrotrophic fungal pathogen *Botrytis cinerea* B05.10 were employed. Both pathogens are established as model pathogens for the plant Arabidopsis (van Kan et al., 2006; Katagiri et al., 2002; Mauch-Mani & Slusarenko, 1993). *Pst* and *B. cinerea* use distinct infection strategies: *Pst* manipulates plant immunity and benefits from living host cells, while *B. cinerea* penetrates and kills host cells to complete its life cycle (Bi et al., 2023; Xin et al., 2018). Hence, plant defence against biotrophic and necrotrophic pathogens includes distinct constitutive and induced immune signaling pathways (Bi et al., 2023; Xin et al., 2018). This offers the possibitity to analyze the impact of different defence responses with the aim to answer the question of whether and how a prior and brief cold exposure affects the resistance of Arabidopsis against pathogens with different infection strategies.

Our study demonstrated that a single 24-day cold exposure (4 °C) was sufficient to immediately enhance the resistance of Arabidopsis (accession: Col-0) against *Pst* and *B. cinerea* (Chapter I, Fig. 1; Chapter III, Fig. 1). In addition and in contrast to the transient cold-mediated resistance against *B. cinerea*, the cold-enhanced resistance against *Pst* was

maintained for a period of five days after the initial cold exposure (Chapter I, Fig 1). Therfore, we concluded that such a cold exposure primes plant defence against *Pst* (Hilker et al., 2016).

6.2 Cold priming on pathogen resistance is rather independent from altered PTI and SA signaling

The enhanced plant resistance against *Pst* after cold priming (Chapter 1, Fig. 1) suggests a cold memory that modifies the pathogen defence/stress response. Such priming memory could be established with epigenetic modifications, changes in gene expression, physiology and metabolism (Hilker et al., 2016). Earlier work showed that also short, repetitive cold exposure treatments enhance resistance against *Pst* and this includes histone 3 acetylation leading to enhanced activation of genes responsive to PAMP-triggered immunity (PTI) and salicylic acid (SA) (Singh et al., 2014; Lusser, 2002). In our study, we could not observe a cold priming enhanced activation of PTI- and SA-responsive genes (Chapter I, Fig. 3). Hence, this distinguishes the signaling in response to short (1.5 h) repetitive cold exposures from single, but longer, (24 h) cold treatments. Additionally, resistance of Arabidopsis against type III secretion system-deficient and PTI-inducing *Pst hrcC*$^{-}$ was not affected by a prior cold exposure (Chapter I, Fig. 1). Taken together, it is unlikely that PTI is responsible for cold mediated resistance due to a single prior cold exposure. Although the stability of the priming memory was not the primary focus of this project, the findings support recent studies that Arabidopsis exhibits a uniform stress memory of approximately five to seven days following diverse stress applications (Chapter I, Fig. 1) (van Buer et al., 2019; Singh et al., 2014; Ding et al., 2012). Cold, however, did not improve the resistance against *B*. *cinerea* with an additional memory phase (Chapter III, Fig. 1). This indicates that the cold priming memory does not impact defence against the necrotrophic fungus *B. cinerea*. We propose that the cold memory effect supports immune responses specific for the defence against biotrophs but weakly contributes to immunity against necrotrophs.

6.3 Cold affects *B*. *cinerea*-triggered plant ROS formation

The next objective of this study was to investigate immune signaling pathways essential for cold-enhanced resistance against *Pst* and *B*. *cinerea*. Reactive oxygen species (ROS) are central plant components for affecting plant stress responses (Peláez-Vico et al., 2024).

As cold modifies ROS accumulation in the chloroplasts (Ensminger et al., 2006; Huner et al., 1993), we assumed that altered ROS levels contribute to cold-mediated resistance against pathogens. One example of a ROS target with potential impact on diverse immune signaling pathways are mitogen-activated protein kinases (MAPKs). MAPKs can be activated/inactivted by ROS and are therefore ROS-responsive (Patterson et al., 2009; Kovtun et al., 2000). MAPKs regulate diverse abiotic and biotic stress responses, including cold, wounding, oxidative stress, and immune signaling pathways (Nakagami et al., 2005). These immune signaling pathways include SA signaling, jasmonic acid (JA) signaling, PTI, effector-triggered immunity (ETI), hypersensitive response (HR), and systemic acquired resistance (SAR) (Lang & Colcombet, 2020; Brodersen et al., 2006; Vilela et al., 2010; Asai et al., 2002; Petersen et al., 2000). The presented example of MAPKs illustrates that a multitude of plant immune responses might be impacted by cold-mediated ROS accumulation through the cold-sensing chloroplasts.

ROS function as stress signals (Peláez-Vico et al., 2024), with putative antimicrobial properties (Juven & Peirson, 1996, Peng & Kuc, 1992), may impact cold mediated resistance against pathogens. Regardless of cold, ROS are involved in plant responses against *Pst* or *B*. *cinerea*. This is indicated by the pathogen usage of effectors and compounds which manipulate plant ROS homeostasis (Li et al., 2014; Rodríguez-Herva et al., 2012; Williams et al., 2011). For instance, the effector HopK1, secreted by *Pst*, targets the chloroplast, resulting in weakened PTI-associated ROS formation and increased susceptibility (Li et al., 2014). It is suggested within a simplified concept that pathogens with a biotrophic phase are susceptible against ROS, while necrotrophic pathogens benefit from elevated ROS levels (Barna et al., 2012). However, this distinction may be to much simplified, as necrotrophic *B*. *cinerea* showes enhanced or decreases susceptibility in response to high and low ROS levels (Rossie et al., 2017; Bliss et al., 2013 L'Haridon et al., 2011). One gap in our understanding is the initial infection phase of *B*. *cinerea*, for which it is hypothesized that *B*. *cinerea* has a short biotrophic phase (Bi et al., 2023; Veloso et al., 2018), as the necrotrophic fungus *Sclerotinia sclerotiorium* has (Kabbage et al., 2013). It is therefore possible that the cold and infection mediated elevated ROS levels observed in Col-0, as demonstrated in this thesis (Chapter III, Fig. 4), occur during the early biotrophic phase of *B*. *cinerea* and correlate with increased resistance against the necrotrophic pathogen. In order to finally determine the role of ROS in *B*. *cinerea* infection, the complex infection process of *B*. *cinerea* must first be investigated in detail.

6.4 Cold-enhanced plant resistance is rather independent from SA signaling

The phytohormone SA is essential for a full activation of the plant immune system against the hemibiotrophic pathogen *Pst* (Thomma et al., 1998). However, SA signaling may also be important in the plant immune response against *B*. *cinerea* for a wildtype-like immune response. Salicylate hydroxylase (NahG-)expressing lines, that do not accumulate SA, exhibit increased susceptibility against *B*. *cinerea* (Ferrari et al., 2003). Our analyses of SA-related transcripts demonstrated that cold exposure did not alter SA signaling in immediately *B. cinerea*-triggered or several days later *Pst*-triggered cold-pretreated plants (Chapter I, Fig. 3; Chapter III, Fig. 3). To further support independency from isochorismate (IC)-synthesized SA as factor for the cold medited resistance against *B. cinerea*, the lesion diameter assay was repeated to determine Arabidopsis resistance against *B*. *cinerea* in a *salicylic acid induction deficient 2* (*sid2-1*) line (Supplements, Fig. 1; Chapter III, Fig. 1). *sid2-1* is impaired in pathogen-triggered SA accumulation via IC (Wildermuth et al., 2001) . The cold pre-treatment enhanced resistance against *B*. *cinerea*, as demonstrated in Col-0 (Chapter III, Fig. 1), was confirmed in the s*id2-1* line, as cold-pretreated *sid2-1* exhibited resistance pattern similar to the wildtype(Supplements, Fig. 1). This suggests that the pathogen-responsive IC pathway is dispensable for cold-enhanced resistance against *B*. *cinerea*. Alternatively, the phenylpropanoid (PAL) pathway could also contribute to the biosynthesis of SA independently of the IC pathway after cold exposure and infection. Strawberries infected with *B*. *cinerea* utilize the PAL pathway rather than the IC pathway to produce SA (Luo et al., 2024). However, PAL and IC pathways lead to SA formation that induce *PR1* expression by NPR1 (Section 1.3.3) and we could not observe cold-related-changes in *PR1* expression after cold and subsequent *Pst* or *B*. *cinerea* infection (Chapter I, Fig. 3; Chapter III, Fig. 3).

6.5 JA signaling may be involved in cold mediated resistance

Jasmonic acid (JA) is an important phytohormone to counteract susceptibility against necrotrophic pathogens like *B*. *cinerea* (Ferrari et al., 2003) but it is dispensable or disadvantageous for resistance against *Pst* (Scalschi et al., 2020; Brooks et al., 2005; Kloek et al., 2001). Accordingly, the investigation of the potential impact of cold exposure on JA signaling was limited to the *B*. *cinerea* Arabidopsis interaction. Cold pre-treatment and subsequent *B*. *cinerea* infection had no significant impact on the transcription of JA responsive *Pathogenesis-related 4* (*PR4)* and *Plant Defensin 1.2a* (*PDF1.2a)* in Arabidopsis

(Chapter III, Fig. 3). This, suggested a JA-independent effect and we extended our experiments to the *jasmonate resistent 1* (*jar1-1)* line, which cannot form the bioactive JA-Ile (Li et al., 2017; Staswick et al., 2002; Staswick et al., 1992). In contrast to the results observed in the wildtype, the lesion diameter results of *jar1-1* were unchanged after cold pre-treatment (Supplements, Fig. 1). This effect was in contradiction to the transcription results of *PR4* and *PDF1.2,* since we would have expected enhanced expression of JA responsive genes if JA signaling is crucial for cold mediated resistance. However, pathogen-triggered transcripts of *PR4* and *PDF1.2* were not affected by the prior cold treatment (Chapter III, Fig. 3). The contrasting results do not allow a definitive conclusion on the role of JA or JA-Ile signaling in cold pre-treatment-enhanced resistance against *B*. *cinerea*. In order to provide a definitive conclusion regarding the role of JA signaling in cold pre-treatment enhanced resistance against *B*. *cinerea*, it is necessary to integrate further JA mutants. These lines should also include mutants with disrupted JA biosynthesis, since the JA precursor 12-oxo phytenoic acid (OPDA) can signal JA-Ile and Coronatine Insensitive 1 (COI1) independently (Jimenez-Aleman et al., 2022). Additionally, phytohormone measurements for JA and SA should be conducted to get a deeper understanding about whether brief cold exposure or crosstalk between cold exposure and pathogen infection alters JA or SA abundance in Arabidopsis.

6.6 Cold exposure-enhanced resistance in Arabidopsis is independent from ETI and HR

Plants provide a robust immune response through HR, which is generally activated via ETI (Lolle et al., 2020). Because biotrophic pathogens depend on living host tissue for disease progression, HR is effective against pathogens with a biotrophic phase (Balint-Kurti, 2019). ETI established via (toll-interleukin-1 receptor-like) TNL receptors necessitates the involvement of nucleocytoplasmatic EDS1 for signaling and the immune response (Dongus & Parker, 2021). Therefor, EDS1 is an important signaling component of TNL-mediated HR. The repertoire of pathogen effectors from virulent *Pst* outruns ETI recognition and prevents ETI-triggered HR (Guo et al., 2009). Nevertheless, it is possible that a prior cold exposure might amplify HR directly or via a priming memory. However, the *eds1-2* null mutant (Chapter I, Fig. 1; Chapter II, Fig. 2) shows a wildtype like increased resistance after cold exposure (CT or CP), which makes HR as the reason for cold-induced

resistance less likely, since TNL-mediated HR does not function in *eds1-2*. Another argument is that the HR-inducing bacterial strains *Pst avrRPM1* and *Pst avrRPS4 (*Hofius et al., 2009) do not exhibit increased resistance after cold treatment (Chapter I, Fig. 1), which could be caused by increased HR. Also, an enhanced cold priming-mediated HR response in the *Pst avrRPM1* Arabidopsis interaction, which is compensated by the attenuation of another immune signaling pathway, is unlikely, as the HR-mediated ion leakage in Col-0 is not affected by cold priming and subsequent *Pst avrRPM1* infection (Supplements; Fig. 2). Finally, in the case of a putative cold-enhanced HR, it can be assumed that a *B*. *cinerea* infection results in greater susceptibility due to the enhanced HR and the necrotrophic behaviour. It has already been demonstrated that Arabidopsis plants, simultaneously infected with the HR-inducing *Pst avrRPM1* and the necrotrophic *B*. *cinerea*, have a higher susceptibility against *B*. *cinerea*, probably due to avrRPM1-tiggered HR (Govrin & Levine, 2000). Taken all arguments together, it can be postulated that HR is not affected by CT or CP and that it is therefore not responsible for cold exposure-enhanced resistance.

6.7 Cold mediated lignification may be a cause of cold mediated enhanced resistance against *B. cinerea*

The plant cell wall, as a part of the apoplast, surrounds plant cells and provides mechanical strength (Zhang et al., 2021). As a physical barrier, it might prevent pathogens from penetrating the cell, for example, by forming transient papillae (including callose) or irreversible lignification (Eynck et al., 2012; Hückelhoven, 2007; Nishimura et al., 2003; Jacobs et al., 2003). Additionally, pathogens and cold exposure alter the composition of the plant cell wall (Lee et al., 2019; Le Gall et al., 2015; Hano et al., 2006; Zhang et al., 2007), which finally might affect the ability of *B*. *cinerea* to penetrate the cell wall. Although it is known that callose can be formed during cold (Wu et al., 2019), the precise role of cold-mediated callose remains unclear. Our findings demonstrated that a 24-hour cold exposure significantly enhanced callose deposition in Arabidopsis (Chapter III, Fig. 5) and therefore confirmed the results of Wu et al., (2019). However, pathogen-induced callose formation was not additionally affected by cold (Chapter III, Fig. 5). Callose formed in response to pathogens is often used as an indication for PTI activation (Luna et al., 2011). Consequently, the pathogen-triggerd callose formation, similar in cold-pretreated and non-cold plamts, provides further evidence that PTI is not the immune signaling layer that

is affected by cold exposure or by the crosstalk between cold exposure and pathogen immunity. It remains disputable whether cold-induced callose contributes to cold-mediated enhanced resistance, given that cold-mediated callose formation was completly absent 24 hours after infection (Chapter III, Fig. 5). Additionally, the probability that cold-induced callose is synthesized at a potential penetration site of *B*. *cinerea* can be considered low as the plant cell callose ratio is very low (chapter III, Fig. 5A). Nevertheless, it is not excluded that the temporary cold-mediated callose contributes to the resistance of *Arabidopsis thaliana* against invading pathogens such as *B*. *cinerea*.

Cold stress has been demonstrated to enhance the accumulation of pectin and lignin in the plant cell wall (Le Gall et al., 2015; Takahasi et al., 2024) (Chapter III, Fig. 6). While pectin can serve as a carbon source for *B*. *cinerea*, as *B*. *cinerea* secretes different pectin-degrading enzymes, it is unlikely that lignin is degradable by *B*. *cinerea* (Choquer et al., 2021; Hörmann et al., 2013; Zhang et al., 2011). The results of this thesis demonstrated that a single 24-hour cold exposure is sufficient to induce significant lignification in Col-0 leaves, and that a subsequent infection by *B*. *cinerea* does not significantly alter the cold-mediated lignification (Chapter III, Fig. 6). The stress-induced lignification of the plant cell wall contributes to the protection of other potential degradable cell wall components, such as cellulose and hemicellulose, against the action of cell wall-degrading enzymes secreted by *B*. *cinerea* (Eynck et al., 2012). Consequently, it can be postulated that cold-mediated, stress-induced lignin may contribute to enhanced resistance against *B*. *cinerea*. Moreover, while the precise regulation of lignification remains elusive, there are reports indicating that JA signaling impacts lignification in various plants (Borah et al., 2023; Scalschi et al., 2020; Denness et al., 2011). In light of these observations and the finding that *jar1-1* is unable to induce cold-mediated resistance against *B*. *cinerea* (Supplements; Fig. 1), we propose that JA-signaling impacts cold-mediated lignification in Arabidopsis. Nevertheless, this hypothesis requires further verification. In contrast to *B*. *cinerea*, *Pst* is restricted to the apoplast as a pathogenic habitat (Xin et al., 2018). Consequently, the cell wall's role as a mechanical barrier against invading *Pst* is less significant for resistance against *Pst*. Nevertheless, Arabidopsis infected with virulent *Pst* or avirulent, HR-inducing *Pst* strains demonstrate a significant enhancement in lignification in the infected leaves, which correlates with enhanced resistance (Lee et al., 2019). Hence, lignification is significantly more pronounced in Arabidopsis infected with HR-inducing strains, and it has been demonstrated that lignification is essential for limiting HR to the infected tissue. It

is noteworthy that HR-inducing *Pst* strains exhibit reduced motility in lignified leaves compared to virulent *Pst*, suggesting that lignification may also impede pathogen motility (Lee et al., 2019). However, whether cold-mediated resistance against *Pst* relies on lignification remains a matter of research, as *Pst avrRPM1* and *Pst avrRPS4* (HR-inducing) infected Arabidopsis cannot benefit from cold priming with enhanced resistance (Chapter I; Fig. 1).

6.8 A prior cold exposure results in a full SAR response against *Pst* in Arabidopsis

SAR is a well-studied immune response that includes cell to cell signaling. Here we focused on the effects of cold on SAR against *Pst* since Arabidopsis SAR fails to enhance resistance against *B. cinerea* (Govrin & Levine, 2002). Contrasting to cold-mediated enhanced resistance against *Pst* in a local immune response (Chapter I, Fig. 1), additional SAR induction immediately or three days after cold exposure abolished the cold mediated resistance in Arabidopsis (Chapter IV, Fig. 3). However, we demonstrated that SAR can be established after cold exposure (Chapter IV, Fig. 3). This contrasts to exposures to enhanced temperature (28 °C) where the establishment of SAR failed (Shields et al., 2023). Because SAR is established and regulated by a variety of compounds including SA, pipecolic acid (Pip), N-hydroxypipecolic acid (NHP), azelaic acid (AzA) and ROS (Section 1.3.8), a variety of cold mediated changes could be responsible for the absence of an additional increase in SAR. SA siganling is crucial for SAR (Section 1.3.8) and we demonstrated enhanced *PR1* transcription in systemic tissue after SAR induction and cold pretreatment (Chapter IV, Fig.1). Thus, in contrast to the local immune response, a cold-mediated change in SA signaling was observed, even if this did not result in an altered systemic resistance of Arabidopsis (Chapter IV, Fig. 1, 3). Another putative candidate which may affect SAR after prior cold exposure is AzA, since AzA is generated in plastids via peroxidation after various abiotic and biotic stressors (Priya Reddy & Oelmüller, 2024). Lastly, we demostrated enhanced susceptibility after SAR induction and cold pre-treatment in *Pst* infected Arabidopsis (Chapter IV, Fig. 3). This prompts the question of why mock treatment leads to increased susceptibility in Arabidopsis? One potential explanation is the wounding caused by the used syringe during mock treatment, which may activate a wound response that induces JA signaling and this response is enhanced due to the cold exposure. JA signaling can enhance the susceptibility of Arabidopsis against *Pst*

(Scalschi et al., 2020; Brooks et al., 2005; Kloek et al., 2001). Furthermore, a local wound response can activates systemic JA signaling within minutes (Koo et al., 2009), making JA signaling a theoretical candidate for the increased susceptibility against *Pst*. Nevertheless, this hypothesis requires further verification.

6.9 Chloroplast-localized sAPX and tAPX are indispensable for cold-enhanced resistance against *Pseudomonas syringae* and contribute to cold-enhanced resistance against *Botrytis cinerea*s

A central aim of this work was to determine the role of the chloroplast as a cold sensing hub in plant immunity and the impact of cold stress-induced ROS formation on plant immune signaling pathways (Section 1.5). We hypothesized that chloroplasts play a role in fine-tuning plant resistance after prior cold exposure, and that this involves the chloroplast-localized ROS scavengers sAPX and tAPX (Section 1.6). To demonstrate that chloroplasts are cold sensing hubs, knockout lines of chloroplast-localized *stroma ascorbate peroxidase* (*sAPX*) and *thylakoid ascorbate peroxidase* (*tAPX*) were analyszed in the CT- and CP-experimental setups. During cold exposure, an increase in ROS formation was observed in chloroplasts. They can migrate as hydrogen peroxide to other cell compartments and may impact various signaling pathways (Ugalde et al., 2021; Ensminger et al., 2006; Huner et al., 1993). These potential impacts on signaling pathways may be fine-tuned through the ROS-scavenging activities of sAPX and tAPX. The aforementioned hypotheses were empirically validated on plant resistance level, wherein it was demonstrated that sAPX and tAPX are indispensable for wildtype-like enhanced resistance of Arabidopsis following prior cold exposure against *Pst* and *B. cinerea* (Chapter I, Fig. 1; Chapter III, Fig. 2).

Pst and *B. cinerea* employ distinct infection strategies and Arabidopsis, therefore, utilizes disparate immune signaling pathways for plant resistance (Section 1.3.3 – 1.3.7). sAPX and tAPX prove beneficial for conferring wild-type-like and cold-mediated resistance against different pathogens. Also, our findings demonstrated that cold priming with a five-day memory phase between the cold treatment and *Pst* infection significantly enhanced resistance in Col-0, but not in *sapx* and *tapx* (Chapter I, Fig. 5). Additionally, we demonstrated enhanced susceptibility in an inducible *tAPX-RNAi* line after cold priming and subsequent *Pst* infection (Chapter I, Fig. 5). This result indicates an involvment of sAPX and tAPX for the priming mediated enhanced resistance, since cold mediated ROS formation

declined rapidly after the cold treatment and cold stress responsive transcripts are allready on non-stress level after 3 days (Chapter I, Fig. 2; Chapter III, Fig. 4 A), suggesting a deacclimated plant. It is noteworthy that the double mutant line *eds1 sapx* exhibited enhanced resistance against *Pst* in the control treatment without cold exposure, in comparison to *eds1-2* (Chapter II, Fig. 2). This indicates a role for sAPX in plant resistance already under control conditions without an additional cold exposure in an immunocompromised background.

With regard to *B. cinerea*, camalexin and JA-mediated defense are crucial for resistance, but SA may also contribute to wildtype-like resistance (Ferrari et al., 2007; Ferrari et al., 2003). However, transcript analysis revealed that *PAD3* (camalexin biosynthesis), JA-, and SA-responsive genes do not exhibit cold-mediated expression in *sapx* and *tapx* (Chapter III, Fig. 3). This result suggests that these defense pathways are not affected by the lack of sAPX and tAPX.

Both *sapx* and *tapx* exhibit comparable levels of resistance against *B. cinerea* after cold pre-treatment, yet only *sapx* displays a distinct ROS formation profile following *B. cinerea* infection (Chapter III, Fig. 4). Previous studies have demonstrated differences in the participation of sAPX and tAPX on cold priming and it has been shown that tAPX alone mediats cold priming-specific transcriptional alterations during a second cold exposure but also the suppression of NADPH dehydrogenase-dependent cyclic electron transport (Seiml-Buchinger et al., 2022; van Buer et al., 2019). Additionally, tAPX is not affected or tends to be downregulated directly after short and long term cold exposure and expression rises only days after cold exposure(Juszczak et al., 2016; van Buer et al., 2016) (Chapter I, Fig. 4). In contrast, sAPX is significantly upregulated directly after a cold treatment (Juszczak et al., 2016; van Buer et al., 2016) (Chapter I, Fig. 4). This indicates a more prominent role of sAPX for cold stress-mediated ROS scavenging. In addition to the interaction of cold-mediated ROS and APX, there is also initial evidence of APX being targeted by pathogens from other studies. The coat protein of Citrus yellow vein clearing virus interacts with Ascorbate Peroxidase 1 of *Citrus limon* and *Nicotiana benthamiana*, enhancing the activity of Ascorbate Peroxidase 1, which promotes the accumulation of Citrus yellow vein clearing virus (Wang et al., 2023). Additionally, the chloroplast-localized Ascorbate Peroxidase 6 (APX6) of *Citrus sinensis* and *Nicotiana benthamiana* is targeted by the secreted effector AGH17488 of *Candidatus* Liberibacter asiaticus, which results in the relocation of APX6 to the cytoplasm and the enhancement of its activity and facilitation of pathogen infection (Du et al., 2023).

6.10 Conclusion

This study demonstrates that a 24 h cold exposure enhances the resistance of *Arabidopsis thaliana* against *Pseudomonas syringae* and *Botrytis cinerea*, independent of PTI, ETI and SA signaling pathways. The findings suggest that ROS management, particularly through chloroplast-localized scavengers like sAPX and tAPX, stress-induced lignification and JA signaling play crucial roles in mediating this cold-induced resistance.

6.11 Supplements

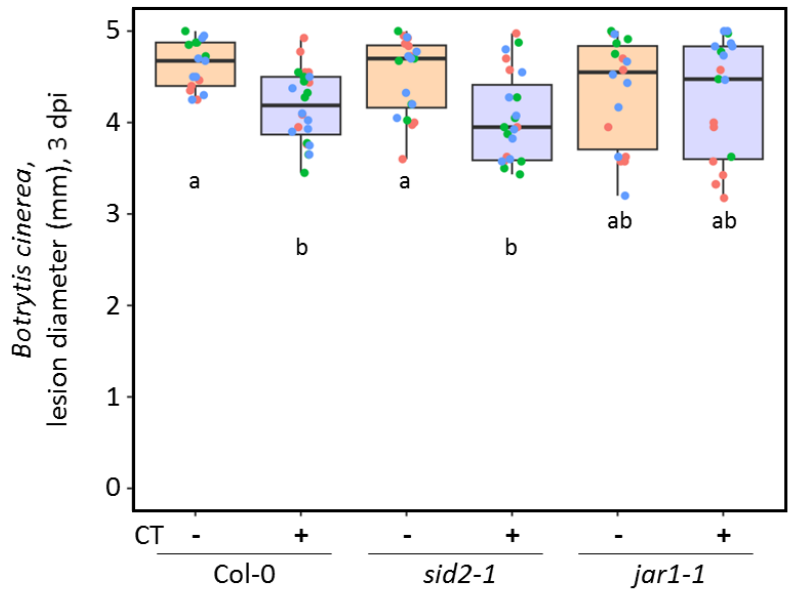

Figure 1:Lesion diameter of *Botrytis cinerea* infected Col-0, *salicylic acid induction deficient 2* (*sid2-1*) knock-out and *jasmonate resistant 1* (*jar1-1*) knockdown lines after prior cold pre-treated (CT; 24 h at 4 °C). Four leaves of each biological replicate were drop-inoculated (5 x 10^4 spores ml^{-1}) and the lesion diameter was measured 3 days post inoculation (dpi). N = 24 (3 independent experiments), different colours of the dots indicate different experiments and different letters denote statistically significant differences (Tukey-HSD: p-value ≤ 0.05).

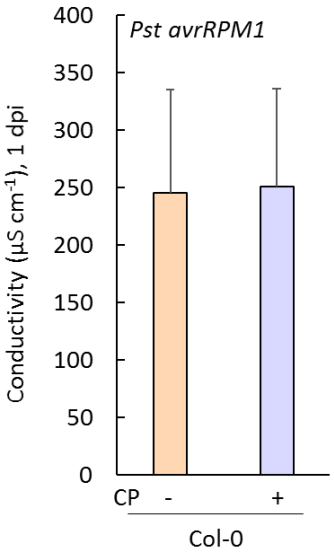

Figure 2: Electrolyte leakage from Arabidopsis leaf discs after cold priming (CP) and inoculation with hypersensitive response-inducing *Pst avrRPM1*. 4-week-old Col-0 plants were cold primed (4°C for 1 day) and inoculated with *Pst avrRPM1* (OD_{600} = 0.005) after a 5-day long memory phase. After the inoculated leaves have dried, two (8 mm Ø) leaf discs per biological replicate were punched out and transferred to a tube containing $_{dd}H20$. Conductivity was measured 1 day post-inoculation (dpi) and was normalized to an initial 2 hpi measurement to minimize the effect of wound-mediated ion leakage. Bars represent mean and standard deviation. N = 11 (3 independent experiments). Two-way t-test; p = 0.05.

6.12 References

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